

Atmospheric pressure photoionization mass spectrometry of fullerenes.

Oscar Núñez¹, Héctor Gallart-Ayala¹, Claudia P.B. Martins², Encarnación Moyano¹, Maria Teresa Galceran^{1*}

¹ Department of Analytical Chemistry, University of Barcelona. Martí i Franquès 1-11, 08028 Barcelona, Spain.

² Thermo Fisher Scientific, c/ Cardenal Reig 19, 08028 Barcelona, Spain.

* Corresponding author: Maria Teresa Galceran

Department of Analytical Chemistry, University of Barcelona.

Martí i Franquès 1-11, E-08028, Barcelona, Spain.

Phone: 34-93-402-1275

Fax: 34-93-402-1233

e-mail: mtgalceran@ub.edu

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Abstract

Atmospheric pressure photoionization (APPI) was evaluated for the analysis of fullerenes. An important response improvement was found when using toluene mediated APPI in negative mode if compared with other API sources (electrospray and atmospheric pressure chemical ionization). Fullerene APPI negative mass spectra were dominated by the isotopic cluster of the molecular ion, although isotopic patterns for M+1, M+2 and M+3 ions showed higher than expected relative abundances. These discrepancies are explained by the presence of two isobaric ions, one due to ^{13}C and the other to the addition of hydrogen to a double bond of the fullerene structure. Triple quadrupole tandem mass spectrometry and ultra-high resolution mass spectrometry and accurate mass measurements were used to confirm these assignments. Additionally, cluster ions M+16 and M+32 were characterized following the same strategy. Ions due to the addition of oxygen and alkyl additions were attributed to the presence of methanol in the mobile phase.

For the fast chromatographic separation of fullerenes (less than 3.5 min) a sub-2 μm C18 column and isocratic elution (toluene:methanol 45:55 v/v) was used. Highly selective-selected ion monitoring (H-SIM) mode (mass resolving power >12,500 FWHM) was proposed monitoring the two most intense isotope ions in the $[\text{M}]^{\bullet}$ cluster. Method limits of quantitation down to 10 pg L^{-1} for C_{60} and C_{70} fullerenes and between 0.75-5.0 ng L^{-1} for larger fullerenes were obtained. Finally, the UHPLC-APPI-MS method was used to analyze fullerenes in river and pond water samples.

INTRODUCTION

Since the existence of the remarkably stable carbon cluster C_{60} was first reported by Kroto et al.¹ in 1985, fullerene nanoparticles have been studied in depth in various fields because of their unique structure and potential as new nanomaterials. Today, interest in the use of the fullerene family in several electronic, biomedical and photovoltaic applications, as well as in personal care products (PCPs) is increasing, and rapid growth in the development, production and use of these compounds is predicted over the next decade, so their dispersion in the environment is to be expected.^{2,3} Fullerenes present unique colloidal properties in water which can affect their behavior in the environment. For instance, it has been reported that although the C_{60} fullerene is practically insoluble in water, it can spontaneously form stable aqueous colloidal suspensions containing nanocrystalline aggregates with effective aqueous phase concentrations many orders of magnitude above that corresponding to the aqueous solubility of molecular fullerenes. Few data on the toxicity of these compounds are available, mainly due to their low aqueous solubility, which requires the combination of chemical dispersants such as THF⁴ with stirring or/and sonication.⁵ Moreover, the ability of carbon nanomaterials to form aggregates could change their bioavailability, producing additional toxicological concerns.⁶ The lack of specific analytical methods for their determination presents a significant limitation when attempting to evaluate the risk these materials present to human health and the environment.^{7,8}

Although liquid chromatography (LC) with UV detection has been proposed for the analysis of fullerenes,⁹⁻¹⁴ liquid chromatography-mass spectrometry (LC-MS) is the most commonly used technique nowadays for the determination and characterization of fullerenes and substituted fullerenes in complex matrices.^{11,15-23} Most of these studies have mainly dealt with the separation and determination of C_{60} and C_{70} fullerenes or some of the C_{60} -substituted fullerenes.^{10-14,21-23} Separation of higher fullerenes by reversed-phase chromatography has also been reported,^{9,15-17,20} but long run times are usually required. For the LC-MS analysis of these compounds, both electrospray ionization (ESI)^{17,19,20,23,24} and atmospheric pressure chemical ionization (APCI)^{11,15,16,18,21,22} have been used as ionization sources.

In 2000, Bruins and co-workers developed atmospheric pressure photoionization (APPI) as a complementary ionization source for LC-MS,²⁵ expanding the application of LC-MS to non-polar compounds and compounds which are difficult to ionize by ESI and/or APCI.²⁶⁻²⁸ There are very few examples in the literature of studies reporting the analysis of fullerenes using APPI as the ionization source. Some authors have used C_{60} as a test analyte to study the performance of APPI, reporting that fullerene ionization occurred through a solvent mediated ionization mechanism

yielding the radical molecular ion $[M]^{\bullet}$ as the base peak of the mass spectrum.²⁹⁻³¹ Kawano *et al.*³² proposed a LC-APPI-MS method for the determination of C₆₀ and C₇₀ in synthetic samples, achieving instrumental limits of quantitation (ILOQs) of 5 and 7 $\mu\text{g L}^{-1}$ for C₆₀ and C₇₀, respectively, in a single-quadrupole mass analyzer. However, the applicability of this method to real samples was not demonstrated. Isaacson and Bouchard³³ used LC-APPI-MS to quantify C₆₀ in different fractions after the separation of aqueous C₆₀ fullerene aggregates by asymmetric flow field flow fractionation and recently, Chen and Ding³⁴ reported an LC-APPI-MS/MS method for the analysis of three aqueous fullerene aggregates (C₆₀, C₇₀ and a functionalized C₆₀ fullerene).

This paper evaluates the applicability of negative ion APPI for the analysis of a wide range of fullerene compounds (C₆₀, C₇₀, C₇₆, C₇₈ and C₈₄) and reports in-depth the study of their APPI MS spectra. These spectra are compared with those found when using other atmospheric pressure ionization (API) sources and possible discrepancies are discussed, taking into account the different ionization mechanisms. Lastly, a sensitive and fast UHPLC-APPI-MS method using enhanced mass resolution is proposed which has been applied to the analysis of fullerenes in surface waters.

EXPERIMENTAL SECTION

Chemicals and consumables

C₆₀ (CAS: 99685-96-8), C₇₀ (CAS: 115383-22-7), C₇₆ (CAS: 142136-39-8), C₇₈ (CAS: 136316-32-0) and C₈₄ (CAS: 135113-16-5) fullerenes were purchased from Sigma-Aldrich (Steinheim, Germany). LC-MS grade methanol (MeOH) and water were supplied by Riedel-de Haën (Seelze, Germany) and toluene (HPLC-grade) from Sigma-Aldrich. *Toluene is toxic and was handled in accordance with the most current material safety data sheets.*

Chromatographic separation was evaluated using two C18 reversed-phase liquid chromatography columns: Ascentis Express C18 (150 x 2.1 mm, 2.7 μm particle size) from Supelco (Gland, Switzerland) and Hypersil GOLD C18 (150 x 2.1 mm, 1.9 μm particle size) from Thermo Fisher Scientific (San José, CA, USA).

Stock standard solutions of fullerenes (10 mg kg⁻¹) were individually prepared by weight in toluene and stored at 4°C. Working solutions were prepared weekly by appropriate dilution of the stock standard solution in toluene:methanol (50:50 v/v). Mobile phases were filtered using 0.22 μm nylon membrane filters (Whatman, Clifton, NJ, US).

