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Evaluation of extraction methods for quantification of aqueous fullerenes in urine

Troy M. Benn,

School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, AZ 85287, USA, troy.benn@asu.edu

Benny F. G. Pycke,

Center for Environmental Biotechnology, The Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA

Pierre Herckes,

Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287, USA

Paul Westerhoff, and

School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, AZ 85287, USA

Rolf U. Halden

Center for Environmental Biotechnology, The Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA

Abstract

There is a growing concern about the human and environmental health effects of fullerenes (e.g., C₆₀) due to their increasing application in research, medicine, and industry. Toxicological and pharmacokinetic research requires standard methods for extraction and detection of fullerenes from biological matrices such as urine. The present study validates the use of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) methods in conjunction with liquid chromatography–mass spectrometry (LC–MS) for the quantitative determination of C₆₀ in human and synthetic urine as compared with ultrapure water. Glacial acetic acid, which is necessary to prevent emulsions during LLE, inhibited C₆₀ detection by LC–MS, but this could be mitigated with evaporation. Aqueous C₆₀ aggregates (nC₆₀) were spiked at 180 µg/L into the components of a synthetic urine recipe to determine their individual impacts on extraction and detection. Urea, creatinine, and a complex protein (i.e., gelatin) were found to impair SPE, leading to a low recovery rate of 43±4% for C₆₀ spiked into human urine. In contrast, C₆₀ was consistently recovered from synthetic matrices using LLE, and recovery in human urine was 80±6%. These results suggest that LLE combined with LC–MS is suitable for studying the clearance of fullerenes from the body. LLE is a robust technique that holds promise for extracting C₆₀ from other complex biological matrices (e.g., blood, sweat, amniotic fluid) in toxicological studies, enabling a better understanding of the behavior of fullerenes in human and animal systems and facilitating a more comprehensive risk evaluation of fullerenes.

Keywords

Solid-phase extraction; Nano; C₆₀; Environment; Fate; Chromatography; Mass spectrometry

Introduction

Fullerenes (C_{60} , C_{70} , etc.), geometric caged arrangements of carbon atoms, are increasingly used in consumer products, photovoltaics, electronics, and medical applications due to their novel physical and chemical properties [1–4]. In addition, fullerenes have been detected in geologic strata [5], combustion soot [6–8], and wastewater treatment effluent [9], suggesting an historical and widespread environmental presence. The unique properties of these nanomaterials coupled with the potential for exposure [10–12] causes concern for their possible impacts on human health [13–15] and the environment [16–20]. Quantitative risk assessment of fullerenes has not yet been achieved, partly due to a lack of standardized fullerene solutions as well as robust extraction and analytical techniques capable of quantifying fullerenes in the complex biological matrices of toxicological studies [21, 22]. With the ability to quantify fullerenes in biological fluids such as urine, blood, plasma, milk, and tissue, researchers could determine the body burden from natural and engineered fullerenes as well as investigate the metabolism and/or clearance of fullerenes from the body, thereby providing a more thorough understanding of potential health risks.

Because the use of carbonaceous nanomaterials in many biomedical and industrial applications will increase human exposure, multiple studies have determined the pharmacokinetics of fullerenes and carbon nanotubes in vivo using murine models [23–26]. Most studies show that, in general, the body readily removes fullerenes and carbon nanotubes from circulation and distributes them throughout organs [24]. However, preliminary findings suggest that ~10% of these carbonaceous nanoparticles partition into urine during short-term exposure studies, as measured directly or indirectly through radioactively labeled compounds [24, 25]. Some fullerene derivatives such as *p,p'*-9-bis(2-aminoethyl)-diphenyl- C_{60} were not detected in urine following exposure, which may indicate the absence of efficient renal clearance mechanisms [27]. However, reliable estimates of excretion rates are scarce because nanoparticle concentrations in urine often are not quantified [26, 28], presumably due to difficulties in nanoparticle analysis.

Quantification of C_{60} in various matrices provides many analytical challenges [21, 22]. The earliest efforts to detect fullerenes in environmental matrices were focused on geologic materials. Exhaustive extractions of soil [29] and meteorite samples [30] in various solvents are capable of extracting individual C_{60} molecules for detection with liquid chromatography (LC) coupled with UV–Vis or mass spectrometry (MS), although extraction efficiency can be low [29]. Often liquid-liquid extraction (LLE) is used to extract individual C_{60} molecules from aqueous solutions of fullerene aggregates (nC_{60}) into toluene for LC–UV or LC–MS quantification [16, 31]. Solid-phase extraction (SPE) is a common method for extracting analytes from urine [32–34] and has been used to extract nC_{60} from larger aqueous sample volumes (liter-scale) into toluene [35, 36]. LLE and SPE should therefore be applicable to the extraction of nC_{60} from urine.

