Quantitative Analysis of Fullerene Nanomaterials in Environmental Systems: A Critical Review

Carl W. Isaacson[a,b], **Markus Kleber**[c], and **Jennifer A. Field**[a,d,*]

[a]Dept. Chemistry, Oregon State University, Corvallis, OR

[c]Dept. Crop and Soil Science, Oregon State University, Corvallis, OR

[d]Dept. Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR

Abstract

The increasing production and use of fullerene nanomaterials has led to calls for more information regarding the potential impacts that releases of these materials may have on human and environmental health. Fullerene nanomaterials, which are comprised of both fullerenes and surface-functionalized fullerenes, are used in electronic, optic, medical and cosmetic applications. Measuring fullerene nanomaterial concentrations in natural environments is difficult because they exhibit a duality of physical and chemical characteristics as they transition from hydrophobic to polar forms upon exposure to water. In aqueous environments, this is expressed as their tendency to initially (i) self assemble into aggregates of appreciable size and hydrophobicity, and subsequently (ii) interact with the surrounding water molecules and other chemical constituents in natural environments thereby acquiring negative surface charge. Fullerene nanomaterials may therefore deceive the application of any single analytical method that is applied with the assumption that fullerenes have but one defining characteristic (e.g., hydrophobicity). [1] We find that analytical procedures are needed to account for the potentially transitory nature of fullerenes in natural environments through the use of approaches that provide chemically-explicit information including molecular weight and the number and identity of surface functional groups. [2] We suggest that sensitive and mass-selective detection, such as that offered by mass spectrometry when combined with optimized extraction procedures, offers the greatest potential to achieve this goal. [3] With this review, we show that significant improvements in analytical rigor would result from an increased availability of well characterized authentic standards, reference materials, and isotopically-labeled internal standards. Finally, the benefits of quantitative and validated analytical methods for advancing the knowledge on fullerene occurrence, fate, and behavior are indicated.

Introduction

Fullerene and surface-functionalized fullerene classes of nanomaterials are proposed for use in optical, electronic, cosmetic, and biomedical applications [1-6]. Fullerenes are hollow carbon cage sp^2 hybridized molecules, the first example of which, C_{60}, was discovered by Kroto *et al.* in 1985 [7]. In the early 1990s, fullerenes ($C_{60}, C_{70}, C_{76}, C_{78}...$) were first produced in macroscopic quantities by condensation of vaporized graphite [8-12]. Additionally, lower order fullerenes (e.g., C_{28} and C_{36}) were also isolated from soot generated by vaporization of graphite [8,9,12-15]. Naturally-occurring fullerenes were detected in the 1990s in materials affected by high energy events such as lightning strikes, meteors [16-18] and meteor-impacted or metamorphic materials [19-23], and in geologic samples [18,23,24]. C_{60} occurs in soot

*Corresponding author, phone 541/737-2265, Jennifer.Field@oregonstate.edu.
[b]Current address: US Environmental Protection Agency, Athens, GA

generated by combustion of hydrocarbons and oxygen [25-28], commercially-available charcoal [29], and soot produced by candle flames [18]. The natural occurrence of C_{60} and C_{70} represent their pre-manufacturing era occurrence and must be understood in order to assess the impact of future industrial discharges. Ultimately, surface-functionalized (e.g., carboxyl and hydroxyl groups) fullerenes may be produced in larger quantities than fullerenes themselves [30-32] in an attempt to create more biologically-compatible forms [31-34].

Over the last two decades, many reports were published on the synthesis and application of fullerene materials, yet quantitative information describing the occurrence, behavior and transport of fullerene nanomaterials in environmental systems is still lacking [35,36]. Studies into the occurrence, behavior and transformation of fullerene and surface-functionalized fullerene nanomaterials require a fundamental understanding of the physical and chemical properties of these materials. However, fullerene nanomaterials exhibit a time-dependent transition from hydrophobic forms present in condensed natural phases (e.g., soot and geological materials) to polar forms that are potentially more mobile in aqueous systems. It is now well documented that the initially hydrophobic C_{60}, upon extended exposure to water, forms water-stable aggregates that are polar in character [37-41]. For example, the aqueous solubility of C_{60} in its hydrophobic, crystalline form is estimated to range from 1.11×10^{-11} M to 1.8×10^{-20} M [42-44]. However, fullerene aggregates suspended in water [37-40, 45-51] lead to measured concentrations of individual fullerene molecules that are more than eight orders of magnitude greater than the water solubility of the hydrophobic form [42-44]. Clearly, the transition from hydrophobic to polar forms has potential implications for fullerene transport, transformation, and biological effects.

Andrievsky *et al.* [45] proposed that C_{60} is stabilized in aqueous solution by electron donor-acceptor (EDA) complexation with water, termed 'localized hydrolysis,' that is represented as $C_{60} + H_2O \leftrightarrow C_{60}(OH)^- + H^+$. The formation of water-stable fullerene aggregates is documented further by UV-spectroscopy, and titration data [37,38,45-47,49-51]. The localized hydrolysis is consistent with the reported formation of negative surface charge on the water-stable fullerene aggregates [38-40,45-47,49]. Chen et al. concluded that water-stable C_{60} aggregates contain dissociable functional groups as determined by X-ray photon spectroscopy, the pH-dependence of electrophoretic mobilities, and their stability in water [41]. However, liquid-state NMR data for water-stable fullerene aggregates indicated a downfield shift (3 ppm) in the single peak for C_{60} (for solid C_{60} in the absence of water) to 143 ppm that is indicative of non-covalent C_{60} interactions with water. Although the nature of the water-stable C_{60} aggregates is under debate, the C_{60} molecule is thought to remain intact, but that a polar shell 'cloaks' the hydrophobic fullerene core molecule [45,52]. To visualize such structures, see figures in Andrievsky et al. [45,52]. While it is not our intention to endorse a particular conceptual model of fullerene-water interactions, it is critical to recognize that contact with water creates forms of fullerene with fundamentally different physical and chemical properties than the hydrophobic forms of fullerenes.

