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Three-phase hollow fiber liquid-phase microextraction of organophosphorous nerve agent degradation products from complex samples

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ABSTRACT

Degradation products of chemical warfare agents are considered as important environmental and biological markers of chemical attacks. Alkyl methylphosphonic acids (AMPAs), resulting from the fast hydrolysis of nerve agents, such as sarin and soman, and the methylphosphonic acid (MPA), final degradation product of AMPAs, were determined from complex matrices by using an emergent and miniaturized extraction technique, the hollow fiber liquid-phase microextraction (HF-LPME), before their analysis by liquid chromatography coupled to mass spectrometry (LC–MS). After studying different conditions of separation in the reversed phase LC–MS analysis, the sample treatment method was set up.

The three-phase HF-LPME was carried out by using a porous polypropylene (PP) hollow fiber impregnated with 1-octanol that separates the donor and acceptor aqueous media. Various extraction parameters were evaluated such as the volume of the sample, the effect of the pH and the salt addition to the sample, the pH of the acceptor phase, the extraction temperature, the stirring speed of the sample, the immersion time in the organic solvent and the time of extraction. The optimum conditions were applied to the determination of MPA and five AMPAs in real samples, such as surface waters and urine. Compounds were extracted from a 3 mL acidified sample into only 6 μL of alkaline water without any other pretreatment of the complex matrices. Enrichment factors (EFs) higher than 170 were obtained for three less polar AMPAs. Limits of quantification (LOQs) in the 0.013–5.3 ng mL^{-1} range were obtained after microextraction of AMPAs from river water and in the range of 0.056–4.8 ng mL^{-1} from urine samples with RSD values between 1 and 9%.

1. Introduction

Following the chemical attacks in the 20th century causing the death of hundreds of thousands of people, the chemical weapon convention (CWC) was established to prohibit the use, the manufacturing and the storage of chemical warfare agents (CWAs) [1]. The organization for prohibition of chemical weapons (OPCW) ensures the respect of the CWC by collecting samples. The analysis can be performed on site by the inspectors or off site by two designated OPCW laboratories. OPCW proficiency tests (PTs) are organized twice a year in order to verify the capability of designated laboratories to deal with CWA and related compound analysis. During OPCW PTs, the analysis of chemical warfare agents is required. However, CWAs are hydrolysable molecules. Therefore, degradation products of CWAs are often as environmental and biological markers of chemical attacks or suspicion of CWA production. This study focuses on the analysis of five alkyl methylphosphonic acids (AMPAs) that are hydrolysis products (also metabolites) of organophosphorous nerve agents, such as Sarin or Soman, and of MPA, the degradation product of AMPAs. Their analysis are still challenging because a sample pretreatment is needed before their separation in order to extract and preconcentrate them from complex aqueous matrices.

Gas chromatography coupled to mass spectrometry (GC–MS) is a widely used technique for phosphonic acid analysis, thanks to its high sensitivity. However, phosphonic acids being not volatile enough, a step of derivatization has to be performed before GC analysis [2–4]. This step of derivatization is still time-consuming and can suffer from matrix effects in real samples [5]. Capillary electrophoresis coupled to mass spectrometry can be used for the direct determination of these compounds [6] but this technique is not sensitive enough, considering its use for the determination of AMPAs at trace level [7–13]. An alternative to GC consists in the use of liquid chromatography [14]. However, due to their low absorbance properties in UV and the absence of fluorescence properties, the direct identification of AMPAs is exclusively performed using mass spectrometry (MS) [7,15–20].

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Considering their acidic properties ($pK_a = 2.5$), AMPAs can be separated in 20 min by an anion-exchange chromatography [19]. A separation based on hydrophilic interaction was reported to separate five AMPAs [21]. Also, the separation of several alkylphosphonic acids was performed in less than 15 min using a porous graphitic carbon (PGC), a stationary phase that induces hydrophobic and electronic interactions [22]. But the reversed-phase liquid chromatography on C18 silica followed by MS identification remains the method of choice for the separation of AMPAs [17,23,24]. For illustration, LOQ from 5 to 50 ng mL^{-1} were obtained for the analysis of MPA and four AMPAs by ESI-LC/MS/MS [24].

The determination of AMPAs, at the trace level in complex samples, requires a method of sample treatment to achieve an efficient cleanup by keeping high enrichment factors. However, the extraction of such polar compounds, especially MPA, from aqueous samples is still a real analytical challenge.

To extract AMPAs from aqueous samples, most sample treatment methods described in publications consist of extraction-derivation procedures to perform GC–MS analysis of the derivatized AMPAs (solid phase extraction [25], solid phase microextraction [2], microemulsion [26], hollow-fiber liquid phase microextraction [27,28]). This study focuses on sample treatment prior to LC–MS analysis.

AMPAs were also extracted under their native from water using a Oasis HLB sorbent and further eluted with acetonitrile before percolation on a molecularly imprinted polymer sorbent in order to favor the interactions between compounds and polar imprinted stationary phase [29]. EFs higher than 1 (2.5–5) were obtained for AMPAs. However, large amounts of solid support were needed to obtain these results, because the interactions between the studied polar analytes and the sorbent were very weak [29].

The miniaturization of the extraction techniques allows a lower solvent consumption, diminishes the cost of the method and makes the transportation of the system possible for field monitoring without causing a loss in enrichment factors or a cross-contamination.

Concerning the microextraction of non-derivatized AMPAs, the use of single drop microextraction (SDME) and HF-LPME were reported [30,31]. SDME was carried out to extract AMPAs by active transport assisted by ion-pair between three phases (aqueous drop introduced into the organic layer put down on the aqueous sample) followed by CE analysis coupled to conductivity contactless detection [30]. LOQs between 0.3 ng mL^{-1} (cyclohexyl methylphosphonic acid, CMPA) and 2.8 ng mL^{-1} (ethyl methylphosphonic acid, EMPA) were obtained but the enrichment factors were not mentioned. Moreover, this method may suffer from a lack of repeatability due to the instability of the suspended drop [30].

To avoid the instability of the droplet, the liquid-phase microextraction can be performed with assistance of a polypropylene (PP) membrane containing the water immiscible solvent to separate aqueous sample, named as donor phase, and the clean acceptor medium. This technique is called supported liquid membrane extraction (SLM). This porous membrane enables to bring selectivity compared to SDME, especially for biological samples which contained macromolecules that are excluded by the membrane from the acceptor medium. The extraction can be carried out in a two-phase mode (water sample/non-miscible solvent) or in a three-phase mode (water sample/non-miscible solvent/aqueous acceptor phase), the three-phase mode being more suitable for LC–MS analysis because the acceptor solution is an aqueous medium.

This mode also allows the use of a power supply to perform an electromigration of compounds from the donor sample to the acceptor phase (electro membrane isolation, EMI) [32]. The EMI system has been used to extract successfully AMPAs before CE-capacitively coupled contactless conductivity detection analysis (CE-C^4D) [33]. The device consisted of two membrane sheets which were heat-sealed at the three edges in order to form a 20 μL-bag. Two platinum wires were introduced in the donor and the acceptor phases. Then, extraction was performed by applying 300 V from pure water spiked with AMPA to pure water inside of the bag. LOQs obtained for four AMPAs are in the range of 0.073–0.367 ng mL^{-1}. Recoveries were very high for methylphosphonic acid (MPA), the most polar compound ($52 \pm 2.8\%$), and cyclohexyl methylphosphonic acid (CMPA), the less polar ($97 \pm 6.5\%$). This method seems to be efficient to extract polar compounds from pure water in 30 min. However, in river water sample, the presence of humic acid seems to significantly reduce performances of extraction. To avoid it, a dilution of the sample with ultrapure water to 10% was performed to obtain recoveries in the range of 39.8 (MPA)–56.7% (ethyl methylphosphonic acid, EMPA) that corresponds to EFs from 80 to 117.

