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Determination of diethylstilbestrol in milk using carbon nanotube-reinforced hollow fiber solid-phase microextraction combined with high-performance liquid chromatography

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ABSTRACT

Carbon nanotube-reinforced hollow fiber solid-phase microextraction (CNTs-HF-SPME) combined with high-performance liquid chromatography (HPLC) was used to extract and determine diethylstilbestrol (DES) in milk products. Wall pores of the hollow fiber were filled with multi-walled carbon nanotubes (MWCNTs) using sol–gel technology. In the proposed method, DES was selectively extracted by MWCNTs, desorbed to methanol, and analyzed by HPLC. The parameters affecting the efficiency of CNTs-HFSPME, such as the length of the hollow fiber, extraction and desorption times, extraction temperature, stirring rate, pH of the sample solution, and the amount of organic solvent and salt in the sample solution, were investigated and optimized. Under the optimized extraction conditions, the method showed good linearity (24–960 $\mu g\ L^{-1}$), a low method detection limit (MDL, 5.1 $\mu g\ L^{-1}$), and good recoveries at four different concentrations. It was proven to be simple, rapid, sensitive, and solvent free for the analysis of DES in dairy products.

1. Introduction

Diethylstilbestrol (DES), a non-steroidal hormone, has been used to treat gynecological diseases and promote animal growth [1,2]. However, DES and its metabolites remain in animal tissues, organs, and excreta; enter the external environment; become environmental estrogen; and then are passed to consumers through the food chain, which can cause cancer in humans [3–5]. Thirty years of extensive research show that DES residues in the human body and environment have obvious detrimental effects; thus, many countries have banned the use of DES [6]. Nevertheless, various other substitutes that can promote animal growth do not have as much potency as DES, so the use of DES in animal production is prohibited but has not completely stopped. Mainly due to economical interests, the abuse in the use of DES continues [7,8]. Therefore, an efficient and sensitive method for the determination of DES should be established to monitor DES residues in food and thereby ensure the safety of food supply.

Generally, the analytical methods for the determination of DES in water or food products are conducted using high-performance liquid chromatography (HPLC) [9–13], liquid chromatography–mass spectrometry [14–16], and gas chromatography–mass spectrometry [17,18]. Most of these methods need solid-phase extraction for sample pretreatment, which is expensive, time consuming, and not sensitive enough for trace analyses. Solid-phase microextraction, developed in 1990 [19], has also been applied for the extraction of DES. However, the use of conventional fibers has several drawbacks, such as their expensive cost, short lifetime, sample carry-over effects, and fiber breakage [20]. Recently, some new sorbent materials have been used successfully as functional coating such as molecular imprinted polymer [12], mesoporous materials [21] and nanomaterials [22,23]. Furthermore, sol–gel technology has also been applied to modify fiber coating in solid-phase microextraction (SPME) [24–26]. It exhibits several advantages, such as good mixing for multi-component systems, strong adhesion of the coating and bed, and high surface areas, among others [27].

Carbon nanotubes (CNTs) are new types of materials discovered by Iijima in 1991 [28]. Since then, they have drawn great attention because of their unique mechanical and thermal properties [29,30]. They have been used in solid-phase microextraction as fiber coating for the extraction of various compounds [31–33]. However, the main drawback of CNTs is that they can form

Abbreviations: DES, diethylstilbestrol; MWCNTs, multi-walled carbon nanotubes; HPLC, high-performance liquid chromatography; CNTs-HF-SPME, carbon nanotube-reinforced hollow fiber solid-phase microextraction

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aggregates due to intermolecular van der Waals interactions [34,35]. Therefore, sol–gel technology is used to solve intermolecular interactions and to disperse CNTs in molecular levels [36]. Nonetheless, the sample carryover effect of SPME fibers still exists.

Es'haghi et al. addressed the problem on carryover using a hollow fiber in solid-phase microextraction (SPME) [37] instead of the conventional fiber. In their experiment, the gel of CNTs was placed on the inner wall of the hollow fiber, which was discarded after extraction, to eliminate the carryover effect [38]. Nevertheless, the gel of CNTs was too thin to be stable on the wall of the hollow fiber. Based on previous work on HF-LPME [39–41], an attempt was made to fix CNTs to the wall pores of the hollow fiber to overcome this disadvantage. In the present study, a novel method of carbon nanotube-reinforced hollow fiber solid-phase microextraction (CNTs-HF-SPME) coupled with HPLC is developed and applied to determine DES in milk samples for food quality control. The relative parameters affecting the extraction efficiency are optimized.