Although the behavior of fullerenes in aqueous matrices has been well studied, less is known about fullerene behavior in complex biological matrices. Individual fullerenes are not soluble in water, but aqueous suspensions of nC_{60} can be prepared by using methods such as solvent exchange, sonication, and extended stirring [21]. The size of nC_{60} aggregates is controlled by many factors including the preparation method [16], ionic strength [37], and pH [38]. Although the relatively high ionic strength of urine would suggest an increase in nC_{60} aggregate size and instability, natural organic matter has been recognized to mitigate aggregation [21]. Further complicating the prediction of their behavior in urine, fullerenes demonstrate highly reactive surface chemistry, in some cases adsorbing radical oxygen species [39–42], photoreacting upon UV-[14] or gamma-irradiation [43], reacting with ozone and oxygen [44], or interacting with biological interfaces [45], including DNA [13,

46] and proteins [47, 48]. nC_{60} has been detected in other biological matrices such as plasma, bovine serum albumin [31, 49], and fish embryos [50], demonstrating that the quantification of C_{60} in complex human and/or animal matrices like urine should be possible, although it is yet to be reported.

Considering the potential increase in human and environmental exposure to fullerenes and the emphasis on risk assessment of nanomaterials by the National Nanotechnology Initiative [51], toxicological studies need the capacity to quantify fullerenes in biological fluids such as urine. The clearance of chemicals in urine can impact water quality, as is evident from the detection of endocrine-disrupting compounds and prescription drugs in receiving waters of treated domestic wastewater [52–54]. Quantification in urine will provide a foundation for estimating the environmental load of fullerenes released from wastewater treatment systems. This study applies existing extraction and analytical techniques to the detection of C_{60} in urine to determine method limitations. It is the first to evaluate LLE and SPE with LC–MS detection for the recovery of nC_{60} spiked into synthetic and human urine matrices at microgram per liter levels relevant to toxicological studies.

Materials and methods

Reagents and materials

Optima grade toluene, methanol, and acetonitrile were used for extractions and liquid chromatography (Fisher Scientific, Hampton, NH). ACS grade glacial acetic acid (GAA) was used during LLEs (Mallinckrodt Chemicals, Phillipsburg, NJ). All samples were prepared by using ultrapure water produced by an EasyPure RoDi Water Purification System (Barnstead, Dubuque, IA). All glassware was cleaned in 10% HCl, rinsed with ultrapure water, and baked at 550 °C for 4 h to remove organic contaminants.

Preparation and characterization of nC_{60} stock solution

Aqueous suspensions of C_{60} aggregates (nC_{60}) were prepared as previously described [55] by adding 245 mg of powdered C_{60} (99% purity, MER, Tucson, AZ) to 500 mL of ultrapure water and sonicating for 3 h at 300 W (Ultrasonic Power Corporation, 2000U, Freeport, IL). The suspension was filtered by using 0.7- μ m-pore-size glass-fiber filters (GF/F, Whatman, Maidstone, England) and stored at 4 °C until use. The nC_{60} suspensions were protected from light in amber glass bottles during the experiments and were wrapped in aluminum foil for storage.

Dynamic light scattering was performed by using a ZetaPals 90Plus analyzer (Brookhaven Instruments, Holtsville, NY) to determine the effective particle diameters of nC_{60} in the aqueous stock solution (270 nm) as well as of nC_{60} spiked into synthetic urine and allowed to equilibrate overnight (1,335 nm). The observed increase in particle size is expected due to the salt content of the synthetic urine matrix (see “Synthetic and human urine sample preparation”), as nC_{60} aggregate size is well known to increase with salt content [16, 56, 57].

