Short communication

Asymmetrical flow field-flow fractionation hyphenated to Orbitrap high resolution mass spectrometry for the determination of (functionalised) aqueous fullerene aggregates

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ARTICLE INFO

ABSTRACT

In this short communication we report on the technical implementations of coupling an asymmetric flow field-flow fractionation (AF4) instrument to a high resolution mass spectrometer (Orbitrap) using an atmospheric photoionisation interface. This will allow for the first time online identification of different fullerenes in aqueous samples after their aggregates have been fractionated in the FFF channel. Quality parameters such as limits of detection (LODs), limits of quantification (LOQs) or linear range were evaluated and they were in the range of hundreds ng/L for LODs and LOQs and the detector response was linear in the range tested (up to ~20 μg/L). The low detection and quantification limits make this technique useful for future environmental or ecotoxicology studies in which low concentration levels are expected for fullerenes and common on-line detectors such as UV or MALS do not have enough sensitivity and selectivity.

1. Introduction

Interest in nanomaterial-related applications is growing due to their novel and unique characteristics compared to "normal scale" materials [1–3]. It can therefore be assumed that nanomaterials including nanoparticles (NP) are emitted into the environment [4]. To assess the environmental risks of NPs, the development of techniques to measure and characterise them in natural environments is a priority issue [5].

Field-flow fractionation (FFF) [6], especially the asymmetrical flow version (AF4), is one of the most promising particle separation techniques that can – especially in combination with different on-line detectors – be used for characterisation of NPs and colloids [7,8]. However, the lack of sensitivity of many detectors commonly used, such as UV or light scattering devices, limits its use under environmentally relevant conditions [7]. Inorganic NPs, such as gold and silver NPs, can be characterised and measured

at environmental concentrations by hyphenation of AF4 to an ICP-MS [9]. Carbon-based NPs, such as fullerenes [10] cannot be characterised using this combination. Several methods have been developed for the determination of concentrations of fullerenes in environmental matrices e.g., LC–UV [11], or LC–MS [12–14] using atmospheric pressure ionisation [15–17], but information about the size of their aggregates in water cannot be obtained as they need to be extracted from the aqueous phase. Information on the aggregate size is, however, crucial as the mobility and deposition of fullerenes in the aquatic environment strongly depends on this characteristic [18–20]. Therefore, up to now samples had to be analysed twice, once by using FFF to receive information on the size of the aggregates and then by using MS to determine the concentration and type of fullerene. Now it is possible to analyse each size fraction. Hence, one can see e.g., if compound A can only be found in size fractions < 50 nm and compound B in fractions > 50 nm. This was not possible with MALS or UV detectors.

It should be mentioned that fullerene clusters are destroyed during ionisation in the MS and size information cannot be obtained by use of APPI-MS alone. FFF is necessary.

To improve, shorten and ease the analysis of samples we suggest coupling AF4 to HRMS (accurate mass). In the present study AF4 was hyphenated with an Orbitrap-HRMS in order to combine

particle/aggregate separation with sensitive concentration detection of three different fullerenes. The description of the technical implementation will not only allow further development of the FFF method but might also open a door to the analysis of other organic particles and aggregates. To the best of our knowledge a combination of FFF, APPI and Orbitrap-HRMS has never been reported on before. The general feasibility of coupling FFF to a MS has been demonstrated before for ICP-MS [21–23] and ESI-MS/MS [24].

2. Experimental

2.1. Reagents and standards

C_{60} (purity > 99.9%) was purchased from Materials and Electrochemical Research Corporation (Tucson, AZ, USA). [6,6]-Phenyl-C_{61} butyric acid methyl ester ([60]PCBM) (purity > 99%) and [6,6]-(bis)phenyl-C_{61} butyric acid methyl ester ([60]bisPCBM) (purity > 99.5%) were purchased from Solenne B.V. (Groningen, The Netherlands). Toluene (ultraresidue analyse grade) and anisole were obtained from J.T. Baker (Boom, Meppel, The Netherlands) and ultrapure water (resistivity > 18 MΩ) was obtained from a Milli-Q water purification system (Millipore, Amsterdam, The Netherlands). Milli-Q-water used as carrier liquid in the FFF was filtered through 0.1 μM membrane filters (Postnova Analytics GmbH, Landsberg, Germany) prior to entering the FFF channel.