2. Experimental

2.1. Chemicals and materials

DES was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Chromatographic-grade acetonitrile was obtained from Merck Co. (Darmstadt, Germany). Seven batches of milk products from four manufactures were bought in local supermarket. Other chemicals were of analytical grade and were purchased from Tianjin Chemical Reagent Co. (Tianjin, China). Water was purified by a water purification system (Shanghai Laikie Instrument Co., Ltd., Shanghai, China). Accurel Q3/2 polypropylene hollow fiber (200 μm wall thickness, 600 μm internal diameter, 0.2 μm pore size) was purchased from Membrana GmbH (Wuppertal, Germany). Multi-walled carbon nanotubes (MWCNTs) were purchased from Chengdu Organic Chemical Co. Ltd., Chinese Academy of Sciences.

2.2. Sample pretreatment

2.2.1. Oxidation of MWCNTs

Crude MWCNTs cannot be used in the further step of sol–gel preparation because their stable structure and pure carbon element inhibit solubility in organic solvents [20]. Therefore, oxidization of MWCNTs is necessary for their dispersion in the organic solvent [42]. In the present study, about 0.1 g of crude MWCNTs was immersed in 20 mL of 8 mol L^{-1} HNO_3 and refluxed in stirring at 140 °C for 4 h. The mixture was cooled and washed with purified water until the pH reached 7.0. Afterward, the oxidized MWCNTs were dried at 70 °C.

2.2.2. Preparation of MWCNTs/silica composite-reinforced hollow fiber

The preparation of the sol solution of MWCNTs/silica composite was taken from the work of Es'haghi [37]. About 1 mL of tetraethylorthosilicate and 1 mL of ethanol were added to a glass vial and stirred at 0 °C for 10 min. Then a buffer solution of Na_2HPO_4–citric acid was added to adjust the pH to 5.0. After 20 min, 300 μL of Poly-(ethylene glycol) 400 was added as surfactant, and the solution was stirred continuously for 120 min. Then 50 mg of the oxidized MWCNTs was added to the resulting mixture via stirring for 30 min. Sol solution of MWCNTs/silica composite was formed.

The polypropylene hollow fiber was cut into 6 mm segments and ultrasonically washed in acetone for 1 min to remove impurities in the fiber. Afterward, the segments were dried in air. The treated hollow fibers were entirely immersed in the sol of the MWCNTs/silica composite and ultrasonically treated at room temperature for 60 min. Finally, MWCNT-reinforced hollow fibers were ultrasonically cleaned with methanol for several times to remove the MWCNTs on the surface and the inner wall, until no CNTs was observed in the cleaning solution; then only the MWCNTs in the wall pores of the hollow fiber were left. Fig. 1 showed the SEM image representing the presence of oxidized MWCNTs in the wall pores of the hollow fiber.

2.2.3. Preparation of standard solutions and milk samples

DES was dissolved in methanol to obtain the stock solution with a concentration of 0.24 mg mL^{-1}. Calibration standard working solutions of DES at six concentration levels were freshly prepared through appropriate dilution of stock solutions with purified water. Fresh milk was purchased from a supermarket in Lanzhou (China) and was stored at 4 °C.

2.2.4. Extraction procedure

Fig. 2 showed schematic illustration of the application of CNTs-HF to extract DES in milk product. Extraction was performed as follows: 6 mm of CNTs-HF, which was impregnated in 1-octanol for 1 min to facilitate the contact of the analyte and sorbent [37], was immersed in 1 mL of the milk sample in a 2 mL glass vial and agitated at 800 rpm of agitation speed for 30 min. After extraction, the fiber was taken out of the milk, cleaned with purified water, and dried with filter paper. It was plunged into a centrifuge tube (0.5 mL) with 50 μL methanol for desorption via the ultrasonic-assisted effect for 10 min. Then 10 μL of the desorbed solution was injected for HPLC analysis. In consideration of the relatively low cost, the used CNTs-HF was discarded after extraction and a fresh one was used for the next experiment to eliminate carry-over effect.

