



Determination of aqueous fullerene aggregates in water by ultrasound-assisted dispersive liquid–liquid microextraction with liquid chromatography–atmospheric pressure photoionization–tandem mass spectrometry

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ABSTRACT

A simple and solvent-minimized method for the determination of three aqueous fullerene aggregates (nC_{60} , nC_{70} , and aqueous [6,6]-phenyl C_{61} butyric acid methyl ester ($nPCBM$)) in water samples is described. The method involves the use of ultrasound-assisted dispersive liquid–liquid microextraction (UA–DLLME) coupled liquid chromatography–tandem mass spectrometry with atmospheric pressure photoionization (LC–APPI–MS/MS). The parameters affecting the extraction efficiency of the analytes from water samples were systematically investigated and the conditions optimized. The best extraction conditions involved the rapid injection of a mixture of 1.0 mL of 2-propanol (as a disperser solvent) and 10 μ L of benzyl bromide (as an extraction solvent) into 10 mL of an aqueous solution (pH 10.0) containing 1% sodium chloride in a conical bottom glass tube. After ultrasonication for 1.0 min and centrifugation at 5000 rpm (10 min), the sedimented phase 5.0 μ L was directly injected into the LC–APPI–MS/MS system. The limits of quantification (LOQs) were 150, 60 and 8 $ng L^{-1}$ for $nPCBM$, nC_{60} and nC_{70} , respectively. The precision for these analytes, as indicated by relative standard deviations (RSDs), were less than 12% for both intra- and inter-day analysis. Accuracy, expressed as the mean extraction recovery, was between 70 and 86%. A standard addition method was used to quantitate three aqueous fullerene aggregates, and the concentrations of these aqueous fullerene aggregates were determined to be in the range from n.d. to 130 $ng L^{-1}$ in various environmental samples including municipal influent and effluent samples, industrial wastewater samples, and surface water samples.

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1. Introduction

Nanoparticles (NPs), which are classified as emerging class of contaminants, enter the environment from both natural (volcanic eruption, forest fires) and industrial processes (diesel exhaust, industrial emissions, and products containing nanomaterials), and are present in soil, surface water, wastewater, and even drinking water [1,2]. A specific term, engineered nanoparticles (ENPs), is used to define NPs that are created and manufactured intentionally through industrial processes [3]. The first ENP, carbon-based nanomaterial, C_{60} (buckminsterfullerene) was manufactured by Kroto and co-workers in 1985 [4]. A series of fullerenes are currently being produced including C_{60} , C_{70} , and the derivatives of C_{60} , and the structures, properties, application, and toxicity of fullerenes

have been reported elsewhere [5–7]. Fullerenes can act as powerful antioxidants to prevent cell damage or death caused by free radicals, and they are extensively utilized in the cosmetics industry and in medicine [6]. Although the solubility of solid C_{60} in water is very poor ($<10^{-9} mg L^{-1}$), it can be dispersed in water by transferring from an organic solvent (i.e., toluene) using sonication [8,9]. Fullerenes can form stable colloidal solutions as aqueous fullerene aggregates (i.e., nC_{60}) in natural water (pH >3), and the letter “ n ” in nC_{60} represents not only the nanoscale in diameter but also the cluster structure of aqueous fullerene aggregates [8–11]. Evidences indicated that contact with nC_{60} could cause oxidative damage to human cells and DNA due to the cytotoxicity and genotoxicity of nC_{60} [12–16], and a genotoxic response on DNA in human lymphocytes has also been observed at a concentration of nC_{60} of 2.2 $\mu g L^{-1}$ [15].

The preferred method for separating these aqueous fullerene aggregates is a high performance liquid chromatography (HPLC) technique using a reversed-phase column with the mobile phase containing a combination of toluene and a polar solvent such as

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methanol (MeOH), acetonitrile (ACN), or tetrahydrofuran (THF) [17,18]. Isaacson and co-workers recently reviewed the methods for the analysis of aqueous fullerene aggregates (nC_{60} and nC_{70}) [18]. It should be noted, however, that little information is available concerning the HPLC separation coupled with a mass analyzer to determine aqueous fullerene aggregates in environmental samples. For sample preparation, liquid–liquid extraction (LLE) with toluene with or without ultrasound-assisted (UA) is the most commonly used extraction method [17–19]. Solid-phase extraction (SPE) has also been applied to evaluate the possibility of extracting aqueous fullerene aggregates from water and urine samples [12,19]. Both LLE and SPE are time-consuming, and LLE is not an eco-friendly technique, because large amounts of organic solvents are used.

Assadi and co-workers recently reported on a novel microextraction method, referred to as a dispersive liquid–liquid microextraction (DLLME) [20]. The method is based on a ternary component solvent system analogous to a combination of LLE and cloud point extraction. The three components of DLLME are the extraction solvent (ES, a high-density and less water miscible solvent), a disperser solvent (DS, a water miscible solvent) and the aqueous sample. In this technique, the extraction is performed between the water sample and a cloud of fine droplets of ES when an appropriate mixture of ES and DS is rapidly injected into the aqueous sample. Since the contact area between the ES and aqueous sample is large, an equilibrium state is rapidly achieved, which shortens the extraction time considerably. DLLME has been successfully applied to the determination of various emerging contaminants in various types of environmental water and urine samples [20–25]. Two review articles on the subject have appeared in 2010 [26,27]. An UA-DLLME method has recently been developed, which accelerates the formation of a fine cloudy dispersive mixture and has proved to be a simple and rapid method for the extraction and subsequent determination of various pesticides, insecticides and semi-volatile pollutants that are found in river waters and wastewaters samples [28–30].

In this study, UA-DLLME coupled with LC–APPI-MS/MS was employed to identify and quantitate three aqueous fullerene aggregates (nC_{60} , nC_{70} , and aqueous [6,6]-phenyl C_{61} butyric acid methyl ester ($nPCBM$)) in environmental water samples. The effects of UA-DLLME parameters (types and volumes of ES and DS, ionic strength, pH value of the water sample, ultrasonic time) on the quantitative extraction of these analytes were systematically investigated and optimized. The accuracy and precision of the developed method were evaluated, and its suitability for use in determining trace levels of these aqueous fullerene aggregates in surface water and wastewater samples was demonstrated.

