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Magnetic nanoparticles of nitrogen enriched carbon (mnNEC) for analysis of pesticides and metabolites in zebrafish by gas chromatography–mass spectrometry

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ABSTRACT

Nanosized carbon based sorbents have been widely used for separation, enrichment and desalting of biological samples because of their distinguished characteristics. In this work, magnetic nanoparticles of nitrogen enriched carbon (mnNEC) have been developed for enrichment of organochlorine pesticide DDT and metabolite DDE that have been accumulated in zebrafish during the course of environmental exposure. Polymerization of pyrrole was performed in the aqueous suspension of Fe_3O_4 nanoparticles. Resultant core–shell nanoparticules coated with polypyrrole were then subjected to a process of carbonization under high temperature and nitrogen atmospheric condition. The presence of nitrogen atoms in carbon nanoparticles increases the hydrophilicity and dispersability in aqueous samples. It has been experimentally demonstrated that mnNEC can be effectively dispersed in aqueous samples and rapidly isolated by the application of an external magnetic field. Recoveries of DDT and DDE from water range from 90% to 102% and 85–97% respectively. In combination with Selected Ion Monitoring (SIM) experiments of gas chromatography–mass spectrometry, the detection limit can be down to low ng/mL level. By using mnNEC approach, two interesting results have been found for zebrafish with 60 days exposure to DDT (1 μg/l). (1) There is higher concentration of DDT (37–143 ng/g) and DDE (173–1108 ng/g) in male zebrafish body tissues than that of female (7–52 ng/g and 146–362 ng/g for DDT and DDE respectively). (2) There is high ratio of DDE/DDT for both female and male zebrafish, implying high environmental persistence and ongoing bioaccumulation.

1. Introduction

Solid phase extraction (SPE) has been the most widely used sample preparation method [1–3]. Compared with liquid–liquid extraction (LLE) approach [4–6], SPE not only significantly decreases the usage of organic solvents but also provides an efficient means with improved recoveries for handling samples that have partial solubility in the aqueous phase. In order to solve more and more emerging analytical problems, research efforts have been continuously focused on the development of different types of SPE formats and sorbents [7–9].

Currently, syringe cartridge [10,11] and disk [12,13] are two major popular SPE formats. Limitations of these techniques include restricted flow rates and plugging of the separation beds, especially when samples containing suspended particles are analyzed. Another direction under development is the sorbent technologies [14,15]. Different kinds of reversed/normal-phase silica [16], copolymer sorbents [17], carbon-based sorbents [18], ion pair and ion exchange sorbents [19,20], immunoaffinity sorbents [21] and molecularly imprinted polymers [22] have been designed for analysis of different samples with different properties. Recent trends even move to simultaneous extraction of analytes with a wide range of polarity, water solubility, ionization property and volatility because of the needs in environment science, pharmaceutics and other research fields in which the identity and quantity of degradation products or metabolites are required [23–25]. Subsequently, mixed-mode sorbents are specially made for such purposes [26]. For example, non-end capped C18 silica was designed so that non-modified silanol groups at the bonded silica surface can be functioned for the secondary polar interactions with analytes [27]. In this work, we are describing a new magnetic SPE based on nitrogen-enriched carbon nanoparticles. The magnetic format eliminates the limitations of flow rates and clogging of packed beds. The presence of nitrogen atoms in carbon nanoparticles provides secondary hydrophilic interactions with analytes in addition to hydrophobic interactions. Because of the enhanced hydrophilicity,

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homogeneous dispersion of nitrogen-enriched carbon nanoparticles in the aqueous phases can be then expected to improve the recoveries of target analytes. The proposed mnNEC has been used to analyze DDT (dichlorodiphenyltrichloroethane) and metabolite DDE (dichlorodiphenyldichloroethylene) in zebrafish that have been subjected to DDT exposure.