2.3. Apparatus and chromatography

The HPLC system (Waters Corp., Milford, MA, USA) was equipped with a Waters quaternary pump (Model Delta 600E), a photodiode array detector (Model 2996), a manual injector, and Waters Millennium^{32} software for peak identification and integration column used was a Kromasil C_{18} column (5 μm, 4.6 × 250 mm

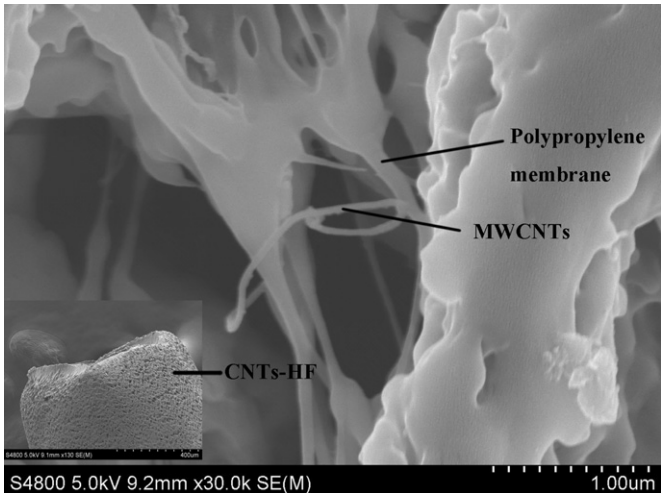

Fig. 1. Scanning electron microscopy of a polypropylene hollow fiber containing oxidized MWCNTs.

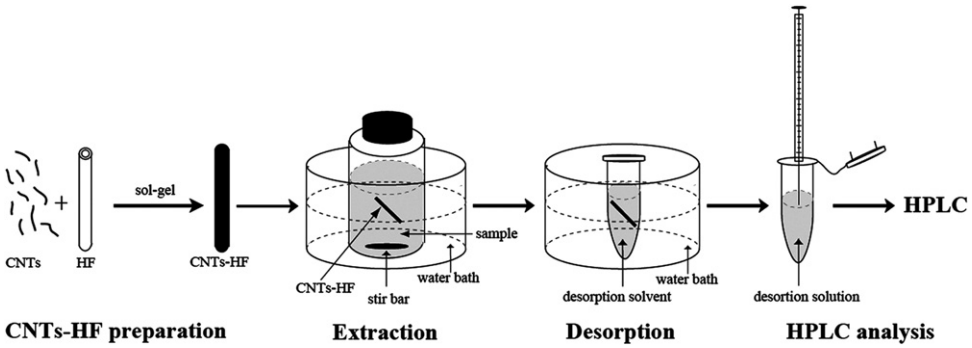

Fig. 2. Schematic illustration of CNTs-HF-SPME combined with HPLC.

i.d.) (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China).The mobile phase consisted of acetonitrile-water (70: 30, v/v) at a flow rate of 1.0 mL min^{-1}, and the wavelength used to measure DES was set at 241 nm. Helium (He) was used for degassing the mobile phase. Temperature of the column during analysis was maintained at 25°C.

3. Results and discussion

3.1. Optimization of the CNTs-HF-SPME procedure

To obtain high extraction efficiency, the main parameters were optimized. In all optimization experiments, each involving three parallel experiments, 1 mL of milk solution containing 240 μg L^{-1} of DES was used.

3.1.1. Organic solvent conditioning

The CNTs and polypropylene membrane are hydrophobic in nature, and low wettability was observed when CNTs-HF was directly exposed to a aqueous solution. So, the wettability of the CNTs-HF needed to be enhanced. In previous reports, the wettability of CNTs, used as SPME sorbent, was improved by conditioning with organic solvents [23,37]. In the present case, CNTs-HF was conditioned with 1-octanol. In the conditioning procedure, trace amounts of 1-octanol was retained on the pores of CNTs-HF. Results showed that wetting with 1-octanol could obtain higher extraction rate and efficiency than that with no conditioning solvent. In addition, to eliminate the possible extraction effect of trace 1-octanol retained, the procedure that CNTs-HF was cleaned with purified water, and dried with filter paper was conducted after extraction. Meanwhile, a comaprative trial was conducted between CNTs-HF, HF and HF conditioned with 1-octanol. Results showed that no analyte was extracted by HF or conditioned HF, which means that the analyte was extracted by CNTs in the pores of hollow fiber, but not by 1-octanol.

