Cyclodextrin-modified nanodiamond for the sensitive fluorometric determination of doxorubicin in urine based on its differential affinity towards β/γ-cyclodextrins

M. Laura Soriano 1✉ Phone+34 957 21 86 16 Emailqa2sodom@uco.es
Carolina Carrillo-Carrion 2
Celia Ruiz-Palomero 1
Miguel Valcárcel 3
1Department of Analytical Chemistry and Institute of Fine Chemistry and Nanochemistry, Marie Curie Building, Campus de Rabanales
AQ1
University of CórdobaE-14071CórdobaSpain
2Bioengineered Particles LabCIC biomaGUNE20009San SebastianSpain
3Spanish Royal Academy of SciencesValverde 24E-28071MadridSpain
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Abstract
The manuscript reports on the preparation of β-cyclodextrin-modified nanodiamonds (βCD-ND) for the extraction and preconcentration of the fluorescent anticancer drug doxorubicin (DOX) from biological samples. The inclusion of DOX into the cavities of the two β- and γ-cyclodextrin (CD) confirms their utility for selective extraction and elution of the drug based on its fitting in the adequate cyclodextrin-sized cavity. Although both larger cyclodextrins (βCD and γCD) accommodate DOX, DOX clearly prefers the biggest γCD cavity. Dispersive micro solid-phase extraction using βCD-ND as sorbent material enables the inclusion complexation of DOX within its βCD cavities. The elution of DOX from βCD-ND cavities occurs with a basic solution of γCD containing 10% acetonitrile owing to the preferential affinity (i.e. optimal fit) of DOX into the larger γCD cavity. DOX is quantified by monitoring its intrinsic fluorescence (exc/em = 475/595 nm). The method can determine DOX in urine with a limit of detection of 18 ng·mL^{-1}. Recoveries (93.2% and 94.0%) and precision (RSDs of 5.9% and 4.7%) at 100 and 400 ng·mL^{-1} DOX levels in urine are satisfactory. The matrix effect is negligible even when working with undiluted urine samples.
Graphical abstract
Nanodiamond functionalized with βcyclodextrin moieties (βCD-ND) were used as sorbent for the determination of nanomolar levels of doxorubicin (DOXox). It is based on host:guest interactions ruled by different stabilities of DOXox within cyclodextrin (CD) cavity-size: βCD/ γCD.

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M. Laura Soriano and Carolina Carrillo-Carrion contributed equally to this work.
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Introduction
The great social interest aroused by nanotechnology in recent decades has promoted the development of carbonaceous nanostructures. Particularly, nanodiamond (ND) [1] consists of a diamond core and a partially graphitized surface. ND possesses a large specific area, chemical inertness and hardness features. ND can be smartly tailored not only by inner or superficial dopings -generally controlled during ND production- but also by surface modification -via covalent attachment of specific groups or (bio)molecules- [2]. Various strategies for surface modification of ND evolves oxidation methods for the attachment of oxygen-containing groups, arylation reactions, Suzuki coupling, phosphonate coupling and click chemistry approaches [3456]. ND is considered as a non-toxic and high-performance scaffold for nanomedicine [378] and for separation processes [9]. However, the number of publications devoted to ND in analytical applications [91011] is rather low if compared to other carbon allotropes such as carbon nanotubes or graphene. ND is mainly involved in solid phase extraction (SPE) and in chromatographic methods [12131415].
Doxorubicin (DOX) is a cytotoxic anthracycline chemotherapeutic agent routinely used to treat diverse cancers. However, its clinical use is limited by their side effects -mainly cardiotoxicity- after long-term treatments [16]. Its determination in body fluids such as urine and plasma has become crucial to assess and control adverse effects coming from cumulative dose. Diverse separation techniques such as electrophoretic

and chromatographic methods have been described for determination of DOX [171819]. In contrast to these expensive instrumentation, voltammetry [20] and fluorometry [21] are also very sensitive for quantifying DOX and mainly involve SPE protocols. Photoluminescence (PL) methods are of great relevance for the fast analysis of DOX in body fluids from its intrinsic fluorescence. However, these methods suffer from serious problems not only related to the low-water solubility of the drug but also its high photochemical decomposition [22].

