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Rapid magnetic solid-phase extraction for the selective determination of isoflavones in soymilk using baicalin-functionalized magnetic nanoparticles

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Abstract

Most protocols of sample preparation for isoflavone determination in soymilk and other liquid soybean products involves tedious freeze drying and time-consuming extraction procedures. We report a facile and rapid magnetic solid phase extraction (MSPE) of isoflavones from soymilk for subsequent HPLC–ESI-MS/MS analysis. The extraction was based on the selective binding of isoflavones to baicalin functionalized core-shell magnetic nanoparticles (BMNPs). The proposed MSPE-HPLC-MS/MS analytical method had a linear calibration curve in the concentration range from 0.3 to 80 mg/L isoflavones. With the use of calycosin, an isomer of one of the isoflavones targeted as internal standard, inter-day (5 days) precisions of the slope and intercept of the calibration curves were found to be in the range between 2.5% and 3.6% (RSD, n = 5). Six isoflavones, i.e. daidzein, glycitein, genistein, daidzin, glycitin, and genistin were detected in commercial soymilk samples and quantified by the proposed analytical method. The results indicated that the method was useful for fast determination of isoflavones in soymilk and other liquid soybean products.

Keywords

soymilk; sample preparation; baicalin-functionalized magnetic nanoparticles; magnetic solid phase extraction; HPLC–ESI-MS/MS

INTRODUCTION

Soybean (*Glycine max*) is one of the most important crops in the world as the staple food in most Asian countries, oil seed crop, and feed for livestock and aquaculture.¹ Isoflavones, namely aglycones (daidzein, genistein and glycitein) and their respective glucosidic conjugates, were important phytoestrogen in soybean responsible for human health.^{2, 3} Previous studies suggested that the isoflavones in soybeans and related products might be the contributing factors in easing symptoms of postmenopausal women,^{1, 4} reducing the risk of osteoporosis,^{5, 6} preventing cardiovascular diseases,^{7, 8} preventing cancer,^{9, 10} and antimutagenic effects.^{11, 12} Meanwhile, the epidemiological studies also revealed that high intake of soy origin foods might have a significant beneficial impact on public health.^{13–15}

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Many analytical methods have been developed for isoflavone determination in soy products.^{16–21} The sample preparation approaches could be divided into two categories according to the sample form: solid or liquid. Solid soy samples, such as soybeans and soy protein, require only grinding before extraction, followed by conventional extraction techniques, such as soxhlet,²² shaking and stirring,²³ using appropriate organic solvents, and/or “modern” extraction methods, such as ultrasound-assisted extraction (UAE),²⁴ pressurized liquid extraction (PLE),²⁵ supercritical fluid extraction (SFE),²⁶ high-speed counter-current chromatography (HSCCC)²⁷ and microwave-assisted extraction (MAE).²⁸ In many cases, in addition to filtration and centrifugation,¹⁸ further purification and/or pre-concentration of the target compound fraction are commonly needed, including evaporation to dryness and re-dissolved in another solvent or solid phase extraction (SPE).²⁹ As for liquid samples such as soymilk and soy beverages, they are often freeze-dried first and then treated as solid soy samples.^{18, 30–32} However, freeze-drying is a tedious, expensive, and more importantly, a non-selective extraction procedure to isolate isoflavones from the sample matrix. As an example, it took 2 days to complete the freeze-drying sample preparation in an UPLC-MS analysis of isoflavones in soymilk.³² The lengthy sample pretreatment may increase variations in analytical results as well. Thus, to develop a rapid, simple, selective, and efficient sample preparation approach for isoflavone determination is highly significant.

Magnetic solid phase extraction (MSPE) is a new type of SPE using superpara magnetic sorbents. It has great advantages in separation science.^{33–37} The sorbent needs not be packed into a cartridge as in traditional SPE. In addition, the phase separation can be easily achieved by using an external magnet placed outside the extraction vessel without the need for centrifugation or filtration, which makes the sample preparation easy and fast. A facile MSPE extraction protocol with high selectivity toward flavonoids has been established in our previous work using baicalin-functionalized magnetic nanoparticles (BMNPs).³³ In this work, the synthetic method of BMNPs was improved, and a rapid MSPE protocol based on BMNPs was developed to selectively extract isoflavones from soymilk without involving a freeze-drying procedure for subsequent HPLC–ESI-MS/MS analysis. The biggest challenge for soymilk isoflavone determination has been to selectively extract the analytes without freeze-drying from liquid soy matrix containing a large amount of interfering substances such as fat, protein and carbohydrate, etc. In this work, selective extraction of isoflavones from soymilk was explored by means of the molecular affinity between baicalin on BMNPs and isoflavones in soymilk due to their common skeleton (C₆–C₃–C₆ great -conjugated system).. Preparation of baicalin functionalized magnetic nanoparticles and MSPE using this material were investigated. Extraction conditions were studied to achieve reproducible and high extraction efficiency. Fast quantification of isoflavones in soymilk by using the proposed MSPE-HPLC-MS/MS analytical method was demonstrated.

