Fullerene nanoparticles in soil: Analysis, occurrence and fate

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Chapter 3

A HPLC-UV method for the analysis of fullerenes in soils

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Fullerenes are carbon-based nanomaterials expected to play a major role in emerging nanotechnology and produced at an increasing rate for industrial and household applications. In the last decade a number of novel compounds (i.e. fullerene derivatives) is being introduced into the market and specific analytical methods are needed for analytical purposes as well as environmental and safety issues. In the present work eight fullerenes (C\textsubscript{60} and C\textsubscript{70}) and functionalized fullerenes (C\textsubscript{60} and C\textsubscript{70} exohedral-derivatives) were selected and a novel liquid chromatographic method was developed for their analysis with UV absorption as a method of detection. The resulting HPLC-UV method is the first one suitable for the analysis of all eight compounds. This method was applied for the analysis of fullerenes added to clayish, sandy and loess top-soils at concentrations of 20, 10 and 5 µg/kg and extracted with a combination of sonication and shaking extraction. The analytical method limits of detection (LoD) and limits of quantification (LoQ) were in the range of 6-10 µg/L and 15-24 µg/L respectively for the analytical solutions. The extraction from soil was highly reproducible with recoveries ranging from 47 ± 5 to 71 ± 4% whereas LoD and LoQ for all soils tested were of 3 µg/kg and 10 µg/kg respectively. No significant difference in the extraction performance was observed depending of the different soil matrices and between the different concentrations. The developed method can be applied for the study of the fate and toxicity of fullerenes in complex matrices at relatively low concentrations and in principle it will be suitable for the analysis of other types of functionalized fullerenes that were not included in this work.
3.1 Introduction

Since their discovery in 1985 by Kroto et al. (Kroto et al., 1985), fullerenes have attracted a lot of interests due to their unique structure and innovative properties and are nowadays considered as some of the most promising materials in nanotechnology. Fullerenes are very versatile compounds already applied in several fields such as optics and electronics as well as cosmetics and in medical research (Tagmatarchis et al., 2001; Guldi et al., 2002; Burangulavet al., 2005; Kim et al., 2006) with a worldwide production estimated in tens of tons per year, that is expected to increase in the near future (Hendren et al., 2011; Piccinno et al., 2012). Furthermore, the possibility to functionalize the closed cage structure by the covalent binding of external groups to the fullerene’s surface (i.e. exohedral fullerene derivatives) increase the solubility of these compounds in organic as well as polar solvents and consequently widens their range of applications and uses. Contrary to pristine compounds such as C\textsubscript{60} and C\textsubscript{70}, that can be naturally produced during highly energetic events such as lightening (Daly et al., 1993) and massive wildfires (Heymann et al., 1994), functionalized fullerenes are in all respect engineered nano-materials (ENMs). Firstly described by Hummelen et al. in 1995 (Hummelen et al., 1995), the fullerene derivative 1-(3-methoxycarbonyl)propyl-1-phenyl[6,6]C\textsubscript{61}, better known as [60]PCBM, is to date one of the most studied in the field of organic photovoltaic (OPV) materials (Dang et al., 2011) and has been proposed for the construction of organic field-effect transistors (OFETs) (Tiwari et al., 2007) and photo detectors (Baierl et al., 2010). In the last decade, a number of PCBM-like chemicals differing in the substituent group (e.g. thienyl analog of [60]PCBM, Popescu et al., 2006), number of substituents (e.g. bisadducts, Lenes et al., 2008) or the functionalization of fullerenes other than C\textsubscript{60} (e.g. 70[PCBM], Wienk et al., 2003) as well as compounds with different functionalization (e.g. C\textsubscript{60}-pyrrolidines, Marchesan et al., 2005) are being produced and studied for their use in novel applications. Despite the broad interest in the development of new engineered nanomaterials, knowledge on the human safety and environmental issues of fullerenes and their derivatives is scarce. Fullerenes entering the environment as consequence of their production and use will presumably accumulate in soil.
and sediments. Although functionalized fullerene derivatives have been recently included in environmental monitoring (Sanchis et al., 2011 and 2013), most of the research so far has been focused on C\textsubscript{60} only and no chromatographic methods have been developed yet for the analysis of the functionalized fullerenes structures. Among the analytical techniques that have been applied to the analysis of fullerenes, liquid chromatography appears to be the most feasible method for routine analysis and the main advancements in this field have already been reviewed elsewhere (Baena et al., 2002; Isaacson et al., 2009). In general, although octadecyl silica (ODS) stationary phases can be used to separate compounds such as C\textsubscript{60} and C\textsubscript{70}, better performance is achieved with other materials that offer a higher surface for the interaction and therefore retention of fullerenes (e.g. 2-(1-pyrenil)ethylsilica or 3-(pentabromobenzyl)oxy-propylsilylsilica) particularly when more compounds are analyzed in a mixture.