The HF-LPME is a similar technique based on the use of a porous PP hollow fiber filled with an organic solvent that separates the donor phase (aqueous sample) and the acceptor phase (extracting medium) [34]. The very low cost of the fiber allows its disposal to prevent carry-over problems. Basically, the fibers are about 3 cm long and admit volumes below 10 μL. Therefore, the resulting extract can be directly injected in the chromatographic system. As SLM, HF-LPME can be used in a two-phase mode where the acceptor phase is an organic medium particularly suitable for a GC-direct injection. A three-phase mode can also be used, with the organic solvent only impregnated in the pores of the fiber, which is then used to separate both aqueous media. This mode is well adapted to LC-direct injection and provides a higher selectivity than the two-phase mode.

Until now, only one study was reported on the application of the three-phase HF-LPME to eight AMPAs [31]. This study focused on the methyl methylphosphonic acid, as the most polar studied compound ($\log K_{ow} = -0.96$ [35]), on isopropyl methylphosphonic acid (iPrMPA), isobutyl methylphosphonic acid (iBMPA), pinacolyl methylphosphonic acid (PMPA) and on four other phosphonic acids that are less polar than PMPA. The three-phase HF-LPME was performed by impregnating the pores of the fiber with 1-octanol and filling the lumen with a basic aqueous phase (pH 14). The fiber was then plunged into the acidified aqueous sample (donor phase). After optimization of several extraction parameters in spiked pure water sample (pH of donor phase, pH of acceptor phase, extraction time, solvent nature, stirring rate and salt concentration), EFs were evaluated for each compound separately. The highest enrichment factor was close to 135 for the hexyl methylphosphonic acid (HMPA) after 60 min of extraction. The EFs obtained for the iPrMPA, the iBMPA and the PMPA were respectively 16 ± 0.2, 68 ± 6 and 85 ± 8. The LOQs obtained for three-phase HF-LPME followed by reverse-phase liquid chromatography–tandem mass spectrometry (LC–MS^n) of the eight alkyl alkylphosphonic acids were in the range of 0.2–200 ng mL^{-1}. To our knowledge, the quantitative aspects (recovery and EF) of this extraction method were only studied in pure water. The determination of PMPA was achieved in a water sample from an official OPCW proficiency test but without quantification. So, the method efficiency was not evaluated in real samples. Moreover, MPA, the most polar alkylphosphonic acid, was not included in the study.

Therefore, the present study focuses on the extraction of MPA, PMPA and four other AMPAs, with intermediate polarities, from aqueous real samples by HF-LPME in a three-phase mode followed by LC–MS analysis. The analytical method used in this study was developed, first by optimizing mass spectrometer parameters, then by testing five different C18 columns with an acidified water/acetonitrile (H_2O/ACN) mobile phase and, finally, by evaluation of the injected volume. The effect of various extraction parameters (volume, pH or salt concentration in the sample, pH of the acceptor phase, temperature of extraction, stirring speed

of the sample, immersion time of the fiber in solvent and time of extraction) was studied. The resulting optimized conditions were applied to environmental samples (surface waters) and for the first time, to a biological sample (human urine) to evaluate the performance of the method in terms of efficiency and repeatability in real media.

2. Experimental

2.1. Chemicals and materials

Pinacolyl methylphosphonic acid (PMPA), ethyl methylphosphonic acid (EMPA), methylphosphonic acid (MPA), sodium chloride (NaCl), formic acid (HCOOH), magnesium sulfate anhydrous ($MgSO_4$) and 1-octanol (99%) were from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Isopropyl methylphosphonic acid (iPrMPA), isobutyl methylphosphonic acid (iBMPA) and cyclopentyl methylphosphonic acid (cPenMPA) were synthesized in DGA CBRN Defense (Vert-le-Petit, France). High-purity water was obtained using an Alpha-Q purification system (Millipore, Saint-Quentin-en-Yvelines, France, 18.2 MΩ). Acetonitrile (ACN) was from Carlo-Erba (Val-de-Reuil, France). Hydrochloric acid (HCl), sodium hydroxide (NaOH) and calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) were from Merck (Darmstadt, Germany). Polyethylene glycol 400 (PEG400) was from EGA-Chemie (Steinheim, Germany). The tetraoctylammonium bromide (tOABr) was purchased from Fluka (Buchs, Switzerland).The evaluated analytical columns were Zorbax eclipse (150 mm × 2.1 mm i.d., 3.5 μm, Agilent Technologies, Massy, France), Symmetry shield (150 mm × 2.1 mm i.d., 3.5 μm, Waters, Saint-Quentin-en-Yvelines, France), Sunfire (150 mm × 2.1 mm i.d., 3.5 μm, Waters), Atlantis T3 (150 mm × 2.1 mm i.d., 5 μm, Waters) and Atlantis dC18 (150 mm × 2.1 mm i.d., 5 μm, Waters).

Accurel Q 3/2 polypropylene hollow fiber was purchased from Membrana (Wuppertal, Germany). The inner diameter is 600 μm, the thickness of the wall is 200 μm and the pore size is 0.2 μm. Hollow fiber was cut into 3 cm segments which were sealed by tweezers at one end.

2.2. Standard solutions and samples

Stock standard solutions of each AMPA and MPA were prepared in acetonitrile at a concentration of 50 μg mL^{-1} and stored at 4 °C. Spiked samples were prepared by dilution of each stock solution in an HCl solution at pH 1 to obtain a concentration of 0.2 μg mL^{-1}, except for MPA (2 μg mL^{-1}). Because of its high polarity that can give rise to low EF, the concentration of MPA in the sample is ten times higher than the AMPAs to be able to detect and quantify it in LC–MS when analyzing the acceptor phase. An appropriate amount of NaCl, from 0 to 30% (w/v) depending on the experiments, was then added.

River water (Marne river, France), simulated samples (250 μg mL^{-1} of $CaCl_2$, 100 μg mL^{-1} of $MgSO_4$ and 250 μg mL^{-1} of PEG400 in pure water) and human urine were spiked at 0.2 μg mL^{-1} for each AMPA and at 2 μg mL^{-1} for MPA. Each sample was adjusted at pH 1 with concentrated HCl and 30% (w/v) of NaCl were added. A volume of 3 mL of each sample was used for the extraction.

2.3. Apparatus and analytical conditions

The AMPAs and the MPA were analyzed by an LC/MS Agilent system composed of an autosampler, a binary gradient pump (1200 series), a triple quadrupole mass spectrometer 6410 controlled by Mass Hunter software. The analytical column, chosen among five C18 columns, was an Atlantis dC18 column (150 mm × 2.1 mm i.d., 3 μm) with a 200 μL min^{-1} flow rate. This column was selected because of the results obtained after an injection of 10 μL of EMPA. In this case, the gradient of the mobile phase begin 98/2 of water/ACN acidified by 0.2% of formic acid to reach 50/50 in 10 min. The analytical separation of the six compounds was performed using a gradient from 100% H_2O acidified with 0.1% formic acid (A) to 90% ACN acidified with 0.1% formic acid (B) during 6 min. Then, this mobile phase composition was kept 3 min before returning to equilibrium. The injection volume was 2 μL.

The ionization was carried out with an electrospray interface (ESI) in a negative ion mode. Acquisition was performed in single ion monitoring (SIM) mode and the deprotonated molecular ion $[M-H]^-$ of each compound was chosen as precursor ion. Capillary voltages were −71 V for the EMPA and the iPrMPA, −106 V for the iBMPA, −86 V for the cPenMPA, −111 for the PMPA and −51 V for the MPA. Nitrogen was used as drying gas (10 L min^{-1}) and as nebulizing gas (20 psi). The temperature of the drying gas was set at 350 °C.