2. Experimental

2.1. Reagents and chemicals

LC-grade ACN and MeOH were purchased from Merck (Darmstadt, Germany). ACS reagent grade sodium hydroxide (NaOH) and spectral-grade acetone (AC) were purchased from Riedel-de Haën (Seelze, Germany). ACS reagent grade ethanol (EtOH), LC-grade 2-propanol (IPA) and sodium chloride (NaCl), and spectral-grade toluene were obtained from J.T. Baker (Phillipsburg, NJ, USA). ACS reagent grade benzyl bromide (BBr), nitrobenzene (NBz), and carbon tetrachloride (CCl_4) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The standard fullerene compounds: C_{60} , C_{70} , and [6,6]-phenyl C_{61} butyric acid methyl ester (PCBM) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (resistance $\geq 18.2 M\Omega cm$) was produced using a Barnstead EasyPure II ultrapure water system (Thermo Fisher Scientific, Hudson, NH, USA).

2.2. Aqueous fullerenes suspension preparation and standardization

The procedure for preparing stock suspensions of the three aqueous fullerenes has been described in a previous report [31], and was performed herein with minor modifications. Taking nC_{60} as an example, powdered C_{60} (2 mg) weighted by a Mettler-Todelo AG104 analytical balance (Columbus, OH, USA) was added to 5 mL of toluene in a vessel, and completely solubilized by ultrasonication. This solution was referred to as a C_{60} -toluene stock solution. The C_{60} -toluene stock solution was transferred to a beaker containing 50 mL of deionized water and 1.5 mL of ethanol, and the suspension sonicated with a Bransonic® 8510 ultrasonic device (Danbury, CT, USA) until the toluene phase disappeared. The resulting solution was filtered through a 0.20- μm membrane filter (Advantec MFS, Inc., Dublin, CA, USA). The resulting filtrate was referred to as the nC_{60} -water stock solution, and was stored in an amber glass container at 4 °C in the dark. The determination of the concentrations of the nC_{60} -water stock solution has been described in a previous report [12], and was performed here with minor modifications. Briefly, 4 mL of the nC_{60} -water stock solution and 4 mL of a 2% aqueous NaCl solution were mixed and then added to 4 mL of toluene. The mixture was shaken vigorously for 60 min with Vortex Genie 2 (Scientific Industries, Inc., New York, USA) and then centrifuged at 5000 rpm (ca. 2680 g) with a CN-3600 centrifuge (Hsiangtai Machinery Industry Co. Ltd., Taiwan) for 5 min to complete separate the aqueous and toluene phases. The upper toluene phase was withdrawn, diluted 20-fold, and the corresponding C_{60} concentration was determined by LC–APPI-MS/MS, and calculated from the calibration curve established from a series of known concentrations of C_{60} in toluene. The same procedures were used for the preparation and standardization of nC_{70} and $nPCBM$ -water stock solutions. The working solutions of nC_{60} , nC_{70} , and $nPCBM$ were prepared by diluting the corresponding aqueous stock solutions with an appropriate volume of deionized water.

2.3. Sample collection

Municipal influent (specific conductance 15,600 $\mu S cm^{-1}$) and municipal effluent (specific conductance 14,800 $\mu S cm^{-1}$) samples were collected from the An-Ping community in Tainan city, Taiwan. Industrial wastewater samples were collected from two sites. An industrial effluent-I (specific conductance 2840 $\mu S cm^{-1}$) sample was obtained at a wastewater discharge point from an electronic company in the Chung-Li Industrial Park in northern Taiwan. The major product of this company is printed circuit boards. The industrial effluent-II (specific conductance 2000 $\mu S cm^{-1}$) was collected at a discharge point from the wastewater treatment plant (WWTP) of Hsinchu Science Park in northern Taiwan. Hsinchu Science Park is a major installation and is the largest industrial park in Taiwan for producing semiconductor, integrated circuit, optoelectronics, and 3C (Computer Communication and Consumer) products. A surface water sample (specific conductance 1430 $\mu S cm^{-1}$) was collected from a creek that received effluents from the WWTP of the Hsinchu Science Park. All samples were collected in duplicate (500 mL each) and shipped to the laboratory in ice-packed containers. Upon arrival, the conductivity and pH value of each sample was measured immediately with a SC-170 conductivity meter (Suntex Instruments Co. Ltd., Taiwan) and a Mettler-Toledo MP220 pH meter (Columbus, OH, USA), respectively, and the sample was passed through a 0.20- μm membrane filter. The filtrates were adjusted to pH 10.0 by adding 1.0 M NaOH solution, and were then ready for extraction.

Table 1
Optimization of LC–MS/MS parameters.

Parameter	Studied range	Optimal parameters for APPI
LC mobile phase	Toluene/MeOH Toluene/ACN	Toluene/MeOH
Mobile phase ratio	65/35 to 40/60 (v/v)	50/50 (v/v)
Ion source	ESI, APCI, APPI	APPI
LC flow rate	0.1–0.3 mL min ⁻¹	0.2 mL min ⁻¹
Capillary voltage	1300–1700 V for C ₆₀ and C ₇₀ 1500–2500 V for PCBM	1600 V for C ₆₀ and C ₇₀ 2100 V for PCBM
Nebulizing gas pressure	30–80 psi	70 psi
Drying gas flow rate	5 and 10 L min ⁻¹	5 L min ⁻¹
Nebulizer temp.	300–400 °C	350 °C
Drying temp.	350 °C	350 °C
Mass transition (<i>m/z</i>)	C ₆₀ (1.0 V) ^a : 720 [M] ^{-•} > 720 [M] ^{-•} C ₇₀ (1.0 V): 840 [M] ^{-•} > 840 [M] ^{-•} PCBM (1.8 V): 910 [M] ^{-•} > 823 [M–(CH ₂) ₂ COOCH ₃] ⁻ 720 [M–C ₆ H ₅ C(CH ₂) ₃ COOCH ₃] ⁻	

^a Fragmentation amplifier for MS/MS.

