Table of content entry

TOC art:
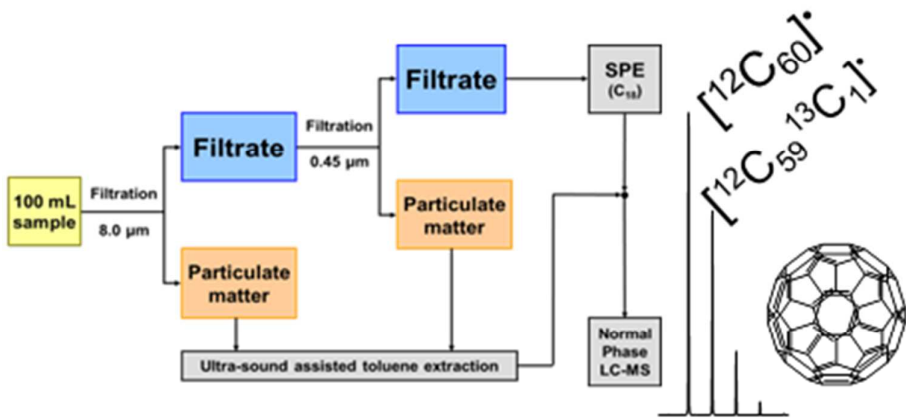
By using a normal phase column this method is capable to unambiguously identify and quantify (functionalised) fullerenes in sewage water.

Environmental Science Nano

ARTICLE

Determination of Several Fullerenes in Sewage Water by LC hr-MS using Atmospheric Pressure Photo Ionisation

E. Emke [a,*], J. Sanchis [1,b,*], M. Farré [b] P.S. Bäuerlein [a], P. de Voogt [a,c]

The main challenge in the mass spectrometric analysis of fullerenes in complex matrices is to proof unambiguously their presence or absence. Usually this can be done by fragmentation but due to the atmospheric interface and solvents commonly used, complex adduct formation will hinder quantification and qualification by distorting the isotopic cluster. In the present study, contrary to other methods in the literature makes use of a 100% toluene isocratic mobile phase is used, employing an Atmospheric Pressure Photo Ionisation (APPI) interface. In this manner the adducts that are formed when methanol is present in the system are almost completely eliminated thus avoiding higher than expected abundances for the isotopic cluster ions. The expected relative abundances of the isotopic clusters correlates well with the ones observed and was shown to be an effective tool for qualifying the presence of fullerenes even at low concentrations. For the analysis of suspended matter for the presence of fullerenes in sewage water an optimised procedure was proposed. Filters with different mesh sizes (8.0, 0.7 and 0.45 μm) were used and the filtrate was analysed by means of solid phase extraction. For a 100 mL influent waste water sample limits of quantitation for the functionalised fullerenes were between 0.22 - 0.28 ng/L and for the pristine fullerenes between 0.7-1.4 ng/L. The method used to analyse sewage water from six different waste water treatment plants, proved to be highly sensitive, able to unambiguously identify and quantify C_{60} in sewage water.

Nano Impact

Detection and characterisation of man-made nanoparticles is an important task. Therefore, analytical techniques that can determine these particles in complex environmental samples are urgently required. Up to now analytical techniques were struggling with matrices containing high amounts of dissolved organic matter. High resolution mass spectrometry for the detection of the organic fullerenes can be a powerful tool to overcome this problem, not only to detect these particles but also to quantify them at low concentrations (ng/L). The application of an atmospheric pressure photo ionisation device reduces the formation of complicated set of adducts. This enables an easy identification and quantification of several fullerenes. Due to the reduced complexity of adducts, the isotope ratio for $^{13}C/^{12}C$ is recommended to be used for identification.

Introduction

Since the discovery of C_{60}-fullerene by Kroto et al[1], as a very stable carbon allotrope, different analytical methods have been developed to determine the presence of fullerenes in the environment. The pristine fullerenes, without any functional group, are naturally present in the environment. These carbon-based nanoparticles have been detected, for example, in 10,000 years old ice cores[2]. Nowadays, the most prominent sources for fullerenes are incidental emissions by different combustion processes[3,4]. However, nanotechnology appears as a new potential source of fullerenes in the environment. Nowadays,

fullerenes are applied in different materials and consumer products such as personal care products[5], drug delivery systems[6] and in particular derivatised fullerenes – which are exclusively of anthropogenic origin – in solar[7] and photovoltaic[8] cells. In addition, their use and production is expected to increase substantially in the next coming years[9]. Therefore, it can be expected that an ever increasing amount will leach into the environment through wastewater discharge, landfills, factories, storm water run-off, erosion from composites and deposition from the atmosphere[10-13].
However, up till now a limited number of studies have determined the presence of fullerenes and in particular

Environmental Science: Nano Accepted Manuscript

functionalised fullerenes in complex environmental samples. Farré et al. detected concentrations up to the μg/L of C_{60}, C_{70} and C_{60}-methylpyrrolidine in the suspended organic matter of wastewater treatment plants[14] and Chen and Ding reported concentrations of 98 and 130 ng/L C_{60} in surface waters and industrial effluent in Taiwan, respectively[15]. Predicted environmental concentrations of fullerenes span from 0.003 ng/L for European surface waters to 4 ng/L for sewage treatment effluents[16]. During a campaign in 2008, grab samples were taken from Dutch surface waters and no fullerenes were detected above a concentration of 5 ng/L (LOQ)[17].

Due to their potential environmental and toxicological risk the properties and behaviour of fullerenes have been thoroughly studied[18-21]. Because of their low . water solubility ($1.3 \cdot 10^{-11}$ for C_{60} and $1.3 \cdot 10^{-10}$ for C_{70} ng/mL)[22], in the aquatic media, one of their main characteristics is the tendency to form (meta)stable colloidal aggregates (nC_{60})[17,18,21]. These aggregates are responsible for their high apparent solubility in aqueous solution media which dwarfs the solubility of the single fullerene molecules[22]. It should be pointed out that nC_{60} aggregates thermodynamically have a metastable character in water solution depending on different environmental factors, such as salinity or organic material. Therefore, environmental changes can revert this aggregates to the single molecules[19].

The environmental behaviour of these analytes should be considered when designing sample preparation and extraction strategies, e.g. organic solvents extraction with toluene has been proposed to extract fullerenes from soils[23,24]. Recently, asymmetric flow field flow fractionation coupled to a mass spectrometer (MS)[25] has been reported for the direct analysis of environmental samples. This method allows the detection of fullerenes in different size fractions. However, so far the most common approach to extract fullerenes from aqueous matrices is solid phase extraction (SPE)[8,14,17,24].