2.4. Three-phase HF-LPME procedure

The acidified sample solution (donor phase) containing the analytes was placed in a 6 mL-hemolysis tube with a PTFE coated magnetic stir bar (10 mm × 3 mm). The extraction was thermostated by a silicon oil bath. In a second time, a volume of 8 μL of alkaline acceptor phase (NaOH, pH 14) was withdrawn into a 50 μL-syringe. The needle end was inserted into the lumen of the sealed hollow fiber segment and the acceptor phase was introduced into it by pressing the plunger. Then, the fiber was immersed in 1-octanol for a few seconds to impregnate the pores. The hollow fiber was then dipped into the donor phase during the extraction time. The extraction device was removed from the sample solution and the syringe plunger was pulled to fill the syringe with the acceptor phase. The used fiber was discarded and a fresh one was used for further extractions. The recovered 6 μL of acceptor phase were then diluted by a three-fold factor to be compatible with automated injections using an LC autosampler.

Enrichment factors were estimated according to the following equation:

$$\mathrm{EF} = \frac{C_{a,f}}{C_{d,i}} = \frac{n_{a,\,\mathrm{final}} \times V_d}{n_{d,\,\mathrm{initial}} \times V_a} = \frac{R}{100} \times \frac{V_d}{V_a}$$

where $C_{d,i}$ is the initial concentration of analyte in the donor phase and $C_{a,f}$ is the final concentration in acceptor phase. $n_{d,\mathrm{initial}}$ and $n_{a,\mathrm{final}}$ are the amounts of analyte present, respectively, in the donor phase and in the acceptor phase. V_d and V_a represent the donor volume and the acceptor volume, respectively. R is the recovery of the extraction given as a percentage.

The limits of quantification (LOQs) after sample treatment were calculated for a signal-to-noise ratio (S/N) of 10 using results obtained by applying HF-LPME followed by LC–MS to the three real samples (RW: river water; SS: simulated sample and U: urine) spiked at low concentrations ($[PMPA]_{RW\ and\ SS}$ = 0.04 ng mL^{-1} and $[PMPA]_U$ = 0.05 ng mL^{-1}; $[iBMPA]_{RW\ and\ SS}$ = 0.15 ng mL^{-1} and $[iBMPA]_U$ = 0.35 ng mL^{-1}; $[cPenMPA]_{RW\ and\ SS}$ = 0.25 ng mL^{-1} and $[cPenMPA]_U$ = 0.75 ng mL^{-1}; $[iPrMPA]_{RW,\ SS\ and\ U}$ = 1.3 ng mL^{-1}; $[EMPA]_{RW,\ SS\ and\ U}$ = 10 ng mL^{-1}; $[MPA]_U$ = 400 ng mL^{-1}).

3. Results and discussion

The aim of this study was to develop a three-phase HF-LPME procedure for the extraction and the enrichment of several polar AMPAs and MPA from aqueous samples, i.e. environmental water and urine. The structure and chemical properties of each target compound are indicated in Fig. 1. However, before evaluating this

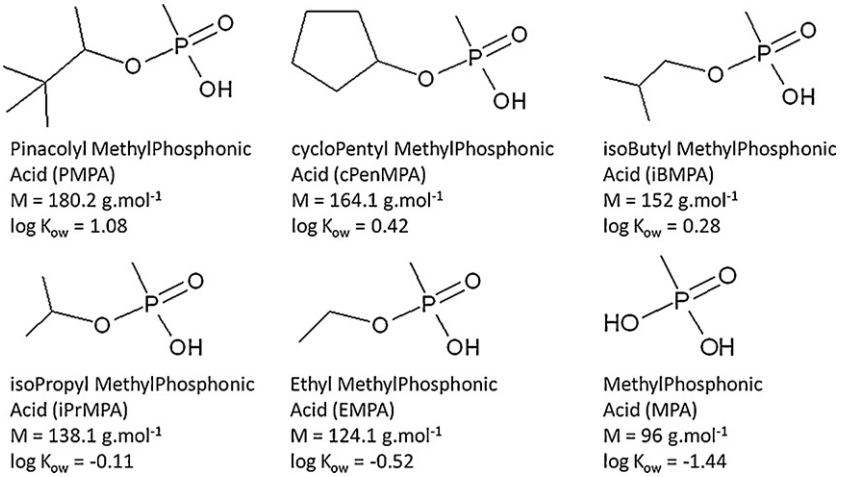

Fig. 1. Structure and physicochemical properties of the studied alkyl methylphosphonic acids and methylphosphonic acid. (log K_{ow} estimated by the Virtual Computational Chemistry Laboratory [35]).

technique, optimal analytical conditions for their separation and their identification by LC–MS analysis had to be determined.

3.1. Selection and optimization of various HF-LPME parameters

HF-LPME is a miniaturized technique of liquid–liquid extraction carried out with the assistance of a hollow fiber as acceptor phase compartment. Both aqueous media (donor and acceptor phases) are separated by an organic solvent maintained in the pores of the fiber. The thickness of the fiber wall should not be lower than 200 μm to ensure the mechanical stability of the solvent inside [34]. The volume of acceptor phase was fixed by the length of the fiber (3 cm). Various parameters, such as the volume of the donor phase, its salt content, the pH of the acceptor phase, the extraction temperature, the stirring speed of the sample, the immersion time in 1-octanol and the extraction time, had to be evaluated to obtain high EFs for AMPAs and MPA. As previously mentioned, a study already showed the potential of HF-LPME across three phases to enrich alkyl alkylphosphonic acids in a clean medium [31]. In the present study, a sealed-end fiber was used and additional parameters were evaluated with additional compounds to obtain the maximal EF. Most parameters were first evaluated with PMPA in pure water, in order to limit the consumption of non-commercially available compounds (iPrMPA, cPenMPA and iBMPA) and because of the very high LC–MS sensitivity of the PMPA compared to EMPA and MPA, which are also commercially available.

3.1.1. Choice of the organic solvent and of the pH of the donor phase

The nature of the organic solvent constitutes an important parameter. Firstly the solvent should be strongly maintained in the pores of the fiber, thus being ensured by a polarity close to the polypropylene of the fiber. However, its nature has to be adapted to the nature of the target analytes in order to favor their transfer [36]. Chlorinated solvents are known to have a better affinity for organophosphorous compounds than commonly-used organic solvents, such as toluene or cyclohexane [37,38]. Furthermore, chloroform was determined to be the best solvent in two-phase mode compared to carbon tetrachloride, trichloroethylene, tetrachloroethylene and their combinations [39]. However, this chlorinated solvent cannot be applied to a three-phase mode, due to its high volatility. Indeed, low volumes of solvent are used in the three-phase mode, therefore low volatility solvents should be used to prevent their loss during extraction. In a previous study, toluene and 1-octanol were evaluated and 1-octanol led to better results for the extraction of the PMPA [31], probably due to its relatively high polarity and hydrogen bonding ability. Furthermore, another study showed that alcohols were the most suitable solvents for the extraction of acidic compounds [40]. Based on these previous studies, the three-phase HF-LPME was performed with 1-octanol in the pores of the fiber.

The acidity constant (pK_a) of MPA and AMPAs being around 2.5, they are under their ionized form in real water. This ionization does not favor their diffusion through the pores of the fiber filled by octanol. Therefore, an acidic donor phase set at low pH was used to get compounds under their molecular form, thus decreasing their polarity and then increasing their affinity for octanol. First experiments were then carried out at pH 1 as previously suggested [31].

3.1.2. Volume of donor phase

In HF-LPME, the volumes of donor (V_d) and acceptor phases (V_a) are key-parameters for EF calculation and their choice results from a compromise. Indeed, as in classical LLE, the acceptor volume/donor volume ratio (V_a/V_d) has to be the highest to obtain the maximal extraction recovery. However, to ensure a high EF, V_a/V_d has to be as small as possible. So, a compromise has to be found between a high extraction recovery and a low V_a/V_d value to reach the maximal EF.