2.4. UA–DLLME extraction procedures

The procedure used for UA–DLLME has been described in previous reports [28,29], and was performed with modifications. A 10 mL sample of water (pH 10.0) containing 0.1 g sodium chloride was placed in a 15-mL screw capped glass tube with a conical bottom (optimized, see Section 3.3), and a mixture of 1.0 mL of isopropyl alcohol (as a DS) and 10 μL of benzyl bromide (as an ES) was then rapidly injected into the aqueous sample (optimized, see Section 3.3). A cloudy suspension was formed containing fine droplets of ES that were completely dispersed in the aqueous phase. This dispersion was subjected to ultrasonication for 1.0 min (optimized, see Section 3.3) to accelerate the extraction of the analytes into the fine droplets. Phase separation was then performed by centrifugation at 5000 rpm for 10 min (ca. 2680 g, optimized, see Section 3.3), and the sedimented phase (6.2 ± 0.5 μL) was collected and prepared for LC–APPI–MS/MS analysis. The method was evaluated using “Industrial effluent-I” samples that were spiked by the standard mixtures of *n*C₆₀, *n*C₇₀, and *n*PCBM (final concentrations: 0.6 μg L⁻¹ for *n*PCBM, 0.3 μg L⁻¹ for *n*C₆₀ and 0.04 μg L⁻¹ for *n*C₇₀). The spiked samples were then subjected to UA–DLLME and LC–APPI–MS/MS analytical procedures for method evaluation.

2.5. LC–APPI–MS/MS analysis

HPLC separation of C₆₀, C₇₀, and PCBM was performed on an Agilent 1100 series HPLC system consisting of a G1311A quaternary pump and a G1313A autosampler (Palo Alto, CA, USA). The HPLC column used in this study was a 5-cm narrow-bore (2.1-mm I.D.) Ascentis® Express C₁₈ column packed with 2.7 μm particles (Supelco, Bellefonte, PA, USA). The injection volume was 5 μL, and isocratic elution was applied at a flow-rate of 0.2 mL min⁻¹ using 50% toluene and 50% MeOH as mobile phases (optimized, see Section 3.2). A baseline separation of C₆₀, C₇₀, and PCBM was achieved in 3 min.

Mass spectrometric detection was performed using an Agilent 1100 series LC–MSD Trap SL mass spectrometer equipped with electrospray ionization (ESI), atmospheric chemical ionization (APCI) and APPI interfaces, and configured in an orthogonal geometry with a negative ionization mode. A photoionization lamp generating 10-eV photons was equipped in the APPI interface. SGE syringes, 1-mL and 10-mL (Ringwood, Australia) were used to evaluate the ionization parameters and to infuse the dopant for the APPI, respectively. Mass spectra were collected in the scan range *m/z* 300–950. Table 1 lists the studied conditions and optimal operating parameters of the capillary voltage, nebulizer gas pressure, drying gas flow rate

and temperature, vaporizer temperature and the LC eluting flow-rate in APPI. Moreover, the APPI system was also optimized with respect to the dopant used. For tandem mass spectrometric detection, collision-induced dissociation (CID) in product ion scan mode was performed using a helium as the background gas and collision gas at a pressure of 6 × 10⁻⁶ torr.

3. Results and discussion

3.1. Comparison of three API interfaces

In this study, at first, we evaluated the behavior and sensitivity of C₆₀, C₇₀, and PCBM in negative ionization mode using ESI, APCI and APPI interfaces in LC–MS. These three fullerenes were ionized in the negative ionization mode to form their radical ions [C₆₀]^{-•}, [C₇₀]^{-•}, and [PCBM]^{-•} with *m/z* 720, *m/z* 840, and *m/z* 910, respectively, which were consistent with a previous report [32]. Solvent-adduct ions [M+CH₃OH–H]⁻ of the three fullerenes were observed in the ESI mode (66–125% compared to [M]^{-•}) and in APPI mode (4–7% compared to [M]^{-•}) when methanol was used as the mobile phase, but were absent when the APCI mode was used. Monoxide adduct ions [M+O]⁻ of C₆₀, C₇₀, and PCBM in three API interfaces were not observed in this study. In a comparison among the ESI, APCI, and APPI modes regarding the ionization efficiency and signal-to-noise ratio (S/N), the best sensitivity was obtained when APPI was used, as shown in Fig. 1. Hence, the APPI interface was used in subsequent experiments, and MS/MS was used to improve the signal identification and quantitation.

3.2. Optimization of LC–APPI–MS/MS

APPI–MS/MS parameters were optimized with respect to the capillary voltage, nebulizer gas pressure, drying gas flow rate and temperature, vaporizer temperature and the LC elution flow rate in APPI. As listed in Table 1, except for the capillary voltage, the operating parameters of the APPI–MS/MS for three fullerenes were the same. The capillary voltage was 2100 V for PCBM, and 1600 V for C₆₀ and C₇₀. Mass transition ions of C₆₀ and C₇₀ for product ion scan were identical to the precursor ions because of the chemical stability of the ions selected in the isolation step [32]. For PCBM, two fragmented ions at *m/z* 720 and *m/z* 823 were observed when fragmentation amplifier was applied, and the characteristic ions were [M–C₆H₅C(CH₂)₃COOCH₃]⁻ and [M–(CH₂)₂COOCH₃]⁻, respectively.

In the LC system, toluene/MeOH and toluene/ACN were tested for use as HPLC mobile phases. The retention times of C₆₀, C₇₀,

