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Simultaneous determination of trace sterols in complicated biological samples by gas chromatography–mass spectrometry coupled with extraction using β-sitosterol magnetic molecularly imprinted polymer beads

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ABSTRACT

In this paper, an efficient and sensitive analytical method for the simultaneous determination of three trace sterols including ergosterol, stigmasterol and β-sitosterol in complicated biological samples was developed by gas chromatography–mass spectrometry (GC–MS) coupled with extraction using novel β-sitosterol magnetic molecularly imprinted polymer (mag-MIP) beads. Physical tests suggested that β-sitosterol mag-MIP beads prepared by a rapid microwave synthesis method possessed the porous morphology, narrow size distribution, stable chemical and thermal property. Due to the greatly enlarging surface area and the strong recognition to the target molecules, β-sitosterol mag-MIP beads have a higher enrichment factor for β-sitosterol (~20-fold) and the higher selectivity for β-sitosterol and its analogs than that of β-sitosterol magnetic nonimprinted polymer (mag-NIP) beads. Under the optimum analytical conditions, all the target compounds achieved good chromatographic separation and sensitive detection without matrix interference. It was interesting that three target sterols were actually found in mushroom samples, and stigmasterol and β-sitosterol were actually found in serum and watermelon samples. The recoveries of spiked sample tests were in range of 71.6–88.2% with RSDs of 2.4–10.0% ($n = 3$). This method is reliable and applicable for the simultaneous determination of trace sterols in real biological samples based on the β-sitosterol mag-MIP bead extraction.

1. Introduction

Sterols and related compounds play essential roles in the hormone signaling and physiology of eukaryotic organisms [1]. Ergosterol, stigmasterol and β-sitosterol are three important sterols in fungal and plant samples [2]. Stigmasterol and β-sitosterol are unsaturated phytosterols which could affect endogenous estrogen levels through receptor competition, alterations in enterohepatic recirculation and estrogen reabsorption [3]. Addition of phytosterols in diets can prevent cholesterol from being absorbed into bloodstream by competing for intestinal absorption and lower the risk of heartsick and other diseases such as colon cancer, breast cancer and prostate cancer caused by the excessive absorption of cholesterol [4–7]. Thus, analysis of trace sterols in biological samples such as serum and food samples would provide useful clues for the nutritional control and healthy evaluation.

Due to trace sterols in complicated biological matrix, the sensitive and in situ analytical method has been one of bottle-necks for the determination of sterol contents in real biological samples. A sensitive analytical method for the determination of trace sterols should include an efficient sample preparation technique and a detection technique. Detection methods for sterol analysis mainly include enzymatic method [8], isotopic dilution mass spectrometry (IDMS) method [9], electrochemical analysis [10] and chromatographic method [11]. Nowadays chromatographic techniques, mainly including high performance liquid chromatography (HPLC) [12,13], gas chromatography–flame ionization detector (GC–FID) [14,15] and gas chromatography–mass spectrometry (GC–MS) [16,17], have been the most popular analytical methods for the determination of sterols owing to the perfect combination of separation and detection process. Especially, HPLC is directly used in most cases for the analysis of sterols. GC–MS is also a powerful tool for the analysis of sterols due to the powerful function of structure identification and relatively higher sensitivity. However, there are still few works focusing on the analysis of sterols by GC–MS, although derivatization should be conducted for some sterols prior to GC–MS analysis [18,19].

Solvent extraction [20], supercritical fluid extraction (SFE) [21], solid phase extraction (SPE) [22] and solid phase microextraction (SPME) [23] have been used as conventional sample preparation methods for the extraction of sterols from animal, plant or fungal

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samples. Solvent extraction always requires the large amounts of solvent and multiple consequent steps. SFE by use of an expensive instrument can easily make sterols inactive during extraction procedure [24]. Although SPE and SPME are efficient and solvent-saving sample preparation techniques, they possess the limited extraction capacity and selectivity for trace sterols in complicated biological samples [25]. Molecularly imprinted polymers (MIPs) show a strong chemical affinity and recognition to the target compounds in complicated matrix, due to the shape recognition, hydrogen bonding, and hydrophobic interaction. The functional mechanism is similar to "key and lock" or "antigen and antibody" [26]. Due to the simple preparation and flexibility of MIPs, molecularly imprinted technique has been widely used in sample preparation techniques especially in SPE and SPME, greatly improving the extraction selectivity for trace target compounds from complicated matrix [27,28].

Magnetic polymer beads are the spherical or particle shape magnetic cores with suitable material on the surface, which have been applied to the study of bio-separation of proteomics, catalysis, drug delivery, etc. [29–32]. Magnetic polymer beads can be quickly separated from a sample solution by a simple and cheap magnet [33–35]. Moreover, the extraction capacity can be greatly enhanced via controlling the amount of magnetic polymer beads added into the sample solution. Combining the molecularly imprinted technique with magnetic polymer beads to produce magnetic MIP (mag-MIP) beads can achieve many advantages during sample preparation such as the higher selectivity, larger extraction capacity and prevention of cross-contamination [36]. Microwave irradiation leads to a very rapid even inner heating of solvent and sample in bulk polymerization compared with conventional methods. Microwave synthesis method for the preparation of magnetic polymer beads has many obvious advantages including the shorter polymerization time, higher yield and narrower size distribution of magnetic polymer beads in the latest reports [37–39].

Until now, there are still few works on the simultaneous determination of trace sterols and actually finding the target compounds in the real biological samples. In this work, novel β-sitosterol mag-MIP beads were prepared by a microwave irradiation method for the simultaneous determination of trace ergosterol, stigmasterol and β-sitosterol in complicated biological samples including mushroom, serum and watermelon samples. These biological samples belong to the microorganic, biochemical and plant sample, and are also considered as typical and complicated biological samples for the human health and food safety analysis. Three target sterols can be actually quantified in real biological samples by use of the β-sitosterol mag-MIP bead extraction coupled with GC–MS detection.

