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Characterisation and determination of fullerenes: A critical review.

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Highlights

- Methodologies for fullerene aggregate characterization are discussed.
- Chromatographic and electrophoretic techniques for fullerene analysis are presented.
- Accurate identification and detection of fullerenes by LC-MS is critically reviewed.

Graphical abstract

Abstract

A prominent sector of nanotechnology is occupied by a class of carbon-based nanoparticles known as fullerenes. Fullerene particle size and shape impact in how easily these particles are transported into and throughout the environment and living tissues. Currently, there is a lack of adequate methodology for their size and shape characterisation, identification and quantitative detection in environmental and biological samples. The most commonly used methods for their size measurements (aggregation, size distribution, shape, etc.), the effect of sampling and sample treatment on these characteristics and the analytical methods proposed for their determination in complex matrices are discussed in this review. For the characterisation and analysis of fullerenes in real samples, different analytical techniques including microscopy, spectroscopy, flow field-flow fractionation, electrophoresis, light scattering, liquid chromatography and mass spectrometry have been reported. The existing limitations and knowledge gaps in the use of these techniques are discussed and the necessity to hyphenate complementary ones for the accurate characterisation, identification and quantitation of these nanoparticles is highlighted.

Abbreviations: C_{60}-pyrr, C_{60} pyrrolidine; PCBM, [6,6] -Phenyl-C61-butyric acid methyl ester; bis-PCBM, [6,6] -Bis-Phenyl-C61-butyric acid methyl ester; C_{70}-PCBM, ([6,6] -Phenyl-C71-butyric acid methyl ester; PCBB, [6,6] -Phenyl-C_{61}-butyric acid butyl ester; ThCBM, [6,6] -Thienyl-C61-butyric acid methyl ester; PCMO, [6,6] -Phenyl-C61-butyric acid octyl ester; MSAD-C_{60}, bis(monosuccimide) derivative of p,p’-bis(2-aminoethyl)diphenyl-C_{60}

Keywords: Fullerenes, Aggregates, Particle size and shape analysis, Identification, detection and quantitation, Complex matrices

1. Introduction

The carbon nanotechnology era was born with the discovery of C_{60}: Buckminsterfullerene by Kroto *et al.* [1] named after the architect Buckminster Fuller who designed geodesic domes in the 1960's. C_{60} and other clusters with a variable number of carbon atoms were produced by carbon vaporization from graphite into a high-density helium flow using a pulsing vaporization laser. The discovery of fullerenes, the third carbon allotrope, resulted in the award of the Chemistry Nobel Prize to Curl, Kroto and Smalley in 1996. Their structure is composed of closed carbon cages formed by 12 pentagons and a number of hexagons that increases with the size of the fullerene ([(2n/2)-10] , n=number of C atoms). Figure 1 shows the structure of C_{60}, C_{70}, and of two surface modified fullerenes.

The sources of nanomaterials in the environment can be natural (*e.g.* forest fires and volcanoes and anthropogenic (*e.g.* fossil fuel combustion and engineered nanomaterials). However, it is very difficult to distinguish between carbon nanoparticles of engineered origin and those from other, natural or anthropogenic, sources [2] . The labelling of engineered nanomaterials using fluorescent, radioactive-labelling and entrapment of rare elements could be useful for this purpose [3,4] .

Despite the fact that fullerenes are very hydrophobic with very low solubility in water, around 8 ng L^{-1} for C_{60} [5] , several methods have been developed to disperse these compounds in aqueous media [6-13] leading to the formation of stable colloidal aggregates (nC_{60}), a highly hydrophilic supramolecular complex consisting of C_{60} enclosed into a hydrated shell containing 24 water molecules: $C_{60}@(H_2O)_{24}$. The aqueous suspensions of nC_{60} are amber coloured and are stable for months to years at very low ionic strength [14,15] . To increase the solubility in water, fullerenes surface chemical derivatisation with polar functional groups has been performed [16-18] . A large number of derivatives that combine the desirable properties of C_{60} with the solubilising power of the side chains have been produced. One common example is the fullerol molecule ($C_{60}(OH)_{24}$), a water soluble compound obtained by the addition of hydroxyl groups to the C_{60} cage. However, even with very polar functional chains, fullerenes undergo aggregation since the spheres agglomerate forming micelle-like aggregates [19] .

Today, fullerenes and surface functionalised fullerenes are used in optical, electronic, cosmetic, and biomedical applications [20-26] and in the last past years surface-functionalised fullerenes have been produced in larger quantities than pristine fullerenes [27-29] in an attempt to create more biologically compatible forms [28-30] . After the discovery of C_{60}, the formation of the endohendral fullerene La@C_{60}, proved for the first time that the interior space of fullerenes can host atoms or molecules [31] . These materials display remarkable electronic and structural properties associated with the charge transfer from the encaged metal or cluster to the carbon cage. During the past decade, intense research lead by several worldwide groups has allowed the inclusion of a variety of atoms and small molecules inside carbon cages, such as metallic nitrides [32] , carbides [33] , sulphides [34,35] and up to three individual metal atoms [36,37] . The new properties of metallofullerenes expand their applications in biomedicine and material sciences fields [38,39] .

The need for an understanding of health and ecological risks of nanotechnology was recently stressed by the EU commission and by the US Environmental Protection Agency. Within the context of the European Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation (EC) 1907/2006 a specific approach for manufactured nanoparticles (MNPs) is under discussion [40] and the European Parliament resolution on (2008/2208(INI)) also calls for legislation to adequately address nanomaterials [41] . Hence, the analysis of these particles is not only a social concern but also a legal necessity. The anticipated market growth of fullerenes in combination with the potential for direct human exposure via several applications [23] has led to widespread concerns about the possibility to cause adverse effects on human health. However, their effects on humans and the environment are poorly understood and there are conflicting reports on their hazard. Risk assessment is necessary in order to produce and use these materials in a responsible and sustainable way.

Early toxicity studies of fullerenes have been focused on the colloidal form of C_{60} (nC_{60}) [42,43] . Some reports indicate that pristine fullerenes are non-toxic, and that fullerene water systems have a wide spectrum of positive and unique biological activities [44] . For instance, there are studies showing that water-soluble fullerenes scavenge reactive oxygen species (ROS) in human skin cells [45] and that they are powerful antioxidants with no acute or sub-acute toxicity [46] . Fullerenes ecotoxicological studies present a major difficulty because of their poor aqueous solubility [47] . The properties and reactivity of fullerenes are affected by the solubilisation method and organic solvent used [6,7] . The choice of a dispersant is problematic, since some of the best dispersants from a chemical point of view are also likely to be toxic to organisms. For example, tetrahydrofuran (THF) is a good dispersant for C_{60} fullerenes in water but there are concerns about its toxic effects [15,48] . Henry *et al.* [49] showed that C_{60} toxic results in zebra fish were attributable to THF metabolites inadvertently formed during the dispersion in THF and this was further confirmed in subsequent research [50] . Regarding the potential toxicity of other fullerenes, Seda *et al.* [51] reported that aqueous colloidal suspensions of gallic acid-stabilized C_{70} reduce significantly the fecundity in Daphnia magna.

In general the water soluble C_{60} derivatives are less toxic than nC_{60} and the toxicity decreases with the number of polar functional groups attached to the C_{60} molecule [52] . Moreover, several positive effects of fullerene derivatives have been described. For example, surface modified fullerenes with pyrrolidine groups showed antibacterial effect and inhibit cancer cell proliferation [53] and polyhydroxyfullerenes are good candidates for the treatment of neuro-degenerative disorders (*e.g.* Parkinson’s and Alzheimer’s disease) [54] . However, some studies indicated that polyhydroxy fullerenes show oxidative eukaryotic cell damage [55] and Roberts *et al.* [56] found that fullerol is both cytotoxic and phototoxic to human lens epithelial cells. Although its acute toxicity was proved to be low, fullerol is retained in the body for long periods [57] , raising concerns over its chronic toxic effect.

Several reviews have been published on methods for the characterisation of nanomaterials (*e.g.* size, surface charge, surface area) [3,58] , their occurrence and behaviour in the environment [59,60] , the potential risk that fullerene nanoparticles pose to the environment and human health [61,62] and the chromatographic separation and quantitative analysis of fullerenes in environmental samples [63-65] . The accurate analysis of particles involves their detection, identification, quantitation and detailed characterisation and each step is an individual challenge in complex and heterogeneous samples. There is a lack of specific analytical methods for fullerenes and especially for functionalised fullerenes which are produced nowadays in larger amounts. The general aim of this review is to provide an overview of the techniques available for the physicochemical characterisation, and for the detection and quantitation of fullerenes. As limited studies have been carried out on real samples, the principal challenges in method development for their characterisation and analysis in complex matrices will be discussed. Given the broad range of particle properties that have to be taken into account, the need for combining complementary techniques for a better understanding of the behaviour and fate of these particles in the environment is highlighted.

2. Characterisation of fullerenes

Fullerene nanoparticles challenge the limits of colloidal science due to their small size, variable shape, structure, composition and potential interaction with organic molecules. One of the most prominent physicochemical properties of these compounds is their tendency to undergo aggregation, leading to the formation of clusters with various shapes and sizes. Knowledge of the aggregation degree, size distribution and shape of fullerenes is crucial for toxicity assessment and to track their transport and fate in the environment [66,67] . In industrial processes the particle size distribution has a critical effect on the quality and performance of the final manufactured product. Up to now, no ideal technique has been found for characterising fullerenes and there are very few reports on their size distribution behaviour in complex matrices [13,68-70] .

In Table 1, the techniques most commonly used for the characterisation of nanoparticles and the measured properties (*i.e.*, size, shape, aggregation, and specific surface area) are summarized. The most popular tools to study the size and morphology of particles are microscopic techniques: transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM). Of the three techniques, TEM provides the highest magnification power covering the widest size range (0.1-1000 nm) and can supply detailed information on particles structure..

High-resolution TEM (HRTEM) has been used to investigate the individual C_{60} fullerene morphology, crystallography, and microstructure. For instance, Goel *et al.* [71] reported a crystalline structure for C_{60} in toluene with a diameter of about 0.7 nm, and a two-fold symmetry. Smith *et al*. [72] and Sloan *et al.* [73] reported HRTEM images of round closed shells of fullerenes with diameters smaller and larger than C_{60} (0.4 to 1.6 nm) encapsulated within the hollow central region of individual single walled carbon nanotubes (SWCNTs) [72,73] and Burden and Hutchison [74] provided real-time evidence of C_{60} and C_{70} formation on the surface of carbon black particles irradiated with an electron beam. However, TEM produces a high level of sample perturbation because the grids must be dried prior to imaging and the particles are likely to aggregate to some degree. Thus, it does not guarantee the original integrity of the particles and aggregates.

For the determination of the size distribution of particles in a fluid, dynamic and static light scattering (DLS and SLS respectively), covering a size range between 10 and 1000 nm, are the most commonly used techniques. These methodologies enable the study of the stability, degree of aggregation, complex formation and conformation of molecules, and moreover, the sample can be recovered at the end of the measurement. DLS was used to study fullerene particle sizes in several organic solvents and binary solvent mixtures showing that they form aggregates of different sizes depending on the solvents used [75,76] . Light scattering techniques are usually combined with microscopic ones and more recently coupled to flow field-flow fractionation (Flow FFF) for both characterisation and separation of particles in aqueous solutions. Flow-FFF is a powerful tool for both the characterisation and separation of aggregates over a wide size range (1 to 1000 nm).Since the separation occurs in a channel which does not contain a stationary phase as in liquid chromatography, there is no mechanical or shear stress applied to the sample components. Asymmetric flow-FFF (AF4) coupled to light scattering techniques has been reported for the determination of the size distribution of fullerene aggregates [77-79] . For instance, AF4 coupled to DLS was used for the characterization of aqu/C_{60} aggregates reporting sizes between 80 and 260 nm [78] and to multi angle light scattering (MALS) for the determination of the size distribution of aqu/nC_{60} and that of two C_{60}-derivatives [79] presenting radii of gyration (Rg) values from 20 to 80 nm. It must be noticed that the Rg radius obtained by MALS is different from the DLS hydrodynamic radii, thus these values cannot be compared.

Additionally, asymmetric flow-FFF combined with AFM was proposed to study the morphology and aggregate sizes of fullerol nanoparticles obtaining sizes of approx. 2 nm [80] . AFM allows the observation of particles in their natural environment with atomic resolution over a wide size range (0.5-1000 nm) and preserves their original morphology. Moreover, this technique gives true 3-dimensional information even in water and in contrast to electron microscopy, specimen preparation is less constringent.

Nanoparticle tracking analysis (NTA) is a laser technique able to simultaneously visualise and determine the particle size distribution and concentration of particles in liquids from 10 to 1000 nm (depending on the material). Recently, Sanchis *et. al.* [70] used NTA for the analysis of fullerenes in river water samples, reporting the presence of C_{60} and C_{70} as both colloidal fractions (< 450 nm) and large agglomerates (< 450 nm). The Brauner, Emmett and Teller (BET) method is also used as an additional tool for the specific surface area and porosity determination of the particles. Recently, thermal-lens spectrometry, a method suitable for optical and thermophysical studies, was used for the characterisation of fullerene aqueous suspensions confirming the formation of large clusters (>130 nm) [81] .