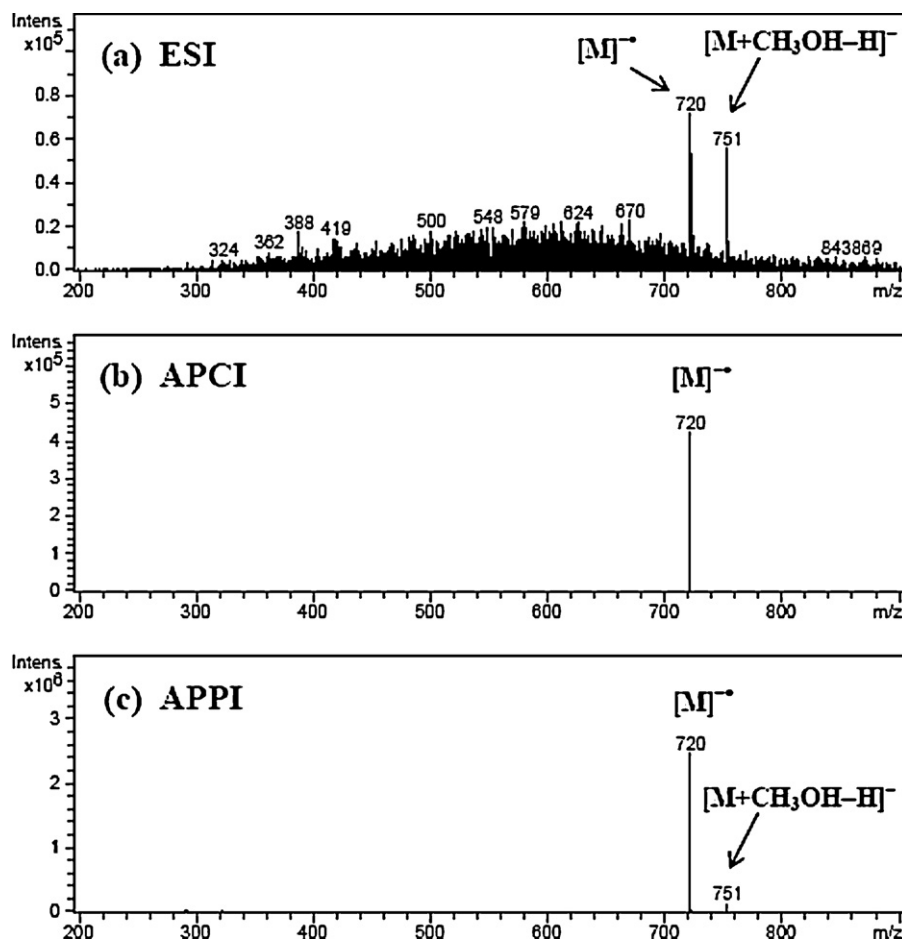


Fig. 1. Mass spectra of C_{60} by (a) ESI, (b) APCI, and (c) APPI.

and PCBM increased as the toluene/MeOH or toluene/ACN ratio decreased. Although no significant differences were found between toluene/MeOH and toluene/ACN regarding of peak shape and resolution, the sensitivity was better when the toluene/MeOH mobile phase was used in the isocratic mode with a ratio of 50/50 (*v/v*). The similar results have also been reported in previous reports [33,34]. Dopant-assisted APPI with various dopants (toluene, acetone, *iso*-octane, heptanes, THF, benzene, pyridine, and anisole) was also evaluated, but no obvious improvement in the S/N for target analytes was found (data not shown). The abundance of three fullerenes in the APPI mode appears to be related to the mechanism for their ionization, which has been described by Kawano and co-workers [33]. In comparing the ionization energy (IE) of toluene (8.83 eV) and the photon energy (10.0 eV) of the krypton lamp in the APPI source, toluene cations and electrons were produced as the first step of ionization process because of the lower IE than photons. Electrons were then captured by fullerene molecules to form molecular ions. As shown in Fig. 2, when the LC flow-rate increased from 0.1 mL min^{-1} to 0.2 mL min^{-1} , the number of electron-donating toluene molecules increased which led to a corresponding increase in the signal intensity. However, when the LC flow-rate was further increased from 0.2 mL min^{-1} to 0.3 mL min^{-1} , the signal abundance remained unchanged. Toluene used in this study can act not only as a LC mobile phase but also a dopant. Because of this, it was unnecessary to add an external dopant to the APPI system. In considering the peak resolution, the sensitivity and solvent consumption, a flow-rate of 0.2 mL min^{-1} with toluene/MeOH as the mobile phase (50/50, *v/v*) in the isocratic elution mode was chosen for the HPLC program.

3.3. Optimization of DLLME

3.3.1. Effect of types and volumes of extraction solvent and disperser solvent

In order to select the significant factors in DLLME process, an orthogonal array experimental design (OAD) with 4 factors and 4 levels was firstly applied to screen the parameters, and this design could be called as an $\text{OAD}_{16} (4^4)$ design, as described elsewhere [35]. This OAD can be used to optimize experimental conditions within a limited number of experiments, and is a type of fractional factorial design in which orthogonal array is used to assign major factors for a series of experimental combinations. The 4 parameters,

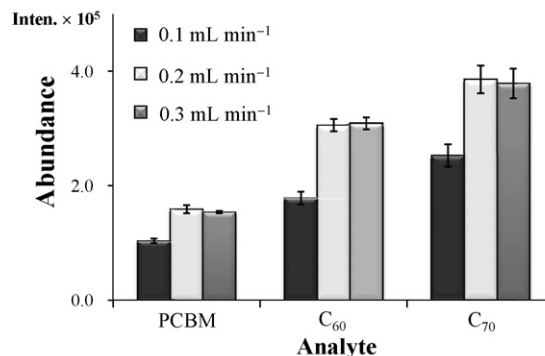


Fig. 2. Abundance of the three fullerenes under various LC flow-rates in LC-APPI-MS/MS system. Three replicate experiments were performed; the error bars represent standard deviations.

Table 2
Results of ANOVA for experimental response.

Parameter	Degree of freedom	Sum of squares	Mean of squares	F-value	P-value ^a
DS	2	4.166×10^9	2.083×10^9	0.63	–
ES	2	3.669×10^{11}	1.834×10^{11}	55.61	<0.05*
DS/ES	1	1.320×10^{10}	1.320×10^{10}	4.00	<0.05*
Error	3	9.894×10^9	3.298×10^9	–	–

^a The critical F-value is 2.8 (* $P < 0.05$).