An interesting approach which bypasses these constrains requires a sorbent material with a large surface area containing multiple highly specific sites to retain DOX. In this direction, it is known that DOX forms inclusion complexes with the cyclodextrins βCD and γCD [23]. The stability of such inclusion complexes is determined by differences in their affinity attributed to the cavity size of the cyclodextrin, being higher for the last one.

It is described the fabrication of a sensitive sorbent towards DOX based on ND covalently tagged with carboxylated β-cyclodextrin (CM-βCD) moieties. The analytical approach to determine DOX using a mere spectrophotometer is described based on the extraction capability of βCD-modified ND towards DOX, and its posterior elution through a straightforward protocol using γCD to form a more stable host-guest inclusion complex. The intrinsic PL signal of DOX enables the monitorization of the drug removal and release during the whole analytical procedure.

This work demonstrates the usefulness of ND functionalized with cyclodextrin as a high-efficiency extracting material and promotes its use as potential nanocarriers of unstable pharmaceuticals in (bio)analytical research.

Experimental

Information of materials and instrumentation are given in the Electronic Supplementary Material (ESM).

Experimental procedures

Nanodiamond derivatization

A novel diazonium approach for the derivatization of nanodiamondsND is applied, following a reaction used for carbon nanotubes [24]. The method is as follows: an amount of ND (514 mg) was ultrasonicated for 3 h in 10 mL of ultrapure water using a 3-mm ultrasonic probe model Vibracell 75,041 (750 W output, 20 KHz) operated at 35% amplitude with on/off intervals of 10:20 s. This suspension was placed in a two-necked flask with a reflux condenser and stirred for 20 min by bubbling nitrogen. Next, 4-[(N-Boc)aminomethyl]aniline (250.5 mg, 1.13 mmol) and isoamyl nitrite (150 µL, 1.11 mmol) were added to the mixture heated to 60 °C. The resulting dispersion was then heated to 80 °C for 24 h, under continuous stirring. After cooling to 30–40 °C the mixture was diluted with dimethylformamide (DMF) and filtered through a nylon membrane (0.45 µm pore size). The resulting solid was first washed repeatedly with DMF-H_2O in sequential cycles of sonication until the filtrate came out colourless. Then, the solid was washed twice with dichloromethane, methanol and diethyl ether and the purified solid was vacuum-dried overnight at room temperature. Afterward, deprotection of Boc was performed by treating the residue with a solution of 4 M HCl in dioxane (2 mL) for 12 h and posterior filtration and washing with ultrapure water until neutral pH. Further washing steps with acetone, ethyl acetate, methanol and diethyl ether were performed for purification. Dryness of sample allows 320 g of amine-ND. To quantify the degree of functionalization, the amount of amine groups per gram of functionalized ND was assessed by Kaiser test (triplicates) using 0.1 mg of sample, yielding a load of 30 $\mu mol \cdot g^{-1}$.

For covalently linkage of βCD moieties, to a suspension of amine-ND (238 mg), triethylamine (5 µL, 0.04 mmol) in 5 mL of dry dimethylformamide, a mixture of NHS (124.4 mg, 1.08 mmol), EDC·HCl (107.5 mg, 0.56 mmol) and CM-βCD (102 mg, 0.08 mmol) in 3 mL of dry DMF was added and stirred at 90 °C for two days under inert atmosphere. The resulting suspension was cooled down to room temperature and filtered. The remaining solid was firstly washed with ultrapure water until neutral pH for then with chloroform, acetone, methanol and diethyl ether. The final solid was dried overnight under vacuum at room temperature. Thermogravimetric analyses and IR spectra of the dried residue were performed. TGA were conducted in a TGA-50 under nitrogen at a heating rate of 5 °C /min from room temperature to 900 °C. TGA profiles indicate a weight loss of approximately 2% as an indicative of cyclodextrin moieties attached to ND surfaces. Kaiser test was used to confirm the absence of free amine groups.

