Salt assisted liquid-liquid extraction combined with dispersive liquid-liquid microextraction for the determination of 24 regulated polycyclic aromatic hydrocarbons in human serum

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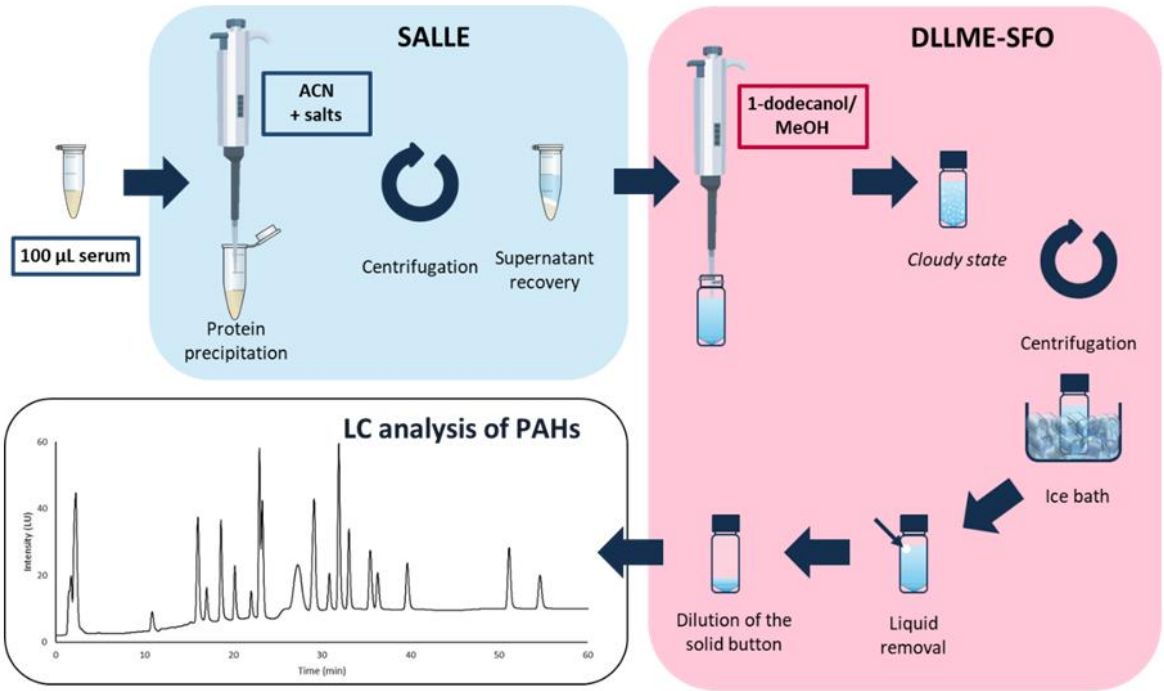

Keywords: Dispersive liquid-liquid microextraction; Liquid chromatography; Polycyclic aromatic hydrocarbons; Salting-out liquid-liquid extraction; Serum

Abstract:
Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants of great concern due to their carcinogenicity and mutagenicity. Their determination in human serum, particularly in at-risk populations, is necessary but difficult because they are distributed over a wide range of polarity and are present at trace level. A new method combining salting-out assisted liquid-liquid extraction (SALLE) and dispersive liquid-liquid microextraction with solidification of floating organic drop (DLLME-SFO) adapted to a reduced volume of sample (100 µl) was developed to determine 24 PAHs in human serum. Some key parameters of DLLME-SFO (volume of extraction solvent, ratio of extraction/dispersive solvent volumes, and salt addition) were first studied by applying it to spiked pure water. For its application to serum, a sample treatment step involving SALLE was optimized in terms of nature and content of salts and applied upstream of DLLME-SFO. It was applied to the extraction of 24 regulated PAHs from spiked serum followed by an analysis by liquid chromatography coupled with UV and fluorescence detection. The extraction recoveries ranged from 48.2 and 116.0% (relative standard deviations: 2.0-14.6%, n=5-9), leading to limits of quantification of PAHs in human serum from 0.04 to 1.03 µg/L using fluorescence detection and from 10 to 40 µg/L using UV detection. This final method combining SALLE and DLLME-SFO showed numerous advantages such as no evaporation step, high efficiency and low solvent-consumption and will be useful for monitoring PAHs in low volumes of serum.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants composed of two to several fused aromatic rings. They are generated by incomplete combustion of organic substances and are emitted in the environment by natural (forest fires, volcanic eruptions) and anthropogenic (industries, road traffic, heating…) processes. They are ubiquitous contaminants of the environment and are detected in air, soil, water, sediment and food leading to a constant exposure of humans via dermal contact, inhalation and ingestion [1]. PAHs are recognized as carcinogenic, mutagenic and reprotoxic substances [1]. Exposition to these compounds has deleterious effects on human health, including the risk of developing diseases such as cancer (breast, lung, gastrointestinal), cardiopulmonary diseases or diabetes [1].

Due to their high toxicity, 24 PAHs are regulated and monitored: 16 by the United States Environmental Protection Agency (US-EPA) in environment and 16 by European Food Safety Authority in food, leading to a total of 24 PAHs of high interest. Their monitoring in biological fluids is important for exposure level assessment among populations, and in this context, the collection of blood samples is one the most common practice [2]. However, access to valuable sample cohorts, such as the ELFE (French Longitudinal Study of Children) cohort is greatly facilitated by having a method that consumes very small sample volumes to conduct different studies on the same sample. Therefore, the choice of the method should prioritize ones requiring low sample volume while reaching a sufficient sensitivity.

Most studies dealing with the analysis of PAHs in blood samples focused on the 16 US-EPA PAHs.The pretreatment of these samples most often consists of a liquid-liquid extraction (LLE) followed by a clean-up using solid phase extraction (SPE) [2]. For LLE, n-hexane is the most used solvent, alone or mixed with dichloromethane or diethyleter; other solvents can also be employed such as dichloromethane or cyclohexane[3–11]. However, one of the main LLE drawbacks is the use of large volumes of these toxic solvents (> 10 mL)[8,9]. SPE was reported to limit solvent consumption and to improve the sample clean-up, alone or after a first LLE step [3,4,8–11], by removing matrix interferents that could compromise PAHs quantification. Another limitation of these sample treatment methods is that they often involved an evaporation step, to concentrate the final extract that may cause the loss of the most volatile PAHs. PAH levels detected in serum ranged from 0.01 to 2.5 µg/L. While these levels have often required sample volumes of between 0.5 and 1 mL [6,11–13], some studies have required volumes of between 2 and 12 mL [5,8,14,15], which limits access to epidemiological cohort samples. Recently, a new SPE procedure was developed by our group which required only 100 µL of serum [16]. The 24 PAHs of interest were targeted for the first time and the extraction yields ranged from 27 to 69% and the enrichment factors between 0.3 and 0.7. These low enrichment factors are explained by this low sample volume fixed to access to epidemiological cohort samples and losses caused by an evaporation step applied to limit the volume of extract. It would be interesting to further improve these results while reducing solvent consumption, avoiding any evaporation step and specific and/or expensive consumable.

Liquid phase microextraction (LPME) may be an interesting alternative for the analysis of small volumes of biological samples while limiting solvent consumption. LPME can be subdivided into three main families of extraction methods: single drop microextraction (SDME), hollow-fiber liquid phase microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME). SDME has few bioanalytical applications related to the instability of the suspended drop that can be lost into the sample [17]. Contrary to SDME, in HF-LPME, the extraction solvent is protected thanks to a supported liquid membrane [18]. Nevertheless, long extraction time and dedicated consumables (fiber) are still an issue [17,18]. DLLME, introduced in 2006 for the analysis of PAHs in water [19], consists of injecting into the aqueous sample a small volume of water-immiscible extraction solvent (5-100 µL), diluted in a dispersive solvent (0.2-1 mL). The analytes are extracted by droplets of solvent from the aqueous phase and recovered in the small volume of organic phase after centrifugation of the mixture. DLLME has been widely applied to the extraction of PAHs from large volumes (10 mL) of environmental samples [18] but also from small volumes (0.25 mL) of biological

samples (plasma) [20]. To avoid the use of toxic halogenated solvents, an alternative DLLME technique based on the solidification of a floating organic droplet (DLLME-SFO) was proposed. It has already been applied for the extraction of PAHs from environmental samples [21,22]. Enrichment factors between 88 and 118 and high extraction recoveries, from 88 to 110%, were reported for the 4 most polar PAHs among the 16 US-EPA allowing the determination of concentrations from 0.046 to 0.4 µg/L in real waters using a sample volume of 10 mL [22]. Only one team used low samples volumes (< 250 µL) to extract by DLLME PAHs from serum [20]. Indeed, Shin et al. achieved a theoretical enrichment factor of 5 for a small sample volume (250 µL) but used non-green solvents such as acetone and methyl tert-butyl ether [20]. The DLLME-SFO method seems promising for application with small sample volumes, requiring minimal solvent consumption and limiting the use of consumables.