Nitrogen (99.98% pure) supplied by Claind Nitrogen Generator N₂ FLO (Lenno, Italy) was used for the API sources and high-purity Argon (Ar1), purchased from Air Liquid (Madrid, Spain), was used as a collision-induced gas (CID gas) in the triple quadrupole instrument.

LC-MS (triple quadrupole analyzer)

An ultra-high performance liquid chromatography (UHPLC) system (Accela system; Thermo Fisher Scientific), equipped with a quaternary pump, autosampler and column oven was used. The final chromatographic separation was performed in the Hypersil GOLD C18 (150 mm x 2.1 mm i.d., 1.9 μ m particle size) column using toluene:methanol (45:55 v/v) as mobile phase, isocratic elution at a flow-rate of 500 μ L min⁻¹ (column back pressure 600 bar) and a column temperature of 25°C.

The UHPLC system was coupled to a TSQ Quantum Ultra AM (Thermo Fisher Scientific, San Jose, CA, US) triple quadrupole mass spectrometer, equipped with hyperbolic rods that permit operation in enhanced mass resolution (isolation window: m/z 0.1-0.04 FWHM, full width at half maximum) and with an Ion Max API source housing (Thermo Fisher Scientific) equipped with ESI and APCI probes. When operating with both ESI and heated-electrospray ionization (H-ESI II, Thermo Fisher Scientific) in negative mode, the electrospray voltage was -3.5 kV and the vaporizer temperature when using the H-ESI II probe was held at 350°C. For negative APCI, the discharge current was 10 μ A and the vaporizer temperature was 350°C. When operating with the APPI, the Ion Max source housing was mounted with a SyagenPhotoMate VUV light source (krypton discharge lamp, 10.0 eV) (Syagen Technology Inc., Tustin, CA, USA), and the APCI probe was used as nebulizer-desolvation device (no corona discharge was applied). Nitrogen (purity > 99.98%) was employed as sheath gas and auxiliary gas at a flow-rate of 60 and 25 a.u. (arbitrary units), respectively. Ion sweep gas was kept at 2 a.u. Both ion transfer tube and vaporizer temperatures were set at 350°C.

Selected ion monitoring (SIM) (Q1 at 0.7 m/z FWHM) and highly selective-selected ion monitoring (H-SIM, Q1 at 0.06 m/z FWHM) were used as acquisition modes for single MS experiments. For all acquisition modes, the scan width was set at 0.1 m/z and a 10 ms scan time (1 μ scan) was used. In order to prevent misunderstandings, M+n ion will be referred to in this paper without indicating the charge or radical when several isobaric ions were present at the same m/z value.

166 To optimize both API source parameters and mass spectrometry conditions, 1 mg L⁻¹
167 fullerene standard solutions prepared in toluene:methanol (1:1) were infused at a flow-rate of 5 µL
168 min⁻¹ using a syringe pump and mixed with the mobile phase (500 µL min⁻¹, toluene:methanol,
169 45:55 v/v) by means of a Valco zero dead volume tee piece (Supelco).

170 For instrument control and data processing, Xcalibur v2.0 (Thermo Fisher Scientific)
171 software was used.

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173 **High resolution mass spectrometry**

174 Two high resolution mass analyzers were used, an Orbitrap instrument (Exactive HCD;
175 Thermo Fisher Scientific, Bremen, Germany) and a hybrid Fourier Transform-Ion Cyclotron
176 Resonance (FT-ICR) instrument (Thermo Fisher Scientific) consisting of a linear ion trap coupled
177 to the FT-ICR mass analyzer. Spectra in both HRMS instruments were obtained by infusion of 1 mg
178 L⁻¹ fullerene standard solutions at a flow-rate of 5 µL min⁻¹.

179 The Orbitrap mass spectrometer operated in negative ion mode, acquiring data in full scan
180 single MS (m/z 100 – 1500) and operating at a mass resolving power of 100,000 at FWHM (m/z
181 200) with a scanning rate of 1 Hz. The FT-ICR mass spectrometer operated in negative ion mode,
182 acquiring data in full scan single MS (m/z 100-1000) and operating at a mass resolving power of
183 400,000 (FWHM, at m/z 400). Instrument mass calibration was performed using the MSCAL5
184 ProteoMassT LTQ/FT-Hybrid ESI Pos/Neg solution purchased from Sigma-Aldrich and external
185 mass axis calibration was always used for accurate mass measurements. For instrument control and
186 data processing, Xcalibur v2.0 (Thermo Fisher Scientific) software was used.

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188 **Sampling and sample treatment**

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190 Water samples were collected at different collection sites along the Congost and Tennes
191 Rivers, close to the Circuit of Catalonia racetrack (Montmeló, Catalonia, Spain), and four water
192 samples were taken from ponds around Barcelona Airport.

193 Extraction of fullerenes was performed following a previously described method²³ with
194 some modifications. Samples were processed in triplicate using aliquots of 500 mL. Briefly, the
195 homogenized water sample was filtered through Glass Microfiber filters (4.7 cm, Whatman,
196 Maidstone, England) to collect the water suspended material and the filters were then dried at 50°C
197 for 4h. The filters were subjected to three batch extractions of toluene (25 mL) by sonication for 15
198 min. Toluene extract (75 mL) was then evaporated to 1 mL using nitrogen in a Turbovap[®] II

Concentration Workstation (Zymark Corporation, Hopkinton, Massachusetts, USA), and finally evaporated to dryness with a Visidry vacuum manifold (Supelco) using plastic vials to prevent adsorption.¹² The dried extract was reconstituted in 1 mL of toluene:methanol (50:50 v/v) solution and transferred to an amber-glass injection vial. Finally, 10 μ L of this extract was injected into the UHPLC-MS system.

RESULTS AND DISCUSSION

Chromatographic and Matrix Effect studies

The chromatographic separation of fullerenes can be achieved by C18 reversed-phase chromatography using conventional LC columns (5 μ m particle size)^{10-13,17,20-23} mainly with toluene-methanol or toluene-acetonitrile mixtures as mobile phase. In order to reduce analysis time, in this study two C18 reversed-phase columns were evaluated, a fused-core (Ascentis Express) and a sub-2 μ m (Hypersil GOLD) column. These column technologies are nowadays the main alternatives for fast UHPLC separations. Figure 1 shows a good chromatographic separation on both columns using toluene:methanol 45:55 (v/v) as mobile phase and isocratic elution mode. However, in contrast to the behavior generally observed for relatively small molecules, which show slightly lower retentions in fused-core columns than in sub-2 μ m particle size columns,³⁵⁻³⁷ fullerenes showed higher retention, and this was particularly the case for larger fullerenes. This can probably be attributed to the fullerenes' size (~10 Å for C₆₀) and symmetry, which facilitated a higher interaction in the fused-core column, with the smallest porous size (90 Å), than in the sub-2 μ m column (175 Å). This effect was also observed when comparing sub 2- μ m columns of different porous sizes. This higher interaction could also explain the slight peak tailing observed for all fullerenes in the fused-core column (for instance, the asymmetry factor for C₇₀ was 1.3 on the fused-core column against 1.0 on the sub-2 μ m column). Although higher flow rates can be used with the fused-core column because of its low pressure, the separation of C₇₆ and C₇₈ worsened. Thus, the sub-2 μ m particle size column (Hypersil Gold) was selected for the chromatographic separation of fullerenes with run times lower than 3.5 minutes.