Toluene is the most common mobile phase applied due to the high solubility of fullerenes in this solvent at room temperature (Ruoff et al., 1993) and can be used as only eluent when C\textsubscript{60} is the only analyte under investigation. When other fullerenes (e.g. C\textsubscript{70}) or functionalized fullerenes such as [60]PCBM were included in the study, more polar solvents such as acetonitrile (Bouchard et al., 2008), hexane or isopropanol (Deye et al., 2008) have been used as modifiers to enhance the separation. Fullerenes absorb light in the 300-350 nm range and UV-vis detection is a powerful tool for their analysis in combination with HPLC because of the broad linearity range and high sensitivity. In a recent study, Wang et al. (Wang et al., 2010) compared UV-vis and mass spectrometry (MS) for the detection of C\textsubscript{60} in HPLC and concluded that, despite the higher selectivity of MS based on the m/z ratio, the two techniques are comparable in terms of sensitivity and UV-vis offers a larger linear range. HPLC-UV methods have been used for the analysis of fullerenes in different matrices such as soil (Shareef et al., 2010; Perez et al., 2013), artificial sediments (Wang et al., 2011), surface and groundwaters (Bouchard et al., 2008) and biological matrices (Moussa et al., 1997; Xia et al., 2006) but most of these studies were focused on C\textsubscript{60} and occasionally higher fullerenes whereas functionalized structures were seldom included. Furthermore, fullerenes and fullerene derivatives have also shown to emit fluorescence at room temperature when dissolved in
organic solvents (Lin et al., 1995; Zhao et al., 2006) but no data are available of fluorescence detection coupled to HPLC.

In the present study we developed a HPLC method with UV detection for the determination of eight selected fullerenes and functionalized fullerenes. After optimization the method was tested for the analysis of the fullerenes in environmental matrices. Soil and sediments might act as a sink for the accumulation of hydrophobic fullerenes after their release into the environment but few studies have addressed yet the issue of analyzing these compounds in these matrices (e.g. Vitek et al., 2009; Shareef et al., 2010; Perez et al., 2013). Furthermore, in the majority of the studies that have addressed the issue, the concentrations tested were relatively high (hundreds µg/kg and above) with the exception of a recent study from Perez et al. (2013). None of these studies included functionalized structures other than [60]PCBM. Thus, in the present work three top-soils differing in their properties as texture and organic matter content, namely sandy, clayey and loess soils were spiked with toluene standard solutions containing all the fullerenes under investigation to a final concentration of 20, 10 and 5 µg/kg for each compound and analyzed using the HPLC-UV method.

3.2 Materials and Methods

3.2.1 Reagents and chemicals

Table 3.1 presents characteristics of the fullerenes in the present study. Toluene and Acetonitrile (Biosolve, Dieuze, France) were both analytical grade. Stock solutions of the individual fullerenes were prepared in toluene at a concentration of 500 mg/L according to the method described by Kolkman et al. (2013). The solutions were placed in the dark overnight on a rotary shaker to achieve complete dissolution of the fullerenes. Diluted solutions for the individual fullerenes and their mixture were obtained by diluting aliquots from the individual stock solutions. The solutions were stored at 4°C in the dark and sonicated for 2 min before use.
3.2.2 Soil sampling, soil characterization and sample treatment

Sandy soil was collected in the Flevopark area, Amsterdam, the Netherlands (52°21'55.09"N, 4°57'3.88"E), the loess soil was collected from an agricultural field in south Limburg, the Netherlands (50°53'58"N, 5°53'16"E) and the clayish soil was collected in Dikkebuiksweg, the Netherlands (50°50'03"N, 5°54'27.7"E). All the soils in the present study were sampled from top soils A horizons within the first 10-15 cm from the surface and their texture was assessed according to WRB 2006. The samples were placed in a freezer at -20°C overnight and lyophilized with a Scanvac Coolsafe freeze-dryer (Labogene, Lynge, Denmark). The dried samples were finely ground with an agate mortar and sieved.