Concerning the extraction of organic compounds from complex liquid samples, salting-out assisted liquid-liquid extraction (SALLE) has been also reported. It allows the extraction of organic compounds from various aqueous matrices by a water-miscible organic solvent rendered non-miscible by the "salting-out" effect. Applied to biological matrices, this "salting-out" effect induced by the presence of a high salt concentration provokes phase separation with the organic solvent, improves the transfer of compounds to the organic solvent by reducing their solubility in the aqueous phase, and also causes the precipitation of proteins, and therefore their elimination from the extract [23].

The main challenge of this work was to develop an extraction method for 24 PAHs belonging to a wide polarity range (log K_{ow} values of 3.3-7.7) from a small volume of serum making the method applicable to low-volume cohort samples, while limiting the extract dilution as much as possible to maintain sensitivity and to avoid any evaporation step to limit losses of the most volatile PAHs. To achieve this, it was decided to combine SALLE and DLLME-SFO. Key parameters were optimized. Finally, to highlight the potential of the developed method, sensitivity, recoveries, and repeatability of the method were determined in spiked human serum.

2. Materials and Methods

2.1 Chemicals and reagents

Methanol (MeOH) and acetonitrile (ACN) were HPLC grade from Carlo Erba (Val de Reuil, France). Purified water was dispensed by a Milli-Q purification system (Millipore, Saint-Quentin-en-Yvelines, France). Sodium chloride, magnesium sulfate, ammonium sulfate, calcium chloride dihydrate, sodium dihydrogen phosphate and glucose were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and zinc sulfate from Fisher Scientific (Illkirch, France). Sodium bicarbonate and 1-dodecanol were from Merck (Darmstadt, Germany). Potassium chloride was purchased from Prolabo (Paris, France).

Standards of PAHs were supplied by LGC Standards (Teddington, UK): 5-methylchrysene (5MCHR, 99.8%), benzo(c)fluoranthene (BcF, 97%), benzo(j)fluoranthene (BjF, 99.7%), cyclopenta(c,d)pyrene (CcdP, 99.7%), dibenzo(a,e)pyrene (DaeP, 99.8%), dibenzo(a,h)pyrene (DahP, 99.8%), dibenzo(a,i)pyrene (DaiP, 99.9%), dibenzo(a,l)pyrene (DalP, 99%) at 1 mg/L in ACN and a standard mixture of the 16 US-EPA PAHs at 10 mg/L in ACN. All 24 PAHs are presented in Table S1.

A stock standard solution mixture containing 100 µg/L of each PAH was prepared in ACN and stored at 4°C until further use. A simulated serum was prepared from Earle's balanced salt solution ($CaCl_2.2H_2O$ 0.265 g/L, $MgSO_4$ 0.098 g/L, KCl 0.4 g/L, $NaHCO_3$ 2.2 g/L, NaCl 6.8 g/L and NaH_2PO_4 0.122 g/L and glucose 1 g/L) and containing human albumin (Sigma-Aldrich) at 30 g/L. A pooled human serum was purchased from Pan Biotech (Aidenbach, Germany).

2.2 Liquid chromatography coupled to UV and fluorescence detection

The PAHs were analyzed using a liquid chromatography coupled to UV and fluorescence detection (LC-UV/FD) system (Agilent 1200 LC, Agilent Technologies, Les Ulis, France), controlled by the

Chemstation software. The separation was performed on a Pursuit PAH column (100 x 2.1 mm, 3 µm, Agilent Technologies) maintained at 35°C with a column oven (Croco-cil, Interchim, Montluçon, France). The mobile phase was composed of water (A) and MeOH/ACN 60:40 (v/v) (B). To separate the 10 PAHs selected for the method development, the gradient started at 60% of B, increased to 98% of B in 21 min, and held for 8 min before returning to equilibrium. To separate the 24 PAHs, the gradient started at 50% of B and increased to 90% of B in 27 min, held for 13 min, and increased to 98% of B in 1 min and held for 10 min before returning to equilibrium. The flow rate was set at 0.2 mL/min and the injection volume at 10 µL. A time program of the excitation and emission wavelengths was performed to detect 20 PAHs in fluorescence. The four low- or non-fluorescent PAHs (ACY, CPcdP, BjF and IcdP) were detected using UV. The wavelengths and the limits of quantification (LOQs) of the LC-UV/FD method are reported in Table S2.

2.3 Optimization of DLLME-SFO in pure media

Initial extraction conditions were fixed according to those described by Vera-Avila et al. [21], for the determination of 29 organic pollutants including 10 PAHs from 10 mL water samples. 1 mL of ultra-pure water containing NaCl 0.5% was introduced in an Eppendorf tube and spiked at 10 µg/L with 10 PAHs (naphthalene (NAPH), acenaphtylene (ACE), phenanthrene (PHE), pyrene (PYR), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DahA), benzo[ghi]perylene (BghiP) and DahP). Then, 40 µL of 1-dodecanol/MeOH 1/3 (v/v) were rapidly injected in this solution with a micropipette forming a homogeneous cloudy solution. The Eppendorf tube was immersed in a water bath at 30°C for 3 min, followed by centrifugation at 4500 rpm for 10 min at room temperature and finally placed in an ice bath. After 5 min, the solid button floating on the solution surface was collected with a spatula and transferred to a glass vial where it melted and was diluted with MeOH to 1 mL. These initial conditions were gradually modified (see discussion in Part 3) and then fixed as follow: 1 mL of ultra-pure water containing NaCl 10% was introduced in a glass vial and spiked at 0.5 µg/L with the 10 representative PAHs. 100 µL of 1-dodecanol/MeOH 1/9 (v/v) were rapidly injected in this solution with a micropipette. The glass vial was then immediately immersed in a water bath at 30°C for 3 min, followed by a centrifugation for 10 min at 4500 rpm at 30°C. Finally, the glass vial was placed in an ice bath and the aqueous phase was removed with a syringe after 5 min. The solid button was diluted with MeOH up to 60 µL in the same vial.

2.4 Optimization of protein precipitation and PAH extraction in simulated serum

Optimized extraction conditions in pure water were applied on 100 µL of simulated serum (a Earle's balanced salt solution and human albumin at 30 g/L) spiked at 4.5 µg/L with the 10 representative PAHs. These conditions in pure water were gradually modified (see discussion in Part 3) and then fixed as follow: 100 µL of simulated serum with the 10 representative PAHs. 100 µL of ACN were added for protein precipitation. A centrifugation at 4500 rpm during 30 min at 4°C was carried out. 150 µL of supernatant was then collected and diluted to 1 mL with water containing NaCl 0.5%. Then, 40 µL of 1-dodecanol/MeOH 1/3 (v/v) were rapidly injected with a micropipette forming a homogeneous cloudy solution. The vial was immediately immersed in a water bath at 30°C for 3 min, followed by an ultrasound bath for 3 min and a centrifugation for 10 min at 4500 rpm at 30 °C. The vial was then placed in an ice bath and the aqueous phase was removed with a syringe after 5 min. The solid button was diluted with MeOH to a volume of 100 µL. Finally, the extract was filtered through a 0.45 µm filter with a diameter of 4 mm in regenerated cellulose (Varian, Torrance, CA, USA) to remove any suspended substances before use.

2.5 I.1.1. Extraction of PAHs using SALLE-DLLME-SFO from serum

First, 150 µL of ACN was mixed with 100 µL of spiked serum with concentration levels ranging from 0.5 to 20 µg/L. 20 µL of a saturated $ZnSO_4$ solution was added and the resulting mixture was vortexed

for 10 s. Then, centrifugation was performed at 12500 rpm for 5 min. The supernatant was dissolved in 1 mL of 0.5% NaCl solution (w/v). Next, 40 µL of 1-dodecanol/MeOH 1/3 (v/v) were rapidly injected with a micropipette forming a homogeneous cloudy solution; the tube was immediately immersed in a water bath at 30°C for 3 min, followed by an ultrasound bath for 3 min and a centrifugation for 10 min at 4500 rpm at 30 °C. Finally, the vial was placed in an ice bath and the aqueous phase was removed with a syringe after 5 min. The solid button was diluted with MeOH to a volume of 80 µL.