The potential toxicity of fullerenes, fullerols and fullerene derivatives is an intensely debated issue. For example, there are reports that "pristine fullerenes are non-toxic, and the fullerene water system has a wide spectrum of positive and unique biological activity" [52]; that "antioxidant effects of water soluble fullerenes scavenge reactive oxygen species in human skin cells"[53] and that "fullerene is a powerful antioxidant with no acute or subacute toxicity"[54]. These views are countered by some more cautious positions pointing to the known ability of C_{60} to both quench and generate reactive oxygen species (ROS)[55,56], which may inflict DNA damage and adversely affect cellular structures through lipid peroxidation [57]. A large part of the uncertainty about fullerene toxicity results from the fact that the ROS producing/quenching ability of functionalized fullerenes depends on the number, structure and

substitution patterns of addends and substituents and appears to synergistically increase as a result of interactions of the fullerenes with functional groups or modifying solvents[55].

An alternative mechanism for the toxicity of water-stable C_{60} fullerenes towards bacteria was suggested by Lyon and Alvarez [58], who propose that water-stable C_{60} exerts ROS independent oxidative stress, with evidence of protein oxidation, changes in cell membrane potential, and interruption of cellular respiration. They further posit that water-stable C_{60} is unique among nanomaterials in that it does not puncture the cell nor release ROS or toxic products, but instead exerts toxicity as a particle via a chemical interaction upon direct contact [58]. While the mechanistic background behind fullerene toxicity remains to be fully elucidated, it is increasingly apparent that the behavior of water-stable C_{60}, as both an oxidizing chemical and as a nanoparticle, is intrinsic to its reactivity [58]. We consider this a strong argument in support of our position that the full exploration of the mechanisms behind fullerene toxicity in terrestrial environments clearly requires analytical information about both their molecular properties and their physical or particulate status.

We conclude that the hydrophobic forms of fullerenes, as well as their water-stable forms, require explicit consideration when considering the overall fate, transport, and toxicity of fullerenes and their relevant forms. The possible presence of fullerenes in hydrophobic and polar forms in natural and engineered systems confounds the notion of developing a single quantitative analytical method for the determination of fullerenes and their 'speciation' in natural and engineered systems. The water-stable fullerenes cannot be recovered from water with toluene because they are no longer hydrophobic in nature [45,50]. While current analytical methods focus on determining fullerenes as particles (*e.g.* aggregate size, mobility, and apparent surface charge), structurally-specific analytical methods also are needed to address the array of molecular or chemical characteristics that affect fullerene and surface-functionalized fullerene nanomaterials and their transport, fate, and effects in aquatic and terrestrial systems.

There are several reviews of fullerene nanomaterials including the chromatographic separation of fullerene nanomaterials [59], the analysis and occurrence of fullerene nanomaterials in geological samples [20], possible mechanisms of water-stable fullerene aggregate formation [60], methods for determining particle characteristics of nanomaterials [61-63] (*e.g.* particle size, surface area, surface charge, and size separation), and the potential risk fullerene nanomaterials pose to human and environmental health [62,64-67]. However, these reviews do not address the analytical challenges, given the tendency of fullerenes to occur as hydrophobic entities and in polar forms in aquatic and terrestrial environments. Consequently, the central objectives of this work are to provide the reader with (i) a report on the state-of-the-art analytical methods for quantifying fullerene nanomaterials, (ii) an exploration of specific challenges to analytical methodologies that must be overcome, (iii) and a discussion of how improvements in analytical methodologies will play an essential role in advancing our understanding of fullerene nanomaterial occurrence, fate, transport, and effects.

Fullerene Analysis: State-of-the-art

This section presents the current state-of-the-art analytical methods that are commonly used for the analysis of fullerene and surface-functionalized fullerenes with a focus on complex environmental matrices. Methods that offer information on fullerenes only as particles such as aggregate size and mobility including transmission electron microscopy (TEM), quartz crystal microbalance, X-ray photon spectroscopy, light scattering, and electrophoretic mobility [37, 39,41,49,68-71] are reviewed elsewhere [61-63] and are not discussed further.

For the purpose of this critical review, attention is focused on methods that are described as quantitative and that can potentially discriminate between the various molecular forms of fullerenes (e.g., C_{60} from C_{70} or C_{60} from $C_{60}O$). Given that analytical methods typically consist of multiple steps (e.g., extract, concentrate, and detect) and are applied in a linear fashion from sample extraction, concentration, followed by separation and detection, an overview of the currently-used techniques is presented in this order. Finally, quantitative methods that are applied to geological, biological, or aqueous matrices for which one or more performance parameters such as accuracy, precision, and detection/quantification limits are presented are described in more detail.

Extraction

Methods for the extraction of fullerenes from environmental solids (Table 1) including sediments and combustion-derived soots currently consist of sonication or Soxhlet extraction with toluene [18,24,72,73]. When extracting C_{60} and C_{70} fullerenes from carbon soot using supercritical CO_2 modified with toluene, Jinno and Kohrikawa found recoveries similar to those obtained by Soxhlet extraction with toluene[74] (Table 1). Approaches for extracting C_{60} from biological matrices such as plasma [75] and homogenized tissues [76-78] also utilize toluene and, in some cases, the addition of salt (Table 1). In contrast, water-stable C_{60} are not efficiently recovered from aqueous solution using toluene alone [45,50] due to their polar nature. Alternatives include toluene with a required addition of salt [79-81], evaporation to dryness [80], and solid phase extraction [80] (Table 1).