2.1. Effect of the preparation method on the morphology and size distribution of fullerene aqueous suspensions

Despite its hydrophobic structure, C_{60} can be solubilised in water forming stable suspensions whose properties differ from those of bulk solid C_{60} [8] . Several procedures were reported (Table 2), mostly involving the solubilisation in water by solvent exchange using different organic solvents (ethanol, toluene, THF and acetone) via sonication or mechanical stirring [6,8,10-15,82] . Additionally, the direct solubilisation of C_{60} in water was also reported via sonication [12] or extended stirring [13-15] . The preparation conditions vary from one study to another in terms of stirring rate, sonication power, time used for sonication and/or stirring, amount of organic solvent and the filtration step.

Several studies have investigated the characteristics of nC_{60} formed via direct addition of C_{60} to water (aqu/nC_{60}) and by solvent exchange [6,7,10,13,68] . In most of the works several characterisation techniques were used in order to increase the amount of information available. Microscopy provides a basis for comparison and is suitable for elucidating particle shape and to some degree aggregate structures but cannot provide information about the size distribution of the aggregates. Hence, for the accurate knowledge of the morphology, aggregation state and size distribution of fullerenes in aqueous solutions, other techniques and mostly light scattering (LS) must be also used [6,7,10,11,15,63,83-86] . For instance, Brant *et al.* [6] investigated the characteristics of nC_{60} obtained using four different preparation procedures by TEM, DLS and SLS (Table 3). The authors studied and compared the sizes and shapes of the nC_{60} obtained using three solvent exchange methods (SON/nC_{60}, THF/nC_{60}, TTA/nC_{60}) (Table 3) and by extended mixing of C_{60} with pure water (aqu/nC_{60}). DLS measurements (Figure 2a) showed that the different preparation procedures produced suspensions of nC_{60} with similar size distributions, (50 to 2000 nm) but with important differences in the size distribution (Figure 2b). Also, TEM micrographs showed differences in the size, shape and structure of the nC_{60} formed (Figure 3). For instance, homogeneous hexagonally shaped structures were found for TTA/nC_{60} and heterogeneous spherical and large elongated aggregates were obtained by mechanical mixing (THF/nC_{60}) and sonication (SON/nC_{60}), respectively. The aqu/nC_{60} suspensions were highly polidisperse in size (20 to 500 nm) appearing as small spherical aggregates and large blunt-angular random structures. Lyon *et al.* [13] also studied the size and morphology (Table 3) of nC_{60} aggregates obtained by four different preparation techniques (THF/nC_{60}, SON/nC_{60}, aqu/nC_{60}

and PVP/C_{60}) using TEM and DLS. TEM micrographs showed more rounded edges and less crystallinity for aqu/nC_{60} compared to the other suspensions in agreement with Brant *et al.* [6] . The DLS average sizes of the THF/nC_{60}, SON/nC_{60} and aqu/nC_{60} suspensions obtained in this study are significantly lower (2-142 nm) than those given by Brant *et al.* [6] (237-354 nm) although the same preparation methods were used (Table 3). The different results obtained with the same technique can be due to the use of different filter sizes for the filtration of the suspensions prior to the analysis, 0.22 µm [13] and 0.45 µm [6] respectively. Besides, the differences in the operation conditions such as stirring/sonication time and power used in each study could also explain the differences in the characteristics of the aggregates formed. Lyon *et al.* [13] also evaluated the effect of the size and morphology on the antibacterial activity of nC_{60} toward the Gram-positive bacterium *Bacillus subtilis* and observed that the smaller aggregates with higher surface areas showed the highest level of antibacterial activity. However, it is unclear whether this behaviour can be attributed to the smaller size or to the presence of an amorphous structure. Even though, the observed bactericidal effects of nC_{60} suggest the need for caution against accidental releases and disposal of fullerenes.

The radical scavenging properties of fullerene C_{60} aggregates stabilised by poly(vinylpyrrolidone) (PVP/nC_{60}) reported by various research groups [13,87,88] have led cosmetic manufacturers to use this nanomaterial as skin ‘anti-aging’ and whitening [89-91] . Dadalt Souto *et al.* [88] studied the characteristics of PVP/nC_{60} suspensions that can affect the distribution and interaction with the skin and its dermal absorption profile by NTA, TEM and DLS since the size distribution of the particles is considered a key parameter in determining its interactions with living systems [92] . The size distributions determined by DLS and NTA were in agreement (116 ± 18 and 129 ± 54, respectively) and TEM micrographs showed polydisperse samples with irregular shapes and sizes between 100 and 200 nm. Moreover, NTA measurements indicated that only 2.7 % of fullerene aggregates were smaller than 40 nm, value that can be correlated with the skin pores (0.4-36 nm) meaning that most of the PVP/C_{60} aggregates will not penetrate the skin. Nevertheless, some concerns about the utilisation of cosmetic formulations containing PVP/nC_{60} still remain because the short- and long-term exposure effects of fullerenes on the skin are not well known yet.

It is clear that many characteristics of the aggregates are dependent upon the preparation method and this makes difficult to reach conclusions about the risks associated with the release of fullerenes into the environment. The lack of standardised procedures for fullerene characterisation makes difficult the comparison of the results reported in the literature on various samples and obtained by different techniques limiting the advance of research in this field. The aggregates formed in the environment will probably be more similar to aqu/nC_{60} than to aggregates produced via solvent exchange methods, but little research has been conducted on this form. Studies on the formation and behaviour of aqu/nC_{60} in aqueous solutions with different properties are essential to help the understanding of the fate of fullerenes upon release in the environment and represent crucial advances in this field.

2.2. Effect of the composition and characteristics of the aqueous media on fullerenes aggregation behaviour

The mobility and toxicity of fullerenes in the environment will depend on the colloidal stability of the formed aggregates. To evaluate the health and environmental risks posed by fullerenes, not only the formation of aggregates but also their stability in natural systems needs to be understood in order to predict their sedimentation rate and to be able to find strategies for their removal. The aggregation of these particles into large clusters will reduce their ability to be transported or to come into contact with aquatic organisms. Knowledge of the stability, mobility and aggregation behaviour of fullerenes as a function of matrix composition, pH and ionic strength will allow us to gain an insight into their potential behaviour when released into the environment. Several studies have been carried out for this purpose for aqu/nC_{60} [6,7,10,15,68,84,85,93] , aqu/nC_{70} [84] and also for some functionalised fullerenes [80,84] .

Independent reports have shown that aqueous fullerene suspensions are negatively charged exhibiting a negative electrophoretic mobility [6,7,10] . Among them, Brant *et al.* [6] studied the effect of the ionic strength (NaCl solutions from 0.01 to 100 mM) on the surface charge of nC_{60} colloidal solutions prepared by different methods (Table 3). All the nC_{60} colloids obtained displayed negative electrophoretic mobility and negative zeta potentials (ζ) (Table 3) which are similar to the ones of fullerol [7,94] . This indicates that C_{60} acquires a charge through charge transfer from the oxygen atoms of the water and/or the organic solvents and/or hydroxylation of its surface through either adsorption of hydroxyl groups or by “localised hydrolysis” [95] .

In general, it is accepted that aqu/nC_{60} will quickly aggregate in natural waters leading to the formation of heteroaggregates settling out of suspension, and as a consequence, reducing its environmental risk [96-98] . However, it has been reported that the presence of natural organic matter (NOM) in waters favours the disaggregation of aqu/nC_{60} causing a significant change in particle size and morphology [85,99,100] . NOM fulvic and humic substances can be attached to the surface of particles in a variety of ways leading to their stabilisation. For instance, Chen *et al.* [85] reported an effective stabilisation of fullerene nanoparticle suspensions through steric repulsion in the presence of humic acid and at high $CaCl_2$ concentration which lead to a dramatic drop in the rate of aggregation, and to an increase in the critical coagulation concentration (CCC).

Moreover, several authors studied the effect of the ionic strength and pH on the degree of aggregation of fullerene in aqueous solutions [7,10,15,80,84,101] . For example, Fortner *et al.* [15] reported that C_{60} heteroaggregates stay stable for months at low ionic strengths similar to those of most surface waters but the average particle size increased up to 360 nm at an ionic strength of 0.05 and values above 0.1 are high enough to precipitate the particles after 48-72 h. Moreover, the average particle sizes also depend on the pH, decreasing at high pH values from 120 nm (pH 3.7) to 60 nm (pH 10). Brant *et al.* [14] also reported the formation of large nC_{60} aggregates with the increase of the ionic strength with average diameters up to 700 nm (0.01 M).

The above mentioned studies combine light scattering techniques with TEM to study the physical and chemical properties of fullerene aggregates. However, as previously mentioned the use of other complementary techniques such as AFM and AF4 has also been reported [77,78,80,99,102] . For instance, AF4 combined with DLS was used to evaluate the effects of sunlight and water composition (NOM) on the size distribution and ζ potential of aqu/C_{60} [99] . The results show that the presence of divalent cations and NOM in the water stabilizes the aqu/nC_{60} nanoparticles leading to sizes lower than the ones observed in deionised water (average sizes of 150-175 nm and 210-230 nm, respectively). Moreover, a decrease in the ζ potential of aqu/nC_{60} with time has been reported under laboratory lighting conditions [84] and under natural sunlight [99] . Thus, the sunlight and the presence of NOM lead to a decrease in the size and zeta potential of aqu/nC_{60} aggregates making them more stable and more available for transport and organism uptake.

Due to the increase in the production and use of functionalised fullerenes their behaviour in aqueous solutions has also been recently evaluated [80,84,94,101] . The studies show that their aggregation is also promoted by the increase of the ionic strength as observed for aqu/nC_{60}, but the aggregates present smaller sizes. For instance, Assemi *et al.* [80] studied by AF4 and AFM the behaviour of a water soluble fullerene compound, $C_{60}(OH)_{24}$, in water showing that this compound exist as very small clusters (doublets or triplets) at basic pH and low salt concentration and its ζ potential is not affected by the pH or ionic strength of the solution as usually happens for aqu/nC_{60}. However, the size of the particles increased with the ionic strength from 1.2 nm at zero ionic strength up to 6.7 nm at 0.1 M NaCl. These small sizes disagree with the ones previously reported by TEM and DLS techniques (~100 nm) [94] . This disagreement could be due to the different methodology used for the size measurements of the particles. The above mentioned studies indicate that surface modified fullerenes with polar groups present much smaller particle sizes than pristine fullerenes. Further studies are needed to establish the toxicity of this family of compounds, their fate and transport in the environment.

Although, the source of the negative electrophoretic mobility is not yet well understood, there are evidences that fullerene aggregation behaviour is in agreement with the classic Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability [6,85] . According to this theory, the attractive and repulsive interactions between colloids are dominated by van der Waals forces and electrostatic double layer forces [103] . Ionic strength controls the extent of the radius of the diffuse layer on the surface. At high ionic strength, the electrical double layer is compressed, the van der Waals attractive interactions dominate and particle aggregation is promoted. Dissolved ionic solutes and pH play crucial roles on the aggregation of fullerenes in engineered and natural systems. Seemingly, the factors that will reduce the degree of aggregation of these materials in the environment include a low ionic strength and the presence of humic-like substances. The typical ionic strengths of many natural waters and the presence of polysaccharide-based natural organic matter, which could be produced by algae or bacteria, will tend to favour deposition and reduce the potential for exposure.

The above mentioned studies have demonstrated that the physicochemical transformations acting simultaneously will affect the behaviour of fullerenes in the environment by encountering a multitude of conditions important for aggregation (*e.g.* pH, ionic strength, NOM). Nevertheless, studies about their aggregation behaviour and properties in complex matrices are lacking. Further research is needed to gain a full understanding of the aggregation of nanoparticles and how to apply this knowledge for industrial applications and risk assessment.

3. Determination of fullerenes

The current section presents the most commonly used methodologies for the determination of pristine and surface-functionalised fullerenes. For this purpose, the analytical separation and detection techniques are discussed.

3.1. Separation

The separation of fullerenes represents a challenge, especially due to their structure similarities, limited solubility in common solvents and tendency to form aggregates. The most widely employed techniques are liquid chromatography (LC) and capillary electrophoresis (CE) in combination with different on-line detectors (*e.g.,* UV-Vis and mass spectrometry). For particle separation, one of the most promising techniques is AF4 coupled to light scattering detection. This technique is suitable for the determination of fullerene aggregate sizes but, to the best of our knowledge, its applicability for the separation of fullerene compounds has not been reported yet.

