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Application of multiwall carbon nanotubes-based matrix solid phase dispersion extraction for determination of hormones in butter by gas chromatography mass spectrometry

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ABSTRACT

The multiwall carbon nanotubes (MWCNTs)-based matrix solid phase dispersion (MSPD) was applied for the extraction of hormones, including 17-α-ethinylestradiol, 17-α-estradiol, estriol, 17-β-estradiol, estrone, medroxyprogesterone, progesterone and norethisterone acetate in butter samples. The method includes MSPD extraction of the target analytes from butter samples, derivatization of hormones with heptafluorobutyric acid anhydride–acetonitrile mixture, and determination by gas chromatography–mass spectrometry. The mixture containing 0.30 g graphitized MWCNTs and 0.10 g MWCNTs was selected as absorbent. Ethyl acetate was used as elution solvent. The elution solvent volume and flow rate were 12 mL and 0.9 mL min^{-1}, respectively. The recoveries of hormones obtained by analyzing the five spiked butter samples were from 84.5 to 111.2% and relative standard deviations from 1.9 to 8.9%. Limits of detection and quantification for determining the analytes were in the range of 0.2–1.3 and 0.8–4.5 μg kg^{-1}, respectively. Compared with other traditional methods, the proposed method is simpler in the operation and shorter in the sample pretreatment time.

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1. Introduction

Butter is a kind of dairy and a valuable nutrient source for humans. However, the previous reports have questioned its safety because of steroid hormone contamination in butter [1,2]. The hormones including estrogen and progesterone are present in milk. The hormones cannot be destroyed or eliminated in the production of the butter with milk. The hormones are lipophilic and can be concentrated in the fat phase of dairy products [3]. The hormones at low-ng L^{-1} concentration could affect biological processes of humans due to their extremely high biological activities [4–6]. The information which comes from a German nutritional study indicate that a man would consume about 0.06 mg hormones daily and dairy products might account for up to 60% [3]. With increasing consumption of dairy products such as butter, amount of hormones to be consumed by humans increased. Bosland et al. reported that estrogens influence prostate development and may also be involved in prostate carcinogenesis [7–10], and other researchers have specifically stated that the estrogens may play a role of increasing the risk of breast cancer of women who consume more dairy [11–13]. It also was considered that estrogens have the sufficient biological activity to lead an increase of mitotic activity of endometrial cells and DNA replication errors [14]. In view of the importance and harmfulness of these compounds, to develop a rapid and sensitive method to detect the compounds in butter is necessary.

Conventional methods for the determination of hormones usually include gas chromatography–mass spectrometry (GC–MS) [15–18], liquid chromatography–MS [19], GC–tandem MS [20,21] and liquid chromatography–tandem MS [22,23]. Most of the researches were focused on environmental water and sediments. Up to now, several researchers quantified estrogens in skim milk [24–26] and whole milk [19,27,28], but few quantified estrogen and progesterone in butter. Actually, 1 kg of milk may produce approximately 3 g of butter. Moreover, most hormones have lipophilic characteristics and the concentrations of the hormones in the fat fraction of butter are higher than those in milk. To separate hormones from matrix of high level fat is difficult. Because of the complexity of biomatrices and the low concentration in samples, the determination of residual hormones in butter became a challenging task.

Currently, the acknowledged sensitive methods for determining steroid hormones are radioimmunoassay (RIA) [28–32] and enzyme immunoassay (EIA) [33,34]. Although RIA and EIA are sensitive, they often suffer from poor specificity, accuracy and/or reproducibility due to the cross-reaction and lot-to-lot variation of antibodies [13]. What is more, the cost of experiment is so expensive that ordinary laboratory cannot afford the expense. Solid phase extraction (SPE) is often used to concentrate and purify hormones

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in tissue, serum and water. Liquid–liquid extraction as a traditional technology was applied to the extraction of hormones from samples frequently [5,35], but usually followed with some obvious disadvantages, including the use of large volume of organic solvent, pollution and relatively high cost [36]. Ultrasonic extraction of hormones using organic solvent was reported, but SPE was required for a further purification [37–41].