The individual aqueous fullerenes suspensions (aqu/nC60) were prepared by extended stirring [25]. 10 mg of each compound was placed in a glass bottle containing 500 mL of Milli-Q water and they were stirred in the dark for more than one month at 25 °C. The exact concentration of the aqueous solution (filtered through 0.45 μm regenerated cellulose (RC) to remove larger particles) was determined by liquid–liquid extraction followed by LC-APPI-HRMS.

2.2. Asymmetrical flow field-flow fractionation

A Postnova AF2000 system (Postnova Analytics GmbH, Landsberg, Germany) was used. The AF4 module was coupled to a UV-detector (Shimadzu) and a Multi Angle Light Scattering detector (Postnova) The AF4 trapezoidal channel was 27.5 cm long from tip to tip, the height of the spacer was 250 μm and the permeable wall consisted of a 10 kDa RC membrane (Postnova). The carrier liquid was Milli-Q water and the injection volume was set to 100 μL using an autosampler device (Postnova). The fractionation conditions are summarised in Table 1.

2.3. APPI-LTQ-Orbitrap measurements

A hybrid LTQ Orbitrap (Thermo Electron) equipped with an atmospheric pressure photoionisation (APPI) interface (Thermo Electron) which uses a Syagen PhotoMate VUV Krypton lamp (20 eV) was employed for HRMS measurements. To optimise the MS conditions, the stock aqu/nC60 solution was infused in the source with a syringe pump using AF4 flow conditions. Toluene was used as a dopant and introduced in the auxiliary gas. The exact mass 720.00055 m/z $[C_{60}]^{\bullet-}$ corresponding to the molecular ion of C_{60} was monitored in full-scan in FTMS analyser at a resolution of 30,000 FWHM over a mass-range of 300–1300 Da. The optimal parameters are summarised in Table 1.

3. Results and discussion

3.1. AF4 optimisation

Information explaining the principle of FFF can be found elsewhere [6,26–28]. Generally, the optimisation of the different

Table 1
Selected parameters for AF4-APPI-LTQ-Orbitrap method.

AF4 conditions	
Membrane	Regenerated cellulose 10 kDa
Carrier liquid	Milli-Q water
Spacer thickness	250 μm
Detector flow	0.1 mL/min
Split flow	0.5 mL/min
Cross flow	1.2 mL/min (0–12 min); 1.2–0 mL/min (12–15 min, exp. 0.2); 0 mL/min (15–20 min)
Injection time	5 min
Injection volume	100 μL
Injection flow	0.2 mL/min
Focusing flow	1.2 mL/min
LTQ-Orbitrap conditions	
Interface	(−)APPI
Probe position	C, 0, 0.75 μm
Capillary temperature	350 °C
Vaporiser temperature	500 °C
Sheath gas	20 AU
Auxiliary gas	10 AU
Sweep gas	0 AU
Dopant	Toluene
Dopant flow	0.1 mL/min
Capillary voltage	−20 V
Tube lens	−200 V

parameters involved in AF4 aims at the separation of monodisperse components resulting in distinct signals for each size fraction. However, the size distribution of aqu/nC$_{60}$ solutions is polydisperse and consequently a broad signal in the fractogram is obtained [17]. Therefore, AF4 parameters were optimised to enhance the MS response but maintaining proper fractionation of fullerene aggregates which was assessed using MALS data obtained by analysing stock solutions of aqu/nC$_{60}$.

The carrier liquid is a limitation for coupling AF4 to MS because the latter should not be used with non-volatile electrolytes and surfactants commonly used in AF4 [29]. Ultrapure water was selected as carrier liquid because it is compatible with the MS interface and its use has been suggested before for fullerene analysis with AF4 [17].

The ratio between cross flow (F_c) and outlet flow (F_{out}) and their absolute values were optimised to separate the void peak from the analyte peak and to minimise the analysis time. Three different ratios were tested (1, 2 and 3) using a constant outlet flow of 0.8 mL/min. A ratio of 2 was selected as it results in good separation of the fullerene aggregates from the void peak, better size distribution than a ratio of 1 and less sample dilution than a ratio of 3. Different settings for F_c and F_{out} were used (all having a ratio of 2). F_{out} = 0.6 mL/min and F_c = 1.2 mL/min were selected as optimal flows. Afterwards, the focusing time was optimised until the peak area was remained constant. The optimum value was 5 min under the aforementioned AF4-conditions.