2. Experimental

2.1. Chemicals and biological samples

β-Sitosterol standard (purity > 98%) was purchased from Zelang Medicine Corporation (Nanjing, China). Stock solution of β-sitosterol was prepared at a concentration of 100 mg/L in methanol and stored at −18 °C in dark. Working solutions were prepared by appropriate dilution of the stock standard solution with methanol and stored at 4 °C in dark. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Aladdin (Shanghai, China). Cholesterol, ergosterol and stigmasterol with the purity > 98% were purchased from Aladdin (Shanghai, China). All other reagents were of analytical grade.

Serum samples were obtained from the Hospital Affiliated to Sun Yat-sen University (Guangzhou, China). Mushroom (*Lentinula edodes*) and watermelon samples (*Citrullus vulgaris Schrad*) were purchased from local markets in Guangzhou.

2.2. Preparation of β-sitosterol mag-MIP beads

The first step for the preparation of β-sitosterol mag-MIP beads was the preparation of Fe_3O_4 nanoparticles. According to our previous works [36,40], the diameters of Fe_3O_4 particles synthesized by the same method were only in range of 30–50 nm as the cores of β-sitosterol mag-MIP beads. Synthesis of magnetite particles by co-precipitation was conducted according to the previous work [41] with some small modifications. Surface modification of Fe_3O_4 nanoparticles was carried out by the reaction of Fe_3O_4 (2.0 g) and polyethylene glycol (PEG)-6000 (10.0 g) dissolved in doubly distilled water (30 mL). After a 20-min sonication, a homogeneously dispersed solution was obtained.

After that, the pre-polymerization mixture was prepared as follows. The functional monomer methyl methacrylate (MMA) (5.00 mL, 47.1 mmol) and template molecular β-sitosterol (0.414 g, 1.0 mmol) were dissolved in toluene (5.00 mL, 47.0 mmol). The solution was sparged with oxygen-free nitrogen and then stored in dark for 12 h. The pre-polymerization solution, PEG-Fe_3O_4 particles, dispersing media (doubly distilled water, 100 mL), styrene (St) (3.00 mL, 26.2 mmol), cross-linker ethylene glycol dimethacrylate (EGDMA) (1.50 mL, 7.2 mmol) and initiator azo(bis)-isobutyronitrile (AIBN) (0.10 g, 0.60 mmol) were well mixed in a 300 mL single-necked flask and dispersed by vigorous agitation (600 rpm) and bubbled with nitrogen throughout the whole reaction. Microwave irradiation was carried out with a programmed temperature control as follows: initial from room temperature to 40 °C within 2 min, from 40 °C to 60 °C within 2 min, from 60 °C to 70 °C within 2 min and finally keeping at 70 °C for 60 min.

Fig. 1 demonstrates the polymerization process for β-sitosterol mag-MIP beads. β-Sitosterol mag-MIP beads were washed extensively with distilled water, 10% (v:v) acetic acid in methanol and methanol under ultrasonic agitation, respectively, until no leakage and residue of polymerization was observed.

2.3. Study of physical characterization and extraction capability

β-Sitosterol mag-MIP beads were placed on aluminum pegs and sputter coated with 15 nm of gold. Scanning electron microscopy (SEM) was conducted by a Philips XL-30 scanning electron microscopy from Philips (Eindhoven, Netherlands). Bead size distribution was examined by a Malvern MasterSizer 2000 particle size analyzer from Malvern (Malvern, Britain). Infrared (IR) absorption spectra of these beads between 400 and 4000 cm^{-1} were obtained by use of an IR-prespige-21 FTIR spectrometer (Shimadzu, Japan). Thermogravimetric analysis was performed under inert atmosphere (N_2) in an STA-409 PC thermogravimetric analyzer (Netzsch, Selb/Bavaria, Germany), over the temperature range of 20–800 °C. The resulting particles were characterized by magnetic analysis using a SQUID-based magnetometer form Quantum Design (San Diego, CA).

Extraction capability of β-sitosterol mag-MIP beads was evaluated based on extraction capacity and selectivity. Extraction capacity was investigated with a series of β-sitosterol standard solutions in range of 1.00–120.00 μg/L. Extraction selectivity was studied by use of the mixed standard solution containing β-sitosterol and three reference compounds including cholesterol, ergosterol, and stigmasterol at a concentration of 20.00 μg/L.

2.4. Extraction performance and GC–MS analysis

2.4.1. Sample preparation

Mushroom, serum and watermelon were selected as biological samples for the method validation. Fifty microliters of serum sample mixed with ergosterol, stigmasterol and β-sitosterol stan-