MATERIALS AND METHODS

Reagents and Chemicals

Soymilk samples were purchased from a local market in Madison, MS, USA and stored at 4 °C till analysis. HPLC grade acetonitrile and formic acid were purchased from Fisher Scientific (Hanover Park, IL). Baicalin was prepared from *Scutellaria baicalensis* in our laboratory and its structure was elucidated on the basis of MS, ¹H NMR and ¹³C NMR spectral evidences. Six isoflavone standards daidzein (De), glycitein (Gle), genistein (Ge), daidzin (Di), glycitin (Gli), genistin (Gi) and internal standard calycosin (IS) (structures shown in Figure 1) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Milli-Q (Millipore Corp., Bedford, MA) water was used throughout the work. Other chemicals and solvents of analytical grade were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA).

Preparation of Baicalin-functionalized Magnetic Nanoparticles (BMNPs)

The multistep procedure for the preparation of BMNPs is shown in Fig. 2. First, amino-functionalized magnetic nanoparticles (AMNPs) were prepared by hydrothermal method as follows: 6.5 g of 1,6-hexanediamine, 2.0 g of anhydrous sodium acetate and 1.0 g of $\text{FeC}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 30 mL of ethylene glycol by vigorously stirring at 50 °C to get a transparent solution. Then, the mixed solution was transferred into a teflon lined autoclave to obtain AMNPs for 6 h at 198 °C. The AMNPs were then rinsed with water and ethanol twice, and then dried at 50 °C. During each rinsing step, the AMNPs were separated from the supernatant by applying an external magnet.³⁸

Finally, 150 mg AMNPs and 50 mg baicalin were dispersed in 10 mL 75% dimethyl sulfoxide (DMSO)/phosphate solution (10 mM, pH 5.5) containing 35 mg 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS). After gently shaking for 2 h, washed by 75% DMSO and water twice successively, BMNPs were obtained by magnetic separation. The preparation procedures are illustrated in Figure 2. The loading mass of baicalin in BMPNs was characterized by elemental analyzer.

BMNPs Characterization

Transmission electron microscope (TEM) images were acquired on a Tecnai G² F20 S-TWIN TEM (200 KeV, FEI, OR, USA). Carbon and nitrogen analyses were performed on a Carlo Erba (Italy) model 1106 elemental analyzer.

MSPE Procedure

To a tube containing 100 μL soymilk sample 5 μL of calycosin at 40 mg/L was added as internal standard (IS). While stirring, 100 μL BMNPs solution prepared above was added. The mixture was vigorously shaken for 5 min using a vortex oscillator. The tube was placed on a magnet for 30 sec to let BMNPs settle down. The supernatant was discarded. After 2 times washing with 100 μL water, isoflavones were eluted out from BMNPs with 100 μL warm methanol (about 60 °C). After magnetic separation, the supernatant was carefully saved, and mixed with 100 μL water. Portions (20 μL) were injected into the HPLC–ESI–MS/MS system for analysis without further purification. The MSPE procedure described above is illustrated in Figure 3.

HPLC–ESI–MS/MS Analysis

The system consisted of two pumps (LC-10ADvp, Shimadzu, Toyoto, Japan), an on-line degasser (DGU-12A, Shi-madzu), and triple quadrupole mass spectrometers equipped with an ESI source (TSQ Quantum, Thermo Scientific, San Jose, CA, USA). Both the LC and mass spectrometer were controlled by Xcalibur software (Thermo Finnigan). A reversed-phase column (C_{18} , 150 mm $\times$ 2.1 mm, 5 μm) was used for separation. A switching-valve was placed after the column directing the effluent either to waste or to the MS detector. Gradient LC elution was carried out with two mobile phases: (A) 10% acetonitrile/0.1% formic acid and (B) 90% acetonitrile/0.1% formic acid at a flow rate of 0.25 mL/min. The elution was programmed as following: time 0–20.00 min, mobile phase B was linearly increased from 10% to 45%; time 20.10–25.00 min, 100% mobile phase A to equilibrate the column for next run.