Taking into account that MS-ionisation performance with an APPI interface is highly affected by the flow rate (see Section 3.2) an interesting option to improve the analyte response is the use of a split pump to remove the upper layer of liquid at the end of the channel (slot outlet). Using this option, the resulting response measured in the MS shows an increase for the following reasons: first, under cross flow conditions the analytes are accumulated close to the membrane and the rest of the channel is void of analytes. Therefore, the upper layer of the carrier liquid is removed in the slot outlet and preconcentration in the detector flow is achieved. Secondly, an ionisation enhancement in MS is obtained when the detector flow is
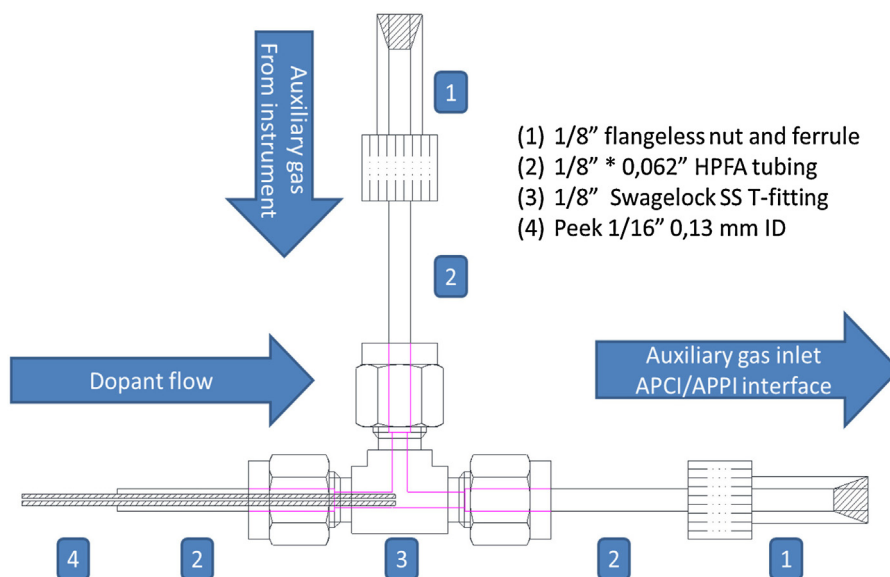
(1) 1/8" flangeless nut and ferrule
(2) 1/8" * 0,062" HPFA tubing
(3) 1/8" Swagelock SS T-fitting
(4) Peek 1/16" 0,13 mm ID

Fig. 1. Scheme of the gas-phase dopant device for coupling AF4 to HRMS using an APPI source.

lowered. Thus, a split flow of 0.5 mL/min (detector flow 0.1 mL/min) was selected based on the response increment observed in MS (Section 3.2). The enrichment factor obtained via stream splitting was 6.

Under the AF4 conditions stated above, the stock solutions of functionalised fullerenes were also analysed by AF4-UV-MALS to determine the size distribution (radius of gyration). The size distribution was very similar for the three fullerenes and the particle radius spans from about 20 nm and to approximately 80 nm. The highest signal intensity can be found for particles around 50 nm.

3.2. AF4-LTQ Orbitrap coupling and optimisation

To couple the AF4 instrument with the mass spectrometer analyser an atmospheric pressure ionisation interface (API) is necessary because the outlet flow of AF4 is a liquid. The APPI interface is the most suitable API interface for fullerenes [16,30].