3.1.2. Selection of the length of the hollow fiber

The length of the hollow fiber determines the amount not only of the MWCNTs but also of the desorption solution. Hollow fibers with lengths of 6, 7, 8, 9, and 10 mm were used to determine the influence of length on the extraction performance of DES, in which equal amounts of desorption solvent were used. Results showed that the peak areas of DES had no apparent increase with the increase in length of the hollow fiber. And the enrichment factors are almost the same by different length of CNTs-HF with values of 18–20. In this kind of SPME, relatively large volume of desorption solvent was needed, so enrichment factor was not too high. Results also indicated that 6 mm of CNTs-HF contained enough MWCNTs to extract DES from the milk sample. By using a shorter hollow fiber, lesser desorption solution would be needed, which can also provide a higher enrichment factor and a lower method detection limit (MDI).

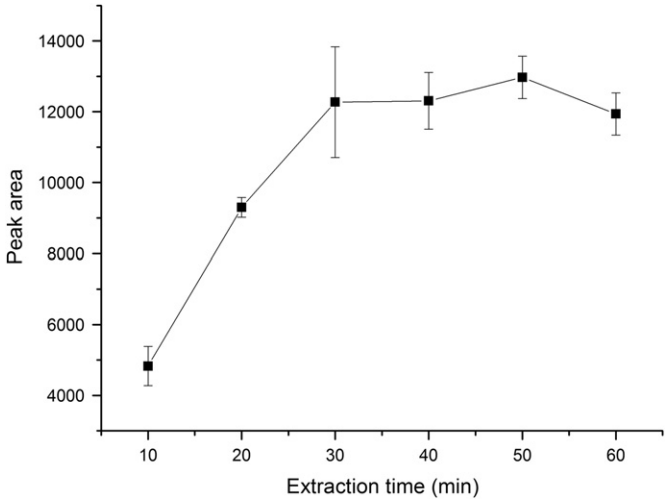

Fig. 3. Effect of extraction time on the peak area of DES extracted with SPME. Extraction condition: length of hollow fiber: 6 mm; desorption time: 10 min; extraction temperature: 50 °C; stirring rate: 800 rpm; pH of sample solution: 2.0.

3.1.3. Selection of extraction time and desorption time

Generally, extraction time is a very important parameter in SPME because it influences the partition of the analyte between the sample solution and the sorbent in the hollow fiber, as analyte needs sufficient time to be transferred from sample solution to CNTs in hollow fiber. Fig. 3 presented the results of the test using different extraction times. Extraction efficiency increased with an increase in extraction time (from 10 to 30 min). This increase was followed by a slight fluctuation but no apparent increase. Prolongation of the extraction time would cause the loss of 1-octanol and eventually hinder the contact between the analyte and the MWCNTs. Moreover, equilibrium does not need to be reached in quantitative analysis as long as the extracting conditions remain constant [43]. Considering the experimental results and compromise between extraction efficiency and analysis time, 30 min was used as the extraction time in the succeeding experiments.

After extraction, the hollow fiber was transferred to a centrifuge tube containing methanol for desorption via ultrasonication. A series of desorption times in the range of 10–60 min was investigated at room temperature to ensure all compound was completely desorbed from CNTs-HF. The highest peak area of DES was obtained at the desorption time of 10 min, and no significant

increase in extraction efficiency can be observed when a longer desorption time was employed. Therefore, 10 min was chosen as the desorption time for succeeding studies.