Several volumes of sample (pure water spiked at 0.2 μg mL^{-1} with PMPA) were investigated (0.5, 1, 2, 3, 4 and 6 mL) keeping the acceptor volume constant (6 μL). All experiments were performed in triplicate. Extraction was carried out during 60 min at 20 °C with a stirring rate of 900 rpm (rotation per minute). The donor phase contained 10% (w/v) of NaCl and the acceptor phase 1 mol L^{-1} of NaOH (pH 14). The fiber was dipped 30 s in organic solvent. These last parameters were chosen according to a previous study [31]. The resulting EFs obtained for PMPA are presented in Fig. 3A. The highest EF (105 ± 14, $n = 3$) was obtained for a volume of 3 mL. This EF corresponds to an extraction recovery of 21%. For larger volumes, the EFs slowly decrease, for instance, an EF of 68 was obtained for 6 mL. For volumes lower than 3 mL, the donor-acceptor volume ratio is the limiting factor. Even if recovery is high for 0.5 mL-sample (34%), the V_d/V_a value is too weak to enhance the EF obtained with 3 mL. Nevertheless, it is important to notice that the EF obtained for 0.5 mL (28 ± 4) is much higher than EFs obtained in SPE [21,29], thus highlighting the great potential of HF-LPME to enrich analytes. Moreover, the extraction step can be performed with only 0.5 mL of sample. So, this device is very attractive for very small volumes of samples, such as biological samples. However, to reach

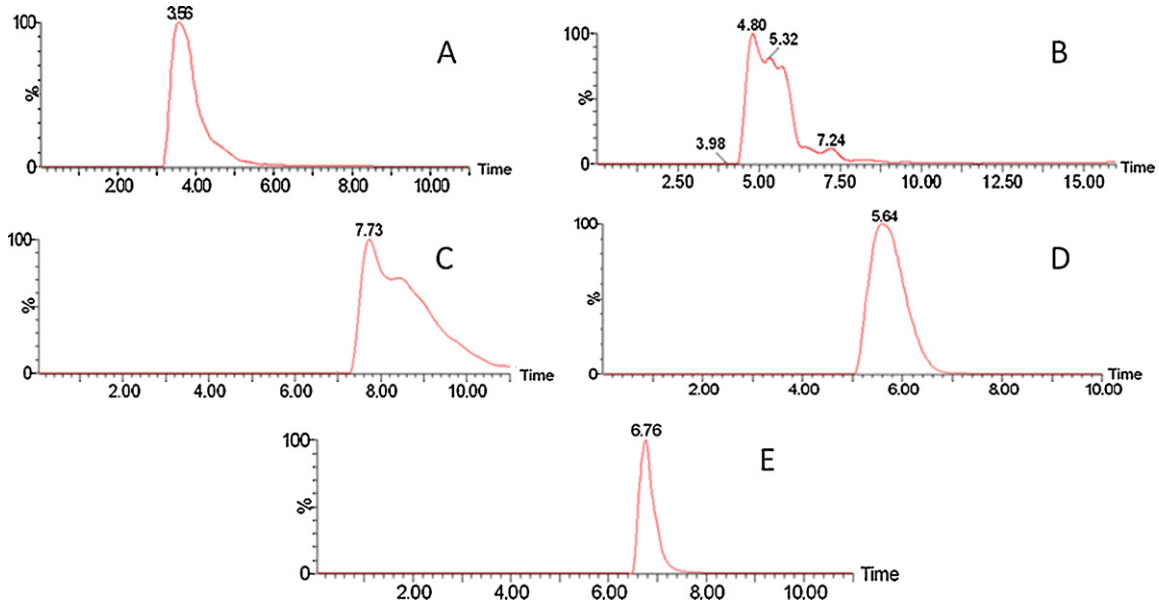

Fig. 2. Shape of the chromatographic peak of EMPA obtained with five different columns (A: Zorbax eclipse, B: Symmetry shield, C: Sunfire, D: Atlantis T3 and E: Atlantis dC18).

the maximum enrichment factors, a volume of sample of 3 mL was fixed.

3.1.3. Salting-out effect

The salting-out effect is widely used to enhance the extraction recovery of polar target compounds from water using conventional LLE. It consists in decreasing the solubility of polar compounds in donor phase by increasing the ionic strength. Indeed, water molecules form hydration spheres around the ionic salt, thus reducing the availability of these molecules for the analytes solvation [41] and favoring the transfer of the analytes from donor phase to the organic solvent. However, a decrease of EFs was also observed with salt addition, in some cases, using the three-phase procedure applied to various compounds, such as hydrophilic basic drugs or hydrophobic acidic and basic drugs [42–45]. This effect could be due to interactions between the salts and the compounds (ion pairs) and also to an increase of the viscosity of the sample with an increase of salt concentration [45]. For these reasons, it was important to study the salt addition. For this, four concentration levels of NaCl were studied: 0%, 10%, 20% and 30% (w/v). The concentration in NaCl was limited to 30% because the solubility of NaCl in water at 25 °C is around 33%. All experiments were performed in triplicate. The same extraction parameters as previously fixed were applied with a volume of donor phase of 3 mL. The resulting EFs are presented in Fig. 3B. The presence of NaCl in the donor medium clearly favors the analyte transfer toward the acceptor phase. For proof, the enrichment factor was around 100 ± 10 without sodium chloride while an EF of 180 ± 12 ($n = 3$) was obtained with 30% NaCl. Therefore, 30% of NaCl in the donor solution was chosen as the most suitable salt content.

3.1.4. pH of the acceptor phase

Considering the acid–base properties of the target molecules ($pK_a = 2.5$), the control of the pH values in the acceptor and donor phases appears to be a key parameter. As previously mentioned, target compounds have to be in molecular form in the donor medium, therefore a pH of 1 is applied to this phase. However, in the acceptor medium, a basic pH must be applied to favor their transfer by a pH gradient and also to avoid the back-extraction through the organic layer.

Therefore, extractions were carried out with three different pH values of the acceptor phase (10, 12 and 14). The alkaline solutions were prepared by diluting NaOH solutions in water. All experiments were performed in triplicate. The results presented in Fig. 3C show that a very high pH of the acceptor phase is necessary to favor the transfer of the compounds into the acceptor phase. Indeed, an EF of 105 ± 14 was obtained at pH 14 while EFs of only 3 were obtained for a donor solution at pH 10 and 12. These results are difficult to explain, because only 0.000003% of the non-ionized form of PMPA ($pK_a = 2.5$) is present in the pH 10 solution. The effect of the ionic strength cannot explain these results because when performing extractions (in triplicate) with an acceptor phase at pH 12 of the same ionic strength as at pH 14 (by adding 1 mol L^{-1} of NaCl), the obtained EF was the same as with an acceptor phase at pH 12 without salt addition ($I = 0.01$ mol L^{-1}). This phenomenon is therefore only due to the pH effect. To ensure a good transfer from the donor phase to the acceptor phase, a large pH difference of these two phases seems to be essential for these highly polar compounds. The pH value of the acceptor phase was then set at 14, which is in good agreement with previous results [31].

3.1.5. Extraction temperature

The temperature of extraction has to be studied because this parameter can affect the partition coefficient of the analytes between the different phases. Moreover, the control of the temperature will ensure a better inter-day repeatability of the extraction recoveries. The previous extractions were performed at 20 °C in an air-conditioned room. To study the effect of the temperature, the sample was placed in an oil bath to heat the donor phase from 25 °C to 80 °C. All experiments were performed in triplicate. The results, reported in Fig. 3D, show that a temperature enhancement favors the transfer of PMPA in the acceptor phase until 60 °C with an EF around 180 (recovery of 36%). A strong decrease of the EF was observed at 80 °C. This can be due to the miscibility of octanol in water at high temperature or to the partial evaporation of this organic solvent. In both cases, the acceptor phase probably leaks into the donor medium. In conclusion, a temperature of 42 °C was chosen as extraction temperature.