At first we obtained an aqueous extract to measure dissolved organic carbon (DOC). Samples of 20 g for each soil were placed in 200 ml polyethylene bottles and 100 ml of ultrapure water were added (dilution 1:5) before to undergo shaking extraction for 2 h at 120 rpm with a Laboshake orbital shaker (Gerhardt, Königswinter, Germany). The samples were then transferred into 50 ml plastic tubes, centrifuged for 15 min at 2000 rpm with a Rotofix 32A (Hettich, Tuttingen, Germany) and the supernatants were transferred into plastic syringes and filtered with 0.2 μm cellulose ester membrane filters (Whatman, Maidstone, United Kingdom).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Purity</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{60} fullerene</td>
<td>C_{60}</td>
<td>&gt;99.9%</td>
<td>MER Corporation. (Tucson, US)</td>
</tr>
<tr>
<td>C_{70} fullerene</td>
<td>C_{70}</td>
<td>&gt;99%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>[6,6]-Phenyl-C_{60}-butyric acid butyl ester</td>
<td>[60]PCBB</td>
<td>&gt;97%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>[6,6]-Phenyl-C_{60}-butyric acid octyl ester</td>
<td>[60]PCMO</td>
<td>&gt;99%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>[6,6]-Phenyl-C_{60}-butyric acid methyl ester</td>
<td>[60]PCBM</td>
<td>&gt;99%</td>
<td>Solenne B.V. (Groningen, NL)</td>
</tr>
<tr>
<td>[6,6]-Bis-Phenyl-C_{60}-butyric acid methyl ester</td>
<td>bis[60]PCBM</td>
<td>&gt;99.5%</td>
<td>Solenne B.V. (Groningen, NL)</td>
</tr>
<tr>
<td>[6,6]-Thienyl-C_{60}-butyric acid methyl ester</td>
<td>[60]ThC8M</td>
<td>&gt;99%</td>
<td>Solenne B.V. (Groningen, NL)</td>
</tr>
<tr>
<td>[6,6]-Phenyl C_{70} butyric acid methyl ester</td>
<td>[70]PCBM</td>
<td>&gt;99%</td>
<td>Solenne B.V. (Groningen, NL)</td>
</tr>
</tbody>
</table>
previously rinsed with ultrapure water. The pH of the final extracts was measured with a Consort C831 electrode (Consort NV, Turnhout, Belgium) and DOC and IC (inorganic carbon) were determined using a TOC-VCPPH (Shimadzu, Kyoto, Japan). The carbon and nitrogen contents in the dried soil samples were measured using a Vario EL Cube (Elementar, Hanau, Germany). All the experiments for the soils characterization were made in triplicate. Three samples (200 g) for each soil were placed into glass jars and fullerenes were added by spiking a fullerene stock solution in toluene to obtain a final concentration of 20 µg/kg, 10 µg/kg and 5 µg/kg. The soils were then homogenized by stirring and left in the dark for 48 h to allow the solvent to evaporate.

3.2.3 Extraction

10 g of soil from each jar were weighed and placed into a glass centrifuge tubes, 10 ml of toluene were added and the samples were placed open into a Branson 12 ultrasonic bath (Branson, Danbury CT, United states) operating at 50 kHz for 30 min. Then, the tubes were closed with a glass stopper and shaking extraction was performed with an orbital shaker at 160 rpm for 90 min. Subsequently, the samples were centrifuged at 2000 rpm and the toluene supernatant was filtered through a 4-7 µm pore size prepleated paper filter (Whatman, Maidstone, United Kingdom) into 40 ml amber glass vials. The filter was rinsed with 3 ml of toluene and the extraction was repeated a second time by adding 8 ml of toluene. In this latter procedure, the samples were not centrifuged and the soil samples were transferred directly to the paper filters. After elution of the solvent, each sample was rinsed with 5 ml of toluene and the extracts were evaporated in a water bath at 60 °C under a gentle nitrogen flow until approximately 3 ml. Finally, the extracts were filtered with 0.45 µm regenerated cellulose filters and concentrated to a final volume of ~ 0.5-1 ml. All experiments were performed in triplicate and non-spiked soils were extracted with the same protocol as reference.
3.2.4 HPLC with UV and fluorescence detection