3.1.4. Selection of extraction temperature

On the one hand, increasing the extraction temperature can lead to an increase in the diffusion coefficient by enhancing the diffusion of analyte to sorbent in hollow fiber. On the other hand, it would cause a decrease in the distribution constant and influence extraction yield [43]. Therefore, the effect of extraction temperatures between 20 and 70 °C was studied. Fig. 4 showed that the extraction efficiency dramatically increased when the extraction temperature increased from 20 to 60 °C. As the temperature becomes higher, there is a possibility of increase in protein denaturation [44], which would block the adsorption of DES and then reduce its peak area. Moreover, the distribution constant dramatically decreased at a higher temperature, and there was the possibility of analyte loss due to higher temperature. Therefore, 60 °C was adopted for subsequent tests.

3.1.5. Selection of stirring rate

Stirring can accelerate extraction kinetics by facilitating mass transfer [45], and then reducing the time required to reach thermodynamic equilibrium, since stirring provides a fresh sample solution to CNTs in hollow fiber and enhances the transportation of analyte from sample solution to CNTs. To evaluate the influence of the stirring rate on the extraction of DES, sample solutions were extracted at stirring rates of 400, 600, 800, 1000, and 1200 rpm. Fig. 5 showed that the peak areas of the analyte increased as the stirring rate increased from 400 to 800 rpm. The analyte, however, cannot be extracted when the stirring rate exceeded 800 rpm, which may be explained as follows: vigorous stirring may increase shearing strength, which is disadvantageous to the adsorption process because the surface of the hollow fiber has a mesoporous coating [46]. And also, increasing linear speed would result in local decrement of pressure. Moreover, in high temperatures, a higher stirring rate would evaporate the analyte from the milk solution. Therefore, 800 rpm was chosen as the optimum stirring rate.

3.1.6. Selection of the pH of the sample solution

In SPME, analytes are generally absorbed by the sorbent in molecular form, and the pH of the sample solution determines whether the state of the analytes is ionic or molecular [47]. At acidic pH, the acid-base equilibrium for DES shifts to molecular state, which has great affinities toward CNTs, therefore enhancing extraction efficiency. Therefore, the influence of the pH, from 1.0 to 5.0, of the sample solution on extraction efficiency was studied. As DES is a weak acid with a pKa of 9.73, the value of the pH should at least be 2–3 units lower than its pKa. Therefore, the pH of sample solution higher than 5.0 was not investigated. According to the results shown in Fig. 6, the peak area of DES increased with an increase in the pH value from 1.0 to 3.0, and then decreased as the pH increased further. Decreasing of extraction efficiency at higher acidic or basic concentration could be due to the increasing the ionic strength of analyte. Therefore, the optimum pH value of the sample solution was 3.0.

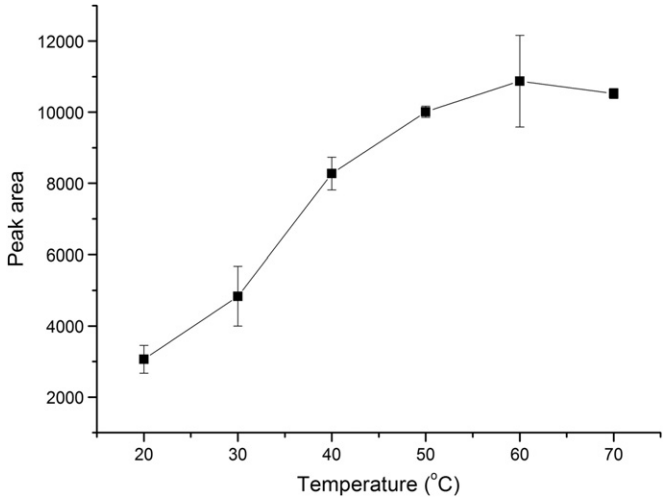

Fig. 4. Effect of extraction temperature on the peak area of DES extracted with SPME. Extraction condition: length of hollow fiber: 6 mm; extraction time: 30 min; desorption time: 10 min; stirring rate: 800 rpm; pH of sample solution: 2.0.