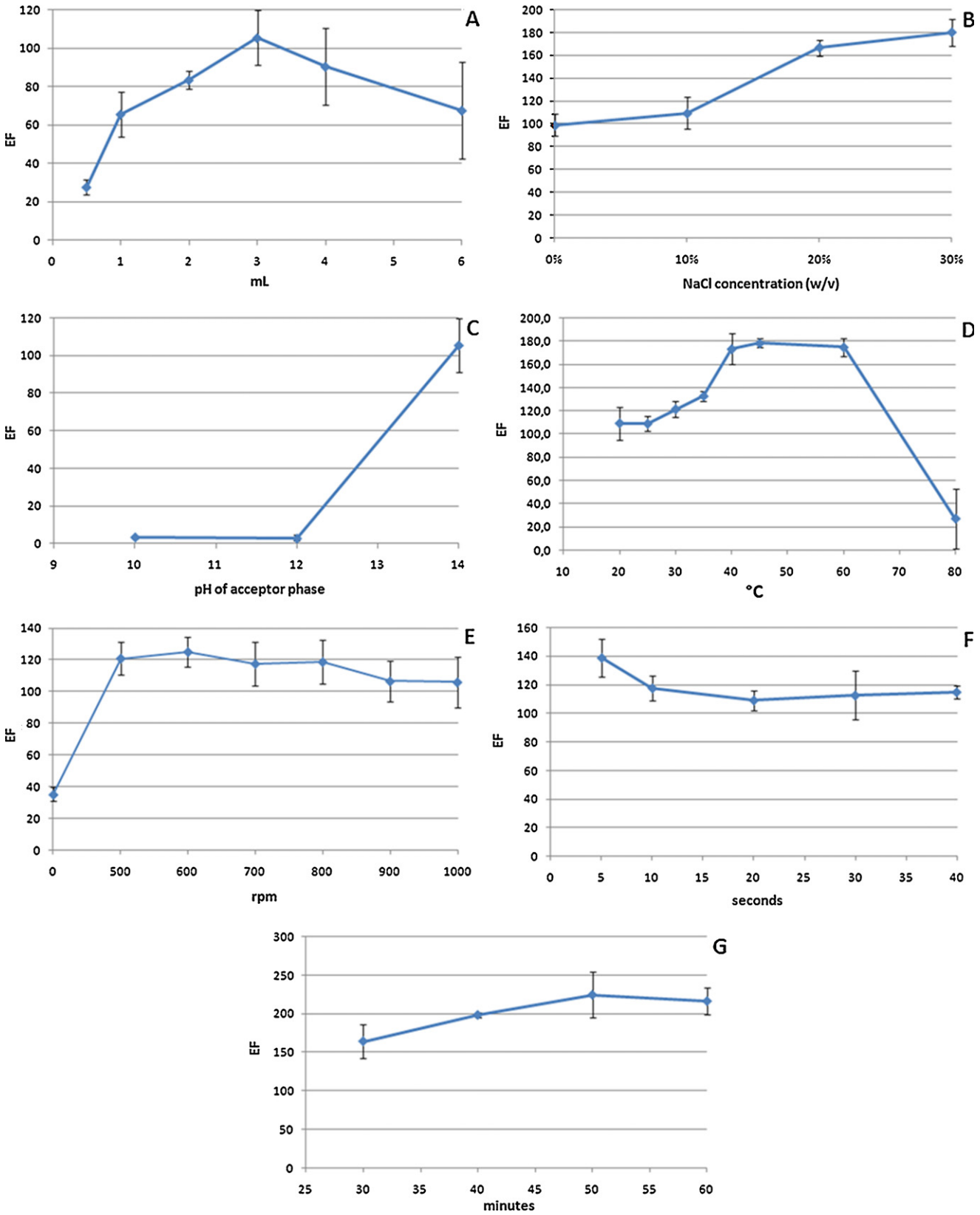

Fig. 3. Effect of the volume of the donor phase V_d (A), the amount of NaCl in the donor phase (B), the pH of the acceptor phase pH_a (C), the temperature (D), the stirring speed (E), the fiber immersion time in octanol t_{imm} (F) and the extraction time t_{extr} (G) on the enrichment factor (EF) of PMPA from pure water by three-phase HF-LPME (A–D, F and G: $n = 3$, E: $n = 6$). Constant parameters from A to F (except when mentioned): donor pH 1; 1-octanol; acceptor volume = 6 μL; extraction time: 60 min; donor volume = 3 mL (except A); 10% NaCl (w/v) (except B); acceptor pH 14 (except C); 20 °C (except D); 900 rpm (except E) and immersion time in octanol: 30 s (except F). Constant parameters for G: donor pH 1; 1-octanol; acceptor volume = 6 μL; donor volume = 3 mL; 30% NaCl (w/v); immersion time in octanol: 5 s; acceptor pH 14; 600 rpm and 42 °C.

3.1.6. Stirring speed

A mechanic stirring is needed to ensure a short extraction time by adding a convection transport to the pure diffusion of molecules. However, this parameter has to be carefully considered because a high stirring speed may cause the fall of the fiber in the sample and may generate air bubbles along the fiber, thus favoring the evaporation of the organic solvent and also limiting the contact area between the sample solution and the fiber. The stirring speed was set in the 0–1000 rpm range and the resulting EFs ($n = 6$) are presented in Fig. 3E. Without stirring, the EF is close to 35 while the EF is almost 4 times higher (121 ± 10, $n = 6$) for a stirring of 500 rpm. No significant changes in terms of enrichment factor or extraction recovery were observed when the stirring speed was increased from 500 to 800 rpm. These results can be explained by the shape of the vial containing the donor phase (6 mL-hemolysis tube), which allows a good transport of the analytes, thanks to the short distance between the inner wall of the tube and the surface of the fiber. Over 800 rpm, a slight decrease of EFs was observed, probably due to the presence of air bubbles. Considering these results, the stirring speed was set at 600 rpm (EF = 125 ± 9, $n = 6$).

3.1.7. Octanol immersion time

After filling the lumen of the hollow fiber with the acceptor phase, the pores must also be filled by immersion in the organic solvent, i.e. 1-octanol, for a few seconds. A too long immersion time can lead to the substitution of the aqueous acceptor phase by the organic solvent in the lumen of the fiber. Therefore, the effect of the immersion time of the fiber in 1-octanol on the EF was studied by immersing the fiber during 5, 10, 20, 30 and 40 s. All experiments were performed in triplicate. The resulting EFs are shown in Fig. 3F. An immersion time of only 5 s provided an enrichment factor of 139 ± 13 ($n = 3$). Longer immersion times caused a decrease of the EFs. After 10 s of immersion, the octanol phase seems to take the place of the aqueous acceptor phase inside the lumen, thus reducing the volume of the acceptor phase, and causing a decrease of the extraction recoveries and, consequently, of the EFs. In conclusion, an immersion time of 5 s was chosen to fill the pores of the fiber with 1-octanol.

3.1.8. Extraction time

For this method based on a partition between different liquid phases, the optimum extraction time is directly related to the equilibrium time. As for other miniaturized techniques based on partition like SPME, it can take from several minutes to hours to reach an equilibrium that ensures an optimal extraction recovery and EF. The equilibrium time strongly depends on the stirring rate, previously fixed at 600 rpm. Other parameters were fixed as follows: donor volume of 3 mL, 30% NaCl, acceptor phase pH of 14, 42 °C and 5 s-immersion time in octanol. In these conditions, four different extraction times were then investigated: 30, 40, 50 and 60 min. All experiments were performed in triplicate. The results presented in Fig. 3G show that the equilibrium is probably reached in 50 min, because, over this extraction time, the enrichment factor remains constant (average value of 220 ± 24). Obviously, to reduce the time of sample treatment, the extraction could be performed in only 30 min with a decrease of only 25% of the EF (164 ± 21, $n = 3$). Extraction capacity of the new HF-LPME method.