Kaiser test protocol

To determine the amount of amine groups at ND surfaces, Kaiser Tests were carried out in triplicate for each sample as follows: Approximately 1 mg of each type of modified ND was weighed accurately in a test tube and treated with 75 µL of solution I, 100 µL of solution II and 75 µL of solution III of the Kaiser Test kit. The test tubes were sonicated and incubated in an oil bath at 100 °C for 7 min and after that removed from the heat bath for the posterior addition of 2.8 mL of 60% ethanol solution. The absorbance of each sample (pale

yellow or purple solutions) was measured at 570 nm and compared to the unmodified ND used as a blank (pale yellow). The amine loading was calculated from the following equation:
1
The values are expressed as micromoles of amino groups per gram of nanomaterial and are an average of at least three replicates.
Extraction and preconcentration procedures
2 mg of βCD-ND (i.e. sorbent) were placed in an eppendorf flask for the preconcentration of the analyte, and 5 mL of the standard/sample solution was added. The resulting mixture was gently stirred (5 min) and centrifuged (10,000 rpm, 5 min). The supernantant was then removed and the sorbent was treated with the elution solution which consisted of γCD (0.5 $mg \cdot mL^{-1}$) in phosphate buffer at pH 8.5 with a 10% of MeCN content. Stirring for 5 min was carried out to assure the complete elution of the analyte, and followed by centrifugation in order to separate the sorbent from the extracted analyte. Fluorescence analyses were performed for each sample at excitation wavelength of 475 nm and maximum emission of 595 nm. Each emission curve was the average of three scans. Between analysis, the sorbent was sequentially washed twice with MeOH (1 mL) and water (1 mL) assisted with sonication and finally vacuum dried. A schematic illustration of the whole procedure is depicted in Scheme 1.
Style3
Scheme 1
Illustration of the analytical process, including details of the extraction, preconcentration and elution of doxorubicin (DOX). The extraction and preconcentration protocol includes mixing the sample with the sorbent material β-cyclodextrin-modified nanodiamonds (βCD-ND). After removal of the supernatant, the residue is treated with the eluent composed of a phosphate buffer containing γ-cyclodextrin (γCD) in 10% of acetonitrile (MeCN). After stirring and centrifugation, the supernatant containing the drug stabilized by γCD is analyzed by fluorometry
Urine samples
20 mL of urine sample was spiked with appropriate amounts of DOX stock solution (10 $\mu g \cdot mL^{-1}$ in water). Samples were spiked at different concentrations (a low one at 0.1 $\mu g \cdot mL^{-1}$ and high one at 0.4 $\mu g \cdot mL^{-1}$) covering the working range. The spiked samples were incubated during 2 h to ensure the complete interaction of the analyte with the matrix sample. Spiked samples were analyzed independently in triplicate to evaluate the repeatability of the analytical method in real samples. After the incubation time, 2 mL of MeCN wereas added to 20 mL of urine sample in order to precipitate the proteins, followed by centrifugation at 5000 rpm for 10 min. The liquid supernatant was taken and directly subjected to the analysis procedure as previously described.
Results and discusion
Choice of materials
Compared to ND, carbon nanotubes or graphene are poorly dispersed in solvents and possess high tendency to aggregate owing to the π-π stacking interactions, making them poor support materials. The low toxicity of ND compared to other nanoparticles (e.g. biocidal metallic nanoparticles) makes it the best choice for sorbent preparation.
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It is known that βCD and γCD form host:guest inclusion complexes with DOX. Considering the higher stability of DOX onto γCD, the ND used as sorbent material need to be tagged by βCD for interacting in less extend with DOX.
Preparation and characterization of βCD-ND
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The detonated ND with size of 40–50 nm is well-known to exhibit oxygen-containing groups but also graphitic structure at the surface. Such distinct features of ND compared to other carbon nanomateriales allows attaching many different functional groups to their surface for creating very sophisticate surface modifications [4]. Due to the high tendency of ND to aggregate, the usefulness of such nanomaterials is quite challenging and most of authors started to incorporate carboxyl groups onto ND by different oxidation procedures. In contrast, some authors used the graphitic shells [325] of ND by means of Diels-Alder reactions [26] and diazonium chemistry [527]. Within the latter case, we describe a more restrictive functionalization procedure to start with diazotization of the areas containing sp^2 carbon graphitic surfaces of ND with aniline monoprotected derivatives in presence of isoamyl nitrite. This procedure does not allow the