Matrix solid-phase dispersion (MSPD) as a sample preparation method was first introduced in 1989 by Barker et al. [42]. MSPD was usually applied to solid and semisolid samples, including animal tissues and foods with a high lipidic content [43]. Application of MSPD in food analysis revealed that MSPD has some advantages over classical extraction methods. MSPD presents a reduced consumption of organic solvents, provides high extraction yields and offers a considerable degree of selectivity. MSPD method comprises sample homogenization, cellular disruption, fractionation, and purification in a single process [44]. There are few papers about using MSPD to extract hormones in fatty foods. Eight free progestogens were extracted from eggs by MSPD [45]. A automated system for extracting acetylgestagens from kidney fat by MSPD [46] was developed. To the best of our knowledge, the simultaneous extraction of estrogen and progesterone in butter by the MSPD is not reported.

Multiwall carbon nanotubes are carbon-based nanomaterials of a kind [43]. As a supporting materials of dispersant sorbents, MWCNTs show great potential to apply to the purification in sample preparation. In theory, MWCNTs have excellent adsorption ability owing to their extremely large surface area and structural characteristics. The high adsorption ability for MWCNTs may be primarily due to their dramatically hydrophobic surface and unique structure with internal tube cavity [47]. In order to simplify pre-treatment of the samples, the mixture of 0.30 g graphitized MWCNTs and 0.10 g MWCNTs was used as adsorbent material of MSPD in this paper. The oxidized MWCNTs were used as the solid-phase micro extraction fiber for extraction of phenols in aqueous samples [48], and the most robust results of the purification ability of carbon nanotubes for oil have also been provided [49]. The main purpose of this work is to evaluate the application of MWCNTs in determining hormones in butter samples by the MSPD extraction combined with GC–MS analysis. MWCNTs were firstly used as adsorbents for the butter sample cleanup and extraction of target hormones in MSPD.

2. Experimental

2.1. Chemicals and materials

The hormone standards (purity, 96.8–99.5%) including 17-α-ethinylestradiol (CEE), 17-α-estradiol (EE_2), estriol (E_3), 17-β-estradiol (E_2), estrone (E_1), medroxyprogesterone (MPG), progesterone (PG) and norethisterone acetate (NEA) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The chemical structures of the compounds are shown in Table 1. Heptafluorobutyric acid anhydride (HFBA, purity > 98%) was purchased from J&K Chemical Ltd. (Beijing, China). HPLC grade acetonitrile, methanol and isooctane were obtained from Fisher Scientific (NJ, USA). Analytical-reagent grade ethyl acetate was obtained from Beijing Chemical Factory (Beijing, China). The mixed standard stock solution containing the eight hormones was prepared by dissolving 10.0 mg of each compound in 10.0 mL methanol. The stock solution was stored at 4 °C and diluted with methanol to give the required concentration.

MWCNTs (10–20 nm outer diameter, 10–30 μm length, >95% purity and >200 $m^2 g^{-1}$ special surface area), Graphitized MWCNTs (GMWCNTs, 8–15 nm outer diameter, ~50 μm length, >99.9% purity and >117 $m^2 g^{-1}$ special surface area) and Carboxyl MWCNTs (<8 nm outer diameter, 10–30 μm length, 3.86 wt.% –COOH content, >95% purity and >500 $m^2 g^{-1}$ special surface area) were purchased from Chengdu Organic Chemistry Company (Chengdu, China).

2.2. Samples

Five kinds of butter samples which were produced in New Zealand (sample 1), France (sample 2), China (sample 3), Argentina (sample 4) and Denmark (sample 5), respectively, were purchased from the supermarkets and stored at −20 °C in refrigerator before analysis. Spiked samples were prepared by adding a proper volume of standard working solution at required concentration into 0.5 g of butter sample. The mixtures were homogenized by grinding for 15 min and then letting stand for 1 h at room temperature in the dark. In this study, all experiments were carried out with sample 1 except for the experiment mentioned in Section 3.4 in which samples 1–5 were used.

2.3. MSPD extraction

0.50 g of butter sample was added into an agate mortar. Then, 0.40 g of adsorbent was added into it. The mixture was ground in the mortar for 10 min until a homogenous paste was obtained. The mixture was transferred into a 5 mL column with a filter paper (Whatman no. 2, Maidstone, UK) at the bottom of the column. Then the mixture in the column was gently compressed using a syringe piston with another filter paper on the top of the sample mixture.