The final concentration of C_{60} in the nC_{60} stock solution concentration was determined by thermal optical transmittance (TOT), an analytical technique for quantifying organic carbon, carbonate, and total carbon in air samples [58] that can be applied to the characterization of carbon nanomaterial solutions [59]. The nC_{60} stock solution was concentrated by evaporating 10 mL of sample under purified nitrogen and low heat (approximately 80 °C) to a gravimetrically measured volume of 1.09 mL. Fifty μ L of concentrated nC_{60} stock was slowly loaded onto a 1-cm² quartz microfiber filter (QM-A, Whatman, Maidstone, England). The filter was held with forceps while the nC_{60} stock was carefully added dropwise, so as not to allow the filter to become saturated and to facilitate deposition of the aggregates onto

the fibrous filter surface. The nC_{60} was dried at 100 °C overnight prior to TOT analysis (Sunset Laboratory, Tigard, OR). TOT analysis of three replicates indicated a total carbon concentration of the stock nC_{60} solution of 18.1 ± 0.2 mg/L, and it is assumed that this represents the fullerene concentration.

Solid-phase extractions (SPE)

Solid-phase extractions were performed manually using 500 mg/6 mL Strata C18-E cartridges with 55- μm particles and 70-Å pore size (Phenomenex, Torrance, CA). Cartridges were conditioned with 5 mL methanol and washed with 5 mL ultrapure water. Then, 5 mL of sample was loaded at a rate of approximately 0.5 mL/min, followed by a wash with 5 mL methanol. The cartridges were then dried in air for 60 min using a vacuum at -500 mmHg. The cartridges were soaked for 5 min in toluene, and then C_{60} was eluted by using 10 mL toluene. One mL of the toluene eluent was sampled for LC–MS analysis.

The ability of the SPE cartridges to retain nC_{60} from water was tested by using UV–Vis spectrometry. A 10-fold dilution of the nC_{60} stock solution (1.8 ppm) was analyzed at a wavelength of 355 nm in a 1-cm quartz cuvette using a DR 5000 spectrophotometer (Hach, Loveland, CO). Five mL of this sample was then loaded on a SPE cartridge and the effluent collected and analyzed by UV–Vis for comparison.

Liquid-liquid extractions (LLE)

LLE extractions were performed as previously described [31]. In brief, 2 mL of 100 mM $\text{Mg}(\text{ClO}_4)_2$ was added to 5 mL of sample as a destabilizing agent. Glacial acetic acid (11.3 mL) was added to organic matrices to reduce the formation of emulsions [31], and to water and salt solution samples to maintain experimental comparability. Five mL of toluene was added, and the two-phase sample was capped and agitated at 280 rpm on a rotary shaker (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Edison, NJ) for at least 1 h. After adequate phase separation occurred (~ 15 min), 1 mL of the toluene phase was sampled for LC–MS analysis.

Liquid chromatography–mass spectrometry analysis

C_{60} was quantified by using LC–MS as described elsewhere [35]. The LC–MS system consisted of two Varian Prostar 210 pumps coupled to a Varian 1200 triple quadrupole mass spectrometer (Varian, Inc., Palo Alto, CA). Samples were eluted isocratically using toluene and acetonitrile at a 55:45 ratio on a Nova-Pak C18 column (3.9 $\times$ 150 mm) (Waters, Milford, MA) with a constant flow rate of 1 mL/min. An autosampler with a 200- μL sample loop loaded 50 μL of sample followed by 75 μL of acetonitrile prior to injection into the LC–MS. The acetonitrile plug was needed to bring the matrix within the sample loop close to the eluent mixture and facilitate a resolved C_{60} peak at 4.5 min. The temperatures for the atmospheric pressure chemical ionization (APCI) housing, N_2 drying gas, and APCI torch were set at 50, 200, and 350 °C, respectively. Shield voltage was set at -600 V, and the mass detector was operated at a fixed voltage of $-1,200$ V. C_{60} was identified and quantified by using the negatively charged ion of m/z 720 with a scan width of 0.70 amu and scan time of 0.2 s. Calibration was achieved by using dilutions from stock solutions of solid C_{60} (99% purity, MER, Tucson, AZ) dissolved in toluene. All C_{60} was assumed to dissolve completely in toluene, and C_{60} concentrations were therefore determined based on the weighed mass of C_{60} powder added to toluene. The instrument detection limit based on three times the background signal was 3 $\mu\text{g/L}$ for C_{60} (0.15 ng of C_{60} injected).

LLE and SPE matrix interference experiments

The matrix interferences of LLE and SPE in C_{60} detection were investigated by spiking a known quantity of a C_{60} -toluene standard into toluene extractions. Blank samples (without C_{60}) of water, synthetic urine, and human urine were extracted by using the LLE and SPE protocols. Five hundred μL of the toluene extract was mixed with 500 μL of a 550- $\mu\text{g/L}$ C_{60} standard in toluene, then analyzed by LC–MS. In addition, potential interferences from the aqueous solutions used in the LLE protocol were investigated. LLE extraction solutions consisted of 5 mL of water, 5 mL of toluene, and the volume of the target component(s) (i.e., 2 mL of 100 mM $\text{Mg}(\text{ClO}_4)_2$, 11.3 mL of acetic acid, and their combination).