Separation

Differentiating between various fullerene forms is accomplished, in part, through chromatographic separation. To date, the emphasis is on C_{60} (Table 1) with a few reports indicating the chromatographic separation of C_{60} from C_{70} (ref from Table 1). The feasibility of identifying and separating a wider range of fullerenes is indicated by Isaacson et al. [76] and Jinno *et al* [9] who report separations for C_{76}–C_{96} and C_{60}-C_{86}, respectively. Few authors report the separation of surface-functionalized fullerenes [81-84]. The most frequently-reported separation conditions for fullerenes and hydrophobic surface-functionalized fullerenes are based on reverse-phase columns such as C_{18} and mobile phases containing toluene and methanol [9,76,77,85,86], acetonitrile [9,75,77,85], acetone [85], isopropanol [85], or THF [75] serving as the polar mobile phase component. Fullerene retention on C_{18} columns is achieved by adding a polar solvent (e.g., methanol, acetonitrile, and THF) to the injection solvent and elution is achieved by increasing the percent of the non-polar solvent, typically toluene [9]. The observed chromatographic behavior is consistent with fullerene solubility, which is greater in aromatic hydrocarbon solvents, such as toluene, and lower in polar solvents such as methanol or acetonitrile [87,88]. For polar surface-functionalized fullerenes, such as *tris*-dicarboxymethanofullerene-C_{60} [83,84] and dendritic monoadduct methano[60]fullerene octadeca acid [82], more polar solvent combinations are employed (e.g., methanol, water, and acetonitrile).

Detection

Currently applied analytical methodology with the ability to differentiate the various molecular forms of fullerenes includes liquid chromatography with ultraviolet (UV) detection [45,47, 49,56,89,90] and ^{13}C NMR [49]. However, mass selective detection offers advantages of selectivity when compared to the aforementioned approaches. Mass spectrometry offers the capability to distinguish fullerenes based on their molecular weight (e.g., molecular specificity), thereby providing structural information that can indicate the presence or absence of surface functional groups. Many procedures with the potential to ionize fullerenes for mass spectroscopy applications are reported and reviewed [20] including laser desorption ionization

(LDI), matrix assisted laser desorption ionization (MALDI) [20,91-94], chemical ionization (CI) [95], thermal desorption ionization (TDI), fast atom bombardment (FAB) [95], atmospheric pressure chemical ionization (APCI) [48,75,77,80], atmospheric pressure photoionization (APPI) [85], and electrospray ionization (ESI) [76,96]. Fewer reports describe ionization for surface-functionalized fullerenes [82,97]. Of all the ionization methods discussed, APCI, APPI and ESI are most readily coupled with LC to provide quantitative data on fullerenes (Table 1) [75-77,80].

Quantitative Methods for Complex Geologic, Biological and Aqueous Matrices

There are relatively few reports on the quantification of fullerene nanomaterials in geological, biological or aqueous matrices. Only those methods for which one or more methodological performance parameters are reported including (i) accuracy (as indicated by recovery), (ii) precision, (iii) method of detection and/or (iv) quantification limits are given special attention here (Table 1).

Most reports of early attempts to quantify fullerenes in geological materials, do not contain method performance characteristics. Exceptions include Heymann et al. [72] who reported recoveries +/- precision of $80 \pm 9\%$ for C_{60} and C_{70} from spiked sediments and Jelickaa et al., 2005 [73] who report recoveries from 0% (below detection) to 114% for spiked geological materials including graphite, shungite, and coal (Table 1). Jinno and Kohrikawa [74] also reported C_{60} and C_{70} recoveries (10-162%) from carbon soot. None of the reports for geologic materials or carbon soot indicated method detection or quantification limits (Table 1).

Quantitative methods for the determination of fullerene concentrations in biological matrices are scarce and centered on extractions with toluene [75-77,79]. Santa *et al* [75] obtained poor recoveries (below detection to 13%) using solvents to extract water-stable C_{60} from rat plasma. Solid phase extraction onto C_{18} increased recoveries to $62 \pm 4\%$ (Table 1). The reported detection limits were 0.5 mg/L (LC-UV) and 5.0 mg/L (LC-APCI-MS). Moussa *et al.* [77] reported recoveries of C_{60} from blood, spleen, and liver with efficiencies from 90-104% with precision ranging from 0.8 to 8% (Table 1). A detection limit of 0.02 μg/L was obtained using LC-UV. Although Moussa et al. [77] used LC-UV for quantification they also carried out analyses by LC-APCI-MS and used C_{70} as an internal standard. Caution is urged when considering the use of C_{70}, which occurs naturally in sediments [8,22,25,98]. Xia et al. [79] obtained recoveries of water-stable C_{60} from 94 – 100% with ± 15% precision when investigating plasma and water containing 5% bovine serum albumin by extraction with toluene and upon the addition of salt (Table 1). With LC-UV for separation and detection, they obtained a detection limit of 0.34 μg/L and a quantification limit of 0.68 μg/L. Isaacson *et al* [76] reported the recovery of C_{60} (prepared in DMSO) from the homogenate of embryonic zebra fish using toluene with recoveries of $90 \pm 3\%$ using LC-ESI-MS and $^{13}C_{60}$ as an internal standard. Detection and quantification limits of 0.02 and 0.04 μg/L were obtained. To the best of our knowledge, this is the only study to incorporate the use of stable isotope-labeled $^{13}C_{60}$ as an internal standard.

Multiple approaches are reported for recovering water-stable C_{60} from aqueous sample matrices including liquid:liquid extraction with toluene with the addition of salt [79-81], evaporation to dryness [80], and by solid-phase extraction [80]. A wide range in recoveries of fullerenes from water by toluene and salt are reported from > 90% [79,81]. Lower recoveries (< 40%) are reported for water-stable C_{60} using toluene and salt, evaporation to dryness, and solid phase extraction [80]. Isaacson *et al* [76] reported the recovery of C_{60} (prepared in DMSO) from aqueous dosing solutions of $110 \pm 10\%$ using toluene alone.

Current Limitations and Research Opportunities

Given the state-of-the-art of fullerene analysis and the role that analytical methods will likely play in illuminating the occurrence, fate, transport, and effects of fullerene nanomaterials, this section focuses on the limitations that must be overcome and the opportunities for future research. The following material is organized in a way that highlights the analytical steps that are likely to be involved in fullerene analysis in the order they are most commonly performed. The topics include the challenges associated with the 1) extraction, the 2) separation and 3) detection, of fullerenes and surface-functionalized fullerenes in complex environmental matrices. Finally, quality control issues will be addressed.