The analytes were eluted with 10 mL of ethyl acetate, and then the eluate was collected in the flask and evaporated to dryness under the low pressure at 40 °C in a Heidolph-Laborata 4000 rotary evaporator (Heizbad WB). Subsequently, the residue was dissolved in 1.0 mL acetonitrile and the flask was washed with 0.4 mL acetonitrile. The resulting acetonitrile solutions were combined and centrifuged at 15,000 rpm at −4 °C for 10.0 min. After centrifugation, white floc was deposited on the bottom of the centrifuge tube at low temperature. And 1.0 mL supernatant was rapidly passed through a 0.22 μm PTFE filter membrane and the resulting solution was referred to as sample solution.

2.4. Derivatization of hormones

The sample solution was evaporated to dryness under a gentle N_2 stream, and the derivatization reaction was performed by adding 360 μL of HFBA–acetonitrile mixture (1:5, v/v) in the residue and then sealing the reaction system. After the reaction was performed at 65 °C for 1 h in water-bath [50,51], the resulting solution was cooled and evaporated to just dryness under a gentle stream of nitrogen. The residue was dissolved in 200 μL of isooctane and the resulting solution was referred to as analytical solution.

2.5. GC–MS analysis

The analytical solution was analyzed using GC–MS QP 2010 (Shimadzu, Kyoto, Japan). The derivatives of hormones were separated with a DB-5MS capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Helium (purity, 99.999%) was used as carrier gas at a constant flow of 1.0 mL min^{-1}. The injection volume of analytical solution was 1 μL in the splitless mode. The oven temperature was programmed as follows: 100 °C for 1 min; ramp to 200 °C at a rate of 30 °C min^{-1}, held for 1 min and then ramp to 280 °C at a rate of 15 °C min^{-1}, held for 10 min.

Mass spectrometric parameters: electron impact ionization mode with an ionizing energy of 70 eV, injector temperature 280 °C, interface temperatures 250 °C, ion source temperature 200 °C. Solvent delay 9.5 min. The mass spectrometer was operated in the

Table 1
Chemical structures and GC–MS acquisition parameters for the analytes.

Analytes	Molecular structure	Retention time (min)	Ions for qualitative analysis (*m*/*z*)	Ions for quantitative analysis (*m*/*z*)
CEE		11.254	474, 459, 446, 431	474
EE_2		11.385	664, 450, 409, 237	664
E_3		11.734	876, 663, 449, 235	876
E_2		11.844	664, 451, 409, 237	664
E_1		12.414	466, 448, 422, 409	466
MPG		13.294	479, 383, 147, 109	479
PG		13.878	510, 495, 425, 147	510
NEA		14.011	536, 468, 341, 270	536

selected ion monitoring (SIM) mode for quantitative analysis and the characteristic ions are given in Table 1. Full-scan MS data were acquired in the range of *m*/*z* 50–900 to obtain the fragmentation spectra of the analytes.

2.6. Box–Behnken design

Box–Behnken design (BBD) is a class of rotatable or nearly rotatable second-order design based on three-level incomplete factorial design [52]. In this work BBD was employed for the optimization of experimental conditions in MSPD. A Design Expert software (Trial Version 7.1.3, Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental design, data analysis and model building.

The BBD involves three variables, including the mass of carbon nanotubes (adsorbent mass, X_1), the elution solvent volume (X_2), the elution solvent flow rate (X_3), and a response value recovery. Accordingly, the low, middle and high levels of each variable were designated as −1, 0, and +1, respectively. The actual design experiment was shown in Table 2. In order to predict the optimal point, all the results obtained in the experiments were used for the computer simulation programming applying the quadratic (second degree) polynomial equation. For three significant independent variables, the equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Table 2
Experimental results based on BBD.