Synthetic and human urine sample preparation

To assess the impact of urine constituents on the detection of C_{60} , nC_{60} was spiked into (1) water, (2) synthetic urine, (3) solutions of synthetic urine constituents, and (4) human urine sample matrices and then recovered by using LLE and SPE with LC–MS quantification. A biologically relevant concentration for the nC_{60} spike was determined through a brief literature review. In toxicology studies, rats are often exposed to nanomaterial concentrations at the level of micrograms per gram of body weight [27, 60]. Although studies of nanomaterial concentrations in excreted urine are rare, it was estimated that up to 10% of intravenously injected carbon nanotubes and fullerene aggregates can be evacuated in urine [24, 25]. Assuming a lower excretion rate of 1% for a 100-g rat exposed to 100 μg of fullerenes, one could anticipate a C_{60} concentration of 200 $\mu\text{g/L}$ in 5 mL of urine (assuming urine production is approximately 5 mL per 100 g per day). Therefore, nC_{60} was spiked at 180 $\mu\text{g/L}$. Five hundred μL of nC_{60} stock solution (1.8 mg/L in ultrapure water) was spiked in 4.5 mL of ultrapure water, synthetic urine, KOVA-Troll III human urine (Hycor, Garden Grove, CA), and the individual constituents of the synthetic urine recipe [61], i.e., aqueous solutions of creatinine (2.0 g/L), urea (25 g/L), and gelatin (1.0 g/L) as well as nutrient broth (0.160 mg/L) (Difco Ltd., Franklin Lakes, NJ, USA), and a salt solution containing 9.0 g/L NaCl, 3.0 g/L $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$, 2.5 g/L Na_2HPO_4 , 2.5 g/L KH_2PO_4 , and 3.0 g/L NH_4Cl . The conductivity of urea, creatinine, gelatin, and nutrient broth solutions was below 55 μS , whereas the conductivity of salt and synthetic urine solutions was approximately 30 mS. The pH of all samples ranged between 5.3 and 7.5. Samples were kept at 4 °C overnight to allow nC_{60} to equilibrate within the matrix before extraction. Synthetic urine and urine component solutions were stored at –20 °C. Lyophilized human urine was reconstituted in 60 mL of nanopure water using gentle mixing and immediately spiked with nC_{60} .

Results and discussion

Determination of extraction matrix interferences on LC–MS detection of C_{60}

Solvent extractions of samples laden with organic compounds (e.g., urine) can potentially lead to matrix interferences in the LC–MS detection of C_{60} . The potential matrix interferences from LLE and SPE were assessed by spiking a C_{60} -toluene standard (240 $\mu\text{g/L}$) into the toluene extract solutions of nanopure water, synthetic urine, and human urine samples (Fig. 1). No interference was observed for the SPE extractions, whereas the LLE solution decreased C_{60} detection by approximately 50%.

To investigate the origin of the LLE matrix interferences, solutions used in the LLE protocol were investigated separately to determine their individual impacts on C_{60} detection. Figure 2a shows that $\text{Mg}(\text{ClO}_4)_2$ alone slightly decreased C_{60} detection ($81 \pm 11\%$), whereas acetic acid decreased detection of C_{60} to approximately 50%. The combined interference of the standard LLE protocol (5 mL aqueous sample, 2 mL 100 mM $\text{Mg}(\text{ClO}_4)_2$, 11.3 mL acetic acid, and 5 mL toluene) decreased C_{60} detection to 35%. Evaporating the sample to dryness