It is well known that the use of toluene as dopant improves ionization efficiency when coupling LC to mass spectrometry using the APPI source.³⁸ Toluene is usually added post-column into the LC eluent flow, but in LC separation of fullerenes it is one of the components of the mobile phase (toluene:methanol 44:55 v/v). This could explain the higher ionization efficiency observed when using solvent mediated negative-ion APPI in comparison to that found with other API sources. As an example, Figure 2 shows the signal obtained for the molecular ion [M][•] of the fullerenes with different API sources (APPI, ESI, H-ESI and APCI). The radical molecular ion [M][•] was always

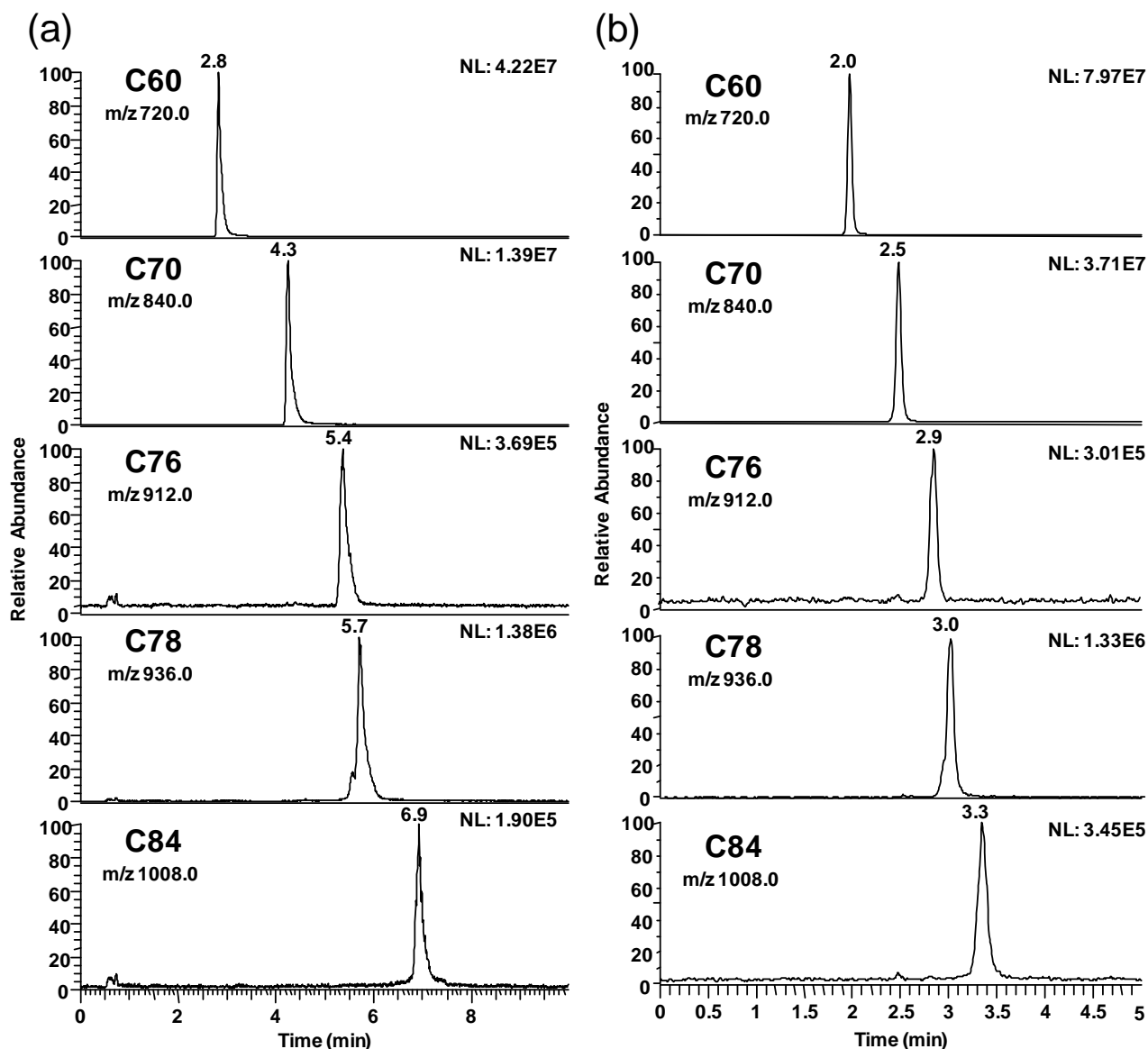


Figure 1. Chromatographic separation of fullerenes ($100 \mu\text{g L}^{-1}$ in toluene:methanol 50:50 v/v) in (a) a C18 Ascentis Express (150 x 2.1 mm, 2.7 μm particle size) column, and (b) a C18 Hypersil Gold (150 x 2.1 mm, 1.9 μm particle size) column. Separation: isocratic elution using toluene:methanol 45:55 (v/v) as mobile phase. Flow-rate: $500 \mu\text{L min}^{-1}$. Acquisition performed in SIM mode and with APPI as ionization source.