3.1.1 Liquid Chromatography

Among the separation techniques, liquid chromatography (LC) is the most effective and its applicability for the separation of fullerenes has been investigated since the 1990s [104-108] . Table 4 summarizes the LC methods reported in the literature for the analysis of this family of compounds in complex matrices. As can be observed, most of the LC methods are focused on pristine fullerenes, mainly C_{60} and C_{70} although recently, some studies have been published describing the separation of functionalised C_{60}-fullerenes, including some water soluble derivatives [109-114] and that of a C_{70}-functionalised fullerene together with other pristine and hydrophobic C_{60} fullerene derivatives [115] . The most common chromatographic separation conditions for hydrophobic fullerenes are reversed-phase chromatography mainly using octadecylsilica stationary phases and mobile phases containing toluene:methanol or toluene:acetonitrile (Table 4). In most of the works, conventional 3.5-5 µm particle size columns are used, resulting in long analysis times (up to 15 min) although recently, columns with sub-2 µm totally porous particles or core-shell partially porous particles have been used (Table 4). A considerable reduction in the analysis time can be achieved with these columns (2.5- 4.3 min), as well as a high column efficiency while maintaining a good chromatographic separation (Figure 4). The elution order of hydrophobic fullerenes in reversed-phase columns is correlated with the presence and number of functional groups on the fullerene cage (functionalised fullerenes eluting earlier than pristine fullerenes), and with the size of the fullerene cage (the greater the size, the higher their retention). Additionally, the retention of this family of compounds on these columns is controlled by adding a polar solvent (*e.g.* methanol, acetonitrile) to a non-polar solvent (*i.e.,* toluene) since the interaction of fullerenes with aromatic solvents such as toluene is stronger than with polar solvents [116] . As regards to these two solvents an interesting chromatographic behaviour in C18 columns has been described in the literature. As an example, Figure 4 shows the chromatograms obtained on a sub-2µm particle size C18 column for C_{60}-C_{84}, C_{60}, C_{60}-pyrr, PCBM and PCBB, using toluene:methanol and toluene:acetonitrile (40:60 v/v) as mobile phases under isocratic conditions. As can be seen, higher retention times were obtained using toluene:acetonitrile than with toluene:methanol. This seems counterintuitive, since the eluotropic strength of the mixture toluene:acetonitrile is higher, hence lower retention times should be expected compared with the toluene:methanol mobile phase. The authors [117] explained this behaviour as being related to the size of fullerene aggregates in each solvent mixture. As previously mentioned, fullerenes can form aggregates in binary mixtures of toluene and a second organic solvent and their sizes decrease with the increase in the polarity of the latter [75,76] . Therefore, bigger aggregates will be formed in toluene:acetonitrile than in toluene:methanol and as a consequence higher retention is observed.

Several other reversed-phase columns such as C30 and C8 have also been evaluated for the separation of pristine fullerenes (C_{60}, C_{70}, C_{76}, C_{78}, C_{82} and C_{84}) [118] and the C30 chains showed to be very effective for the interaction with fullerene molecules, achieving a strong retention, although with very long analysis times (> 40 min). Moreover, several chromatographic columns have been especially designed for the separation of these compounds [119] . Among them, Cosmosil Buckyprep (pyrenylpropyl group) and Cosmosil PYE (pyrenylethyl group) columns are the most commonly used for the analysis of fullerenes. These columns have been employed by several authors for the separation of fullerenes and several derivatives using 100 % toluene as mobile phase [120-123] , toluene mixtures with acetonitrile [109,115,124] or methanol [78] , and acetonitrile-dichlormethane mixtures [106] . For instance, Carboni *et al.* [124] reported the separation of C_{60}, C_{70} and several functionalised fullerenes (PCMO, bis-PCBM, ThCBM, C_{70}-PCBM, PCBM and PCBB) (Figure 5). The first eluting compound in the chromatogram (bis-PCBM) presented a jagged peak shape that was attributed to either the presence of different isomers or to the formation of micelles in the solution. As depicted in Figure 5, long analysis times are usually obtained with the Cosmosil Bulkyprep column (25 min), which is disadvantageous in comparison to the short analysis times that can be achieved with sub-2µm or core-shell C18 columns, as previously commented. This specially designed column is ideal for preparative-scale separations since it exceeds the injection volume of a standard C18 column by a factor of 35 [119] .

Among the other stationary phases reported for the analysis of pristine fullerenes [125,126] the ones based on phenylporphyrin immobilized on silica, such as tetraphenylporphyrin-silica [108] and hydroxyphenyltriphenylporphyrin-silica [127] provide very good selectivity and efficiency. However, the use of these columns is limited by the high cost of phenylporphyrin materials. In addition, chiral columns have been used [110,128] for the enantiomeric separation of chiral fullerene derivatives.

Recently, there is a manifested interest in developing methods for the analysis of water soluble fullerenes with potential industrial, biomedical and cosmetic applications. Different stationary phases and polar solvent combinations (*e.g.* water, methanol, acetonitrile) are employed for the analysis of water soluble surface-functionalised fullerenes, such as $C_{60}(OH)_{24}$ [111,112] , chiral hexakis adducts ($C_{60}(COOEt)_{12}$) [110] , (C3, D3)tris-dicacrboxymethanofullerene-C_{60} [114] , dendritic monoadduct methano [60] fullerene octadeca acid [113] and MSAD-C_{60} [129] . Among them, the chromatographic separation of $C_{60}(OH)_{24}$ is challenging because of the high hydroxylation and the associated negative surface charges that make difficult the use of conventional C18 separation approaches. Despite this, Silion *et al.* [112] used a Zorbax SB-C18 reversed-phase column and 100 % water as the mobile phase for the retention of fullerol (retention time of 2.6 min). In contrast, Chao *et al.* [111] were not able to retain fullerol in a XBridge C18 column regardless the mobile phase composition used. Therefore, the authors proposed the use of hydrophilic interaction liquid chromatography (HILIC) employing a XBridge amide column with water:acetonitrile (10:90 *v/v*) as mobile phase achieving a good efficiency and a retention time of 3 min for fullerol.

Although advances have been made in the analysis of water soluble fullerenes, there is still a limited number of studies, and most of them have been performed using standard mixtures [46,110-112,114] . In comparison, a significantly higher number of studies have been reported for the analysis of hydrophobic fullerenes, and most of them are applied to complex matrices (*i.e.*, environmental, biomedical, and cosmetic) (Table 4).

3.1.2 Capillary electrophoresis

Capillary electrophoresis (CE) techniques with UV-Vis detection have also been proposed to separate fullerene compounds [130-136] although its employment for the analysis of these compounds in real samples is very limited. Table 5 summarises the reported methods and the experimental conditions used.

CE is an analytical technique that separates ions inside a fused-silica capillary according to their differences in electrophoretic mobility under the influence of a strong electric field. The main problems to be addressed when using CE for the separation of hydrophobic fullerenes is their lack of charge and electrophoretic mobility and their low solubility in aqueous buffers currently used as electrophoretic media in CE. To overcome these drawbacks two approaches have been proposed: non aqueous capillary electrophoresis (NACE) by employing charged compounds and organic solvent mixtures as separation medium and micellar electrokinetic capillary chromatography (MECC) by adding a surfactant above the critical micellar concentration to an aqueous electrophoretic buffer in order to form micelles.

The separation of hydrophobic fullerenes by NACE has been studied by several authors [130-132] . Different compounds able to interact with the target analytes to form charged complexes which can migrate under the electric field are added to the background electrolyte to accomplish their separation. For instance, pristine fullerenes (*i.e.*, C_{60}, C_{70}) and several hydrophobic C_{60} derivatives have been separated using tetraalkylammonium (TAA) salts in a solvent mixture containing acetonitrile, chlorobenzene, methanol and acetic acid as background electrolyte (BGE) [130,131] . A long chain TAA salt (tetradecylammonium bromide, TDAB) was used to induce the electrophoretic migration of the analytes via solvophobic interactions while the short chain TAA salt (tetraethylammonium bromide, TEAB) reduced the electro-osmotic flow (EOF) improving the separation. Among these studies, Astefanei *et al.* [130] applied a NACE method for the analysis of C_{60} in a commercial cosmetic product as an alternative to conventional LC methods. NACE has also been proposed for the separation of three open-cage fullerenes using a mixture of acetonitrile and methanol with trifluoroacetic acid and sodium acetate in order to provide the compounds with an overall positive charge via protonation [132] .

The separation of C_{60} and C_{70} using an aqueous buffer by MECC using sodium dodecylsulphate (SDS) as surfactant has also been reported [133] . To overcome solubility problems, the fullerenes were first solubilised in the aqueous media via complexation with SDS. Nevertheless, this involves an additional time-consuming sample preparation step to the analytical procedure. Moreover, the encapsulation process and the sizes of C_{60} and C_{70} aggregates within the SDS micelle are yet to be understood.

CE techniques have also been proposed for the separation of water soluble fullerenes [134-136] . For instance, Chan *et al.* [134] evaluated the use of capillary zone electrophoresis (CZE) and MECC with SDS micelles, for the analysis of two water soluble fullerene derivatives (dendro[60] fullerene (DF) and carboxy fullerene (C3)) in human serum samples. For the CZE experiments, bare and dynamically coated fused-silica capillaries were used. Using the coated capillaries, the EOF was suppressed allowing faster migration times but with a slight decrease in resolution between both nanoparticles. The authors recommend using CZE for the quantitation of both C3 and DF in human serum because this technique allowed to obtain good results for DF and presented some advantages over MECC for C3 such as lower analysis time and lower detection limits due to less baseline noise and better reproducibility. However, dilution of the human serum with SDS or addition of SDS to the running buffer to break the interactions between nanoparticles and proteins within the serum sample is required. The behaviour of DF in CZE as a function of pH, ionic strength, solvent amount and concentration of additives to reduce EOF has been also reported [135] . The parameters which showed the most important effect on the migration time and separation efficiency of the analytes were the pH and the ionic strength. The migration time of DF increased with the pH and decreased with the salt concentration.

The above mentioned studies indicated that CE could be a suitable technique for the analysis of both hydrophobic and water soluble fullerenes as an alternative to LC, in samples with relative high concentrations such as cosmetic and pharmaceutical products.

3.2. Detection

For the detection of fullerenes, either UV-Vis or mass spectrometry (MS) have been used, due to the excellent response obtained with both techniques. UV-Vis detection is commonly used for CE techniques and to the best of our knowledge its coupling to MS has not been described in the literature. Nevertheless, MS provides better selectivity, allows differentiating fullerenes by their molecular weight and provides structural information that can indicate the presence of surface functional groups. Different mass analysers (Table 4) such as single quadrupole (Q), triple quadrupole (QqQ), ion-trap (IT), and quadrupole-linear ion-trap (QqLIT) have been used for their analysis. High-resolution mass spectrometry (HRMS) either using time-of-flight (TOF), hybrid quadrupole-time-of-flight (Q-TOF), and more recently hybrid linear trap-Orbitrap (LTQ-Orbitrap) and hybrid quadrupole Orbitrap (Q-Orbitrap)analysers has also been reported (Table 4).

Among the atmospheric pressure ionisation (API) techniques, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) in negative ionisation mode are the sources most frequently used for both pristine and functionalised fullerenes (Table 4). Atmospheric pressure photoionisation (APPI) [137] allowed to expand the application of LC-MS to non-polar compounds and compounds difficult to be ionised by ESI or APCI such as polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers, naphtalene, nitropyrenes and lipids [138-140] and it has been also proposed for the LC-MS analysis of fullerene and fullerene derivatives (Table 4). The relatively high number of papers that used APPI for the analysis of fullerenes is related to the use of toluene in the chromatographic mobile phase which favours the formation of fullerene molecular ions through a solvent-mediated ionisation mechanism by electron capture.

Mass spectra of fullerenes in negative mode are simple providing the isotopic pattern of the molecular radical anion $[M]^{-\bullet}$ (Figure 6a). However, the presence of other radical anions in negative ESI and APPI, such as the oxidized ions $[M+O]^{-\bullet}$ (*i.e., m/z* M+16 and *m/z* M+32) [141-144] and solvent adduct ions $[M+CH_3OH-H]^{-}$ [145,146] has also been reported. Núñez *et al.* [144] studied and characterised the cluster ions present in the MS spectra of fullerenes at *m/z* M+16 and *m/z* M+32 by tandem MS, HRMS and accurate mass measurements using an Orbitrap mass analyser at a mass resolving power of 100,000 full width at half maximum (FWHM). As an example, the spectrum obtained for C_{60} cluster ion *m/z* M+16 is shown in the Figure 6b where it is shown the presence of two ions, one at *m/z* 735.9945 (mass error -1.36 ppm) due to the addition of oxygen ($[M+O]^{-\bullet}$) [147] and another at *m/z* 736.0314 (mass error -0.57 ppm) assigned to $[M+CH_4]^{-\bullet}$ and explained by the gas-phase ion molecule reaction of $[M]^{-\bullet}$ with a radical $^{\bullet}CH_3$ and subsequent stabilization by hydrogen addition.

Several studies compared the ionisation efficiency of pristine fullerenes [144] with that of hydrophobic C_{60}-derivatives [117,145] using different API sources (ESI, heated-ESI (H-ESI), APCI and APPI). Better sensitivity is always reported by using an APPI in negative mode which is, as previously mentioned, related to the use of toluene in the mobile phase that improves ionisation efficiency in APPI [148] .

Regarding the other API sources, H-ESI showed better results than ESI for pristine fullerenes, mainly for larger fullerenes, while APCI showed worst performance for the ionisation of these compounds [144] . In contrast, for several functionalised C_{60} fullerenes (C_{60}-pyrr, PCBM and PCBB) better results were obtained with APCI in comparison to ESI since for these specific compounds electron capture is favoured in the APCI plasma [117] .

