



Quantifying fullerene C₆₀ including transformation products in water with LC LTQ Orbitrap MS and application to environmental samples

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Summary

Development of analytical techniques and application to environmental samples is essential for human and environmental risk assessment of the growing use of engineered nanoparticles. The purpose of this study was to develop a sensitive analytical method to quantify nC₆₀ in water, using accurate mass screening liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry. The transformation of parent (n)C₆₀ by oxidation, reduction and photochemical reaction is well-known, but the exact nature of the transformation products is not well studied. A second aim was therefore to study and identify transformation products of C₆₀. Finally, the method was applied to surface water samples from several locations in the Netherlands. The detection limit as found in the present study is considerably more sensitive than existing analytical methods for nC₆₀ in water. It is possible to detect nC₆₀ and transformation products in water at concentrations as low as 5 ng/L. C₆₀ transformation products are observed, and can exceed parent C₆₀. The accurate mass allows identifying these transformation products. No nC₆₀ or transformation products are detected in any of the surface waters monitored. This might be due to low emissions, and/or to high loss factors by sedimentation and removal due to further transformation processes.

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1 Introduction

Engineered nanoparticles (ENP) are a large group of carbon-based and inorganic compounds that are smaller than 100 nm in at least one dimension. Their nanoscale and large surface to volume ratio gives them special properties, with potential application in different areas such as pharmacy, food, energy, and water treatment. ENP are currently used in various consumer products, but exact production volumes are not known and official emission data are lacking. Estimated production volumes are hundreds to thousands tons per year per ENP, estimated economic impact of ENP is \$292 billion dollars by 2010 towards \$1 trillion dollars in 2015 (1,2). Based on estimated future emissions, environmental aqueous concentrations in the ng/L to µg/L range are expected for several ENP (3,4). ENP represent a minor fraction of the nanoparticulate matter that occurs naturally in the environment, which is in the mg/L range (5,6).

Worldwide there is a large demand for a better understanding of human and environmental risks by the growing use of ENP. Total estimated costs for toxicity testing of existing nanoparticles in the US range from \$250 million to \$1.2 billion dollar (7). Several large national and international research programs focus on risk assessment of ENP, e.g. by the OECD, EPA and EU. A common priority in these programs is further development of analytical methods to determine environmental fate and exposure to ENP. As long as environmental concentrations are not known, a quantitative risk assessment of ENP for humans and for the environment is hampered. Development of analytical techniques and application to environmental samples is a key for understanding environmental fate processes of ENP, for a proper modeling of environmental concentrations and for risk assessment.

An important group of carbon ENP is the fullerenes (8). Their cage-like structure allows encapsulating other molecules. They are used for drug delivery, sensors and photonic devices, coatings and pigments, cosmetic products and electronic applications. Further applications are likely to emerge, and rapid increase of fullerene production has been reported for example in Japan. Fullerenes are produced in factories with capacities up to 1500 tonnes/yr (9), but they also occur naturally, e.g. from combustion processes such as forest fires and volcanoes (10). C₆₀ is one of the most common fullerenes; others are C₇₀, C₈₂, C₈₈ and so forth. Based on potential use in consumer products and assuming 10% market penetration, environmental concentrations of engineered fullerenes in the UK were predicted of 0.31 µg/L in water and 13.1 µg/kg in soil (4). Up to now, there are no published data on measured concentrations of fullerenes in the aqueous environment.