functionalization of the complete surface, thus preserving most of their main superficial structure for further modification in order to achieve multifunctional platforms depending on the desired applications.
Starting with pristine ND (originated via detonation production) the surface loading with monoprotected aminomethylaniline is only achieved when isoamyl nitrite was added to the reaction media, as a result of a covalent diazotization of the sp^2 superficial zones of ND. To identify the number of molecules attached to the surface, a deprotonation of the Boc-protected amines was performed in acidic treatment with 4 M of HCl, as previously described [24]. Kaiser tests were carried out to estimate the amount of free amine groups per gram of ND. As expected, the amount of amine groups given by Eq. 1 was of 30 $\mu mol \cdot g^{-1}$.
For the second step, the amine-ND was added to a degassed activated CM-βCD solution for facilitating the formation of amide bonds. After purification, the resulting βCD-ND was subjected to Kaiser tests confirming the absence of free amine groups at their surface.
Differences in charge after functionalization with CM-βCD were determined by Zeta potential measurements, observing a significant change for a-ND (being close to 0 mV at the working pH value) and for βCD-ND (−30.4 mV). Interestingly, no isoelectric point was registered for βCD-ND. A good colloidal stability is suggested for the high negatively charged CD-ND.
TGA profiles for the modified ND are depicted in Fig. 1a, in which a weight loss of 2% is due to the cyclodextrin moieties attached onto ND surface.
Style3
Fig. 1
a Thermogravimetric profiles of unmodified (raw ND), amine- (a-ND) and βcyclodextrin-modified (CD-ND) nanodiamonds under N_2 atmosphere. b X-ray diffraction patterns of raw nanodiamonds (raw ND) and βcyclodextrin-modified nanodiamonds (βCD-ND). c–d Micrographic images of cyclodextrin-modified nanodiamonds performed by TEM
The crystal size and crystallinity of βCD-ND were examined by TEM and XRD, as depicted in Fig. 1. The mean peaks at angles of $2\theta = 43.9$ {111}, 75.6 {220} and 91.5° {311} reflexions shown in the XRD pattern (Fig. 1b) are indexed to the cubic carbon diamond structure. By applying the Scherrer equation, the ND mean size resulted to be ca. 20 nm ($n = 70$). Comparing with raw material (pristine-ND), XRD data did not show significant changes of the diamond lattice parameters, but the crystallinity of functionalized ND increased considerably whereas their nanoparticle size decreased. Thus, the functionalized ND displayed prominent peaks at angles corresponding to the crystalline structure of
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diamonds. TEM images depicted in Fig. 1c–d showed nanoparticles of mean sizes between few tens of nanometers while biggest pieces were mainly discarded during functionalization.
Here, the new strategy for the stable grafting of cavitand moieties onto the surface of ND using Tour reaction opens the possibility for conjugating other cavitands and even bioactive moieties, which may be very useful in the field of drug search and delivery.
Sorbent capability towards doxorubicin
The role of the cyclodextrin moiety is to form host-guest inclusion complexes with specific molecules with a high affinity to be accommodated in the cyclodextrin cavity. Only those with specific size and shape are accommodated and stabilized inside the cavity.
In order to investigate the active surface area of βCD-ND, nitrogen adsorption and desorption measurements (Fig. S1). BET specific surface area of βCD-ND resulted to be of 70.7 $m^2 \cdot g^{-1}$. The total volume of pores resulted to be of 29.7 $cm^3 \cdot g^{-1}$. Respective information and Fig. S1 are shown in ESM.
Extraction of DOX was performed only for βCD-ND, while no extraction occurred for oxidized ND either amine-modified ND. Results from the SPE preliminary experiments confirmed the inclusion complexation of the drug by the suitable βCD-ND host. In fact, the suitable host displayed no isoelectric point in the range of pH spanning from 2 to 10. For more details, see ESM.
Elution solution
Optimization of diverse parameters to get the most suitable eluent was carried out. Information about the selection of the solution composition, concentration and pH are given in the ESM. Owing to the recognized destabilizing effect of organic solvents on host-guest complexes, the addition of small amounts of MeCN resulted to be crucial for the desorption of DOX from the sorbent.
The experimental conditions shown in ESM indicated that best results for the elution of DOX are given with a phosphate buffer at pH 8.5 containing γCD (at 0.5 $mg \cdot mL^{-1}$) with a 10% of MeCN.
Analytical figures of merit

The analytical method was characterized by determining their analytical performance in order to evaluate its usefulness for quantitative analysis. DOX was detected based on its intrinsic fluorescence, with maximum emission at 595 nm under excitation at 475 nm.