The ionisation of fullerenes is enhanced using toluene as dopant. For this reason, a lab-made device to introduce toluene in the APPI ionisation chamber was constructed (Fig. 1 and SI1). The auxiliary gas tube was connected to the toluene flow (pumped with an HPLC pump) using a metal "T" junction. The AF4 outlet stream was connected to the auxiliary gas inlet port in the APPI probe creating a gas phase dopant delivery system. Without this device it is almost impossible to introduce toluene to the aqueous effluent from the AF4, due to their liquid-phase immiscibility. Also, the AF4 instrument operates at low pressures (<15 bar). If the toluene is mixed with the aqueous outlet of AF4 this results in an increase of pressure. Moreover, the introduction into the APPI probe of two immiscible solvents can result in a poor stability of the ionisation. Mixing toluene and water in the gas phase does not lead to an increase of the AF4 system pressure while the ionisation under APPI conditions is enhanced. For the optimisation of the APPI interface, the aqu/nC_{60} stock solution was infused (10 μL/min) into the probe, together with an AF4 flow rate (0.1 mL/min of Milli-Q water) by a "T" junction under the aforementioned conditions. The initial parameters of the interface were selected based on our previous experience and were as follows. Capillary temperature 350 °C, vaporiser temperature 500 °C, sheath gas 50 AU, auxiliary gas 25 AU, sweep gas 2 AU, tube lens −200 V, capillary voltage −20 V and toluene flow rate at 50 μL/min. The mass of $[C_{60}]^{\bullet-}$ (720.00055 m/z) was monitored. Different parameters were optimised taking into account

signal intensity and signal stability. Thus, the probe position (horizontal (−1 to +1), vertical (B, C or D) and axial (0.5–2 μm)), capillary and vaporiser temperature (from 350 to 500 °C), sheath (from 10 to 100 AU), auxiliary (from 5 to 25 AU) and sweep gas (from 0 to 10 AU) and capillary (−5 to −120 V) and tube lens voltage (−10 to −250 V) were tested. Two different dopants (toluene and anisole (5% (v/v) in toluene)) and their flow rates (10–200 μL/min) were tested. The optimum parameters are listed in Table 1.

First, the probe position was adjusted and the best results were obtained at position C (vertical), 0 (horizontal) and 0.75 μm (axial). The latter was the most important parameter and a closer position between the probe and lamp enhances the ionisation. Different vaporiser temperatures were tested (Fig. 2) The best result was obtained at 500 °C due to the best vaporisation of fullerene under water flow conditions and the thermal stability of this kind of compounds. Capillary temperature was changed but no differences in signal intensity were observed along all of the range tested and therefore, it was maintained at 350 °C. Next, the gas flow rates were optimised. A lower sheath gas flow rate results in an increase of ionisation but less spray stability was also observed. 20 AU was selected for the sheath gas flow rate. The same effect was observed by the auxiliary gas flow rate and therefore was kept at 10 AU. The sweep gas was turned off because its use reduces the number of ionised molecules which can enter to the analyser. Furthermore, it was observed that a lower capillary voltage and a higher tube lens improves the signal intensity for fullerenes. Dopant flow rate was optimised under these conditions (Fig. 2). A flow rate below 75 μL/min reduces the ionisation. At higher flow rates, an ionisation enhancement was not observed as the ionisation chamber atmosphere becomes sufficiently saturated with dopant molecules. In addition, a dopant solution consisting of a 5% (v/v) anisole in toluene was tested. For some compounds, the use of anisole can result in a significant increase in the ionisation [31]. However, no differences between both dopants were observed. Therefore, 100 μL/min of pure toluene was selected. Moreover, APPI/APCI dual mode ionisation was tested, but less ionisation efficiency was observed.