UV-vis and fluorescence spectra of the fullerenes were obtained analyzing stock solutions of the single compounds in quartz cuvettes with an Olis DW-2000 spectrophotometer and an Olis DM45 spectrofluorimeter (Olis, Bogart GA, United States), both equipped with Olis SpectralWorks software. Liquid chromatography was performed with a Shimadzu Prominence system (Shimadzu, Kyoto, Japan) equipped with a diode-array detector and a fluorescence detector. The wavelengths monitored for UV detection were 305 nm and 332 nm. For fluorescence detection, emission wavelengths at 400 nm, 550 nm and 700 nm were monitored with excitation wavelength set at 286 nm, 332 nm, 400 nm or 463 nm. The data were collected with the LCsolution software. The separation was achieved with a Cosmosil® Buckyprep column consisting of 3-(1-pyrenyl)propyl groups stationary phase (4.6 mm ID x 250 mm, Nacalai-Tesque, Kyoto, Japan) equipped with a C18 silica pre-column at a flow rate of 1 ml/min and an injection volume of 20 µl. External calibration curves were obtained analyzing standard solutions in toluene at concentrations ranging from 4 µg/L to 1 mg/L and quantification was based on chromatographic peak areas whereas limits of detection (LoD) and quantification (LoQ) were assessed observing the signal to noise ratio (S/N) and considering LoD as the concentration with S/N=3 and LoQ as the concentration with S/N=10.

3.3 Results and discussion

3.3.1 HPLC-UV method

The separation of fullerenes in liquid chromatography necessitates the use of an apolar mobile phase able to dissolve and elute the compounds in a relatively short time. In this study toluene was applied as the main eluent in the mobile phase in combination with a specific stationary-phase, composed of pyrenyl-propyl functionalized silica (Buckyprep), that enhances the retention of fullerenes as a result of the large ligand that can interact with the aromatic structure of the fullerenes. This non-aqueous
chromatographic system can be nominally referred to as normal-phase liquid chromatography due to the apolarity of both the mobile and stationary phases. Since the isocratic elution with toluene as only eluent resulted in a partial or total co-elution of some of the compounds in the mixtures a more polar solvent, in this case acetonitrile, was added to the mobile phase in different percentages to enhance the separation of the analytes. The final optimized method (fig. 3.1) consisted in a gradient elution starting with 75:25, toluene:acetonitrile (% volume) and the gradual conversion after 6 min to 100% toluene to allow a faster elution of the more apolar compounds. With these settings the elution of all the analytes is obtained within 25 min while the whole method lasted 32 min to allow the system to equilibrate prior to the next analysis.

Figure 3.1. HPLC-UV chromatogram of fullerenes and functionalized fullerenes in toluene containing 20 ng of each analyte. [1]: bis[60]PCBM, [2]: [60]PCBO, [3]: [60]PCBB, [4]: [60]PCBM, [5]: [60]ThCBM, [6]: [70]PCBM, [7]: C_{60} and [8]: C_{70}.