3. Results and Discussion

The objective of the study was the development of a new miniaturized technique allowing the extraction of the 24 regulated PAHs from small volumes of serum samples before their analysis in LC/UV-FD. First, to simplify the method development, 10 PAHs were selected among the 24 regulated ones: NAPH, ACE, PHE, PYR, CHR, BbF, BaP, DahA, BghiP and DahP. They were chosen according to their number of aromatic rings and their hydrophobicity (log K_{ow}) to be representative of the 24 ones and for their good fluorescence detectability.

3.1 Extraction of PAHs using DLLME-SFO from ultra-pure water

3.1.1 Choice of the extraction and dispersive solvents

In DLLME-SFO, the extraction solvent must meet several requirements [24]. It must be immiscible with water but miscible with the dispersive solvent, it must have a low melting point (below room temperature), a density lower than water and a low volatility. It must favor high partition coefficients for target compounds and its presence in the final extract must not interfere with the chromatographic separation. Finally, a low cost and low toxicity solvent should be preferred. 1-undecanol, 1-dodecanol and 2-dodecanol have been used in most DLLME-SFO applications [24]. Their properties are summarized in Table S3. They all have a density below 1 mg/L and a melting point temperature below 25°C. As previously mentioned, PAHs are hydrophobic compounds (log K_{ow} values higher than 3.5). 1-dodecanol is the less polar solvent among the three. Moreover, it was reported that due to their low melting point, 1-undecanol and 2-dodecanol melted quickly, making them difficult to draw them out [25]. Therefore, 1-dodecanol was selected as the extraction solvent for this method development.
Regarding the dispersive solvent, it must be miscible with both the extraction solvent and water. Solvents like MeOH, ethanol, acetone and ACN are commonly employed as dispersive solvents [24]. Xu et al. evaluated the efficiency of these solvents combined with 1-dodecanol for the extraction of 4 PAHs (NAPH, ACE, ANT, and FLU) [22]. They concluded that MeOH presented the best extraction efficiency. Moreover, 1-dodecanol and methanol were often used together in DLLME-SFO [24]. So, MeOH was chosen as dispersive solvent for this method development.

3.1.2 Preliminary experiments

The use of a mix of 1-dodecanol/MeOH was reported by Vera-Avila et al. for the extraction from environmental waters of 10 PAHs regulated by US-EPA (log K_{ow} values of 3.4-6.9) [21]. They were extracted from 10 mL of water samples (spiked with 100 µL of PAHs in MeOH) using a 1-dodecanol/MeOH 1/2, v/v mixture (*i.e.* a total volume of 300 µL of organic mixture for 10 ml of water) with extraction yields between 85 and 94% for concentrations ranging from 25 to 500 µg/L. Our objective was to adapt this method to a reduced serum volume and to extract a greater number of PAHs belonging to a slightly wider polarity range (log K_{ow} of 3.4-7.7). The sample volume was first reduced from 10 to 1 mL of water, the extraction solvent from 100 to 10 µL and the dispersive solvent from 300 to 30 µL. Since Vera-Avila et al. introduced 50 mg of NaCl in a 10-mL sample (*i.e.* a concentration of 0.5% NaCl), 0.5% NaCl was also introduced in the sample. The other experimental

conditions were kept similar: after the dispersion of the mix of solvents in water spiked with the 10 representative PAHs, the 1.5 mL Eppendorf tube was immersed in a water bath at 30°C for 3 min, followed by centrifugation at 4500 rpm for 10 min at room temperature and finally placed in an ice bath. After 5 min, the solid button floating on the solution surface was collected and transferred to a glass vial where it melted and was diluted to 1 mL with MeOH.

It is well known that the most hydrophobic PAHs can adsorb onto the tube wall which may reduce their extraction rate. This phenomenon can be accentuated when handling small volumes as the surface-to-volume ratio increases. In addition, the less hydrophobic PAHs such as NAPH are likely to remain in the aqueous phase. To evaluate these different risks of loss, PAHs were quantified in the final extract but also in the remaining aqueous fraction and in 500 µL of ACN used after the extraction process to rinse the Eppendorf tube's wall. Moreover, as the introduced volumes of extraction/dispersive solvents were smaller (about 10 times less than in the original study of Vera-Avila et al.), the collection of the reduced size solid button containing the extracted PAHs also appeared to be a critical point. Two different techniques were therefore used to collect it. The first consisted of collecting the solid floating button from the Eppendorf tube using a spatula as often described, and dissolving it in a glass vial [21,26]. The second involved removing the liquid from the Eppendorf tube with a syringe before dissolving the residual solid by adding MeOH in the same tube. Recoveries obtained in the aqueous phase after the extraction, on the tube wall (ACN washing fraction) and in the final extract for both collection methods are reported in Figure S1. The comparison of both methods shows that the collection method based on removing the liquid phase from the tube almost doubled the extraction recoveries for the most hydrophobic PAHs. Indeed, it allows to add directly methanol into the tube used for the DLLME-SFO to directly solubilize the solid button which allows to recover some PAHs adsorbed on the tube wall. These preliminary results also confirmed that, for the most polar PAHs, i.e. NAPH, ACE, and PHE, a loss of 30-40% occurred because of their higher solubility in the aqueous media. As adsorption was observed on the Eppendorf tube wall, DLLME-SFO experiments were then carried out in glass tubes (of the same volume as Eppendorf tubes, *i.e.* 1.5 mL) while applying the same extraction conditions, including the removal of the liquid by a syringe before dissolving the residual solid. Figure S2 presents the obtained recoveries in the liquid phase, on the tube wall and in the final extract. This comparison shows that the use of glass tubes seems to slightly reduce the quantity of PAHs adsorbed on the wall. Glass tubes were therefore retained for further study.

Ultrasonication can improve the mass transfer of analytes from the sample to the extraction solvent and generates smaller droplets of dispersed solvent, thus increasing the contact surface between the dispersed phase and the sample. Consequently, the same extraction procedure was applied, but with the addition of an ultrasonication step prior to the centrifugation one. The results are shown in Figure S3. By using ultrasonication, the residual content of the 3 most polar PAHs (NAPH, ACE and PHE) in the aqueous phase tends to decrease and consequently their extraction recoveries increase. Moreover, this step does not bring tedious handling constraints. This is why, the use of the ultrasonic bath prior to the centrifugation step was chosen.

The last parameter investigated during this preliminary study was the centrifugation temperature. For all the results previously discussed, the centrifugation step was carried out at ambient room temperature. However, it may be subject to variations. The melting temperature of 1-dodecanol being 24°C, the centrifugation temperature was set at 30°C. As shown by results provided in Figure S4**,** this higher centrifugation temperature improved the extraction recoveries for all the PAHs. Consequently, this preliminary study enabled us to set the following conditions, slightly different from those set by Vera-Avila et al. in order to adapt it to small sample volumes: use of glass tubes, removal of the liquid with a syringe and dissolution of the residual solid in the same tube, and addition of an ultrasonication step prior to centrifugation carried out at 30°C.

3.1.3 Volume and ratio of extraction and dispersive solvents

These preliminary experiments were carried out by adding 40 µL of 1-dodecanol/MeOH 1/3, i.e. 10 µL of extraction solvent, to 1 mL of water. The extraction solvent volume and the ratio of extraction and dispersive solvents having a great influence on extraction efficiency in DLLME-SFO, different volumes of 1-dodecanol, 5, 10, and 20 µL, were evaluated with a 1-dodecanol/MeOH ratio of 1/3 or 1/9. It is interesting to note that a 1/1 ratio led to a viscous mixture difficult to handle [21] and to the formation of a non optimal cloudy state resulting in low extraction efficiency [22]. In addition, Vera-Avila et al. showed no difference between 1/2 and 1/3 ratios, the ratio 1/2 having finally been chosen to favor the extraction of molecules that are more polar than PAHs, such as sulfonamides. Figure 1 presents the effect of the volume of the extraction solvent and the extraction/dispersive solvent ratio on the extraction recoveries of the 10 representative PAHs from 1 mL of water containing 0.5% NaCl.