Pesticides have been used world-widely in order to prevent or control pests, weeds, plant pathogens or diseases [28]. Serious concerns have been raised about agricultural product safety and public health risks resulting from environmental exposure or pesticide residues in food [29]. Because most pesticides such as organochlorine DDT and metabolite DDE have strong resistance to metabolism and very low solubility in water, they tend to accumulate in fat tissue [30]. Enrichment of target molecules in such samples by using conventional types of SPE such as cartridges and disks is challenging due to the presence of greasy tissue particles. It has also been found that fat tissue particles are difficult to remove by centrifugation because they float on the surface or suspend in the aqueous medium [31]. Activated carbon or recently developed different kinds of carbon nanotube have been developed for adsorption of such environmental pollutants [32–35]. The major difficulties include: (1) difficulties in uniform dispersion of highly hydrophobic carbon or carbon nanotube in aqueous samples due to increased surface area and consequently increased attractive forces among nanoparticles. Surfactants [36] and surface chemical modifications [37] have to be used in sample preparation. (2) Difficulties in sedimentation of solid sorbents. It usually takes time to collect nanosized particles by centrifugal forces [38]. (3) Difficulties in desorption of analytes by suitable solvents due to the entrapment of analytes inside the nanopores [39]. The proposed mnNEC approach takes the advantages of lone-pair electrons in nitrogen atoms that not only increase hydrophilicity and dispersibility in aqueous samples but also provide the additional affinity to charge-deficient atoms in target molecules. Large surface to volume ratio of nanoparticles further enhances the adsorptive capacity without entrapment of analytes inside nanopores. Finally, enriched analytes can be rapidly isolated from the bulk background of samples by the application of an external magnetic field and desorbed by suitable solvents. The mnNEC approach has been applied to analysis of not only standard DDT and DDE spiked in water but also those accumulated in zebrafish after 60 days of exposure to DDT.

2. Materials and methods

2.1. Reagents and apparatus

LC–MS grade water and *n*-hexane (HPLC grade) were purchased from Fisher Scientific (NJ, USA). Acetone, ethanol, $FeCl_3$, $FeCl_2$, anhydrous Na_2SO_4, NH_4HCO_3, and ammonia hydroxide of analytical reagent were purchased from Guoyao, China. Pyrrole and ammonium persulfate were purchased from Sigma–Aldrich (MO, USA). Standard 4,4′DDT and DDE were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Cell lysis buffer was purchased from Shenergy Biocolor BioScience & Technology (Shanghai, China). Ultrasonicator was purchased from Kunshan (China).

2.2. Preparation of magnetic nanoparticles of Fe_3O_4

Stock solutions of 1 M $FeCl_3$ and $FeCl_2$ were prepared in 2 M HCl respectively. Mix 10 ml of 1 M $FeCl_3$ solution with 5 ml of 1 M $FeCl_2$ solution at room temperature. Add ammonia hydroxide drop by drop with stirring to the mixture until the pH value of the solution reached 11–12. Keep stirring at room temperature for 30 min and then wash the magnetic nanoparticles three times by water and three times by ethanol. Resultant magnetic nanoparticles were kept in ethanol. Before use, these nanoparticles were washed by pure water.

2.3. Coating of Fe_3O_4 nanoparticles by polypyrrole

Magnetic nanoparticles of Fe_3O_4 were suspended in 20 ml of pure water and subjected to 5 min of ultrasonication. Then 7.3 mmol pyrrole was added to the suspension drop by drop with stirring. The mixture was ultrasonicated for 10 min again in order to mix pyrrole with nanoparticles thoroughly. Then 3.6 mmol ammonium persulfate was added to the mixture drop by drop and then the solution was subjected to ultrasonication for another 1 h. Resultant nanoparticles were washed by three cycles of pure water and ethanol until pH ~7. These particles were dried at 60 °C and characterized by FT-IR (Fourier Transform Infrared, Thermoscientific, USA) spectroscopy. Supplementary Fig. 1 (A–C) are FTIR spectra of bare Fe_3O_4, bare polypyrrole, and polypyrrole coated Fe_3O_4 respectively. In the IR spectrum of bare Fe_3O_4, absorptions at ~3400 cm^{-1} and ~1630 cm^{-1} attribute to the stretching and bending vibrations of O—H or N—H groups respectively that are absorbed on the surface of Fe_3O_4 nanoparticles. The featured very strong peak at ~580 cm^{-1} results from the vibration of Fe—O bond [40]. In the IR spectrum of bare polypyrrole, absorptions at ~1550 cm^{-1} and 1450 cm^{-1} attribute to the backbone ring vibrations and the peak at ~890 cm^{-1} results from the out-plane vibration of =C—H, indicating the polymerization of pyrrole. Instead, peaks at ~1320 cm^{-1} and 1050 cm^{-1} attribute the in-plane vibration of =C—H bond. The peak at ~1180 cm^{-1} comes from the N—C stretching vibration [41,42]. It has been shown that the IR spectrum of polypyrrole coated Fe_3O_4 nanoparticles combines both the features of Fe_3O_4 and polypyrrole. So it has been experimentally demonstrated that polypyrrole has been successfully coated on the surface of Fe_3O_4 nanoparticles.