Experiments	Coded levels			Response: hormone recoveries (%)							
	X_1 Adsorbent amount (g)	X_2 Elution solvent volume (mL)	X_3 Elution solvent flow rate (mL min^{-1})	CEE	EE_2	E_3	E_2	E_1	MPG	PG	NEA
1	−1 (0.2)	+1 (14.0)	0 (1.0)	26.9	21.2	26.9	24.0	24.1	21.8	33.3	37.1
2	0 (0.4)	0 (10.0)	0 (1.0)	90.3	83.2	85.2	67.6	113.0	95.3	86.5	94.7
3	+1 (0.6)	0 (10.0)	−1 (0.5)	33.6	26.4	33.2	13.0	20.0	10.3	36.0	44.1
4	0 (0.4)	−1 (6.0)	+1 (1.5)	44.8	19.4	52.3	18.4	17.3	13.8	77.9	36.4
5	−1 (0.2)	−1 (6.0)	0 (1.0)	52.2	20.8	33.2	12.9	16.0	18.9	56.6	50.7
6	0 (0.4)	0 (10.0)	0 (1.0)	99.3	66.6	102.7	64.5	73.3	65.4	89.4	87.1
7	0 (0.4)	0 (10.0)	0 (1.0)	109.0	68.3	95.0	84.4	114.1	94.3	93.4	88.7
8	0 (0.4)	+1 (14.0)	−1 (0.5)	48.5	14.2	16.4	16.2	22.6	15.2	42.8	31.9
9	0 (0.4)	−1 (6.0)	−1 (0.5)	51.5	17.1	33.5	46.0	34.6	22.3	91.9	52.3
10	+1 (0.6)	0 (10.0)	+1 (1.5)	38.1	14.3	23.5	14.3	13.7	12.7	35.5	48.6
11	−1 (0.2)	0 (10.0)	+1 (1.5)	54.5	25.6	32.8	22.1	25.0	23.0	66.8	63.0
12	0 (0.4)	0 (10.0)	0 (1.0)	83.6	83.2	92.1	86.0	114.5	88.2	102.5	91.0
13	+1 (0.6)	−1 (6.0)	0 (1.0)	41.8	43.4	64.0	50.3	69.8	11.9	40.4	58.3
14	−1 (0.2)	0 (10.0)	−1 (0.5)	36.6	17.3	26.9	49.9	73.1	16.2	36.2	41.5
15	+1 (0.6)	+1 (14.0)	0 (1.0)	80.6	55.2	44.2	43.6	48.0	60.1	42.1	53.8
16	0 (0.4)	+1 (14.0)	+1 (1.5)	38.8	36.3	32.0	14.4	12.3	13.0	67.2	64.4
17	0 (0.4)	0 (10.0)	0 (1.0)	99.3	80.2	90.1	89.3	103.5	85.1	99.2	80.9

where Y is estimate response, β_0 is constant; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are interaction coefficients between the three factors (X_1, X_2 and X_3); β_{11}, β_{22} and β_{33} are quadratic coefficients.

3. Results and discussion

3.1. The optimization of MSPD conditions

In this study, two parameters, including types of adsorbents and elution solvents for MSPD were studied separately and other three relevant parameters, including the amount of adsorbent, volume of the elution solvent, and flow rate of elution solvent, were studied with the BBD. For the optimization of the experiment parameters the spiked butter samples were analyzed.

3.1.1. Types of adsorbents

In order to achieve an adequate extraction performance for MSPD, several kinds of MWCNTs were evaluated. In principle, MWCNTs–COOH can provide a better interaction between analyte and adsorbent for determining phenols than MWCNTs [48]. However, the sample solution obtained by the MWCNTs–COOH contained so much impurity that may not be suitable for GC analysis. Compared with MWCNTs, the GMWCNTs have more perfect crystal lattice and higher purity. The adsorption capability of MWCNTs was compared with that provided by GMWCNTs. Fig. 1 shows that compared with the adsorption capacity of MWCNTs that of GMWCNTs is markedly better for CEE, EE_2, E_3, E_2, E_1, and worse for MPG, PG and NEA. Because the functional groups of carbonyl produced steric hindrance, the interaction between analyte and GMWCNTs (perfect crystal lattice) increase. Meanwhile the MWCNTs with damaged lattice provided enough space to support the interaction between analyte and adsorbent. The influence of the mass ratio of GMWCNTs to MWCNTs from 1:3 to 3:1 on the recoveries was evaluated. The experimental results were obtained in triplicate and shown in Fig. 1. As can be seen from Fig. 1, the recoveries for most analytes have the maximal values when the ratio is 3:1. Therefore, the mixture of 0.30 g GMWCNTs and 0.10 g MWCNTs was selected as the adsorbent.