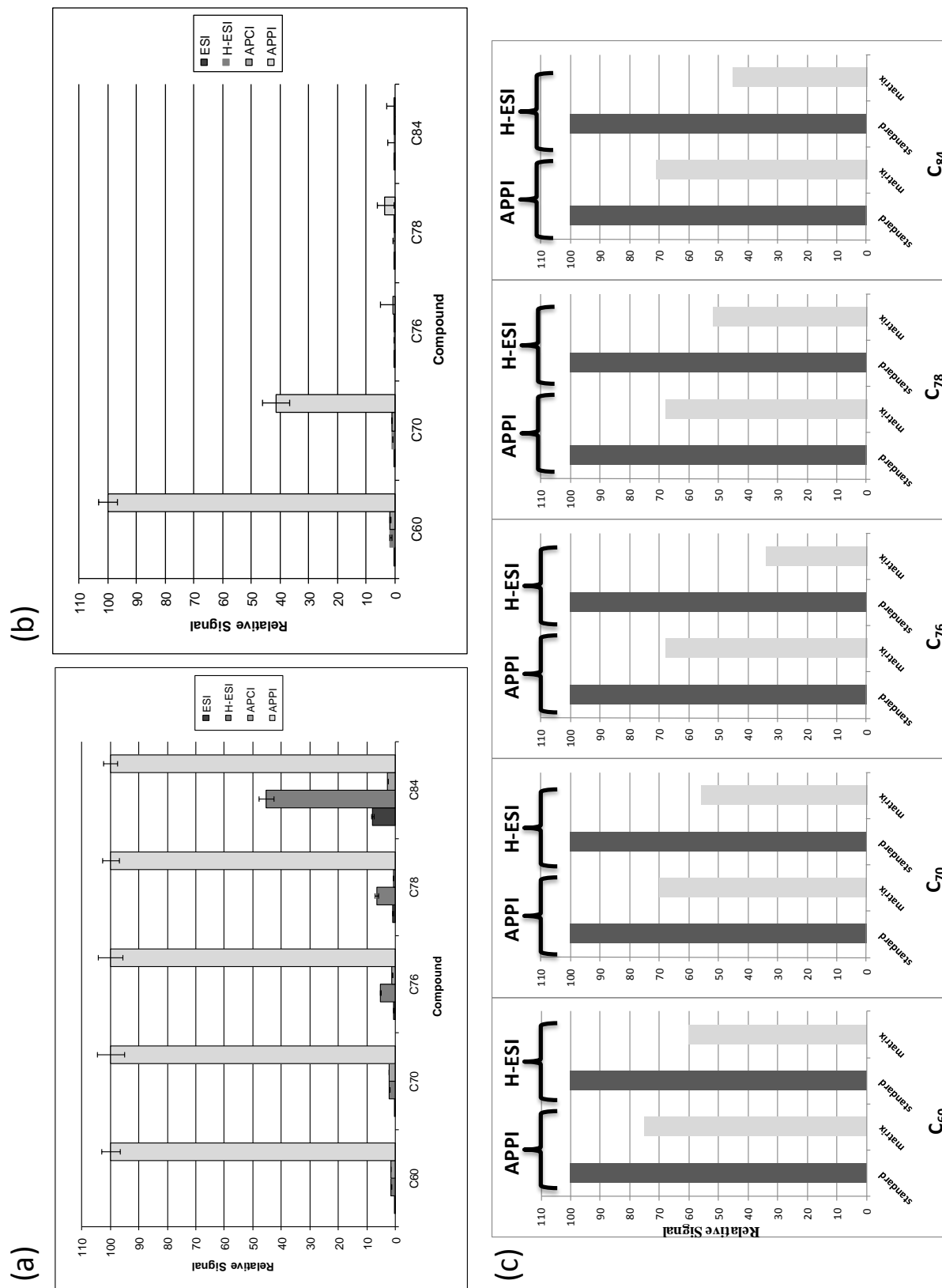


Figure 2. Signal of the $[M]^+$ ion for all fullerenes with different API sources. (a) Absolute response values normalized to the signal observed for APPI with each fullerene, and (b) absolute response values normalized to the highest signal observed (the one for C_{60} with APPI). (c) Comparison of the matrix effect between APPI and H-ESI when a standard solution and a blank matrix extract (both at $10 \mu\text{g L}^{-1}$ for C_{60} and C_{70} and at $100 \mu\text{g L}^{-1}$ for C_{76} , C_{78} and C_{84}) were analyzed. Absolute response values are normalized to the signal observed for the standard solution in all cases.

the base peak of the mass spectrum. In Figure 2a, the absolute response values for each compound have been normalized to the signal observed in the APPI source, while in Figure 2b the same data have been normalized to the highest signal (C_{60} with APPI). As can be seen, APPI provided the highest responses (Figure 2a), and the signal for C_{60} and C_{70} was more than 20 times higher than that observed for the larger fullerenes (Figure 2b). Regarding H-ESI, this source gave better results than ESI mainly for larger fullerenes, while APCI showed a bad performance for the ionization of this family of compounds.

In general, it has been reported that APPI generates more reproducible signals and it is less susceptible than APCI and ESI to ion suppression by matrix effects or salt buffers.³⁹ Nevertheless, some authors have found matrix effects when using APPI, as happened for instance in the analysis of hexabromocyclododecane enantiomers in environmental samples (1-20% matrix effect)²⁷ or of pharmaceuticals in wastewater samples.⁴⁰ In our study, we evaluated the matrix effect on those API sources providing better ionization efficiencies for fullerenes (APPI and H-ESI). The comparison was carried out by analyzing a standard solution of the target fullerenes ($10\text{ }\mu\text{g L}^{-1}$ for C_{60} and C_{70} and $100\text{ }\mu\text{g L}^{-1}$ for C_{76} , C_{78} and C_{84}) in toluene:methanol 50:50 v/v and a blank sample extract spiked at the same concentration. This blank sample extract was obtained by subjecting a river water sample free of fullerenes to the sample treatment procedure described in the experimental section. Figure 2c shows the comparison between the signal obtained with APPI and H-ESI when analyzing both the standard solution and the spiked blank sample extract. As can be seen, H-ESI showed between 40 to 65% ion suppression for the analysis of fullerenes due to matrix effects. This matrix effect was significantly reduced when using APPI (25 to 35%).

Mass spectrometry studies

Fullerene APPI single MS spectra were acquired in both negative and positive modes in a triple quadrupole instrument (full scan m/z 50-1100) by infusing individual standard solutions (1 mg L^{-1}) prepared in toluene:methanol (50:50 v/v) as indicated in the experimental section. MS spectra of fullerenes were dominated by the isotope cluster of the molecular ions m/z M, M+1, M+2 and M+3 (see Figure 3a and Figure 1S in the supporting information for C_{60} negative and positive MS spectra, respectively), although negative MS spectra signals were more than 200 times higher than those of positive MS spectra. However, these isotope patterns did not match those obtained with ESI and APCI or with the theoretical ones, showing higher than expected relative abundances of m/z M, M+1, M+2 and M+3 for all the studied compounds.^{11,19} These discrepancies increased with fullerene size, and for fullerenes with more than 70 carbon

