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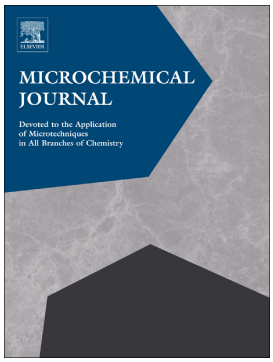

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Determination of nitrated and oxygenated polycyclic aromatic hydrocarbons in water samples by a liquid–liquid phase microextraction procedure based on the solidification of a floating organic drop followed by solvent assisted back-extraction and liquid chromatography–tandem mass spectrometry

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Abstract

A methodology was optimized for the determination of nitrated-PAHs (nitro-PAHs) and oxygenated-PAHs (oxy-PAHs) in natural waters. The extraction/preconcentration procedure was performed by liquid–liquid phase microextraction based on the solidification of a floating organic drop followed by a novel solvent assisted back-extraction (DLLME-SFO-SBE) combined with liquid chromatography-tandem mass spectrometry. The solvent assisted back extraction into a suitable solvent enabled the direct injection of nitro and oxy-PAHs into the UHPLC-(+)APCI-MS/MS system. Parameters affecting the efficiency of the back-extraction procedure were evaluated and optimized, including the nature of the back-extractant volume, temperature and agitation effect. Additionally, various strategies related to the emulsion formation process were assayed.

Detection and quantification limits were in the range of 0.02 - 0.85 ng mL^{-1} and 0.15 – 1.10 ng mL^{-1}. Acceptable extraction recoveries between 95.1 and 100 % and enrichment factors between 192 and 200-fold, with relative standard deviations < 7.6 %, were obtained. The method was successfully applied to the analysis of different types of water samples. In addition, concentration levels of nitro-PAHs and oxy-PAHs ranging from 0.97 to 7.16 ng mL^{-1} and from 0.69 to 2.36 ng mL^{-1}; respectively, were detected in lake water. The proposed methodology is an easy, sensitive, and accurate analytical approach for determining nitrated and oxygenated PAHs of environmental concern in water samples.

Keywords: *Nitrated Polycyclic Aromatic Hydrocarbons; Oxygenated Polycyclic Aromatic Hydrocarbons; Water samples; DLLME-SFO; Solvent assisted back-extraction; Liquid chromatography–tandem mass spectrometry*

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) and their derivatives including oxy-, nitro- and alkyl-PAHs are widespread environmental pollutants. Recently, attention has been paid to typical substituted PAHs due to their toxic effects. In this context, carcinogenicity and mutagenicity of these compounds have also been demonstrated [1]. Moreover, previous studies suggest that some nitro-PAHs have more mutagenic and carcinogenic properties than unsubstituted PAHs [2-4].

Nitro- and oxy-PAHs can be emitted by incomplete combustion processes [5] and also by reactions of gas-phase PAHs and oxidants in the atmosphere or other precursor molecules [6]. Because of their hydrophobicity and low solubility, the presence of nitro-PAHs and oxy-PAHs in water samples has been associated with suspended particles and subsequent deposition in sediments [7, 8]. In addition to the mentioned process, these compounds may also enter into aquatic environments through industrial and domestic sewage effluents, exhaust of gasoline and diesel combustion engines, industrial discharges and wastewater treatment plants and especially from spillage of petroleum and petroleum products [8]. Although a recent study reported pg L^{-1} to ng L^{-1} levels of nitro-PAHs derivatives in lake and river water samples [7, 9-12], information regarding of nitrated and oxygenated PAHs contents in aquatic samples is limited. As a consequence, research devoted to understand the behavior of PAHs derivatives, concentrations in water compartments, sources and occurrence, mutagenic and carcinogenic activities, has recently gained importance, and the development of analytical methods for identification and quantification is crucial for the above mentioned purposes.

The analysis of environmental extracts containing nitro- and oxy-PAHs is often complex and requires clean-up steps and multiple liquid or gas chromatographic methods. A major improvement in sensitivity has been observed with liquid chromatography–tandem mass spectrometry (LC-MS/MS) interfaces operating at atmospheric pressure, e.g. with atmospheric pressure chemical ionization (APCI) and with electrospray ionization (ESI) [13-15].

In the last years, there have been limited methods developed for the extraction of nitrated and oxygenated-PAHs in water samples, these include liquid–liquid extraction (LLE) and solid phase extraction (SPE) [8, 16, 17]. Both LLE and SPE are efficient approaches that have been widely applied to the trace determination of these compounds for many years, but these strategies require large amount of toxic organic solvent. A recent study has reported the applicability of solid-phase microextraction (SPME) and gas chromatography–mass spectrometry detection (GC-MS) for the detection of nitro-PAHs [12, 18, 19]. However, this method presents some drawbacks such as high cost, sample carry-over and time. As an alternative to this popular technique is dispersive liquid–liquid microextraction (DLLME) [20, 21].

DLLME provides high recoveries and enrichment factors within a short period of time. In contrast to conventional extraction techniques, DLLME is fast, simple, and inexpensive [20, 21]. The extraction solvents adopted for DLLME require to have a greater density than water, such as chlorobenzene, carbon tetrachloride, and chloroform. Obviously the mentioned solvents are toxic and environment-unfriendly. To overcome these disadvantages, DLLME based on solidification of floating organic drop (DLLME–SFO) was introduced in 2008 by Leong *et al.* [22] for the extraction and preconcentration of some halogenated organic compounds from water samples. In

contrast to DLLME, the SFO variant is simple, environmentally friendly and a fast extraction is achieved. The extractant often used are of lower density than water and of lower toxicity [23]. In DLLME-SFO, an extraction solvent with low density, low toxicity and proper melting point near room temperature dissolved in a water miscible dispersive solvent is rapidly injected into an aqueous sample by syringe. Then an emulsion solution containing fine droplets of extraction solvent dispersed entirely in the aqueous sample phase is formed. After centrifugation, the extractant droplet floating can be quickly solidified on an ice bath and taken with a spatula spoon, after melting, the droplet is used for analytes determination. In several works, prior to GC or LC analysis, the enriched phase is mixed with an adequate solvent to decrease its viscosity [24, 25]. Ethanol, methanol and acetonitrile are the solvents most commonly used for this purpose. The volume of the diluted extract is conditioned mainly by the minimum volume necessary to carry out the analytical measurement.

In relation to environmental analysis, DLLME-SFO has been applied to water samples for determining specific groups of pollutants such as organochlorine pesticides [26, 27], dinitrobenzenes [4], herbicides [28], heterocyclic aromatic amines [29] and PAHs [25, 30]. However, as far as we know, DLLME-SFO and LC-MS/MS has not been developed for the simultaneous extraction and determination of nitrated and oxygenated PAHs in water samples.

Considering the related problems associated to low density and high viscosity of the solvent used in DLLME-SFO, a back-extraction (SBE) step is proposed prior to LC-MS/MS analysis. In this work, an original dispersive liquid-liquid microextraction based on the solidification of a floating organic drop followed by an innovative solvent assisted back-extraction (DLLME-SFO-SBE) approach coupled to ultra high

performance liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (UHPLC-(+)APCI-MS/MS) for the extraction/enrichment/quantitative determination of nitrated and oxygenated-PAHs of ecotoxicological importance-1-nitropyrene, 2-nitrofluorene, 3-nitrofluoranthene, 9-nitroanthracene, 5,12-naphthacenedione, 9,10-anthracenedione, and 2-fluorenecarboxaldehyde- in environmental water samples is proposed.

Part of the study and optimization of DLLME-SFO herein proposed was previously developed for the extraction of only two nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) followed by fluorescence detection [31]. In this work, after evaluating the experimental conditions, the methodology proved to be suitable for the efficient extraction of all nitro-PAHs and oxy-PAHs above mentioned. However, in contrast to fluorescence, the determination of compounds by UHPLC-APCI-MS/MS after DLLME-SFO has a crucial step to guarantee compatibility, the solvent assisted back-extraction (SBE) procedure herein developed. Thus the various parameters that could affect mostly the solvent assisted back-extraction efficiency were studied and optimized.