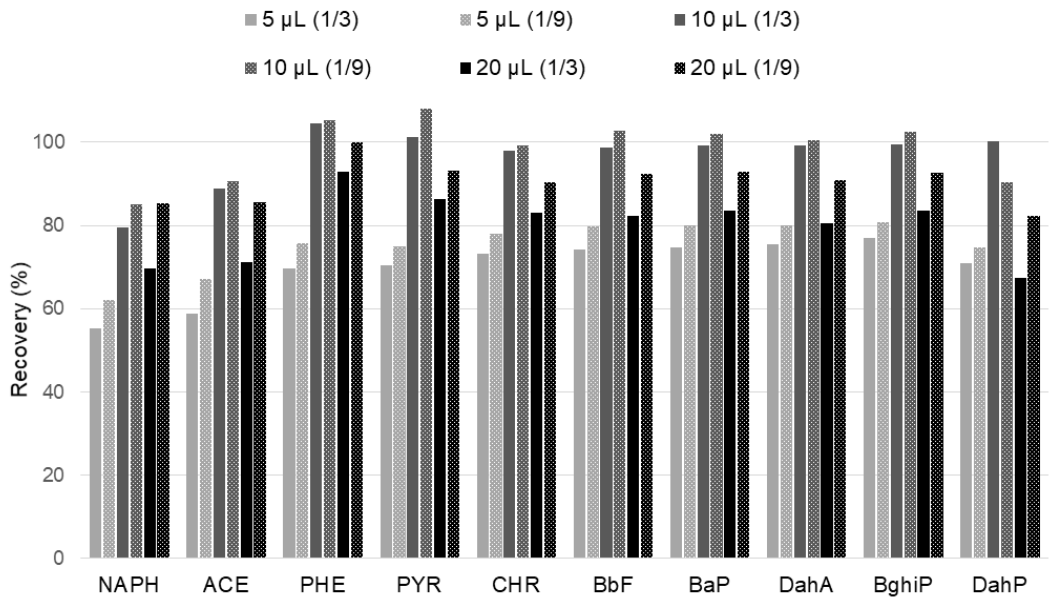

Figure 1 Effect of the volume of the extraction solvent (5 or 10 µL) and the extraction/dispersive solvent ratio (1/3 or 1/9) on the extraction yields of the 10 representative PAHs from spiked ultra-pure water (1 mL) containing NaCl 0.5% by DLLME-SFO (n=1). *Extraction conditions: sample spiked at 0.5 µg/L (50 µg/L, 10 µL in ACN); 5, 10 or 20 µL of the extraction solvent (1-dodecanol) mixed with a ratio 1/3 or 1/9 of the dispersive solvent (MeOH); water bath (3 min, 30°C); ultrasonic bath (3 min); centrifugation (4500 rpm, 10 min, 30°C); ice bath (5 min); liquid removal and dissolution of the solid keeping the same glass vial.*

Extraction yields tended to increase from 5 to 10 µL of 1-dodecanol and then to decrease for 20 µL. For 10 µL of 1-dodecanol, the ratios 1/3 and 1/9 seem to give similar results. A few replicates would certainly have confirmed that 10 µL of 1-dodecanol lead to higher extraction yields than 20 µL. However, with an extraction solvent volume of 20 µL, 150 µL of MeOH are required to dissolve the final solid button and obtain a liquid extract ready to be analyzed, compared with 60 µL of MeOH for 10 µL. Consequently, the use of 20 µL of 1-dodecanol leads to lower enrichment factors, reducing the final sensitivity of the method by a factor 2.5. The volume of extraction solvent was therefore fixed at 10 µL. As the difference between results obtained using the 1/3 and 1/9 ratios was not significant, this parameter was again studied by varying also the salt concentration in the sample.

3.1.4 Salt addition

In liquid-liquid extraction, salt is added into aqueous samples to increase the ionic strength, which generally leads to the salting out effect, inducing a decrease in the analyte solubility in the sample and an enhanced extraction efficiency, especially for the most polar compounds. Nevertheless, the resulting effect of salt addition on extraction efficiency of DLLME-SFO can be variable [24]. In some

studies, the addition of salt into the sample had no effect or slightly decreased extraction efficiency and enrichment factor [22,27], while in others, the ionic strength had a positive effect on extraction yields [21,28]. It was then decided to investigate this parameter. The previous tests were carried out with 0.5% NaCl, as described by Vera-Avila et al [21]. The influence of the percentage of NaCl on extraction efficiency was therefore studied by comparing the extraction recoveries obtained using 0.5, 5 and 10% of NaCl added in 1 mL of spiked water extracted with 1-dodecanol/MeOH (10 µL) for both ratios (1/3 and 1/9). No trend was observed on extraction efficiency for these NaCl contents, whether with ratio of 1-dodecanol/MeOH of 1/3 or 1/9 (Figure S5). To conclude this study of DLLME-SFO applied to spiked ultra-pure water, the repeatability of the procedure was evaluated under the following conditions: NaCl set at 10%, 10 µL of 1-dodecanol, extraction/dispersive solvent ratio of 1/9 and dilution of the final extract in 60 µL of MeOH. The recoveries for the 10 representative PAHs, reported in Figure 2, were between 71 and 115% with RSD values between 3 and 10% (n=3), leading to enrichment factors between 12 to 19.

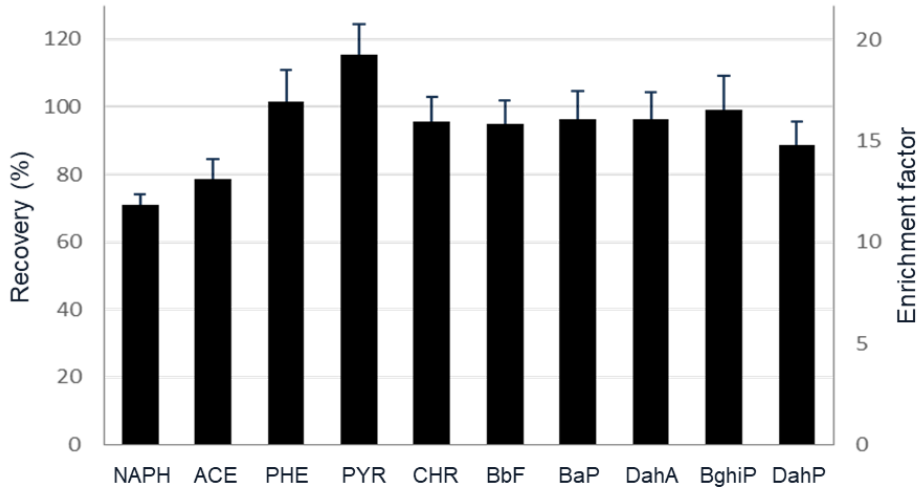

Figure 2 Extraction recoveries and enrichment factors of the 10 representative PAHs from ultra-pure water (1 mL) containing NaCl 10% by DLLME-SFO (n=3). *Extraction conditions: sample spiked at 0.5 µg/L (50 µg/L, 10 µL in ACN); 1-dodecanol (10 µL)/MeOH (90 µL) (1/9). Other DMLLE-SFO conditions: see Fig. 1.*

3.2 Optimization of the extraction method on simulated serum samples

As already mentioned in a recent review [29], the application of DLLME to complex matrices such as biological fluids requires a prior sample treatment before extraction. It can be achieved for example by dilution, protein precipitation or SPE [29]. To maintain high enrichment factors and avoid an evaporation step, protein precipitation was chosen. The first tests on protein precipitation and DLLME-SFO condition adjustment was conducted with simulated serum, *i.e.* human albumin at 30 g/L in a Earle's balanced salt solution (i.e. a mixture of salt and glucose at a concentration close to human serum composition).

3.2.1 Selection of the protein precipitation solvent

The main problem for serum analysis is its content in proteins, which must be eliminated while minimizing losses of PAHs that could be adsorbed to them. To achieve this, a precipitation agent combined with a cold centrifugation step was considered. This precipitation agent can be an organic solvent (ACN, MeOH, ethanol or acetone), an organic acid (perchloric, trichloroacetic or phosphoric acid), a concentrated salt, a metal ion or a surfactant such as sodium dodecyl sulfate [30]. After some preliminary tests with ACN and MeOH on simulated serum (data not shown), acetonitrile was chosen as precipitation agent. A serum/ACN ratio of 1/1 (v/v) was chosen to limit the sample dilution. However, the addition of ACN alone was not sufficient to remove certain components from the

supernatant, which caused significant matrix effects (ie., coelution, loss of signal, increase of noise) and clogging of the column [23]. Consequently, an ultrafiltration step of the final extract after the DLLME-SFO was introduced to solve these problems and allow the injection of the extract in LC.