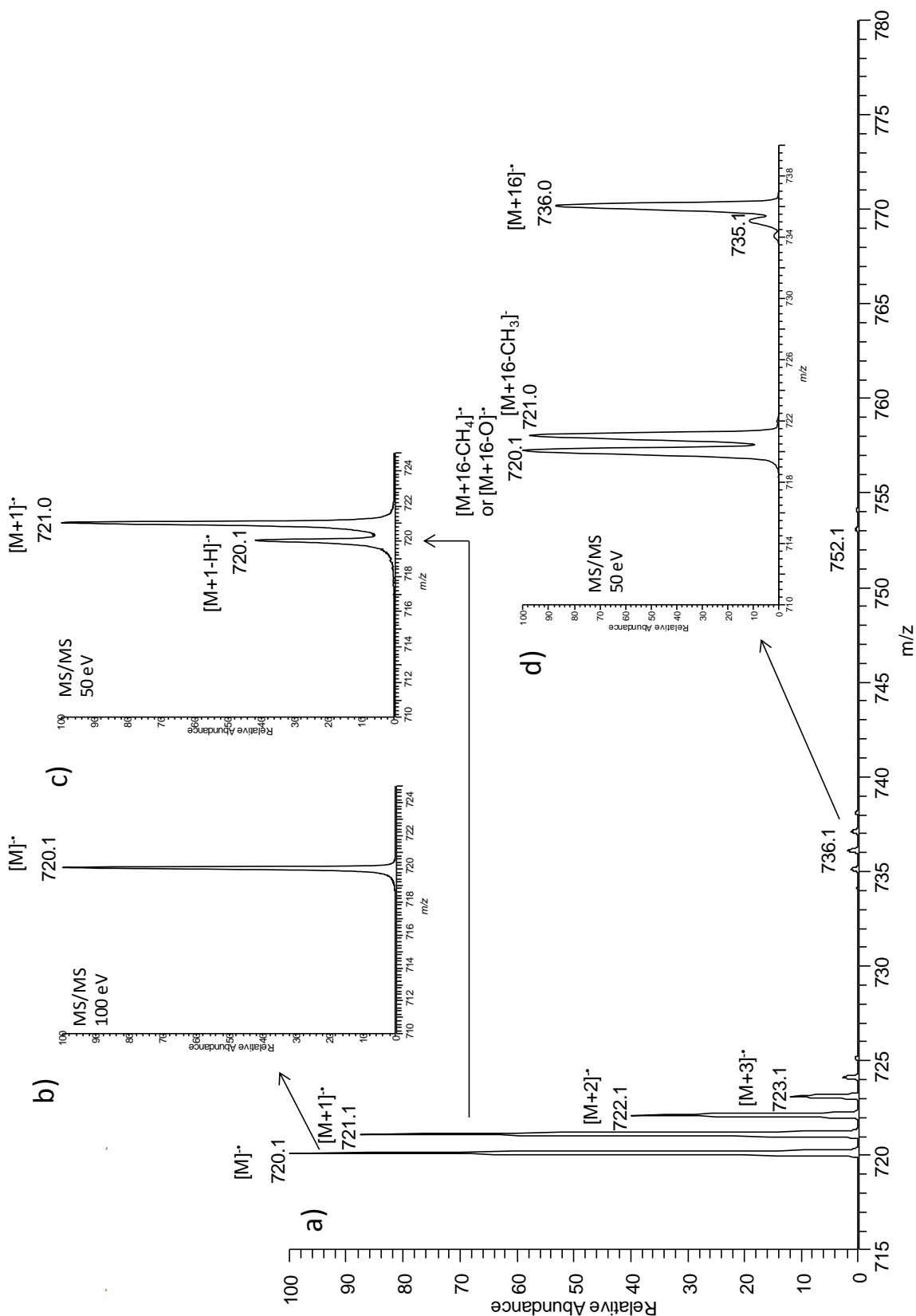
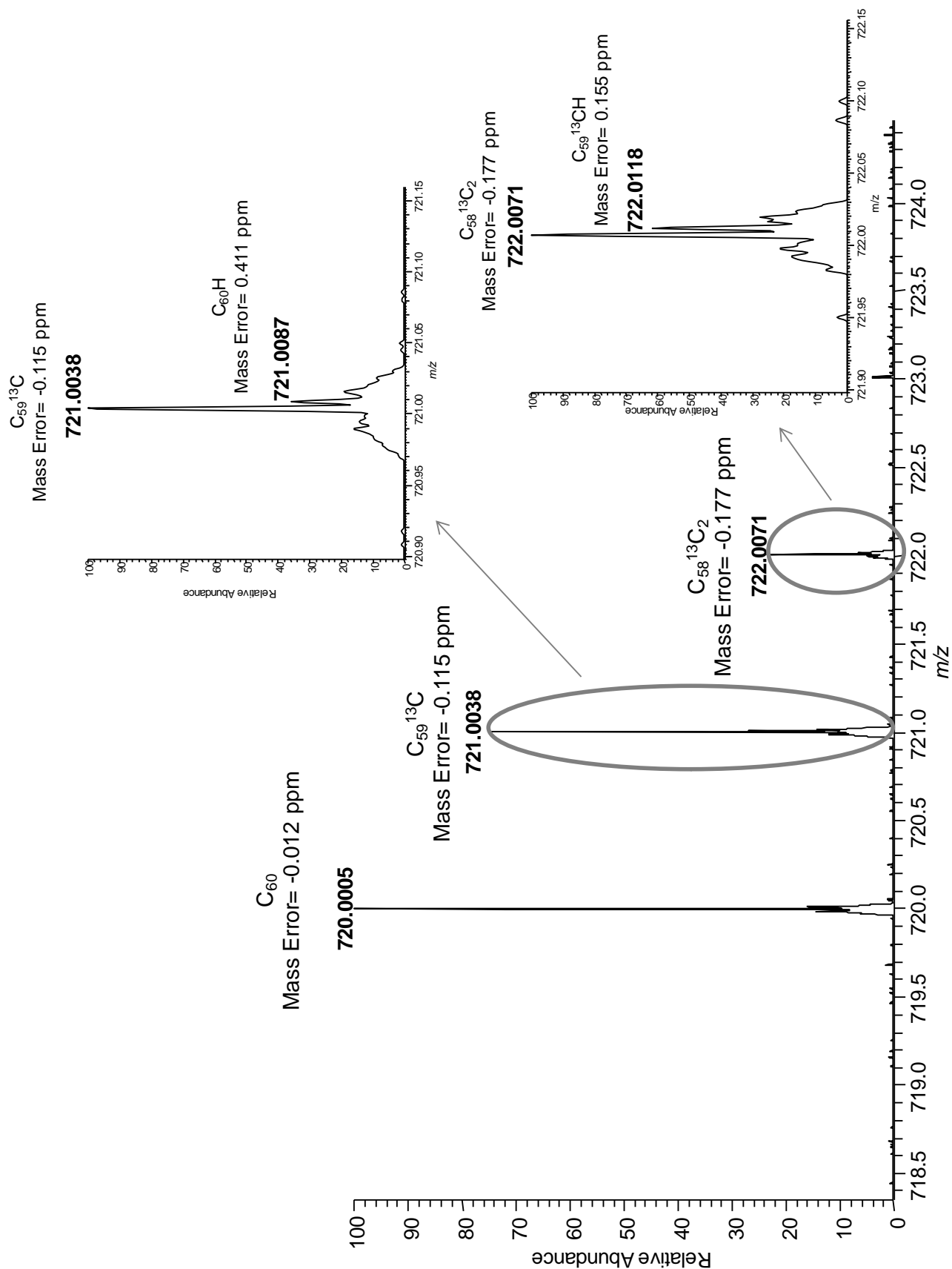


Figure 3. LC-APPI-MS spectra of C_{60} fullerene in triple quadrupole instrument. A) Full scan MS spectrum, B) MS/MS spectrum of the precursor ion at m/z 720 (CE: 100 eV), C) MS/MS spectrum of the precursor ion at m/z 721 (CE: 50 eV) and D) MS/MS spectrum of the precursor ion at m/z 736 (CE: 50 eV).

atoms the relative abundance of m/z $M+1$ was even higher than m/z M . This behavior can only be explained if two isobaric ions are contributing to the abundance of m/z $M+1$. In order to investigate this possibility, tandem mass spectrometry of the molecular cluster ions, m/z M and m/z $M+1$, was performed in the triple quadrupole instrument. As an example in Figure 3b tandem mass spectrum of fullerene C_{60} in negative mode is given. As can be seen the molecular ion $[M]^\bullet$ (m/z 720) was not fragmented even when working at high collision energies (>100 eV). On the other hand, m/z 721 could be fragmented, giving an ion at m/z 720 (Figure 3c) by applying a collision energy of 50 eV. This suggests that in addition to ^{13}C , another ion contributed to the relative abundance of $[M+1]^\bullet$. In positive mode this ion can be assigned to the protonated molecule. To determine the identity of this new ion in negative mode, high resolution mass spectrometry (HRMS) and accurate mass measurements in a FT-ICR instrument were used. Figure 4 gives the HRMS spectrum of the C_{60} fullerene at a mass resolving power of 400,000 FWHM, showing that two ions are present in the signal at m/z $M+1$, the most intense one due to the contribution of ^{13}C at m/z 721.0038 (mass error: -0.115 ppm) and the other one at m/z 721.0087 (mass error: 0.411 ppm), assigned to the addition of hydrogen to the fullerene double bonds. Two ions were also observed at m/z $M+2$ and $M+3$ as a result of the overlapping of both isotope clusters. All the studied fullerene compounds presented the same behavior.

Additionally, in the single MS spectra of these compounds other cluster ions at high m/z values were also observed one of them at m/z $M+16$ and the other one at m/z $M+32$. Two of these ions, $[M+16]^\bullet$ and $[M+32]^\bullet$, have been found in the ESI and APCI mass spectra and are generally explained by the addition of oxygen to the fullerenes.^{20,23,24,32} Although these ions are present at low relative abundances, we intended to characterize them in order to better understand the behavior of this family of compounds in APPI. For this purpose, tandem mass spectrometry experiments in a triple quadrupole instrument were carried out. When the ion at m/z $M+16$ was selected as precursor ion, two product ions shifted in mass 15 Da and 16 Da were observed. These results revealed that the ion at m/z $M+16$ cannot be assigned to the addition of oxygen alone, $[M+O]^\bullet$, as previously described in the literature³², but must also be assigned to $[M+CH_4]^\bullet$ or to the presence of both isobaric ions. As an example, Figure 3d shows the product ion spectrum in negative mode corresponding to the precursor ion at m/z 736 (fullerene C_{60}) that yields the product ions at m/z 721 and 720. In order to correctly assign the ion at m/z 736, HRMS and accurate mass measurements were performed using an Orbitrap mass analyzer operating at a mass resolving power of 100,000 FWHM. As can be seen in Figure 5a, two ions were observed for $M+16$. The ion at m/z 735.9945 (mass error: -1.36 ppm) assigned to $[M+O]^\bullet$ could be generated via the radical addition



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322 Figure 4. Full scan MS spectrum of fullerene C_{60} using a FT-ICR instrument at a resolving power of
 323 400,000 FWHM.