The separation of fullerenes is commonly achieved by high performance liquid chromatography (HPLC) using a C18 analytical column with toluene and methanol or acetonitrile as mobile phases[14,17,26]. In spite of that, the use of more specific stationary phases, e.g. with a pyrenyl-propyl group has not been reported extensively[8,23,27]. The main disadvantage of using acetonitrile or methanol is that a complex set of by-products (adducts) are formed during the atmospheric pressure ionisation in the negative mode; mainly with electrospray but also to some extent with photoionisation[8,17,28], the isotopic abundance pattern, mainly ^{13}C is distorted due to protonation[28]. Since pristine fullerenes hardly fragment, identification based solely on the presence of the accurate mass and retention time is not sufficient. For confirmation, the ratio between the ^{12}C and ^{13}C isotope can be an alternative option but the ^{13}C needs to be free from interferences (adducts).

Kolkman et al. have reported an analytical method for the detection of C_{60}, C_{70} and six functionalised fullerenes in waters at environmentally relevant concentrations (LOD = 0.3 ng/L and LOQ = 1 ng/L). This analytical method was based on the use of HPLC combined with an Orbitrap MS using electrospray ionisation (ESI) in negative mode. The main advantage of this method is the possibility to characterise other (unknown) fullerenes thanks to the application of full-scan high-resolution (HR) MS.

In the present work we present a new analytical approach for the determination of various fullerenes in sewage water influents. The present method is based on the previous method developed by Kolkman et al.[8] but with enhanced sensitivity and selectivity (10 times less sample volume is needed). The present approach makes use of a pyrenyl-propyl based analytical column, with toluene as mobile phase in isocratic conditions in combination with an APPI interface. This combination reduces the adduct formation and ion suppression. In addition, toluene acts as dopant in the APPI process ion source enhancing the sensitivity.

Materials and methods

Chemicals and Reagents Standard compounds were purchased to table 1

Table1: Standard compounds

Name	Abbreviation used	Empirical formula	Standard purity	Supplier Reference
C_{60} fullerene	[60]	C_{60}	99.90%	A: 572500
C_{70} fullerene	[70]	C_{70}	99%	A: 482994
[6,6]-Phenyl-C_{61}-butyric acid octyl ester	[60]PCBO	$C_{79}H_{28}O_2$	≥99%	A: 684481
[6,6]-Phenyl-C_{61}-butyric acid butyl ester	[60]PCBB	$C_{75}H_{20}O_2$	>97%	B
[6,6]-Phenyl-C_{61}-butyric acid methyl ester	[60]PCBM	$C_{72}H_{14}O_2$	>99.5%	B
[6,6]-Bis-Phenyl-C_{61}-butyric acid methyl ester	bis[60]PCBM	$C_{84}H_{28}O_4$	99.50%	B
[6,6]-Phenyl-C_{71}-butyric acid methyl ester	[70]PCBM	$C_{82}H_{14}O_2$	99%	B
[6,6]-Thienyl-C_{61}-butyric acid methyl ester	[60]ThCBM	$C_{70}H_{12}O_2S$	>99%	B

Supplier and reference: A: Sigma-Aldrich (Steinheim, Germany), B: Solenne B.V. (Groningen, The Netherlands).

Toluene (Ultra residue analysed), methanol and acetone (HPLC analysed) were supplied by Mallinckrodt Baker B.V. (Deventer, The Netherlands). Petroleum ether (Puriss boling point 40-60°C) were supplied Sigma-Aldrich (Steinheim, Germany), Cellulose nitrate filters of 47 mm diameter with pore sizes of 8.0 μm (Ref. 11301-50-AHN) and 0.45 μm (Ref. 11306-50-ACN) were purchased from Sartorius Stedim Biotech (Göttingen, Germany). Glass fibre filters of 0.7 μm pore size were obtained from Whatman (Maidstone, Kent, UK). Solid phase extraction (SPE) cartridges with Reversed Phase endcapped octadecylsilane (C_{18}) material, bonded to Silica Gel (40 μm, 60Å), were supplied by Mallinckrodt Baker B.V..

Sample collection. Influent (collected after the sand-trap) and effluent 24-h flow dependent composite wastewater (WW) samples were obtained from six different locations. All samples were stored in high density polyethylene containers and immediately after collection kept at 4°C until analysis.

Preparation of fullerene suspensions. Stock toluene suspensions of individual fullerenes were prepared at ~500 mg/L and shaken overnight. A fullerene mix stock suspension was prepared by diluting the previous stock solutions to 40 mg/L in toluene. Finally, the external calibration standard curve and the toluene spiking suspensions employed for method development were prepared from the fullerene mix stock suspension. A stock water dispersion of the fullerenes mixture was prepared by solvent transfer method, following the procedure which is fully described in Kolkman et al. (2013)[8]. Briefly, a toluene stock solution was slowly dispersed under controlled temperature conditions in water under continuous ultrasonic sonication until the dispersion was clear and yellow, finally the fullerene suspensions were filtered. The final concentration was confirmed by LLE[8] n=7 and ranged between 80-140 µg/l in water with a relative standard deviation of 2%.

Sample Handling. Sample preparation and extraction was based on the method developed by Farré et al.[14] with some modifications. Briefly, wastewater samples were filtered through a 0.45µm filter, dried and extracted three times with 20 mL of toluene. To optimise the filtration and extraction of fullerenes from the particulate matter different experiments were performed: (1) The amount of toluene to extract fullerenes from spiked filtered particulate matter is varied. 50 mL of blank wastewater were consecutively filtered through 8.0 µm, 0.7 µm and, finally, through 0.45 µm filter dried (3h, 50°C) and spiked with 100 ng of fullerene stock solution in toluene, dried and extracted by ultrasonic assisted extraction for 45 min by single extraction (10, 20 and 30 ml) and also through a double extraction with the same volumes, all in triplicate. (2) The extraction time needed with toluene under sonication is varied. Filters with particulate matter were spiked with 100 ng of fullerene stock solution in toluene,, dried, and extracted with 10 mL of toluene for different times (30 to 240 min). (3) The distribution of the fullerenes associated with the particulate matter of sewage water was investigated. 50 mL of wastewater were spiked with the aqueous stock water suspension were filtered consecutively through three filters (8.0 µm, 0.7 µm and, finally, through 0.45 µm mesh filters) and the particulate matter fraction of each filter was analysed separately. The filtered aqueous phase was analysed with SPE[8]. The experiment was carried out in triplicate at a concentration of 2 ng/L The filtration of the wastewater samples was carried out using a custom-made stainless steel filtration unit in order to avoid adsorption of fullerenes to the glass frit. A 50 ml ultra pure water sample were spiked with the same volume of water dispersion and directly extracted via LLE and set to 100% recovery.