Extraction

Although endogenous concentrations of fullerenes are reported for geological samples [18, 23,24] and combustion-derived solids [8,74] there is only one attempt to optimize the extraction of fullerenes from geologic material; however, recoveries of less than 5 % were obtained [73]. Although several reports [8,74,76] indicate compelling evidence of a range of fullerenes (e.g., C_{60} to C_{98}) in carbon soots, no optimized methods are yet reported for the extraction of a wider range of fullerenes from combustion-derived solids. The challenge is to understand if the reported concentrations are representative.

Recovery data for fullerenes from geologic materials are based on the recovery of spiked fullerenes and are therefore not likely to represent the actual recovery of endogenous fullerenes. Moreover, the true range of fullerene forms that occur in geological and other environmental condensed phases is not well understood due to the nearly universal exclusive application of toluene as the extraction solvent [14]. For example, the solubility trends of C_{70} for a series of solvents mirror those of C_{60}, although C_{70} solubility is typically less than that of C_{60} for any given solvent [99]. To the best of our knowledge, the solubilities of fullerenes larger than C_{70} have not been reported. However, Jinno et al. [8] first extracted C_{60} and C_{70} from carbon soot and then used 1,2,4-trichlorobenzene to recover larger fullerenes (e.g., C_{76}-C_{94}). Smaller fullerenes, such as C_{36}, are not soluble in toluene but rather are soluble in pyridine and carbon disulfide [14]. Therefore, a single solvent may not be suitable for the recovery of the full range of fullerenes that may exist in environmental samples. When attempting to develop and validate extraction methods for a wide suite of fullerenes in environmental solids, different solvents may be needed to exhaustively extract the range of naturally-occurring fullerenes.

The conventional approach for maximizing analyte extraction from condensed natural phases such as soils and sediments is to optimize the temperature of solvent extraction. However, in contrast to most environmental analytes of interest that are recovered more efficiently at higher extraction temperatures, C_{60} exhibits anomalous solubility – temperature trends. The temperature of maximum C_{60} solubility ranges from 3°C to 37°C, depending on the solvent used, and diminishes with further increases in temperature [100,101]. The observed solubility maxima result from the negative enthalpy of solution for C_{60} and highly-stable solvated state of C_{60} crystals [99]. Therefore, application of the conventional wisdom of applying higher temperatures for enhanced recovery of environmental analytes will likely result in decreased C_{60} extraction efficiencies. It is not yet known if this trend in solubility/extraction recovery will apply to surface-functionalized forms of fullerenes as well.

The selection of solvent extraction conditions potentially confounds the recovery of fullerenes from aqueous systems. Inconsistent recoveries from aqueous systems of fullerenes prepared by various methods may be indicative that different molecular forms of fullerenes are present. For example, C_{60} prepared in tetrahydrofuran and transferred to water was recovered from aqueous media by toluene but only when salt (NaCL 20% w/w) was added [80], while C_{60} prepared in DMSO and transferred into water was quantitatively extracted with toluene alone

[76]. We hypothesize that the C_{60} extracted by toluene directly [76] remained in the hydrophobic form, while C_{60} prepared via THF was converted to a water-stable form as indicated by Xia et al. [79]. From these observations, we conclude that the method of fullerene preparation is important, not only for toxicological investigations but also when developing and validating analytical methods for fullerenes in environmental matrices. Further, we suggest that sequential extractions involving a first extraction step using toluene and a subsequent addition of salts can serve to operationally define the fullerene forms present in aqueous solutions. For example, it is now well documented that the hydrophobic form of C_{60} is recovered from aqueous solutions by toluene whereas the addition of salt recovers water-stable fullerenes [47,49,52,77]. Consequently, we propose that sequential extractions can be tuned to differentiate the various forms present in environmental samples. A robust protocol to achieve this has yet to be developed and demonstrated.

Separation

Among the current challenges facing the separation of fullerenes is the necessity that methods of separation be able to differentiate fullerenes of varying sizes that may occur in both hydrophobic and polar fullerene forms, from those that may have surface-functional groups. Although smaller fullerenes (e.g., C_{28}, C_{36}, and $C_{50}Cl_{10}$) are reported to occur, chromatographic separations of the lower-order fullerenes have yet to be demonstrated. In addition, the most common approach for the chromatographic separation of fullerenes includes the addition of a polar solvent to the toluene extracts, which basically dilutes the fullerenes and adversely impacts sensitivity. In the work reported by Isaacson et al. [76], up to 70% of the final volume consisted of the polar solvent in order to retain and separate fullerenes on a C_{18} column [76]. Therefore, due to the potential range in molecular weights and polarities, it is likely that more specific stationary and mobile chromatographic devices and phases will be required to separate the individual species that make up fullerene nanomaterials. The development of alternative columns [102,103] will reduce the percentage of (diluting) polar solvent needed to achieve separation [81,103]. By minimizing the dilution of toluene extracts, lower detection limits can be achieved.

There are relatively few reports of the separation of surface-functionalized fullerenes, as there are relatively few reports for their separation [81-84]. For polar functionalized fullerenes, such as tris-dicarboxymethanofullerene-C_{60} [83,84] and dendritic monoadduct methano[60] fullerene octadeca acid [82], more polar solvent combinations are employed (e.g., methanol, water, and acetonitrile). In order to address the potential diversity of fullerene forms likely present in environmental samples, a spectrum of extraction as well as separation conditions must be considered and a single 'one size fits all' approach is therefore not likely to succeed.

Detection

Methods based on Raman spectroscopy [104,105] and ^{13}C-NMR [49] are not suitable for routine analysis (e.g., monitoring). Radiocarbon labeled compounds, such as [14]C_{60}, can be used in controlled laboratory experiments [50] but are not useful for determining ambient environmental concentrations of fullerene nanomaterials. While approaches including TEM, quartz crystal microbalance, X-ray photon spectroscopy, light scattering, and electrophoretic mobility that detect fullerene aggregates are clearly valuable, such analytical approaches are applied largely to 'clean' laboratory systems that lack potentially interfering naturally-occurring particulate matter.