Other ionisation techniques such as matrix-assisted laser desorption ionisation (MALDI) have also been proposed for the characterisation of pristine fullerenes [108] and fullerol [111] , although the utility of MALDI for quantitative analysis is limited. For instance, Xiao *et al.* [108] used MALDI-TOF-MS to identify several fullerenes, from C_{76} to C_{94} in a pyridine extract of crude soot. More recently, Chao *et al.* [111] used MALDI-TOF-MS to characterise fullerol using 2,5-dihydrobenzoic acid as matrix. In the MALDI-MS spectra, the authors found a major peak at *m/z* 720, corresponding to the molecular mass of C_{60} and a peak series from *m/z* 720 to 576 with a spacing of 24 *m/z*. These ions have also been observed in early studies of fullerol and have been attributed to sequential C_2 losses [149] . The authors also studied the same sample by LC-ESI-MS(/MS) in positive mode obtaining (Figure 7) a complex full scan spectrum with a weak peak corresponding to the protonated molecular mass of fullerol $[C_{60}(OH)_{24}+H]^+$ at *m/z* 1129. In contrast to MALDI-TOF spectra, no C_2 losses were found although a decreasing series spaced by 74 *m/z* units from *m/z* 1129 to 537 ($[M\text{-}n74+H]^+$, n=1-8) was observed, which was explained by subsequent neutral losses of $C_2H_2O_3$ groups.

4. Analysis of complex matrices

This section presents the existing sample treatment strategies (*i.e.*, extraction and preconcentration) and the proposed methods for the identification and quantitation of fullerenes. The reported recoveries, detection/quantitation limits and detected levels in complex matrices (*i.e.*, environmental, cosmetic and biological) are discussed and included in Table 4.

4.1. Sampling and sample treatment

Sampling and sample preparation is always a critical step in an analytical method but in the analysis of fullerenes additional problems arise because nanoparticles physicochemical changes that modify their dispersion state can occur and the presence of other nanoparticles and/or organic matter complicates the sample treatment. Hence, attention should be given to minimize and prevent changes in the aggregation state of the particles [59] especially when the objective of the study is the characterisation of fullerene aggregates in complex samples as undetected changes could lead to incorrect conclusions about their properties. Since the aggregation state of fullerenes is unstable and difficult to be preserved during sample treatment, most of the reported analytical methods quantify the total amount of compounds without taking into account their different aggregation states. Recently, Herrero *et al.* [79] addressed this problem by coupling AF4 to high resolution mass spectrometry allowing both size and concentration determination of fullerene aggregates.

On the other hand, for the extraction of fullerenes from complex matrices, procedures that fit with the nature of the matrix but also with the characteristics of the particles must be applied. For instance, despite the fact that C_{60} presents a very high solubility in toluene, it cannot be readily extracted from water samples where it is present as stable colloidal aggregates (nC_{60}) without the presence of oxidizing agents or salts to disrupt the stability of the colloidal aggregates and enhance the partitioning into the toluene phase [10,15,150] . Moreover, care must be taken during the analysis to prevent losses. For example, it has been reported that C_{60} in methanol adsorbs to glassware, being 100 % adsorbed in few hours [63] .

Liquid-liquid extraction (LLE) is the method typically used for C_{60} extraction from environmental [63,109,117,120] biological samples [142,150,151] and cosmetics [152] . The extraction methods and the recoveries reported for fullerenes in complex matrices are included in Table 4. LLE [109,117,120] and solid phase extraction (SPE) [141,153] are the methods most commonly used for the extraction of fullerenes from environmental waters. In LLE, salts are currently added to destabilize the aqueous nC_{60} aggregates and facilitate its transfer into the organic solvent [109,120] . In general, LLE provides higher recoveries than SPE (Table 4) and it can be applied to a wider range of water samples. For instance, Wang *et al*. [120] reported the use of LLE for the determination of nC_{60} in different wastewater matrices, while SPE required filtration and was only applicable to reclaimed wastewater and effluents with little suspended solids. Some authors only analysed the suspended solids obtained after the filtration of the water samples [143,144] . Nonetheless, taking into account the characteristics and complexity of the aggregates formed in aqueous media, the procedure proposed in these two studies does not seem to be the best approach to ensure that the maximum possible amount of the analytes is extracted. Ultrasound assisted extraction (UAE) has also been used to extract fullerenes from water samples. For example, Emke *et. al.* [154] proposed recently the combined analysis of the water particulate matter by SPE and its aqueous phase by UAE for C_{60} and C_{70} and several functionalised fullerenes in environmental waters. In this context, Chen *et al*. [145] reported the use of ultrasound assisted dispersive liquid-liquid micro-extraction (UAE-LLME) with benzyl bromide as the extraction solvent and 2-propanol as the dispersive solvent for the extraction of surface water and wastewater samples (Table 4).

The proposed methods for the extraction of fullerene compounds from environmental solids such as sediments, soils, airborne particulate and water suspended solids used UAE [105,122,124,143,144,155-158] , Soxhlet [105] , and pressurized liquid extraction (PLE) [117,159] with toluene as extraction solvent. Similar recoveries were reported for these methods (> 70 %) except for two studies regarding the extraction of fullerenes by UAE from soils and sediments that found lower recoveries [124,157] (Table 4). It was reported that the extraction efficiency may be affected by the adsorption of fullerenes to different soil components (*e.g.*, clay minerals, natural organic matter, etc.) [159,160] . For functionalised fullerenes, Sanchis *et al.* [157] obtained significantly lower recovery yields (35-59 %) which was attributed to their lower affinity to toluene. PLE was also proposed for the extraction of pristine and functionalised fullerenes from sediment samples and allowed to achieve higher recovery values (87-92 %) than those reported by UAE (35-59 %) for the same functionalised fullerene compounds (Table 4). Regarding the extraction of fullerenes from airborne particulate, recoveries between 60 and 70 % were reported by using UAE with toluene [156] . For the extraction of C_{60} and C_{70} from cosmetic samples, Benn *et al*. [152] obtained recoveries between 96 and 107 % by LLE whereas SPE yielded recoveries from 27 to 42 % (Table 4). Additionally, mechanical mixing with toluene and salt was reported for the extraction of C_{60} from cosmetics yielding recoveries between 75 and 86 % [121] .

Regarding the extraction of fullerenes from biological samples, the conventional LLE protocols cannot be used due to the large amounts of proteins, lipids and surfactants commonly present in these kind of samples [150] . Magnesium perchlorate ($Mg(ClO_4)_2$) and/or sodium dodecyl sulfate (SDS) together with the addition of glacial acetic acid (GAA) are usually used as destabilizing agents to solubilise the proteins and surfactants and to eliminate emulsion problems during the LLE with toluene. In general, recoveries higher than 90 % are reported [129,142,150,151] .

It should be mentioned that although several procedures have been proposed for the extraction of fullerenes from complex matrices, a robust protocol has not been developed yet, and the extraction mechanisms involved are not fully understood. Moreover, studies related to the extraction of water-soluble fullerenes (*i.e.,* fullerols) in complex matrices are lacking. Currently, there are no reports for the combined extraction of hydrophobic and hydrophilic fullerenes which may be present in some samples. Therefore, the development of an extraction protocol able to extract fullerenes of different polarities is needed. For this purpose, the existing LLE and SPE strategies should be extended by using serial extractions with several solvents of increasing polarity and by combining different SPE sorbents to differentiate and extract all the fullerenes present in the selected samples. Another potential method to separate the fractions of hydrophobic fullerenes from the hydrophilic ones could be the use of porous membranes such as polyethersulphone ones. In the presence of salts, the aggregation of hydrophobic fullerenes will increase enabling their retention on the membrane while the fraction of the hydrophilic ones could be collected after filtration. It has been reported that hydrophobic membranes can induce the adsorption of aromatic compounds (*e.g.* polycyclic aromatic hydrocarbons, humic substances, aromatic pesticides) onto the surface by hydrophobic and electrostatic interactions [161-163] and this fact has been used for the removal of organic pollutants during water treatment [162] .

4.2. Identification, quantitation and detected levels

The necessity to control and analyse fullerene nanoparticles in complex matrices, and the legal requirements for unequivocal identification and confirmation of positive/negative samples posed by the European Union directive 2002/657/EC [164] drives the need to make use of sensitive and reliable analytical methods. Nowadays, the best technique to fulfil these requirements is liquid chromatography coupled to mass spectrometry (LC-MS).

In MS/MS with triple quadrupole instruments the four identification points per analyte required by the EU legislation to guarantee confirmation can be achieved by using two selected reaction monitoring (SRM) transitions. However, pristine fullerenes cannot be fragmented because of their highly stable buckyball structure, thus other strategies need to be used for confirmation purposes. Despite this fact, several authors used a “pseudo” SRM mode by selecting the same ion as both precursor and product ion. For instance, Sanchis *et al.* [122,157] proposed the monitoring of $[M]^{-\bullet} \rightarrow [M]^{-\bullet}$ (quantitation) and $[M+1]^{-\bullet} \rightarrow [M+1]^{-\bullet}$ (confirmation) SRM transitions for the analysis of pristine fullerenes in river sediments and soils. Alternatively, monitoring the transitions of $[M]^{-\bullet} \rightarrow [M]^{-\bullet}$ for quantitation, and of the oxidized ions $[MO]^{-\bullet} \rightarrow [MO]^{-\bullet}$ for confirmation has been reported for the analysis of pristine fullerenes in wastewater [143] , tap and surface water [115] and aerosol [156] samples though in this case the detection limits increased due to the low abundance of the oxidized ions (Figure 6a). Since no fragmentation occurs in this “pseudo” SRM mode, the four identification points required by the EU legislation are not fulfilled although potential coeluting isobaric interference compounds can be eliminated. The use of HRMS by selecting two ions from the obtained spectra represents a good strategy to guarantee confirmation for this family of compounds. For instance, highly selective-selected ion monitoring (H-SIM) by monitoring two ions of the pristine fullerene isotopic cluster, $[M]^{-\bullet}$ and $[M+1]^{-\bullet}$, has been proposed [117,144] . Nevertheless, to apply this strategy MS instruments with high enough mass resolving power (at least higher than 12,500 FWHM) must be used [144] .

In contrast to pristine fullerenes, C_{60}-functionalised fullerenes can be fragmented at relatively high collision energies. In general, the most intense peak of the resulting MS/MS spectra is the ion at *m/z* 720 corresponding to the buckyball structure (C_{60}) caused by the loss of all the functional groups of these derivatives. In some cases, additional fragments with lower abundances have also been reported, such as the cleavage of the carboxylate ester for fullerenes with large alkyl moieties and the loss of both the propyl acid alkyl ester group $(CH_2)_2COO(CH_x)H$ and the butyl acid alkyl ester group $(CH_2)_3COO(CH_x)H$ for PCBM and PCBB, respectively [115,117,145] . For PCBB, the loss of the butyl group of the carboxylate ester and of the propene group has also been described [117] . Hence, for functionalised fullerenes SRM mode, by monitoring the transition $[M]^{-\bullet} \rightarrow [C_{60}]^{-\bullet}$ for quantitation, and a transition providing a characteristic fragment ion for confirmation is generally used, although the isotopic cluster has also been proposed for confirmation purposes ($[M+1]^{-\bullet} \rightarrow [C_{60}+1]^{-\bullet}$) [117,157] .

Most of the LC and LC-MS methods proposed in the literature for the quantitation of fullerenes in complex matrices were developed for the analysis of environmental samples, and only a limited number are addressed to other sample matrices such as cosmetics [121,152] or biological fluids and tissues [142,150,151] (Table 4). Without any doubt, isotope dilution is the best quantitation method for trace level analysis of organic contaminants in complex samples. However, for fullerenes it only exists a single commercially available stable-isotope labelled fullerene, $^{13}C_{60}$ [165] and to the best of our knowledge, there is only one study reporting its use as internal standard in a toxicological assay using *Zebrafish embryos* [142] and one study using it as a surrogate in the extraction of soil samples [122] . This situation is expected to change in the near future since the demand for stable-isotope labelled fullerene standards for the quantitative detection, fate and toxicity studies of these compounds is likely to increase. In general, the quantitation method used in most of the studies is external calibration (Table 4).

As previously mentioned, C_{60} is being considered for drug delivery and recently it has begun to be used in a number of cosmetic products. Hence, methods suitable for the analysis of fullerenes in cosmetic products are needed. For instance, LC-UV has been reported for the quantitation of C_{60} in different commercial creams by standard addition calibration [121] . C_{60} was found at low concentration levels, between 0.002 and 0.005 % (*w/w*), in all the analysed products. Additionally, the ROS production of the cosmetic products was studied under different illumination conditions and the photochemical reactivity of the creams containing C_{60} in deionised water was compared with that of fullerol which is a well-known ROS generator [166] . The results showed that although radical scavenging occurs in the dark, a significant amount of ROS is generated by the aged cosmetic products raising concerns about their effect when released into the environment. However, the relationship between the amount of fullerene present in the product and the ROS generation and their impact on the environment is unknown yet. Recently, Benn *et al.* [152] analysed C_{60} and C_{70} in anti-aging commercial cosmetic products by LC-(-)APCI-MS after their extraction by LLE with toluene, in order to provide potential exposure levels for future risk assessment of fullerenes. The quantitation was performed by standard addition calibration and C_{60} was found at concentrations ranging from 0.04 to 1.1 µg/g, while C_{70} was only qualitatively detected in some samples. The authors indicated that a single-use quantity of cosmetic (around 0.5 g) may contain up to 0.6 µg of C_{60}, which can be a pathway for human exposure. In addition, CE with UV detection was reported for the analysis of C_{60} in a cosmetic product (serum) after LLE with toluene [130] and a C_{60} concentration level of 2.10 mg L^{-1} in the analysed serum was reported.