2. Materials and methods

2.1. Reagents and chemicals

Seven environmentally relevant chemical standards of nitrated and oxygenated PAHs were selected. Thus chemical standards of 1-nitropyrene (1-NPYR), 2-nitrofluorene (2-NFLU), 3-nitrofluoranthene (3-NFLUANTH), 9-nitroanthracene (9-NANTHR), 5,12-naphthacenedione (5,12-NAPHTONE), 9,10-anthracenedione (9,10-ANTHRONE), and 2-fluorenecarboxaldehyde (2-FLUCHO) were purchased from Sigma Chemical (St.

Louis, MO, USA). The nitrated and oxygenated PAHs derivatives studied are shown in **Fig. 1**.

Acetonitrile (ACN), methanol (MeOH), acetone and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, New Jersey) and the extractant 1-dodecanol (99%) were purchased from Sigma Chemical (St. Louis, MO, USA). Formic acid and nitric acid (HNO_3) were obtained from Fisher Scientific (Loughborough, UK).

2.2. Preparation of standard solutions

Standard working solutions at different concentrations were prepared daily in methanol by appropriate dilution of a 10 mg L^{-1} stock standard solutions of each compound. All water samples and stock standard solutions were protected from light and stored at −4°C to prevent degradation.

2.3. Instrumentation and conditions

2.3.1. Ionization and MS conditions

Mass spectrometry analyses were performed on a Quattro Premier™ XE Micromass MS Technologies, triple quadrupole mass spectrometer with a ZSpray™ equipped with APCI interface (Waters, Milford, USA) configured in positive ion mode. The source was operated in a positive mode at 400 °C with N_2 as the nebulizer and the source temperature was kept at 120 °C. The corona discharge current was maintained at 3.0 μA and the extractor voltage was set at 4.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 200 L h^{-1}. Argon was used as collision gas at a flow of 0.18 mL min^{-1} achieving and collision gas pressure was 0.00358 mbar.

Experimental parameters for the APCI source were adjusted and some of the operational variables were optimized in full scan mode. Quantitative determination was performed in multiple reaction monitoring (MRM) mode of the selected ion at the first (Q_1) and

third (Q_3) quadrupole. To choose the fragmentation patterns of *m/z* (Q_1) → *m/z* (Q_3) for the analytes under study in the MRM mode, direct infusion (via syringe pump) into the mass spectrometer of each standard solution in methanol was performed, the product ion scan mass spectra was recorded. A standard solution of each PAHs derivative at a concentration of 500 µg L^{-1} and at a flow rate of 25 µL min^{-1} were injected directly. The data were acquired using Mass Lynx Spectrometry Software (Waters, Milford, USA).

2.3.2. Chromatographic separation

An Acquity™ Ultra High Performance LC system (Waters, Milford) equipped with autosampler injection and pump systems (Waters, Milford) was used. The needle was washed with proper mixtures of acetonitrile and methanol. The separation was performed with an ACQUITY UPLC® BEH Phenyl (Waters, Milford, USA) analytical column. Variations of the flow rate (flow gradients) combined with solvent gradients were used for the compounds separation. Under these conditions, no sample contamination or sample to sample carryover was observed. General conditions are summarized in **Table 1**.

2.4. Sampling and sample preparation

In this assay, two local environmental water samples, including drinking water and lake water, were collected. Drinking water samples were obtained from our lab (33º 17' 29.5368"S, 66º 20' 24.7194"W, San Luis province, Argentina) and the lake water was collected from different places of the Potrero de Los Funes reservoir (33º 14' 6.2376"S, 66º 13' 59.8908"W, San Luis Province, Argentina). These samples were collected in large 1 L dark-glass bottles and all filtered through a 0.45 µm filter and stocked in amber glass at 4 °C avoiding light. Before sample collection, the bottles were cleaned.

Aqueous samples pH was adjusted to 2 with HNO_3 to suppress all the microbiological activity.

2.5. DLLME-SFO-SBE procedure

The method DLLME-SFO is based on a previously developed approach [31], with some modifications. The procedure considers now the extraction of seven nitro- and oxy-PAHs. The extraction strategy was developed as follows: a mixture of the disperser solvent (MeOH, 125 µL) and the extraction solvent (1-dodecanol, 50 µL) were injected rapidly. A cloudy mixture was formed with dispersion of fine organic droplets into the water sample. At this step, nitrated and oxygenated-PAHs molecules were extracted into 1-dodecanol in a few seconds. After centrifugation at 3000 rpm (1106.8 g) for 10 min, the organic droplet was floating at the top of the glass tube because of the low density of the extraction solvent. Following, the floating organic drop is pulled into a Hamilton syringe (at room temperature) and then placed into a glass test tube with screw cap. Another difference with our previous proposed methodology is the incorporation of the solvent assisted back-extraction stage (SBE). This back-extraction was carried out by adding 400 µL of ACN into the organic solvent phase kept at room temperature. After that, the mixture was vortexed for 0.5 min and centrifuged at 3000 rpm (1106.8 g) for 2 min. Finally, the obtained solution was transferred into an ice bath again to remove the solidified solvent and the remaining ACN phase, about 300 µL, was transferred into amber vials and stored for subsequent UHPLC-MS/MS analysis. The extraction steps are illustrated in **Fig. 2**. It is important to mention that no group-dependent compounds extraction behaviors were observed, which correlates with the findings previously reported for the DLLME-SFO extraction of a group of ten PAHs [24].

2.6. Method validation

2.6.1. Enrichment factor and recovery

There are typically two ways to display and compare data attained during an optimization process, by means of the enrichment factor (EF) and/or the recovery (Recovery (%)). Thus, the enrichment factor can be defined as the ratio between the analyte concentration in the floating phase solvent ($C_{flotaed}$) and the initial concentration of analyte ($C_{initial}$) within the sample:

$$EF = \frac{C_{flotaed}}{C_{initial}}$$

On the other hand, recovery was obtained from the following equation:

$$Recovery\ (\%) = \frac{(C_{found} - C_{real})}{(C_{added})}\ x\ 100$$

Where: C_{found} represents the concentration of the analyte after adding a known amount of standard to the real sample, C_{real} is the concentration of the analyte in the real sample, and C_{added} is the concentration of known amount of standard that was spiked to the real sample [25, 29, 32].

2.6.2. Limit of detection and limit of quantification

Taking into account the behavior of the compounds during ionization (i.e. ion suppression due to matrix components) and its influence on the variability and calibration results, an approach with spiked samples was preferred instead of using blank samples (from which the signal-to-noise ratio is commonly obtained and used for the calculation of the LoD and LoQ). Thus, a calibration based on spiked samples was performed. As result, the figures of merit were calculated as follows:

$$LoD = \frac{3.3S_{y/x}}{b}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}}$$

$$LoQ = \frac{10S_{y/x}}{b}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}}$$

Where $\bar{x}$ corresponds to the mean concentration, $S_{y/x}$, the residual standard deviation, *b*, the slope of the calibration curve, *m*, the number of replicates per concentration level of the spiked samples, and *n*, the number of concentration levels for spiked samples: $i = 1, 2 \ldots I$.

2.6.3. Precision and recovery

In order to evaluate the methodology, precision and recovery were calculated. Precision of the whole method was evaluated in terms of repeatability (intraday precision) and reproducibility (inter-day precision). Reproducibility was evaluated with a similar procedure in five different days. Water spiked samples composed by 3 blanks, and 3 replicates at 2.5 ng mL^{-1}; 5 ng mL^{-1} and 10 ng mL^{-1} were analyzed under the conditions mentioned above.

2.6.4. Linearity

Calibration curves were obtained by least-squares linear regression analysis of the intensity of signal versus nitro-PAHs and oxy-PAHs concentrations. Linearity was evaluated from values closer to the LoQ up to approximately 500 ng mL^{-1}.