To understand environmental fate and model environmental concentrations, next to emission also physico-chemical properties and transformation pathways of fullerenes are of importance. The aqueous solubility of C₆₀ in solvent-saturated water is approximately 8 ng/L (11). However, fullerenes can form a stable aqueous suspension of crystalline aggregates of nC₆₀ (12). Aqueous stocks of nC₆₀ can be prepared by solvent exchange (contacting organic solvent with C₆₀ with water and sonication afterwards), or by direct dispersion (mixing dry C₆₀ with water for a longer period) (13,14). Parent C₆₀ can be transformed by oxidation, reduction and photochemical reactions. After UV irradiation C₆₀ is excited to a singlet state and subsequently to a triplet state, producing reactive oxygen species when returning to ground state (15,16). In short term experiments aqueous nC₆₀ loses its photochemical reactivity (17), but more amorphous nC₆₀ clusters formed e.g. under influence of natural organic matter (NOM) do show a potential to produce reactive oxygen species (16). Aggregated nC₆₀ in water transforms under influence of short wavelength UV light or sunlight, transformation rates are slower in the absence of oxygen (14,18). Resulting transformation products are more hydrophilic and have a higher surface charge density and electrophoretic mobility than the parent nC₆₀, and the original aggregated structure is lost. Fortner et al. (19) showed the formation of oxygen and/or hydroxyl functionalities of nC₆₀ after exposure in the aqueous phase to ozone. After longer irradiation times (65 days) parent nC₆₀ concentration decreased by two orders of magnitude (20). Reaction rates were not influenced by trace amounts of solvents in the aqueous solution, nor by pH or fulvic acid. The several studies mentioned did not identify the exact nature of the transformation products.

Several analytical methods for C₆₀ are described in literature (21). Quantification methods for C₆₀ in organic solvents, mainly toluene, are well known. These are based on high performance liquid chromatography with UV detection (HPLC/UV) or with mass spectrometric detection (HPLC/MS). Different ionization methods are used for mass spectrometry of C₆₀: electro spray ionization (ESI), atmospheric pressure ionization (APCI) and atmospheric pressure photo ionization (APPI) (22-25). To quantify C₆₀ in environmental aqueous samples at trace concentrations, a HPLC/APCI/MS method using solid phase extraction (SPE) is described with a detection limit of 0.3 µg/L (26). Other methods using liquid/liquid extraction (LLE) and HPLC/UV, or LLE/SPE and HPLC/UV respectively found a detection limit of 0.4 µg/L (27) and limit of quantification of 2.48 µg/L (28).

The purpose of this study was to further develop sensitive analytical methods to quantify nC₆₀ in water at environmentally relevant concentrations, using SPE and accurate mass screening liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry (29). Chemical analysis was optimized by testing both ESI and APCI as ionization method, including two eluent combinations. A second aim was to study and identify transformation products of C₆₀. Finally, the method was applied to quantify C₆₀ concentrations in real surface water samples from several locations in the Netherlands.

2 Experimental section

Chemicals

Fullerene C₆₀ (99.5%) was purchased from Aldrich (Steinheim, Germany). Toluene (98%), methanol (99.8%) and acetonitrile (HPLC Grade) were purchased from Baker (Deventer, The Netherlands). Ethanol (absolute HPLC grade) was purchased from Biosolve B.V (Valkenswaard, The Netherlands). Tap water came from Nieuwegein, the Netherlands, with 1.96 mg/L total organic carbon, a pH of 7.9 and an electric conductivity of 39 mS/m. Humic acid (HA, ash 20%) was purchased from Fluka.

Preparation of aqueous nC₆₀ solution

An aqueous stock solution was prepared by dissolving 30 mg C₆₀ in 50 mL of toluene using a shaker platform. Then 10 mL of this deeply purple C₆₀ toluene solution was added to 40 mL ultrapure water, after sonication for 2.5 hr (Branson 3210) the toluene was evaporated. This aqueous solution was filtered over a 0.2 µm regenerated cellulose filter (Sartorius, Goettingen, Germany), the volume of the aqueous stock solution was adjusted to 100 mL with ultrapure water. The filter was rinsed with 1.5 mL ethanol to dry. The dried filter and beaker were rinsed with toluene, and the C₆₀ that did not dissolve in the stock solution was quantified with HPLC/UV/MS. The concentration of nC₆₀ in the aqueous stock solution was derived by mass balance calculations, to be 24 mg/L. The stock solution was very stable in time during months, as also observed in literature (12, 30,31).