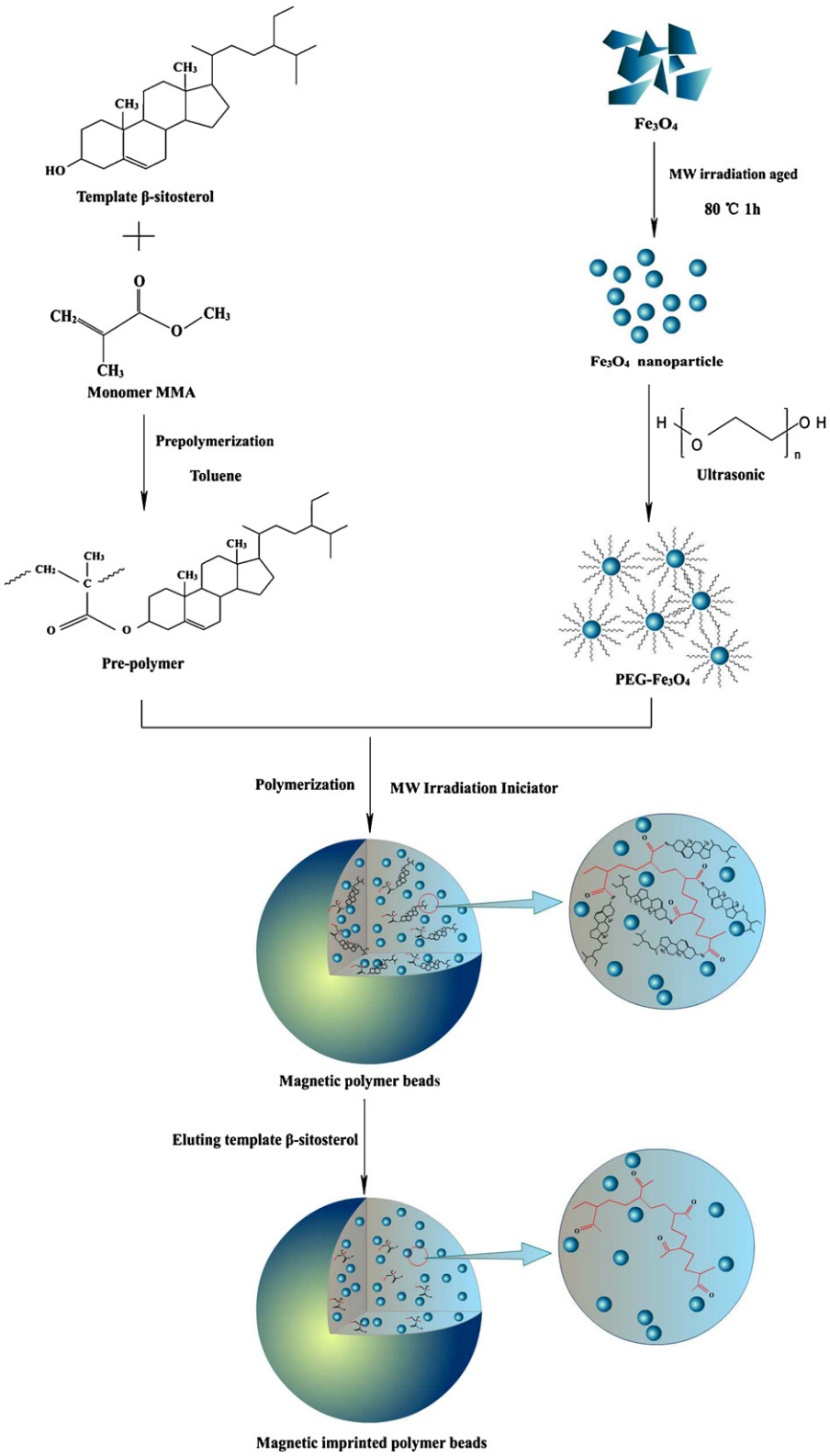

Fig. 1. Schematic representation of polymerization of β-sitosterol mag-MIP beads.

dard solutions were well-balanced and then dissolved in 5.0 mL 0.4 mol/L KOH–methanol and 1.0 mL n-heptane. After that, samples were extracted by microwave assisted extraction (MAE) under the extraction power of 600 W for 30 s. The solution and twice 1 mL n-heptane cleaning solutions were diverted into a centrifuge tube and centrifuged with 3000 rpm for 4 min. Supernatant organic phase was dried with nitrogen, and then dissolved in 10 mL toluene. Two hundred milligrams of mushroom and watermelon samples, respectively, mixed with ergosterol, stigmasterol and β-sitosterol standard solutions were well-balanced and then dissolved in 10 mL 0.4 mol/L KOH solution using H_2O–methanol (2:8, v/v) as the mixed solvent. After that, samples were extracted by MAE under the

extraction power of 420 W for 90 s. Then the sample was filtrated, the extraction and twice 1 mL cleaning solutions were dried by vacuum distillation. After being partitioned by chloroform twice, the organic layer was concentrated by vacuum distillation at 45 °C and dissolved in 10 mL toluene in which the analytes were redissolved. Three milliliters of toluene solution containing analytes was extracted by 40.0 mg of β-sitosterol mag-MIP beads.

2.4.2. mag-MIP bead extraction

The β-sitosterol standard solutions and biological sample extraction solutions were extracted by β-sitosterol mag-MIP beads. Before each use, the recycled beads were regenerated at 120 °C for 12 h. Forty milligrams of beads were added into a 50 mL conical flask and immersed in 3.0 mL standard or sample extraction solution under a reciprocating shaking-table at the room temperature. After being incubated for 40 min with the shaking rate of 120 rpm, 40.0 mg beads were magnetically separated and then eluted for 30 min in 1.0 mL acetonitrile as desorption solvent. The analyte elution was dealt with nitrogen drying step and then derivatizated with 70 μL of BSTFA in 50 mL pyridine. The mixture was heated at 80 °C for 30 min. After cooling, the derivatization solution was dried with a nitrogen stream and redissolved in 100 μL of ethyl acetate. 1.0 μL of sample solution was used for GC–MS injection and consequent analysis.

The validation for the analysis of mushroom, serum and watermelon was investigated by spiked experiments. The amounts of ergosterol standard added to mushroom samples were set at 10.00 and 20.00 μg/L. The amounts of ergosterol standard added to serum and watermelon samples were set at 20.00 and 40.00 μg/L. The amounts of stigmasterol and β-sitosterol standard added to mushroom, serum and watermelon samples were set at 5.00 and 10.00 μg/L, respectively.