(thus removing the GAA) and reconstituting in toluene mitigated the effect of acetic acid on detection of C_{60} in extracts of water and synthetic urine (Fig. 2b). When 1 mL of the toluene phase was analyzed from LLE of water and synthetic urine, recoveries were approximately 40%, demonstrating the interference of the acetic acid. But when 500- μ L aliquots of the same samples were evaporated under nitrogen and then reconstituted in 500 μ L of toluene, recoveries of the nC_{60} increased to $70\pm 4\%$ and $82\pm 3\%$ for water and synthetic urine, respectively. Signal suppression and enhancement are known issues for APCI-LC-MS [62]. Furthermore, the *Varian Operator's Manual* suggests that acetate can interfere with APCI, and it is likely the matrix interference observed here that is mitigated with evaporation. Other investigations using acetic acid during LLE [31] included an evaporative concentration step during which residual GAA might have been removed, potentially explaining why a matrix interference was not observed. Previous research has shown that evaporating to dryness can impede the recovery of C_{60} at trace levels [31]. To test this loss of recovery due to evaporation, 800 μ L of C_{60} standard in toluene (225 μ g/L) was evaporated under nitrogen in an LC-MS vial and reconstituted in 800 μ L of toluene, and recovery was $93\pm 17\%$. Evaporation of C_{60} to dryness in pure toluene does not appear to affect recovery at these spiked levels (180 ng), which are nine times higher than in a previous investigation (20 ng) [31]. Therefore, LLE samples of synthetic and human urine were evaporated to dryness and reconstituted in toluene prior to C_{60} quantification.

Extraction efficiencies of nC_{60} from water

Inefficient extraction of nC_{60} from aqueous samples could impair detection of C_{60} . The C_{60} recovery efficiencies from water of LLE and SPE were compared with a third extraction technique, that of an aqueous nC_{60} sample evaporated to dryness under nitrogen and reconstituted in toluene. Compared with the $76\pm 8\%$ recovery by this evaporation technique, LLE had a similar recovery of $70\pm 4\%$, whereas SPE had a significantly lower recovery of $40\pm 13\%$. Although evaporation followed by toluene reconstitution recovered the highest percentage of nC_{60} from water, it is not a suitable method for more complex matrices, as C_{60} recovery significantly decreased in the synthetic urine matrix ($17\pm 5\%$).

The inhibited recovery of SPE is most likely due to extraction inefficiencies, as no matrix interference was observed in the toluene eluent of SPE (see previous section). The water sample was collected from the outlet of the SPE cartridge for UV-Vis analysis (355 nm) to determine the quantity of nC_{60} not retained on the SPE cartridge. When 5 mL of 1.8 mg/L nC_{60} solution was subjected to SPE, the water effluent contained 1.0 ± 0.1 mg/L nC_{60} . Therefore, the SPE cartridge retained 45% of the nC_{60} loaded under these conditions. The recovered C_{60} in the toluene eluent as determined by LC-MS was $35\pm 3\%$, suggesting that approximately 10% of the nC_{60} adsorbed to the SPE column was not recovered.

Impact of urine constituents on recovery of C_{60}

The recovery of nC_{60} spiked into water, synthetic urine, human urine, and the isolated components of synthetic urine varied between LLE and SPE (Fig. 3). An equilibration time for the nC_{60} spike in synthetic urine of up to 6 days did not impact the recovery of C_{60} with either LLE or SPE (data not shown). Also, C_{60} recovery from synthetic urine was reproducible (within 3%) over the urinary pH range (5.0–8.0) of healthy subjects (data not shown). Recovery via LLE was relatively consistent at approximately 76%, except for samples containing salt and gelatin solutions, which had recoveries of 50 and 107%, respectively. The negative effect of the salt matrix is unexpected as salts are often used to facilitate the transfer of nC_{60} from the aqueous to the toluene phase in LLE. The increased fullerene recovery from the gelatin solution is currently unexplained and warrants further investigation. SPE exhibited C_{60} recoveries for the different sample matrices ranging from 3 to 60%. As previously shown, the lower C_{60} recoveries of SPE compared with LLE are

attributed to inefficient extraction from the sample as no matrix interference occurred during the detection of C_{60} after the SPEs.

The recovery efficiency of nC_{60} by SPE was severely inhibited by gelatin, urea, and creatinine, as recoveries were $14\pm3\%$, $16\pm5\%$, and $3\pm1\%$, respectively. Gelatin is used in synthetic urine matrices to represent a suite of complex proteins that may be present in human or animal urine, and was isolated to determine its role in inhibiting recovery. Interestingly, the recovery of the synthetic urine matrix including gelatin was $29\pm1\%$, greater than the $14\pm3\%$ recovery of gelatin alone. This demonstrates that the components of the synthetic urine matrix can synergistically reduce the effect of gelatin on C_{60} recovery. Recovery from synthetic urine further increased to $65\pm5\%$ when gelatin was omitted. Interestingly, this recovery was even higher than that from water. This could be attributed to the salt content, which facilitates the formation of larger nC_{60} aggregates that may lead to better retention during loading of the SPE cartridge. These results suggest that organic compounds (gelatin, urea, creatinine) and salts are in competition to control the SPE of C_{60} from biological matrices. LLE provides an alternative extraction method to avoid these complications.