Chemical analysis

HPLC/UV/MS analysis was performed with an LC-DAD-LTQ-FT Orbitrap MS system, consisting of a Surveyor autosampler model Plus, a Surveyor quaternair gradient LC-pump, a Surveyor Photo Diode Array (DAD) model Plus detector and an LTQ-FT Orbitrap mass spectrometer (Thermo Electron GmbH, Bremen, Germany). Samples were injected through a 5 µL injection loop. Separation was achieved using a C18 column (150 X 3.9 mm; Varian, Ireland), in isocratic mode at a constant flow rate of 1 mL/min. After UV detection at 333 nm, 200 µL/min of the eluate was sent to the MS system. Negative mode was selected for MS detection, the capillary temperature was 400°C. Accurate masses (200 to 2000 Da) were obtained at high resolution (100,000 FWHM) after external calibration, and processed using Xcalibur v.2.0. software.

Two eluent combinations were tested; toluene/methanol and toluene/acetonitrile, boths at a ratio of v/v 60/40. In addition, both ESI and APCI were used for ionization. The spray voltage of ESI was set at 2.8 kV. For APCI, source voltage was set at 6 kV and vaporizer temperature was 400°C. The MS peak for negatively ionized pure C₆₀ with accurate mass m/z=720.00055±5 ppm was used for HPLC/UV/MS optimization. Calibration curves were analyzed with standards ranging from 0.002 to 5 µg/L solvent, the solvent mixture equals the eluent combination. After preparation, standards were stored in the dark and in a fridge.

Solid phase extraction

Aqueous samples were extracted using C18 SPE columns (1000 mg, 6 mL), surmounted by filtration columns (4 mg see sand). Both columns were preconditioned with 6 mL toluene, 12 mL methanol, and 6 mL ultrapure water, and filled with ultrapure water. Aqueous samples of 1 L were passed over the columns under vacuum for approximately 3 hr. The columns were then rinsed with 6 mL ultrapure water, subsequently dried under vacuum for 1 hr, and eluted with 15 mL of toluene. Concentration of the toluene extracts to a volume less then 0.6 nL followed by evaporation under nitrogen flow in a 56°C bath. The volume was adjusted to 0.6 mL by weighing. Prior to HPLC/UV/MS injection, 0.4 mL methanol was added and the extracts were vortexed. After preparation, samples were stored in the dark in a fridge.

SPE recovery was measured by spiking nC₆₀ from the aqueous stock in ultrapure or tap water. In addition, the influence of HA on SPE recovery was studied at different concentrations in demineralised water.

Surface water samples

In November 2008, surface water samples (Table 1) were collected throughout the Netherlands in glass bottles of 1 L. In the rivers the Dommel and the Meuse, samples were taken before and after industrial sites where release of ENP can be expected, though information on production and emission at these sites is not available. Rotterdam Harbor was included as a reference for a contaminated situation, while the Drentsche Aa was a reference for a relatively uncontaminated situation. Each sample was analyzed three times: once with spiking nC₆₀ aqueous stock to measure recoveries of the analytical procedure and twice without spiking.

Table 1. Collected surface waters

Surface water	Place	Description
Drentsche Aa		Reference for a clean Dutch surface water
Rhine	Lobith	
Lekkanaal (Rhine estuary)	Vork	
Meuse	Brakel	
	Esloo	Before a chemical plant
	Obbich	After the chemical plant
Dommel	Eindhoven	
	Boxtel	After Eindhoven and a plant where emission of ENP can be expected
Rotterdam harbor		At rising tide

3 Results and Discussion

HPLC/UV/MS optimization and formation of C₆₀ transformation products

The combination of toluene/methanol as eluents and ESI as ionization method gives the highest peak areas for C₆₀ (accurate mass $m/z=720.00055\pm 5$ ppm). Therefore this combination is the most sensitive for C₆₀ detection and quantification using liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry, compared to the other combinations tested (Table 2). The calibration curve is nonlinear with ESI/MS detection above a concentration of 100 µg/L, whereas linearity remains obtained using UV-detection (Figure 1a).

Table 2. HPLC/MS optimization, peak areas for two eluent combinations and two ionization modes

	toluene/methanol 60/40 ^a	toluene/acetonitrile 60/40 ^a	Calibration curves for ionization methods
ESI	390	1	Nonlinear > 100 µg/L
APCI	65	120	Linear up to 5 mg/L

^a Normalized area of the MS peak at $m/z = 720.00055 \pm 5$ ppm.