As shown in fig. 3.1, the order of elution is correlated with: 1) the number and presence of functionalization on the cage, that increase the solubility of the compounds in the mobile phase and decrease the surface available for the interaction with the pyrenyl-propyl groups in the stationary phase (functionalized elute earlier than pristine fullerenes) and 2) the size of the
cage ($C_{60}$ structures elute earlier than $C_{70}$). Thus, the double functionalized bis[60]PCBM (Fig. 3.1, peak 1) is the first compound to elute with a relatively broad peak, between 3.5 and 4.7 min. Its jagged peak-shape might be due to either the presence of different isomers (60 positions are available on the structure for the attachment of the two functional groups) or by the formation of micelles in the solution. The four $C_{60}$ derivatives (fig. 3.1, peaks 2, 3, 4 and 5), which are not baseline resolved, eluted in a cluster between 6 to 7.5 min followed by the [70]PCBM (fig. 3.1, peak 6) at time 11.8 min. This latter peak has a shoulder that might be due to oxidized products or the presence of different isomers. $C_{60}$ and then $C_{70}$ (fig. 3.1, peaks 7 and 8 respectively) are fully resolved and elute in the end of the analysis after 17.0 and 24.8 min respectively.

The separation of the mono-functionalized $C_{60}$ fullerenes is challenging because of the high similarity in the structures (Table A.1 in appendix A) that results in the co-elution of the compounds in between 6 and 7.5 min as shown in fig. 3.1 (peaks 2-5). Since the absorption spectra of the compounds is very similar (discussed below) and because of the lack of selectivity, UV detection alone cannot help in the determination of these non-fully resolved peaks. If a more selective detection method such as mass spectrometry is not available, the determination of the respective compounds that are co-eluting must be achieved by improving the chromatographic separation. In general, the elution time of the functionalized $C_{60}$ structures in this study is correlated with the aromatic ring in the functionalizing group (phenyl-functionalized eluted before thienyl-functionalised) and is inversely proportional with the length of the alkyl chain in it. Therefore [60]PCBO and [60]PCBB (octyl ester and butyl ester respectively) eluted before than [60]PCBM and [60]ThCBM (both methyl esters but with different aromatic rings).

When a mobile phase composition of 75:25, toluene:acetonitrile was applied (fig. 3.2B), the four compounds created two clusters, the first one including [60]PCBO and [60]PCBB (Rs < 1) which was fully resolved from the second one composed by [60]PCBM and [60]ThCBM (also Rs < 1). A better resolution of the peaks in one of the clusters was obtained by modification of the mobile phase composition, i.e the ratio between acetonitrile and toluene, but resulted in a lower resolution in the other cluster. For instance,
increasing the percentage of acetonitrile (fig. 3.2A) and therefore the polarity of the eluent resulted in a better separation of the compounds based on the different aromatic rings ($R_s \geq 1$ for [60]PCBM and [60]ThCBM) but decreased the resolution between [60]PCBO and [60]PCBB. On the contrary, increasing the percentage of toluene in the mobile phase (fig. 3.2C) allowed a better separation of the compounds depending on the alkyl length, thus improving the resolution between [60]PCBO, [60]PCBB but resulted in the co-elution of [60]PCBM and [60]ThCBM. In addition, the variation in the polarity of the mobile phase affected the peak shape and retention times of the analytes. A larger percentage of acetonitrile (fig. 3.2A) enhanced the separation of the jagged peaks of bis[60]PCBM but also caused a slower elution of all the compounds whereas a more apolar eluent (fig. 3.2C) leaded to a faster elution of all the compounds. These results suggest that a complete separation of very similar structures such as the ones included in this study might be achieved by the variation of the physical parameters (e.g. length of the column, particles size) more than chemical parameters such as the polarity of the mobile phase.
Figure 3.2. Chromatographic separation of fullerene derivatives at mobile phase composition of Toluene:acetonitrile; 65:15 (A), 75:25 (B) and 85:15 (C) (volume %). The fullerenes structures are numbered according to the caption of fig. 3.1.

When fullerenes are dissolved in organic solvents such as toluene, spectrophotometric detection is a powerful tool for their analysis owing to the strong absorption of these compounds in the UV range. The absorption spectra of the functionalized fullerenes included in this study (fig. 3.3) are
comparable to those of the pristine $C_{60}$ and $C_{70}$ fullerenes from which they are derived.