2.4.3. GC–MS analysis

GC–MS analysis was carried out by a Shimadzu GC-MS 2010 (Shimadzu, Japan) in this study. A DB-5MS (Agilent Scientific, USA) capillary column (30 m length × 0.25 mm I.D. × 0.25 μm film thickness) was used for the chromatographic separation with the following instrumental conditions: helium flow 1 mL/min; injector temperature 280 °C; transfer line temperature 280 °C; energy of electron 70 eV; ion source temperature 250 °C; and MS Quad 150 °C. Oven temperature programming was as follows: initial 100 °C (1 min) to 280 °C (4 min) at a ramp rate of 30 °C/min and from 280 °C to 300 °C (6 min) at a ramp rate of 20 °C/min. MS analysis was carried out in selected ion mode (SIM), and quantification ions for monitoring ergosterol, stigmasterol and β-sitosterol at *m*/*z* were the ion fraction groups of (468, 378 and 363) with the retention time ranging from 10.01 to 15.80 min (484, 394 and 355) with the retention time ranging from 15.81 to 16.50 min and (486 and 396) with the retention time ranging from 16.51 to 18.00 min, respectively.

3. Results and discussion

3.1. Preparation and physical characterization of β-sitosterol mag-MIP beads

3.1.1. Preparation procedure

Prior to the suspension polymerization, it is imperative to make the surface modification of Fe_3O_4 nanoparticles by PEG as surfactant. According to our previous experience [35], PEG can disperse homogeneously in water and modify Fe_3O_4 well. Moreover, the procedure for the modification of Fe_3O_4 by use of PEG was simple and easily controlled during microwave irradiation.

During the preparation of β-sitosterol mag-MIP beads, some essential factors including polymerization solvent, the types and amounts of functional monomer and cross-linker, the amounts of copolymer monomer were optimized to achieve the optimum preparation conditions according to size distribution and surface morphology of beads observed by microscope (see supplementary materials Tables 1s–3s). First, the whole synthesis process occurred in a suitable organic solvent. Thus, toluene, ethyl acetate, chloroform and DMS were used for the selection of polymerization solvent. The result suggested that β-sitosterol could dissolve well in toluene, and toluene was chosen as the optimum polymerization solvent in the study. Second, functional monomer is a key factor for the powerful molecular recognition of MIPs. Herein, some common functional monomers including acrylamide (AM), methacrylic acid (MAA), 4-vinylpyridine (4-VP) and MMA and the amount of each functional monomer were optimized systematically in the study. By use of MMA as functional monomer, the resultant mag-MIP beads possessed the better homogeneous morphology and stronger molecular recognition for the target compounds. Also, the amount of MMA would further affect the productive yield, homogeneity and morphological structure. The optimization for the amount of MMA suggested that the use of 5.0 mL (47.1 mmol) resulted in the higher productive yield and narrower diameter distribution of β-sitosterol mag-MIP beads. Thus, 5.0 mL (47.1 mmol) MMA was chosen as the optimum functional monomer in the study. Finally, in order to improve the mechanical strength of β-sitosterol mag-MIP beads, cross-linker and copolymer monomer were optimized in the study. When 1.5 mL EGDMA was used as cross-linker, the higher productive yield and better surface homogeneity of the beads were achieved with a narrower size distribution. The use of styrene as copolymer monomer could avoid the leakage of Fe_3O_4 nanoparticles and fragility of the resultant beads. Thus, styrene was chosen as the optimum copolymer monomer in this study with the optimum amount of 3 mL (26.2 mmol).

3.1.2. Physical characteristics

Homogeneous surface morphology is the precondition for the good extraction capacity and reproducibility by use of β-sitosterol mag-MIP beads during the analytical procedure. SEM was employed for the analysis of the particle size and morphology of β-sitosterol mag-MIP beads under different magnifications of 200-fold and 10,000-fold (Fig. 2), respectively. SEM results suggested that β-sitosterol mag-MIP beads were well-shaped spherical particles with a narrow size distribution (Fig. 2a), and the corresponding MIP coating was homogeneously fabricated on the surface of the resultant beads (Fig. 2b). β-Sitosterol mag-MIP beads have the rough and porous surface morphology, greatly enlarging surface area and accelerating the distribution of molecules between sample solution and coating. Therefore, β-sitosterol mag-MIP beads would guarantee good extraction efficiency for trace sterols. Particle size analysis further suggested that homogeneous mag-MIP beads possess a narrow size distribution of 40–200 μm (see supplementary materials Fig. 1s). Moreover, particle size distribution of 80% mag-MIP beads is in the range of 60–160 μm. This result is consistent to SEM analysis.

β-Sitosterol mag-MIP and magnetic nonimprinted polymer (mag-NIP) beads possess similar IR absorption spectra (see supplementary materials Fig. 2s). IR analysis result suggested that free radical polymerization reaction occurred involving the functional monomer MMA, copolymer monomer styrene and cross-linker EDGMA during the preparation of β-sitosterol mag-MIP beads. Thermogravimetric analysis was performed to test the thermal stability of β-sitosterol mag-NIP and mag-MIP beads. There is no obvious weight loss below 300 °C. Dramatic weight loss happens from 300 to 450 °C, and the fastest mass loss occurs at 390.2 and 398.4 °C for β-sitosterol mag-NIP and mag-MIP beads, respectively (see supplementary materials Fig. 3s). Thermogravimetric analysis results suggested that these mag-MIP beads are thermally stable and suitable for HPLC and GC–MS analysis.