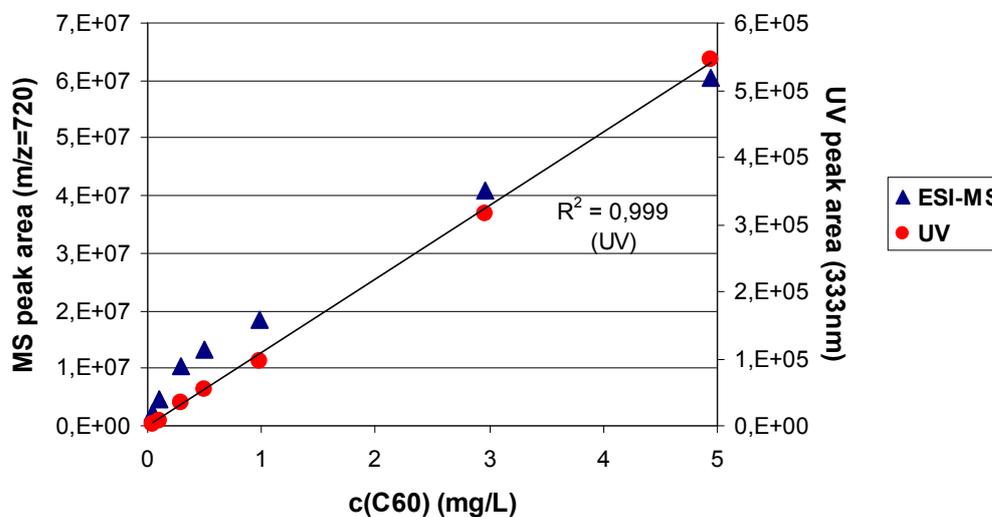


Figure 1a.

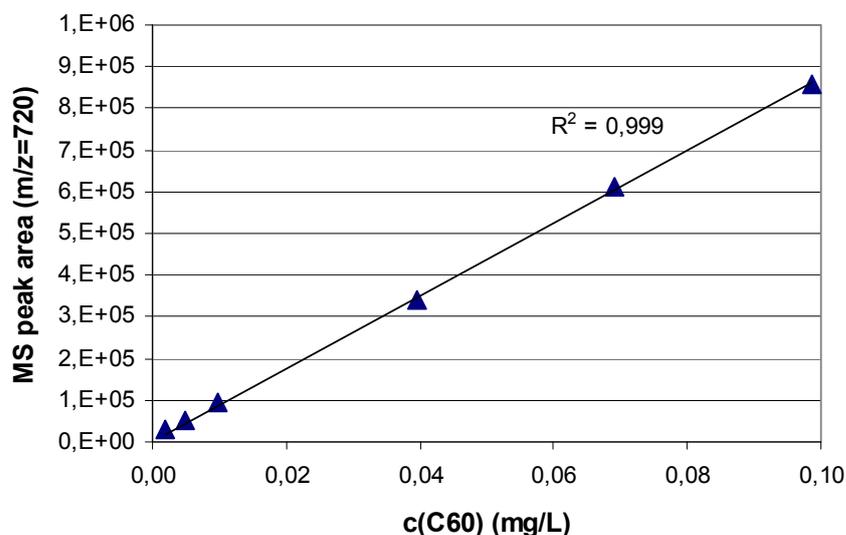


Figure 1b. Calibration curve for C₆₀ in toluene/methanol (0.002 to 5 µg/L), using ESI-MS and UV detection and toluene/methanol (60/40) as eluent. At high concentrations ESI-MS detection shows a nonlinear calibration curve (a), below 100 µg/L ESI-MS detection shows linearity (b).