Regarding the analysis of biological samples, the number of papers related to the presence of fullerenes in these samples is rather limited. In general, LLE with toluene followed by LC-MS is performed. For instance, this procedure has been reported for the analysis of C_{60} in blood, tissues [142,150,151] and in protein containing media [150] . Some of these works are devoted to evaluate fullerene's *in vivo* toxicity. For example, Moussa *et al.* [151] developed an LC-(-)APCI-MS method for the quantitation of C_{60} in blood, liver and spleen samples to evaluate its possible acute toxicity. The method limit of detection was 0.1 ng of C_{60} (injected amount) and the results showed that C_{60} is well absorbed in tissues and its concentration in different organs varied strongly between the animals. As respects functionalised fullerenes, Rajagopalan *et al.* [129] developed a LC-UV method for the analysis of MSAD-C_{60} in order to perform pharmacokinetic studies in plasma and urine samples of rats. MSAD-C_{60} is a pharmaceutical compound that possess antiviral activity against human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) *in vitro* and has virucidal and anti-human immunodeficiency virus protease activities [167] . The study was focused to evaluate the binding of MSAD-C_{60} to plasma proteins (>99 %) and the tolerated dose (15 mg kg^{-1}).

With respect to the analysis of environmental samples, LC-UV and LC-MS methods have been used to determine pristine fullerenes and some functionalised fullerenes in geological materials [105] , soot extracts [106] , soils and sediments [117,122,124,157,158] , aerosols [156] , and water samples [109,115,117,120,141,143-145,154] , although in some of these works only blank matrices spiked with fullerenes have been used. To the best of our knowledge, there are still no LC-UV or LC-MS methods reported for the analysis of water soluble fullerenes such as fullerol in environmental samples. As regards the presence of fullerenes and fullerene derivatives in aerosols, soils and sediments, Sanchis *et al.* [122,156] reported for the first time the presence of C_{60} and C_{70} in airborne particulate at concentration levels of 0.06 ng m^{-3} and 0.48 ng m^{-3}, respectively. Several fullerene derivatives (C_{60}-pyrr, C_{60} pyrrolidine tris-acid ethyl ester (CPTAE), PCBM, and ThCBM) were not detected in the analysed samples at concentrations higher than the method limits of detection. The same research group [157] also analysed C_{60}, C_{70} and three C_{60}-fullerene derivatives in soils collected from different urban and industrial areas in Saudi Arabia and river sediments from the Llobregat River Basin (Spain). C_{60} was the only fullerene detected and was quantitated in 19 % of the soil samples (0.15 to 6.83 ng g^{-1}) and 33 % of the sediments (0.13-0.70 ng g^{-1}). More recently, these authors [122] found C_{60}, C_{70} in sediments and soils from Santa Catarina (Brasil) at concentration levels from low fg g^{-1} up to 154 pg g^{-1} whereas none of the functionalised fullerenes were detected. Astefanei *et al.* [117] reported the presence of C_{60} and also of three C_{60} fullerene derivatives (C_{60}-pyrr, PCBM and PCBB) in sediment samples collected from several ponds located around Barcelona's Airport (Spain). In this work C_{60} and C_{70} fullerenes were detected in 85 % and 71 % of the analysed samples, respectively, at levels from 0.8 to 7.2 ng Kg^{-1} while the PCBM, PCBB and C_{60}-pyrrwere found at levels from 0.14 to 5.1 ng Kg^{-1} and the highest concentration was found for PCBB. The presence of fullerenes in sediment and soil samples [117,122,157] could be related to the location of the sampling points which generally are close highly industrial areas or international airports.

The number of publications dealing with the analysis of fullerene compounds in environmental waters is higher than for soils and sediments (Table 4). Most of the works are focused on the analysis of several pristine and functionalised fullerenes simultaneously and one is devoted to the screening of nC_{60} and some of its transformation products in water [141] . The method was able to detect and quantitate concentrations of nC_{60} as low as 5 ng L^{-1} but, despite its high sensitivity no nC_{60} or transformation products were detected in the analysed surface waters. Regarding the simultaneous analysis of pristine and functionalised fullerenes, two recent works reported methods for the determination of up to 8 fullerene compounds. Kolkman *et al.* [115] used a LC-(-)ESI-Orbitrap system for the analysis of C_{60}, C_{70} and six functionalised fullerenes in spiked tap water after SPE (C18) achieving very low MLODs ranging from 0.17 ng L^{-1} (C_{70}-PCBM) to 0.28 ng L^{-1} (C_{60}). However, the developed method was not applied for the analysis of environmental water samples and its main disadvantage was the long chromatographic analysis time (27 min). Astefanei *et al.* [117] reported an LC-(-)APPI-MS(/MS) method for the analysis of river water samples achieving MLODs ranging from 0.4 pg L^{-1} for PCBM to 1.6 ng L^{-1} for C_{84}, with a run chromatographic time of only 4.3 min. The method was applied to the analysis of pond water samples collected around the airport of Barcelona (Spain) where C_{60} and C_{70} were found at concentration levels between 9 and 330 pg L^{-1} and three C_{60}-fullerene derivatives (C_{60}-pyrr, PCBM, and PCBB) were detected (50-83 % of the samples) at levels between 2 and 8.5 pg L^{-1} (Table 4). This is the first report of the presence of these functionalised fullerenes in water samples, except for C_{60}-pyrr which has been also found in wastewater effluents (Table 4).

The presence of pristine fullerenes in environmental water samples has been reported by several authors being usually present at low ng L^{-1} levels [143,144,157] although in one of these works [143] C_{60} was found in some of the analysed samples at µg L^{-1} levels. To the best of our knowledge, the presence of C_{84} in water samples has only been reported once, and also at low ng L^{-1} levels, when analysing water particulate suspended material using a LC-(-)APPI-MS method [144] meanwhile C_{76} and C_{78} have never been found in environmental samples.

5. Conclusions and future trends

Fullerenes occupy a prominent place among nanomaterials. Furthermore, these particles display a high reactivity potential that has led to their use in a wide array of applications (*i.e.*, biomedicine, cosmetic products, solar cells and catalysts). Their anticipated market growth in combination with the potential for direct human exposure via several applications has led to widespread concerns about their potential adverse effects on human health. There are a number of factors that appear to be implicated in fullerene behaviour and toxicity, including chemical structure, surface functionalisation and size distribution. The particle sizes and size distribution are important characteristics of nanoparticles and determine their mobility and deposition in the aquatic environment, *in vivo* distribution, biological fate, and toxicity. Therefore, a thorough physicochemical characterisation of fullerene aggregates in terms of size distribution, shape and ζ potential is required in order to know which attributes are responsible for the observed toxic responses. Moreover, these parameters should be taken into account for health and environmental regulations. To determine these characteristics, a great number of complementary techniques such as TEM, DLS, SLS, Flow-FFF, NTA, and AFM should be combined. However, it has been demonstrated that the solubilisation procedures used to prepare the samples affect the physicochemical properties of the fullerenes, thus the measured sizes and shapes would not be totally representative of the actual degree of aggregation of the particles in the aqueous media. The aggregates found in the environment will probably be more similar to those obtained by direct solubilisation in water via extended stirring (aqu/nC_{60}) than to those obtained via solvent exchange methods. Hence, the solubilisation of fullerenes via extended stirring is recommended for the studies devoted to their behaviour and toxicity.

Efforts have been made to investigate the fate of fullerenes under laboratory and environmental conditions and the current literature stresses the physicochemical transformations that occur to fullerenes after being released in the environment. So far, it has been demonstrated that the degree of aggregation is affected by several conditions such as the ionic strength, pH, the presence of natural organic matter and sunlight but further research is needed to determine how aggregate size and surface charge affect the ability of environmental test organisms to uptake the aggregated forms. While there are a significant number of studies on the biological effects of fullerenes, there are very few attempts to rigorously quantitate them in biological matrices. Due to the potential interaction between fullerenes and organs and tissues, the still unknown incubation time, and the possible DNA damaging, thorough studies are recommended before using them in cosmetic, biomedical and other industrial applications.

Each step involved in the analysis of fullerenes (*i.e.*, extraction, separation, identification and quantitative detection) represents a specific challenge. The extraction procedure used could affect the physicochemical properties of fullerenes. Hence, it is crucial to develop methodologies which preserve their properties and degree of aggregation. Moreover, the analytical methodologies should not only address the characterisation and analysis of hydrophobic fullerenes but must be extended to a broader range of compounds including the water soluble derivatives and related transformation products. To date, most of the reported methods are focused on the analysis of the total amount of fullerene compounds, mainly the hydrophobic ones, without taking into account their different aggregation states. Sample treatment procedures for hydrophobic fullerenes are based on their extraction with toluene (LLE, PLE, SPE and UAE) and disrupt the fullerene aggregates present in the matrix. Moreover, they cannot be applied for the extraction of the water soluble fullerenes. Therefore, combined extraction methods enabling the analysis of the entire family of compounds need to be developed.

Chromatographic separation could also be problematic due to the differences in the mobile and/or stationary phases which must be used to achieve proper separation of a wide range of compounds. In this context, it would be useful to evaluate the applicability of comprehensive two-dimensional liquid-liquid chromatography (normal-phase x reversed-phase). Moreover, in order to cope with the necessity of performing a high number of analyses in complex matrices, a short analysis time is ideal. Therefore, the use of UHPLC column technology either with sub-2µm particle size totally porous columns or core-shell partially porous columns is recommended.

Finally, the specific detection of a maximum range of fullerenes and transformation products is challenging. Although it has been reported the formation of transformation products via oxidation and photochemical reactions generating reactive oxygen species (ROS), very little is known about their chemical identities and there are still no methods for their detection in real samples. Information about the presence of these products is important since they may possess inherent toxicity or may alter that of the fullerenes under investigation. Thus, methodologies for the identification and quantitation of these products are necessary.

LC-MS is without any doubt the best option when dealing with the analysis of this family of compounds, although LC-UV and few CE-UV methods have also been reported and can be used for the analysis of specific fullerenes in samples such as cosmetic and pharmaceutical products. However, these latter methods do not fulfil the requisite for unambiguous detection of fullerenes in complex samples that contain potential interferences. Although fullerenes have been detected in a wide variety of samples, including airborne particulate, sediments, soils, and water, the available data is still limited to correctly asses the occurrence, fate and behaviour of fullerene nanoparticles in the environment. Hence, both the scientific community and stakeholders must address these knowledge gaps and provide tools for the risk assessment legislation.

Although LC-MS methods are a powerful tool for the quantitation of fullerenes in complex samples, these techniques alone cannot provide detailed qualitative information. Therefore, these methods need to be adapted for the identification and quantitation of these particles while preserving the information of their degree of aggregation and cluster size. The hyphenation of techniques able to separate the natural aggregate particles with LC-MS seems to be a suitable approach to overcome this problem. In this context, the recent paper of Herrero *et al.* [79] reporting the coupling of AF4-MALS to high resolution mass spectrometry for the characterisation and quantitation of aqu/nC_{60} and aqueous functionalised fullerene aggregates can be an example of future research direction. This hyphenation represents a powerful methodology for both characterisation and accurate analysis of fullerene aggregates in aqueous samples at environmentally relevant conditions.

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Figure captions

Figure 1. C_{60}, C_{70}, C_{60}-pyrr and $C_{60}(OH)_{24}$ structures

Figure 2. (a) Intensity-weighted size distributions for the nC_{60} suspensions produced using the four different techniques by DLS (pH) 5.6, (T) 20 °C. The x-axis is plotted on a semilogarithmic scale for clarity; (b) Number-weighted size distributions for the nC_{60} suspensions produced using the four different techniques by SLS (pH) 5.6, (T) 20 °C. A refractive index of 2.20 was used for the nC_{60}. The x-axis is plotted on a semilogarithmic scale for clarity. Reprinted with permission from reference [6] . Copyright (2006) American Chemical Society

Figure 3. Representative TEM images of the (a) TTA/nC_{60}, (b) THF/nC_{60}, (c) SON/nC_{60}, and (d) aqu/nC_{60} (pH) 5.6) suspensions. The insets in each figure are magnifications of the respective nC_{60} clusters. Reprinted with permission from reference [6] . Copyright (2006) American Chemical Society

Figure 4. Chromatographic separation of three C_{60}-fullerene derivatives and five pristine fullerenes in a sub-2µm C18 Hypersil Gold (150 x 2.1 mm, 1.9 µm particle size) column using (a) toluene:methanol (40:60 *v/v*) and (b) toluene:acetonitrile (40:60 *v/v*) as mobile phases under isocratic elution. Flow rate: 500 µL/min. Acquisition performed by LC-(-)APPI-MS(/MS). Reprinted with permission from reference [117] . Copyright (2014) Elsevier.

