Fullerene nanoparticles in soil: Analysis, occurrence and fate

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Chapter 4

An UHPLC-HRMS method for the analysis of fullerenes in soil

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Abstract

The increasing production of fullerenes likely means a release of these chemicals in the environment. Since soils and sediments are expected to act as a sink, analytical tools are needed to assess the presence of fullerenes in these matrices. In the present work, a method was developed for the determination of fullerenes at environmental relevant levels employing Ultra High Performance Liquid Chromatograph coupled with High Resolution Mass Spectrometry (UHPLC-HRMS). Chromatographic separation was achieved with a core-shell biphenyl stationary phase that provided fast analysis with complete baseline separation. Ion Booster Electro Spray Ionization (IB–ESI) resulted in higher ionization efficiency and was much less susceptible to adduct formation in comparison with standard ESI, whereas Quadrupole Time of Flight (QTOF) MS granted high resolution mass spectra used for accurate identification. The Instrumental method limits of detection (ILoD) and quantification (ILoQ) were 6 and 20 fg, respectively, for $C_{60}$ and 12 and 39 fg, respectively, for $C_{70}$. Matrix effects related to co-extractants were systematically investigated in soil and sediments extracts through standard addition method (SAM) and monitoring the signal response during the chromatographic run of these samples. Consequently, minor chromatographic modifications were necessary for the analysis of matrices with high organic carbon content. The method limit of detection (MLoD) ranged from 84 pg/kg to 335 pg/kg, whereas limit of quantification (MLoQ) ranged from 279 pg/kg to 1.1 ng/kg. Furthermore, the method was successfully applied for the analysis of functionalized fullerenes (i.e. methanofullerenes). To the best of our knowledge, this is the first analytical method for the analysis of fullerenes in soils and sediments that employ core-shell biphenyl stationary phase as well as IB-ESI-QTOF MS hyphenated with UHPLC.
4.1 Introduction