Additionally, the effect of flow rate on ionisation efficiency was checked and optimised. The lower the flow rate, the higher the sensitivity of the APPI interface (Fig. 2). For this reason a split of the flow of the AF4 outlet stream is necessary since low flow rates are not the most suitable option for AF4. Better resolution and
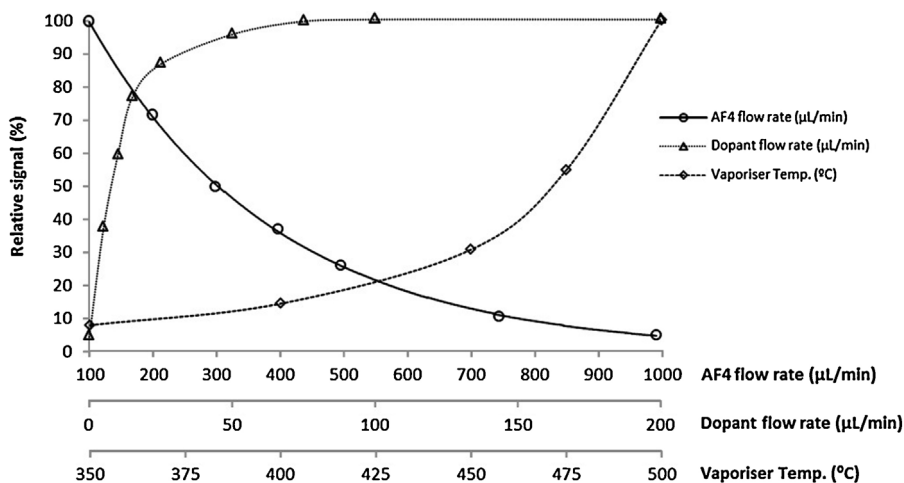
Fig. 2. Absolute response values normalised to the highest signal observed during the optimisation of flow rate, dopant flow rate and vaporiser temperature in APPI for C_{60}.

efficiency are obtained under high flow conditions [26]. The highest ionisation efficiency was obtained at 0.1 mL/min of AF4 detector flow and then a split flow of 0.5 mL/min (to waste) and a detector flow of 0.1 mL/min (to MS) were used for the fractionation (Section 3.1).

3.3. Mass spectral characterisation

The ionisation mechanisms in negative (fullerenes ionise as $[M]^{\bullet-}$) mode are not properly understood, but probably the fullerenes were ionised via an electron capture mechanism [32,33]
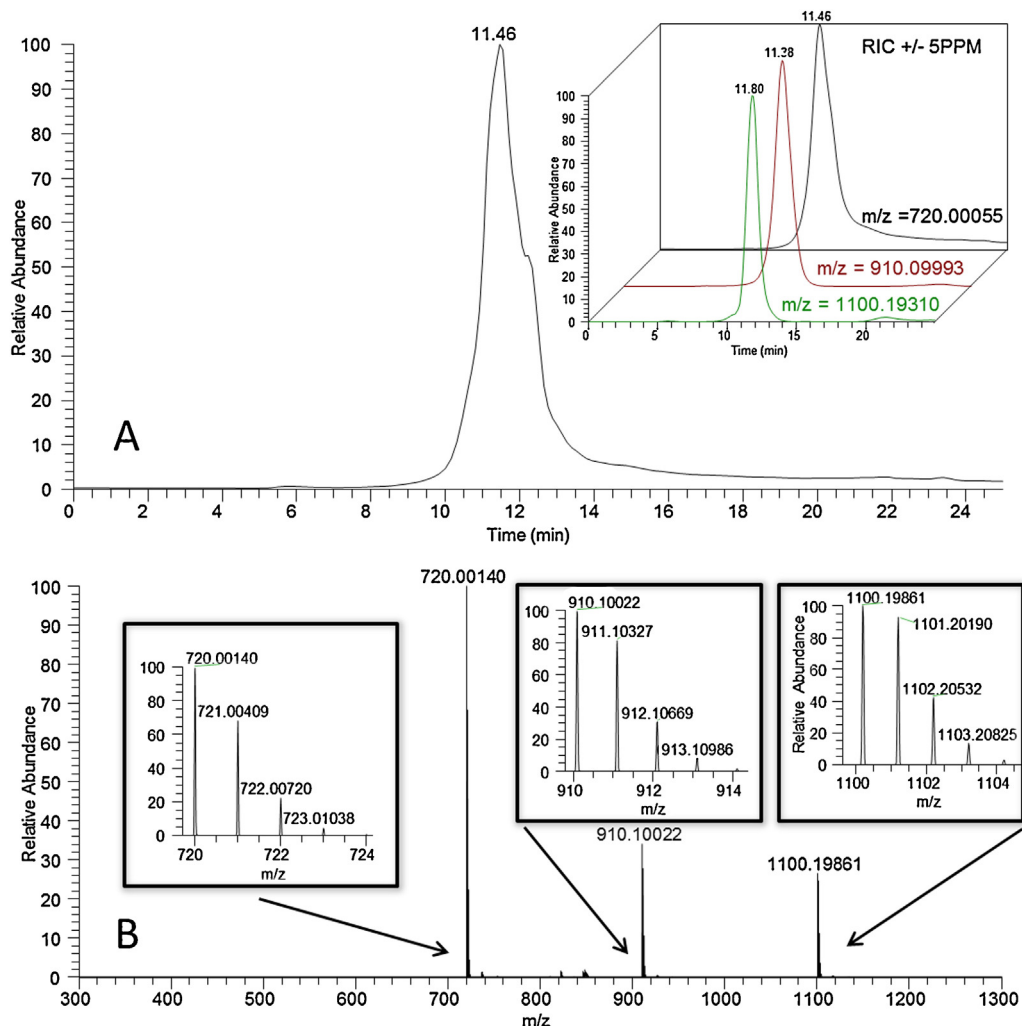