i.e., DS, ES, pH value of water sample and ionic strength were chosen for DLLME process in this study. Preliminary ANOVA (analysis of variance) results revealed that the contribution of ES was significant. Notably, previous studies also proved the volumes and types of ES and DS are two important parameters that affect the extraction efficiency of the DLLME technique [20–27]. Afterward, a three-level full factorial design with 2 factors (the 3^2 design) was performed to optimize DLLME process, and 2 factors were ES and DS [36]. To enhance the extraction efficiency, the ES should have a higher density than that of water, a low solubility in water and a high extraction capability for the target compounds. In our experiences, an efficient dispersive and cloudy suspension of ES fine droplets could be formed when the density of the ES ranged from 1.2 g cm^{-3} to 1.8 g cm^{-3} . If the ES density was too high, the fine droplets of ES would settle to the bottom of conical tube so quickly that the extraction efficiency was poor. In considering the density and the solubility for the target compounds and availability, BBr and NBz were selected as extraction solvents [37], and CCl_4 was also chosen because of it is frequently used in DLLME [20–27]. For DS, five solvents (methanol, EtOH, AC, IPA, acetonitrile) which were commonly used in DLLME were tested, and among these solvents, EtOH, AC, and IPA performed the best and were chosen as DS in this study. The 3^2 design was conducted with Stat-Ease Design-Expert 7.0.0 software (Stat-Ease, Inc., Minneapolis, MN, USA), and the design table with the factors of ES and DS was applied to evaluate the possibility of DLLME for aqueous fullerene aggregates. The procedure consisted of an experimental protocol involving 9 runs to evaluate the optimal combination of the three ES and three DS simultaneously in 10-mL water samples (pH 10.0). However, when NBz was applied to DLLME, no cloudy suspension was formed when a mixture of ES and DS was quickly injected into the aqueous sample. Therefore, BBr and CCl_4 were used as ES for extraction evaluation. The volumes of ES and DS used in this study were $10 \mu\text{L}$ and 1 mL, respectively. Fig. 3 shows the effect of the various DS and ES used. The optimal ES was BBr due to the better solubility of fullerenes in this solvent (i.e., C_{60} : 4.94 g L^{-1}) than that in CCl_4 (0.27 g L^{-1}) [37], and the disperser solvent was IPA. The ANOVA results revealed that F-values of the DS and ES were 0.63 and 55.61, respectively, indicating that the ES was a significant term for DLLME (p -value < 0.05), as shown in Table 2.

The volumes of ES and DS required were also evaluated. As shown in Fig. 4(a), small amounts of extraction solvent ($10 \mu\text{L}$) were utilized, in order to obtain the highest extraction efficiency (refer to peak abundance). However, the peak abundances decreased significantly with increasing volume of ES from 15 to $40 \mu\text{L}$ due to a reduction in the extraction efficiencies, and the dispersive ES droplets becoming too large to form a cloudy suspension. When the volume of ES was $5 \mu\text{L}$ or less, it was difficult to incorporate the sedimented phase into the micro-syringe and reproducibility was drastically reduced. As shown in Fig. 4(b), a better peak abundance was achieved when the volume of DS (IPA) was 1.0 mL. This can be attributed to the fact that a larger volume of DS formed a more homogenous cloudy suspension, thus permitting the ES to be more efficiently dispersed (as droplets) in the aqueous solution. However, the volumes of the sedimented phases were decreased with increasing IPA, and no phase separation was observed when

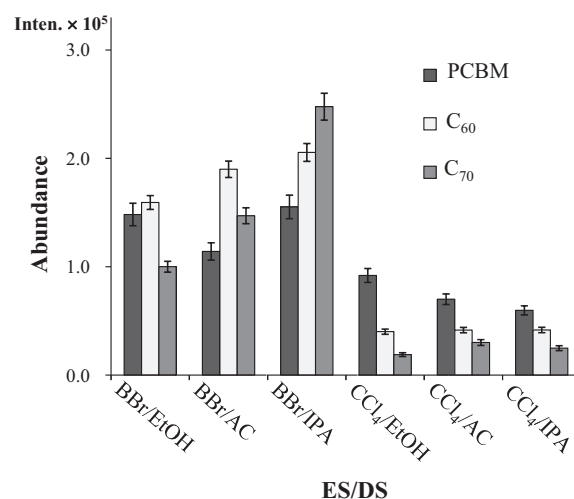


Fig. 3. Effects of the type of extraction and disperser solvent on peak abundances obtained using the UA-DLLME technique. CCl_4 : carbon tetrachloride; BBr: benzyl bromide; IPA: 2-propanol; MeOH: methanol; EtOH: ethanol. Three replicate experiments were performed; the error bars represent standard deviations.

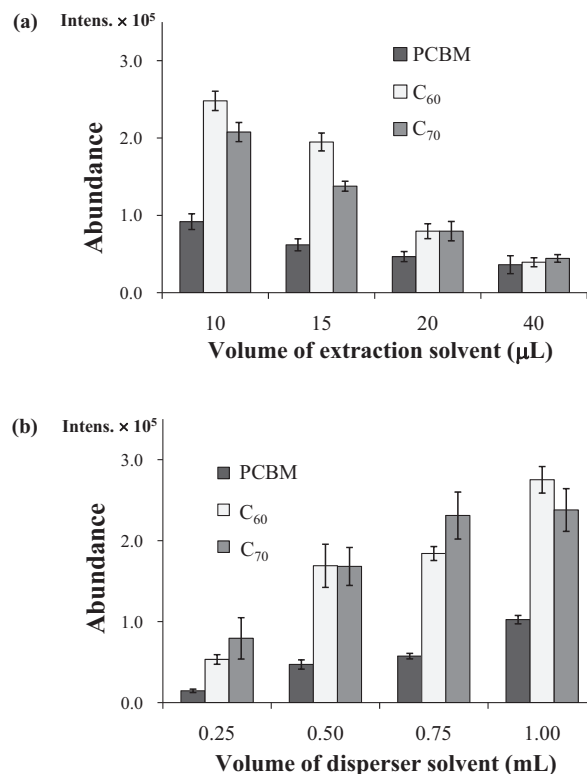


Fig. 4. Effects of the volume of (a) extraction solvent and (b) disperser solvent on the peak abundances obtained using the UA-DLLME technique. Three replicate experiments were performed; the error bars represent standard deviations.