The MS detector was operated in the positive mode with the following settings: spray voltage of 3 KV, vaporizer temperature of 300°C, tube lens voltage of 150 V; capillary voltage of 35 V, capillary temperature of 270°C, and sheath and aux gas flow rates of 35 and 10, respectively. The optimized relative collision energies for collision-induced dissociation (CID) were 30 V, using m/z 255 199 for daidzein, m/z 271 153 for genistein, m/z

285 242 for glycitein and calycosin (IS), m/z 417 255 for daidzin, m/z 433 271 for genistin, and m/z 447 285 for glycitin SRM detection, respectively.

RESULTS AND DISCUSSION

Characterization of BMNPs

A facile two-step synthesis of BMNPs was developed in this work. Firstly, AMNPs were prepared by one-pot strategy using $\text{FeC}_3 \cdot 6\text{H}_2\text{O}$ and 1,6-hexanediamine as reported previously.³⁸ Then, the acid amide was condensed through the $-\text{NH}_2$ on AMNPs and the $-\text{COOH}$ on baicalin using EDC and Sulfo-NHS as dehydrating agents. Sulfo-NHS was used to prepare amine-reactive esters of carboxylate groups for crosslinking, i.e. carboxylates ($-\text{COOH}$) of baicalin reacted to Sulfo-NHS in the presence of a carbodiimide (here is EDC), to form a semi-stable Sulfo-NHS ester, the latter of which then reacted with primary amines ($-\text{NH}_2$) to form amide crosslinks (as shown in Figure 2).

The TEM image of BMNPs with 400 nm in diameter is shown in Figure 4. According to the BMNPs structure (Figure 2), the carbon came from baicalin and AMNP. And the ratio of carbon to nitrogen in AMNPs part was constant (3:1). Thus, the mass percentage of loaded baicalin in BMNPs was calculated as $5.13 \pm 0.06\%$ which was determined indirectly by the quantities of carbon and nitrogen using Eq. (1), in which C% and N% are the content of carbon and nitrogen in BMNPs; Ar (C), Ar (N) and Mr ($\text{C}_{21}\text{H}_{18}\text{O}_{11}$) are the relative atomic mass of carbon and nitrogen, and relative molecular mass of baicalin, respectively.

$$\text{Baicalin \%} = \left(\frac{\text{C\%}}{\text{Ar(C)}} - \frac{\text{N\%}}{\text{Ar(N)}} \times 3 \right) \times \text{Ar(C)} / \left(\frac{\text{Ar(C)} \times 21}{\text{Mr}(\text{C}_{21}\text{H}_{18}\text{O}_{11})} \right) \quad (1)$$

MSPE Procedure

Baicalin-functionalized magnetic-nanoparticles (BMNPs) may serve as effective magnetic SPE sorbents for selective extraction of isoflavones. The affinity may be attributed to the following factors: the polar functional groups in baicalin molecule, such as $-\text{OH}$ and $-\text{CHO}-$, can form hydrogen bonds with isoflavones; the planar structure of baicalin molecular skeleton ($\text{C}_6-\text{C}_3-\text{C}_6$, a great conjugated system) is prone to absorb similar planar structures through $\pi-\pi$ and/or $n-\pi$ charge transfer effects; and the great conjugated π system in baicalin molecule enhances the charge transfer capacity through the increment of the electron density in the ring. Therefore, the interaction of BMNPs with polar aromatic compounds such as isoflavones is particularly strong. The interaction is comparable to SPE cartridges based on divinylbenzene,³⁹ which are very effective to selectively extract soy isoflavones. In the present MSPE procedure, sorbents (BMNPs) were added into the soymilk solution. The targeted analytes (isoflavones) in the soymilk were adsorbed onto the sorbent surface under vigorously shaking. The sorbents with captured analytes were then easily recovered from the suspension using an external magnetic separator. The analytes were consequently eluted from the sorbents and analyzed by HPLC-ESI-MS/MS. Compared to widely used freeze-dried methods, the application of MSPE greatly simplified the sample pretreatment which needed no more than 10 min for sample preparation.