Since their discovery (Kroto et al., 1985), fullerenes and especially $C_{60}$ have been subject of a large number of studies mostly focused on their innovative physical-chemical properties and possible applications. Fullerenes are already used in cosmetics (as antioxidants) and in photovoltaics (as electron acceptors) and possible fields of application include electronics and optics as well as biomedical engineering (Tagmatarchis et al., 2001; Guldí et al., 2002; Burangulov et al., 2006; Kim et al., Maynard et al., 2006). Furthermore, the possibility of derivatization of their structure e.g. with surface functionalization (Hummelen et al., 1995) as well as the encapsulation of other chemical species (Chai et al., 1991), is likely to increase the range of applications of these chemicals. Precise data regarding the manufactured amounts are missing but the worldwide production was estimated in tens of tons per year and is expected to increase in the near future (Hendren et al., 2011; Piccinno et al., 2012). This will inevitably mean a release into the environment and there are several concerns about their possible accumulation and toxic effects. Besides, fullerenes can also be naturally produced (e.g. in energetic events such as volcanic eruptions (Heymann et al., 1994) or flame generation (Howard et al., 1991)) but data are limited and often contradictory (Elsila et al., 2005). Thus, the presence of fullerenes in the environment may be due to both natural and anthropogenic origins as well as resulting from transformation of engineered nanoparticles (e.g. fullerene derivatives). In this context, fullerenes are nowadays considered as emerging contaminants and analytical methods are needed for both environmental and toxicological assessment. However, only in the last few years have environmental monitoring and modeling studies been carried out and the knowledge is still limited. Data modeled based on the estimated production suggested that the concentrations (Predicted Environmental Concentration, PEC) of $C_{60}$ in the environment should range between parts per billion and parts per trillion with higher concentrations in soil and sediments than water and air (Gottschalk et al., 2009). Thus, soil may act as a sink for the fullerenes whose source may be direct release and atmospheric deposition but also water transport (in the form of $nC_{60}$ and/or
associated with organic matter) and application of biosolids (Navarro et al., 2013). In the last few years, a number of studies have been published focused on the extraction of fullerenes and methods are now available that allow the extraction of these chemicals in a large number of matrices with acceptable if not excellent recoveries (e.g. Carboni et al., 2013; Kolkman et al., 2013; Perez et al., 2013). High Performance Liquid Chromatography (HPLC) in combination with UV-vis and/or mass spectrometry (MS) is the most widely used technique for the determination of fullerenes and issues related to the chromatographic separation of the fullerenes have been recently reviewed elsewhere (Saito et al., 2004; Astefanei et al., 2014a). Briefly, mobile phases usually consisted of toluene as a main eluent in combination with a more polar solvent, such as acetonitrile or methanol, whereas stationary phases commonly employed include standard octadecyl silica (ODS) as well as functionalized silica. However, these methods are often time consuming and seldom optimized for the analysis of complex environmental samples (e.g. Carboni et al., 2013; Kolkman et al., 2013). In this context, core-shell biphenyl is a novel stationary phase, never applied for the analysis of fullerenes, which may provide a retention mechanism similar to that of the pyrenylpropyl silica (Lomas et al., 2015). Regarding the detection, although UV-vis showed promising performance (Wang et al., 2010) for the determination of fullerenes in environmental matrices (Carboni et al., 2013), its relatively low sensitivity and its lack of selectivity in comparison with mass spectrometric detection make it unsuitable for the analysis of the low concentrations expected in the environment. Thus, mass spectrometry is considered as the detection method of choice and low as well as high resolution methods have been described. Instruments commonly employed make use of triple quadrupole (Sanchis et al., 2013; Astefanei et al., 2014b), LTQ Orbitrap (van Wezel et al., 2011; Nunez et al., 2012; Kolkman et al., 2013; Emke et al., 2015), and FTICR (Nunez et al., 2012) coupled with HPLC systems and Quadrupole time of flight (QTOF) MS with direct injection (Song et al., 2007). Atmospheric pressure ionization (API) methods were usually employed in negative mode. Specifically, the use of ESI in negative mode allowed the detection of fullerenes in soils and water at relatively low concentrations (Kolkman et al., 2013; Sanchis et al., 2013) but APPI was recently proposed as a better option due to the higher sensitivity and less complex mass spectra (Nunez et al., 2012; Astefanei et
Heated interfaces such as H-ESI were seldom applied and showed efficiency of ionization generally higher than that of standard ESI, but lower in comparison with other systems such as atmospheric pressure photo (APPI) and chemical (APCI) ionization (Nunez et al., 2012; Astefanei et al., 2014). In this context, the Ion Booster-ESI (IB-ESI) consists of a modified ESI source that was recently employed in the screening of drugs in serum, showing better performance than standard ESI (Huppertz et al., 2014; Kempf et al., 2014). The additional soft voltage and vaporizer temperature are expected to enhance the ionization of the fullerenes in comparison with standard ESI, i.e. to improve the sensitivity. The analysis of fullerenes in the environment is challenging because of the low concentrations expected and only recently monitoring studies have highlighted the presence of these chemicals in environmental samples (e.g. soil (Sanchis et al., 2013 and 2015), sediments (Sanchis et al., 2013; Astefanei et al., 2014b), surface waters (Nunez et al., 2012; Astefanei et al., 2014b), wastewater (Farre et al., 2010; Emke et al., 2015) and air (Sanchis et al., 2012)). Furthermore, the complexity of some of the matrices that have to be analyzed (e.g. WWTPs, soil) is an issue during both the extraction and analysis of fullerenes (Jehlicka et al., 2005). The presence of co-extractants, with special regard to organic matter content, could hamper a correct quantification and it may have hindered the detection in other recent works focused on soil (Perez et al., 2013) and water (van Wezel et al., 2011). To date a few studies addressed the challenges of a clean-up procedure (Sanchis et al., 2013) or accounted for the matrix effect during the detection and quantification of fullerenes. Although fortification (i.e. standard addition) of environmental samples under investigation was recently included in monitoring studies, this issue was never systematically approached and analytical methodologies such as matrix matched calibration were recommended (Astefanei et al., 2014b; Sanchis et al., 2015). Thus, in the present work we developed an analytical method that is specifically aimed for the analysis of fullerenes in complex environmental samples such as soil and sediment. The method included the use of a novel stationary phase, i.e. biphenyl functionalized silica, and of a heated interface (IB-ESI) in combination with high resolution mass spectrometry (QTOF-MS).
4.2 Materials and Methods

4.2.1 Reagents and chemicals

Toluene, methanol, isopropanol and acetonitrile (Biosolve, Valkenswaard, The Netherlands) were all analytical grade (LC-MS). The mobile phase modifiers tested were acetic acid (Merck, Darmstadt, Germany), formic acid (Biosolve, Valkenswaard, The Netherlands) and ammonium acetate (Sigma-Aldrich, Zwijndrecht, The Netherlands) whereas sodium hydroxide was purchased by Merck KGaA (Amsterdam, The Netherlands). Fullerenes C$_{60}$ (CAS: 99685-96-8) and C$_{70}$ (CAS: 115383-22-7) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Stock solutions of the individual fullerenes were prepared in toluene, at a concentration of 5 mg/L, and placed on an orbital shaker (Laboshake orbital shaker, Gerhardt, Königswinter, Germany) in the dark overnight. Further solutions needed for the experiments, including mixtures of the fullerenes, were obtained by dilution of the stock solutions, stored at 4°C in the dark and sonicated for 2 min before use (Branson 12, Branson, Danbury CT, United states). The analyses were performed using an UHPLC system (Nexera, Shimadzu, Den Bosch, The Netherlands) equipped with a binary pump, autosampler and column oven. Retention of the chemicals was achieved with a core-shell Kinetex 2.6 µm biphenyl 100 Å chromatographic column (Phenomenex, Utrecht, The Netherlands) consisting of a biphenyl stationary phase. The UHPLC system was coupled to a high resolution Quadrupole-Time of Flight mass spectrometer (Q-TOF; maXis 4G upgraded with HD collision cell, Bruker Daltonics, Wormer, The Netherlands) equipped with either an electro spray (ESI) or an Ion Booster electro spray (IB-ESI) ionization sources (Bruker Daltonics, Wormer, The Netherlands). High purity nitrogen was supplied by a N$_2$ generator (Avilo, Dirksland, The Netherlands) and used for ionization and collision gas.
4.2.2 Analytical method settings