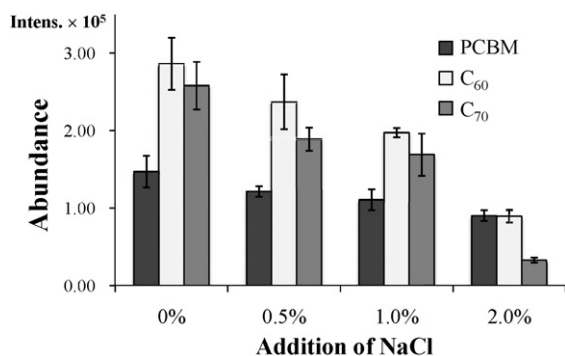


Fig. 5. Effects of added sodium chloride on the relative abundances obtained using the UA-DLLME technique. Three replicate experiments ($n=3$) were performed; the error bars represent standard deviations.

the volume of IPA exceeded 1.0 mL, which is probably due to the higher solubility of BBr in the aqueous phase when a larger volume of DS was used [29]. Collectively, considering the best extraction efficiency and the volume of the sedimented phase, a mixture of 1.0 mL of IPA (as the DS) and 10 μ L of BBr (as the ES) were chosen for subsequent experiments.

3.3.2. Effect of NaCl content and pH value

According to previous reports [20–30], the effect of salting-out (or ionic strength) for DLLME is dependent on the analytes and the ES/DS combination, such as (i) negligible effect for the extraction of UV filters (a group of organic UV absorbers commonly used in sunscreen, cosmetic and skin care products) by chlorobenzene used as ES and acetone used as DS [1,23]; and (ii) the extraction efficiency increased significantly when the extraction of different UV filters by chloroform as ES and acetone as DS [24]. However, various effects on the extraction efficiency of DLLME have been also observed when the addition of NaCl to extract organophosphorus flame retardants (OPFRs) [21]. When aromatic solvent (i.e., C₆H₅Cl) has been used as the ES for some OPFRs, the extraction efficiency was decreased when the amount of NaCl was increased. Moreover, the surface potentials (ζ -potentials) of aqueous fullerene aggregates were sensitive to ionic strength and the pH of the background solution [11,15]. Generally, stable aqueous fullerene aggregates were formed when the absolute ζ -potentials were greater than 30 mV, since the negatively charged particles with strong electrostatic repulsion would not be expected to bind to other negatively charged species in aqueous samples. The mobility of stable aqueous fullerene aggregates in aqueous media increased with increasing ζ -potentials [11]. However, when the ionic strength (addition of NaCl) was increased, a decrease in ζ -potentials was observed for the aqueous fullerene aggregates [15]. Therefore, the effect of the addition of NaCl in aqueous samples was evaluated from 0% to 2% in this study. Fig. 5 shows that the peak abundances decreased as the percentage of NaCl approached 2%. Although a higher peak abundance was observed in the absence of salt, the presence of NaCl was required to avoid the emulsion problems caused by surfactants, proteins and lipids in environmental water samples during the DLLME process [12,31,38]. Furthermore, when DLLME was applied to environmental water samples, the addition of 0% and 0.5% of NaCl failed to improve the emulsion phenomenon until 1% of NaCl was added to the actual water samples. Hence, the addition of 1% of NaCl was chosen to avoid emulsion problems.

The pH value of an aqueous sample also affects the ζ -potentials of aqueous fullerene aggregates. The ζ -potential of *n*C₆₀ changed from -45.8 mV to -62.2 mV when the pH value was increased from 4 to 10 [11]. The pH value of water samples was evaluated in the ranges of 3–10. Fig. 6 shows that higher peak abundances

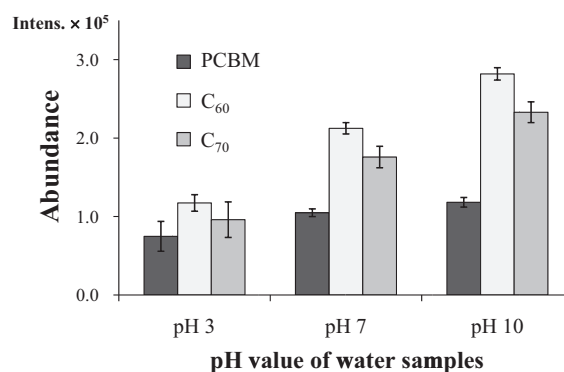


Fig. 6. Effects of the pH of water samples on the peak abundances obtained using the UA-DLLME technique. Three replicate experiments were performed; the error bars represent standard deviations.

were observed when the pH value of the water samples was increased to 10. This can be attributed to the higher mobility of aqueous fullerene aggregates and their stability (do not bind to other species) in water samples during the DLLME process.

3.3.3. Effect of ultrasonic time

According to previous studies [20,24,26,27], the extraction by DLLME is very rapid for some analytes (less than 2 min) due to the extremely large area of contact between the ES and the aqueous phase. Recent studies also indicated that UA-DLLME improves extraction efficiency because finer ES droplets are produced when ultrasonic vibration is used [28–30]. Therefore, the impact of ultrasonic time was examined in the range of 0–15 min. As shown in Fig. 7, the peak abundances changed dramatically when the ultrasonic time was increased from 0 min to 1.0 min. This can be attributed to the formation of finer ES droplets during ultrasonic vibration in the first 1.0 min and the assistance conferred by ultrasound accelerated the rate of mass transfer of analytes from the aqueous phase to the ES droplets. However, when the ultrasonic time exceeded 1.0 min, more sedimented phase was observed in the bottom of the tube and the extraction efficiency was decreased. This can be attributed to the result of increase the volume of the droplets of ES in aqueous phase during the longer ultrasonic vibration. Notably, the extraction efficiency of PCBM did not change dramatically in various solvent volume, pH, ionic strength and sonication time were applied. They may attribute to the different ζ -potential of PCBM in aqueous condition [11], and the higher solubility of PCBM in organic solvents comparison with C₆₀ and C₇₀ in the same solvent [37,39]. The solubilities of C₆₀, C₇₀ and PCBM in

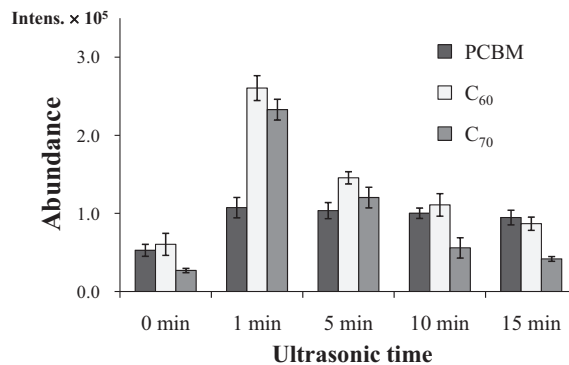


Fig. 7. Effects of ultrasonic time on peak abundances obtained using the UA-DLLME technique. Three replicate experiments were performed; the error bars represent standard deviations.