The optimised method is based on the combined analysis of the wastewater particulate matter and its aqueous phase. 100 mL of influent wastewater is filtered through 8.0 and 0.45 µm mesh size cellulose nitrate filters in that order. Each filter is placed in a 100 mL Erlenmeyer flask and dried at 50 ºC, overnight. 10 mL of toluene is added to each Erlenmeyer flask, fully covering the filter. Next the filters are extracted in an ultrasound bath for a period of 90 min. The toluene extracts of the particulate matter are combined with the extract of the aqueous phase (extraction according to Kolkman et al[8]) in a glass tube. The mixture is evaporated under a heated nitrogen flow, by using an automated blow-down apparatus (Barkey, Germany) until ~0.5 mL of solvent is left. Finally, extracts are transferred to a vial and the volume is adjusted to 1000 mL by weight.). For all optimizations experiments with sewage water, influent grab wastewater samples from Utrecht WW treatment plant (Utrecht, The Netherlands) were used as field blanks. They were analysed and none of the targeted fullerenes were detected. For the procedural blanks ultra pure water was used instead of sewage water. When the stock water dispersion was used the same spiked amount was extracted by LLE and the concentration set to 100%

Safety Precautions. The HPLC system setup was placed in a custom-built fume hood and the exhaust from the atmospheric pressure interface was directly vented into the exhaust system to prevent toluene vapours entering the laboratory atmosphere.

High-Resolution Mass Spectrometry Measurements. A hybrid LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) was interfaced to a Surveyor HPLC system (ThermoElectron, Bremen, Germany) for the chromatographic separation. The initial method used a HPLC gradient with toluene and acetonitrile with post column infusion of methanol[8] and an ESI. The ESI interface was replaced by an Ion Max API source housing (Thermo Fisher Scientific) containing; an APCI probe (used as a nebuliser/desolvation device) and a SyagenPhotoMate VUV light source (krypton discharge lamp, 10.0 eV) (Syagen Technology Inc., Tustin, CA, USA) forming the Atmospheric Pressure Photo Ionisation interface (APPI) with a 100% isocratic gradient of toluene (20 min). 25 µL of extract was injected using the chromatographic system. Separation was achieved using a custom made Cosmosil® Buckyprep column (250 mm length, 2.0 mm internal diameter, 5 µm particles) from Nacalai Tesque, Japan, with 100% of toluene as mobile phase at 0.200 mL/min (Toluene also acts as the dopant). For comparison experiments methanol was infused post-column.

The APPI source parameters were optimised by infusing a solution of C_{60} in toluene and further fine-tuned with sequential injections of the mixture stock with the complete set of fullerenes. Vaporiser temperature was set at 350ºC. Sheath gas, auxiliary gas and swept gas were set at 50 Arbitrary Anits (a.u.)., 20 a.u. and 2 a.u. respectively. The tube lens was set to 200 V and the capillary temperature and voltage were maintained at 350ºC and -7.5 V, respectively, no discharge voltage was applied. Full-scan high accuracy mass profile spectra were acquired in the range of 100−1300 m/z with the resolution set at 30 000 (FWHM m/z 400). For confirmation purposes accurate MS/MS spectra were recorded at a resolution of 7500 at normalised collision energy (NCE) of 40.

Quality assurance. In order to avoid sample contamination, all materials, in contact with fullerenes were cleaned and rinsed (three times) with acetone, petroleum ether and finally toluene prior to use. Since fullerenes are prone to radical-oxidation in contact with UV light[29], light exposure was avoided by using amber glassware or either covering with aluminium foil. All quantification was done by measuring external calibration standards between 10 and 500 ng/L of all fullerenes in toluene, Instrumental interday and intraday repeatability were calculated at three concentration levels as relative standard deviation, which are obtained when analysing 5.0 µL of 2.0, 16 and 128 pg/µL toluene standards. Instrumental limits of detection and quantification (ILOD and ILOQ, respectively) were defined as the concentration that provides a signal-to-noise (S/N) of 3 and 10 for the quantifier ion of each analyte and they were experimentally assessed by consecutive dilution of the same standard.

The validation of the method was carried out by analysing spiked wastewater. The method's limits of detection and quantification, recovery yields, linearity range and interday precision were assessed as follows: (1) recovery rates and interday repeatability were calculated at 10, 50 and 500 ng/L, (2) the limits of detection (MLODs) and quantification (MLOQs) for fullerenes were calculated for the quantifier ion of each analyte as 3 and 10 times its signal-to-noise (S/N) value.

Results and discussion.