3.2.2 DLLME-SFO condition adjustment in simulated serum

As the first tests carried out with spiked ultra-pure water did not allow to conclude on the extraction/dispersive solvent ratio and the salt content, their influence on extraction yields was again investigated, but on spiked simulated serum. The simulated serum (100 µL) was spiked at 4.5 µg/L with the 10 representative PAHs and protein precipitation was carried out using an equivalent volume of ACN. The supernatant was recovered after a centrifugation step and diluted by a factor 6.7 (150 µL in 1 mL) to decrease the ACN content that could affect the DLLME-SFO extraction process that was next achieved without NaCl or by adding 0.5, 5 or 10% of NaCl and using 1-dodecanol/MeOH ratios of 1/3, 1/6 or 1/9. The results are reported on Figure 3 for 0.5 and 10% of NaCl and on Figure S6 for all the tested conditions. First of all, these DLLME-SFO conditions led to lower recoveries with the simulated serum than with water, with the exception of PHE for which, as it will be discussed later, quantification is affected by the presence of a co-eluted compound. These lower yields could be due to the strong adsorption of PAHs on removed proteins. As the extraction recoveries decrease with the hydrophobic character of the compounds, contrary to what was obtained in spiked pure water, the hypothesis seems correct. Nevertheless, the highest recoveries were obtained by adding 0.5% NaCl, which is close to the salt concentration of isotonic solution (0.9% NaCl), and a 1-dodecanol/MeOH ratio of 1/3 or 1/6. To continue the study, a 1/3 ratio was preferred to limit the organic solvent input.

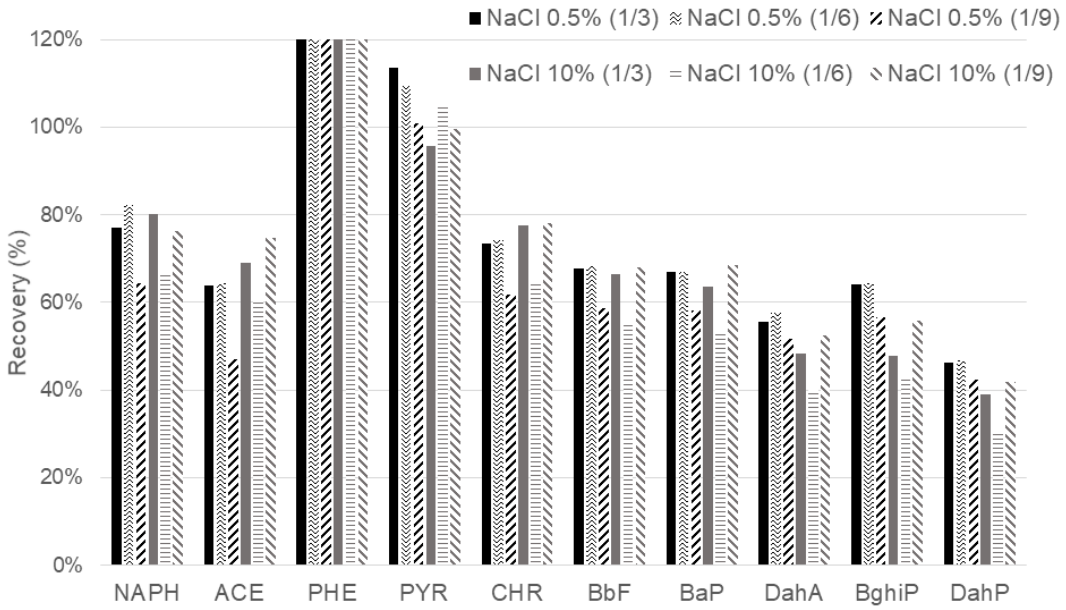

Figure 3 Effect of the salt addition and the extraction/dispersive solvent ratio on the extraction recoveries of the 10 representative PAHs from simulated serum (100 µL) by DLLME-SFO after protein precipitation (n=1). *Extraction conditions: sample spiked at 4.5 µg/L (50 µg/L, 10 µL in ACN); protein precipitation: ACN (100 µL); centrifugation (4500 rpm, 30 min, 4°C); supernatant (150 µL) diluted to 1 mL with water containing 0.5% or 10% of NaCl; 1-dodecanol/MeOH (1/3, 1/6 or 1/9), extraction solvent volume (10 µL); syringe filter (cellulose, diameter 4 mm, porosity: 0.45 µm). The figure is truncated at 120%. Other DMLLE-SFO conditions: see Fig. 1.*

During these experiments, co-elutions in LC between some PAHs (NAPH, ACE, PHE, PYR and BghiP) and unknown compounds were noticed. For most of these PAHs, these co-elutions were explained by a contamination coming from the cellulose filter used for the DMLLE-SFO extract before its injection in LC as illustrated in Figure S7. As an example, a peak arises at the retention time of PHE

when analyzing a MeOH fraction used for the washing of a cellulose filter, which prevents from a correct quantification of some PAHs and explains the high recoveries reported on Figure 3. Therefore, different filters were evaluated by analyzing filtered simulated final extract (prepared by mixing 1-dodecanol with methanol) spiked with the 10 PAHs. As seen in Figure S8, recovery yields higher than 100% were obtained for NAPH, ACE, PHE and PYR for filters made of PVDF and PVDF-HL. In return, recoveries close to 100% were obtained with PTFE-HL filter. Pre-washing the filters with methanol eliminates these interfering compounds. Nevertheless, this pre-washing step slightly increases the final volume of extract and may decrease the extraction yield repeatability according to variation of the residual volume of methanol in the filter. So, for the rest of the study, PTFE-HL filter without any prewashing step with methanol was chosen.

3.2.3 Selection of salt for protein precipitation in human serum: SALLE

The conditions of precipitation and extraction developed on simulated serum were applied to real human serum and led to a decrease of the extraction recoveries, particularly for the most hydrophobic PAHs (DahA, BghiP, and DahP) with a decrease between 30 and 50%. Improving the efficiency of the protein precipitation step was thus necessary. The use of ACN alone was therefore replaced by a salting out liquid-liquid extraction (SALLE) method. According to Tang et al., sulfate salts combined with ACN lead to high extraction yields for a wide range of compounds (hydrophobic drugs, steroids, and cannabinoids) from biological samples such as plasma, urine and brain [23]. Therefore, different volumes (10, 20 or 30 µL) of solutions saturated with sulfate salts, $(NH_4)_2SO_4$, $ZnSO_4$, or $MgSO_4$, were tested and results are provided in Figure 4. $(NH_4)_2SO_4$ led to the co-extraction of many compounds that co-elute with the most polar PAHs thus giving rise to an overestimation of their recoveries. $ZnSO_4$ showed better extraction efficiency than $MgSO_4$ and the use of 20 µL of the saturated solution seems to provide the highest extraction efficiency.