The detection method based on LC-UV lacks the requisite specificity for the unambiguous determinations of fullerenes in complex heterogeneous environmental samples that contain potential interferences such as natural dissolved and particulate matter. In the case of fullerenes, the use of LC/UV for determining fullerenes in geologic samples was challenged due to the

possibility of interferences [106]. Additionally, UV *per se* is not able to distinguish between fullerenes and surface-functionalized fullerenes, nor can peak purity be verified by UV if mixtures of unknown composition are present in complex environmental matrices.

Mass spectrometry offers the capability to distinguish fullerenes based on their molecular weight (e.g., molecular specificity), thereby providing structural information that can indicate the presence or absence of surface functional groups. However, with so many ionization approaches that can be used to ionize fullerenes, the question is what approaches offer the greatest opportunity to advance our understanding of the occurrence, fate, transport, and effects of fullerenes in environmental system? For example, while MALDI, FAB, LDI, TDI, and CI ionization techniques are useful for determining molecular identity of fullerene nanomaterials, the utility of these ionization methods for quantitative analysis is limited because these ionization methods are not easily interfaced with separation instrumentation such as liquid chromatographs. For this reason, we recommend mass spectrometers and ionization modes that are readily interfaced with liquid chromatographs, such as APCI, APPI, and ESI.

Current mass spectrometric approaches that appear useful for quantifying fullerenes in complex systems containing natural organic and inorganic matter do not preserve information on aggregate size. Therefore, there is a need to develop mass spectrometric or other approaches that analyze the fullerene composition of aggregates while preserving information on aggregate solvation state and cluster size. Andrievsky et al. [45] suggested that mass spectrometric approaches such as those described by Wei et al. [107] have the potential for investigating cluster dynamics and bonding. However, such approaches have yet to be demonstrated as suitable for the detection of water-stable fullerenes.

We encourage that such investigations include the wide range of fullerenes that may occur (e.g., C_{36} to C_{98}). Increased emphasis on the development and validation of selective and sensitive separation and quantification is needed to capture the range of fullerene forms that may occur as hydrophobic and polar forms. Such methodology is needed to identify transformation products in natural and engineered systems because they may be of equal or greater importance in terms of risk. Moreover, concomitant with the development and application of methodology for detecting and describing fullerenes as particles, analytical methods that offer chemically-explicit information on the components within fullerene aggregates are needed to rationalize observed distributions and any transformation products. Mass selective detection will most likely play a significant role because it can be used to identify and quantify individual fullerenes present in environmental systems containing natural dissolved and particulate organic as well as inorganic matter.

Quality Control

Given the ambiguous behavior of fullerene nanomaterials in environmental systems, quality control will play a critical role. The issues surrounding quality control are not unique to fullerenes. However, it appears that few studies employ 'gold standard' practices for quality control. As for other emerging contaminants of concern, accurate and precise measures of concentration are needed for investigations that determine the occurrence, fate, transport, and toxicity of the various possible forms of fullerene. Indications of method performance including quality control parameters such as recovery/accuracy, precision, and detection/quantification limits are used to communicate reliability and uncertainty surrounding reported concentrations. Transparency is provided by detailed descriptions of analytical standard purity/source, preparation and storage of standards. Each element of analytical methodology must be described so that methods can be reproduced and for communicating expectations surrounding method performance.

Well-characterized standards exist for C_{60} and C_{70} fullerenes, but not for lower- and higher-order forms. Without a full array of well-characterized standards, it will be difficult to fully validate methodologies for the various fullerenes that are environmentally relevant. The lack of authentic or certified reference materials is particularly problematic. For example, during initial attempts to develop quantitative methods for hydroxylated C_{60} fullerenes (fullerenols), the present authors purchased $C_{60}OH_{24}$ (Materials and Electrochemical Research Corporation, Tucson, AR) and $C_{60}(OH)_{20-28}$ (Alfa Aesar, Ward Hill, MA). Experiments using LDI-MS and MALDI-MS analyses of commercially-available $C_{60}OH_{24}$ and $C_{60}(OH)_{20-28}$ using ionization-inducing matrices recommended for hydroxylated fullerene identification [33,108] produced only molecular ions for C_{60} at m/z 720 and no ions were observed that corresponded to either form of the hydroxylated C_{60}s (unpublished data). Furthermore, experiments using ESI-MS and APCI-MS with $C_{60}OH_{24}$ and $C_{60}(OH)_{20-28}$ dissolved in acidic, basic, and neutral solutions did not produce ions diagnostic of $C_{60}(OH)_x$ nor C_{60} (unpublished data). In the end, we were unable to verify the presence of hydroxylated fullerenes in the commercial 'standards' using four independent ionization/MS techniques. Unfortunately, the only manufacturer-supplied data for the $C_{60}OH_{24}$ was limited to non-specific infrared spectra and thermogravometric data while the manufacturer of the $C_{60}(OH)_{20-28}$ was unable to provide any characterization information to establish authenticity. Therefore, commercially-available test materials must be well-characterized, ideally with certificates that unequivocally demonstrate authenticity.

Internal standard quantification using stable isotope-labeled internal standards is the *de rigueur* for trace level analysis of organic contaminants in environmental matrices. Transparent and rigorous validation of analytical methods is advanced through the use of internal standard calibration, which is widely-recognized as the 'gold standard' for the quantification of analytes in complex environmental matrices [109]. To the best of our knowledge, there currently exists only a single, commercially available stable-isotope labeled fullerene internal standard, $^{13}C_{60}$ [76]. In the absence of well-matched internal standards, standard-addition is a widely-accepted approach for quantifying analytes in complex environmental samples or extracts when employing LC/MS [109]. Demand for quantitative information on fullerene occurrence, behavior, transport, and toxicity is likely to increase the demand for well-characterized internal standards.