![UV-vis absorption spectra](image)

**Figure 3.3.** UV-vis absorption spectra of functionalized fullerenes at concentrations ranging from 1.5 to 2 mg/L.

As reported by Bouchard et al. (2008), the wavelength selected for the detection during the chromatographic runs were 332 nm for $C_{60}$, $C_{70}$ and the $C_{60}$ mono-derivatives whereas the optimum for bis[60]PCBM and [70]PCBM was found at 305 nm (fig. A.2 in appendix A), despite the fact that the maximum absorbance for all the compounds was recorded at 286 nm. This latter wavelength was not applied in the measurements owing to the toluene absorbance in the same range that resulted in a greater baseline noise. Thus, at these wavelengths selected the detector response was linear (correlation coefficients $> 0.99$) over more than two orders of magnitude of
mass. The analytical method limits of detection (LoD) and limits of quantification (LoQ) were assessed to be 120 pg (LoD) and 300 pg (LoQ) for C\textsubscript{60} and the C\textsubscript{60} mono-derivatives respectively and 200 pg (LoD) and 480 pg (LoQ) for C\textsubscript{70} and its derivative. The presence of an interference peak at retention time 4.0 min precluded an accurate detection and quantification of bis[60]PCBM below 2 ng injected. Fluorescence emission spectra were collected for all the compounds dissolved in toluene and the wavelengths tested for the excitation were 286 nm, 332 nm and 463 nm, because of the absorption of fullerenes at these values and 400 nm which had been reported to excite C\textsubscript{60} with consequent emission at 700 nm (Zhao et al., 2006). C\textsubscript{70} and [70]PCBM showed a weak fluorescence emission at 700 nm when excited at 463 nm while the other compounds did not display any clear emission signal at none of the excitation wavelengths tested. Furthermore, the chromatograms collected recording the fluorescence emission at 700 nm for the excitation wavelengths tested showed a very high baseline noise and no clear chromatographic peak. Therefore, fluorescence detection was not considered for further analysis in this study.

3.3.2 Application of the method

The analysis of fullerenes in environmental matrices can be problematic because of the presence of matrix components in the extracts that can absorb in the same range of wavelengths affecting the detection. The properties of the soils used in the present study are reported in table 3.2 whereas the HPLC chromatograms corresponding to the analysis of the three soil matrices spiked at 20 µg/kg are shown in figure 3.4.
Table 3.2. Physico-chemical properties of the soils used in the present study (DOC, dissolved organic carbon; IC, inorganic carbon).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Texture</th>
<th>TOC (mg/L)</th>
<th>IC (mg/L)</th>
<th>pH</th>
<th>%Carbon</th>
<th>%Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandy</td>
<td>Sand</td>
<td>19.1</td>
<td>9.2</td>
<td>8.4</td>
<td>1.076</td>
<td>0.021</td>
</tr>
<tr>
<td>Loess</td>
<td>Silty Loam</td>
<td>23.0</td>
<td>2.2</td>
<td>6.7</td>
<td>0.941</td>
<td>0.098</td>
</tr>
<tr>
<td>Clay</td>
<td>Silty Clay Loam</td>
<td>26.9</td>
<td>11.9</td>
<td>7.4</td>
<td>1.768</td>
<td>0.174</td>
</tr>
</tbody>
</table>