Internal mass calibration was performed automatically during each analysis in order to assure good mass accuracy for all the samples independently of the total analysis time. This was achieved at the beginning of the analysis (0.1-0.4 min) by infusing a 2 mM sodium acetate solution in a water isopropanol mixture (1:1, v:v), with a loop injection of 20 µl and a loop rinse of 20 µl. A temperature gradient was employed for the ionization source because of the different optimal temperatures needed for the analysis of the mass calibration solution and the analytes under investigation. Specifically, the temperature was set at 325°C at the beginning of the run and was gradually increased after one minute in order to reach 450°C at 2 min (Figure B.1 in appendix B). The sodium acetate cluster provided ten points of calibration ranging from \( m/z \) 387 to \( m/z \) 1207 (Table B.2 in appendix B) of which at least eight points (standard deviation ≤ 0.3 ppm) were taken for the mass calibration of each sample. The final chromatographic methods were optimized with regard to the methanol (eluent A) and toluene (eluent B) amounts employed and consisted of the following programs. Method A: flow rate of 400 µl, 40 sec at 100% eluent A (focusing step), a linear gradient up to 50% eluent B in 0.3 min, an isocratic step of 3.50 min at 50% eluent B (elution step) and then a linear gradient of 20 sec to reach 100% eluent B which was maintained for 2.5 min (cleaning step). Method B: flow rate of 600 µl starting with 40 sec at 100% eluent A (focusing step), a linear gradient up to 35% eluent B in 20 sec, an isocratic step of 4 min at 35% eluent B followed by a linear gradient of 1.5 min to reach 60% B (elution step), a linear gradient of 10 sec in order to reach 100% eluent B which was maintained for 2.5 min (cleaning step). The chromatograms were divided into four segments: segment 1 (from 0 min to 0.1 min) to assure the correct position of the loop, segment 2 (from 0.1 min to 0.4 min) dedicated to the mass calibration, segment 3 (from 0.4 min to 3.4 min in method A and 4.4 min in method B) for the SRM analysis of \( \text{C}_{60} \) (\( m/z \) 720.0005) and segment 4 (from 3.4 min to 7.6 min for method A and from 4.4 min to 9.6 min for method B) for the SRM analysis of \( \text{C}_{70} \) (\( m/z \) 840.0005). After the mass calibration was achieved, the LC flow was diverted to the waste (0.5 min) in order to avoid the exposure of the interface to the more polar co-extractants that eluted at the beginning of the chromatogram. The LC flow was redirected to the MS (1.5 min in
method A and 2.5 min in method B) to allow the interface to equilibrate before the elution of the chemicals and then switched again to waste after the elution of the analytes (4.5 min in method A and 6.5 min in method B). Fullerenes ESI and IB-ESI spectra were obtained by infusing both individual and mixture standard solutions in toluene:methanol (1:1,v:v) at varying concentrations. The optimized working conditions for the ESI and IB-ESI in negative mode were: capillary voltage 1000 V, end plate offset -400 V, charging voltage 300 V, nebulizer gas 4.1, dry gas 3.0 l/min and dry heater 200°C. Further settings optimized for the MS analysis were: funnel radio frequency (RF) at 325.0 Vpp (voltage point to point), Multipole RF at 300.0 Vpp, collision cell RF at 1600.0 Vpp, transfer time at 50 µs and prepulse storage time at 25 µs. The mass range analyzed in MS1 was 300 – 2000 m/z whereas the spectra rate frequency was set at 2 Hz during all the analysis performed. Collision energy for MS/MS was set at 100 V whereas the isolation width was 8.00 m/z. The present settings allowed a mass resolving power up to 80000 (0.03 m/z FWHM). The [M]" and [M+1]" abundances relative to C_{60} and C_{70} were determined by analysing a standard solution at 500 ng/L repeatedly with both method A (n=7) and method B (n=7). More details about the molecular ions and isotopic clusters during the detection are provided in the discussion section. The Bruker Compass 1.7 software was employed in both the data collection and data processing.