2.6.5. Matrix effect

The matrix effect was evaluated because different constituents present in the matrix can modify the instrumental response of the analyte under interest, resulting in a suppression or enhancement of the signal. Matrix effect was calculated by the use of the

matrix-assisted calibration curves of each compound. In this sense, the slopes of the calibration curves of compounds standards in both pure solvent (ACN) and spiked samples were compared. The percentage of the quotient of the slopes (*b*) in the spiked and solvent samples was used as an indicator of the extent of the ion suppression or signal enhancement, which was calculated as *100 - (b spiked / b solvent × 100)*.

3. Results and discussion

3.1. Optimization of instrumental conditions

Nitro-PAHs and oxy-PAHs standard solution (5 μg L^{-1}) in methanol was introduced into the MS system at a flow rate of 25 μL min^{-1} via a syringe pump. The APCI method was optimized with respect to dominant conditions such as corona current, source temperature, probe temperature, drying gas flow rate, and drying gas temperature, as were mentioned in "Ionization and mass spectrometry conditions" (see section 2.3.1). The MRM conditions were further optimized for each analyte to obtain maximum sensitivity and they are shown in **Table 2**. Thus the most sensitive transitions for each compound were selected for quantification purposes.

Many chromatographic separation variables were optimized to provide good peak shapes and short retention times for the studied PAHs derivatives. Screening experiments showed that to obtain an efficient separation and determination of the nitro-PAHs and oxy-PAHs, various linear gradients of aqueous solution with methanol, acetonitrile, at different concentrations of formic acid in the mobile phase, were investigated. A mixture of water/acetonitrile as gradient solvents was the best choice. In addition, variations of the flow rate (flow gradients) as well as its combination with solvent gradients were studied for the compounds separation to obtain the required

selectivity within a short time period. The optimal solvents gradient and flow gradients were mentioned in the "Chromatographic separation" section (see section 2.3.2). In this study, variable formic acid concentrations ranging from 0.01 to 0.3 % (v v^{-1}) were tested to enhance the signal response. The use of 0.1% (v v^{-1}) formic acid improved retention time, peak shape, and sensitivity in comparison with the results obtained without buffer. The optimal conditions were compatible with the APCI interface.

The effect of temperature over the separation of nitrated and oxygenated PAHs derivatives was evaluated. Thus the chromatographic column was thermostated between 20 and 60 °C. For all the studied compounds the retention's behavior decreased when increasing the column temperature. The optimal retention conditions were obtained when the temperature was fixed at 35 °C.

Under optimum conditions (**Table 1**), the seven analytes eluted from the column within a 5.5 min total run cycle, which was considerably shorter than the chromatographic runs reported in recent works [4, 15, 33] and the herein optimized cycle was similar to the one reported by Fujiwara *et al.* [14]. Representative MRM chromatograms of the nitro- and oxy-PAHs are shown in **Fig. 3.**

The use of UHPLC–MS/MS combines the separation capability of UHPLC and the selective detection power of MS/MS, which facilitates the identification of unresolved peaks even if coeluted peaks are present. For instance, as shown, 1-NPYR and 3-NFLUANTH coeluted and they have the same parent ion (m/z 248). Bearing in mind that retention time and precursor ion were similar for both analytes, the product ions were selected taking into account that they must be different in order to avoid interferences. In this sense, both nitro-PAHs generated an intensive product ion at m/z 218, and this could not be used for quantification purposes because, as mentioned, both

compounds can interfere each other during determination, so specific transitions for each nitro-PAHs were selected to monitor these two compounds (see **Table 2**).

3.2. Optimization of the back-extraction in DLLME-SFO

The optimization of DLLME-SFO method had previously been developed for the extraction of only two nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) in water samples followed by fluorescence detection [31].

In order to evaluate the efficient extraction of all nitro-PAHs and oxy-PAHs herein studied, further studies based on the performance of several extraction solvents, including not only 1-dodecanol, but also 1-undecanol and 2-dodecanol, were developed. For this and the following optimization studies, sample aliquots of 10 mL spiked with 40 ng mL^{-1} of each analyte were used to investigate extraction conditions and the corresponding peak area was used to evaluate the extraction efficiency of the proposed DLLME-SFO method. From the findings, the major extraction capability was observed for 1-dodecanol, which agreed with the results obtained for several persistent organic pollutants and PAHs. However, due to compatibility issues, the determination of compounds by UHPLC-APCI-MS/MS after DLLME-SFO has a crucial and unavoidable step, the back-extraction (SBE) strategy. In this work, the back-extraction procedure consisted in the transfer of the analytes from the floating organic drop, after extraction DLLME-SFO, to a solvent compatible with the system of analysis.

There are two reasons for using DLLME-SFO with SBE to extract and determine these compounds: extraction solvents commonly used exhibit low melting point, consequently, temperature of both laboratory and instrumental setup should be carefully controlled. Secondly, these solvents are too viscous and exhibit low volatility to directly

inject them into the ionization/mass spectrometry system. Therefore, the back-extraction protocol plays an important overpassing the above mentioned drawbacks.

3.2.1. Selection of the nature and volume of back-extraction solvent

Major criterion in the selection of a solvent for back-extraction are: (i) compatibility of the solvent with LC-MS (ii) greater solubility of the nitro- and oxy-PAHs in this solvent than in the organic extractant and (iii) the selected solvent should be immiscible with the organic extractant solvent so that the organic phase can be discarded easily, before injecting into the LC-APCI-MS/MS system. Taking these considerations into account for back-extraction, several solvents were tested. From the results, similar volumes of acetonitrile (ACN) and 1-dodecanol satisfied only the condition of to be partially immiscible [25]. Since density of 1-dodecanol (0.830 g cm^{-3}) is higher than acetonitrile (0.786 g cm^{-3}), after mixing and centrifuging these solvents, 1-dodecanol solidified itself in the bottom of the conical tube and the upper acetonitrile phase was taken up with a micropipette and stored in an amber vial for UHPLC-MS/MS analysis (as shown in **Fig. 2**). Thus, as mentioned, ACN was selected as the back-extraction solvent.

Other important factor was the selection of the volume of the back-extraction solvent. To achieve this purpose, different volumes of ACN solvent were evaluated: 100, 200, 400, 600 and 800 µL. Results on the back-extraction efficiency are shown in **Fig. 4**. From the obtained results, it was observed that the recovery (Recovery (%)) was poor when the back-extraction solvent volume was lower than 200 µL. In all cases, the phase originated after back-extraction was difficult to separate and, as a consequence, irreproducible results were obtained. In contrast, at higher ACN volumes, greater than 600 µL, the solubility of 1-dodecanol in ACN increased, which resulted in deficient

phases separation, extraction efficiency and EF. Consequently, a volume of 400 µL of ACN was used as optimal.

3.2.2. Temperature effect

A series of experiments were designed for the optimization of the back-extraction temperature. Sample tubes containing the floating phase/acetonitrile were placed for 10 min in a water bath maintained at 15, 25, 35, 45 and 50 °C. The results are shown in **Fig. 5**. As can be seen, when decreasing the temperature from 25 to 15 °C or increasing this variable above 35 °C, extraction efficiency and EF decreased. On the other hand, when the temperature was kept in the range between 25 and 35 °C, optimal extraction recoveries were achieved for all of the compounds. Degradation of nitro-PAHs at high temperature is most likely the reason of low efficiency at high temperatures [8]. According to these results, SBE laboratory temperature was also set at 25 ± 5 °C, which was suitable for performing the mentioned procedure.

3.2.3. Ultrasound application and vortex agitation time

Application of ultrasound and vortex agitation are efficient treatments to enhance liquid-liquid microextraction [12] and [23]. These strategies might also affect the back-extraction efficiency because their effect on the mass transfer process. Both treatments were compared and the ER was monitored with back-extraction times varying from 0.25 to 1.5 min (**Fig. 6**). Evidently, the surface mass transfer process was more rapid and efficient with vortex agitation. The results showed that there were no significant differences in recovery values by increasing the time from 30 s to 1.5 min of vortex agitation. Thus vortex agitation for a 30 seconds was adopted in the subsequent experiments.