Daidzein, one of the isoflavones in soymilk, was selected as the model compound to evaluate the extraction efficiency. The extraction efficiencies remained almost the same for extraction times of 5, 10, and 20 min (from $88.7 \pm 0.2\%$ to $89.5 \pm 0.3\%$); while the eluting efficiencies remained almost the same for elute times of 1, 3, and 5 min. Therefore, 5 min and 1 min were selected for extraction and eluting time, respectively. Isoflavones are soluble in methanol, ethyl acetate, pyridine, slightly soluble in warm water, and practically insoluble

in chloroform and hexane. Therefore, water-based soymilk was directly used as the loading solvent. On the other hand, warm methanol at about 60°C was chosen as eluting solvent due to its high miscibility with water as well as high suitability for HPLC analysis. To improve the accuracy and reproducibility of the quantification of isoflavones in soymilk, calycosin (IS), an isomer of one of the isoflavones targeted (i.e. glycitein) was used as internal standard. Calycosin exhibited behaviors similar to the isoflavone analytes in both MSPE and MS detection processes. Calycosin does not occur naturally in soy beans. It is also easily available compared with isotope labeled isoflavones. An equal amount of water was added into the elution solution prior to HPLC–ESI-MS/MS analysis to minimize the solvent effects on the separation.

Analytical Figures of Merit

After the sample preparation, isoflavones in soymilk were quantitatively analyzed using calycosin as internal standard (IS). LC–MS analysis of authentic daidzein, genistein, glycitein, daidzin, genistin, glycitin and internal standard was performed. The behaviors of chromatographic retention and product ion spectra of these six isoflavones are shown in Figure 5. Using m/z 255 → 199 for daidzein, m/z 271 → 153 for genistein, m/z 285 → 242 for glycitein and calycosin (internal standard), m/z 417 → 255 for daidzin, m/z 433 → 271 for genistin, and m/z 447 → 285 for glycitin SRM MS/MS mode, the quantification was carried out by means of the signal ratio of analyte to internal standard. Five-point calibration curves were prepared with authentic isoflavones solutions at concentrations ranging from 0.3 mg/L to 80 mg/L while keeping internal standard concentration constant at 1 mg/L. Peak areas were used for the calculation. Linear regression analysis of the results yielded the following equations for the six isoflavones:

daidzein	$Y=0.8505 X+0.0366$	$r^2=0.993$
genistein	$Y=1.0713 X+0.0323$	$r^2=0.995$
glycitein	$Y=0.6809 X+0.0230$	$r^2=0.993$
daidzin	$Y=5.0499 X+0.0650$	$r^2=0.998$
genistin	$Y=5.1745 X+0.0560$	$r^2=0.994$
glycitin	$Y=4.9001 X+0.0183$	$r^2=0.997$

where Y is the peak area ratio of the analyte to internal standard, X is the concentration of the analyte in mg/L, and r^2 is the correlation coefficient. Interday (5 days) precisions of the slope and intercept of the calibration curves were found to be in the range between 2.5% and 3.6% (RSD, $n = 5$). From the calibration curves, the limits of detection were estimated to be in the range from 0.03 mg/L for genistin to 0.05 mg/L for glycitein (signal/noise = 3). These results indicated that the present method was sufficiently sensitive for the analysis of isoflavones in soymilk. Comparing with other sample preparation approaches previously reported for soymilk analysis, the present MSPE procedure was easy to carry out and much faster (less than 10 min). As shown in Table 2, analytical figures of merit are compared for several HPLC-based quantitative methods reported^{31, 32, 40} for analysis of isoflavones in soymilk.

HPLC–ESI-MS/MS determination of isoflavones in soymilk samples

Monitoring the levels of isoflavones in commercial soymilks is of importance. Using the present HPLC–ESI-MS/MS method, six isoflavones (daidzin, genistin, glycitin, daidzein, genistein, and glycitein, respectively) were detected and quantified simultaneously from soymilk samples obtained from local supermarkets. Typical chromatograms from these analyses are shown in Figure 6. As can be seen, peaks corresponding to isoflavones were well identified. Besides, a minor peak with same MS and MS² behavior to genistein was

observed at retention time 5.2 min (Figure 6). Because it was well separated from genistein by HPLC, it didn't interfere with the quantification. All the analyses were conducted three times, and the analytical results are summarized in Table 1. Isoflavone concentrations in the soymilk samples were found at the mg/L level which was consistent with the results reported previously.⁴¹ Recovery of this isoflavones from soymilk matrix by the present HPLC-MS method was studied. Three soymilk samples were spiked with authentic isoflavones at 20.0 mg/L and analyzed. Recoveries were found to be in the range of 97.5 ± 0.7% and 102.0 ± 1.2%.

