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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-