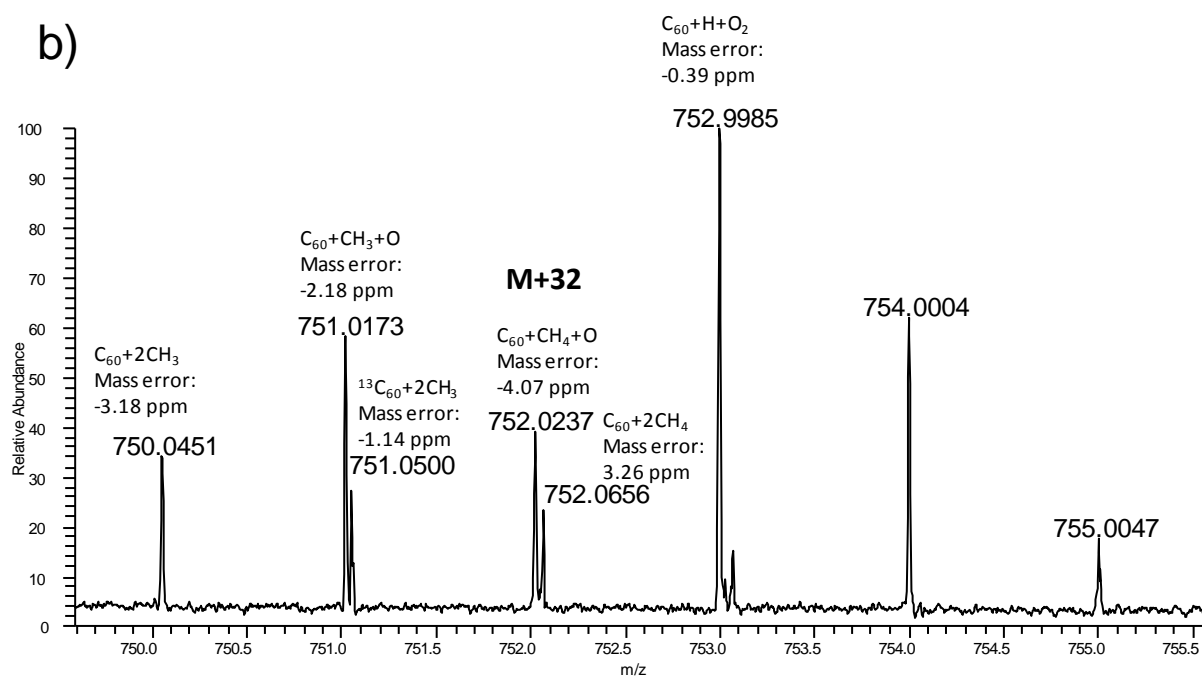
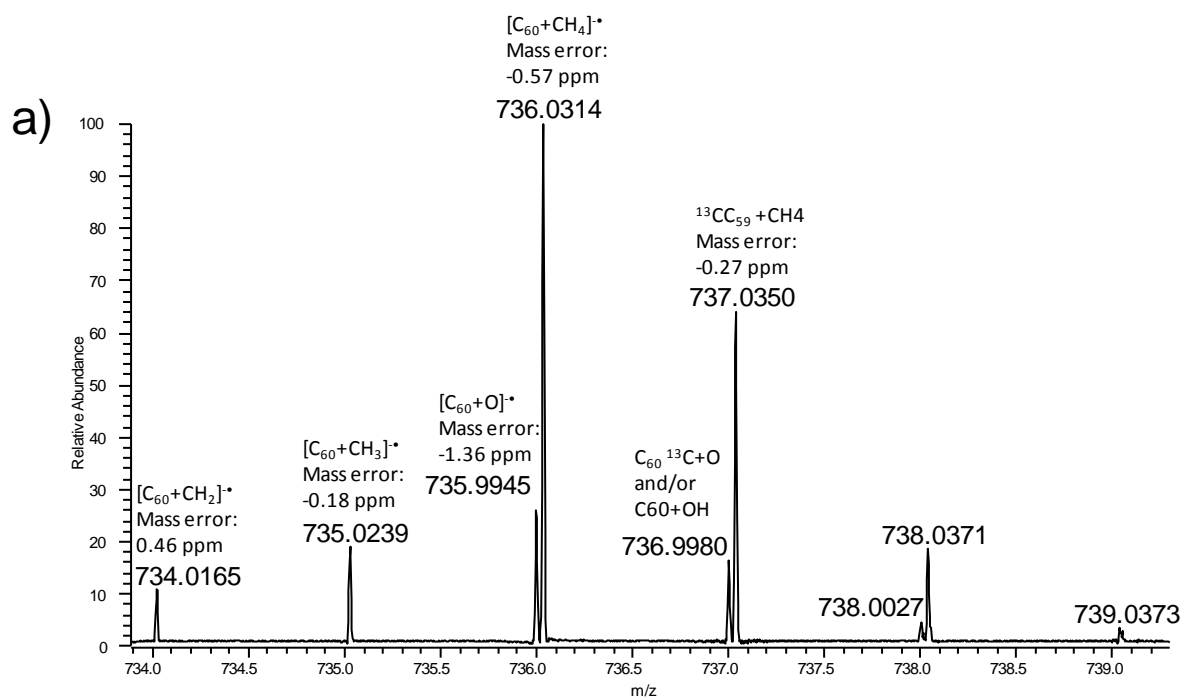


Figure 5. Orbitrap-MS spectra at a resolving power of 100,000 FWHM of the cluster ions a) M+16, and b) M+32.

329 of oxygen to yield an epoxy group as indicated by the ring double bond (RDB) value calculated.
330 The other ion at m/z 736.0314 $[M+CH_4]^{\bullet}$ could be generated by the gas-phase ion molecule
331 reaction of $[M]^{\bullet}$ with a $\bullet CH_3$ and subsequent stabilization by hydrogen addition.⁴¹ Among other
332 ions present in the cluster, the one at m/z 735.0239 can be explained by the addition of $\bullet CH_3$, which
333 can lose one hydrogen giving as a result the ion at m/z 734.0165 (Figure 5a). The ions at higher m/z
334 values can be assigned to the corresponding isotopic cluster ions of both $M+16$ ions, although
335 isobaric ions due to the addition of hydrogen can also be present. To separate these isobaric ions a
336 mass resolving power higher than 300,000 FWHM is needed but the limited sensitivity of the FT-
337 ICR at this mass resolving power prevented the confirmation of their presence.

338 The observed alkyl additions can be explained by the presence of methanol in the gas-phase.
339 In order to verify this hypothesis, the mass spectra of the fullerene C_{60} in 100% toluene and in
340 toluene:acetonitrile (1:1) were obtained. When 100% toluene was used, only the ion corresponding
341 to $[M+O]^{\bullet}$ at m/z 735.9950 (mass error: -0.62 ppm) was observed (Figure 2Sa included in the
342 supporting information), while when toluene:acetonitrile (1:1) was used, both $[M+O]^{\bullet}$ (m/z
343 735.9948, mass error -0.90 ppm) and $[M+CH_4]^{\bullet}$ (m/z 736.0310 mass error: -1.15 ppm) were
344 observed, indicating that the presence of acetonitrile also favored the alkylation mechanism (Figure
345 2Sb). Furthermore, when acetonitrile was used, the addition of CN (m/z 746.0035; mass error -0.16
346 ppm) was also observed (Figure 1Sb). The alkyl additions were probably due to the reaction
347 between fullerene radical ions and the alkyl radicals generated by the photodissociation of methanol
348 and acetonitrile in the APPI source. This behavior was not observed when using ESI in negative
349 mode with toluene:methanol (1:1), where only the addition of oxygen took place .