Figure 5. HPLC-UV chromatogram of fullerenes and functionalised fullerenes in a Cosmosil Buckyprep (250 x 4.6 mm, 5µm particle size) column. Separation under gradient elution from toluene:acetonitrile (75:25 *v/v*) to 100% toluene in 6 min, and then isocratic elution at 100% toluene. Peak identification: (1) bis-PCBM; (2) PCMO; (3) PCBB; (4) PCBM; (5) ThCBM; (6) C_{70}-PCBM); (7) C_{60}; and (8) C_{70}. Reprinted with permission from reference [124] . Copyright (2014) Elsevier.

Figure 6. (a) Full scan MS spectrum of C_{60} (b) HRMS spectrum of C_{60} cluster ion at M+16 at a resolving power of 100,000 FWHM. Reproduced with permission from reference [144] . Copyright (2012) American Chemical Society.

Figure 7. Overview (Q1) scan spectrum of a fullerol sample. Peaks of the two 74 *m/*z series (A and B) are indicated by the arrows. Reproduced with permission from reference [111] . Copyright (2011) American Chemical Society.

Table 1. Nanoparticle characterisation techniques

Method	**Measured property**	**Approximate size range (nm)**	**Level of perturbation**
TEM, SEM	Size (r_H), degree of aggregation, morphology	0.1 to 1000	High
AFM	Size and morphology	0.5 to 1000	Minimum
DLS,SLS	Size distribution (r_H, R_g) and degree of aggregation	10 to 1000	Minimum
Flow FFF	Size separation (r_H)	1 to 1000	Low
NTA	Size distribution, degree of aggregation, zeta potential and concentration	10 to 2000	Minimum
BET	Surface area and porosity	1 to 1000	High

r_H: hydrodynamic radius

R_g: Radius of gyration

Table 2. Preparation methods of fullerene aqueous suspensions

Preparation method		**Ref.**
Abbreviation	**Procedure**	
Krätschmer method	A solution of C_{60} and C_{70} (ratio 10:7) in toluene is added to water and subjected to sonication until the complete evaporation of toluene, then filtered (0.22 µm)	[8]
Hand ground C_{60}	Hand ground C_{60} is added to pure water and sonicated or stirred for 1 day and then filtered (5 µm)	[12]
Hand ground C_{60} with SDS	Hand ground C_{60} is mixed with SDS in water followed by ultrasonic treatment (30 min), and subsequent filtration (5 µm).	
Toluene and ethanol	A solution of C_{60} in toluene is added to water and ethanol mixture. The solution is sonicated for 3h to evaporate the toluene and ethanol, filtered through 0.45 µm/ 0.22 µm	[11]
THF/nC_{60}	C_{60} is added to THF, stirred (overnight) and the excess filtered through 0.45 µm filter. Water is added to this solution and then purged with nitrogen to remove the THF. The solutions are filtered (0.45 µm / 0.22 µm)	[6,7,10, 13,15]
TTA/nC_{60}	C_{60} in toluene is added to THF and acetone. Then water is added slowly and then the solvents are evaporated and the solutions filtered (0.45 µm / 0.22 µm)	[6,7,14, 82]
SON/nC_{60}	C_{60} in toluene solution is mixed with water (sonication for several hours). After the evaporation of toluene the solutions are filtered (0.45 µm / 0.22 µm)	[6,13]
PVP/nC_{60}	PVP in chloroform is added to C_{60} in toluene and stirred/sonicated at 45° C/25° C for several hours until the solvents evaporated and then is suspended in water and filtered (0.45 µm /0.22 µm or 0.1 µm)	[13,87, 88]
Aqu/nC_{60}	C_{60} powder is added to water, stirred for several weeks and then filtered (0.45 µm / 0.22 µm)	[6,7,13]

Table 3. Effect of the preparation method on the formed aggregates

Preparation method	**Methodology used for characterisation and results**				**Ref.**
	TEM	**SLS**	**DLS**	**Zeta potential (mV)**	
TTA/nC$_{60}$ (unfiltered)	Hexagonal shapes with faceted edges (100 to 200 nm)	170±20nm Rg: 57.22 nm	Average size: 237 nm (unfiltered)	0.01-100 mM NaCl: -31 to -4	[6]
SON/nC$_{60}$ (sonication for 3h) Unfiltered and filtrated solutions (0.45 µm)	Spherical particles (5-86 nm)	160±20nm Rg: 57.87 nm	Average size: 277 nm (unfiltered and filtered)	0.01-100 mM NaCl: -34 to -16	
THF/ nC$_{60}$ (12 h stirring) Unfiltered and filtrated solutions (0.45 µm)	Spherical particles (≤50nm) and elongated aggregates (≈ 200nm)	160±20nm Rg: 70.47 nm	Average size: 271 nm (filtered); 300nm and 5 µm (unfiltered)	0.01-100 mM NaCl: -52 to -26	
Aqu/nC$_{60}$ (Stirring for 2 weeks at 500 rpm) Unfiltered and filtered solutions (0.45 µm)	Spherical particles and random shaped aggregates (20-500 nm)	180±20nm Rg: 80.10 nm	Average size: 357 nm (filtered); 300 nm and 5 µm (unfiltered)	0.01-100 mM NaCl: -32 to -17	
SON/nC$_{60}$ (sonication at 80-100 W for 15 min)	Spherical particles (10-25 nm)		Average size ≈2 nm* (small and large)		[13]
THF/nC$_{60}$ (12h stirring and heated at 60°C)	Random shapes (hexagonal, tubular, square) (50-150 nm)		Average size: 74.6 nm Small: 39.1 nm Large:97.4 nm		
Aqu/nC$_{60}$ (Stirring for 2-4 weeks at 1000 rpm and filtered (0.45 µm and 0.22 µm)	Spherical and elongated shapes (30-100 nm)		Average size: 74.9 nm Small: 2 nm* Large: 142.3 nm		
PVP/C$_{60}$	Spherical and uniform particles (10-25 nm)		Average size ≈2 nm* (small and large)		

Rg: radius of gyration
*Bellow DLS detection limit

Table 4. Analysis of fullerenes in complex matrices: chromatographic methods

Fullerenes	**Sample**	**Extraction, Recovery range (%)**	**Column**	**Chromatographic conditions**	**Detection/ Quantitation**	**Detected levels**	**MLODs/MLOQs**	**Analysis time**	**Ref.**
C_{60}, C_{70}, PCBM	UPW, surface and ground water (spiked)	LLE with toluene and $Mg(ClO_4)_2$, 89-99 %	Cosmosil PYE 150 x 4.6 mm, 5 µm, 120 Å	Toluene:acetonitrile (80:20, *v/v*) 1.3 mL/min	UV-Vis 330 and 333nm External calibration	-	MLODs: 2.9-6.5 µg/L	6.5 min	[109]
C_{60}	Wastewater, secondary effluent (spiked)	LLE with toluene and NaCl, 88-97 %	Cosmosil Buckyprep 250 x 4.6 mm, 5 µm, 120 Å	Toluene (100%) 1 mL/min	UV-Vis 332 nm Mass spectrometry (-) APCI-MS (single quadrupole) External calibration	-	MLODs (µg/L): 3-4 (LC-UV); 4-11 (LC-MS).	-	[120]
nC_{60}	UPW, surface water (spiked)	C18-SPE with toluene, 32-42 %	Nova-Pak C18 150 x 3.9 mm, 4 µm, 60 Å	Toluene:methanol (50:50, *v/v*) 1 mL/min 0.2 mL/min	UV-Vis 333 nm Mass spectrometry (-) APCI/ESI-MS (LTQ-FT Orbitrap) External calibration	-	MLOD: 5 ng L^{-1}	-	[141]
C_{60}, C_{70}, PCBM, C_{70}-PCBM, bis-PCBM, PCBB, ThCBM, PCBO	UPW, tap water, surface water (spiked)	C18-SPE with toluene and NaCl, >78 %	Cosmosil Buckyprep 250 x 2 mm, 5 µm, 120 Å	(A)Toluene;(B)Acetonitrile (from 75 % to 100 % (A)) 0.2 mL/min	Mass spectrometry (-) ESI-MS (LTQ Orbitrap) External calibration	-	MLODs: 0.17-0.28 ng L^{-1}	30 min	[115]
C_{60}, C_{70}, C_{76}, C_{78},C_{84}	Pond water	UAE with toluene (suspended solids), 80-88 %	Hypersil GOLD C18 150 x 2.1 mm, 1.9 µm , 175 Å	Toluene:methanol (45:55, *v/v*) 0.5 mL/min	Mass spectrometry (-) APPI-MS (triple quadrupole) Dopant: toluene Matrix-matched calibration	C_{60}: 0.01-0.02 ng L^{-1} C_{70}: 0.01-0.03 ng L^{-1} C_{76}: 1.04 ng L^{-1} C_{84}: 5-19.2 ng L^{-1}	MLOQs: 0.01-5 ng L^{-1}	3.5 min	[144]
C_{60}, C_{70}, PCBO, PCBB, PCBM, bis-PCBM, C_{70}-PCBM, ThCBM	Sewage water	UAE and C18-SPE with toluene, 49-104 %	Cosmosil Buckyprep 250 x 2 mm, 5 µm, 120 Å	Toluene (100%) 0.2 mL/min	Mass spectrometry (-) APPI-MS (LTQ-Orbitrap) External calibration	C_{60}: < 0.1 – 19 ng L^{-1}	MLOQs: 230-1400 pg L^{-1}	-	[154]
C_{60}, C_{70}, C_{60}-pyrr	Wastewater	UAE with toluene (suspended solid), 58-84 %	Purospher STAR C18 125 x 2 mm, 5 µm	Toluene:methanol (50:50, *v/v*) 0.4 mL/min	Mass spectrometry (-) ESI-MS (QqLIT) External calibration	C_{60}: 0.5-14,400 ng L^{-1} C_{70}: 181-1650 ng L^{-1} C_{60}-pyrr: 60-65,900 ng L^{-1}	MLODs: 1-20 ng L^{-1}	4 min	[143]
C_{60}, C_{70}, PCBM	Surface water, effluent, influent	UAE- LLME with benzyl bromide (extraction	Ascentis Express C18	Toluene:methanol (50:50, *v/v*) 0.2mL/min	Mass spectrometry (-) APPI-MS/MS	C_{60}: 56-130 ng L^{-1} C_{70}: 25-83 ng L^{-1}	MLODs: 3-40 ng L^{-1}	2.5 min	[145]

Fullerenes	**Sample**	**Extraction, Recovery range (%)**	**Column**	**Chromatographic conditions**	**Detection/ Quantitation**	**Detected levels**	**MLODs/MLOQs**	**Analysis time**	**Ref.**
		solvent), 2 propanol (dispersive solvent) and NaCl, 70-86 %	50 x 2.1 mm, 2.7 µm, 90 Å		(ion trap) Dopant: toluene Standard addition calibration				
C_{60}, C_{70}, C_{76}, C_{78}, C_{84}, PCBM, PCBB, C_{60}-pyrr	Pond water, sediments	LLE with toluene and NaCl, 83-96 % PLE with toluene, 70-92 %	Hypersil GOLD C18 150 x 2.1 mm, 1.9 µm , 175 Å	(A) Toluene;(B) Methanol (from 30 % to 55 % (A)) 0.5 mL/min	Mass spectrometry (-) APPI-MS (triple quadrupole) Dopant: toluene Matrix-matched calibration	Pond waters (pg L^{-1}): C_{60}: 9-22; C_{70}: 22-330; PCBM: 5.2-8; PCBB: 5; C_{60}-pyrr: 2-8.5 Sediments (ng Kg^{-1}): C_{60}: 0.8-1.1; C_{70}: 2.1-7.2; PCBM: 0.15-2.6; PCBB: 0.18-5.1; C_{60}-pyrr: 0.5-2.7	MLODs: 1.4-1600 pg L^{-1} (pond water), 0.03-158 ng Kg^{-1} (sediments)	4.3 min	[117]
C_{60}, C_{70}	Soils and sediments	UAE with toluene, 72-104 %	Luna C18 150 x 4.60 mm, 5 µm, 100 Å	Toluene:acetonitrile (60:40, *v/v*) 0.75 mL/min	UV-Vis 334 nm External calibration	Not detected	MLOQs: 0.2-0.3 ng g^{-1}	9 min	[158]
C_{60}, C_{70}	Sediments	UAE with toluene, 80 %	Nova-Pak C18 300 x 3.9 mm, 4 µm, 60 Å	Toluene:methanol (40:60, *v/v*) 2 mL/min	Uv-Vis, 310-430 nm External calibration	0.1-11.9 ng g^{-1} C_{60}; C_{70}/C_{60}: 0.2-0.3	MLODs: 0.2-0.3 ng injected	11 min	[155]
C_{60}, C_{70}, C_{60}-pyrr, PCBM, ThCBM	Soils and sediments	UAE with toluene, 35-108 %	Purospher STAR RP-18 125 x 2.0 mm, 5 µm	Toluene:methanol (50:50, *v/v*) 3 mL/min	Mass spectrometry (-) ESI-MS (triple quadrupole) External calibration	C_{60}: 0.15-6.83 ng g^{-1}	MLODs: 8.7-290 pg g^{-1}	-	[157]
C_{60}, C_{70}, C_{60}-pyrr, PCBM, ThCBM, C_{70}-PCBM, CPTAE	Soils and sediments	UAE with toluene, 68-106 %	Cosmosil Buckyprep, 150 x 2 mm, 120 Å	Toluene (100 %) 0.4 mL/min	Mass spectrometry LC-APPI (-)-HRMS (Q-Orbitrap) Matrix matched calibration	C_{60}, C_{70}: low fg g^{-1}-154 pg g^{-1}	MLODs: 0.02-0.4 pg g^{-1}	10 min	[122]
C_{60}, C_{70}, PCBB, PCMO, PCBM, bis-PCBM,ThCBM, C_{70}-PCBM	Clayish, sandy and lower top-soils (spiked)	UAE and shaking with toluene, 47-71 %	Cosmosil Buckyprep 250 x 4.6 mm, 5 µm, 120 Å	(A)Toluene;(B)Acetonitrile (from 75 % to 100 % (A)) 1 mL/mmin	UV-Vis 305 and 332 nm External calibration	-	MLODs: 6-10 µg L^{-1}	25 min	[124]
C_{60}, C_{70}	Geological materials	UAE and Soxhlet with toluene (demineralisation with HCl and HF), 90 %	Nova-Pak C18 3.9 x 300 mm, 4 µm , 60 Å	Toluene:methanol (40:60, *v/v*) 2 mL/min	UV-Vis 330 nm External calibration	C_{60}: 2 mg Kg^{-1} C_{70}: 0.68 µg Kg^{-1}	-	12 min	[105]
C_{60}, C_{70}, C_{60}-pyrr, CPTAE, PCBM, ThCBM	Airborne particulate	UAE with toluene, 60-71 %	Luna C18 (2)-HST 50 x 2.0 mm, 2.5 µm, 100 Å	(A) Toluene;(B) Methanol (from 50 % to 80 % (A)) 0.3 mL/min	Mass spectrometry (-) ESI-MS (triple quadrupole) Standard addition calibration	C_{60}: 0.03-49.3 ng m^{-3}; C_{70}: 0.08-233.8 ng m^{-3}	MLODs: 5.4-20.9 pg m^{-3}	-	[156]
C_{60}	Plasma (porcine),	LLE with toluene,	Delta Pak C18	Toluene:acetonitrile (60:40, *v/v*)	UV-Vis	-	MLOD(ng mL^{-1}):	3 min	[150]