All soil extracts analyzed showed different but consistent matrix interferences which are probably due to the extracted constituents of the soil (e.g. hydrophobic fraction of the organic matter) that are not retained in the column and eluted with the void peak in the beginning of the chromatograms. The matrix effect was particularly evident in sandy soil extracts (fig 3.4A) where the co-extractants eluted until 8 minutes and in loess soil extracts (Fig 3.4B) with a number of small signals in the first 12 minutes of elution. In clay soil extracts (fig. 3.4C) the fullerenes peaks were relatively clear (with higher S/N ratios) in comparison with the other two matrices although this soil had the highest content in organic matter, DOC and clay. The matrix constituents seemed not to affect the order of elution, separation and retention times of the compounds. However, they interfered with the detection of the fullerenes eluting in the beginning, i.e. several of the functionalized C₆₀S. Thus, while the last three fullerenes to elute, [70]PCBM, C₆₀ and C₇₀, seemed not to be affected by any strong interference in comparison with the chromatogram obtained running pure standard solutions (fig. 3.1), bis[60]PCBM (Rt: ~4 min.) was not detected in any of the soil extracts and the C₆₀ mono-functionalized peaks were difficult to quantify. Except for bis[60]PCBM, all the analytes could be detected (S/N ≥ 3) and quantified (S/N ≥ 10) in the extracts for all the three soils spiked at
20 and 10 µg/kg. When samples were spiked at 5 µg/kg, detection of the compounds was still possible but no quantification could be made. In general, C\textsubscript{60} and C\textsubscript{60} derivatives were more easy to detect than C\textsubscript{70} and [70]PCBM owing to the lower sensitivity of the detector for these latter compounds and because of the slope in the baseline that affected the determination in the end of the chromatograms. These results suggest that the use of the optimized method is suitable for the analysis of fullerenes in soils differing in clay and organic matter content especially for pristine fullerenes such as C\textsubscript{60} and C\textsubscript{70}. A sample clean-up before the injection may help to remove the impurities in the extract that interfere with the analysis of the fullerene derivatives in the beginning of the chromatograms.
Figure 3.4. Chromatograms of fullerenes extracts from sandy (A), loess (B) and clay (C) soils spiked at 20 µg/kg (blue, continues line). The red non-continues lines represent the non-spiked soils. The fullerenes structures are numbered according to the caption of fig. 3.1. Note that [70]PCBM was detected at 305 nm.
The recoveries of extraction for all the compounds spiked in the three soils tested are reported in table 3.3. Several methods of extraction have already been applied for the extraction of fullerenes from soil samples (e.g. microwave-assisted extraction, sonication, soxhlet and accelerated solvent extraction). However, it is not possible to establish which, among these techniques, is the better because of the differences in the experimental settings (e.g. kind of soil, concentrations) reported. Ultrasonication is a robust method that was already investigated by Jehlicka et al. (2005), Vitek et al. (2009) and Perez et al. (2013) and was applied in the present study in combination with shaking extraction. As shown in table 3.3 the recoveries for all other compounds were acceptable with good repeatability (n = 3) except for bis[60]PCBM, that could not be recovered in any of the samples because of the co-extracted interferences. The good repeatabilities (on average less than 5% for the 20 µg/kg level and less than 6% for the 10 µg/kg level) demonstrate that the method developed in the present study is robust. Increasing the injection volume in the HPLC or extracting a larger sample intake could further improve the recovery of extractions because of the higher amount of fullerenes in the extracts and the limited interference of the co-extractants for the other compounds except for bis[60]PCBM.

Fullerenes are expected to absorb to the soil matrix (Jehlicka et al., 2005) and different soil components (e.g. clay minerals, organic carbon etc.) may affect the extraction efficiency. In the present work, all analytes except for one were recovered from the three soils to similar extents and this is consistent with what already reported by Shareef et al. (2010) who did not observe any real difference in the recovery from six soils tested in their study. Statistical analysis performed (two way ANOVA) on the mean recoveries (combining all concentrations tested) from the present study revealed that there is no significant difference (P > 0.05) between the three soils tested. Jehlicka et al., (2005) highlighted the role of fullerenes concentration in the extraction efficiency of fullerenes from carbonaceous matrices. They observed that the extraction efficiency decreased at decreasing concentration of the C60 in soil and concluded that the possible reasons for the reduction might be a decomposition or transformation of the compounds and/or the absorption of fullerenes to the soil components. This effect was not observed in the present study where the difference between the recoveries for all the compounds at the two quantifiable

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concentrations tested (table 3.3) is not significant at the 5% confident level ($P > 0.05$).