2.4. Carbonization of polypyrrole coated Fe_3O_4 nanoparticles

Carbonization of polypyrrole coated Fe_3O_4 nanoparticles was carried out under nitrogen atmospheric condition at different temperatures including 250 °C, 300 °C, 350 °C, 400 °C, 450 °C, 500 °C, 550 °C and 600 °C. The morphology of resultant particles was characterized by Scan Electron Microscope (SEM, JEOL, Japan). Supplementary Fig. 2 shows the SEM pictures of these particles treated at different temperature. When temperature was increased to more than 400 °C, particles start to aggregate. In particular, particles with flower shapes were observed at 600 °C. In order to evaluate the carbonization process, resultant nanoparticles have also been subjected to an element analysis of C, N and H by using Vario EL III (Elementar GmbH, Germany). The spontaneous breakdown of chemical bonds and formation of carbonized residues were demonstrated by FT-IR.

2.5. Sample preparation for standard DDT and DDE analysis

Quantification of target analytes was performed in Selected Ion Monitoring (SIM) mode. Fragment ions at m/z = 235, 237 and 165 were selected for quantitative analysis of DDT. Fragment ions at m/z = 246, 248 and 318 were selected for quantitative analysis of DDE. A series of standard solutions of *n*-hexane containing 0.01, 0.05, 0.11, 1.00, 4.97 ng/μl DDT and 0.005, 0.01, 0.05, 0.50 and 2.50 ng/μl DDE respectively were prepared and 1 μl of each standard solution was injected into GC–MS (gas chromatography–mass spectrometry) for preparation of calibration curve. To investigate the recoveries of DDT and DDE from water by using the proposed method, standard DDT (2.05 ng/μl) and DDE (2.12 g/μl) have also been prepared as stock solutions in ethanol. Spike 200 μl DDT stock solution into 150 ml of water and the concentration of DDT residue is 2.7 μg/l, which is lower than

the environmental concentration of DDT residue reported in literatures [43]. For DDE experiments, 200 μl DDE stock solution was spiked into 100 ml of water and the concentration of DDE residue is 4.24 μg/l. Then about 5 mg synthesized magnetic nanoparticles of nitrogen enriched carbon were suspended in 10 ml of DDT or DDE spiked water. The sample was vortexed for about 5 min so that the magnetic sorbents can be dispersed uniformly into the aqueous sample thoroughly. And then an external magnetic field was applied to isolate sorbents from the bulk sample. Enriched DDT or DDE was eluted by 80 μl of elution solution (acetone:*n*-hexane = 1:1 in volume). Pipette supernatant solutions into clean glass vials and several anhydrous particles of sodium sulfate were added in vials in order to absorb trace water in the samples. The presence of water may cause GC column bleeding.

2.6. Sample preparation for analysis of DDT and metabolite DDE residues in zebrafish

Female and male zebrafish that have been subjected to 60 days exposure of DDT (1 μg/l) were chosen for demonstration of the proposed approach. In this work, only body tissues were analyzed and internal organs have been taken out for other research purposes. Each fish body was weighted before homogenation with 5 ml of lysis buffer on ice. Take 1 ml of the tissue suspension from each sample to mix with 5 mg magnetic sorbents and vortex 5 min. Enriched analytes were eluted by 80 μl of elution solution and stored in clean glass vials. Several particles of anhydrous sodium sulfate were added in the samples to absorb trace water.

2.7. GC–MS analysis of DDT and DDE

Inject 1 μl *n*-hexane solution of DDT or DDE samples prepared by the previously described procedure into a capillary gas chromatography (Rtx-5MS, 0.25 μm in thickness, 30 m × 0.25 mm i.d., USA)–mass spectrometry system (QP2010PLUS, Shimadzu, Japan). The column oven temperature was optimized by using standard DDT and DDE. The initial temperature was set as 60 °C and then it was programmed to 200 °C at the rate of 20 °C per minute (held at 200 °C for 10 min), then from 200 °C to 250 °C at the rate of 10 °C per minute. The column was maintained at 250 °C for 10 min before completing the whole program. During the whole process of analysis, the injector temperature was kept at 200 °C. Splitless injection was performed with 1 min sampling time. The interface temperature was set at 270 °C and the ion source was maintained at 200 °C. 70 eV ionization energy of impact ionization (EI) was used to fragment the eluents from the capiliary GC. Mass spectra were recorded in either full scan or SIM modes with mass-to-charge ratio (m/z) ranging from 50 to 600 unit.