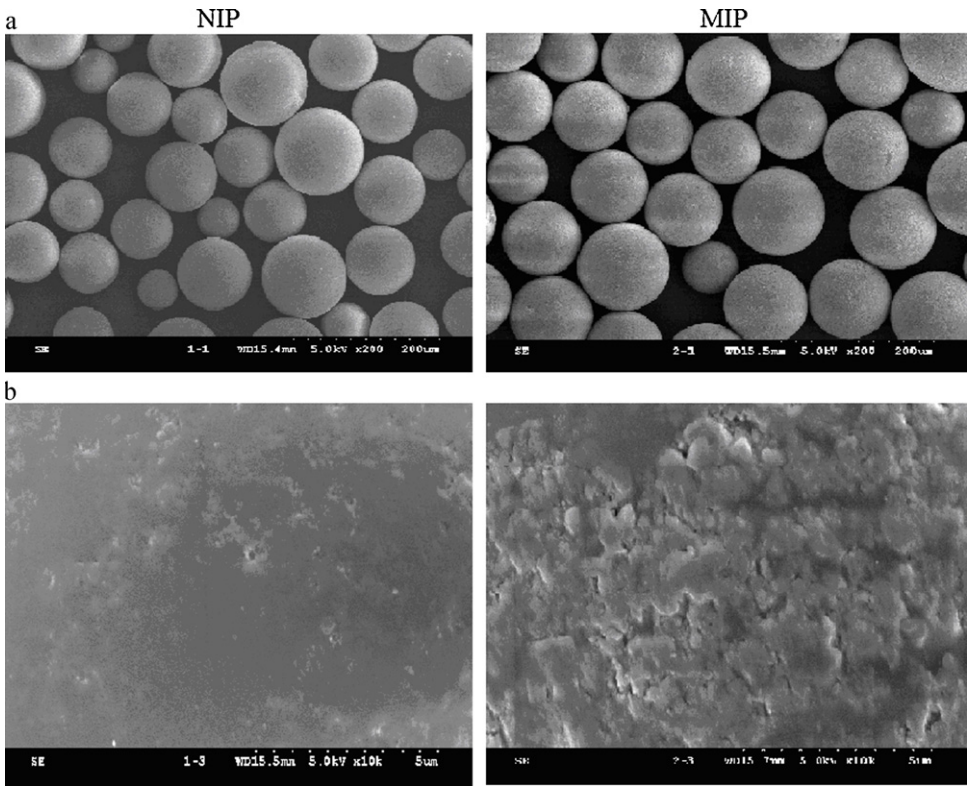

Fig. 2. Scanning electron micrographs of the mag-NIP (left) and mag-MIP (right) beads prepared by microwave irradiation. Magnifications: (a) 200× and (b) 10,000×.

Magnetic hysteresis loops analysis was conducted to test the magnetic property of β-sitosterol mag-NIP and mag-MIP beads (see supplementary materials Fig. 4s). Symmetrical general shape curves suggest that there is no magnetic retentivity, so these mag-NIP and mag-MIP beads show superparamagnetism with saturation magnetization values of 0.24 and 0.10 emu/g, respectively, and can fully meet the requirement of magnetic separation. Solvent-resistant property of β-sitosterol mag-MIP beads was tested by the solvents with different polarity including methanol, acetonitrile, acetone, chloroform, ethyl acetate, tetrahydrofuran, benzene, toluene, 10% (v:v) acetic acid in methanol and 10% (v:v) acetic acid in acetonitrile. The beads were immersed in these solvents followed by ultrasound for 30 min, and no any desquamation or crack phenomenon occurred. Therefore, β-sitosterol mag-MIP beads possess the excellent chemical stability and could remain the good surface morphology and extraction efficiency after the use of more than 100 times.

3.2. Extraction capability of β-sitosterol mag-MIP beads

3.2.1. Extraction capacity

Extraction capacity of enrichment medium is directly related with analytical sensitivity. In this study, a series of β-sitosterol standard solutions in range of 1.00–120.00 μg/L were prepared to evaluate the extraction capacity of β-sitosterol mag-NIP and mag-MIP beads under optimum extraction conditions. Fig. 3 shows the gradually increasing trends of extraction yields by β-sitosterol mag-NIP and mag-MIP bead extraction with the increasing concentrations of β-sitosterol standard solutions in range of 1.00–120.00 μg/L. Mag-NIP and mag-MIP bead extraction for β-sitosterol reaches the equilibrium when standard concentrations are up to 50.00 and 80.00 μg/L, respectively. Finally, extraction capacity of β-sitosterol mag-NIP and mag-MIP beads was calculated to be around 115.4 and 327.5 pmol for β-sitosterol. β-Sitosterol mag-MIP beads demonstrate a strong molecular recognition to β-sitosterol, while mag-NIP beads have only the nonspecific adsorption to the target compound. Thus, it can be seen that β-sitosterol mag-MIP beads have the higher extraction capacity than mag-NIP beads. The proposal method provided a high enrichment factor, about 20-fold for β-sitosterol.

3.2.2. Extraction selectivity

Quantitative analysis of trace sterols in biological samples is difficult owing to the complicated matrix. Novel β-sitosterol mag-MIP beads can achieve the good selective separation of trace β-sitosterol and its analogs from complicated matrix, and extraction selectivity

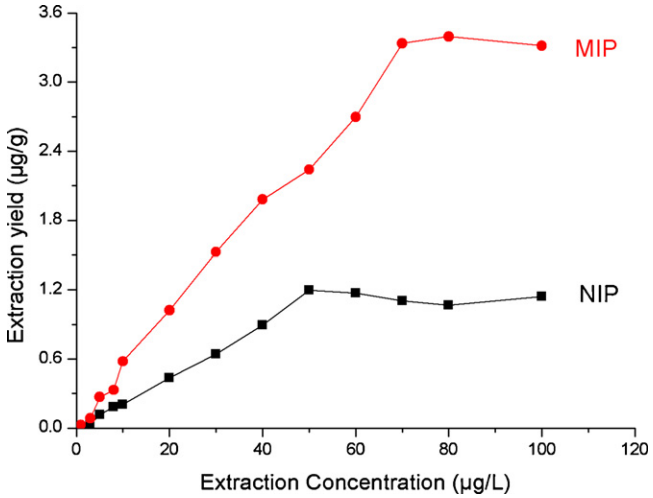

Fig. 3. Extraction yield curve by the mag-NIP and mag-MIP extraction for β-sitosterol in range of 1.00–120.00 μg/L.

is a key factor influencing the specific recognition to target compounds with the similar structures as β-sitosterol [27].