350 The other cluster ion $M+32$ observed in the full scan MS was also studied following the
351 same strategy. As can be seen in Figure 5b, the base peak of this cluster was the ion at m/z 752.9985
352 assigned to $[C_{60}+H+O_2]^{\bullet}$ and its corresponding isotope pattern. Moreover, other ions at lower
353 masses were also observed. Two ions were obtained for the ion at m/z $M+32$, one at m/z 752.0237
354 (mass error: -4.07 ppm) assigned to $[C_{60}+CH_4+O]^{\bullet}$ due to the addition of oxygen and $\bullet CH_3$ and
355 subsequent stabilization by the addition of hydrogen, and the other one at m/z 752.0656 (mass error:
356 3.26 ppm) generated by the addition of two $\bullet CH_3$ groups and two hydrogens, $[C_{60}+2CH_4]^{\bullet}$. The
357 ions at m/z 751.0173 (mass error: -2.18 ppm) and 750.0451 (mass error: -3.18 ppm) were assigned
358 to $[C_{60}+CH_3+O]^{\bullet}$ and $[C_{60}+2CH_3]^{\bullet}$, respectively (Figure 5b).

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362 UHPLC-MS method

363 In this work an LC-APPI-MS method in negative mode based on the monitoring of the two
364 most intense ions of the fullerene isotope clusters ($[M]^{\bullet}$ and $M+1$ ion) in H-SIM mode in the
365 quadrupole analyzer (Q at 0.06 m/z FWHM with a mass resolving power higher than 12,500
366 FWHM) was developed. Ion-ratios ($[M]^{\bullet}/M+1$ ion) were also monitored and calculated for
367 confirmation purposes (Table 1). Although several isotope ions are present as has been commented
368 above, good ion ratio precision was obtained permitting us to avoid the use of ultrahigh resolution
369 mass spectrometry for the analysis. For C_{60} $[M]^{\bullet}$ and $M+1$ ion were used for quantitation and
370 confirmation, respectively, while for the other compounds with ion ratios <1 , $M+1$ ion was used
371 for quantitation and $[M]^{\bullet}$ for confirmation.

372 Instrumental limits of detection (ILODs) based on a signal-to-noise ratio of 3:1 for the ion
373 that showed the lowest ion intensity were obtained by analyzing solutions of the five fullerenes at
374 low concentration levels prepared in both, toluene:methanol 50:50 v/v and in a blank sample extract
375 obtained as described in the experimental section. ILODs down to 0.5 $ng\ L^{-1}$ in toluene:methanol
376 solutions were obtained for C_{60} and C_{70} by monitoring two ions in H-SIM mode, while values
377 between 60 and 650 $ng\ L^{-1}$ were found for larger fullerenes (Table 1). The slightly higher values
378 obtained in the sample matrix (Table 1) can be related to the matrix effects observed in APPI.
379 Alternatively, a SIM method (Q at 0.7 m/z FWHM) monitoring the three most intense ions of the
380 fullerene isotope clusters ($[M]^{\bullet}$, $M+1$ and $M+2$) to fulfill confirmatory requirements⁴² was also
381 tested although in this case worse ILODs (22- to 29-fold higher) were found for C_{60} and C_{70} (Table
382 1S, supporting information). So, H-SIM mode and matrix-matched calibration is proposed for the
383 analysis of fullerenes.

384 Calibration curves based on the peak area for the five fullerenes at concentrations between
385 instrumental limits of quantitation (ILOQ., based on a signal-to-noise ratio of 10:1) and 100 $\mu g\ L^{-1}$,
386 and prepared in sample matrix, were obtained using UHPLC-APPI-MS in H-SIM mode, showing
387 acceptable linearity ($r^2 > 0.996$). Five replicate determinations of a blank sample matrix that
388 contained the five fullerenes at concentration levels of 100 $ng\ L^{-1}$ (C_{60} and C_{70}) and 10 $\mu g\ L^{-1}$
389 (higher fullerenes) were performed under the optimized conditions to determine instrumental run-
390 to-run precision. Day-to-day precision was determined by performing 15 replicate determinations
391 on three non-consecutive days (five replicates each day). The relative standard deviations (RSDs)
392 based on concentration ranged from 5.7 to 6.9% (run-to-run) and from 8.9 to 9.6% (day-to-day),
393 showing very good performance. Moreover, comparison of the calculated concentration of the

Table 1. Instrumental and method quality parameters.

Compound	Instrumental Quality Parameters					Method Quality Parameters			
	ILODs (ng L ⁻¹) H-SIM		Ion ratio [M] ⁺ /M+1 ion	Run-to-run precision ¹	Day-to-day precision ²	MLOQs (ng L ⁻¹)	Recovery (%)	Run-to-run precision ¹	Day-to-day precision ²
	Standard	Matrix							
C ₆₀	0.5	0.7	1.09 ± 0.08	6.8	9.1	0.01	80	8.1	12.1
C ₇₀	0.5	0.8	0.73 ± 0.06	5.9	8.9	0.01	85	7.5	11.6
C ₇₆	500	600	0.82 ± 0.07	6.0	9.6	4.6	82	9.1	12.8
C ₇₈	60	140	0.76 ± 0.06	6.9	9.2	0.75	85	8.5	12.5
C ₈₄	650	800	0.63 ± 0.05	5.7	9.4	5.0	88	8.7	12.2

¹ %RSD (n=5)
² %RSD (n=3x5)

replicates with that of the target values provided relative errors lower than 3.5%, also showing a good trueness.

In order to evaluate the applicability of the UHPLC-APPI-MS method, water samples collected in different rivers and ponds close to Barcelona (Spain) were analyzed. Recoveries were determined by subjecting a river water sample free of fullerenes spiked at four concentration levels (between 1 and 10 ng L⁻¹ for C₆₀ and C₇₀ and between 5 and 25 ng L⁻¹ for larger fullerenes) to the sample treatment described in the experimental section, and performing analysis by external calibration. Recoveries were then calculated from the slope obtained by plotting the calculated amount versus the added concentration, and values higher than 80% (Table 1) were obtained. Method limits of quantitation (MLOQs) using H-SIM acquisition mode were determined, obtaining values down to 10 pg L⁻¹ for C₆₀ and C₇₀ and between 0.75 and 5.0 ng L⁻¹ for larger fullerenes. The high sensitivity provided by APPI enabled us to obtain MLOQs much lower than those previously described in the literature for this family of compounds in environmental water samples.^{22,23} Method reproducibility was determined by performing analyses of a river water sample free of fullerenes spiked at 1 ng L⁻¹ for C₆₀ and C₇₀ and at 100 ng L⁻¹ for larger fullerenes, and RSD (%) values lower than 12.8% for both run-to-run and day-to-day precisions (Table 1) were obtained, showing that sample treatment did not considerably affect method reproducibility. These results allow us to propose the developed method for the analysis of fullerenes in water suspended material.

A total of six river and pond water samples were analyzed and the results obtained are summarized in Table 2. As an example, Figure 6 shows the chromatogram obtained for a blank sample (showing H-SIM signal corresponding to the [M]^{•+}) and a pond water sample (pond sample 1) showing H-SIM signals corresponding to the two ions monitored for each compound. Quantitation was performed using matrix-matched calibration by preparing the standards in sample matrix and the results were corrected with recoveries. The high sensitivity and selectivity of the UHPLC-APPI-MS (H-SIM) method proposed has allowed to detect for first time fullerenes in surface water although generally at concentrations near the quantitation limits. Fullerenes C₆₀ and C₇₀ were detected in almost all the samples but could only be quantified in some of them. These compounds have also

Table 2. Analysis of fullerenes in water by UHPLC-APPI-MS.