4.2.3 Samples collection and treatment

In table 4.1, the properties of the soil and sediment samples included in the present study are reported.
Table 4.1. Properties of the soil and sediment samples included in the present study.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Substrate</th>
<th>N [%]</th>
<th>S [%]</th>
<th>Inorg C [%]</th>
<th>Org C [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Loess soil</td>
<td>0.11</td>
<td>0.02</td>
<td>n.d.</td>
<td>0.90</td>
</tr>
<tr>
<td>2</td>
<td>Urban park Soil</td>
<td>0.18</td>
<td>0.03</td>
<td>n.d.</td>
<td>2.68</td>
</tr>
<tr>
<td>3</td>
<td>River Clay Sediments</td>
<td>0.14</td>
<td>0.03</td>
<td>n.d.</td>
<td>1.03</td>
</tr>
<tr>
<td>4</td>
<td>Sea Clay Sediments</td>
<td>0.21</td>
<td>0.06</td>
<td>0.64</td>
<td>1.69</td>
</tr>
<tr>
<td>5</td>
<td>Carbon-rich Sandy Soil</td>
<td>0.14</td>
<td>0.04</td>
<td>0.84</td>
<td>2.24</td>
</tr>
<tr>
<td>6</td>
<td>Dune Sand</td>
<td>0.03</td>
<td>0.01</td>
<td>0.08</td>
<td>0.20</td>
</tr>
</tbody>
</table>

n.d. not detected

The Loess soil (matrix 1) was collected from an agricultural field in South Limburg, The Netherlands (50°53’58”N, 5°53’16”E). Matrix 2 consisted of an urban park soil sample collected in Vondelpark, Amsterdam, The Netherlands (52.3580° N, 4.8680° E). River clay and sea clay (matrix 3 and matrix 4 respectively) were available from the collection of the Earth Surface Science research group at the University of Amsterdam. The sandy top soil rich in organic carbon (matrix 5) was collected in Flevopark, Amsterdam, The Netherlands (52.3611° N, 4.9492° E) whereas the dune sand (matrix 6) was sampled in a natural park near Castricum, The Netherlands (52.5500° N, 4.6667° E). The samples were placed in a freezer at -20°C overnight and lyophilized with a Scanvac Coolsafe freeze-dryer (Labogene, Lynge, Denmark) in order to remove traces of water. The dried samples were finely ground with an agate mortar and sieved. The total carbon, sulfur and nitrogen contents of the dried soil samples were measured using a Vario EL Cube (Elementar, Hanau, Germany). The inorganic carbon content was determined as reported by Wesemael (1955) as follows: 1 g of soil was weighed into a 250 ml Erlenmeyer provided with a silica gel lid and an excess of HCl 4 M was added. Control samples consisted of 0.250 g of CaCO₃. The samples were weighed again after 24 h of shaking at 60 rpm (Laboshake orbital shaker, Gerhardt, Königswinter, Germany) and the inorganic carbon content was calculated accordingly. The soil and sediment samples were extracted with the protocol already
reported by our group (Carboni et al., 2013) with minor modifications, i.e. the temperature was set at 20 ± 2 °C as recommended by Perez et al. (Perez et al., 2013). Briefly, 10 to 15 g of each sample underwent two cycles of extraction with toluene with each cycle consisting of a combination of sonication for 20 min with a ultrasonic bath (Branson 12, Branson, Danbury CT, United states) and shaking for 2 h (Laboshake orbital shaker, Gerhardt, Königswinter, Germany). The extracts were concentrated evaporating the solvent under a gentle nitrogen flow (enrichment factor 20x) and stored in a refrigerator at 4 ºC in the dark. A repeatability test for the extraction procedure was achieved by spiking and extracting the fullerenes into matrix 6 at the concentration of 100 ng/kg (n=8) as reported by Carboni et al. (2013).

4.2.4 Sample analysis

Methanol (25% in volume) was added to each sample and the analyses were performed with an injection volume of 10 µl. External calibration curves were obtained by analyzing standard solutions at concentrations ranging from 1 to 500 ng/L and quantification was based on chromatographic peak areas. Instrumental method limits of quantifications (ILOQs) were assumed as the lowest measured concentration in the linear range with a deviation less than 30% of the theoretical concentration injected (EC commission, 2009). The instrumental method limit of detection (ILoD) was calculated as 3/10 of the ILoQ observed. The method limits of detection (MLoDs) and quantification (MLoQs) in soil and sediment samples were extrapolated from the signal-to-noise ratio observed in the chromatograms assuming a 70% recovery of extraction (Carboni et al., 2013). In case of absence of matrix effect and noise, these values were assumed to be equal to the ILoD and ILoQ. The criteria used for the identification of the analytes were: 1) the expected chromatographic retention time (± 0.2 min), 2) a mass accuracy threshold (≤ 5 ppm) and 3) an isotopic pattern fit threshold (≤ 50 mSigma), where mSigma represents the goodness of fit (the smaller the better) between the measured and theoretical isotopic pattern (Gago-Ferrero et al., 2015). For the standard addition method (SAM) experiments, each
matrix was spiked with both C$_{60}$ and C$_{70}$ fullerenes at increasing concentrations (25, 50, 100, 250 and 500 ng/L) in order to obtain concentration versus response curves (fig. B.3 and B.4 in appendix B). Standard solutions at the same concentration were also analyzed to allow a comparison in terms of linearity, slope and intercept. In addition, the ion suppression/enhancement due to the matrix effect was evaluated in terms of response recovery by comparing the peak areas obtained for C$_{60}$ and C$_{70}$ spiked into the extracts with those obtained analyzing standard solutions at the same concentrations. The matrix effect (ME) was further investigated by continuously infusing a fullerenes standard (1 µg/L in toluene) into the MS source while injecting the matrices extracts. This allowed us to monitor the detector response during the chromatographic run, i.e. the matrix suppression/enhancement during the analysis. For this purpose a 5 ml glass chromatographic syringe and syringe pumps (KD Scientific, Holliston MA, United States) operating at 0.2 ml/h were employed.