In this study, extraction selectivity was investigated based on the comparison of extraction yields of the mixed standard solutions containing cholesterol, ergosterol, stigmasterol and β-sitosterol at a concentration of 20.00 μg/L extracted by β-sitosterol mag-MIP and mag-NIP beads. Four compounds belong to the sterol family and have a very similar structure with a hydroxyl group at the 3-position of A-ring. It can be seen from Fig. 4 that mag-MIP bead extraction yields of stigmasterol and β-sitosterol in water are 118.8 and 122.9 pmol, respectively, which are much higher than mag-NIP bead extraction yields. However, the extraction yields of cholesterol and ergosterol by these mag-MIP and mag-NIP beads have no significantly difference. The comparison result of the extraction yields by β-sitosterol mag-MIP and mag-NIP beads is attributed to the molecular analog degree of target compounds to the template molecule [42]. Stigmasterol has almost the same molecular structure to β-sitosterol except for a double bond in side chain, whereas the structures of cholesterol and ergosterol have some difference to the structure of template molecule. The strong interaction between β-sitosterol mag-MIP beads and target compounds is based on the specific molecular recognition, so the excellent extraction yields for stigmasterol and β-sitosterol are presented. The extraction mechanism of cholesterol and ergosterol by β-sitosterol mag-MIP beads likely depends on the nonspecific absorption, which results in the lower extraction yields than those of stigmasterol and β-sitosterol. The results suggested that β-sitosterol mag-MIP beads possessed the good extraction selectivity for β-sitosterol and its analogs.

3.3. Development of an analytical method for trace sterols

3.3.1. Study on extraction conditions by β-sitosterol mag-MIP beads

During the β-sitosterol mag-MIP bead extraction, some important influencing factors were studied to achieve the good extraction efficiency, including the shaking rate, extraction solvent, extraction time, desorption solvent and desorption time (see supplementary materials Fig. 5s). Appropriately stirring the sample solution could accelerate the diffusion of analytes from sample solution to mag-MIP beads and improve the extraction efficiency. Finally, 120 rpm was selected as the optimum stirring rate. Extraction procedure consists of thermodynamic and dynamic processes. Apart from stirring rate, extraction solvent, extraction time, desorption solvent and desorption time are essential factors greatly influencing the extraction and desorption process. In the study, different polar solvents including hexane, toluene, tetrahydrofuran (THF), acetic ester, chloroform, acetone, acetonitrile, methanol and water were used for the optimization of extraction solvent by mag-MIP bead extraction. Finally, toluene was chosen as extraction solvent in the study with the optimum extraction time of 40 min. Moreover, desorption solvent and time was also optimized in detail. Two series of mixed desorption solvents were used for the selection by use of methanol, acetic acid in methanol (1% or 10%, v:v), acetonitrile and acetic acid in acetonitrile (1% or 10%, v:v). Finally, acetonitrile was selected as the optimum desorption solvent with the optimum desorption time of 30 min.

3.3.2. GC–MS analysis

Analysis of sterols by GC–MS based on an SIM mode can achieve excellent analytical sensitivity, but derivatization is needed prior to GC–MS detection due to the low volatility of target compounds. BSTFA as an efficient derivative reagent was used prior to GC–MS analysis in this study. In order to make sterols derived completely and achieve the excellent analytical sensitivity, derivative conditions were optimized including the amount of derivative reagent, derivative temperature and derivative time (see supplementary materials Fig. 6s). The results suggested that the optimal derivatization procedure should be carried out by use of 70 μL derivative reagent BSTFA for 30 min under the temperature of 80 °C.

A series of mixed standard solutions involving ergosterol, stigmasterol and β-sitosterol were prepared and extracted by β-sitosterol mag-MIP beads under the optimum extraction conditions followed by GC–MS detection. The linear ranges for ergosterol, stigmasterol and β-sitosterol were 5.0–60.0 μg/L, 2.0–30.0 μg/L and 2.0–30.0 μg/L, respectively. The precision of the analytical method was evaluated by triplicate measurements using three batches of β-sitosterol mag-MIP beads for the extraction of 20.00 μg/L mixed standard solution, and the relative standard deviations (RSDs) were achieved in range of 6.0–6.2%. The detection limits were calculated to be 3.6, 1.2 and 1.1 μg/L based on a signal-to-noise ratio of 3 (S/N = 3) for ergosterol, stigmasterol and β-sitosterol, respectively. According to the previous paper [43], the detection limits (S/N = 3) of β-sitosterol and stigmasterol were 3.40 and 1.06 μmol/L, respectively, by HPLC. The limit of quantification of ergosterol was 40 μg/L by HPLC [44]. The method sensitivity by GC–MS is equivalent or even higher than the previous HPLC method. All the analytical parameters suggested the reliability of the method for the simultaneous determination of trace sterols.

3.4. Application for the simultaneous determination of sterols in biological samples

In this study, the proposal analytical method was applied for the simultaneous determination of trace sterols in the complicated mushroom, serum and watermelon samples. Typical chromatograms for the simultaneous determination of ergosterol, stigmasterol and β-sitosterol in the real and spiked biological samples by β-sitosterol mag-MIP bead extraction coupled with GC–MS detection are illustrated in Fig. 5. It can be seen from the chromatograms that all the three target sterols achieve a good chromatographic separation without matrix interference.