3.2.4. Centrifugation and ice bath times

Centrifugation is important in SBE for phase separation. The effect of centrifugation time and speed was studied for the range from 2 to 10 min and from 2000 to 3500 rpm; respectively, finding that, in general, the EFs for all the analytes were higher within the interval between 2 and 5 min. At higher centrifugation times, the analytical response of the studied compounds diminished since the sedimented 1-dodecanol in the BE could be partly dissolved in the acetonitrile phase by the generated heat due to an inappropriate centrifugation time. In addition, centrifugation time could also affect phase separation lowering this way the obtained recoveries. Consequently, centrifugation time of 2 min was selected since this time was enough for complete phase separation and longer periods of times did not demonstrate improvements on analyte extraction (**Fig. 7-A**).

During back-extraction and after centrifugation, the tube was immersed in an ice bath, thus the 1-dodecanol solvent solidified after a short period of time at the bottom of the tube. The upper, non-solidified phase was formed by ACN, the back-extraction solvent, and PAHs derivatives. Therefore, ice bath time was another important parameter to optimize and efficient separation of phases. To investigate these effect, ice bath periods from 3 to 15 min were evaluated. The results showed that the ice bath times above 5 min have no significant effects on back-extraction efficiency (**Fig. 7-B**).

3.3 Analytical performance

The figures of merit for the proposed DLLME-SFO-SBE coupled to UHPLC-MS/MS method, including enrichment factor factors (EF), linear ranges (LR), limit of detections (LOD) and quantification (LOQ), and inter-day and intra-day precisions were calculated. In addition, recoveries (Recovery (%)) for nitro-PAHs and oxy-PAHs were attained under optimum conditions. The results are summarized in **Table 3**. Good

linearity for each compound was observed, with correlation coefficients (r^2) higher than 0.990 for all of them. The F-test demonstrated that linear regression was statistically acceptable in the working range and this model showed goodness of fit. Limits of detection and quantification were from 0.02 to 0.85 ng L^{-1} and from 0.15 to 1.10 ng L^{-1}, respectively. The EFs and ERs for the nitro-PAHs and oxy-PAHs ranged from 192 to 200-fold and from 95.2 to 100%, respectively (**Table 3 and 4**). Thus the herein proposed methodology showed highly satisfactory analytical performance based on the LODs, LOQs, and ERs obtained for nitro- and oxy-PAHs. In addition, the EFs achieved were considerably higher than those obtained in the DLLME extraction of dinitro-aromatic compounds (EF ~ 85), nitrophenols (EF ~ 90), and PAHs (EF ~ 88-118) reported in the literature [4, 25, 34]. Moreover, a higher analytical performance of the proposed methodology was observed in relation to that presented in our previous work [31], this is mainly due to the separation and detection UHPLC-MS/MS system used, which provided greater selectivity and sensitivity for the simultaneous determination of the PAHs derivatives under study, which allowed a significant improvement in the sensitivity for the determination of this group of contaminants in water samples.

As mentioned in section 2.6.5, the matrix effect was evaluated because different constituents present in the matrix can modify the instrumental response of the analyte under interest, resulting in a suppression or enhancement of the signal. From the obtained results, after applying SBE, sample matrices have negligible effect on the PAHs derivatives studied. Therefore, quantification was performed by means of external calibration.

3.4 Application to real samples

The samples were then spiked with nitrated and oxygenated PAHs standards at concentration levels from 2.5 to 10 ng mL^{-1}. The sample analysis and recovery studies were performed in triplicate. The obtained recoveries for the nitro-PAHs and oxy-PAHs in lake and drinking water samples are summarized in **Table 4**. As expected, most of the targeted nitrated and oxygenated PAHs were not detected in drinking water samples, except for 1-NPYR and 2-FLUCHO, which were found at concentrations of approximately 1 ng mL^{-1}. To the best of our knowledge, this work constitutes the first report in which some of the PAHs derivatives were found in drinking water samples.

On the other hand, the concentrations of nitro-PAHs and oxy-PAHs in lake water were approximately 1.5 ng mL^{-1} and 0.72 ng mL^{-1}, respectively. These samples were collected from small water reservoirs strongly influenced by boats and local and tourist traffic. Additionally, the surrounding areas of the lake from where the samples were collected were recently affected by wildfires, which could be one of the reasons of the presence of nitrated and oxygenated PAHs in these samples. Thus, the obtained results demonstrated the presence of nitro-PAHs at concentrations significantly higher than those reported in previous studies for river, sea and wastewater samples [8, 11, 12, 19, 35]. On the other hand, although literature regarding determination of oxy-PAHs in water samples is still scarce, this work's findings are in agreement with the results reported by other authors [8, 12, 16].

3.5 Comparison with other methods

Separation and determination of nitrated and oxygenated PAHs by the developed DLLME-SFO-BE and LC-MS/MS method was compared with the other methods used for the determination of these compounds and the results are shown in **Table 5**. As

shown, in general, most of the methods require large amount of toxic organic solvent (10-200 mL), high sample volumes (100 – 8000 mL) and long extraction times (~45 min). Thus the optimized DLLME-SFO-BE procedure appears as an advantageous alternative when compared to these approaches, showing the potential of DLLME-SFO-BE to be coupled to LC-MS/MS for the routine monitoring of pollutants in water samples.

4. Conclusions

In this work, a method based on DLLME-SFO-SBE coupled to LC-MS/MS was developed for efficient extraction, clean-up, enrichment and simultaneous determination of nitrated and oxygenated PAHs in drinking water and lake samples. All compound concentrations found were in the ng mL^{-1} range, except for 9,10-ANTHRONA that was not detected. Additionally, the proposed DLLME-SFO-SBE procedure resulted simple, fast, and effective, and presented eco-friendly advantages. The satisfactory obtained results indicated that the proposed method would be a valuable alternative for the routine analysis of these and others typical pollutants in real-not only environmental-samples. The proposed methodology was found to have a suitable performance in terms of accuracy, linearity, repeatability and limits of detection and quantification. The presence of nitrated and oxygenated PAHs derivatives demonstrated the importance and necessity of a continuous monitoring of these compounds in water samples.

Acknowledgements

Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica and Instituto de Química de San Luis (INQUISAL, UNSL-CONICET) are gratefully acknowledged for financial support.

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Fig. 1 Structures and abbreviations of the nitro-PAHs and oxy-PAHs studied

Fig. 2 Scheme of the experimental SBE-DLLME-SFO procedure applied for extraction and enrichment of nitrated and oxygenated PAHs followed by UHPLC-(+)APCI-MS/MS

Fig. 3 Chromatograms of the seven compounds determined by UHPLC-(+)APCI-MS/MS: **(A)** 9,10-ANTHRONE (tr: 2.72 min); **(B)** 2-FLUCHO (tr: 2.83 min); **(C)** 2-NFLU (tr: 3.17 min); **(D)** 5,12-NAPHTONE (tr: 3.30 min); **(E)** 1-NPYR (tr: 4.10 min); **(F)** 3-NFLUANTH (tr: 4.17 min); **(G)** 9-NANTHR (tr: 4.49 min)

Fig. 4 Influence of the back-extraction solvent volume, ACN, on the EFs of the nitro-PAHs and oxy-PAHs. Conditions of extraction: concentration of the mixture standard solution: 40 ng mL^{-1}; sample volume: 10 mL; volume of 1-dodecanol (extracting solvent): 50 µL. Back-extraction conditions: vortex time: 0.5 min; centrifugation rate/time: 3000 rpm/2 min

Fig. 5 Effect of temperature on the back-extraction efficiency. Concentration of mixture standard solution: 40 ng mL^{-1}; sample volume: 10 mL; 1-dodecanol (extracting solvent) volume: 50 µL. Back-extraction conditions: volume of ACN (back-extraction solvent): 400 µL; vortex time: 0.5 min; centrifugation rate/time: 3000 rpm/2 min

Fig. 6 Ultrasound application **(A)** and vortex agitation **(B)** effect on the extraction efficiency. Concentration of mixture standard solution: 40 ng mL^{-1}; sample volume: 10 mL; 1-dodecanol (extracting solvent) volume: 50 µL. Back-extraction conditions: volume of ACN (back-extraction solvent): 400 µL; vortex time: 0.5 min; centrifugation rate/time: 3000 rpm/2 min