Unambiguous identification of DDT and DDE was achieved by searching against NIST database and comparison of the chromatographic retention time of standard DDT and DDE with that of experimental results. The search results were also interpreted manually according to mass spectra characteristics including molecular weight, isotopic patterns and fragment ions.

3. Results and discussion

3.1. Principle and feasibility of mnNEC approach

Nanosized carbon-based sorbents including every kind of carbon nanotubes are attracting more and more interests as an efficient solid phase extraction approach because of its unique features such as stability in acidic and basic experimental conditions, tolerance in most organic solvents and high surface area-to-volume ratio. While these features are in favor for adsorption of analytes, the elution of analytes from nanopores is difficult. In addition, the highly hydrophobic property of carbon-based sorbents makes it difficult to uniformly disperse in the aqueous samples. It is also hard to take the formats of either packed cartridge due to high backpressures or isolation by centrifugation due to the slow sedimentation process. The mnNEC approach is a new mixed mode SPE with magnetic format and nanosize characteristics. It is based on the following principles. Nitrogen atoms introduced in the carbonized materials increase the hydrophilicity and thus dispersability in aqueous samples, and also enhance the affinity to charge deficient atoms in samples. Particle size synthesized in this work is about 20–30 nm, providing high surface area-to-volume ratio that can afford enough force for mass diffusion. The magnetic format allows rapid separation of nanoparticles from bulk sample solutions by applying an external magnetic field.

As described in the experimental section, magnetic nanoparticles of Fe_3O_4 were synthesized by a co-precipitation chemical method. Then pyrrole was added to the suspension of Fe_3O_4 nanoparticles and undergo *in situ* polymerization. The reason to choose polypyrrole for coating of nanoparticles is that the polymerization reaction of pyrrole monomer on the surface of Fe_3O_4 nanoparticles is simple and can form uniform coating. FT-IR experiments confirm the successful coating as shown in Supplementary Fig. 1. Carbonization was carried out in the nitrogen atmospheric condition at different temperature as described in the experimental section. All SEM pictures were shown in Supplementary Fig. 2 and element contents of N, C and H were listed in the figures. It was shown that nanoparticles obtained at 350 °C are almost spherically shaped and about 20–30 nm in size. Increased temperature such as 550 °C and 600 °C not only causes agglomeration but also results in losses of N, C and H elements. Formation of carbonized materials was demonstrated by FT-IR shown in Supplementary Fig. 1(D). Featured vibrational absorptions of polypyrrole at 1550 cm^{-1}, 1450 cm^{-1}, 1320 cm^{-1}, 1050 cm^{-1}, 1180 cm^{-1} and 890 cm^{-1} all significantly eliminated. Broad absorptions at ~1590 cm^{-1} and 1360 cm^{-1} correspond to featured absorptions of carbon.

Because of the presence of nitrogen atoms and adsorbed O—H groups in the surface, synthesized magnetic nanoparticles of Fe_3O_4 can be readily dispersed in the aqueous sample without addition of any surfactants as shown in Supplementary Fig. 3(A). And thus maximum interactions between sorbents and analytes can be achieved. Isolation of these magnetic nanoparticles can also be rapidly carried out within 10 s (Supplementary Fig. 3(B)). In contrast, Supplementary Fig. 3(C) shows that carbon nanotubes cannot be dispersed in the sample solution due to strong hydrophobic interactions with the vial wall.

3.2. Recoveries of DDT and DDE from water by mnNEC approach

In this work, quantification of DDT and DDE was performed in SIM mode by using a GC–MS system. A series of standard solutions of DDT and DDE with different concentrations has been prepared as described in the experimental section. Standard samples were first analyzed in a full scan mode in order to select suitable ions for SIM experiments. Mass spectra of DDT and DDE are shown in Fig. 1(A) and (B) respectively. Set-in figures are standard calibration curves that show excellent linearity in 0.005–2.50 ng and 0.01–4.97 ng ranges with correlation coefficients 0.99998 and 0.99929 for DDT and DDE respectively. Because DDT concentration in biological samples is usually lower than that of DDE, we have chosen lower level of DDT for preparation of DDT calibration curve. All standard samples have been injected and analyzed automatically.