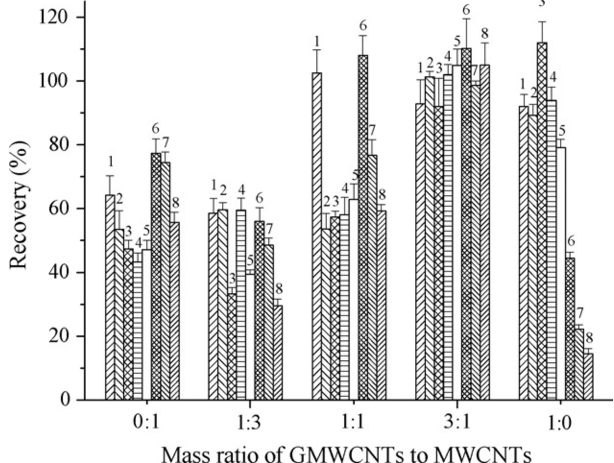


Fig. 1. Effect of type of adsorbents on the recoveries of hormones. 1. CEE, 2. EE_2, 3. E_3, 4. E_2, 5. E_1, 6. NEA, 7. PG, 8. MPG. Adsorbent amount: 0.40 g. Elution solvent: ethyl acetate. Elution solvent flow rate: 1.0 mL min^{-1}.

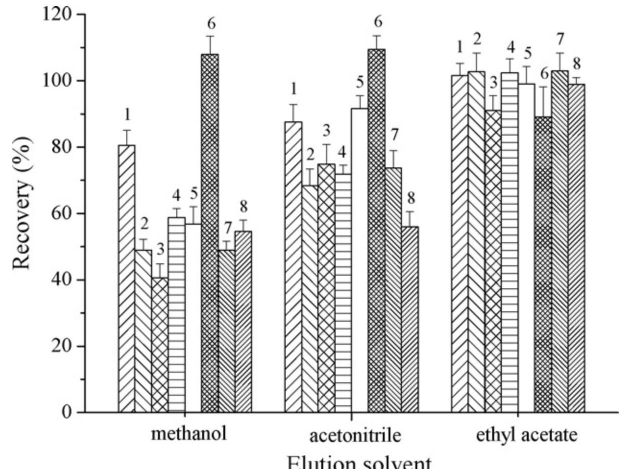


Fig. 2. Effect of elution solvent. 1. CEE, 2. EE_2, 3. E_3, 4. E_2, 5. E_1, 6. NEA, 7. PG, 8. MPG. Adsorbent amount: 0.40 g (GMWCNTs:MWCNTs = 3:1 m/m). Elution solvent volume: 10 mL. Elution solvent flow rate: 1.0 mL min^{-1}.

3.1.2. Types of elution solvents

In order to obtain high recoveries of the analytes, methanol, acetonitrile and ethyl acetate were used as the elution solvents and examined. All the experiments were performed in triplicate. Fig. 2

shows that when ethyl acetate is used as elution solvent the recoveries are highest. Therefore, ethyl acetate was chosen as the elution solvent because of its compatibility with derivatization reaction and its strength to elute the hormones from the solid phase sorbents [53,54].

3.1.3. Amount of adsorbent, volume of elution solvent and flow rate of elution solvent

Three variables that could be potentially affected on the hormone recoveries, such as amount of adsorbent, elution solvent volume and elution solvent flow rate were chosen with BBD. The experimental design is shown in Table 2.

The summary of the analysis of variance (ANOVA) is shown in Table 3. The lack-of-fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression [55]. The non-significant values of lack-of-fit (≥0.0616) for eight analytes revealed that the quadratic model is statistically significant for the response. The significant values of *P*-values (≤0.0069) for analytes indicate the model fitness is good. The value of R^2 (0.9031–0.9664) which also is a measurement of the degree of fitness indicates a good correlation between the experimental and predicted values of the recoveries.

Three response surfaces obtained based on the results in the BBD are illustrated in Fig. 3. Each figure shows the 3D response surface based on two variables of MSPD and quality of the extraction method at center level of the third variable. Fig. 3 shows the effect of different parameters on the E_3 recovery. E_3 was chosen as a model analyte for the hormones because the results obtained by the eight analytes were similar.

From Fig. 3(a), it can be concluded that adsorbent amount and extraction solvent volume have the most significant effect on the hormones recoveries. With increase of the adsorbent amount, the response first increases and then decreases. Because when the adsorbent was too much it was difficult to elute the analytes completely and the signal decreased. The elution solvent volume has a positive effect on hormone recovery. The large volume of elution solvent is beneficial to extraction efficiency. The influence of flow rate of elution solvent on the recovery is shown in Fig. 3(b), the recovery increases first and then decreases with the increase of the flow rate. The 3D response surfaces present a campanulate shape and this result predicates that the interactions between the parameters are significant.

As a result of compromising, the following variables were chosen for the experiment: adsorbent amount, 0.40 g; elution solvent volume, 12 mL and elution solvent flow rate, 0.9 mL min^{-1}.