Fig. 7 Centrifugation **(A)** and ice bath **(B)** time effect on the extraction efficiency. Concentration of mixture standard solution: 40 ng mL^{-1}; sample volume: 10 mL; 1-dodecanol (extracting solvent) volume: 50 µL. Back-extraction conditions: volume of ACN (back-extraction solvent): 400 µL; vortex time: 0.5 min; centrifugation rate: 3000 rpm

Table 1 UHPLC experimental conditions

Injected sample volume (µL)	10
Temperature column (°C)	35
Mobile phases	(A): H_2O - 0.1 % (v/v) formic acid (B): ACN - 0.1 % (v/v) formic acid
Solvent gradient (A:B)/run time(min)	60:40 (0.0); 10:90 (3.0); 0:100 (3.7); 60:40 (5.0-5.5)
Flow rate (mL min^{-1})/run time (min)	0.25 (0.0); 0.20 (3.7); 0.15 (4.0); 0.25 (5.0-5.5)
Autosampler temperature (°C)	25

Table 2 MRM conditions for (+)APCI-MS/MS determination

Compounds	Cone (V)	Precursor ion (m/z)	Collision (V)	Product ion (m/z)
nitro-PAHs				
1-NPYR	30	248	16	218
			25	202*
			30	190
2-NFLU	30	212	12	195
			17	165*
3-NFLUANTH	19	248	17	231*
			16	218
			20	190
9-NANTHR	19	224	8	207
			30	178*

oxy-PAHs				
			35	242
5,12-NAPHTONE	10	259	21	231
			23	203*
			20	181
9,10-ANTHRONE	35	209		
			20	153*
2-FLUCHO	32	195	16	167*

*Quantification transition

Table 3 Analytical figures of merit of the DLLME-SFO-SBE methodology combined with UHPLC-(+)APCI-MS/MS

Compounds	r^2	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	DLLME-SFO		Back-extraction		Intra-day precision	Inter-day precision
					Recovery (%)	EF	Recovery (%)	EF	(RSD%, n = 3)	
nitro-PAHs										
1-NPYR	0.9999	0.25-250	0.05	0.23	99,9	200	98,3	24,6	3.29	6.21
2-NFLU	0.9969	1.00-500	0.26	0.87	96,2	192	95,3	23,8	5.41	7.01
3-NFLUANTH	0.9983	1.00-500	0.85	1.10	94,9	190	94,5	23,6	2.90	3.30
9-NANTHR	0.9902	1.00-500	0.28	0.69	99,1	199	97,3	24,3	5.09	7.53
oxy-PAHs										
5,12-NAPHTONE	0.9984	0.50-500	0.17	0.51	96,5	193	95,4	23,9	3.97	4.94
9,10-	0.9978	1.00-500	0.14	0.76	98,6	197	97,5	24,4	3.20	4.33

ANTHRONE										
2-FLUCHO	0.9999	0.25-250	0.02	0.15	99,6	200	97,8	24,4	1.38	2.98

Table 4. Analysis of water samples using the proposed methodology[a]

Compounds	*Drinking water samples*					*Lake water samples*				
	Sample concentration (ng mL^{-1})[b]	Added Concentration (ng mL^{-1})	Found concentration (ng mL^{-1})	R (%)[c]	RSD (%) (n=3)	Sample concentration (ng mL^{-1})[b]	Added Concentration (ng mL^{-1})	Found concentration (ng mL^{-1})	R (%)[c]	RSD (%) (n=3)
nitro-PAHs										
1-NPYR	1.07±0.15	2.50	3.55	99.2	2.3	7.16±0.15	2.50	9.54	95.2	2.3
		5.00	5.92	97.0	0.9		5.00	12.08	98.4	1.5
		10.00	10.90	98.3	1.0		10.00	16.77	96.1	3.6
2-NFLU	n.d.(0.26)[d]	2.50	2.41	96.4	2.5	6.25±0.56	2.50	8.68	97.2	3.4
		5.00	4.88	97.6	3.5		5.00	11.02	95.4	2.1
		10.00	9.80	98.0	1.2		10.00	15.70	94.5	4.5
3-NFLUANTH	n.d.(0.85)	2.50	2.39	95.6	5.3	1.57±0.17	2.50	4.06	99.6	5.6
		5.00	4.86	97.2	4.5		5.00	6.32	95.0	5.7

		10.00	9.62	96.2	2.6		10.00	11.19	96.2	4.3
9-NANTHR	n.d.(0.28)	2.50	2.47	98.8	1.2	0.97±0.19	2.50	3.45	99.2	1.1
		5.00	4.85	97.0	1.9		5.00	5.82	97.0	1.9
		10.00	9.63	96.3	2.3		10.00	10.82	98.5	1.0
oxy-PAHs										
5,12-NAPTHONE	n.d.(0.17)	2.50	2.38	95.2	3.5	0.69±0.23	2.50	3.09	96.0	2.3
		5.00	4.85	97.0	3.4		5.00	5.54	97.0	5.6
		10.00	9.60	96.0	2.9		10.00	10.32	96.3	4.7
9,10-ANTHRONE	n.d. (0.14)	2,50	2.50	100.0	2.4	n.d (0.14)	2,50	2.50	100.0	2.1
		5.00	4.91	98.2	3.7		5.00	4.82	96.4	3.6
		10.00	10.04	100.4	1.9		10.00	10.00	100.0	1.9
2-FLUCHO	0.82±0.17	2.50	3.22	96.0	2.3	2.36±0.56	2.50	4.78	96.8	2.7
		5.00	5.70	97.6	4.2		5.00	7.21	97.0	3.6

	10.00	10.82	100.0	1.9		10.00	11.85	94.9	3.9

[a]Conditions as described in Section 2.5

[b] Mean value ± standard deviation

[c] Recovery, $n = 3$ replicates

[d] n.d., not detected (detection limit)

Table 5. DLLME-SFO-BE extraction compared with other methods reported for nitrated and oxygenated PAHs determination