4.3 Results and discussion

4.3.1 UHPLC-HRMS

The first aim of the present work was to assess the performance of the biphenyl stationary phase selected for the separation of the fullerenes. When method A was applied, C$_{60}$ and C$_{70}$ eluted at 3.1 min and 3.8 min respectively (fig. 4.1) with fully resolved (Rs>1.5) and highly symmetric chromatographic peaks.
Similar to what is observed with columns that are specifically designed for the separation of fullerenes, e.g. the pyrenylpropyl functionalized silica (Buckyprep), with biphenyl groups the main interactions are expected to be the pi-pi-interactions and pi-stacking between the aromatic rings in the fullerenes and those of the stationary phase. However, as suggested by Nunez et al. (2012) the size of the buckyballs may play a role in the retention mechanism. The biphenyl stationary phase was an optimal compromise for selectivity and rapidity when compared with other materials that were usually employed providing higher retention than the standard octadecyl silica and shorter analysis time in comparison with the pyrenylpropyl functionalized silica. It must be noticed that the analysis time may be shortened when using a shorter column with smaller particle size (i.e. 1.7 µm) that is currently available on the market. The “focusing step” i.e. the application of a 100% methanol at the beginning of the chromatographic run, resulted in a better peak shape and sensitivity and may be explained with the fact that the analytes accumulated at the start of the column (focused) until a stronger solvent was provided. The use of ESI-HRMS was already investigated by some of the authors of the present work (Kolkman
et al., 2013). In those studies, a consistent formation of adducts during the ionization process was reported, consisting of both oxidized products ([M + O]•, [M + OH]•) and methanol and/or toluene adducts (mostly [M + OCH₃]• and [M + C₇H₇O₂]•). In contrast, IB-ESI-HRMS provided mass spectra that were dominated by the isotope cluster of the molecular ions with an only minor abundance of [M+16]• adducts as shown in figure 4.2. Similar to what has already been reported in other studies, the method was more sensitive for C₆₀ than C₇₀ (a factor of 2) at all the concentrations tested. Furthermore, in the present study the IB-ESI signal response was several orders of magnitudes higher than that of ESI stand-alone for both C₆₀ and C₇₀ (data not shown). Thus, ESI was not taken into consideration for further analysis.

In the present work, sodium acetate was selected for internal mass calibration because it can stand higher temperatures in comparison with other commonly applied calibration solutions (e.g. sodium formate). This allowed the determination of the accurate masses of fullerenes with errors lower than 5 ppm. In comparison with APPI, where toluene was used as a dopant to improve the ionization efficiency, ESI analysis required the presence of methanol during ionization that can be added post-column as already described (Kolkman et al., 2013). The post column addition was not needed in the present work since methanol was one of the constituents in the mobile phase during the elution of the chemicals. It must be noted that fragmentation of the fullerenes could not be obtained even at extremely high collision energies (> 100 eV). Thus, although multiple reaction monitoring (MRM) mode was applied in the present work, the MS2 analysis corresponded to [M]*→[M]• transitions (pseudo-MRM) and the MS2 spectra resembled the MS1 ones (fig. 4.2b and 4.2e) in which adducts and background masses (e.g. m/z 731.4124 in fig 4.2a) were removed.
Nunez et al. (2012) reported higher than expected relative abundances of the isotopic cluster ions when using APPI with special regard to the \( m/z \) \([M+1]^+\) and an enhanced effect on larger fullerenes (Nunez et al., 2012). In their work they demonstrated that the addition of hydrogenated products to the peaks corresponding to the \( ^{13}\text{C} \) natural abundance may account for the phenomenon. Furthermore, Emke et al. (2015) showed that the unexpected isotopic distribution was due to the presence of methanol during the ionization, a phenomenon not observed when toluene was the only solvent employed. If highly resolving systems, such as FTICR MS, are not available, anomalous isotopic abundances may hinder a qualitative detection of fullerenes. Especially when analyzing non-functionalized structures, such as \( \text{C}_{60} \) and \( \text{C}_{70} \), the lack of MS/MS fragmentation implies that the main identification points must be provided by the accurate mass and cluster distribution. In the present work the \( \text{C}_{60} \) spectra presented...
molecular ions at $m/z$ $[M]^*$, $[M+1]^*$, $[M+2]^*$ and $[M+3]^*$ consistent with the theoretical isotopic patterns (fig. 4.1c) and those obtained by ESI, whereas $C_{70}$ showed slightly higher than expected $m/z$ $[M+1]^*$ abundance in MS1 fig (4.2a and 4.2f). In detail, $C_{60}$ presented a $[M+1]^*/[M]^*$ ratio of 0.64 ± 0.05 (versus the expected value of 0.65) whereas $C_{70}$ presented a ratio of 0.77 ± 0.03 (versus the 0.74 expected).