Recovery of C_{60} from synthetic and human urine

C_{60} recovery from human urine was $80\pm6\%$ with LLE compared with $43\pm4\%$ using SPE (Fig. 3). The C_{60} recoveries from synthetic and human urine for LLE are similar to those from the nanopure water matrix, which suggests the urine matrix does not inhibit the extraction of C_{60} . Although SPE performed similarly for water and human urine samples, recovery of C_{60} from synthetic urine (without gelatin) was much higher. This would suggest that a SPE protocol could be optimized to improve the quantity and consistency of C_{60} recoveries from various sample matrices. Unlike SPE, LLE proved to be a robust method for extracting nC_{60} from complex biological matrices such as urine.

Conclusions

This research has investigated the capability of common analytical techniques to detect nC_{60} in urine. Although destabilizing agents such as $Mg(ClO_4)_2$ and glacial acetic acid are necessary during LLE to facilitate transfer of C_{60} into toluene and prevent the formation of emulsions, they interfered with LC–MS detection of C_{60} using negativemode APCI under these analytical conditions. The interference from acetic acid was mitigated through evaporation, yielding C_{60} recoveries from urine matrices of approximately 75%. The recovery efficiency of SPE was less consistent than that of LLE, and was severely impacted by gelatin, urea, and creatinine. The inconsistent performance of SPE suggests further optimization is necessary to make it a suitable method for the extraction of nC_{60} from biological matrices. For example, complex proteins such as gelatin may need to be removed from solution prior to sample loading onto the SPE cartridge to mitigate interferences with C_{60} extraction.

It is important to note that these experiments do not necessarily mimic the form in which fullerenes would be present in real urine samples. In these experiments, stable aqueous C_{60} aggregates were spiked and allowed to equilibrate in urine. In mammalian systems, fullerenes may be oxidized, functionalized, or adsorbed to organic material during metabolism or clearance from the body, and these varying forms of fullerenes will most likely require different detection methods. For example, oxidation of fullerenes within the body may produce fullerene epoxide ($C_{60}O$) molecules, which require specific mass spectrometric detection [21, 63]. LLE followed by LC–MS quantification of C_{60} excreted in urine during toxicological studies would help determine if fullerene transformation within the body is significant.

LLE combined with LC–MS offers a robust detection method for quantifying C₆₀ in urine and should be applicable to other complex biological matrices. The instrument detection limit for this research was 3 µg/L but could be lowered with an increase in the LC–MS sample injection volume. In combination with sample preconcentration steps, method detection limits can reach the biologically relevant nanogram per liter range. Evaluation of drug delivery systems and personal care products (e.g., cosmetics) could benefit from determining the quantity of fullerenes contained in bodily fluids such as blood, sweat, amniotic fluid, and urine. As novel applications for fullerenes are continually discovered, the potential for increased human exposure will create a demand for detection methods applicable to biological samples like those described here.

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Fig. 1. Interference of the extraction solution on the detection of C₆₀ using LC-MS. Blank samples of water, synthetic urine, and human urine were subjected to SPE and LLE. C₆₀ was spiked into the extraction solutions at 240 µg/L. Detection of C₆₀ spiked into LLE matrices was inhibited by approximately 45%. Error bars represent three LC-MS analyses of one sample

Fig. 2.

Impact of LLE solutions on the detection of C_{60} using LC–MS. **a** The four columns on the *left* represent the detection of a C_{60} -toluene standard (240 $\mu\text{g/L}$ final conc.) spiked into the extracted toluene phase of the various components of the LLE protocol. $Mg(ClO_4)_2$ had a marginal impact on detection of C_{60} , whereas the presence of acetic acid lowered the signal by approximately 50%. *Error bars* represent three LC–MS analyses of one sample. **b** nC_{60} was spiked into water and synthetic urine prior to LLE. The recoveries of nC_{60} without (*white*) and with (*blue*) evaporation of the toluene extract indicate the importance of removing acetic acid from the toluene sample to mitigate signal suppression

Fig. 3. Recovery of C_{60} from synthetic and human urine matrices using LLE and SPE. C_{60} was spiked to a final concentration of 180 $\mu\text{g/L}$ and allowed to equilibrate in the media overnight. *Error bars* indicate the variability in quantification between three extractions of each sample matrix