When C_{60} was introduced into aqueous systems with dimethyl sulfoxide (DMSO), over the relatively short time scales of laboratory experiments (hours to days), up to 50-60% was 'lost' from solution [76]. However, careful attention to achieving mass balance revealed that the C_{60} 'loss' from solution was due to association of with the glass surfaces [76]. Xia et al. [79] also report loss onto glass when evaporating extracts to dryness; however, they found that the C_{60} could not be recovered from the glassware. Alternatively, when fullerene nanomaterials are suspended in water by extended stirring in water or sonication, the observation that some fraction cannot be recovered by toluene is a clear indication that not all of the fullerene materials were present in their hydrophobic forms. Given the change in fullerene properties depending on the method of preparation or the type of experiment conducted, it will be critical to use analytical methodologies for extraction and quantification that differentiate between, and account for, the relevant forms and mass of fullerenes present in complex heterogeneous engineered and natural systems.

Fullerene Occurrence, Fate, Transport, Effects and the Role for Analytical Chemistry

Presented in this section is the critical role analytical chemistry will play in determining the occurrence, fate, transport and biological effects of fullerene nanomaterials. Methods for determining the occurrence of fullerenes in environmental solids must be optimized to fully understand environmental fullerene distribution. The fate of fullerenes in environmental and

engineered systems is just beginning to be explored and methods must be developed to determine the identity, transport and effects of transformation products. Finally, reports on the transport and effects of fullerene nanomaterials are discussed.

Occurrence

The earliest work on determining the natural occurrence and concentration of fullerenes (endogenous) in natural systems was in the geological sciences. Fullerene concentrations up to 10 μg C_{60}/g are reported for geologic materials, [18,23,24]. The occurrence, concentration, and species of fullerenes present in combustion-derived solids (*e.g.* domestic wood burning stoves, diesel exhaust, and black carbon) will provide information on the natural background levels of fullerenes and on human exposure to fullerenes. However, the fullerene recoveries reported are for spiked geological and soot-type materials so it is difficult to assess if the concentrations reported are representative of the natural abundance of fullerenes in geological materials. It is also likely that our knowledge of the full range of fullerenes that occur naturally is incomplete since the ability to concentrate lower and higher-order fullerenes or surface-functionalized fullerenes using toluene is not known.

To the best of our knowledge, there are no data on naturally-occurring fullerenes in biota or water from natural or engineered (non-laboratory) systems. However, we anticipate that the number of reports will increase significantly as the methods indicated in Table 1 are used, modified, and demonstrated for the purpose of describing the occurrence, concentration, and range of fullerene forms present in surface waters, ground waters, wastewaters.

Fate

Relatively little is known about the fate of fullerenes in natural and engineered systems. To the best of our knowledge, there is one study that has demonstrated fullerenol decomposition by fungi [110]. Recent research focuses on reaction with ozone and in sunlight (UV). There is a single report of the reaction of water-stable C_{60} with ozone in which a reduction in the parent molecule, C_{60}, concentration is observed and the formation of transformation products is reported [111]. These transformation products are identified by NMR, IR, MS and XPS methods and are reported to be of the composition $C_{60}(O)_x(OH)_y$ with $x + y = 29$ with 11 being monooxygenated and the remaining 29 being dioxygenated, as determined by XPS [111].

There are an increasing number of reports of the increased mass of C_{60} suspended in water upon exposure to sunlight [112,113] and the transformation of C_{60} by sunlight [114-116]. Techniques including x-ray photon spectroscopy, FT-IR, and mass spectrometry [112,113] were used to document reductions in the mass of C_{60} suspended, the role oxygen plays in the degradation, and the formation of oxidized products; however, no degradation products have yet been identified.

Outside the growing literature on reactions with ozone and photo-induced transformation of fullerenes, other types of transformation processes affecting fullerenes under environmental conditions have yet to be reported. Such investigations are lacking, in part, because appropriate analytical methods for such investigations are in their infancy. The situation is analogous to attempting to study polycyclic aromatic hydrocarbon transformation prior to the development of gas chromatography and selective detectors. As more selective and sensitive analytical became available for PAHs, investigations yielded mechanistic information on the processes affecting PAH fate.

While current attention within the science community is on C_{60}, structure-activity relations are yet to be reported even for these molecules. The spherical-shaped C_{60} may be less reactive than the ovoid-shaped C_{70}, which is considered less aromatic with greater potential reactivity

at the poles than at its equator [117]. Additionally, C_{70} has a greater number of bond types (eight for C_{70} vs. two for C_{60}) and greater local differences in the aromaticity of constituent rings [117]. Currently, it is not known how the size and shape of individual fullerenes affect reactivity and aggregation in environmental waters. Therefore, analytical methods that differentiate between individual fullerene constituents within aggregates are needed to observe and to explain fullerene reactivity and any transformation products formed.

The lack of adequate analytical tools has the potential to generate misleading conclusions regarding processes and pathways. Analytical methods that can differentiate between the myriad of fullerene forms are needed to rationalize transformation product formation and pathways because reactions will occur at the scale of individual molecules that make up fullerene aggregates. Vigilance is required to minimize artifacts and to enable investigators to differentiate loss due to reaction from 'loss' onto laboratory materials, and to explicitly differentiate between, and account for, hydrophobic and polar fullerene forms and their transformation products. For example, the use of oxidants including $Mg(ClO_4)_2$, H_2O_2, and HClO [49,68,118] gave increases in recovery as compared to the extraction with toluene and no oxidant [49]. The explanation offered was that the oxidants promoted 'reduction' of the water-stable fullerene species and that this effect, in turn, improved extraction efficiencies into toluene. Improved recoveries are attributed to salting out upon the addition of salts (e.g., NaCl) [47,80,119]. The present authors compared the recovery of C_{60} prepared by extended (3 weeks) stirring in deionized water using toluene as the extraction solvent with recoveries from salt solutions of similar ionic strength (e.g., 0.1 M $MgClO_4$ and 0.1 M NaCl). The C_{60} concentration $\pm 95\%$ CI obtained by the addition of $MgClO_4$ (54 ± 24 ng/mL; n=4) was statistically equivalent to the concentration obtained using an equivalent molarity of NaCl (66 ± 25 ng/mL; n=4). Although this simple extraction experiment does not preclude the possibility that oxidation occurs upon the addition of $MgClO_4$, equivalent recoveries indicate that a salting out mechanism can account for the increased recovery. Salting out is consistent with the observations that increased ionic strength increases aggregate size [39] and deposition in column studies [120] of water-stable C_{60} aggregates.