Known amounts of DDT and DDE have been spiked in tap water in order to demonstrate the performance of mnNEC approach. The final concentrations of DDT and DDE in prepared water samples are 2.73 ng/ml and 4.24 ng/ml respectively. Each experiment was repeated three times under the same experimental conditions and

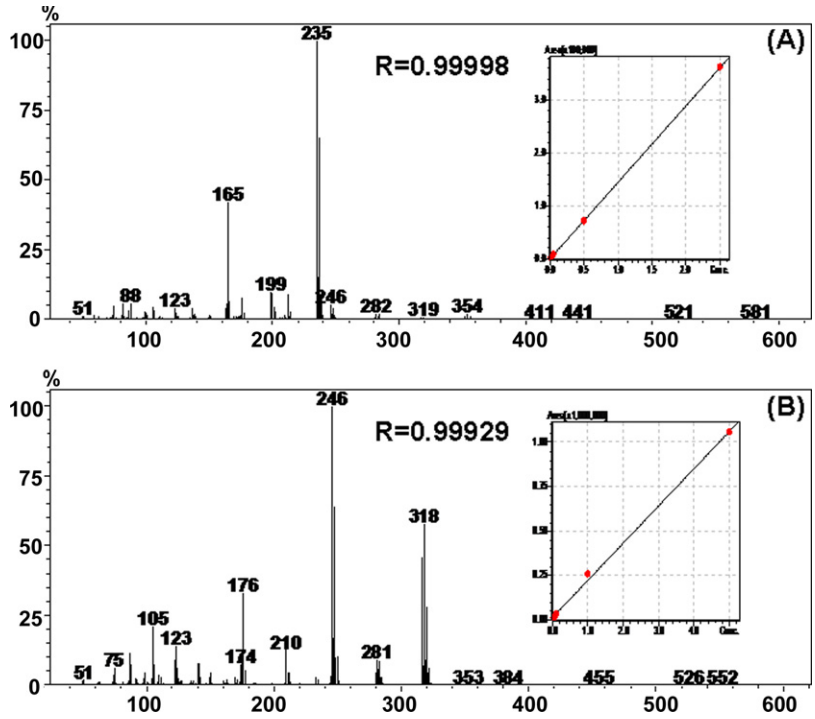

Fig. 1. Mass spectra of standard DDT (A) and DDE (B). Set-in figures are standard calibration curves. Correlation coefficients are shown in the figures.

Table 1
Recoveries of standard DDT and DDE in prepared water samples.

No.	DDE[a]				DDT[b]			
	Expt. (ng/μl)	Obs. (ng/μl)	Recovery (%)	RSD (%)[c]	Expt. (ng/μl)	Obs. (ng/μl)	Recovery (%)	RSD (%)[c]
1	0.53	0.51	97	7	0.34	0.35	102	6
2		0.47	89			0.31	90	
3		0.45	85			0.32	93	

[a] DDE concentration in samples is 4.2 ng/ml.
[b] DDT concentration in samples is 2.7 ng/ml.
[c] RSD: relative standard deviation.

all results were listed in Table 1. Recoveries of DDT range from 90% to 102% and RSD is 6%. Similar results were observed for DDE whose recoveries ranges from 85% to 97% and RSD is 7%.

3.3. Analysis of DDT and metabolite DDE residues in zebrafish

The mnNEC approach has been applied to the analysis of DDT and metabolite DDE residues in zebrafishes that have been exposed to DDT (1 μg/l) for 60 days. Body tissues were homogenated with lysis buffer and magnetic nanoparticles were added to the tissue suspension. Enriched analytes were separated and analyzed by a GC–MS system. Fig. 2 shows the TIC of enriched compounds. Compared with the retention time of standard DDT and DDE (Supplementary Fig. 4(A and B)), DDE was indicated as a black arrow but DDT was indicated as a dotted black arrow according to its standard retention time although it was not detected in full scan mode. By using the featured fragment ions, both DDT and DDE were detected in SIM mode. Peaks labeled by black balls were identified as fatty acids by searching against NIST database and manual interpretation. It has been experimentally demonstrated that DDT and metabolite DDE co-exist with lipids in biological samples. The proposed mnNEC approach is convenient to handle such greasy samples. It only needs less than 15 min to complete the whole process of sample preparation without worrying about damages on sorbents. Quantities of DDT and DDE were summarized in Table 2. It should be kept in mind that different approaches for sample preparation may result in different results. Therefore, samples should be prepared in parallel for quantitative comparison.