Table 3
Linear range, linearity, and limits of detection and quantification.

Analyte	Retention time (min)	Linear range (ng L ⁻¹)	r ²	LOD (ng L ⁻¹)	LOQ (ng L ⁻¹)
nPCBM	0.9	300–5000	0.9927	40	150
nC ₆₀	1.6	150–3200	0.9918	20	60
nC ₇₀	2.3	20–1000	0.9965	3	8

toluene at 25 °C are 2.9 g L⁻¹, 1,406 g L⁻¹ and 10 g L⁻¹, respectively. The solubilities of C₆₀, C₇₀ and PCBM should have the same trend found in benzyl bromide, which leads PCBM has slight different behaviors as C₆₀ and C₇₀. Hence, for achieving a rapid procedure and a stable cloudy suspension, a 1.0-min ultrasonic time was applied in the UA-DLLME process to extract the aqueous fullerene aggregates from water samples.

3.4. Method performance

The analytical characteristics of the optimized UA-DLLME method in terms of its linear response range, reproducibility, limits of detection (LODs) and limits of quantification (LOQs) were investigated, in order to determine the efficiency and feasibility of the application for the analysis of environmental water samples. Table 3 reveals that good linearities (coefficient of determination (r²) greater than 0.9918) were found in the five-level calibration curve in the range from 300, 150, and 20 to 5000, 3200, and 1000 ng L⁻¹ for nPCBM, nC₆₀, and nC₇₀, respectively. The LODs, defined at a signal/noise (S/N) ratio of 3, were determined through a UA-DLLME analysis of spiked “Industrial effluent-I” samples; the values were 40, 20, and 3 ng L⁻¹ for nPCBM, nC₆₀, and nC₇₀, respectively, for 10-mL water samples. The LOQs, defined as the concentration required for S/N ratios equal or above 10, were 150, 60, and 8 ng L⁻¹ for nPCBM, nC₆₀, and nC₇₀, respectively, for 10-mL water samples.

Precision, accuracy and stability were evaluated by intra- and inter-day analyses. Intra-day precision was determined by analyzing five spiked “Industrial effluent-I” samples, spiked with two levels of the standard mixture on the same day (n = 5). Inter-day precision was evaluated by determining five replicates on 4 consecutive days (n = 20). Intra-day precision (RSD) for these three aqueous fullerenes was 11% or less, whereas the inter-day precision was 12% or less, as summarized in Table 4. Accuracy, determined as the mean recovery, ranged from 70 to 86%. These data reveal that the combination of UA-DLLME and LC-APPI-MS/MS is capable of achieving a high reproducibility with excellent linearity and sensitivity for the analysis of wastewater samples of these three aqueous fullerene aggregates.

Table 4
Precision and accuracy data.

Analyte	Intra-day (n = 5)		Inter-day (n = 20)
	Low level Recovery (%RSD)	High level Recovery (%RSD)	High level Recovery (%RSD)
nPCBM	72 ^a (9) ^b	70 (11)	70 (12)
nC ₆₀	84 (4)	86 (2)	84 (7)
nC ₇₀	81 (6)	83 (8)	75 (11)

Final spiked concentration of low level: 0.6 μg L⁻¹ for nPCBM, 0.3 μg L⁻¹ for nC₆₀ and 0.04 μg L⁻¹ for nC₇₀.

Final spiked concentration of high level: 3 μg L⁻¹ for nPCBM, 2 μg L⁻¹ for nC₆₀ and 0.7 μg L⁻¹ for nC₇₀.

^a Mean spiked recovery (n = 5 or n = 20).

^b The relative standard deviation (%RSD) is given in parentheses (n = 5 or n = 20).

Table 5
Spiked recovery (%) and concentrations (ng L⁻¹) of three aqueous fullerenes detected in real water samples using the optimized UA-DLLME.

Sample	Analytes		
	nPCBM	nC ₆₀	nC ₇₀
Industrial effluent-I			
Spiked recovery (%)	70 ^a (11) ^b	86 (2)	83 (8)
Background conc. (ng L ⁻¹)	n.d.	n.d.	10
r ²	0.9927	0.9918	0.9965
Industrial effluent-II			
Background conc. (ng L ⁻¹)	n.d.	130 (8)	25 (3)
r ²	0.9992	0.9937	0.9908
Municipal influent			
Background conc. (ng L ⁻¹)	n.d.	n.d.	37 (5)
r ²	0.9918	0.9942	0.9906
Municipal effluent			
Background conc. (ng L ⁻¹)	n.d.	n.d.	n.d.
r ²	0.9937	0.9925	0.9914
Surface water			
Background conc. (ng L ⁻¹)	n.d.	98 (7)	n.d.
r ²	0.9948	0.9935	0.9864
RSD of the slopes (%)	12	10	12

n.d.: not detected at the LOQ listed in Table 2. r²: the coefficients of determination of the method of standard addition curves.

^a Mean spiked recovery (n = 5), final spiked concentration: 3 μg L⁻¹ for nPCBM, 2 μg L⁻¹ for nC₆₀ and 0.7 μg L⁻¹ for nC₇₀.

^b The relative standard deviation (%RSD) is given in parentheses (n = 5).

3.5. Application

The developed method was employed in conjunction with the standard addition method to quantitatively determine the concentrations of these aqueous fullerene aggregates in various environmental water samples. Five tubes, each containing 10-mL of a water sample, were spiked with standard solutions to obtain final concentrations of 0–5.0 μg L⁻¹ (i.e., 0, 0.6, 1.2, 3.0, and 5.0 μg L⁻¹) for nPCBM, 0–3.2 μg L⁻¹ (i.e., 0, 0.3, 0.8, 2.0, and 3.2 μg L⁻¹) for nC₆₀, and 0–1.0 μg L⁻¹ (i.e., 0, 0.04, 0.30, 0.70 and 1.0 μg L⁻¹) for nC₇₀. Table 5 lists the spiked recoveries and the concentrations of the three analytes detected in the environmental samples and the coefficients of determination (r²) for the standard addition curves. Fig. 8 displays the LC-APPI-MS/MS chromatograms for (a) the non-spiked and (b) the spiked Industrial effluent-II samples. Except for the “Municipal effluent” sample, aqueous fullerene aggregates were detected in the other four environmental samples. The recoveries of these analytes from the spiked “Industrial effluent-I” sample ranged from 70 to 86%, and the RSDs ranged from 2 to 11%. Preliminary results show that, except for nPCBM, nC₆₀ and nC₇₀ were detected in these environmental samples, and the total concentrations ranged from n.d. to 130 ng L⁻¹ with RSD% less than 8%. The coefficients of determination (r²) of the standard addition curves were all greater than 0.9864, and the RSD of the slopes from the

Table 6
The results of the recovery function for matrix test, y = r₀ + r₁x.