Identification points in the present work were provided by the chromatographic retention times and the accurate mass detection and isotopic clusters in both MS1 and MS2. Calibration curves, obtained analyzing standard solutions of the fullerenes, showed high linearity ($R^2 > 0.998$) and the ILoD and ILoQ were 0.6 and 2 ng/L, respectively, for $C_{60}$ and 1.2 and 4 ng/L, respectively, for $C_{70}$. These results indicate that the IB-ESI-HRMS system allowed the determination of fullerenes at concentrations lower than those of other ESI-MS and heated ESI methods applied (van Wezel et al., 2011) and similar to those of APPI-MS methods recently developed (Astefanei et al., 2014b). Finally, the method was also tested for the determination of fullerene derivatives to assess the possibility to include functionalized structures investigated in our previous works (Kolkman et al., 2013; Carboni et al., 2013). The method was suitable for the analysis of the methanofullerenes that were completely resolved ($R_s > 1.5$) from the related non-functionalized structures at the beginning of the chromatogram (Fig. B.5 in appendix B).

### 4.3.2 Matrix effects

The method we developed was tested for the determination of fullerenes spiked into extracts of soils and sediments. Soil and sediment samples were chosen in order to represent a range of possible environmental matrices and textures (e.g. clay, sand) and the selection included samples with varying inorganic and organic carbon contents whereas nitrogen and sulfur concentrations were similar between the samples (table 4.1). After the extraction, the sample extracts were spiked with fullerenes in order to investigate the analysis of the chemicals in presence of the co-extractants. The use of fullerene standards in organic solvent for the spiking of samples has often been the subject of debate since it is not expected to represent
the real conditions at which the chemicals are present in the environment (Shareef et al., 2010; Carboni et al., 2013). However, in the present work, standard solutions in toluene were directly added to the toluene extracts, thus the results hereby presented will help in understanding the behavior of fullerenes that are already present in the extracts with no effect of the extraction procedure applied to the original sample. According to a previous study, the texture of the soil samples should be of a minor concern when analyzing fullerenes (Carboni et al., 2013). On the contrary, the presence of carbon and specifically organic matter in the samples is expected to play a major role, e.g. by hindering the detection and quantification of the chemicals (matrix effects, ME). The ME may either cause the enhancement or the decrease of the method’s accuracy and sensitivity. It must be noted that during the sample preparation, an addition of methanol at percentages higher than 25% of the final volume caused precipitation in the extracts with the highest carbon content (matrix 2). This was due to the large content of non-polar co-extractants resulting from the extraction with 100% toluene. Therefore, although the initial optimization was achieved with a toluene:methanol ratio of 1:1 (v:v), the final composition of each sample injected into the UHPLC was a toluene:methanol ratio of 3:1 (v:v). The high collision energy values (100 V) applied during the analysis led to a “Background clean-up”, i.e. the degradation of the co-extractants, with resulting extremely clean MS2 spectra. This allowed the unambiguous determination of the fullerenes in all the samples, even at concentrations as low as 25 ng/L (fig. 4.3).
Fig. 4.3. MS1 (top) and MS2 (bottom) spectra of C₆₀ spiked at the concentration of 25 ng/L in presence of high organic carbon content (matrix 2).