**Table 3.3.** Comparison of the extraction recoveries of fullerenes from sandy, loess and clay soil at the concentrations of 20 µg/kg (left) and 10 µg/kg (right).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sandy</th>
<th>Loess</th>
<th>Clay</th>
<th>Sandy</th>
<th>Loess</th>
<th>Clay</th>
</tr>
</thead>
<tbody>
<tr>
<td>[60]PCBO</td>
<td>70 ± 4</td>
<td>66 ± 5</td>
<td>60 ± 4</td>
<td>71 ± 4</td>
<td>66 ± 6</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>[60]PCBB</td>
<td>59 ± 2</td>
<td>61 ± 2</td>
<td>53 ± 4</td>
<td>56 ± 2</td>
<td>61 ± 2</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>[60]PCBM</td>
<td>65 ± 2</td>
<td>66 ± 3</td>
<td>55 ± 4</td>
<td>63 ± 3</td>
<td>62 ± 3</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>[60]ThCBM</td>
<td>58 ± 2</td>
<td>60 ± 1</td>
<td>55 ± 3</td>
<td>55 ± 2</td>
<td>63 ± 4</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>[70]PCBM</td>
<td>59 ± 1</td>
<td>52 ± 1</td>
<td>55 ± 1</td>
<td>55 ± 2</td>
<td>47 ± 5</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>C$_{60}$</td>
<td>64 ± 4</td>
<td>65 ± 3</td>
<td>57 ± 4</td>
<td>65 ± 3</td>
<td>66 ± 6</td>
<td>63 ± 1</td>
</tr>
<tr>
<td>C$_{20}$</td>
<td>61 ± 3</td>
<td>63 ± 3</td>
<td>65 ± 4</td>
<td>57 ± 1</td>
<td>60 ± 11</td>
<td>66 ± 3</td>
</tr>
</tbody>
</table>
This could be explained by the fact that the concentrations in our study are relatively similar (factor of 2 difference) in comparison with those tested by Jehlicka et al. (factor of 10) and that the effect of the fullerenes concentration on the extraction recovery is not appreciable in this small range. Since the concentrations tested in the present work were lower than those reported in the majority of previous studies, absorption and general losses of the compounds during the sample treatments are a possible explanation for the lower recoveries (from 47% to 71%) in comparison with those already reported (e.g. 83-107% recovery with ASE, Shareef et al., 2010). Recently Perez et al. (2013) reported a temperature dependency on the recoveries of extraction for C_{60} and C_{70} spiked into soil at concentrations similar to those tested in the present study. The spiking of the samples at low concentrations in the present study allowed an accurate determination of the LoDs and LoQs, estimated to be 3 µg/kg and 10 µg/kg respectively for all the soil tested. Although more sensitive LC-MS methodologies have been developed very recently, that allow the determination of fullerenes in environmental matrices at even lower concentrations (Kolkman et al., 2013; Sanchis et al 2013), the HPLC-UV method in the present study is a valid alternative, cheaper and easier to interpret. Finally it must be noted that, despite the fact that spiking of concentrated solutions in toluene is a common procedure, it also represents a limitation because it could not reproduce the real conditions at which fullerenes are present in the soil environment and further efforts will be needed in the development of alternative and more representative spiking techniques.

3.4 Conclusions

In this study a new chromatographic method was developed and optimized for the analysis of fullerenes and functionalized fullerenes in soil using HPLC with UV as method of detection. UV-detection showed very high linearity for the compounds under investigation and allowed their detection at concentrations as low as 6 µg/kg whereas fluorescence detection did not fulfil the prerequisite for the analysis when coupled with HPLC in the
present study. This is the first time that such a number of functionalized and non-functionalized fullerenes are analyzed by HPLC-UV in a single run and in principle, other functionalized structures, similar to those included in this study, can be analyzed with the method. The analytical settings can be optimized depending on the analyte(s) of interest e.g. by modification of the mobile-phase composition or wavelength of detection.

The analysis of fullerenes including the functionalized derivatives extracted from real soil samples spiked with the compounds, showed that the method is robust and suitable for the determination of these compounds in complex environmental matrices at concentrations in the range of µg/kg. The extraction of the compounds with a combination of sonication and shaking in two steps, with toluene as extracting solvent, is highly reproducible and relatively efficient (from 47% to 71% recovery) and the method limit of detection and limit of quantification (3 µg/kg and 10 µg/kg respectively) are lower than those already reported by other works.

This method would allow the study of the fate and toxicity of fullerenes and their functionalized derivatives in environmental samples at concentrations close to those expected in the real environment. However, since the predicted environmental concentration of fullerenes is expected to be in the range of ng/kg (Gottshalk et al., 2009), further developments are needed in order to apply the method to environmental monitoring, especially for the functionalized structures whose UV detection in the present study was affected by the matrix components. The current limitations of the HPLC-UV method developed in the present study may be overcome by applying preconcentration methods in combination with clean up or using more sensitive but also more expensive detection methods such as high resolution mass spectrometry.