After the evaluation of the extraction parameters to obtain high enrichment factors, the method should be evaluated with the whole target compounds and for different types of samples. First of all, the separation and detection method should be optimized to have a high sensitivity and a high efficiency too. Then, the evaluation of the HF-LPME method was carried out in pure water and in real samples (environmental water and urine).

3.1.9. Optimization of the LC–MS analysis

Mass spectrometry detection was performed with an ESI source in SIM mode. Both positive and negative ionization modes were evaluated and all compounds were observed with negative ions $[M-H]^-$. The capillary voltages applied to each compound were estimated at −71 V for the EMPA and the iPrMPA, −106 V for the iBMPA, −86 V for the cPenMPA, −111 V for the PMPA and −51 V for the MPA.

In order to limit the consumption of non-commercially available AMPAs, the optimization of the LC–MS conditions was first carried out with the two commercially available compounds, the EMPA and the PMPA. For the chromatographic separation of alkyl methylphosphonic acids, five reversed-phase analytical columns (C18, 150 mm × 2.1 mm i.d.) were studied: Zorbax eclipse, Symmetry shield, Sunfire, Atlantis T3 and Atlantis dC18. The flow rate of the mobile phase was set at 0.2 mL min^{-1}. The EMPA was chosen to compare peak efficiency because it is more polar than PMPA, so less retained on C18 column. The chromatograms obtained by injecting EMPA on different columns with a 10 min-gradient from 98/2 to 50/50 of water/ACN acidified by 0.2% of formic acid were compared. Fig. 2 shows that the best peak efficiency was obtained using the Atlantis dC18 column. The shape of peaks is really different from a column to another, including various degrees of asymmetry. The peak asymmetry is due to two different retention mechanisms, one of which being limiting in terms of capacity. All studied columns are octadecyl carbon chain (C18) bonded silica thus favoring hydrophobic interactions. However, the residual silanols may favor additional polar interactions with the polar EMPA thus giving rise to peak tailing problems. The five studied columns result from different synthesis processes in order to limit the silanol contribution. Among the different columns tested, good results were only obtained with the Atlantis dC18, the only one prepared using a surface treatment with polar groups. It appears that the polar interactions with this stationary phase were not limiting. This treatment seems to be more appropriate for the analysis of the alkyl methylphosphonic acids, so the Atlantis dC18 column was chosen for further analysis.

The separation conditions were then optimized to obtain a fast separation for MPA and the five AMPAs studied. The resulting gradient was from 0% to 90% of ACN in water (acidified by 0.1% of formic acid) during 6 min. A high pH value of the acceptor phase being considered, the effect of the injection of a basic solution containing alkyl methylphosphonic acids was studied. It was shown that the injection of a volume of pH 14-solution higher than 2 μL affects the shape of the peak of the most polar compounds (EMPA and MPA). The injection volume was therefore set at to 2 μL.

A linear response was observed when injecting nine solutions of PMPA, cPenMPA, iBMPA and iPrMPA in a very broad concentration range from 0.002 to 40 μg mL^{-1} due to the high EFs expected after extraction. The squared regression coefficients were of 0.9923 for PMPA, 0.9945 for cPenMPA, 0.9907 for iBMPA and 0.9946 for iPrMPA. The linearity of the EMPA response was observed with six solutions in a concentration range from 0.002 to 5 μg mL^{-1} with a squared regression coefficient of 1. For MPA, five solutions in a concentration range from 0.1 to 5 μg mL^{-1} were used to obtain a squared regression coefficient of 0.9992.

3.1.10. Extractions of AMPAs and MPA from pure aqueous media

After the study of the different HF-LPME parameters, optimal extraction conditions were determined for the PMPA and are summarized in Table 1. The EFs of the MPA and of each AMPA were first determined for spiked pure water samples. All experiments were performed in triplicate. The results are shown in Fig. 4. Enrichment factors are directly related to the polarity of the compounds. Indeed, PMPA, the less polar compound ($\log K_{ow} = 1.08$), was extracted from pure water with an EF of 225 ± 30 and MPA, the most polar

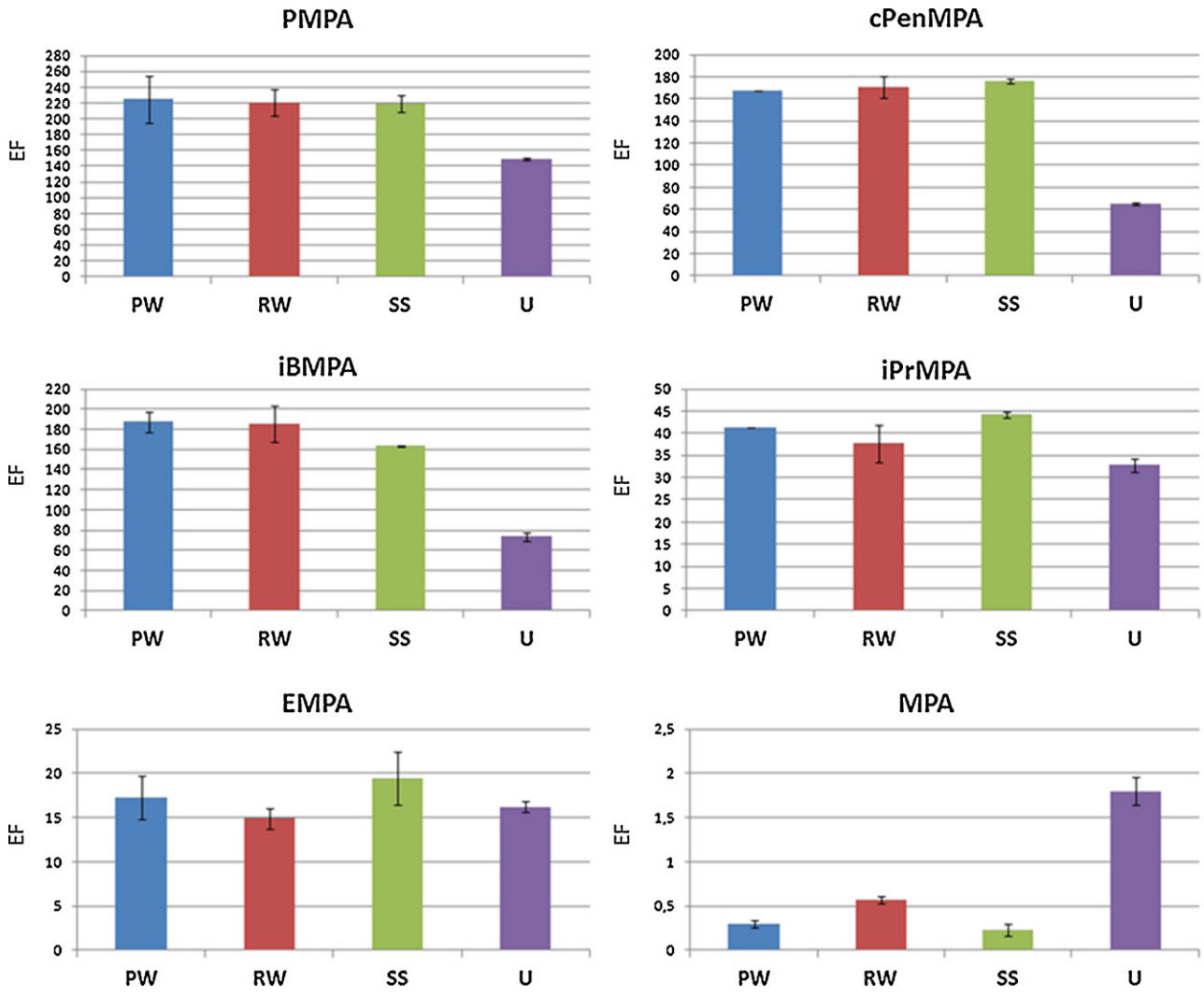

Fig. 4. Enrichment factors ($n = 3$) obtained for 5 alkyl methylphosphonic acids (PMPA, iBMPA, cPenMPA, iPrMPA and EMPA) and the MPA in four aqueous media: pure water (PW), river water (RW), simulated sample (SS) and urine (U). Conditions: see Table 1.

compound ($\log K_{ow} = -1.44$), was extracted with an EF lower than 1. The high polarity of MPA leads to a poor transfer from the donor aqueous phase to the acceptor phase caused by a limited diffusion through the pores filled by octanol. As expected, EMPA and iPrMPA provided lower EFs (17 and 41 respectively), considering their $\log K_{ow}$ values, than cPenMPA and iBMPA (167 and 188 respectively).