Fig. 3. Fractograms and HRMS spectrum obtained for a mixture of C_{60} (2.12 μg/L), [60]PCBM (0.88 μg/L) and [60]bisPCBM (0.66 μg/L) aqueous suspensions by AF4-APPI-LTQ Orbitrap method.

Table 2
Validation parameters obtained with AF4-APPI-LTQ-Orbitrap method.

Compound	LOD		LOQ		Linear range		r^2
	μg/L	pg	μg/L	pg	μg/L	pg	
C_{60}	0.42	42	0.85	85	LOQ–21	LOQ–2120	0.9992
[60]PCBM	0.18	18	0.35	35	LOQ–18	LOQ–1770	0.9989
[60]bisPCBM	0.13	13	0.26	26	LOQ–22	LOQ–2202	0.9989

enhanced by the use of toluene. The effect of water in APPI ionisation is not well studied and even less in negative ionisation. Nonetheless, the proposed ionisation mechanisms in negative mode via electron capture suggest that the use of water as mobile phase does not have effect on the ionisation performance as the proton affinity of analyte and solvent are not involved in the ionisation reaction.

The mass spectra of fullerenes are dominated by the $[M]^{\bullet-}$ and their corresponding ^{13}C isotopic pattern (Fig. 3). In addition, the oxidised adducts $[M+O]^{\bullet}$ are also observed and for functionalised fullerenes, a small in-source fragmentation is observed (less than 5%) resulting in a weak signal of the $[C_{60}]^{\bullet}$ ion in HRMS spectra. Fig. 3 also shows that the size distribution of C_{60} (m/z 720) is the broadest one. This information could not have been deduced from MALS or UV fractograms.

3.4. Method validation

Linear range, limit of quantification and detection for C_{60}, [60]PCBM and [60]bisPCBM aqueous fullerene aggregates were validated. These parameters were calculated by injecting 100 μL of aqueous standard solutions prepared from the aqueous stock solutions. The results are presented in Table 2, expressed as both mass amount injected and concentration (μg/L) because the injection volume is not a limiting factor in AF4 due to the focusing step. The linear range was between the LOQ and around 20 μg/L. The regression coefficient (r^2) was higher than 0.998 for all compounds. The LOQs were defined as the lowest point of the calibration curve and were between 0.3 and 0.8 μg/L. LODs corresponded to a signal/noise ratio better than 3 and were between 0.1 and 0.4 μg/L. The repeatability of the method was assessed by injecting 5 replicates of a standard solution and the %RSD was lower than 4% for peak area variation and lower than 0.4% for the retention time at peak maximum.

4. Conclusions

The coupling of AF4 to a LTQ Orbitrap MS using an APPI interface for the determination of aqueous (functionalised) fullerenes aggregates was successfully accomplished. The use of the slot outlet to reduce the flow to the detector and the gas-phase dopant device were two of the most important requirements. The former does not only reduce the flow to the MS, but also increases the signal intensity. Quality parameters such as LODs, LOQs or linear range were evaluated and they were in the range of hundreds ng/L and the detector response was linear in the range tested (up to ∼20 μg/L). The low detection and quantification limits make this technique useful for future environmental or ecotoxicology studies in which low concentration levels are expected for fullerenes. Common online detectors such as UV, or MALS do not have enough sensitivity and selectivity. Due to the successful coupling of the FFF to an Orbitrap HRMS it is now possible to develop methods for the analysis of fullerenes in various aqueous samples at environmentally relevant conditions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.06.068.

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