One will note that the errors associated with the assigned concentrations obtained upon the addition of $MgClO_4$ and NaCl are large and we attribute this to our use of extraction methodology that is not yet optimized for maximum recovery and precision. However, attention to quality control and mass balance, as well as how the analytical methods or steps selected, can influence data interpretation.

Transport

This section will first discuss the fullerene – specific limitations of current methods for calculating properties beneficial to understanding a chemical's environmental behavior (e.g. K_{oc} and K_{ow}). Then, the implications of increased or decreased suspendability of fullerene nanomaterials in the presence of organic or black carbon or mineral phases will be considered.

Due to the lack of quantitative analytical studies on fullerene nanomaterials in complex environmental systems, it is not currently known to what extent existing paradigms for describing organic chemical partitioning between particulate matter, dissolved organic matter, and the aqueous phase apply to fullerene nanomaterials. It is important to determine what particulate and aqueous-phase characteristics determine the magnitude of fullerene partitioning to natural sediments, soils, and biosolids.

Moreover, it should be determined if partition coefficients can be appropriately normalized to organic or black carbon contents. Because a range of hydrophobic fullerenes occur in soot [25-27,121,122], it is reasonable to assume that fullerenes are among the constituents that make up some forms of black carbon. Partitioning of hydrophobic 'dissolved' organic contaminants

such as natural organic matter are shown to partition to fullerenes [69,113,119,123] through hydrophobic and π-π interactions that are commonly used to rationalize adsorption of hydrophobic contaminants, such as PAHS, to black carbon [124-126]. The possibility that hydrophobic fullerenes form part of the carbon black fraction of natural soils and sediments and so may be one cause of deviations between model estimates and field data on biota-sediment accumulation factors for hydrophobic organic chemicals (e.g., PAHs) [127] should be explored.

Octanol-water partition coefficients (K_{ow}) are recognized as a useful property for predicting other physical properties of chemicals such as the organic carbon-water (K_{oc}) and lipid-water partition coefficients. K_{ow} values prove useful when water solubility values are not readily available or are of questionable accuracy. In the case of C_{60}, its range of extremely low, estimated aqueous solubilities from 10^{-11}-10^{-20} M [42-44] should be treated with caution. To date, there is but one reported log K_{ow} value for C_{60} of 6.67 [44]. Values of log K_{ow} and K_{oc} are not yet reported for aggregated C_{60} suspensions created by extended stirring in water or for lower- or higher-order fullerenes or surface-functionalized fullerenes. Fragment-contribution methods for estimating K_{ow} values [128] cannot be applied to fullerene nanomaterials since the training sets of compounds used to develop existing LFER did not include fullerene-type molecules nor molecules that exist in aggregate form. Inappropriate application of K_{ow} estimation methods to chemical classes outside the training sets can lead to substantial errors [129].

Linear free energy relations (LFERs), used to estimate partition coefficients from K_{ow} values, are predicated on the notion that the mechanisms of chemical partitioning into a solvent (e.g., octanol) are similar to that for partitioning into the phase of interest (e.g., dissolved or particulate-phase organic matter) [130]. Since the mechanisms that govern fullerene nanomaterial partitioning into organic carbon phase or onto mineral surfaces are not well known, the suitability of single or poly-parameter LFERs cannot be established *a priori*.

Although not established, we hypothesize that polyparameter LFERs will likely be needed to explain and predict fullerene interactions with environmental phases. The Lewis acid (electron acceptor) nature of fullerenes [99,131] may result in specific interactions with organic matter and mineral constituents of natural soils and sediments due to EDA interactions. EDA interactions with water were proposed early to explain the formation of water-stable fullerene aggregates [47,49,52,132].

As is the case for nitroaromatic compounds, fullerenes are likely to interact with the accessible electron-donating siloxane oxygens of phyllosilicates. In order to gain a more complete understanding of the effect EDA properties, surface-site density, surface charge, and functional group density of environmental solids have on mobility of fullerene nanomaterials, the EDA properties must be explicitly addressed.

Once EDA interactions are addressed, models (LFERs) can potentially be developed for rationalizing and predicting fullerene transport and bioavailability in environmental systems. While one can speculate on the role of such mechanisms, selective and sensitive analytical methodology will be needed to carry out experiments to test hypotheses regarding the mechanisms of fullerene interactions with natural sorbents. Such experimentation will require the use of model sorbents as well as complex, heterogeneous sorbents and analytical methodology that provide quantitative and interference-free data. Only when analytical tools are available that probe the fundamental molecular nature of all relevant fullerene forms can single or poly parameter LFERs be tested for their validity towards predicting partitioning of fullerenes.

Dissolved organic carbon may also play a role in determining the transport of fullerenes in porous media. Currently there are no data that quantify fullerene partition coefficients between the aqueous and dissolved organic carbon phases. However, there is evidence that the type of organic matter in solution impacts the apparent aqueous solubility of C_{60} in both hydrophobic and water-stirred forms. For example, increases in the apparent aqueous solubility of water-stable C_{60} were observed in the presence of fulvic acids [119] and to a greater extent in the presence of humic acids [112,113,119]. The greater aromaticity and lower charge density of humic acids [119] consequently lead to increased transport of C_{60} [70,123]. Alternatively, the presence of alginate, a polysaccharide-based organic material, increased deposition and decreased transport. This increased deposition is attributed to the large size of the alginate polysaccharide, which is able to encapsulate the suspended polar C_{60} and aid in deposition [123]. Therefore, the type of organic carbon occurring in natural aquatic systems may impact the fraction of fullerene nanomaterials in the dissolved organic carbon phase and in the 'free' fraction in the aqueous phase and their relative mobilities.