Relative standard deviations (RSDs) were in the range of 5–14% in pure water for experiments carried out in triplicate (PMPA: 13.3%, iBMPA: 5.4%, cPenMPA: 5.8%, iPrMPA: 10.4% and EMPA: 14.0%). These values are satisfactory when considering the low acceptor volume to handle.

Table 1
Extraction parameters.

Parameters	Fixed values
Donor/acceptor volume	3 mL/6 μL
NaCl content in donor phase	30% (w/v)
pH of donor/acceptor phase	1/14
Immersion time in octanol	5 s
Stirring speed	600 rpm
Temperature	42 °C
Extraction time	50 min

Compared to the three-phase HF-LPME procedure already described in the literature and applied to less polar compounds [31], the enrichment factors obtained for PMPA, iBMPA and iPrMPA (85, 68 and 16 respectively) are more than 2.5 times higher in this present study. This significant improvement results from the optimization of additional parameters (the volume of the sample, the salt concentration in the donor phase, the temperature of extraction, the immersion time of the fiber in the solvent and the extraction time).

However, to improve the enrichment of the more polar compounds, two additional parameters were evaluated in pure media spiked with the six analytes. First, the presence of an ion-pairing reagent in the octanol was evaluated in order to improve the transfer of the compounds through the organic phase, especially for the MPA. The results obtained by adding 2% of tetraoctylammonium bromide (tOABr) in the octanol have demonstrated that the EFs for all compounds decreased. Therefore, pure octanol was used for further experiments.

Secondly, the pH in the donor phase was reduced to 0.3 and 0.7 to favor the transfer of the target compounds in the octanol layer. At the concentration of 1 mol L^{-1} of HCl (pH 0), the dissolution of the NaCl (30%) was not complete. Therefore this value was not evaluated. For pH 0.3 and 0.7, the six compounds were investigated with the parameters presented in Table 1. All experiments were

Table 2
Limits of quantification (S/N = 10) of the HF-LPME followed by LC–MS analysis of each AMPA in river water, simulated sample and urine (see Section 2 for calculation). n.r.: not relevant.

Analytes	LOQs in river water ($ng\,mL^{-1}$)	LOQs in simulated sample ($ng\,mL^{-1}$)	LOQs in urine ($ng\,mL^{-1}$)
PMPA	0.013	0.013	0.056
cPenMPA	0.28	0.31	0.36
iBMPA	0.13	0.14	0.19
iPrMPA	0.34	0.42	1.18
EMPA	5.3	6.3	4.8
MPA	n.r.	n.r.	63.5

pH 1 in donor solution; NaCl content: 30% (w/v); immersion time in 1-octanol: 5 s; pH in acceptor solution: 14; agitation: 600 rpm; temperature: 42 °C; extraction time: 50 min.

performed in triplicate. The results are shown in Fig. 5. The EFs obtained at pH 0.3 were divided by a factor higher than 2 for PMPA, cPen and iBMPA compared to those obtained at pH 1. The three other compounds seemed to be not affected by the change of pH. By analyzing the results obtained at pH 0.3, the evaluation of pH 0 was not relevant even with a lower amount of NaCl. The adjustment to 0.7 of the donor pH leads to the same results than pH 1. So these two pH values have to be evaluated in real media to determine the optimum one.

Nevertheless, all the good results obtained in pure water have to be confirmed by applying the procedure to the extraction of the target analytes from real media.

3.1.11. *Application to environmental and urine samples*

AMPAs and MPA were determined in two environmental samples consisting in a river water sample and a simulated sample of water spiked with PEG400, $CaCl_2$ and $MgSO_4$. The method was also evaluated by applying it to the extraction of the target analytes from urine considering the increasing demand for this kind of sample. The pH of the different types of samples was set at 1. The EFs obtained by applying the extraction conditions described in Table 1 are presented in Fig. 4. All experiments were performed in triplicate.

For all compounds, the EFs were rather the same for pure water and for environmental samples (river water and simulated samples), thus highlighting that no matrix effect was observed.

Compared to SPE results (recovery > 95% for PMPA, cPenMPA, iBMPA and iPrMPA, 30% for EMPA [29]), the recovery in HF-LPME are lower, the highest recovery being 45% for the PMPA. In return, the interest of the HF-LPME is to enrich analytes in a clean medium without including evaporation or other enrichment steps that can provide loss of compounds. The EFs obtained in HF-LPME of AMPAs (15 < EFs < 225 for environmental samples and urine) are much better than in SPE (EF of 2 and 20 without or with the introduction an evaporation step to concentrate the SPE elution fraction respectively for the less polar compounds) [21,29]. Furthermore, the three-phase HF-LPME has the advantage to extract compounds from aqueous sample to a reduce volume of water that can be directly analyzed by LC–MS and to avoid the high consumption of organic solvent.

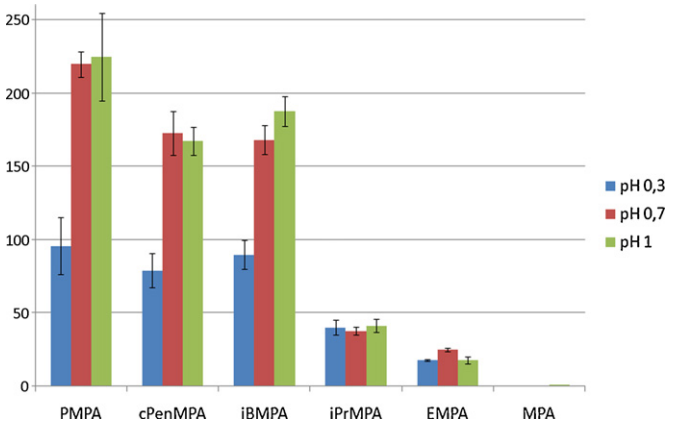

Fig. 5. Effect of the donor pH on the EF of the six target compounds in pure water sample. Constant parameters: 1-octanol; acceptor volume = 6 μL; donor volume = 3 mL; 30% NaCl (w/v); immersion time in octanol: 5 s; acceptor pH 14; 600 rpm; 42 °C and extraction time of 50 min.

The LOQs of HF-LPME from environmental samples followed by LC–MS are reported in Table 2. LOQs of $0.013\,ng\,mL^{-1}$ were obtained for PMPA, around $0.13\,ng\,mL^{-1}$ for iBMPA and around $0.30\,ng\,mL^{-1}$ for cPenMPA in the environmental samples. For EMPA and iPrMPA, the LOQs in simulated samples (6.3 and $0.42\,ng\,mL^{-1}$ respectively) were slightly lower than in river water (5.3 and $0.34\,ng\,mL^{-1}$ respectively). These LOQs obtained for the five AMPAs are very low in particular for the less polar compounds. Enrichment factors obtained with MPA being lower than 1, LOQs of the procedure were not relevant to be calculated knowing that they will be less than LOQ of the analytical method.