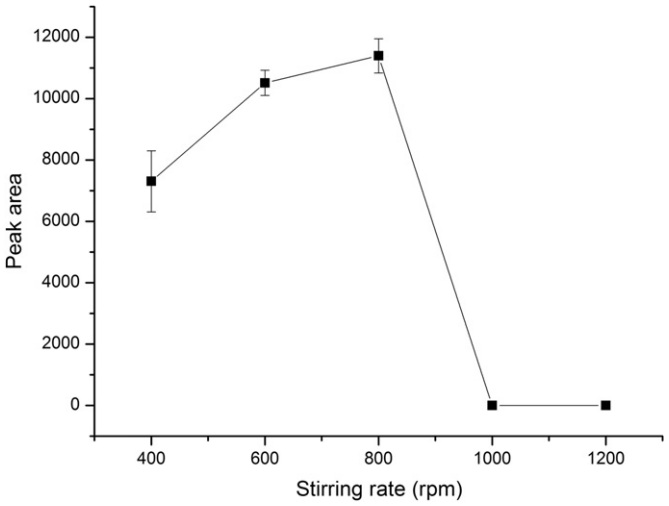

Fig. 5. Effect of stirring rate on the peak area of DES extracted with SPME. Extraction condition: length of hollow fiber: 6 mm; extraction time: 30 min; desorption time: 10 min; extraction temperature: 60 °C; pH of sample solution: 2.0.

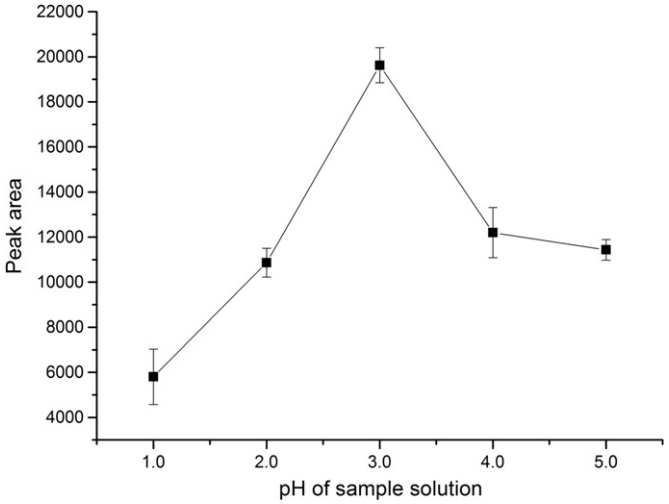

Fig. 6. Effect of pH of sample solution on the peak area of DES extracted with SPME. Extraction condition: length of hollow fiber: 6 mm; extraction time: 30 min; desorption time: 10 min; extraction temperature: 60 °C; stirring rate: 800 rpm.

3.1.7. Selection of the amount of organic solvent and salt in the sample solution

For complex samples, such as milk, organic solvent is often added to enhance extraction efficiency. Whether or not the addition of an organic solvent was beneficial for the proposed

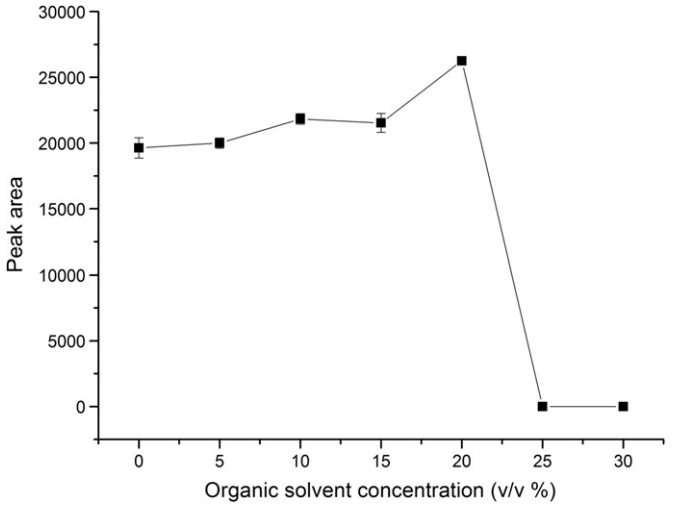

Fig. 7. Effect of the amount of organic solvent on the peak area of DES extracted with SPME. Extraction condition: length of hollow fiber: 6 mm; extraction time: 30 min; desorption time: 10 min; extraction temperature: 60 °C; stirring rate: 800 rpm; pH of sample solution: 3.

method was examined. Results in Fig. 7 showed that the addition of methanol resulted in an improvement in extraction efficiency, with the effect being most significant when 20% methanol was added. Possible reason is that a small proportion of methanol can help analyte molecules transfer to the MWCNTs in the wall pores of the hollow fiber [48]. And also, the addition of organic solvent could release analyte from matrix protein in milk. However, when the amount of methanol is higher, analyte molecules are prone to be dissolved in the organic solvent and not be absorbed by the MWCNTs.