Transport tests in columns containing clean glass beads or quartz sand indicate that water-stable C_{60} were less mobile as the ionic strength of the media increased [39,123,133], which is consistent with the decreased transport of other particles with increasing ionic strength [134]. The decreased transport results from a salting out mechanism that leads to increased aggregation and deposition as the ionic strength increases [39,120]. Fullerols show greater mobilities and greater degrees of passage (99%) than water-stable fullerenes (55%) in columns packed with glass beads [135,136]. In contrast, fullerols applied to the columns were recovered while the water-stable fullerenes were retained as indicated by breakthrough experiments. While valuable, complementary data on fullerene transport are needed for more complex heterogeneous systems containing interferences such as other particulate, colloidal, or dissolved organic matter phases. For such investigations, selective analytical methods are needed to validate or 'ground truth' the mobility of the various forms of fullerenes in these more complex aquatic and terrestrial systems containing both background organic and particulate matter.

Biological Effects

In any risk assessment determining the exposure concentration (dose) of the toxicant is of critical importance [35,36]. While there are increasing numbers of reports on the biological effects of fullerene nanomaterials [48,56,68,76,118,137-140], there are few attempts to rigorously quantify the concentrations of fullerenes nanomaterials used in biological exposure studies. Typically, studies are performed with concentrations that are based on the mass of fullerene nanomaterial suspended in a given volume of dosing solution, or are quantified by non-specific total organic carbon or UV measured of the initial suspension. In addition, toxicological studies attempt to make use of the commercial reference materials, which in our experience, are sold with little chemically-explicit information from manufacturers or guarantees of form or purity. Given the infancy of investigations into the occurrence and toxicity of fullerenes, it is not surprising that there is a lack of certified reference materials. Historically, this is typically the case for other contaminants as they 'emerge' into public view. Because 'concentration makes the dose' as well as the form or speciation of the toxicant, exposure concentrations and the form of fullerene should be verified and the methodological approaches to verification should be documented.

To understand transport and the potential for accumulation in biota, it is important to determine the fraction of a chemical that is potentially mobile in aquatic and terrestrial environments. In the case of fullerene nanomaterials, aggregates are not considered truly dissolved and so the term 'freely dissolved' loses its conventional meaning. Conventional wisdom of environmental exposure assessment is that only toxicants that are 'dissolved' are available to elicit biological

responses. Therefore, additional research is needed to determine how aggregate size and surface charge affect the ability of environmental test organisms to uptake aggregated forms. Furthermore, determining the fraction of fullerene nanomaterials in association with particulate and dissolved organic material will permit a more thorough understanding of potential exposure pathways and transport of fullerene nanomaterials.

We conclude that the full exploration of the mechanisms behind fullerene toxicity in terrestrial environments clearly requires information about the molecular properties of fullerenes that make up aggregates. This emphasizes the necessity for analytical protocols that are able to provide chemically-explicit information including molecular weight and the number and identity of surface functional groups.

Acknowledgments

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Table 1

Current quantitative methods for fullerene nanomaterials classified by fullerene species quantified, sample matrix, mode of detection and quantification, recovery ± precision, and method detection and quantification limits.

Author	Species	Matrix	Extraction	Separation/Detection and internal standard (IS)	Recovery ±precision	Method detection/ quantification limits (µg/L)
Heymann et al., 1996 [72]	C_{60}, C_{70} (spiked)	Sediments	Sonication with toluene	LC-UV	80±9 %	NR
Jehlička, et al. 2005 [73][d]	C_{60} (spiked)	Graphite	Soxhlet, Toluene	LC-UV	6-88±NR%	NR
		Bituminous Coal	"	"	0	NR
		Shungite	"	"	0-4.6%	NR
		Quartz	"	"	0-114%	NR
Jinno and Kohrikawa, 1998 [74]	C_{60}-C_{96}	Carbon soot	Toluene Extraction	LC-UV	10-162±NR%[a]	NR
Santa et al. 1995 [75]	Water-stable C_{60} (solvent exchange from benzene and chloroform)	Plasma (rat)	Liquid:liquid extraction with benzene, heptane-isoamylalcohol, or chloroform:methanol Solid phase extraction (C_{18})	LC-UV LC-APCI-MS	ND to 13% 62±4%	500 (LC-UV) 5,000 (LC-APCI-MS) (quantification)
Moussa et al. 1997 [77]	C_{60} (prepared in toluene and injected into mice)	Blood	Liquid:Liquid with Toluene with 0.1 M sodium dodecyl sulfate	LC-UV	90-103±0.8-6%	0.02 (detection)
		Spleen	"	"	100-104±3-8%	"
		Liver	"	"	96-102±2-8%	"
Xia et al. 1999 [79]	Water-stable C_{60} (from THF)	Skin permeation media (5% bovine serum albumin-BSA) Plasma (porcine)	Liquid:Liquid with Toluene and salt	LC-UV	94-100±15%	0.34 (aqueous with BSA) (detection) 0.68 (plasma) (detection)
Isaacson et al. 2007 [76]	C_{60} (prepared in toluene → DMSO)	Embryonic zebrafish homogenate	Liquid:Liquid with Toluene (no salt)	LC-ESI-MS with $^{13}C_{60}$ as IS	90±3%	0.02 (detection) 0.04 (quantification)
		Aqueous exposure solution	"	"	110±10%	"
Chen et al., 2008 [80]	Water-stable C_{60} (solvent exchange from toluene)	Tap water (spiked)	Evaporation to Dryness	LC-APCI-MS	2-35±0.4-5%	2.78 (detection)
			Liquid:Liquid with Toluene with salt	"	32-39±3-7%	3.33 (detection)
			Solid Phase Extraction	"	33-42±3%	0.3 (detection)
Bouchard and Ma [81]	Water-stable C_{60}, C_{70}, & surface-functionalized C_{61} (no organic solvent)	Surface and ground water (spiked)	Liquid:Liquid with Toluene with salt	LC-UV	>85±10%	3-6 (detection) 0.8-2 (quantification)

IS = internal standard; ND= Not detected; NR= Not Reported

[a] recovery reported relative to the recovery obtained by 18 hr Soxhlet extraction with toluene, see Table 5 [74],

[b]LD-MS laser desorption – mass spectrometry,

[c]Electron-impact ionization mass spectrometry,

[d]Sonication and pressurized liquid extraction also performed, but achieved lower recoveries.