In river water, RSDs were 7.6% for the PMPA, 9.7% for the iBMPA, 1.2% for the cPenMPA, 1.5% for the iPrMPA and 8.0% for the EMPA. In simulated samples, RSDs were from 0.5% (iBMPA) to 15.5% (EMPA) with 5.1% for the PMPA, 0.6% for the cPenMPA and 3.2% for the iPrMPA. Therefore, the high sensitivity and the good repeatability of the method show the great potential of HF-LPME for the determination of AMPAs from environmental matrices.

For the first time, a determination of AMPAs and MPA by HF-LPME from urine samples was carried out. The extraction from urine samples lead to a decrease of the EFs of 30% for PMPA and 60% for cPenMPA and iBMPA, as seen in Fig. 4. For these compounds, the matrix effects affect the EFs that nevertheless keep high values (>60). In return, EF values obtained for iPrMPA (33 ± 1) and EMPA (16 ± 0.6) were very close to those obtained for environmental samples. Moreover, for the most polar analyte (MPA), the EF obtained for urine (1.8 ± 0.2) was higher than the EF value obtained in pure water. For the less polar molecules, the EFs were lower in urine than in environmental samples, due to the complexity of this biological medium. This decrease can be explained by various phenomena such as an increase of the viscosity or a saturation of the low volume of organic phase. However, this matrix effect, that causes a decrease of the EFs, is less visible for the most polar compounds. This phenomenon can be explained by the salting out effect. Indeed, urine is a very complex medium containing a lot of ionized molecules that can reduce the solubility of the target compounds in the donor phase. The more polar the compounds, the more they are positively affected by the salting out effect. For the MPA, the negative effect, caused by the complexity of the matrix, seems to be less significant than the positive salting out effect that enhances its EF obtained in environmental matrices.

This study in urine was also carried out at pH 0.7. For five compounds, the EFs were unchanged between the two pH values. But

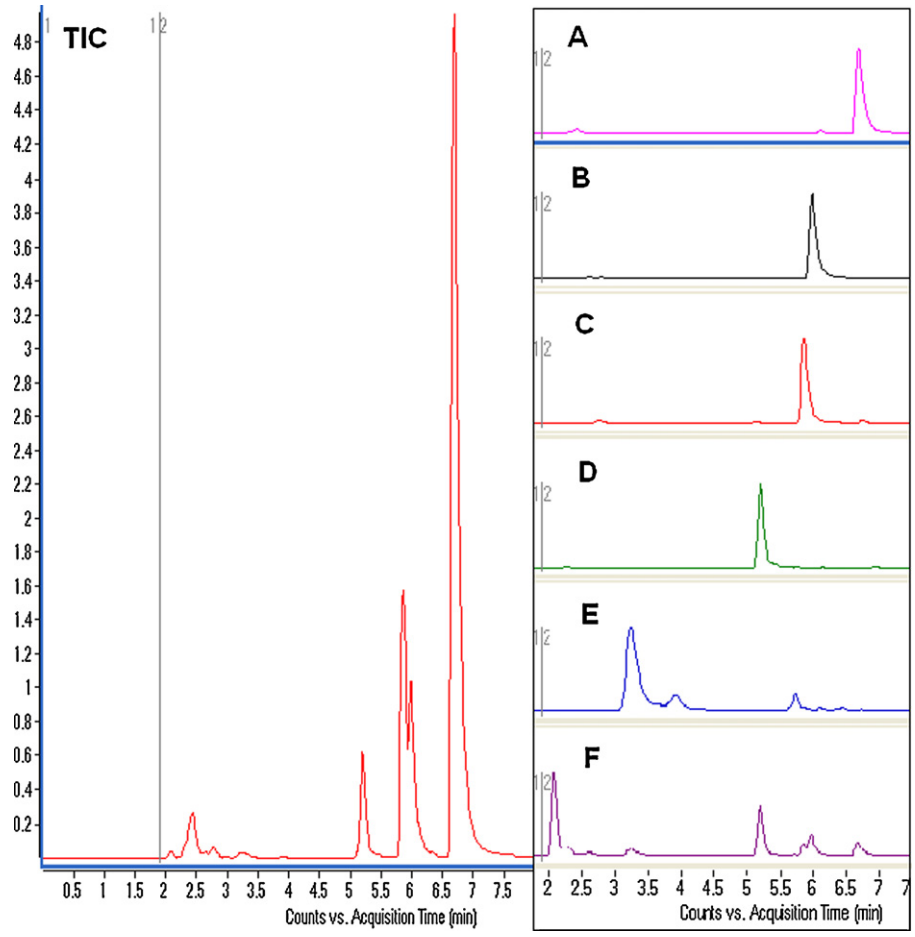

Fig. 6. Chromatogram of target molecules obtained after extraction from urine samples by HF-LPME and analysis by LC–MS in negative ionization mode, TIC and SIM mode (compounds A: PMPA *m/z* 179.2; B: cPenMPA *m/z* 163.1; C: iBMPA *m/z* 151; D: iPrMPA *m/z* 137.1; E: EMPA *m/z* 123.1; F: MPA *m/z* 95).

for the extraction of the PMPA from a urine sample adjust to pH 0.7, the EF decreased of almost a factor 2 compared to pH 1 (results not shown). Therefore, the samples have to be adjusted to pH 1 to obtain the best EFs in real complex samples.

The chromatograms, obtained after injection of the acceptor phase in LC–MS of the urine extract, are presented in Fig. 6, the TIC and the SIM with the individual compounds. After HF-LPME was applied to a urine sample, the resulting extract was very clean, thus allowing the quantification of the AMPAs and MPA. Moreover, the urine treatment by HF-LPME made it possible to introduce a clean medium in the mass spectrometer, without salt, thus preventing damages to the device.

As indicated in Table 2, the LOQs of AMPAs in urine are between 0.056 ng mL^{-1} for PMPA and 4.8 ng mL^{-1} for EMPA, thus demonstrating the potential of HF-LPME for the extraction of AMPAs. The LOQ of 63.5 ng mL^{-1} obtained for the extraction of MPA from urine also highlights the potential of HF-LPME compared to methods already developed. Furthermore, RSD values are very low for the urine samples: 1.1% for the PMPA, 6.4% for the iBMPA, 4.0% for the cPenMPA, 2.7% for the iPrMPA, 3.7% for the EMPA and 8.9% for the MPA. Therefore, the repeatability of HF-LPME is still very satisfactory considering the error sources of manual handling and by taking into account the matrix complexity.

4. Conclusions

HF-LPME in three-phase mode was successfully applied to the extraction of alkyl methylphosphonic acids from aqueous samples followed by a LC–MS analysis without necessitating a tedious derivatization step. The easy use and the low cost of this method make HF-LPME very attractive. Significant parameters affecting the extraction process were assessed to obtain very high enrichment factors, especially for the alkyl methylphosphonic acids that are less polar studied compounds. The results obtained in pure water demonstrate the great potential of this technique to extract AMPAs. The application of HF-LPME in three-phase mode to real samples such as surface water and urine confirmed the performances of this miniaturized technique with a very satisfactory repeatability (0.6% < RSD < 15.5%, all compounds and matrices taken together). The EFs obtained for the extraction of AMPAs from real samples (from 15 to 225) allowed the development of a very sensitive analytical method for the determination of AMPAs, with LOQs between 0.013 ng mL^{-1} and 6.3 ng mL^{-1} for all matrices taken together. HF-LPME also led to an effective clean-up of the sample even for the most polar analyte, MPA, with a LOQ of 63.5 ng mL^{-1} in urine despite the low EF obtained for this compound.

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