Extraction optimisation. The most common way for determining the amount of fullerenes associated with the particulate matter is to filter the sample. Since raw sewage is a highly complex matrix with high amounts of suspended matter a filtration directly through 0.45 µm will result in clogging and therefore a prefilter (>0.45 µm) is needed. This the reason why several different sized filters were used (8.0, 0.7, and 0.45 µm) to optimise the extraction. Starting point was a method developed by Farré et al. (2010)[14]. In addition to the optimisation a larger number of fullerenes were included.
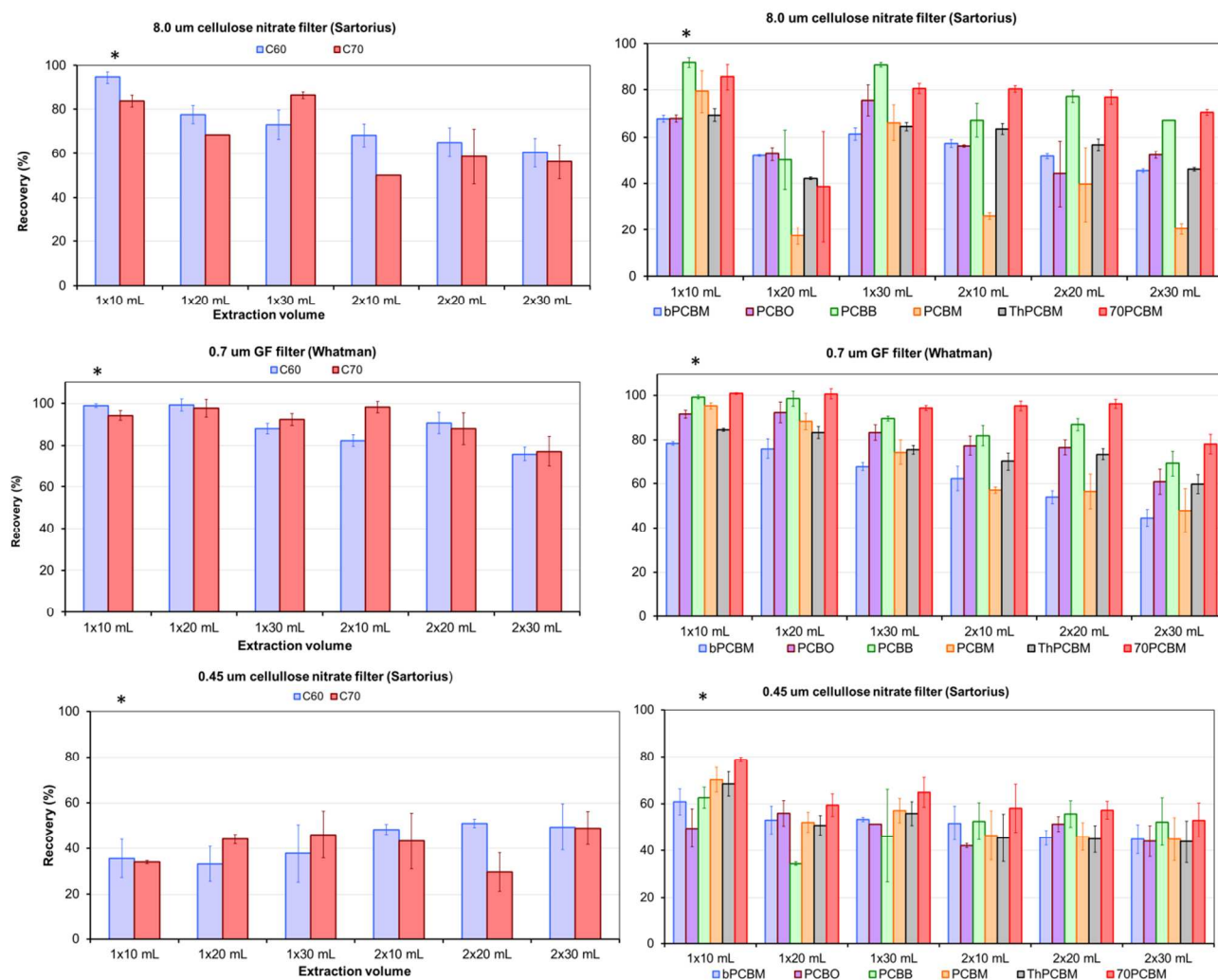

Figure 1: Recoveries of (functionalised) fullerenes with different filters and extraction volumes(asterisk showing the chosen volume) the extraction time was kept constant.

Environmental Science Nano

ARTICLE

The main goal was to optimise the extraction of the set of (functionalised) fullerenes and to aim at a reduction of the amount of toluene to create a one-step extraction. First of all the amount of toluene to extract fullerenes from spiked filtered particulate matter were varied In order to obtain spiked samples of particulate matter three aliquots (100 mL each) of a blank influent were passed over the three filters. The particulate matter collected on each of the filters was then spiked with 50 µL of fullerene mixture stock suspension at 2 ng/µL, dried and extracted with toluene in an ultrasonic bath. The extracts were evaporated and transferred to a vial. All experiments were performed in triplicate and the results are summarised in Figure 1.As can be seen 10 mL of toluene were enough for obtaining a good recovery in case of 0.7 µm glass fiber filters (≥80% for all the fullerenes, >95% for C_{60} and C_{70}) with only 45 min of extraction. Recoveries for all fullerenes did not improve significantly using 20 mL or 30 mL of toluene and decreased when applying two sequential extractions with toluene, probably due to an increase in evaporation steps and resulting manipulation of the sample. Similar trends were observed with 8.0 µm cellulose nitrate filters, although recoveries were slightly lower. The poorest recoveries were obtained with 0.45 µm cellulose nitrate filters and no significant differences between extraction volumes could be identified.
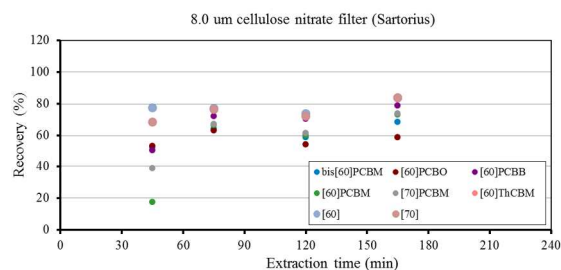
Therefore, longer extraction times were tested for the cellulose nitrate 8.0 µm (Figure 2) and 0.45 µm filters (Figure 3), to which fullerenes seemed to attach more strongly.. Optimum times were 60 min, 45 min and 90 min for 8.0 µm, 0.7 µm and 0.45 µm, respectively. Extraction times longer than these ones did not result in a significant improvement, or even caused a slight decrease of the recovery. Extraction time was set to 60 minutes for all filters

Fullerene distribution in wastewater samples. The distribution of the fullerenes in the wastewater was studied by separately analysing each of the filtrate fractions of a spiked influent wastewater free from fullerenes at 2 ng/L. Furthermore the distribution was analysed at different equilibration times allowed after spiking: from 1 min 180 min.

The results are summarised in Figure 4.

Figure 2: Recoveries of fullerenes on 8.0 µm cellulose nitrate filter, obtained with different extraction times

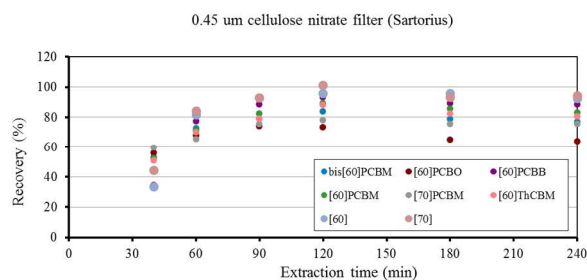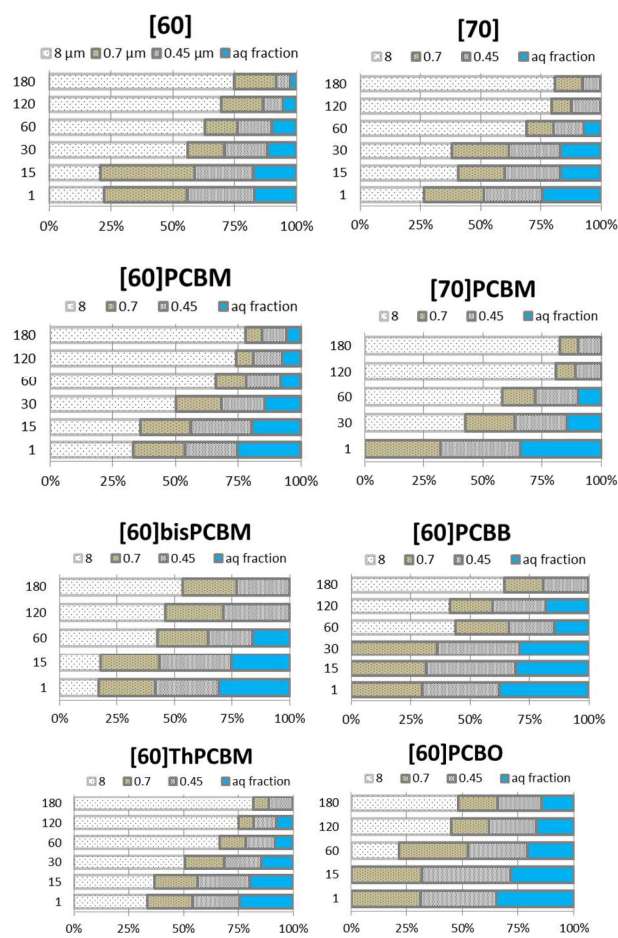Figure 3: Recoveries of fullerenes on 0.45 µm cellulose nitrate filter, obtained with different extraction times