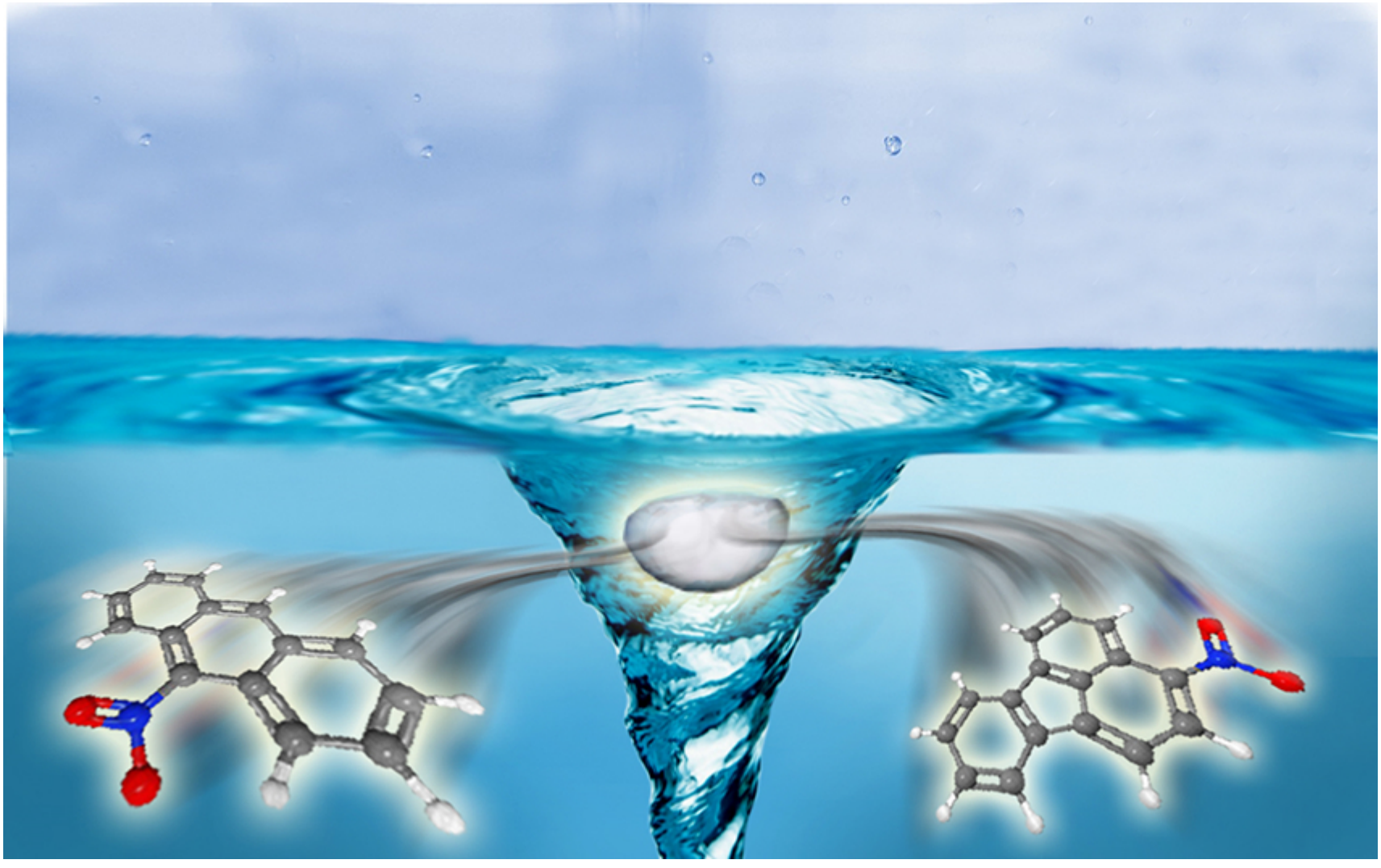
Method	Separation-detection technique	Compounds	Sample volume (mL)	Extraction solvent (volume, mL)	LOD (ng L^{-1})	R (%)	Total procedure time (min)	Sample	References
SPE	GC-MS	nitro-PAHs and oxy-PAHs	4000	Dichloromethane (60)	0.02 – 7.40	45-158	[a]	river and wastewater	[7, 8]
C18-disk	HPLC-fluorescence	nitro-PAHs	2000	Dichloromethane ([a])	0.18 -6.24	87-104	[a]	river water	[17]
SPE	HPLC-chemiluminescence	nitro-PAHs	1500	Dichloromethane (20)	0.009 – 0.041[b]	71-103	40	river water	[10]
SPE	GC-MS	oxy-PAHs	500	Dichloromethane (20)	0.2 – 4.8	78-149	[a]	seawater	[16]
SPE	µLC-UV	nitro-PAHs	100	Dichloromethane (10)	0.008 – 0.058[b]	80-97	[a]	river water	[34]
SPME	GC-MS	nitro-PAHs	10	[a]	0.004 – 0.059	91-102	45	tap and well water	[18]

PA/HS-SPME	GC-MS	nitro-PAHs	10	[a]	0.01 – 0.11	[a]	45	river water	[19]
SDME	GC-MS	nitro-PAHs and oxy-PAHs	10	Toluene (1)	0.6 - 468 [b]	23-134	30	river, sea and groundwater	[12]
DLLME-SFO	Fluorescence detection	nitro-PAHs	10	1-dodecanol (0.025)	2.3 – 5.0 [b]	95-100	~ 10	tap and lake water	[31]
DLLME-SFO-BE	**UHPLC-MS/MS**	**nitro-PAHs and oxy-PAHs**	**10**	**1-dodecanol (0.05)**	**0.02 – 0.85 [b]**	**95-100**	**~ 13**	**tap and lake water**	**This method**

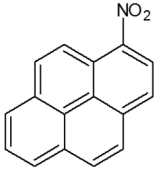
[a] n.m: not mentioned; [b]LOD (ng mL^{-1})

Highlights

- *Original DLLME-SFO-SBE strategy for extraction/enrichment of nitro and oxy-PAHs.*
- *Solvent assisted back-extraction step followed by UHPLC-(+)APCI-MS/MS analyses.*
- *Optimization of variables affecting the experimental system performance.*
- *Application to lake and drinking water samples.*



Graphics Abstract



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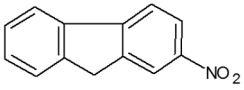
(1-NPYR)



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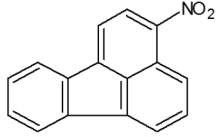

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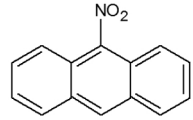
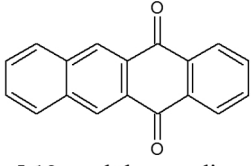
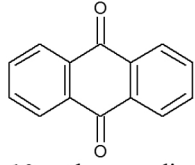
(3-NFLUANTH)



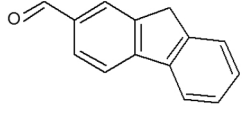
9-nitroanthracene
(9-NANTHR)



5,12-naphthacenedione
(5,12-NAPHTONE)

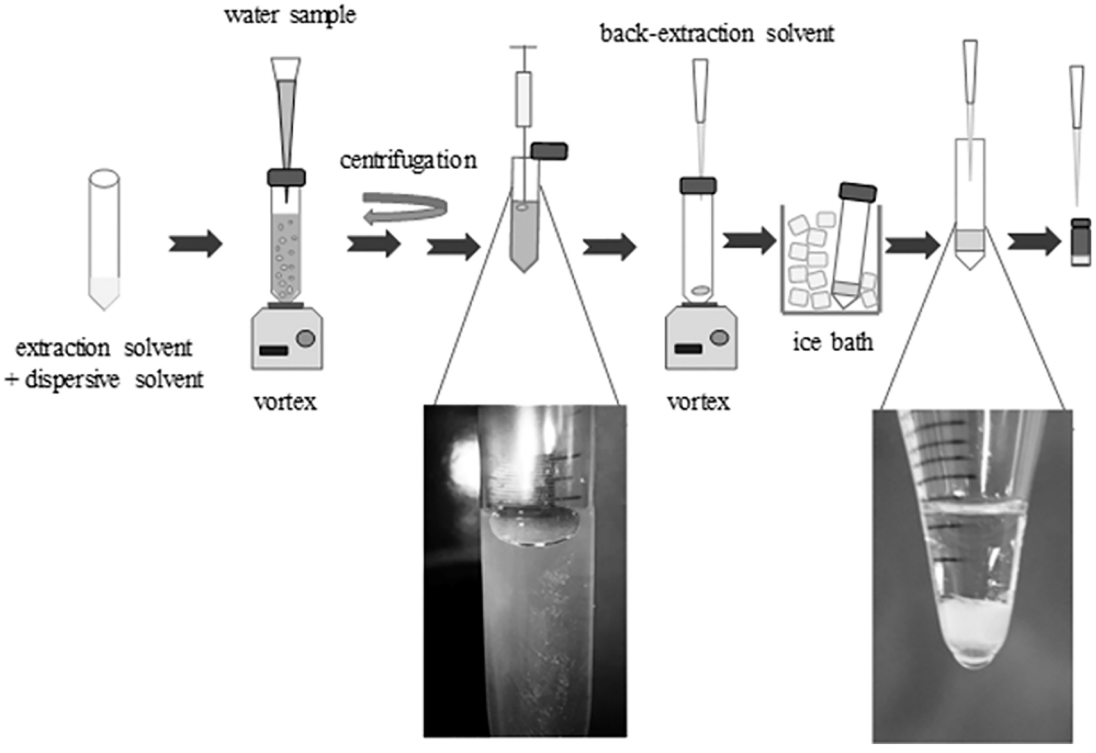


9,10-anthracenedione
(9,10-ANTHRONE)



2-fluorenecarboxaldehyde
(2-FLUCHO)