In conclusion, baicalin functionalized core-shell superparamagnetic nanoparticles (BMNPs) of high water suspensibility were successfully prepared. The synthetic method is easy to carry out, and the amount of baicalin loaded to the MNPs is larger than previous one³³. Using BMNPs as magnetic solid phase extract sorbent, a selective extraction of isoflavones from soymilk was developed, and the extracted isoflavones were subsequently subjected to HPLC-ESI-MS/MS analysis. This is the first time that BMNPs was used in quantitative analysis. As demonstrated in this work, the proposed MSPE method is effective, easy to carry out, and applicable to fast HPLC-MS/MS quantification of isoflavones in soymilk and other liquid soybean products, such as soy beverages.

Acknowledgments

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Abbreviations Used

BMNPs	baicalin-functionalized magnetic nanoparticles
MSPE	magnetic solid phase extraction
De	daidzein
Di	daidzin
Ge	genistein
Gi	genistin
Gle	glycitein
Gli	glycitin
IS	internal standard

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Baicalin

	Mw.	R ₁	R ₂	R ₃	R ₄	R ₅
Daidzein (De)	254	H	H	H	H	OH
Glycitein (Gle)	284	H	OCH ₃	H	H	OH
Genistein (Ge)	270	OH	H	H	H	OH
Daidzin (Di)	416	H	H	glucosyl	H	OH
Glycitin (Gli)	446	H	OCH ₃	glucosyl	H	OH
Genistin (Gi)	432	OH	H	glucosyl	H	OH
Calycosin (IS)	284	H	H	H	OH	OCH ₃

Fig. 1.
Chemical structures of baicalin and six isoflavones detected in soymilk and the internal standard

Fig. 2. Illustration of the preparation of baicalin-functionalized magnetic-nanoparticles (BMNPs) (upper) and the mechanism for EDC/Sulfo-NHS crosslinking of baicalin with AMNPs (lower)

Fig. 3.
Illustration of the proposed magnetic solid phase extraction (MSPE) procedure.

Fig. 4.
TEM image of BMNPs prepared with ~400 nm in diameter.

Fig. 5. HPLC-MS TIC chromatograms and MS² spectra of authentic isoflavones (De, daidzein; Di, daidzin; Ge, genistein; Gi, genistin; Gle, glycitein; Gli, glycitin; IS, internal standard).

Fig. 6. HPLC–ESI-MS/MS determination of isoflavones in a commercial soymilk sample (De, daidzein; Di, daidzin; Ge, genistein; Gi, genistin; Gle, glycitein; Gli, glycitin).

Table 1

Concentrations of isoflavones in commercial soymilk samples (mg/L)

Sample	Di	Gli	Gi	De	Gle	Ge
Soy milk 1	9.30±0.83	3.41±0.31	74.80±6.01	53.31±3.34	2.96±0.38	58.46±2.96
Soy milk 2	6.05±0.79	3.66±0.34	72.23±0.92	47.20±2.57	1.88±0.11	64.26±3.73
Soy milk 3	2.58±0.19	0.55±0.09	25.99±0.75	13.32±1.87	2.06±0.09	14.12±1.13

Table 2

Comparing analytical methods reported for quantification of isoflavones in soymilk

Method	Sample pretreatment	LOD	Recovery	Ref.
HPLC-MS	Magnetic solid-phase extraction using BMNPs	0.03–0.05 mg/Kg	97.5%–102.0%	This work
HPLC-UV-MS	Freeze-drying and removal of lipids	0.1 mg/Kg	not mentioned	Ref. 31
UHPLC-UV	Freeze-drying	0.02–0.05 mg/Kg	95.8%–101.5%	Ref. 32
HPLC-UV	Removal of proteins with a precipitation mixture	0.2–0.3 mg/L	90.0%–110.0%	Ref. 40