Figure 4: Fullerene distribution on 8, 0.7 and 0.45µm filters and the aqueous phase as a function of time (min) allowed for equilibration after spiking

Due to the complexity of the matrix, homoaggregates (solely one type of fullerenes) with a size above 450 nm (unpublished results) are unlikely to occur. In general heteroaggregate (fullerenes with other particles form the matrix) formation[30] appears to be quite fast in the samples except for [60]PCBO and [60]PCBB probably due to their longer butyl and octyl-ester group. As can be seen, fullerenes distributed widely and they could be detected initially in almost all the fractions. When the equilibration time reached 180 minutes, the contribution of the aqueous fraction to the total mass balance reduced to (almost) zero. The fullerenes aggregated with the respective particulate fractions. For example, after 2h of equilibration time 41 to 79% was recovered in the particulate fraction above 8.0 μm, 7 to 25% of the fullerenes were recovered in the particulate fraction between 8.0 μm> x > 0.7 μm. The amounts of fullerenes were slightly lower, in the particulate fraction between 0.7 μm> and > 0.45 μm 8 to 22%. Finally, around 0 to 18% of the fullerenes passed through the three filters and were detected in the aqueous phase after two h. The present data suggest that most of the fullerene aggregates are likely to be captured by the filters due to hetero aggregate formation with the particulate matter. This implies that after some considerable time almost 100 percent of the fullerenes are likely to precipitate. Pakarinen[31] demonstrated that a portion of nC_{60} aggregates may remobilise from sediment. Figure 5 shows the method scheme that was used to quantify all possible fractions.
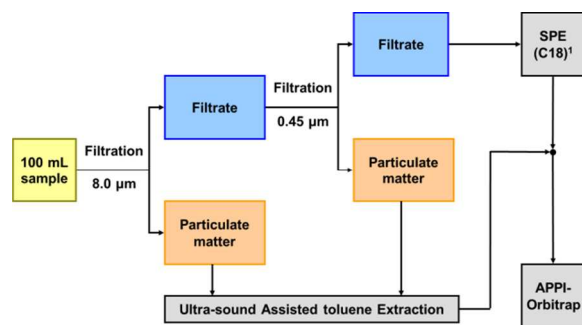

Figure 5: Final method scheme

Atmospheric Pressure ionisation Mass spectrometry Using an ESI interface and Orbitrap-MS in negative mode, methanol or acetonitrile are most often used to generate the electrons needed to produce a molecular ion. Depending on the HPLC stationary phase they can be added to the mobile phase (reversed phase) or post-column (normal phase). The ESI process induces the complex adduct formation[32] with the inconveniences previously stated[8,17]. This unfavourable behaviour hinders the process of qualifying and quantifying. For fullerenes analysis equal adduct patterns are obtained. Pristine fullerenes form adducts with oxygen, hydrogen, methanol and toluene (Figure 6). For the functionalised fullerenes a minor deprotonation product can be observed (<5% relative to the molecular ion peak $[M-H]^{-\bullet}$) (Figure 7).
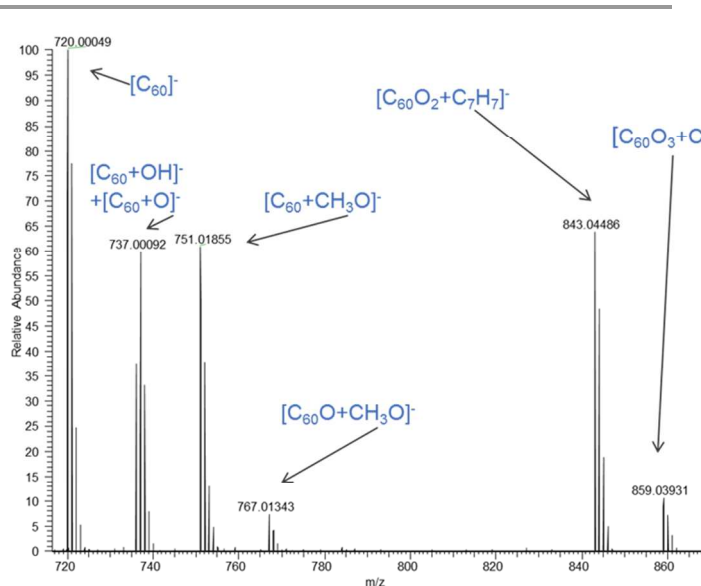
Figure 6: Mass spectrum of C_{60} with post column infusion of methanol generated by ESI-OrbitrapMS
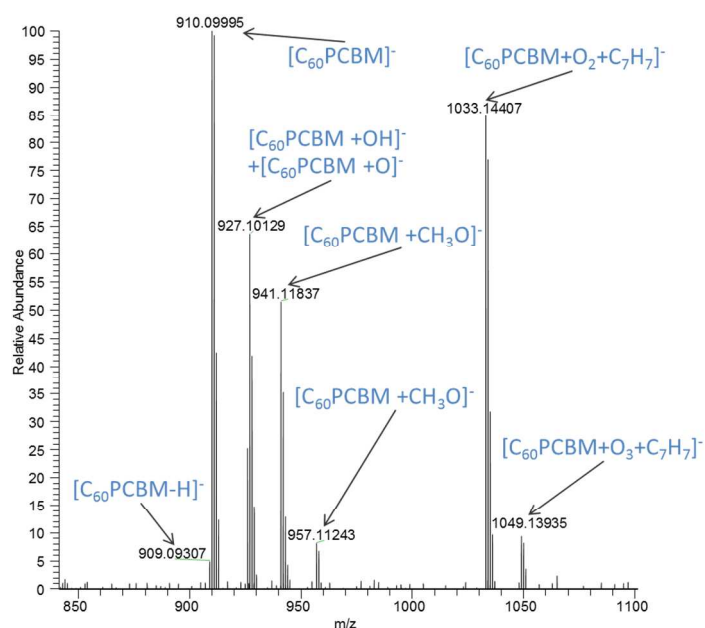
Figure 7: Mass spectrum of C_{60}PCBM with post column infusion of methanol generated by ESI-OrbitrapMS