The addition of salt was also investigated. However, the results showed that the extraction efficiency of DES decreased with an increase in NaCl concentration. Addition of salt could have two effects. On the one hand, it decreased the solubility of analyte, so enhanced extraction efficiency by salting-out effect. On the other hand, it caused a change in the physical properties of the Nernst diffusion film, which reduced the diffusion of the analytes to the MWCNTs comparing with pure water [49]. Therefore, no NaCl was added to the milk sample in the following experiments.

3.2. Method evaluation

Under the optimized conditions, the maximum peak area, 23,096 could be achieved for 240 μg L^{-1} of DES. Linearity range, method detection limit (MDI), method qualification limit (MQL), precision, repeatability, and accuracy of the proposed method were studied.

The linearity of the calibration plot was investigated over a concentration range of 24–960 μg L^{-1} using six spiked levels of DES. These spiked samples were extracted using an established procedure and analyzed via HPLC. Each concentration of DES in the milk sample was analyzed three times. Results had a good correlation coefficient of 0.9933, and the linear regression equation was $y=92.7x+848.6$, where y represents the peak area of DES, and x denotes the concentration of DES in the milk sample. MDI of DES, which was investigated in the milk sample, was calculated at a signal-to-noise ratio of 3 (S/N=3) and was as low as 5.1 μg L^{-1}. And MQL was 15.3 μg L^{-1} at S/N=10.

The precision of the proposed method was investigated by performing intra- and inter-day assays via repetitive injection of extract from 240 μg L^{-1} DES-spiked milk by using CNTs-HF-SPME. Precision of the intra-day assay was determined using five continuous injections within the same day, and that of the inter-day assay was determined in three consecutive days. Results are shown in Table 1.

Table 1
Precision of the developed method.

Analyte	Intra-assay RSD (%, $n=5$)		Inter-assay RSD (%, $n=3$)	
	Peak area	Retention time	Peak area	Retention time
DES	0.2	1.3	1.8	2.5

Table 2
Spiked recoveries of the DES in the milk ($n=3$).

Analyte	Original amount (μg L^{-1})	Added amount (μg L^{-1})	Found amount (μg L^{-1})	Recovery (%)	RSD (%)
DES	240	120	69	57.50	11.85
	240	192	114	59.38	12.04
	240	240	289	120.42	8.67
	240	288	305	105.90	7.36

Repeatability was studied by extracting the milk sample spiked with 240 μg L^{-1} of DES with the same batch produced fiber under the optimum conditions. The relative standard deviation ($n=4$) was 7.51%.

The batch-to-batch reproducibility of CNTs-HF preparation was evaluated by extracting the milk sample spiked 240 μg L^{-1} of DES with four different batch fibers prepared in the same procedure. The relative standard deviation was 9.86%.

The accuracy of the analytical method was confirmed by the spiked recovery test. 240 μg L^{-1} of DES was added to the blank milk sample as the original amount, then four different quantities, i.e., 50% (lower), 80% (low), 100% (medium), and 120% (high), of the above concentration of DES were spiked to the original sample. Then the four sets of milk samples were extracted and analyzed. A summary of the recovery data obtained with HPLC is shown in Table 2. Recoveries were in the range of 57.50% to 120.42%, and the RSDs were from 7.36% to 12.04%.

3.3. Application in the real sample

The proposed procedure was applied to anlalyse seven batches of milk products from four manufactures. Contents of fat, protein and carbohydrate in these milk samples were in the range of 25–40, 30–35, 50–100 μg mL^{-1}, respectively. About 0.1 mL of milk, 0.2 mL of methanol, and a buffer solution of Na_2HPO_4-citric acid were added to a total volume of 1 mL, and the above solution was extracted under the conditions previously optimized. Results shown in Table 3 indicated that DES were found in two batches of milk, but could not be quantified. In addition, real blank milk was extracted and analyzed to investigate the specificity of the method. Fig. 8 showed the typical chromatograms obtained from the analysis of the real blank milk and milk samples containing 240 μg L^{-1} of DES after extraction via the proposed procedure. No peaks were found at the retention times of the corresponding analyte in blank sample. This indicates that other coexisting species and sample matrix components could not interfere with precise analysis of the analyte.