Analytes	Slope r ₁ ± S _{r1} ^a	t-Calculated	t-Tabulated ^a (df = 4)
nPCBM	0.923 ± 0.090	1.8912	2.13
nC ₆₀	1.010 ± 0.070	2.0386	2.13
nC ₇₀	1.044 ± 0.078	1.2434	2.13

^a Confidence intervals at 95%.

Table 7
Comparison of the developed method with previous studies.

Analyte	Extraction method	Analysis method	LOQ	Application	Reference
nC_{60} , nC_{70} and $nPCBM$	UA-DLLME (10 μ L BBr)	LC-APPI ⁻ -MS/MS	nC_{60} : 60 ng L ⁻¹ nC_{70} : 8 ng L ⁻¹ $nPCBM$: 150 ng L ⁻¹	Influent Effluent Surface waters	This study
nC_{60} to nC_{98}	LLE (0.5 mL toluene)	LC-ESI ⁻ /SIM-MS	0.04 μ g L ⁻¹	Zebrafish for in vivo toxicity assays	[41]
nC_{60}	LLE (4.5 mL toluene)	LC-UV (333 nm)	LOD: 0.34 μ g L ⁻¹	Protein-containing samples	[42]
nC_{60} , nC_{70} and $nPCBM$	LLE (20 mL toluene) + SPE (2.5 mL toluene)	LC-UV (333 nm)	nC_{60} : 2.48 μ g L ⁻¹ nC_{70} : 6.54 μ g L ⁻¹ $nPCBM$: 2.87 μ g L ⁻¹	Ground water Surface water	[43]
nC_{60}	–	LC-APPI ⁻ /QqQ-MS	–	nC_{60} size discussion	[44]
nC_{60}	LLE (4 mL toluene) + SPE (10 mL toluene)	LC-UV-APCI ⁻ /MS (PDA at 332 nm)	LLE: 3.4 μ g L ⁻¹ SPE: 0.42 μ g L ⁻¹	Reclaimed wastewater Secondary effluent	[12]

UA-DLLME: ultrasound-assisted dispersive liquid–liquid microextraction; BBr: benzyl bromide; LLE: liquid–liquid extraction; SPE: solid-phase extraction.

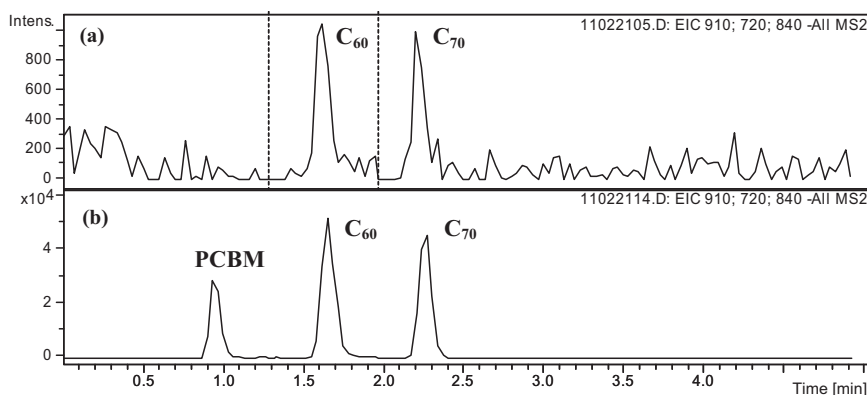


Fig. 8. LC-APPI-MS/MS chromatograms of (a) the non-spiked and (b) the spiked Industrial effluent-II samples collected at the WWTP of Hsinchu Science Park in northern Taiwan.

different samples were less than 12%, indicating that the accuracy and repeatability of this developed UA-DLLME method is acceptable. This UA-DLLME method appears to be an appropriate technique for analyzing aqueous fullerene aggregates in various wastewater samples.

The influence of the matrix effect was investigated by the so-called “recovery function” procedures with the addition of standards in our final extraction extracts, as described elsewhere [40]. The recovery functions were calculated by plotting the “found concentrations” versus the standard addition calibration curves at five concentration levels. The slope and intercept of the recovery functions calculated for all three analytes were compared, respectively, with unity and zero, by means of a *t*-test (with confidence intervals at 95%). As listed in Table 6, the *t*-calculated values were lower than the *t*-tabulated values, it can be inferred that the results obtained by the addition of standards in the final extraction extract of real sample were not significantly different from the results obtained using the standard addition method [40]. Hence, absence of matrix effect allowed us an accurate quantitative determination of $nPCBM$, nC_{60} and nC_{70} in real environmental samples by using the standard addition method.

A comparison with previous studies (Table 7) show that the traditional liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are commonly used to determine aqueous fullerene aggregates in water samples. A longer extraction time (at least 20 min for both LLE and SPE procedures) was required and it was necessary to concentrate the resulting extract by evaporation. UA-DLLME

consumed less than 10 μ L of organic solvent, but for LLE and SPE, the value was more than 0.5 mL [12,41–43].

4. Conclusion

This represents the first application of a liquid microextraction technique combined with LC-APPI-MS/MS to determine aqueous fullerenes aggregates in water samples. A fast and reliable UA-DLLME method was developed and optimized to permit the identification and quantitation of trace levels of three aqueous fullerene aggregates in various environmental samples. The UA-DLLME accelerates the formation of a fine cloudy dispersion. Overall, UA-DLLME appears to be a good alternative extraction method for the determination of aqueous fullerene aggregates in environmental water samples, in that it is a rapid, simple, low cost, effective, and eco-friendly analytical method.

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