By using APPI instead of ESI this complex adduct formation is reduced dramatically and hence the sensitivity is enhanced[28,32,33]. Still, adduct formation can occur by using methanol[32] or acetonitrile in combination with toluene[28], which is indispensable in a reversed phase HPLC system. Nuñez et al. (2012)[28] demonstrated that the ^{13}C isotope is influenced by the addition of a hydrogen to the fullerene double bonds. Switching to a normal phase system with a 100% isocratic toluene system eliminates most of the adduct formation and solely results in a minor oxygen adduct (0.23%) (Figure 8).
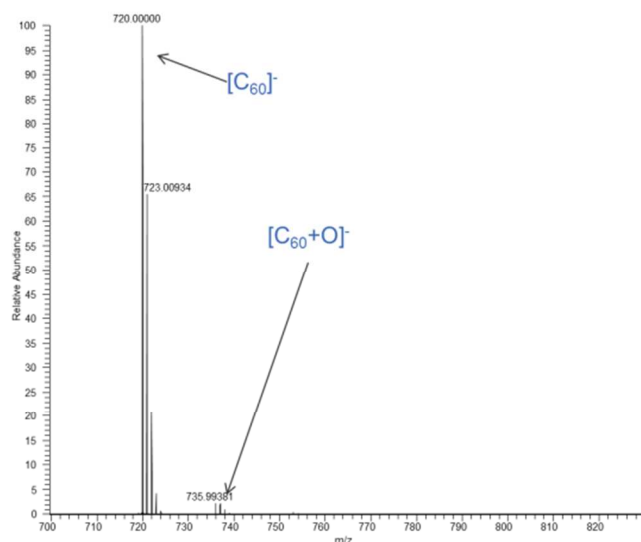
Figure 8: Mass spectrum of C_{60} generated by a mobile phase consisting of 100% toluene and APPI-Orbitrap-MS

This is resulting in an experimental $^{13}C/^{12}C$ ratio which correlates well with the theoretically predicted ratio (Figure 9) By using a 100% isocratic elution with toluene and eliminating adduct formation (through ionisation of methanol or acetonitrile) the quantification becomes more straightforward compared to the common approach to sum up all adducts. For identification of pristine fullerenes, since they do not exhibit any fragmentation even at high collision energies, the most common approach is to use the oxidised ion as confirmation $[C_{60}O]^{-\bullet}$. As demonstrated in Figure 9, with 100% toluene APPI-Orbitrap the $^{13}C/^{12}C$ ratio correlates well with the theoretical one and could be used for qualifying fullerenes instead of the very low abundant oxygen adduct. This would comply with to the Commission Decision 2002/657/EC[34] which states that the ratio of the intensity of a diagnostic ion from the isotopic pattern such as a $^{13}C_{N-1}$ with a relative intensity higher than 10% of the $^{12}C_N$ should be within ± 20% of the theoretical ratio withthe base peak. Figure 9 demonstrates that if methanol is used in the mobile phase this criterion will often not be met. Hence identification was achieved for all analytes (4 points) by: retention time agreement for the standard and sample (within 2.5%), monitoring the parent mass in high resolution (2 points) and confirmation of the $^{13}C/^{12}C$ ratio (2 points).
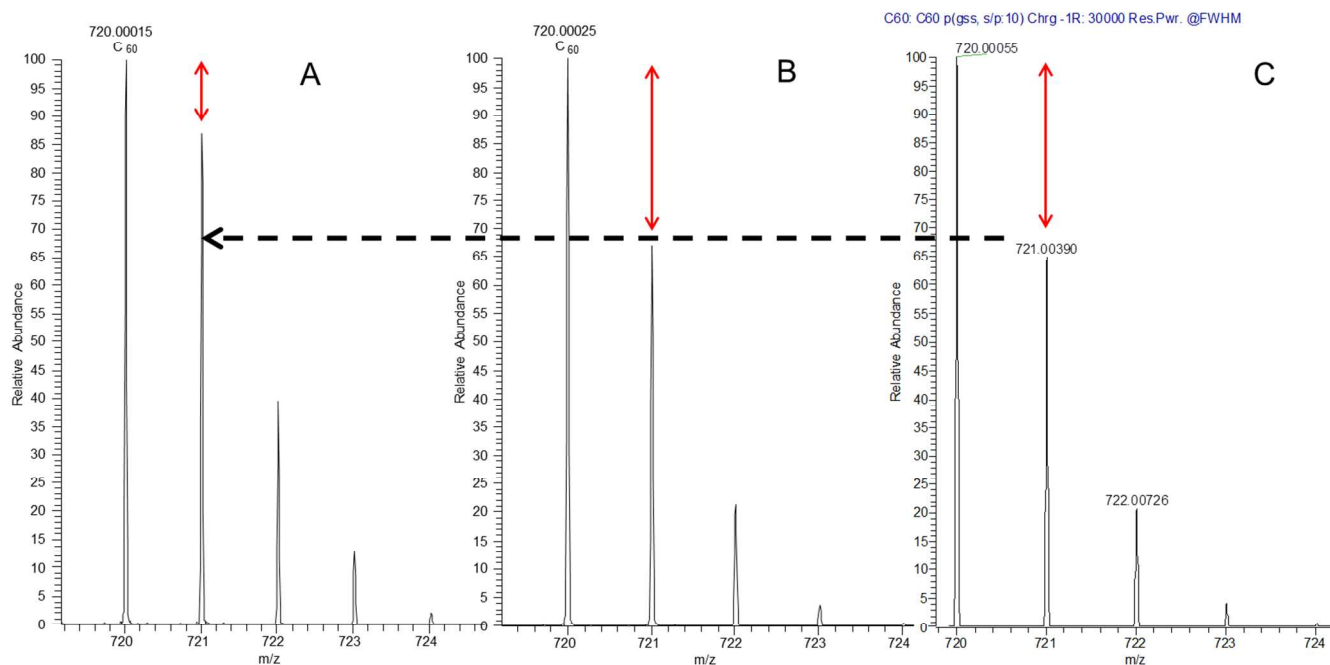

Figure 9: Mass spectra of C_{60} obtained with APPI-Orbitrap;, A: isotopic pattern with Methanol/toluene (25/75 v%) mobile phase - APPI-Orbitrap, B: isotopic pattern 100% toluene APPI-Orbitrap, C: Theoretical isotopic pattern C60. Resolution 30,000 FWHM 400m/z

The use of APPI-HRMS instrumentation results in better limits of detection and quantification than those obtained in previous works. For instance, they are one order of magnitude lower than those described in Farré et al. (2010)[14] using ESI-MS/MS, highlighting the suitability of the APPI-HRMS configuration for these compounds. Also, these parameters are of the same order (or slightly lower) than those obtained by Kolkman et al. (2013)[8], analysing spiked ultrapure water by ESI-HRMS.