3.2. Study of stability of derivatives and matrix effect

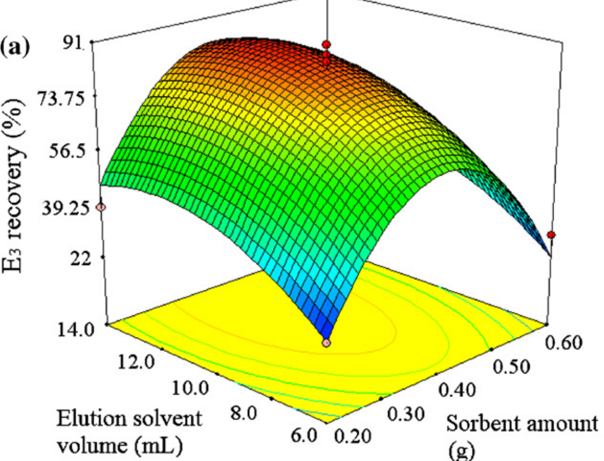
The stability of derivatives were assessed by determining the hormones at the concentration of 20 μg kg^{-1}. The derivatives were determined within 0 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, and 2.0 h after derivatization reaction, respectivly. It was found that the derivatives were stable under room temperature for 2 h. Therefore the analytes could be determined within 2 h.

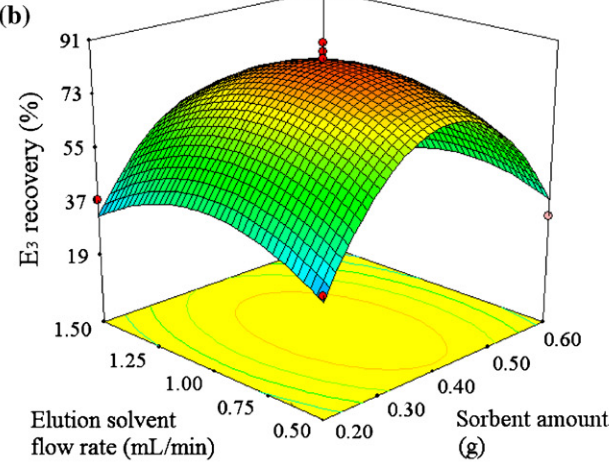
It is well known that matrix effect due to co-extracting and co-eluting matrix substances can seriously affect the analyte signals. This phenomenon can be prevented and resolved by using the ionization technologies, such as the electron impact. Usually, the matrix effect was evaluated by using the following equation:

$$\text{Matrix effect (\%)} = \left[\frac{A_{m+s} - A_m}{A_0} - 1\right] \times 100$$

where A_{m+s} is the peak area response of analyte in the spiked sample, A_m is the peak area response of the analyte in the unspiked sample and A_0 is the peak area of the analyte spiked in purified water. The concentrations of spiked analytes are 10.0, 50.0 and 200.0 μg kg^{-1}. The matrix effect value of zero indicates that there is no matrix effect, a positive value means signal enhancement and a negative value represents signal suppression.

The results as shown in Table 4 indicate that the matrix effect values for eight analytes range from −11.0 to 7.3. The results are





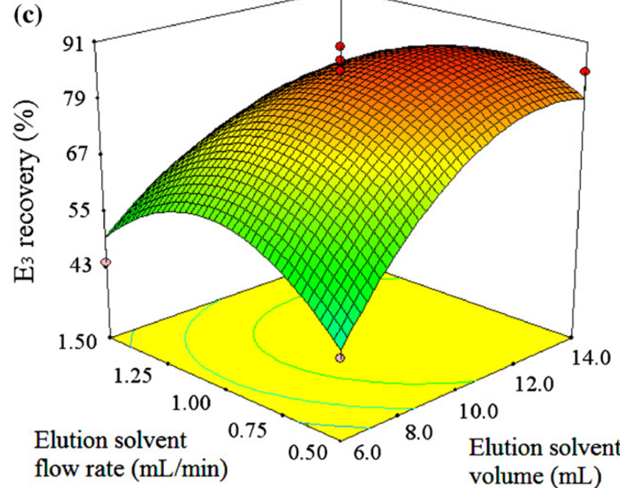


Fig. 3. 3D response surfaces. Variables for extraction of E_3: (a) adsorbent amount and elution solvent volume (solvent flow rate, 1.0 mL min^{-1}); (b) adsorbent amount and extraction solvent flow rate (elution solvent volume, 10 mL) and (c) elution solvent volume and elution solvent flow rate (adsorbent amount, 0.40 g).