Under optimized conditions, analytical features such as linearity, precision and limits of detection and quantification were determined. The calibration curve came of using standard solutions of DOX at different concentrations over the range 50–500 $ng \cdot mL^{-1}$, which were subjected to the optimized analytical procedure described in the Experimental

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section (Fig. 2). A good linear relationship over the whole working range was observed, showing a correlation coefficient of 0.996. The limits of detection and quantification, calculated following the 3σ and 10σ IUPAC criteria, respectively, were 18 $ng \cdot mL^{-1}$ and 59 $ng \cdot mL^{-1}$. Therefore, the method has enough sensitivity to be applied to the determination of DOX in biological samples.

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Fig. 2

Calibration curve made from photoluminescence (PL) measurements of doxorubicin after being subjected to the analytical procedure, considering the excitation and emission wavelengths of 475 and 595 nm, respectively. Inset: Representative emission spectra at the different concentrations assayed in $\mu g \cdot mL^{-1}$ (emission and excitation slit widths fixed at 5 nm)

The enrichment factor, calculated as the ratio of slopes of the calibration graphs made from the measurements of the standard solutions before and after being subjected to the preconcentration procedure, was 9.2.

The precision of the method expressed as relative standard deviation (RSD) was determined in terms of repeatability (n = 3) at concentration level 0.1 $\mu g \cdot mL^{-1}$ obtaining a RSD value of 4.0%. The batch-to-batch reproducibility using three different batches of βCD-ND was also evaluated, obtaining a RSD of only 4.5%.

It is well-known that DOX is chemically unstable in aqueous solutions and is subject to photochemical decomposition [22]. However, the physicochemical properties of free drug molecules are different from those bound to the cyclodextrin molecules. Apart from others, the chemical stability and photostability is a property which can be strongly affected by the inclusion of a molecule in a host-guest complex. To test the photostability of the γCD-DOX formed during the elution step, the PL intensity at the maximum emission (595 nm) was recorded during 3000 s under continuous illumination at 475 nm, which is the maximum excitation wavelength of the DOX. The kinetic of photodegradation was also measured for the free DOX molecules under the same conditions. As shown in Fig. S2, it is clear the photostabilization of the DOX after their complexation with the γCD. As the drug is stabilized in the elution solution, its measurement has not to be carried out immediately after elution, thus facilitating the analysis of several samples in parallel. This fact contributes also to the good repeatability of the method.

Application to urine samples

In order to validate the method, spiked urine samples (prepared as described in the method section) were analyzed under the optimized conditions of the analytical method. In order to cover the whole working range, two concentrations levels (low and high) were tested. In both cases, the recovery was very similar and quite satisfactory, with values of 93.2% and 94.0% at 0.1 and 0.4 $\mu g \cdot mL^{-1}$, respectively. The precision was determined as the relative standard deviation (RSD) from three independently analyzed samples (n = 3). Additionally, the accuracy of the method was calculated to indicate the closeness of the measured result (i.e. resulting concentration found) to the true value (i.e. spiked concentration), and expressed in terms of relative error. Both parameters were evaluated at two concentration levels. From the results depicted in Table 1, the method presents both good precision and good accuracy working with real samples.