Figure 1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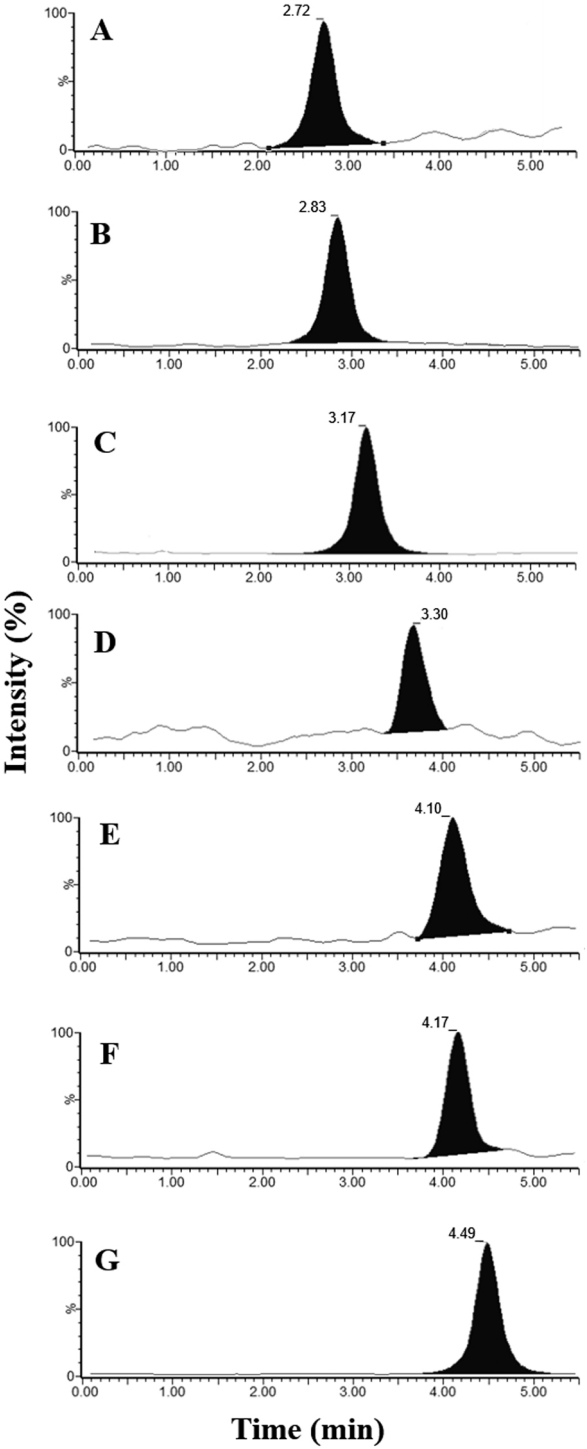
back-extraction solvent
centrifugation
extraction solvent
+ dispersive solvent
vortex
vortex
ice bath

Figure 2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B
2.83
C
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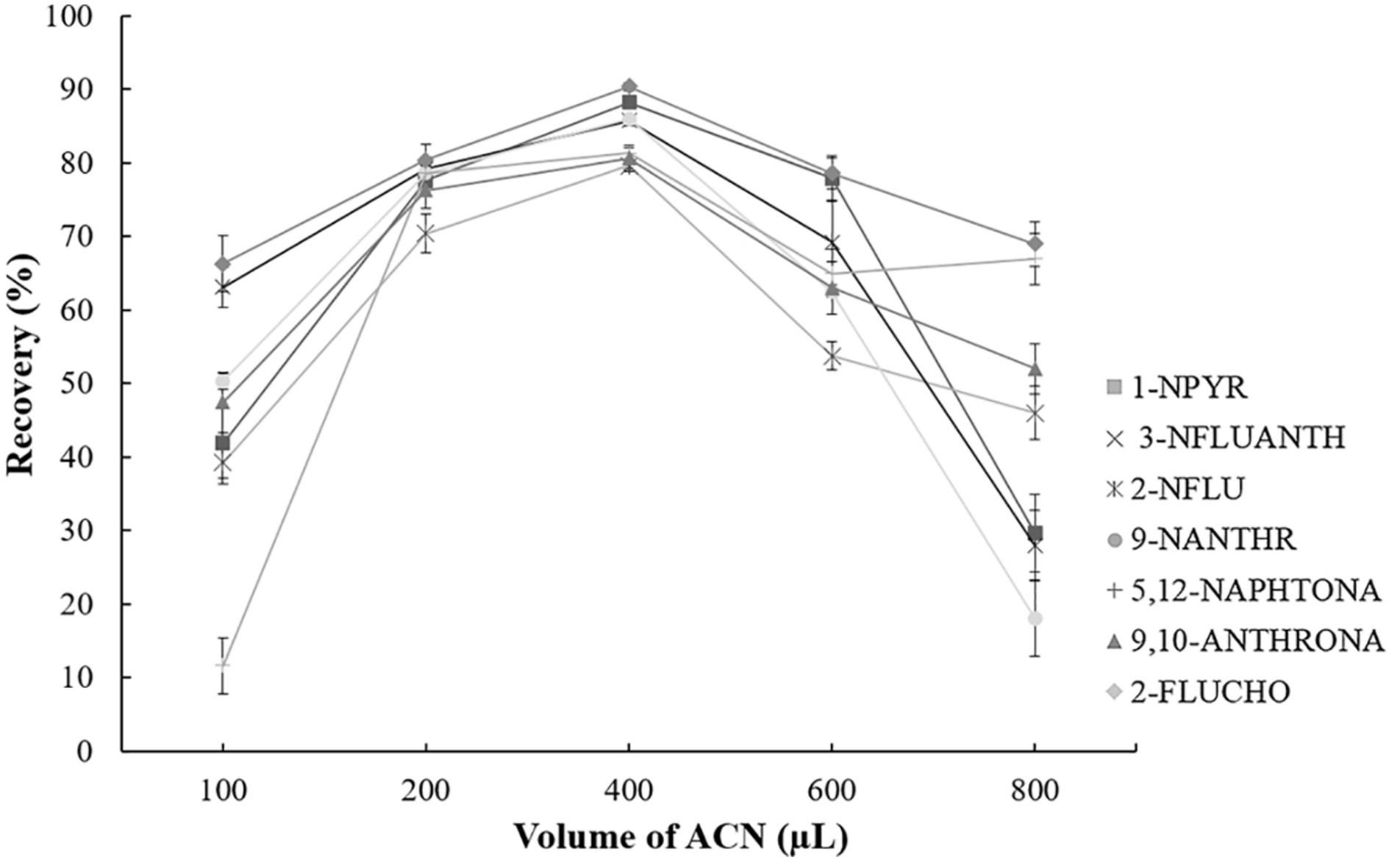
D
3.30
E
4.10
F
4.17
G
4.49
100
%
0
0.00
1.00
2.00
3.00
4.00
5.00
Intensity (%)
Time (min)