Fullerenes	**Sample**	**Extraction, Recovery range (%)**	**Column**	**Chromatographic conditions**	**Detection/ Quantitation**	**Detected levels**	**MLODs/MLOQs**	**Analysis time**	**Ref.**
	Skin permeable media (bovine serum albumin-BSA)	$Mg(ClO_4)_2$ and GAA, 94-100 %	150 x 3.9 mm, 5 µm, 300 Å	1 mL/min	333 nm External calibration		0.68 (plasma); 0.34 (BSA)		
C_{60}	Blood, Spleen, Liver	LLE with toluene, SDS and GAA, 90-103 %	LiChrocart C18 20 x 4.6 mm, 5 µm, 100 Å	Toluene:acetonitrile (60:40, *v/v*) Toluene:acetonitrile (55:45, *v/v*) Toluene:methanol (55:45, *v/v*) 1 mL/min	UV-Vis 333 nm Mass spectrometry (-) APCI-MS (ion trap) Internal standard calibration (C_{70})	-	MLOD: 0.1 ng/injection	6 min	[151]
C_{60}	Embryonic zebrafish homologeate	LLE with toluene (no salt), 90 %	Targa C18, 150 x 2 mm, 5 µm, 120 Å	Toluene:methanol (45:55, *v/v*)	(-) ESI-MS Internal standard calibration ($^{13}C_{60}$)	-	MLOD: 0.02 µg L^{-1}	15 min	[142]
MSAD-C_{60}	Plasma	LLE with ethylacetate, 73-100 %	Alltech, Deerfield, Ill C18, 150 x 4.5 mm, 5 µm	5 mM dibasic sodium phosphate–methanol–acetonitrile (35:4:61; *v/v*), pH 7.5	UV-Vis, 270 nm Internal standard calibration (DESD)	7 µg/L (after 3h of intravenous administration of 15 mg Kg^{-1})	-	15 min	[129]
C_{60}, C_{70}	Cosmetic products	LLE with toluene, $Mg(ClO_4)_2$, and GAA, 96-107 % C18-SPE with toluene, 27-42 %	Nova-Pak C18 150 x 3.9 mm, 4 µm , 60 Å	Toluene:acetonitrile (55:45, *v/v*) 1 mL/min	Mass spectrometry (-) APCI-MS (triple quadrupole) Standard addition calibration	0.04-1.1 µg g^{-1} (C_{60})	-	6 min	[152]
C_{60}	Cosmetic products	Mechanically mixing with toluene and KCl, 75-86 %	Cosmosil Buckyprep 250 x 4.6 mm, 5 µm, 120 Å	Toluene (100 %)	UV-Vis 285 nm Standard addition calibration	0.002-0.005 % (*w/w*)	-	-	[121]

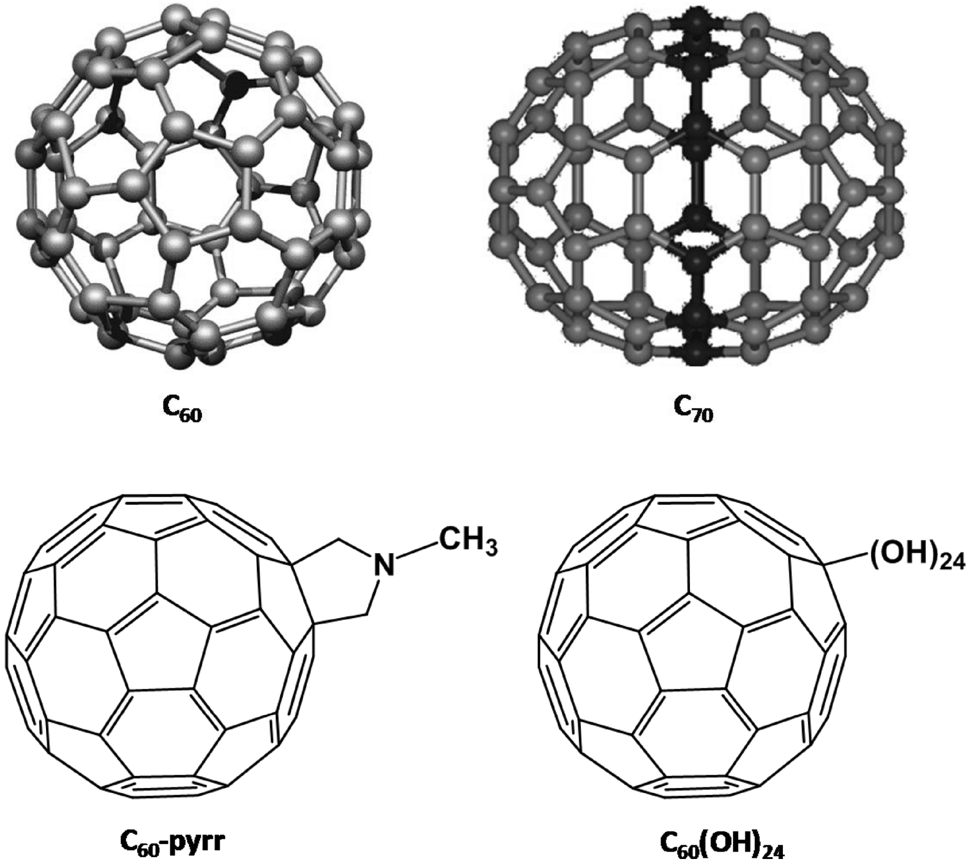
UPW: ultra-pure water

DESD: Diethylstilbestrol dipropionate

Table 5. Analysis of fullerenes: Capillary electrophoresis techniques

Compound	**Sample**	**Experimental conditions**	**Detection**	**Ref.**
C_{60}, C_{70}, C_{84}	Standard solution	(NACE) BGE: 100 mM TDAB, 50 mM TEAB, 6% MeOH, 1 % acetic acid in chlorobenzene-acetonitrile 1:1 (v/v), V= +30 kV Capillary: 75 µm x 60 cm	UV: 313 nm	[131]
C_{60}, C_{70}, C_{60} pyrr and C_{60} COOH	Commercial cosmetic product (C_{60})	(NACE) BGE: 200 mM TDAB, 40 mM TEAB, 6% MeOH, 10% acetic acid in chlorobenzene-acetonitrile 1:1 (*v/v*), V= +30 kV Capillary: 50 µm x 50 cm	UV: 350 nm	[130]
Open cage fullerenes	Standard solution	(NACE) BGE: 200 mM Trifluoroacetic acid, 20 mM sodium acetate in acetonitrile-methanol (90:10, v/v), V= +25 kV Capillary: 75 µm x 60 cm	UV: 200 nm	[132]
C_{60},C_{70}	Standard solution	(MECC) BGE: 100 mM SDS in 10 mM sodium tetraborate- phosphate, pH=9.5, V= +10 kV Capillary: 75 µm x 28 cm	UV: 254 nm	[133]
Dentro[60] fullerene (DF)	Standard solution	(CZE) BGE: 75 mM phosphate buffer pH 6, V= +25 kV Capillary: 50 µm x 40 cm	UV: 254 nm	[135]
Carboxy fullerene (C3) and DF	Serum matrix	DF: (CZE) BGE: 40 mM sodium tetraborate, pH=9.2, V= - 14 kV C3 : (MECC) BGE: 10 mM sodium tetraborate and 150 mM SDS, pH= 9.2, + 14 kV ; (CZE) BGE: 40 mM sodium tetraborate, pH=7.4, V= +14kV Capillary: 50 µm x 40 cm, dynamic coating	UV: 260 nm	[134]
Th-symmetric hexakis-adduct $C_{66}(COOH)_{12}$	Standard solution	(CZE) BGE:15mM sodium tetraborate, pH 9.3, V= +20 kV Capillary: 75 µm x 50 cm	UV: 330 nm	[136]

C_{60} COOH : (1,2-methanofullerene C_{60})-61-carboxylic acid



C_{60}

C_{70}

C_{60}-pyrr

$C_{60}(OH)_{24}$

Fig. 1

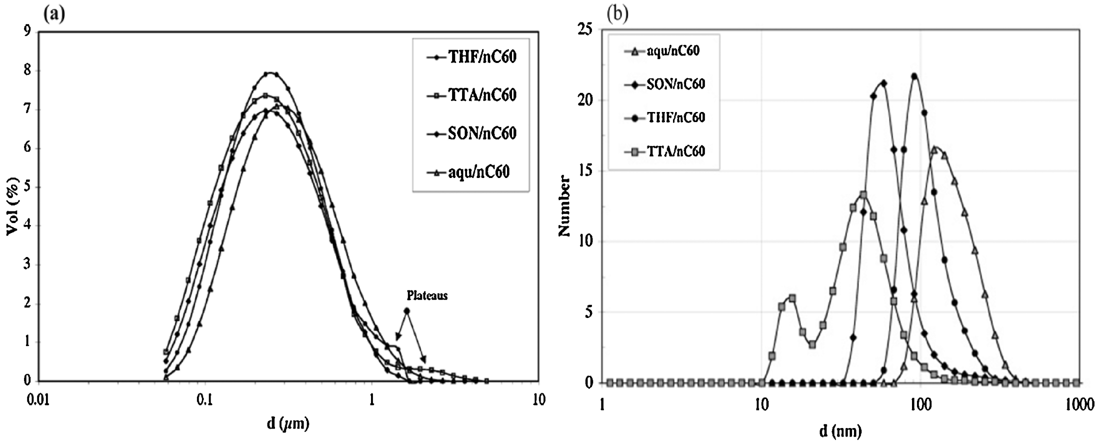

(a)
Vol (%)
0
1
2
3
4
5
6
7
8
9
0.01
0.1
1
10
d (μm)
THF/nC60
TTA/nC60
SON/nC60
aqu/nC60
Plateaus
(b)
Number
0
5
10
15
20
25
1
10
100
1000
d (nm)
aqu/nC60
SON/nC60
THF/nC60
TTA/nC60