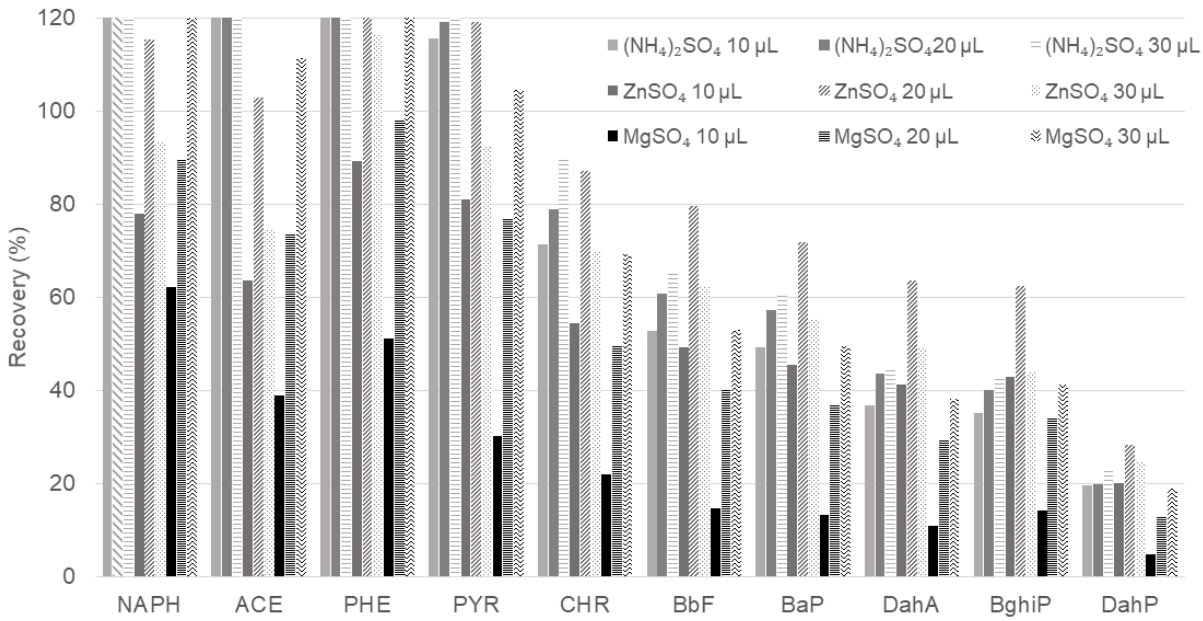

Figure 4 Effect of salt addition in human serum (100 µL) during the precipitation step with ACN on the extraction recoveries of the 10 representative PAHs (n=1). *Extraction conditions: sample spiked at 4.5 µg/L (50 µg/L, 10 µL in ACN); protein precipitation with ACN (100 µL) + saturated solution of $MgSO_4$, $ZnSO_4$ or $(NH_4)_2SO_4$ (10, 20 or 30 µL); centrifugation (4500 rpm, 30 min, 4°C); supernatant sample (150 µL) diluted to 1 mL with NaCl 0.5%; extraction solvent/dispersive solvent : 1-dodecanol/MeOH (1/3, 40 µL); syringe filter (PTFE-HL, diameter: 4 mm, porosity: 0.2 µm). The figure is truncated from 120%. Other DMLLE-SFO conditions: see Fig. 1.*

It was also observed that this SALLE method introduced upstream of the DLLME-SFO was sufficiently efficient to remove the ultrafiltration step of the final extract before its injection in LC thus limiting the risk of introduction of interfering compounds and loss of PAHs that could be caused by this filtration step. In order to improve extraction efficiency of the most hydrophobic compounds, the volume of ACN used for SALLE was increased from 100 to 150 µL, resulting in a larger volume of supernatant, 180 versus 150 µL. Centrifugation conditions were also modified: the duration was reduced from 30 to 5 min and the speed increased from 4500 to 12500 rpm. Under these conditions, as shown in Figure 5**,** the SALLE-DLLME-SFO method led to extraction yields ranging from 44 to 91% with RSD from 3 to 9% (n=3) and enrichment factors between 0.35 and 0.73, *i.e.* a dilution ratio between 1.5 and 2.8. For the three least hydrophobic PAHs (NAPH, ACE and PHE), extraction yields were similar between ultra-pure water and serum: NAPH: 71 ± 3% in ultra-pure water vs 79 ± 9% in serum; ACE: 79 ± 6% vs 90 ± 5%; PHE: 101 ± 9 vs 84 ± 8%. However, a decrease in the extraction yields of the most hydrophobic PAHs was observed: DahA: 96 ± 3% in ultra-pure water vs 73 ± 3% in serum; BghiP: 99 ± 10% vs 71 ± 3%; DahP: 89 ± 7% vs 44 ± 4%, respectively. Nevertheless, these results are still very satisfactory given the complexity of the sample, the small volumes handled and the number of steps required to extract/purify these compounds, which are spread over a very wide range of polarity.

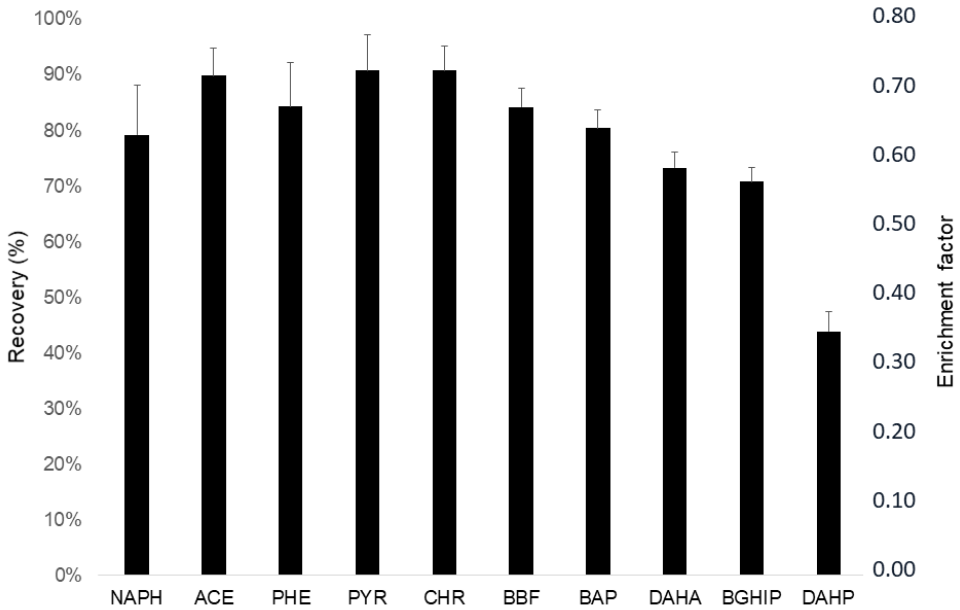

Figure 5 Extraction recoveries of the 10 representative PAHs from spiked human serum (100 µL) using SALLE-DLLME-SFO (n=3). *Extraction conditions: sample spiked at 4.5 µg/L (50 µg/L, 10 µL in ACN); solvent for protein precipitation ACN (150 µL) + saturated solution of $ZnSO_4$ (20 µL); centrifugation (12500 rpm, 5 min, 4°C); supernatant sample (180 µL) diluted to 1 mL with NaCl 0.5%; extraction/dispersive solvent: 1-dodecanol/MeOH (1/3, 40 µL). Other DMLLE-SFO conditions: see Fig. 1.*

3.3 Extraction of the 24 PAHs from spiked human serum using SALLE-DLLME-SFO

The SALLE-DLLME-SFO protocol developed for the 10 representative PAHs was applied to the 24 regulated PAHs in spiked serum. Figure 6 shows the chromatograms obtained by spiking serum at levels close to the LOQ, i.e. 2 µg/L for the 20 PAHs monitored by fluorescence and 40 µg/L for the 4 PAHs monitored by UV. It is worthwhile to notice that the identification of the compounds based on their retention times were confirmed in UV by their UV spectrum (Figure S9). In LC-FD, except for the isomers DahA/DalP, the compounds were well separated (Rs > 1.3).
Nevertheless, it was noticed that the presence of 1-dodecanol in the final extract injected in LC modified the retention time and/or peak shape for some compounds, which has not been reported

in literature yet, as far as we know. Indeed, it affects both the peak shape and the retention time for compounds arising between 25 and 30 min, i.e. BaA, CHR, 5MCHR, BcF and CPcdP.

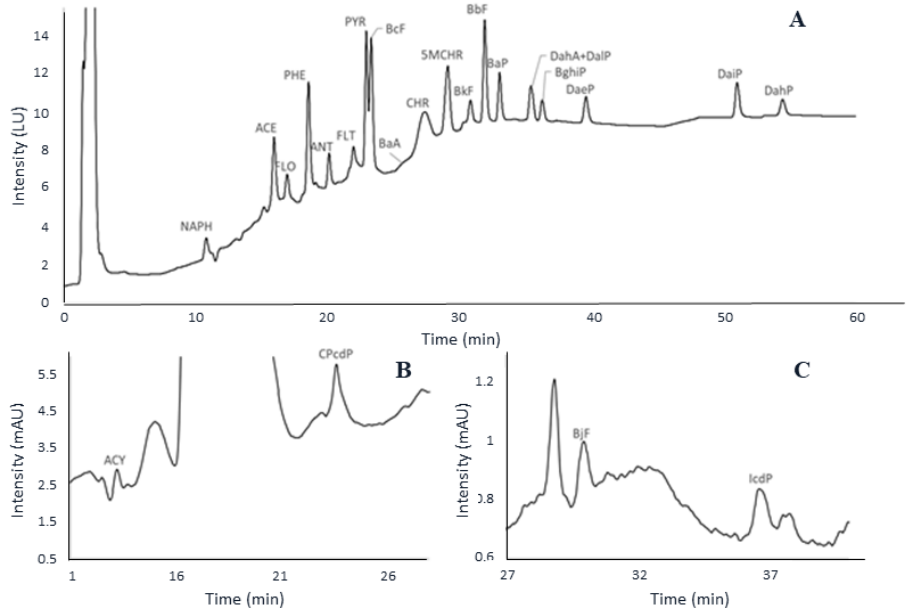

Figure 6 LC-UV/FD chromatograms obtained for the analysis of the 24 PAHs extracted by the SALLE-DLLME-SFO method from spiked human serum. Spiking levels: 2 µg/L for the 20 PAHs monitored by fluorescence (A) and 40 µg/L for the four PAHs monitored by UV at 230 nm (B) and at 300 nm (C).