Table 1

Precision and accuracy of the method in urine samples (n = 3)

Concentration ($\mu g \cdot mL^{-1}$)	**Precision (RSD %)**	**Accuracy (error %)**
0.1	5.9	- 6.4
0.4	4.7	- 7.3

From a practical point of view, it is important to note that the application of the method to real urine samples involves only a single step of pretreatment procedure for protein precipitation. The sorbent is not able to extract metal ions either larger biomolecules. Even when urine also displays intrinsic fluorescence, this is not a problem in our method owing to two facts. First, the native or intrinsic fluorescence emission of the urine is in the blue-green region but not at the wavelengths (around 600 nm) used in the present work. Second, the

analytical procedure comprises removing the sample after their extraction with the βCD-ND, and therefore the analyte is finally measured in the elution solution, free of potential interferences from the urine sample. The accuracy of results is confirmed after a comparison with other fluorometricanalytical methods as well as with including voltammetric and chromatographic techniquesmethods (see Table 2). It is clearly observed that voltammetry is indeed a very sensitive for detecting DOX although the fluorometer methods gave rise to similar or better recoveries. Although the sensitivity of methods that combined both SPE and liquid chromatography coupled to tandem mass spectrometry (HPLC-MS7MS) [19], the fluorometry methods gave rise to better recoveries. The superior performance of the βCD-ND towards this drug is attributed to their high affinity to the inner cyclodextrin cavities located at ND surface.

Table 2

An overview on recently reported methods for the determination of doxorubicin in biological samples

Method	Material	Role	Sample	Figures of merits		Ref.
				LOD	Recovery (%)	
Differential-pulse voltammetry	GQD-glassy carbon electrode	Sensor	Human plasma	16 nM	≥90	[28]
Voltammetry	MWCNTs & $CoFe_2O_4$ NPs	Sensor	Urine	0.01 nM	≥93	[29]
HPLC	C-18	Reverse phase	Rat plasma	500 $ng \cdot mL^{-1}$	≥91	[18]
SPE + HPLC/MS/MS	C-18	Sorbent	Urine	0.2 $ng \cdot mL^{-1}$	≥72	[19]
Frequency-domain fluorometry	$Ru(dpn)_3$ NPs	Lifetime reference	Urine	400 nM	≥97	[30]
SPE + Fluorometry	CD-ND	Sorbent	Urine	33 nM 18 $ng \cdot mL^{-1}$	≥93	This work

It should be also pointed out that the matrix effect was negligible working with non-diluted urine samples, so it could be easily extended to other clinical samples such as plasma.

Conclusions

ND has been modified with cyclodextrin cavitands and used as an analytical tool for sensing of DOX in biological samples. The analytical method consists of SPE with βCD-ND as sorbent material and detection of DOX intrinsic fluorescence using a mere spectrofluorometer. The potential of the method has been demonstrated for the analysis of human urine samples. The large surface area of ND containing multiple βCD, combined with the differential affinities of βCD and γCD towards the drug, allowed a sensitive method. The good batch-to-batch reproducibility of the method is outlined, which has allowed its transfer to real applications. Other important contribution lies on the stabilization of chemically unstable drugs, which integrity is preserved until the analysis or other further uses. This method is limited by possible interferences like fluorescent molecules with similar structure and size than DOX, only if they exhibit an orange-red fluorescence.

In the future, we will apply this useful nanoplatform for grafting cavitands with high affinity to other drugs. Besides, this work does not only open the way for novel applications of ND as nanotools in the detection of anticancerogenic drugs as targets but also in the stabilization and transportation of chemically unstable pharmaceuticals as nanocarriers for drug delivery.

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Compliance with ethical standards

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The author(s) declare that they have no competing interests.

Abbreviations

ND	Nanodiamonds
CD	Cyclodextrin
βCD	Beta-cyclodextrin

γCD	Gamma-cyclodextrin
βCD-ND	β-cyclodextrin-functionalized nanodiamonds
DOX	Doxorubicin
NHS	N-hydroxysuccinimide
EDC·HCl	1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride
PL	Photoluminescence
FT-IR	Fourier Transform infrared spectroscopy
TEM	Transmission electron microscopy
XRD	X-ray diffraction
TGA	Thermogravimetric analysis
MeCN	Acetonitrile
RSD	Relative standard deviation
HPLC	High-performance liquid chromatography
HPLC-MS/MS	Liquid chromatography tandem-mass spectrometry
SPE	Solid phase extraction

Electronic supplementary material
ESM 1
(DOCX 206 kb)

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