Apart from the mother compound C₆₀, related ions are observed at the same retention time in both freshly prepared standards and in standards that were stored at room temperature in light for a week (Figure 2). The ions are identified as C₆₀ transformation products, due to the characteristic isotopic pattern of carbon-based ions MS-spectra. The response of the C₆₀ transformation products can exceed the parent C₆₀. The accurate mass allows identifying these transformation products (Table 3). Fullerene oxidized ions (C₆₀+O and C₆₀+OH) are systematically observed, in a lower response than C₆₀. The relative abundance of the individual transformation products is not constant in time, leading to huge variations in quantification for individual transformation products. Therefore quantification is based on the sum of all C₆₀ related accurate masses. This quantification is linear from 1 to 100 µg/L and reproducible in time (data not shown). The detection limit of the overall HPLC/UV/ESI/MS was 1 µg/L in toluene/methanol, the detection limit of the HPLC/UV in toluene/methanol was 5 µg/L.

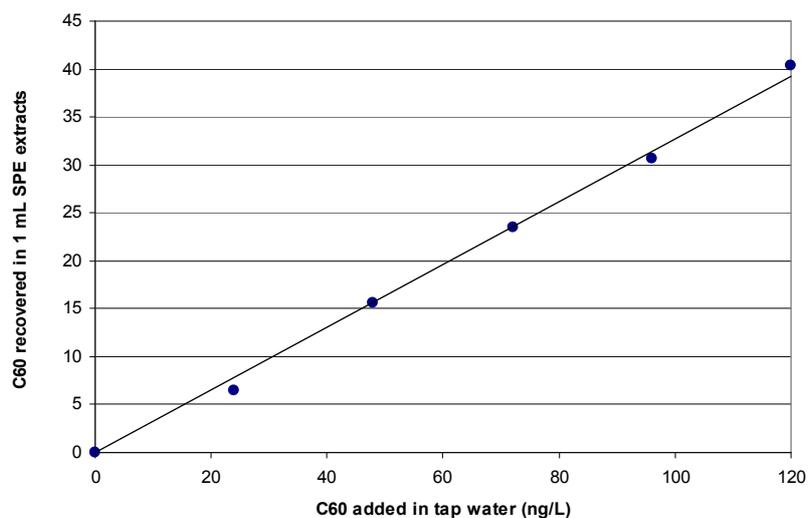


Figure 3. Calibration curve for C₆₀ and its adducts after addition to tap water and after SPE extraction.

Table 3. C₆₀ transformation products formed in freshly prepared solvent standards

	Ion	accurate mass (m/z)
Pure and oxidized fullerene ions	C ₆₀	720.00055
	C ₆₀ +O	735.99546
	C ₆₀ +OH	737.00329
Pure and oxidized adducts with methanol	C ₆₀ +OCH ₃	751.01894
	C ₆₀ +OCH ₃ +O	767.01385
	C ₆₀ +OCH ₃ +OH	768.02168
Oxidized adducts probably with toluene	C ₆₀ +C ₇ H ₇ O ₂	843.04515
	C ₆₀ +C ₇ H ₇ O ₃	859.04007
	C ₆₀ +C ₇ H ₇ O ₄	875.03498

The transformation of parent (n)C₆₀ by oxidation, reduction and photochemical reaction is well-known, but the exact nature of the transformation products is not well studied (15-20). The suggestions in literature on the nature of the transformation products - more hydrophilic, a higher surface charge density and electrophoretic mobility - are in line with the findings in the present study. The described approach to determine accurate mass with LC-DAD-LTQ-FT Orbitrap MS using ESI ionization seems promising for a more in-depth study on various transformation processes under environmental circumstances and identification of the transformation products. These reactions are of relevance for the environmental occurrence of nC₆₀, because nC₆₀ in water will be exposed to sunlight and eventually during drinking water treatment to UV irradiation. The physicochemical properties and environmental fate will vary for the different individual C₆₀ transformation products. However, with the method described it is difficult to separate the transformation processes that take place in the solvent after the extraction of nC₆₀ from the aqueous sample from transformation processes that take place in the water itself. Obviously, this also holds true for many of the cited literature, where solvents are being used during sample preparation and analysis.

Recoveries in ultrapure and tap water, influence of humic acids and detection limit

Recoveries of nC₆₀ and transformation products in ultrapure water after SPE appeared to be below 3%, however in tap water recoveries are higher with 32 ± 2% (Table 4). In both ultrapure and tap water samples without spiking, no nC₆₀ is detected. In demineralised water recoveries are comparable to ultrapure water, but increase with the concentration of HA (Figure 4). However, recoveries remain low with 5% at a HA concentration of 12 mg/L.