Fig. 2

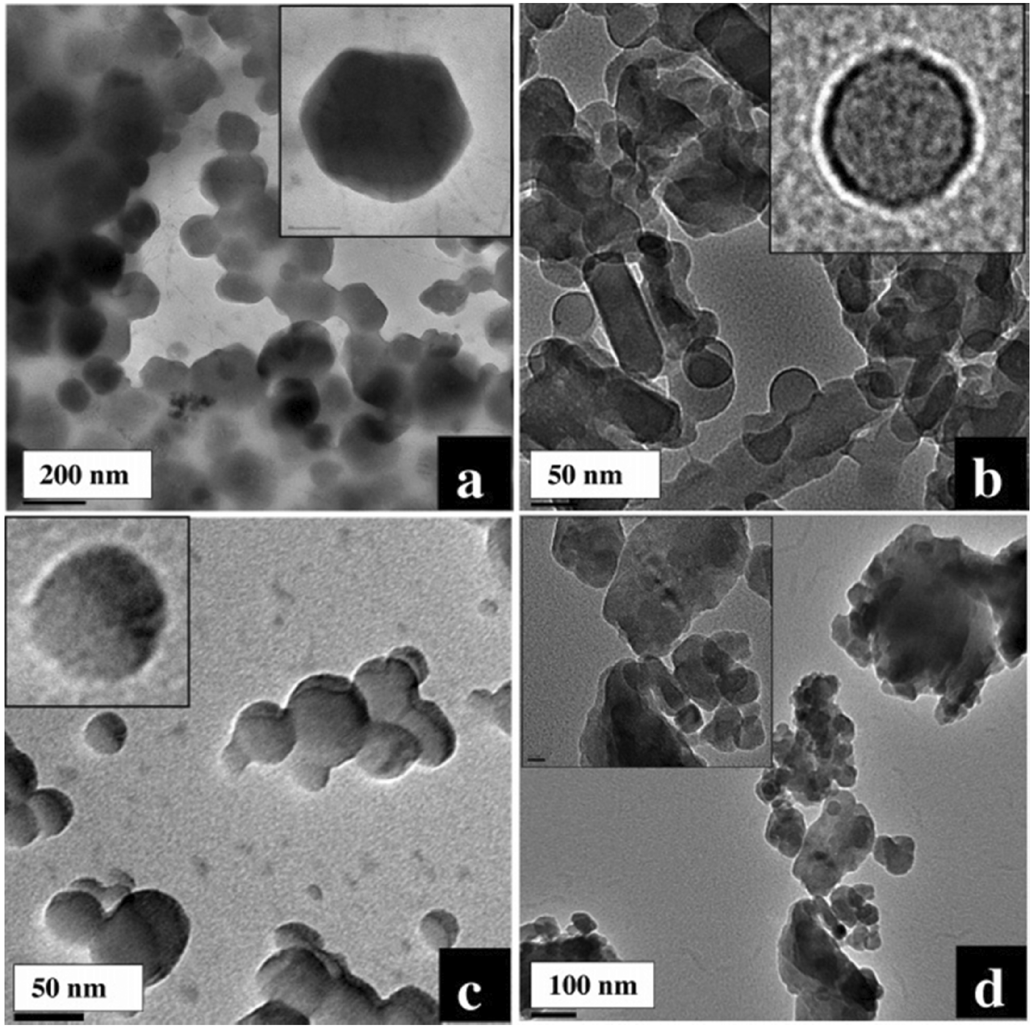


Fig. 3

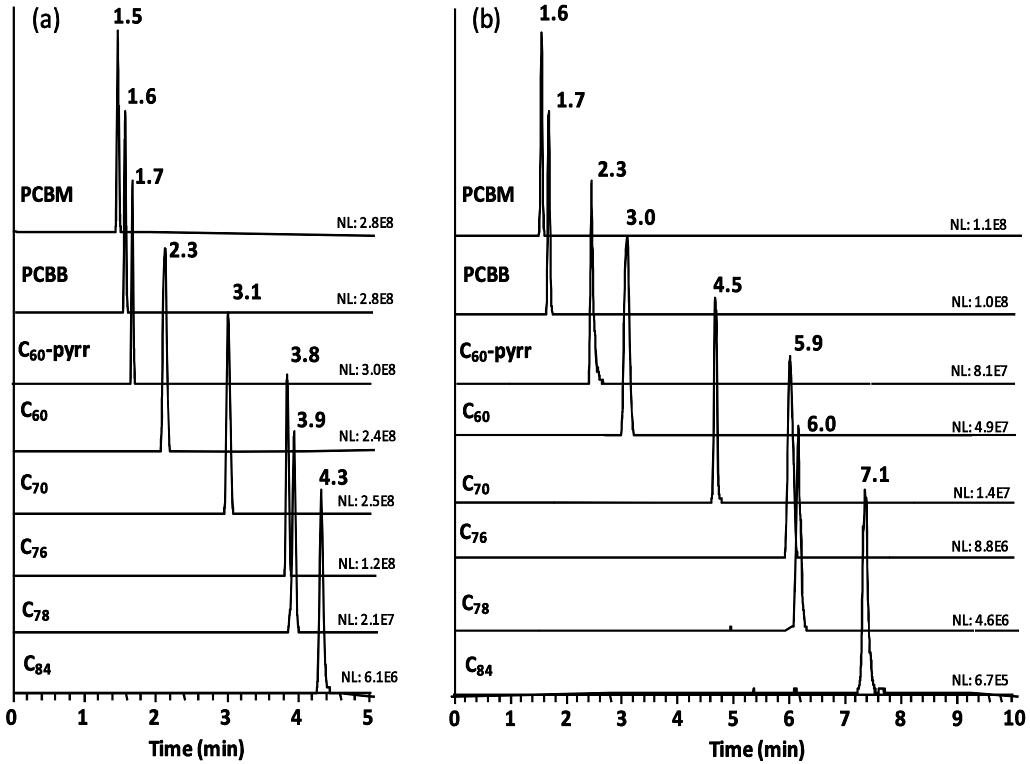
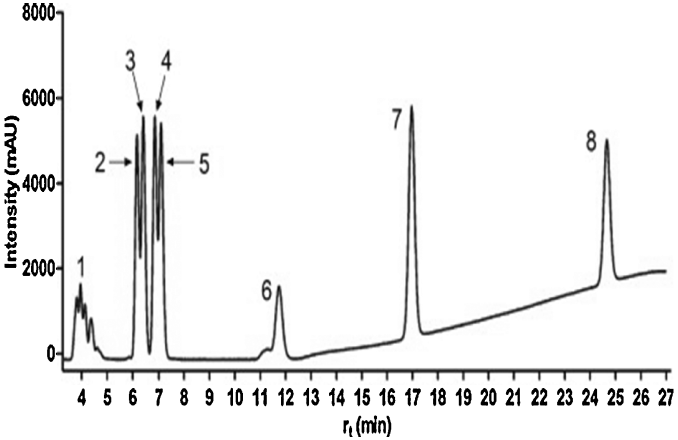

(a)
1.5
1.6
1.7
2.3
3.1
3.8
3.9
4.3
PCBM
PCBB
C60-pyrr
C60
C70
C76
C78
C84
NL: 2.8E8
NL: 2.8E8
NL: 3.0E8
NL: 2.4E8
NL: 2.5E8
NL: 1.2E8
NL: 2.1E7
NL: 6.1E6
0
1
2
3
4
5
Time (min)
(b)
1.6
1.7
2.3
3.0
4.5
5.9
6.0
7.1
PCBM
PCBB
C60-pyrr
C60
C70
C76
C78
C84
NL: 1.1E8
NL: 1.0E8
NL: 8.1E7
NL: 4.9E7
NL: 1.4E7
NL: 8.8E6
NL: 4.6E6
NL: 6.7E5
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Fig. 4



8000
6000
4000
2000
0
Intensity (mAU)
1
2
3
4
5
6
7
8
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
r_t (min)

Fig. 5

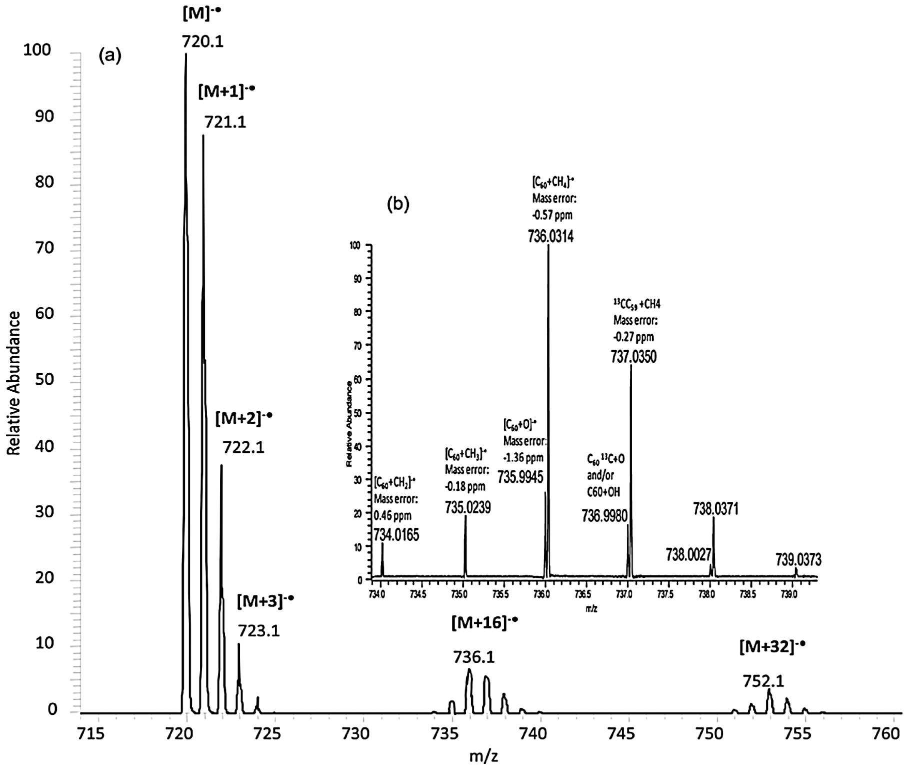
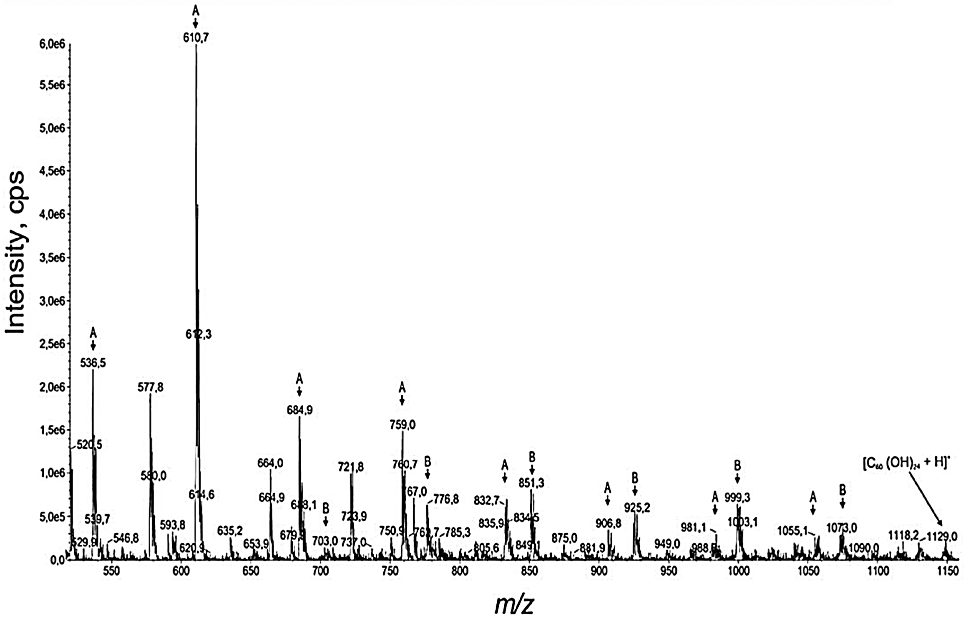

(a)
[M]-•
720.1
[M+1]-•
721.1
[M+2]-•
722.1
[M+3]-•
723.1
[M+16]-•
736.1
[M+32]-•
752.1
Relative Abundance
m/z
0
10
20
30
40
50
60
70
80
90
100
715
720
725
730
735
740
745
750
755
760
(b)
[C60+CH4]-•
Mass error:
-0.57 ppm
736.0314
13CC59+CH4
Mass error:
-0.27 ppm
737.0350
[C60+O]-•
Mass error:
-1.36 ppm
735.9945
[C60+CH3]-•
Mass error:
-0.18 ppm
735.0239
[C60+CH2]-•
Mass error:
0.46 ppm
734.0165
C60 13C+O
and/or
C60+OH
736.9980
738.0371
738.0027
739.0373
Relative Abundance
m/z
734.0
734.5
735.0
735.5
736.0
736.5
737.0
737.5
738.0
738.5
739.0

Fig. 6


Intensity, cps
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5,5e6
5,0e6
4,5e6
4,0e6
3,5e6
3,0e6
2,5e6
2,0e6
1,5e6
1,0e6
5,0e5
0,0
550
600
650
700
750
800
850
900
950
1000
1050
1100
1150
m/z
A
610,7
612,3
A
536,5
520,5
539,7
529,9
546,8
577,8
580,0
593,8
620,9
614,6
635,2
653,9
664,0
664,9
679,9
A
684,9
688,1
B
703,0
721,8
723,9
737,0
A
759,0
760,7
750,9
767,0
762,7
B
776,8
785,3
805,6
A
832,7
835,9
834,5
B
851,3
849,1
875,0
881,9
A
906,8
B
925,2
949,0
A
981,1
988,8
B
999,3
1003,1
A
1055,1
B
1073,0
1090,0
1118,2
1129,0
[C60 (OH)24 + H]+

Fig. 7