It is satisfactory that these three target compounds were actually found in mushroom sample simultaneously by the analytical method proposed, and also stigmasterol and β-sitosterol can be actually detected in real serum and watermelon samples. The contents of ergosterol, stigmasterol and β-sitosterol in mushroom samples were calculated to be 23.08, 4.33 and 3.52 μg/L, respectively. The contents of stigmasterol and β-sitosterol in serum samples were calculated to be 3.05 and 7.21 μg/L, respectively. And the contents of stigmasterol and β-sitosterol in watermelon samples were calculated to be 8.72 and 3.64 μg/L, respectively. According to the previous reports [45–47], the contents of three target sterols are almost consistent to the reference values (Table 1). Then, spiked experiments were conducted for a further method validation. As shown in Table 1, the contents of ergosterol, stigmasterol and β-sitosterol in spiked samples were set at the corresponding levels according to the contents of sterols in the real biological samples. The recoveries of spiked mushroom samples were found to be 80.9–86.0% for ergosterol, 75.8–77.2% for stigmasterol and 74.2–80.8% for β-sitosterol, respectively, with RSDs of 2.4–9.3% (n = 3). The recoveries of spiked serum samples were found to be 75.4–75.5% for ergosterol, 86.2–88.2% for stigmasterol and 72.4–79.4% for β-sitosterol, respectively, with RSDs of 4.4–9.7% (n = 3). The recoveries of spiked watermelon samples were found to be 71.6–74.9% for ergosterol, 81.0–84.5% for stigmasterol and 71.6–76.9% for β-sitosterol, respectively, with RSDs of 4.1–10.0% (n = 3). From all the results mentioned above, it is clear that the method proposed is reliable and applicable for the simultaneous determination of trace ergosterol, stigmasterol and β-sitosterol in complicated biological samples.

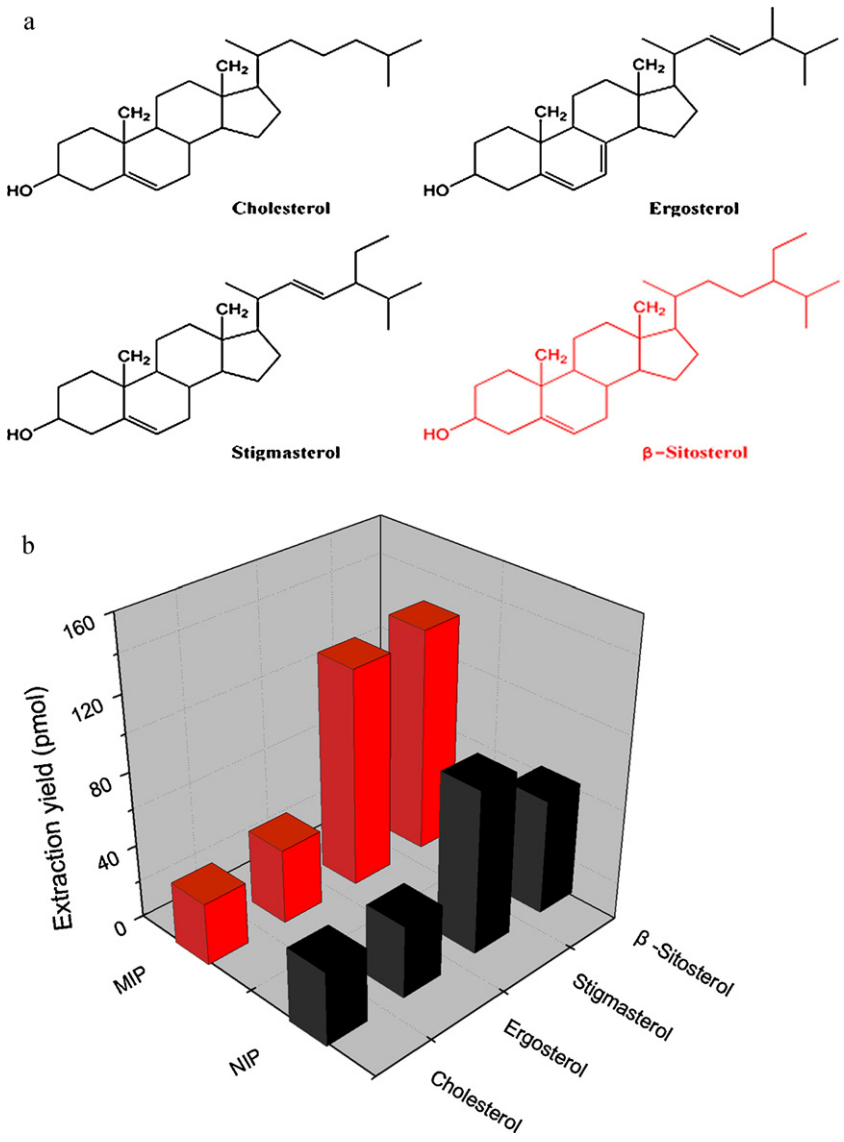

Fig. 4. Extraction yields of β-sitosterol, cholesterol, ergosterol and stigmasterol by mag-MIP and mag-NIP beads at 20.00 μg/L. (a) Structure of β-sitosterol and other three reference compounds and (b) extraction selectivity for β-sitosterol by mag-MIP beads based on extraction yields.

Table 1
Recoveries of sterols in biological samples by the mag-MIP bead extraction ($n = 3$).