Table 1: Instrumental and method quality parameters

			bis[60]PCBM	[60]PCBO	[60]PCBB	[60]PCBM	[60]ThCBM	[70]PCBM	C_{60}	C_{70}
Instrumental Parameter	**ILOD (fg)**		15	11	22	16	31	21	7.9	10
	ILOQ (fg)		50	37	74	52	100	69	26	34
	Instrumental linearity (R^2)[a]		0.9999	0.9996	0.9995	0.9999	0.9982	0.9997	0.9998	0.9979
	Sensitivity [a.u.]		15.8	14.0	15.4	16.9	15.1	10.4	8.85	8.18
	Intraday (n=7) repeatability (%)	**10 pg**	2.34	3.40	3.27	2.62	4.17	3.22	2.60	4.58
		80 pg	1.34	1.49	1.54	1.36	1.81	1.83	1.65	1.31
		640 pg	3.56	3.73	3.48	2.74	2.79	1.51	4.44	3.03
	Interday repeatability (%)	**10 pg**	8.44	8.32	9.36	9.47	9.50	8.51	7.53	6.68
		80 pg	10.7	9.55	7.52	8.35	8.94	7.15	4.89	4.09
		640 pg	6.70	6.81	5.42	7.72	6.44	5.84	8.00	4.01
Method quality parameters	**MLOD (pg/L)**		77	68	75	83	74	66	210	440
	MLOQ (pg/L)		260	230	250	280	250	220	710	1,400
	Method linearity (R^2)[b]		0.997	0.998	0.995	0.994	0.995	0.991	0.991	0.991
	Method interday repeatability (%)	**10 ng/L**	8.51	11.3	14.9	9.98	14.9	11.3	13.5	12.2
		50 ng/L	3.91	4.24	5.20	10.9	8.60	8.24	11.5	7.90
		1,000 ng/L	3.94	2.58	4.76	3.03	3.03	1.00	3.19	7.24
	Recovery (%)	**10 ng/L**	87.0	88.1	99.6	104	97.8	103	104	106
		50 ng/L	48.3	67.7	74.0	80.3	82.4	79.0	105	105
		1,000 ng/L	50.1	72.1	101	97.6	98.9	88.9	98.6	102
	Accurate Mass [M]·-		1100.19931	1008.20948	952.14688	910.09993	916.05635	1030.09993	720.00055	840.00055
	Theorethical $^{13}C/^{12}C$ ratio (%)		91	86	81	78	77	89	65	76
	experimental $^{13}C/^{12}C$ ratio (%± RSD) [N=14]		94.2 ± 0.4	88.1 ± 0.3	83.7 ± 0.6	80.8 ± 0.5	78.9 ± 0.3	91.9 ± 0.4	67.2 ± 0.4	78.6 ± 0.5

[a]: Instrumental linearity was assessed between 125 fg and 1.28 ng, [b] Method linearity was assessed between 10 and 500 ng/L, [c] Method repeatability was assessed by quintuplicate and each of the replicates was extracted in a consecutive day.

Moreover, the method presented here requires a sample volume that is 10 times smaller. The quality parameters were obtained with spiked wastewater matrix. Similarly, Nuñez et al. [28] described a MLOD. of 0.01 ng/L for both C_{60} and C_{70}, although in freshwater matrix and using a triple quadrupole MS with enhanced mass resolution.

Method validation. Instrumental quality parameters are summarised in Table 1. As can be seen, instrumental limits of detection were in the fg range (between 7.9 and 31 fg). Interday repeatability was below 11% for all the compounds (between 4 and 11%) and it was significantly better when considering intraday runs (between 1.3 and 4.5%). Linearity ranges were acceptable through the studied range assessed between 128 fg and 1.28 ng. The sensitivity was slightly better for those fullerenes containing functional groups that allow an easier ionisation. Detection limits expressed as sample concentrations were in the pg/L order (between 66 and 440 pg/L) which implies that the method can be applied to real samples. Recoveries for C_{60} and C_{70} for the filters and filtrate of the samples were near 100% at all the tested concentrations and they were acceptable for the functionalised fullerenes (49-104%) (Table 1). Interday repeatabilities were found to be between 1 and 15%.

Table 2: Results showing concentrations of C_{60} (ng/l) in six different sewage treatment plants. All other fullerenes were below the LOD

STP	Type	[60] ng/L	$^{13}C/^{12}C$ ratio (%)	deviation from standard
1	Influent	19	64	-2%
1	Effluent	< (0.1)[a]	70	8%
1	Sediment receiving river	12ng/kg	68	5%
2	Influent	5	61	-6%
2	Effluent	< (0.2)[a]	73	12%
3	Influent	4	65	0%
3	Effluent	< (0.1)[a]	75	15%
4	Influent	7	68	5%
4	Effluent	<	69	6%
5	Influent	9	65	0%
5	Effluent	< (0.1)[a]	67	3%
6	Influent	2	66	2%
6	C-stage	1	72	11%
6	N-stage	<	n/a	-
6	Effluent Nereda™ reactor	<	n/a	-
6	Effluent	<	n/a	-

[a] < LOQ>LOD

Analysis of field samples. To evaluate the applicability of the method to determine fullerenes by APPI-HRMS, 24 h flow dependent composite influent samples were analysed from six different sewage water treatment plants. The results are shown in Table 2.

In order to establish the efficiency of the STPs the total concentration of fullerenes in the effluents was determined according to Kolkman et al[8] disregarding possible fractionation (because of a much lower suspended particle concentration). The highest level of C_{60} (19 ng/L) was observed in the influent of STP 1. In the effluents no appreciable concentrations of fullerenes could be observed. A sediment sample was collected downstream from the Treatment plant 1, extracted according to Carboni et al (2013)[23] and analysed with the toluene-APPI-HRMS method. A level of 12 ng/kg was observed.