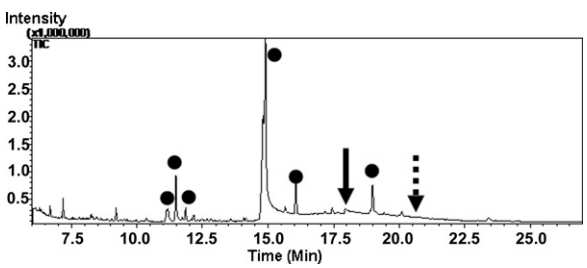

Fig. 2. Total Ion Chromatogram (TIC) of enriched analytes from zebrafish body tissues. Black balls: fatty acids. Black arrow: DDE. Dotted black arrow: DDT.

Examination of Table 2 reveals two important facts. First, although there are unavoidable individual variations in biological samples, it is shown that averaged DDT and metabolite DDE detected in male zebrafish body tissues are more than that detected in female zebrafish body tissues under the exposure to DDT (1 μg/l) for 60 days. So far, it is still not clear how DDT residues have been involved in the biological pathways. Since DDT has been extensively used world-widely, it is still detected in human blood even after it was banned many years ago due to its resistance to metabolism and bioaccumulation through food chain [44]. Continuous research efforts are necessary for epidemiologic and experimental studies

Table 2
Analysis of DDT and metabolite DDE in zebrafish[a]

No.	Weight (g)[b]	DDE		DDT	
		Obs. (ng/μl)	DDE in body (ng/g)	Obs. (ng/μl)	DDT in body (ng/g)
F1[c]	0.6880	0.34	199	0.04	25
F2	1.2640	0.46	146	0.02	7
F3	0.7720	0.70	362	0.10	52
F4	0.7750	0.44	226	0.02	10
M1[d]	0.5950	1.65	1108	0.21	143
M2	0.6640	0.41	245	0.06	37
M3	0.6810	0.29	173	0.07	44
M4	0.7750	0.94	487	0.11	57

[a] Zebrafish have been exposed to DDT (1 μg/l) for 60 days.
[b] Wet body weights without internal organs.
[c] F: female.
[d] M: male.

of carcinogenesis of DDT residues in order to understand progressions and mechanisms of many complex human diseases. Lots of researches have shown that DDT can induce ferminization and cause health problems [45–48]. It has been found that the mean blubber organohalogen concentration of the male gray seals was significantly higher than that of female gray seals [49], which is in agreement with the experimental results described in this work. Secondly, the concentrations of DDE are higher than that of DDT in body tissues for both male and female zebrafish. DDT is slow to convert into other metabolites and DDE is its major metabolite [50]. It was shown in this work that the concentration of DDE is several times or even more than twenty times higher than that of DDT. This experimental result indicates that DDE is much more resistant to metabolism than DDT and persistently accumulate in body tissues. A high ratio of DDE/DDT indicates a high environmental persistence and ongoing bioaccumulation. On the other hand, a high DDT/DDE ratio implies a chronic but ongoing exposure to DDT [51].

4. Conclusions

The mnNEC approach is an efficient SPE format for enrichment of target analytes from aqueous samples. It is a mixed mode SPE combined the hydrophobicity of carbon with the hydrophilicity of nitrogen atoms. Features of uniform dispersion in aqueous samples, nanosized spheric shapes and rapid magnetic isolation make it possible for mnNEC to handle greasy biological tissue samples. Adsorption and desorption of analytes on mnNEC process quickly compared with other carbon nanotubes based approaches. By using GC–MS, the detection limit can be down to low ng/ml level. Experimental results for analysis of DDT and metabolite DDE in zebrafish body tissues are in accordance with those reported in the literature. Additionally, mnNEC can be readily synthesized in any lab. The cost is low and the experimental procedure is easy to perform. It should be able to provide a wide application means for analysis of environmental pollutants in tissues or body fluids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.12.021.

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