4. Conclusion

A novel method using CNTs-HF-SPME prior to HPLC was developed for the determination of DES in milk products. In the

Table 3
Analytical results of DES from the 7 milk products ($n=3$).

Manufacture	Batch no.	Fat content ($\mu g\ mL^{-1}$)	Protein content ($\mu g\ mL^{-1}$)	Carbohydrate content ($\mu g\ mL^{-1}$)	DES content ($\mu g\ L^{-1}$)
A	J20120307R11	25	30	50	Found[a]
B	20110626	31	31	100	Unfound
	20120101	31	31	100	Unfound
	20120203	31	31	100	Unfound
C	20120120A5	35	30	50	Unfound
	20111113EV9	35	30	50	Found
D	20111128B31E	40	35	50	Unfound

[a] DES could be found, but could not be quantified.

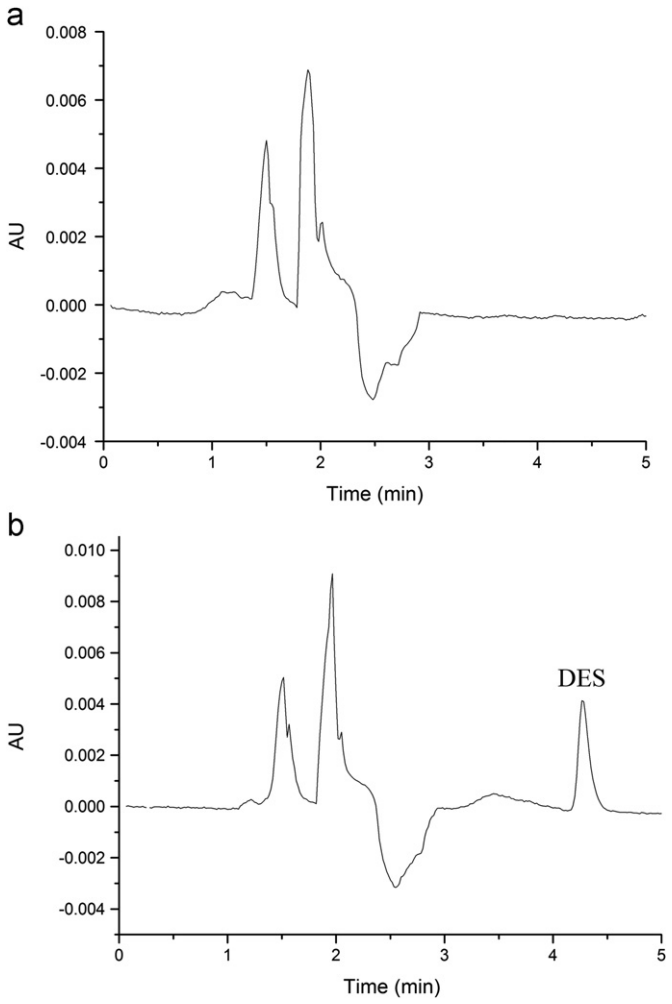

Fig. 8. Chromatograms of (a) blank milk (b) milk sample spiked with 240 $\mu g\ L^{-1}$ of DES extracted by SPME. Mobile phase: acetonitrile–water (70:30, v/v); flow rate: 1.0 $mL\ min^{-1}$; detection wavelength: 241 nm.

present experiment, the wall pores of hollow fibers were filled with MWCNTs using sol–gel technology. Hollow fibers can prevent large molecules, such as proteins, from entering small pores. The MWCNTs in the wall pores of the hollow fiber can absorb target molecules, thus effectively and selectively extracting DES from milk products. After the optimization of the extraction conditions for DES, effective sample clean-up, good linearity, and recovery were obtained. Results of the present experiments showed that CNTs-HF-SPME combined with HPLC is a simple, rapid, and cost-effective technique to monitor DES residues in milk products.

Acknowledgements

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