This is illustrated for the four fluorescent PAHs by the comparison of LC/FD chromatograms resulting from the injection of a mix of PAHs in MeOH (Figure 7A) and of a SALLE-DLLME-SFO extracts of spiked serum and spiked pure water (Figure 7B and Figure S10). These phenomena of peak shifting and shape modification affect the LOQs of these 5 compounds and would require a calibration in the presence of 1-dodecanol in order to validate the method. This problem could also be solved by using GC-MS which could also improve the sensitivity for the 4 PAHs only detectable in UV [20]

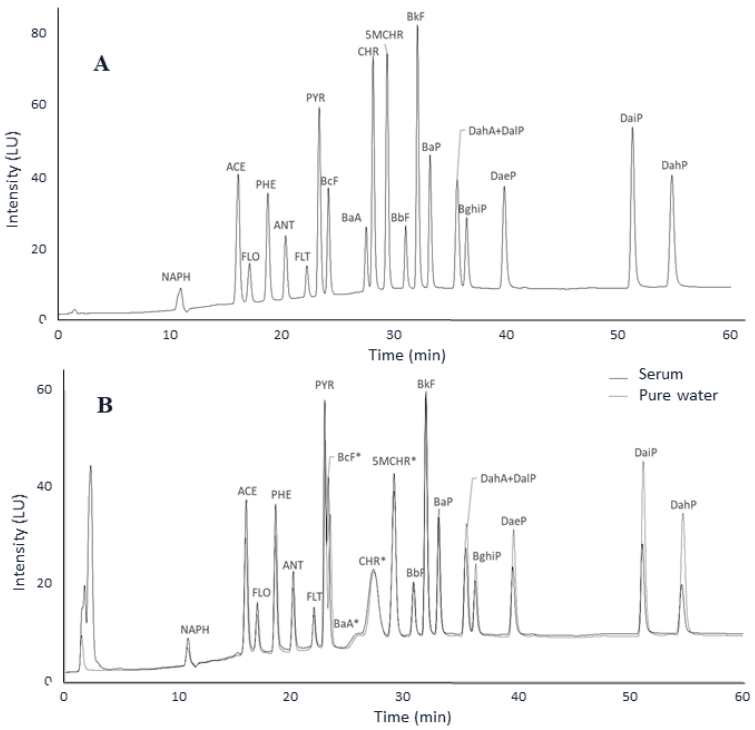

Figure 7 LC-FD chromatograms corresponding to the analysis of the 20 PAHs at 15 µg/L in pure MeOH (A) and in the SALLE-DLLME-SFO extract from spiked human serum (full line) and spiked pure water (dashed line) (B). *: compound having a shifted retention time when 1-dodecanol is present.

The linearity of the method applied to spiked serum was evaluated at 7 concentration levels (0.5, 1, 2, 5, 10, 15 and 20 µg/L) for PAHs detected in FD. For UV detectable compounds, the linearity was evaluated at 6 concentration levels for ACY, BjF and IcdP (10, 15, 20, 30, 40 and 50 µg/L) and at 5 concentration levels for CPcdP (40, 50, 80, 100 and 200 µg/L). The results are shown in Table 1 and Table 2 respectively. The R^2 are ranging from 0.995 to 0.9993. For all the fluorescent compounds, Table 1 presents mean extraction recoveries calculated by integrating the different spiking level (n=7, 8 or 9 depending on the compounds) and calculated for a spiking level of 15 µg/L (n=3). Extraction yields ranged from 44 to 112%, with an average of 84 ± 10% which is a quite high mean value, but which does not consider the impact of the presence of 1-dodecanol in the extract on several PAH peak shapes, particularly on BaA peak shape. Indeed, when recoveries are calculated not with a calibration curve obtained by injecting a standard solution of PAHs into MeOH, but in relation to a SALLE-DLLME-SFO extract of spiked pure water containing 1-dodecanol, higher values are obtained between 48.2 and 116.0% with an average of 92.4%. The enrichment factor for 100 µL of serum sample was between 0.35 to 0.90 (i.e. limited dilution factor between 1.11 and 2.83). The LOQs were estimated for a signal-to-noise ratio (S/N) of 10 using results obtained by applying SALLE-DLLME-SFO to the serum spiked at the lowest concentration of the linearity range for each PAH. They are between 0.04 and 1.03 µg/L for PAHs detected by FD and at 10 µg/L for ACY, BjF and IcdP and at 40 µg/L for CPcdP detected by UV.

Table 1 Analytical performance of the SALLE-DLLME-SFO method to extract the 20 PAHs detected by fluorescence from spiked human serum

PAH	Concentration range (µg/L)	Equation	Linearity (R^2)	Recovery[(a)] (RSD) (%)	Recovery[(b)] (RSD) (%)	EF[(c)]	Recovery[(d)] (RSD) (%)	LOQ serum[(e)] (µg/L)
NAPH	1-20	6.44x-4.53	0.996	67.8 (12.5)	70.3 (5.1)	0.54	106.0 (5.1)	1.00
ACE	0.5-20	39.51x-8.73	0.996	95.2 (5.0)	95.4 (2.5)	0.77	116.0 (2.5)	0.45
FLO	1-20	12.81x-8.67	0.996	99.5 (10.4)	103.1 (3.8)	0.80	115.6 (3.8)	0.91
PHE	1-20	31.08x-19.94	0.9992	92.8 (11.5)	100.2 (2.8)	0.75	99.5 (2.8)	0.64
ANT	2-20	18.58x-7.15	0.998	102.3 (2.0)	103.3 (2.1)	0.82	112.8 (2.1)	1.03
FLT	0.5-20	8.94x-1.52	0.9990	111.6 (7.2)	109.6 (2.0)	0.90	113.9 (2.0)	0.27
PYR	0.5-20	48.14x-20.00	0.998	92.2 (13.7)	90.7 (12.1)	0.74	99.6 (12.1)	0.36
BcF	0.5-20	29.77x-9.02	0.997	103.2 (14.0)	119.0 (5.3)	0.83	100.4 (5.3)	0.50
BaA	2-20	9.01x-8.55	0.997	53.1 (11.2)	57.0 (4.5)	0.42	95.2 (4.5)	2.00
CHR	0.5-20	60.13x-4.26	0.998	110.9 (5.8)	109.8 (0.7)	0.89	107.4 (0.7)	0.08
5MCHR	0.5-20	54.04x-7.15	0.9992	97.9 (7.2)	98.9 (2.7)	0.79	102.7 (2.7)	0.22
BbF	0.5-20	14.20x-2.95	0.9992	97.5 (9.7)	100.1 (3.8)	0.78	99.8 (3.8)	0.29
BkF	0.5-20	57.94x-9.15	0.9993	97.4 (9.7)	98.3 (3.9)	0.78	98.0 (3.9)	0.06
BaP	0.5-20	28.30x-5.87	0.9992	90.3 (9.0)	92.0 (3.9)	0.73	92.1 (3.9)	0.05
DahA/DalP	0.5-20	27.44x-3.30	0.9990	81.7 (12.5)	83.6 (7.1)	0.66	83.4 (7.1)	0.06
BghiP	0.5-20	15.36x-3.55	0.9990	77.6 (10.7)	81.2 (7.2)	0.62	80.8 (7.2)	0.04
DaeP	0.5-20	22.91x-3.44	0.998	71.3 (10.5)	72.7 (9.9)	0.57	71.8 (9.9)	0.04
DaiP	0.5-20	28.91x-1.53	0.998	59.4 (11.9)	60.7 (10.7)	0.48	61.2 (10.7)	0.14
DahP	0.5-20	17.95x-6.41	0.995	44.0 (14.6)	47.3 (13.1)	0.35	48.2 (13.1)	0.10

[(a)] Mean recoveries and relative standard deviation (RSD) for the different concentration levels; n=9 except for NAPH, FLO, PHE, BjF, IcdP (n=8) BaA and ANT (n=7)
[(b)] Mean recoveries and RSD values for a spiking level of 15 µg/L (n=3)
[(c)] Enrichment factor (EF) calculated for n=9 except for NAPH, FLO, PHE BjF, IcdP (n=8) BaA and ANT (n=7)
[(d)] Extraction recovery in serum calculated using the spiked water extract as reference
[(e)] Limit of quantification (LOQ)

The comparison of the chromatograms, obtained for spiked human serum and spiked pure water, both submitted to SALLE-DLLME-SFO and provided in Figure 7B, illustrated that recoveries of extraction by this method are not much affected by the serum matrix. This is also illustrated by the recoveries provided in Table 1 and Table 2 that ranged from 48 to 116%. Except PAHs with log K_{ow} superior to 6.5, all compounds were extracted close to 100%. The lowest extraction yields could be explained by the formation of protein adducts of PAHs with albumin [31].