Sample	C ₆₀		C ₇₀		C ₇₆		C ₇₈		C ₈₄	
	Conc. (ng L ⁻¹)	Ion-ratio	Conc. (ng L ⁻¹)	Ion-ratio	Conc. (ng L ⁻¹)	Ion-ratio	Conc. (ng L ⁻¹)	Ion-ratio	Conc. (ng L ⁻¹)	Ion-ratio
Congost	0.021±0.001	1.12±0.05	0.028±0.002	0.78±0.05	n.d.	--	n.d.	--	~LOD	--
Tennes	0.013±0.001	1.06±0.05	0.022±0.001	0.79±0.04	n.d.	--	n.d.	--	5.0±0.4	0.65±0.05
Pond sample 1	0.022±0.002	1.11±0.04	0.020±0.001	0.77±0.05	n.d.	--	1.04±0.06	0.80±0.05	19.2±1.1	0.68±0.06
Pond sample 2	~LOD	--	~LOD	--	n.d.	--	n.d.	--	n.d.	--
Pond sample 3	<MLOQ	--	0.012±0.001	0.78±0.06	n.d.	--	n.d.	--	~LOD	--
Pond sample 4	n.d.	--	<MLOQ	--	n.d.	--	n.d.	--	n.d.	--

Ion-ratio: [M]⁺/M+1 ion

n.d.: not detected

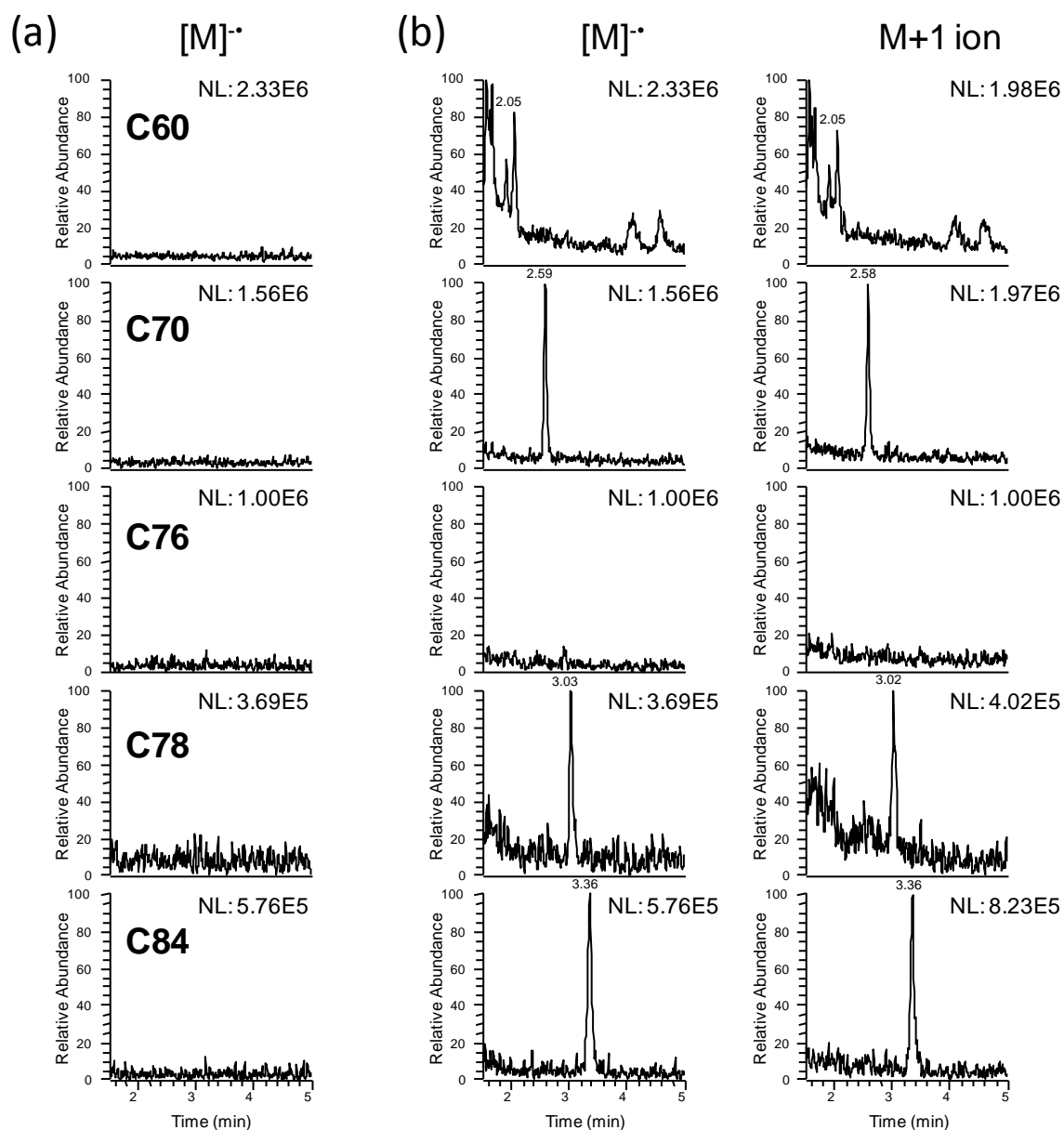


Figure 6. Chromatographic separation of (a) a blank sample, and (b) a pond water sample (pond sample 1) using the proposed UHPLC-APPI-MS (in H-SIM mode) method.

been found in effluents of wastewater treatment plants.²³ Regarding larger fullerenes no data about concentration levels in environmental samples have been reported in the literature until now. Our results show that these compounds, mainly C₈₄, can also be present in surface water at concentration higher than fullerenes more frequently studied, C₆₀ and C₇₀. The presence of fullerenes in the analyzed surface water could be related to the location of the sampling points near to the landing strips of an airport and a racetrack because of the formation in combustion processes of fullerene-like carbon particles in petrol soot.^{43,44}

Conclusions

The advantage of using negative ion APPI to improve the ionization of a wide range of fullerene compounds (C₆₀, C₇₀, C₇₆, C₇₈, and C₈₄) has been demonstrated. A considerable improvement in fullerene sensitivity was observed in APPI compared to other API sources, which was attributed to the use of toluene for solvent-mediated APPI ionization. Moreover, APPI showed a reduced matrix effect (ion suppression lower than 35%) in comparison to that observed for H-ESI (40-65% ion suppression). The addition of hydrogen to the double bonds of the fullerene structure explains the relative abundances, higher than expected, of the [M][•] isotope ions. The combined information of tandem mass spectrometry and ultra-high resolution mass spectrometry allowed the correct assignment of [C_{n-1}¹³C][•] and [C_n+H][•]. Other APPI gas-phase reactions such as the addition of oxygen and methyl groups have been attributed to the presence of methanol in the mobile phase. These processes took place via the reaction between fullerene radical ions and the alkyl radicals generated by the photodissociation of methanol in the APPI source.

A sensitive and fast UHPLC-APPI-MS method is proposed for the analysis of fullerenes using enhanced mass resolution in H-SIM mode. Method quality parameters were established, with MLOQs down to 10 pg L⁻¹ for C₆₀ and C₇₀ fullerenes (between 0.75 and 5.0 ng L⁻¹ for larger fullerenes), recoveries higher than 80%, and good method precisions (RSD values lower than 9 %). The UHPLC-APPI-MS method proposed was applied to the analysis of fullerenes in river and pond water samples. This is the first paper reporting the presence of fullerenes in surface water. Larger fullerenes were found at concentration higher than C₆₀ and C₇₀.

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