Table 3
Parameters for the BBD.

	β_0	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{11}	β_{22}	β_{33}	*P*-value of the mode	Lack of fit value	R^2
CEE	92.24	1.85	7.56	−2.14	16.28	−5.88	−9.00	−29.54	−12.57	−19.52	0.0010	0.0845	0.9486
EE_2	78.70	6.81	10.03	−0.91	−2.15	−10.13	−8.55	−35.71	−2.84	−17.11	0.0006	0.1949	0.9550
E_3	84.22	−1.22	10.67	−4.26	−0.26	−4.09	−6.27	−38.81	−11.71	−14.29	0.0002	0.1239	0.9664
E_2	86.16	0.77	11.45	−9.12	−7.95	−7.20	−2.80	−35.56	−14.41	−22.26	0.0080	0.1314	0.9031
E_1	92.88	4.39	11.68	−7.89	−2.55	−11.88	−4.25	−37.43	−14.45	−25.23	0.0014	0.1607	0.9434
MPG	85.66	5.62	12.89	−6.44	3.80	−6.10	−0.93	−34.02	−15.99	−31.09	0.0023	0.5634	0.9343
PG	90.80	2.02	14.20	−4.68	−5.01	−12.27	−5.39	−35.17	−16.31	−19.52	0.0069	0.1572	0.9074
NEA	86.68	8.31	9.19	−7.43	−0.48	−8.00	−6.65	−37.95	−13.50	−20.68	0.0003	0.0616	0.9655

Table 4
Analytical performances and matrix effect.

Analyte	Linear range ($\mu g\,kg^{-1}$)	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)	*R*	Matrix effect (%)
CEE	5–300	1.3	4.5	0.9982	−11.0
EE_2	2–300	0.5	1.8	0.9989	−8.2
E_3	2–300	0.5	1.7	0.9986	−6.4
E_2	1–300	0.3	0.9	0.9985	1.8
E_1	1–300	0.2	0.8	0.9981	−4.7
MPG	2–300	0.4	1.4	0.9995	1.3
PG	2–300	0.5	1.7	0.9961	7.3
NEA	2–300	0.4	1.2	0.9995	−5.1

acceptable and it can be concluded that the matrix effect of the method is not serious, and the sample pretreatment is successful.

3.3. Method performance

In order to evaluate the performances of the proposed method for quantitative determination of hormones in butter, some spiked samples were used for constructing calibration curves and obtaining other analytical performances under the optimized condition. As shown in Table 4, limit of detection (LOD) and quantification (LOQ) are regarded as the lowest concentrations of the analytes that can be confidently identified and quantified by the proposed method, respectively. The LOD and LOQ are the analyte concentrations producing signal/noise ratio of 3 and 10, respectively. As can be seen in Table 4, the LODs and LOQs are in the range of 0.2–1.3 and 0.8–4.5 $\mu g\,kg^{-1}$, respectively. To establish the linearity of the method, five replicates were done and the correlation coefficients are higher than 0.9961 for 8 target analytes.

3.4. Analysis of samples

Five butter samples were analyzed to evaluate the applicability of the proposed method. In the five samples, no hormones residues at detectable levels were found except for sample 2. In sample 2, the concentration of MPG was found to be 4.1 $\mu g\,kg^{-1}$. The accuracy and repeatability of this method were also evaluated by analyzing the spiked samples. The mean recoveries ($n = 3$) obtained by the proposed method are listed in Table 5. The recoveries range from 84.5 to 111.2% with relative standard deviations from 1.9 to 8.9%. Fig. 4 shows GC–MS chromatograms of five different blank samples and a spiked sample at analytes concentration of 10 $\mu g\,kg^{-1}$.