Table 2 Analytical performance of the SALLE-DLLME-SFO to extract the 4 PAHs detected by UV from spiked human serum

PAH	Concentration range (µg/L)	Equation	Linearity (R^2)	Recovery[(a)] (RSD) (%)	Recovery[(b)] (RSD) (%)	EF[(c)]	Recovery[(d)] (RSD)[(c)] (%)	LOQ serum (µg/L)
ACY	10-50	0.38x-0.98	0.996	57.1 (3.9)	58.6 (2.1)	0.49	72.4 (2.1)	10
CPcdP	40-200	0.70x-9.81	0.997	91.1 (13.0)	nd	0.73	nd	40
BjF	10-50	0.13x-0.60	0.997	82.2 (14.3)	75.6 (0.9)	0.66	84.8 (0.9)	10
IcdP	10-50	0.12x-0.36	0.995	66.8 (4.9)	68.9 (3.7)	0.54	70.9 (3.7)	10

nd: not determined
[(a)] n=8 for all except CPcdP (n=5)
[(b)] Mean recoveries and RSD values for a spiking level of 15 µg/L (n=3)
[(c)] Enrichment factor (EF) calculated for n=8 for all except CPcdP (n=5)
[(d)] Extraction recovery in serum calculated using the spiked water extract

All the extractions by SALLE-DLLME-SFO were done by spiking a pooled serum for which the initial PAH content was unknown. Therefore, the developed SALLE-DLLME-SFO method was carried out without spiking the serum but also with ultra-pure water for comparison and Figure S11 shows the resulting chromatograms. The chromatographic profiles of serum and pure water treated with DLLME-SFO are very similar. Some peaks arise at the same retention time as those of NAPH, PHE and PYR in both matrices while peaks arise at the same retention time as FLO, FLT and 5MCHR only in blank serum. For peaks with retention times corresponding to PHE and PYR, it was challenging to determine their presence in the serum since their peak areas were similar in both serum and pure water extracts. Moreover, all the peaks detected only detected in blank serum (FLO, FLT and 5MCHR) were under LOQs. The peak at the retention time of NAPH was more intense in the serum than in pure water, which could suspect the presence of NAPH in the serum. However, its presence could not be conclusively verified using the UV spectrum, so confirmation through mass spectrometry would be necessary. Nonetheless, an approximate concentration found in the blank serum could be estimated to be around 1 µg/L. This magnitude would be consistent with values found in other articles from Chinese [9,14,32], Iranian [12], American [33] or European [13] populations. For ACY, CPcdP, BjF and IcdP, UV detection was insufficiently sensitive for accurate identification (Figure S11).

3.4 Comparison with previous studies

The SALLE-DLLME-SFO method developed here was compared to other methods already reported in literature and listed in Table 3. It appears that the developed method displays many advantages. The volume of sample used (0.1 mL) is 2.5 to 40 times lower than that usually reported in literature. As with the other studies mentioned, several samples can be simultaneously extracted in 1h40. These two characteristics are valuable advantages when analyzing samples from a cohort of patients. Moreover, the consumption of extraction solvent is strongly reduced, less than 0.3 mL for 100 µL of serum sample, compared to volumes used in other studies ranging from 0.8 to 140 mL. Moreover, the extraction solvent, 1-dodecanol, is much less toxic than those used previously such as n-hexane, dichloromethane, methyl-tert butyl-etheror tetrahydrofuran. All the methods listed in Table 3 involved an evaporation step under nitrogen, which is time-consuming and could promote the loss of the most volatile PAHs. The SALLE-DLLME-SFO method developed here avoids this evaporation step. In addition, the use of consumables is reduced to two vials by sample. The method developed in this

work enabled the extraction of the 24 regulated PAHs. Only two studies looked for more than 16 PAHs (the ones targeted by US-EPA) in serum samples [7,16]. Swiha et al. analyzed the same 24 PAHs (those regulated in food and in environment) from a low volume of serum (0.1 mL), but the mean extraction recoveries were significantly lower than the ones obtained in this study (21-69 ± 1-11% vs 48-116 ± 2-15%) and the LOQs were 3 to 5 times higher (0.2-3.1 µg/L vs 0.04-1.03 µg/L) [16]. Conversely, Wang et al. achieved similar mean extraction recoveries (67-109 ± 7-25%) for 27 PAHs, but they used between 0.6 and 1.5 mL of serum and more than 140 mL of solvents [7]. Except for PAHs only detectable in UV, the LOQs were in the same order of magnitude in LC-FD and GC-MS (hundredth µg/L) but from a higher serum volume (0.6-1.5 mL versus 100 µL) [7].

Table 3 Comparison of this SALLE-DLLME-SFO procedure with previous methods for determining PAHs in serum

PAH	Sample pre-treatment	Treatment techniques	Sample volume (mL)	Solvent consumption (mL)	Analytical method	Recovery (RSD) (%)	LOQ (µg/L)	Ref
27[(a)]	MASE (ACN, 110°C, 20 min)	LLE + SPE	0.6-1.5	> 140	GC-MS	67-106 (7-25)	LOD: 0.05-0.10	[7]
16 US-EPA	Filtration Treatment with ACN	PT-SPE	0.2	0.80	GC-MS	85-115 (2-9)	0.007-0.013	[34]
16 US-EPA	-	DLLME	0.25	1.55	GC-MS/MS	95[(b)] (10)	0.001-0.039	[20]
22[(c)]	Treatment with SDS and ACN	SPE	0.1	0.89	LC-UV/FD	27-69 (1-11)	FD: 0.2-3.1 UV: 7-14.5	[16]
24	SALLE	DLLME-SFO	0.1	0.29	LC-UV/FD	48- 116 (2-15)	FD: 0.04-1.03 UV: 10-40	This study

[(a)] ACY, ACE, FLO, PHE, ANT, FLT, PYR, CPcdP, BaA, CHR, BbF, BkF, BaP, IcdP, DahA, BghiP, DalA, DaeP, DaiP, DahP and others PAHs not regulated
[(b)] Average recovery
[(c)] NAPH, ACY, ACE, FLO, PHE, ANT, FLT, PYR, BcF, BaA, CHR, 5MCHR, BjF, BbF, BkF, BaP, DahA, DalP, BghiP, DaeP, DaiP, DahP
MASE: microwave-assisted solvent extraction; SDS: sodium dodecyl sulfate; n.a.: not available

4. Conclusion

A new miniaturized extraction method, combining SALLE and DLLME-SFO, was developed for the determination of the 24 regulated PAHs in serum. The main challenge was to extract compounds belonging to a wide range of polarity from a volume of serum reduced to 100 µL so that this method could eventually be applied to cohort samples available in reduced volumes. The handling of such a small sample volume has shown the importance of the choice of the nature of the consumable to limit adsorption and loss of compounds as well as the way to collect the resulting floating extract. Combined with ACN, sulfate salts were used to improve protein precipitation and extract clean-up before DLLME-SFO. Key parameters such as volume of extraction solvent, extraction/dispersive solvent ratio or salt content were studied and set according to the extraction yields obtained, as well as their effect on the dilution ratio of the final extract. The green solvent used, 1-dodecanol, exhibited good extraction recoveries for PAHs in serum, but also showed some chromatographic limits (peak shifting and peak shape modification) which affect quantification of some compounds. The proposed procedure is now ready to be used to evaluate the contamination by PAHs in real serum samples after its validation, allowing environmental monitoring and retrospective surveys of at-risk populations. For this, the association of LC-UV/FD with GC-MS, as previously described in blood [4], would allow the identification and confirmation of the presence of PAHs while lowering the LOQ of some compounds.

Author Contributions

Conceptualization: V. Pichon, N. Delaunay. Formal analysis and investigation: C. Boughanem. Writing-original draft preparation: C. Boughanem. Writing-review and editing: V. Pichon, N. Delaunay. Funding acquisition: V. Pichon. The manuscript was written through contributions of all authors.

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