Figure 3


100
90
80
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50
40
30
20
10
0
Recovery (%)
100
200
400
600
800
Volume of ACN (µL)
1-NPYR
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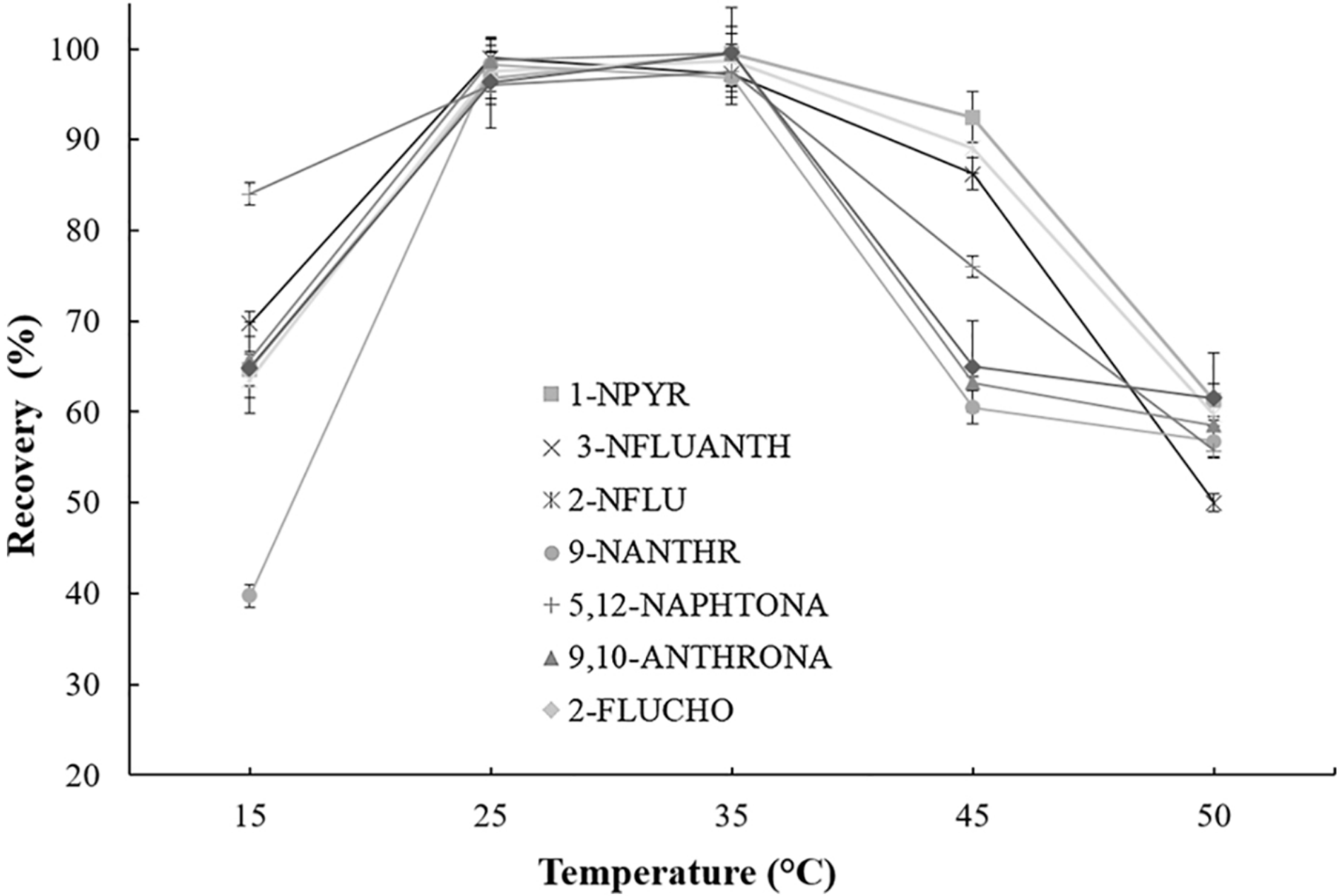
2-NFLU
9-NANTHR
5,12-NAPHTONA
9,10-ANTHRONA
2-FLUCHO

Figure 4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80
70
60
50
40
30
20
Recovery (%)
15
25
35
45
50
Temperature (°C)
1-NPYR
3-NFLUANTH
2-NFLU
9-NANTHR
5,12-NAPHTONA
9,10-ANTHRONA
2-FLUCHO

Figure 5

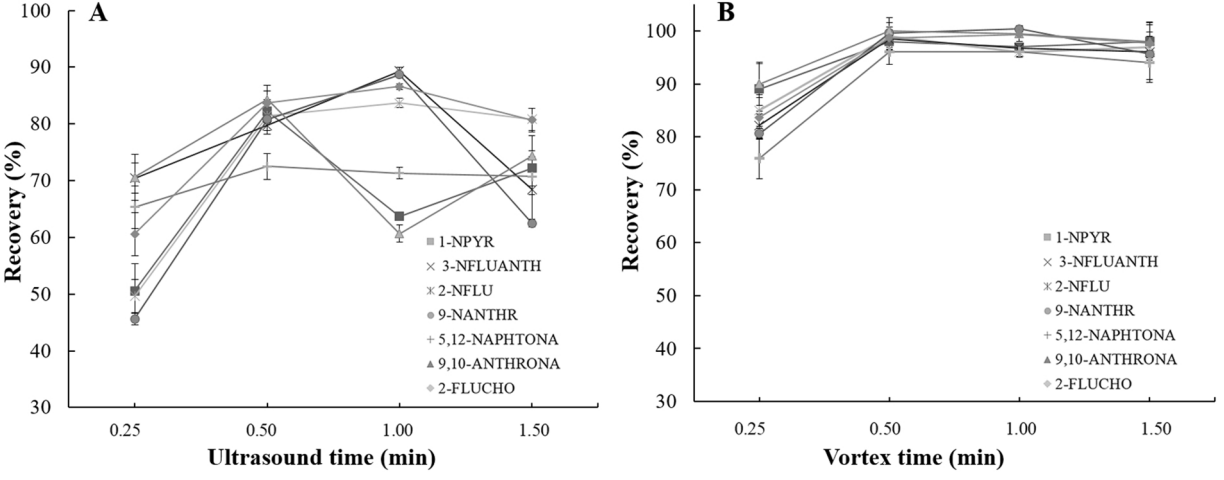
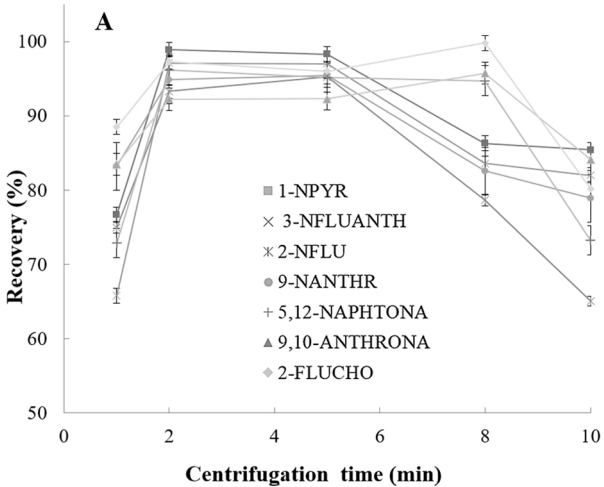

A
Recovery (%)
100
90
80
70
60
50
40
30
0.25
0.50
1.00
1.50
Ultrasound time (min)
1-NPYR
3-NFLUANTH
2-NFLU
9-NANTHR
5,12-NAPHTONA
9,10-ANTHRONA
2-FLUCHO
B
Recovery (%)
100
90
80
70
60
50
40
30
0.25
0.50
1.00
1.50
Vortex time (min)
1-NPYR
3-NFLUANTH
2-NFLU
9-NANTHR
5,12-NAPHTONA
9,10-ANTHRONA
2-FLUCHO

Figure 6


A
Recovery (%)
100
90
80
70
60
50
0
2
4
6
8
10
Centrifugation time (min)
1-NPYR
3-NFLUANTH
2-NFLU
9-NANTHR
5,12-NAPHTONA
9,10-ANTHRONA
2-FLUCHO

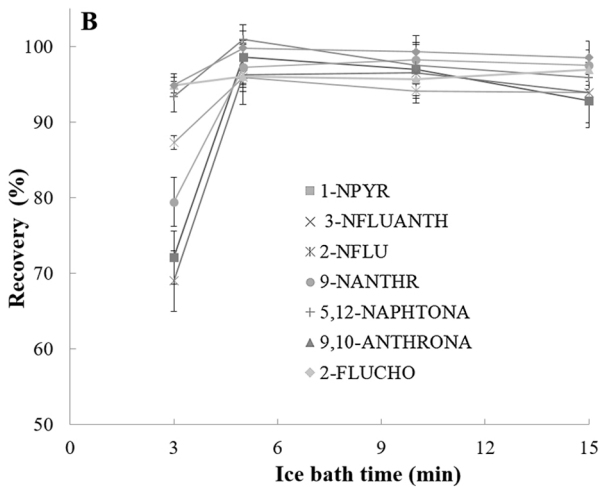

B
Recovery (%)
100
90
80
70
60
50
0
3
6
9
12
15
Ice bath time (min)
1-NPYR
3-NFLUANTH
2-NFLU
9-NANTHR
5,12-NAPHTONA
9,10-ANTHRONA
2-FLUCHO

Figure 7