Table 4. nC₆₀ (including transformation products) recoveries in tap water (TW) and ultrapure water (UW) after C18 solid phase extraction

	ng C60 spiked in 1 L water	ng C60 in SPE extracts	Recovery (%)
C18, TW	120	36.4	30
	72	24.6	34
	120	36.7	31
	120	38.3	32
	120	40.3	34
	100	30.6	32
	72	23.5	33
	48	15.6	33
	24	6.5	27
	120	36.7	31
<i>Average</i>			32±2
C18, UW	120	0.9*	1
	72	1.8	3
	120	0.5*	0.4

*below the detection limit of the HPLC/MS

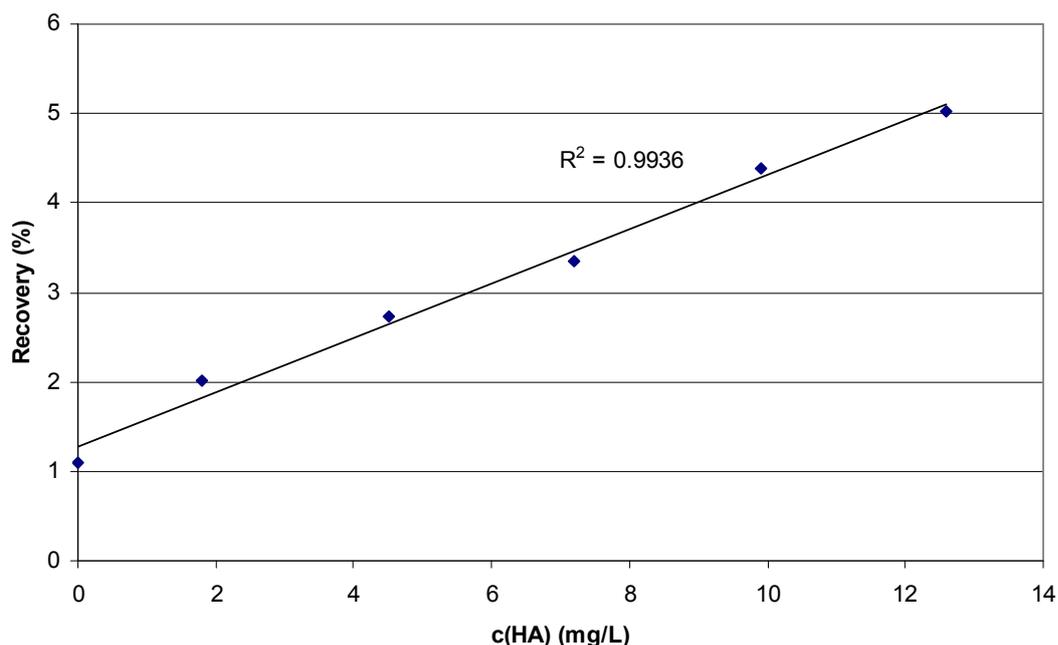


Figure 4. Influence of addition of humic acid on recoveries of C₆₀ and its adducts in demineralised water.

The recovery in tap water is comparable to Chen et al. (26) who found a recovery of $33 \pm 2.6\%$ in tap water. These authors found much higher recoveries ($42 \pm 2.9\%$) in ultrapure water than the present study. The presence of humic and fulvic acids stabilizes the concentrations of nC₆₀ in water, as NOM molecules absorbed on nC₆₀ reduce surface hydrophobicity and increase steric hindrance and thus enhance colloidal stability (14). The introduction of NOM in water leads to smaller nC₆₀ aggregates (30). Next to NOM, electrolyte conditions also influence particle size and electrostatic repulsion of nC₆₀ (14,32). The difference in recovery between tap and ultrapure water might be explained by the higher content of NOM and salts in tap water, stabilizing nC₆₀ and decreasing loss factors. Elution of tap water on SPE with 15 mL of toluene appears sufficient, as a second elution with 15 mL toluene yields less than 2% of the mass recovered during the first elution (data not shown). The recoveries using SPE extraction are concentration independent from 24 to 120 ng/L in tap water (Figure 3).