The results of the SAM experiments are reported in Table 4.2 and more comprehensively in Appendix B (Table B.6). The response was linear (R² > 0.99) for both C₆₀ and C₇₀ in all the matrices tested (with the only exception of C₆₀ in matrix 5 when analyzed in MS2), and no significant difference (P>0.05, F-test for covariances) was observed between MS1 and MS2 measurements in the same matrix. It should be noted that, although relative small intercepts were observed, these were not significantly different from zero (P>0.05).
Table 4.2. Results of the standard addition experiment for $C_{60}$ and $C_{70}$ with the use of the method A (left) and method B (right)\textsuperscript{a}. For matrix descriptions, see section 2.3

<table>
<thead>
<tr>
<th></th>
<th>Method A</th>
<th></th>
<th>Method B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>%Rec</td>
<td>$r^2$</td>
<td>%Rec</td>
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<td>$r^2$</td>
<td>%Rec</td>
<td>$r^2$</td>
<td>%Rec</td>
</tr>
<tr>
<td>Matrix 1</td>
<td>0.997</td>
<td>122 ± 15</td>
<td>0.999</td>
<td>123 ± 13</td>
</tr>
<tr>
<td>Matrix 2</td>
<td>0.998</td>
<td>67 ± 10</td>
<td>0.999</td>
<td>13 ± 10</td>
</tr>
<tr>
<td>Matrix 2 dil 10x</td>
<td>0.989</td>
<td>141 ± 16</td>
<td>0.997</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>Matrix 3</td>
<td>1.000</td>
<td>102 ± 6</td>
<td>0.997</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>Matrix 4</td>
<td>0.999</td>
<td>112 ± 7</td>
<td>0.997</td>
<td>115 ± 7</td>
</tr>
<tr>
<td>Matrix 5</td>
<td>1.000</td>
<td>119 ± 6</td>
<td>0.997</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>Matrix 6</td>
<td>0.999</td>
<td>113 ± 1</td>
<td>0.998</td>
<td>113 ± 3</td>
</tr>
</tbody>
</table>

Matrix 2 dil 10x = matrix 2 diluted ten times; method A and method B differ in the mobile phase composition during the isocratic step (50:50, MeOH:Tol and 65:35, MeOH, respectively) and flow rate (400 and 600 ul/min, respectively).

\textsuperscript{a} $r^2$ = regression coefficient of the standard addition lines; Rec\% = recovery; Matrix 2 dil 10x = matrix 2 diluted ten times; method A and method B differ in the mobile phase composition during the isocratic step (50:50, MeOH:Tol and 65:35, MeOH, respectively) and flow rate (400 and 600 ul/min, respectively).

However, a significant difference (P<0.05) was present when comparing the response recovery of different samples. Indeed, although the majority of the matrices showed recoveries ranging from 100% to 122%, i.e. featuring either no ME or a slight enhancement of the response, a large signal suppression (specifically 6% recovery for $C_{60}$ and 29% recovery for $C_{70}$) was found in the matrix with the highest carbon content (matrix 2). This effect was not observed in other matrices with a high organic carbon content (matrix 4 and matrix 5) indicating that the percentage of organic matter in the samples was not the only property determining the ion suppression and that other feature such as its chemical composition may play a role. However, when the extract from matrix 2 was diluted ten times (matrix 2 dil 10x in table 4.2), an enhancement of the response was found, which is consistent with that observed in matrices that have a lower organic carbon content. This suggests that although the chemical composition of the co-
extractants played a role in the ion suppression, their concentration was likely the main reason for the response loss observed in the extract of matrix 2. The MLoDs were extrapolated assuming a 70% recovery of extraction from the matrices (see materials and methods) and ranged from 84 pg/kg for C\textsubscript{60} to 168 pg/kg for C\textsubscript{70} whereas MLoQs ranged from 279 pg/kg for C\textsubscript{60} to 559 pg/kg for C\textsubscript{70} (method A). However, in the case of matrix 2, which showed a strong matrix effect, these values were a factor of 30 higher for C\textsubscript{60} and a factor of 5 higher for C\textsubscript{70}. The smaller suppression of the C\textsubscript{70} signal in comparison with C\textsubscript{60} may be explained by the fact that, being more retained during the analysis, C\textsubscript{70} eluted in a position of the chromatogram that was less affected by the more polar co-extractants. The matrix effect due to the co-extractants in the samples was monitored by injecting the matrices while infusing the fullerenes in the MS system. The signal suppression was due to the more polar fraction of the co-extractants, eluting at the beginning of the chromatographic run where a higher percentage of methanol was present.

![Graph](image)