Sample	Target compounds	Original content (μg/L)	Added (μg/L)	Found (μg/L)	Recovery (%)	RSD (%)
Mushroom	Ergosterol	23.08	10.00	31.17	80.9	3.6
			20.00	40.29	86.0	2.4
	Stigmasterol	4.33	5.00	8.12	75.8	9.3
			10.00	12.05	77.2	6.5
	β-Sitosterol	3.52	5.00	7.23	74.2	4.0
			10.00	11.60	80.8	4.4
Serum	Ergosterol	N.D.[a]	20.00	15.08	75.4	4.4
			40.00	30.21	75.5	5.8
	Stigmasterol	3.05	5.00	7.36	86.2	7.4
			10.00	11.87	88.2	6.2
	β-Sitosterol	7.21	5.00	10.83	72.4	8.6
			10.00	15.15	79.4	9.7
Watermelon	Ergosterol	N.D.[a]	20.00	14.33	71.6	10.0
			40.00	29.97	74.9	4.1
	Stigmasterol	8.72	5.00	12.77	81.0	6.4
			10.00	17.17	84.5	5.6
	β-Sitosterol	3.64	5.00	7.22	71.6	6.2
			10.00	11.33	76.9	6.3

[a] N.D. means not detected.

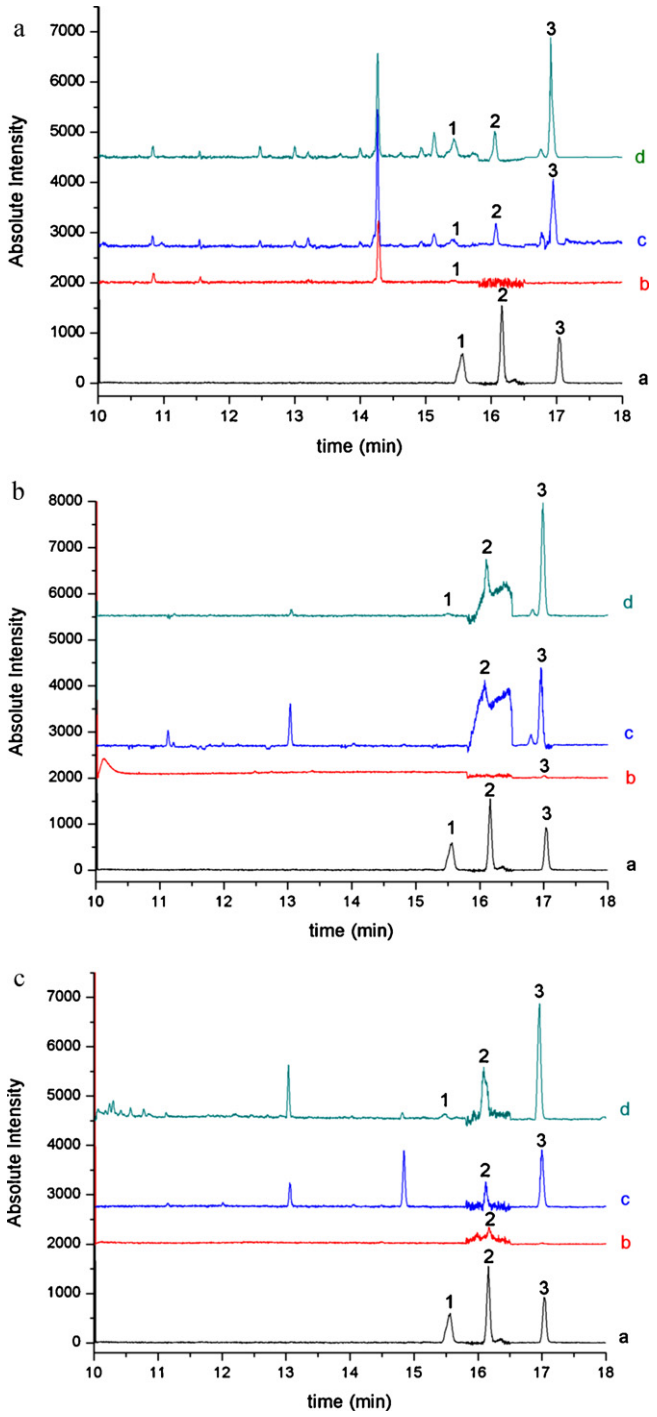

Fig. 5. GC–MS chromatograms of the mushroom (a), serum (b) and watermelon samples (c) by β-sitosterol mag-MIP bead extraction. 1. ergosterol, 2. stigmasterol, and 3. β-sitosterol. Curve *a* is the chromatogram of direct injection of mixed standard solution (200.0 μg/L); curve *b* is the chromatogram of sample solution after MAE; curve *c* is the chromatogram of sample solution extracted by mag-MIP beads; and curve *d* is the chromatogram of spiked sample solutions (20.00 μg/L for ergosterol in mushroom, and 40.00 μg/L for ergosterol in serum and watermelon; 10.00 μg/L for stigmasterol and β-sitosterol in three samples) extracted by mag-MIP beads.

4. Conclusion

Simultaneous determination of trace ergosterol, stigmasterol and β-sitosterol in complicated biological samples were conducted by a novel β-sitosterol mag-MIP bead extraction coupled with GC–MS detection. β-Sitosterol mag-MIP beads possessed the porous morphology, narrow size distribution, stable chemical and thermal property, which guaranteed the good extraction capability for the target sterols. β-Sitosterol mag-MIP beads provided the higher enrichment factor and selectivity for β-sitosterol and its analogs than that of corresponding mag-NIP beads. Then, the proposal analytical method based on the β-sitosterol mag-MIP bead extraction and GC–MS detection was established and applied for the simultaneous determination of trace sterols in real biological samples. Owing to the strong recognition power of mag-MIP beads, all the target compounds achieved the good chromatographic separation without matrix interference. It is satisfactory that these three target sterols were actually found in mushroom sample simultaneously by the analytical method proposed, and stigmasterol and β-sitosterol were actually detected in serum and watermelon samples. The spiked experiments achieved the recoveries in range of 71.6–88.2% with RSDs of 2.4–10.0% ($n = 3$). The results suggested the reliability of this new method for the simultaneous determination of trace ergosterol, stigmasterol and β-sitosterol in biological samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.022.

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