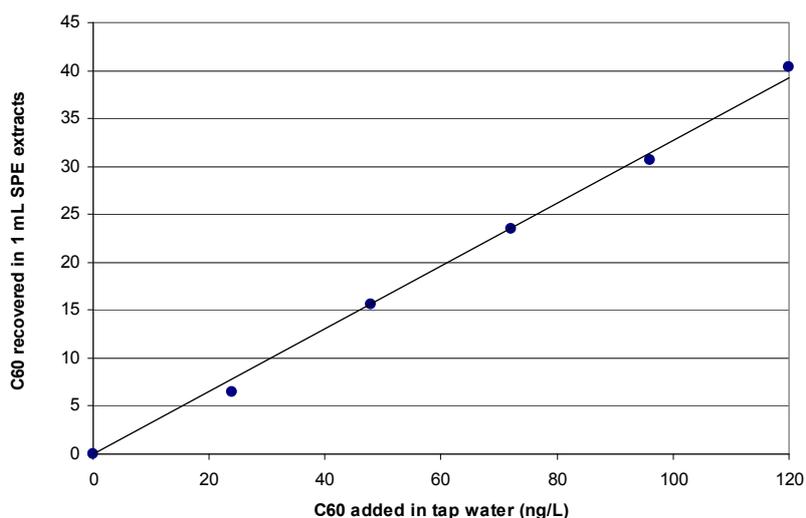


Figure 3. Calibration curve for C₆₀ and its adducts after addition to tap water and after SPE extraction.

The detection limit as found in the present study is below 24 ng/L in tap water, and is thereby over an order of magnitude more sensitive than existing analytical methods for nC₆₀ in water. A concentration of 24 ng/L in water corresponds to a concentration of 6.5 µg/L in toluene/methanol, as the detection limit of the HPLC/MS is 1 µg/L in toluene/methanol it is possible to detect nC₆₀ and transformation products in water at concentrations as low as 5 ng/L. Sensitivity can be improved further by increasing the injection volume, or by using other solid phase materials that obtain higher recoveries. A difference between Chen et al. (26) and the current study is the use of ESI compared to APCI, a more accurate mass determination by the use of LTQ-FT Orbitrap mass spectrometry, and the use of the sum of C₆₀ and its transformation products.

Surface water samples

Recoveries in the various surface waters are slightly lower than in tap water (Table 5). Bouchard and Ma (28) found higher recoveries (78.2±3.22%) for nC₆₀ after SPE extraction of ground and surface water, which was spiked with a C₆₀ suspension in toluene at high concentrations (250 mg/L water).

Table 5. nC₆₀ (including transformation products) recoveries in surface water samples

Samples	recovery (%)
Lekkanaal	28
Rijn	32
Maas at Brakel	23
Maas at Esloo	26
Maas at Obbich	27
Dommel at Boxtel	20
Dommel at Eindhoven	28
Drentsche Aa	21
Rotterdam Harbor	27
Average	26±4

Without spiking, nC₆₀ nor transformation products are detected in any of the surface waters analysed in this study. Therefore, if present, the concentration of the sum of nC₆₀ and the transformation products is below 5 ng/L. A fullerene concentration reaching 0.31 µg/L in water was predicted assuming 10% market penetration, mainly for cosmetic applications (4). As yet, evidence is lacking that fullerenes enter the cosmetic and pharmaceutical markets in this amount, for controversial issues on their toxicity. Besides that, industrially produced “fullerenes” include a variety of pure and functionalized fullerenes.

The fact that we do not find nC₆₀ in natural waters might be due to low emissions, and/or to high loss factors by sedimentation and removal due to further transformation processes than observed in the analytical procedure. As recoveries in environmental surface water samples are comparable to recoveries in tap water, any processes that may explain low concentrations of nC₆₀ in water do not occur in the time frame of one day -which was the time frame of the recovery experiments. This conclusion holds true for the aqueous aggregates of nC₆₀ as prepared; nC₆₀ aggregates in environmental surface water may differ from the laboratory situation.

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