**Fig. 4.4.** Response of C\textsubscript{60} post-column infused directly into the IB-ESI-MS during chromatographic runs of the soil or sediments extracts using a gradient elution of methanol:toluene (method A). The black line represents a pure solvent injection in comparison with matrix 1 (light blue), matrix 2 (red), matrix 3 (orange), matrix 4 (yellow), matrix 5 (blue) and matrix 6 (green). The start of section b corresponds to the moment the gradient reaches 100% toluene, at the start of c the gradient program returns to initial solvent composition (see 6.2.2).
In particular, as shown in Figure 4.4, the matrix 2 (red line) suffered a large signal suppression at the beginning of the analysis (Fig. 4.4a) whereas signals from other matrices with a high organic carbon content were not suppressed (matrix 4 in yellow) or were affected to a lesser extent (matrix 5 blue line). Flushing with toluene (100%) (Fig. 4.4b), in order to remove the more non-polar fraction that may remain in the column and affect the next injections, ultimately restored the response to the maximum levels as shown in Figure 4.4c. During the flushing stage of the chromatogram (b), however, no signal was present because of the absence of any methanol and consequent lack of ionization of the fullerenes (Fig. 4.4b) in the ESI. Matrices 2 and 5 were selected for further experiments aimed to improve the analytical method for the determination of fullerenes in complex matrices with mitigation of the ion suppression. Three mobile phase modifiers: acetic acid, ammonium acetate and formic acid, were tested at varying concentrations with the purpose of attenuating the ion suppression. However, since the addition of formic acid resulted in a near or complete loss of the response whereas both acetic acid and ammonium acetate suppressed the signal to a large extent, these modifiers were not taken into account for further analysis. The large suppression may be due to the susceptibility of the ESI to buffer salts in comparison with APPI as mentioned by Nunez et al. (2012). Also the modification of the mobile-phase composition with increasing amounts of isopropanol and acetonitrile did not lead to any improvement in recovering the response and, in the case of acetonitrile, caused the formation of adducts at the $m/z \ [M+40]^+$ for both C$_{60}$ and C$_{70}$. Subsequently, the modification of the mobile phase composition was tested with regards to the methanol:toluene ratio. In detail, weakening the eluotropicity, i.e. increasing the amount of methanol, delayed the elution of the fullerenes and enhanced the separation of interfering compounds in the biphenyl column, with a general improvement of the signal response (Fig. B.7 in appendix B). Optimal conditions were found at 65:35 (methanol:toluene, v:v) whereas further addition of methanol (e.g. 70:30, v:v) increased the retention time of the fullerenes and broadened the peak, with no further enhancement of the response. Finally, the original method (method A) was modified accordingly by changing the methanol:toluene ratio in the isocratic step from 50:50 (method A) to 65:35 (method B). Further optimization of the methods consisted in the addition
of a washing step (100% toluene) at the end of the analysis and to divert the LC flow to the waste in order to prevent the exposure of the source to the co-extractants at the beginning of the analysis and provide higher sensitivity and better peak shape of the fullerenes. In the resulting method B, the retention times of the fullerenes were delayed, with C\textsubscript{60} and C\textsubscript{70} eluting at 4 and 6 min respectively (Fig. B.8 in appendix B) and the ILOD and ILOQ were 12 and 39 fg, respectively, for C\textsubscript{60} and 23 and 78 fg, respectively, for C\textsubscript{70}. When method B was applied, a considerable improvement in the response recovery of C\textsubscript{60} was observed for the matrix 2 (from 6\% to 67\%) and minor but consistent improvements were obtained for C\textsubscript{70} (from 29 to 45\%), as reported in Table 4.2. This corresponded to a MLoD and MLoQ of 168 pg/kg and 559 ng/kg, respectively, for C\textsubscript{60} and 335 pg/kg and 1.1 ng/kg, respectively, for C\textsubscript{70}. Furthermore, no significant difference (P<0.05) was found when comparing the performances of the two methods for the analysis of the other matrices. Eventually, we recommend the use of method B (10 min) for monitoring studies, since it can provide a more reliable measurement of the fullerenes in a wider range of matrices. In particular, method B should be employed in the analysis of soils and sediments as well as wastewater samples that can consist of complex matrices possessing high organic carbon contents. However, method A can provide a faster (8 min) and more sensitive analysis for the determination of fullerenes in standard solutions and less complex samples such as sandy soils with low organic carbon contents.

4.4 Conclusions

In the present work we developed a sensitive and fast method for the determination of fullerenes in soils and sediments. The biphenyl-coated stationary LC phase provides an adequate retention and represents a valid alternative to other stationary phase materials currently applied with promising perspectives for the analysis of complex mixtures including functionalized fullerenes. We also showed how heated ESI, i.e. the IB-ESI employed in the present work, can grant an ionization efficiency higher than
that of standard ESI and comparable to that of other recently developed APPI methods, with the advantage of producing isotopic patterns that resemble the theoretical ones. In general, the high resolution and the clarity of the mass spectra allowed an unambiguous determination of the fullerenes in all the samples under investigation, without the need of an additional clean up, and at low concentrations that are environmentally relevant. The matrix effect due to the presence of co-extractants was investigated and the method is robust and flexible with regard to the analysis of very complex matrices such as soils having high organic carbon contents. However, the matrix effect and specifically the ion suppression/enhancement during the analysis remains an issue and the use of internal standards (e.g. isotopically labeled), if available, as well as matrix matched calibration are recommended for a more precise determination. Due to its favorable qualitative and quantitative features, the method developed in the present study is a valid tool for the monitoring of fullerenes in soils and sediments and for the study of the fate of these novel contaminants at environmentally relevant concentrations.