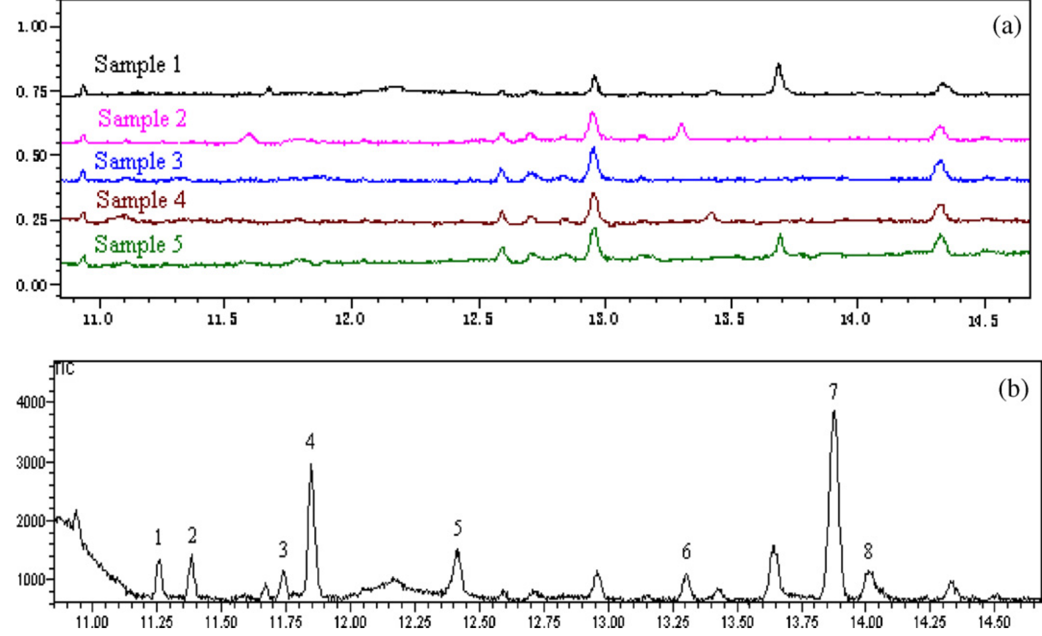


Fig. 4. GC–MS chromatograms of five different blank butter samples (a) and spiked sample 1 at concentration of 10 $\mu g\,kg^{-1}$ (b). 1. CEE; 2. EE_2; 3. E_3; 4. E_2; 5. E_1; 6. MPG; 7. PG; 8. NEA. Adsorbent amount: 0.40 g (GMWCNTs:MWCNTs = 3:1 m/m). Elution solvent: ethyl acetate. Elution solvent volume: 12 mL. Elution solvent flow rate: 0.9 mL min^{-1}.

Table 5
Recoveries and relative standard deviations ($n = 3$) for the analytes in spiked samples.

Sample	Added ($\mu g\,kg^{-1}$)	CEE		EE_2		E_3		E_2		E_1		MPG		PG		NEA	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
1	2	96.3	7.6	87.7	2.1	98.8	8.7	106.7	3.8	84.0	8.1	105.8	2.3	98.5	5.9	88.9	3.7
	50	92.7	5.4	98.7	6.2	91.0	7.3	85.6	5.8	90.6	8.2	92.3	5.6	104.5	6.8	87.6	5.1
2	2	90.1	7.6	95.3	4.3	89.8	6.4	91.5	1.6	98.0	8.0	99.9	6.8	110.5	3.9	109.2	4.7
	50	96.0	6.1	99.1	5.8	92.1	1.9	106.9	2.7	91.0	9.1	101.0	2.7	94.6	9.0	87.8	5.8
3	2	103.8	7.2	86.5	2.4	91.5	7.1	105.0	3.6	93.1	6.6	100.6	8.3	92.8	7.6	103.8	7.1
	50	93.6	7.0	105.9	3.6	93.3	5.6	94.2	4.0	87.7	8.6	91.8	8.9	86.4	4.5	102.9	6.4
4	2	94.3	2.9	97.5	3.0	98.6	6.0	89.2	5.1	93.5	8.4	87.9	6.4	105.4	7.3	100.9	3.9
	50	99.8	7.4	96.6	4.8	102.9	6.2	99.4	6.7	93.1	5.7	91.3	4.5	96.8	4.0	104.1	7.0
5	2	89.1	4.5	88.6	3.5	94.0	4.4	86.0	5.7	89.8	5.8	90.8	6.4	88.2	3.6	84.5	6.3
	50	94.8	2.6	106.6	7.9	95.8	5.1	90.9	7.9	94.6	8.4	96.8	5.3	102.5	6.8	111.2	8.6

4. Conclusion

In this study, MWCNTs were used for extraction of the hormones from butter samples based on MSPD. The results indicated that the proposed method has some advantages in respect of extraction efficiency and extraction time. Compared with the conventional LLE and SPE, it is obvious that the proposed method is much simpler. The method shows potential to be extended to other types of fatty food samples by varying the extraction conditions.

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