Conclusions

In the present work, a simple isocratic HPLC method for analysing eight fullerenes in wastewater samples by APPI-Orbitrap MS has been developed and validated. The method allows the accurate mass identification of fullerenes aggregated with the particulate matter and those aggregated in the aqueous phase. The application of a normal phase column in combination with APPI avoids the use of methanol thus minimising adduct formation in the interface, and allows a straightforward identification with unambiguous mass spectra never demonstrated before. Compared with ESI the method is more sensitive and more robust, mass spectra are free from adducts, and experimental isotopic patterns match the theoretical ones. In all influent samples measured only C_{60} was encountered at levels up to 19 ng/L. The levels of C_{60} observed in the effluents were all above the method detection limit but below the quantification limit.

Acknowledgements

Notes and references

[a] KWR Watercycle Research Institute, P.O. Box 1072, 3430 BB Nieuwegein, The Netherlands.
[b] Department of Environmental Chemistry, CIDCSIC c/Jordi Girona, 18-26, 08034 Barcelona
[c] Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam
* co-first authors
[1] Visiting Scientist at KWR.

1. H. W. Kroto, J. R. Heath, S. C. O'Brien, R. F. Curl and R. E. Smalley, *Nature*, 1985, **318**, 162-163.
2. R. D. Handy, R. Owen and E. Valsami-Jones, *Ecotoxicology*, 2008, **17**, 315-325.
3. S. Utsunomiya, K. A. Jensen, G. J. Keeler and R. C. Ewing, *Environmental Science & Technology*, 2002, **36**, 4943-4947.
4. T. Laitinen, T. Petäjä, J. Backman, K. Hartonen, H. Junninen, J. Ruiz-Jiménez, D. Worsnop, M. Kulmala and M.-L. Riekkola, *International Journal of Mass Spectrometry*, 2014, **358**, 17-24.
5. T. M. Benn, P. Westerhoff and P. Herckes, *Environmental Pollution*, 2011, **159**, 1334-1342.
6. Z. Chen, R. Mao and Y. Liu, *Current Drug Metabolism*, 2012, **13**, 1035-1045.
7. C. J. Brabec, S. Gowrisanker, J. J. M. Halls, D. Laird, S. Jia and S. P. Williams, *Advanced Materials*, 2010, **22**, 3839-3856.
8. A. Kolkman, E. Emke, P. S. Bäuerlein, A. Carboni, D. Truc Tran, T. L. Ter Laak, A. van Wezel and P. de Voogt, *Analytical Chemistry*, 2013, **12**, 5867-5874.
9. F. Piccinno, F. Gottschalk, S. Seeger and B. Nowack, *Journal of Nanoparticle Research*, 2012, **14**, 1-11.
10. S. K. Brar, M. Verma, R. D. Tyagi and R. Y. Surampalli, *Waste Manage.*, 2010, **30**, 504-520.
11. T. Y. Sun, F. Gottschalk, K. Hungerbühler and B. Nowack, *Environmental Pollution*, 2014, **185**, 69-76.
12. M. R. Wiesner, G. V. Lowry, P. Alvarez, D. Dionysiou and P. Biswas, *Environmental Science & Technology*, 2006, **40**, 4336-4345.

13. J. Sanchís, N. Berrojalbiz, G. Caballero, J. Dachs, M. Farré and D. Barceló, *Environmental Science & Technology*, 2011, **46**, 1335-1343.

14. M. Farré, S. Pérez, K. Gajda-Schrantz, V. Osorio, L. Kantiani, A. Ginebreda and D. Barceló, *Journal of Hydrology*, 2010, **383**, 44-51.

15. H. C. Chen and W. H. Ding, *Journal of Chromatography A*, 2012, **1223**, 15-23.

16. F. Gottschalk, T. Sonderer, R. W. Scholz and B. Nowack, *Environmental Science & Technology*, 2009, **43**, 9216-9222.

17. A. P. van Wezel, V. Morinière, E. Emke, T. ter Laak and A. C. Hogenboom, *Environmental International*, 2011, **37**, 1063-1067.

18. D. Bouchard, X. Ma and C. Isaacson, *Environmental Science & Technology*, 2009, **43**, 6597-6603.

19. J. D. Fortner, D. Y. Lyon, C. M. Sayes, A. M. Boyd, J. C. Falkner, E. M. Hotze, L. B. Alemany, Y. J. Tao, W. Guo, K. D. Ausman, V. L. Colvin and J. B. Hughes, *Environmental Science & Technology*, 2005, **39**, 4307-4316.

20. W.-C. Hou, L. Kong, K. A. Wepasnick, R. G. Zepp, D. H. Fairbrother and C. T. Jafvert, *Environmental Science & Technology*, 2010, **44**, 8121-8127.

21. Z. Wang, J. Chen, Q. Sun and W. J. G. M. Peijnenburg, *Environmental International*, 2011, **37**, 1078-1082.

22. D. Heymann, *Fullerene Science and Technology*, 1996, **4**, 509-515.

23. A. Carboni, E. Emke, J. R. Parsons, K. Kalbitz and P. de Voogt, *Analytica Chimica Acta*, 2014, **807**, 159-165.

24. C. Wang, C. Shang and P. Westerhoff, *Chemosphere*, 2010, **80**, 334-339.

25. P. Herrero, P. S. Bäuerlein, E. Emke, E. Pocurull and P. de Voogt, *Journal of Chromatography A*, 2014, **1356**, 277-282.

26. C. W. Isaacson, C. Y. Usenko, R. L. Tanguay and J. A. Field, *Analytical Chemistry*, 2007, **79**, 9091-9097.

27. J. F. Anacleto and M. A. Quilliam, *Analytical Chemistry*, 1993, **65**, 2236-2242.

28. O. Núñez, H. Gallart-Ayala, C. P. B. Martins, E. Moyano and M. T. Galceran, *Analytical Chemistry*, 2012, **84**, 5316-5326.

29. X. Qu, Y. S. Hwang, P. J. J. Alvarez, D. Bouchard and Q. Li, *Environmental Science & Technology*, 2010, **44**, 7821-7826.

30. J. T. K. Quik, I. Velzeboer, M. Wouterse, A. A. Koelmans and D. van de Meent, *Water Res.*, 2014, **48**, 269-279.

31. K. Pakarinen, J. Akkanen, M. T. Leppänen and J. V. K. Kukkonen, *Chemosphere*, 2014, **108**, 320-325.

32. L. Li, S. Huhtala, M. Sillanpää and P. Sainio, *Anal Bioanal Chem*, 2012, **403**, 1931-1938.

33. S.-i. Kawano, H. Murata, H. Mikami, K. Mukaibatake and H. Waki, *Rapid Communications in Mass Spectrometry*, 2006, **20**, 2783-2785.

34. EU, *Official Journal of the European Communities*, 2002, **L 221**, 29.