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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 high-performance liquid chromatography electrospray ionization tandem mass spectrometry (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 an internal standard, 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). Six isoflavones, that is, 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, baicilin-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 an 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]

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 preconcentration of the target compound fraction are commonly needed, including evaporation to dryness and being redissolved 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 nonselective extraction procedure to isolate isoflavones from the sample matrix. As an example, it took two 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 superparamagnetic 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

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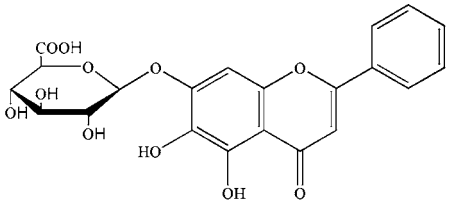
extract isoflavones from soymilk without involving a freeze-drying procedure for subsequent high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) analysis. The biggest challenge for soymilk isoflavone determination has been to selectively extract the analytes without freeze-drying from liquid soy matrix containing a large amount of interfering substances such as fat, protein, carbohydrate, and so forth. 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 ($C_6-C_3-C_6$ great $\pi$-conjugated system). Preparation of BMNPs 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, and stored at 4 °C until 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, $^1$H NMR, and $^{13}$C NMR spectral evidence. Six isoflavone standards daidzein (De), glycitein (Gle), genistein (Ge), daidzin (Di), glycitin (Gli), and genistin (Gi) and internal standard (IS) calycosin (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).




| | 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 | $OCH_3$ |

**Figure 1.** Chemical structures of baicalin and six isoflavones detected in soymilk and the IS.

**Preparation of BMNPs.** The multistep procedure for the preparation of BMNPs is shown in Figure 2. First, amino-functionalized magnetic nanoparticles (AMNPs) were prepared by the hydrothermal method as follows: 6.5 g of 1,6-hexanediamine, 2.0 g of anhydrous sodium acetate, and 1.0 g of $FeC_3{\cdot}6H_2O$ 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 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 of AMNPs and 50 mg of bacialin were dispersed in 10 mL of 75% dimethyl sulfoxide (DMSO)/phosphate solution (10 mM, pH 5.5) containing 35 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS). After gently shaking for 2 h and washing 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 baicilin in BMPNs was characterized by elemental analyzer.

**BMNPs Characterization.** Transmission electron microscope (TEM) images were acquired on a Tecnai G$^2$ F20 S-TWIN TEM (200 KeV, FEI, OR). Carbon and nitrogen analyses were performed on a Carlo Erba (Italy) model 1106 elemental analyzer.

**MSPE Procedure.** To a tube containing 100 $\mu$L of soymilk sample, 5 $\mu$L of calycosin at 40 mg/L was added as IS. While stirring, 100 $\mu$L of 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 s to let the BMNPs settle down. The supernatant was discarded. After washing 2 times with 100 $\mu$L of water, isoflavones were eluted out from the BMNPs with 100 $\mu$L of warm methanol (about 60 °C). After magnetic separation, the supernatant was carefully saved and mixed with 100 $\mu$L of water. Portions (20 $\mu$L) 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 online degasser (DGU-12A, Shi-madzu), and triple quadrupole mass spectrometers equipped with an ESI source (TSQ Quantum, Thermo Scientific, San Jose, CA). Both the liquid chromatograph and the mass spectrometer were controlled by Xcalibur software (Thermo Finnigan). A reversed-phase column ($C_{18}$, 150 mm × 2.1 mm, 5 $\mu$m) 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 follows: 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 the 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. First, AMNPs were prepared by one-pot strategy using $FeC_3{\cdot}6H_2O$ and 1,6-hexanediamine as reported previously.[38] Then, the acid amide was condensed through the $-NH_2$ on the AMNPs and the −COOH on baicalin using EDC and sulfo-NHS as dehydrating agents. Sulfo-NHS was used to prepare amine-reactive esters of carboxylate groups for cross-linking, that is, carboxylates (−COOH) of baicalin reacted to sulfo-NHS in the presence of a carbodiimide (here is EDC), to form a semistable sulfo-NHS ester, the latter of which then reacted with primary amines ($-NH_2$) to form amide cross-links (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

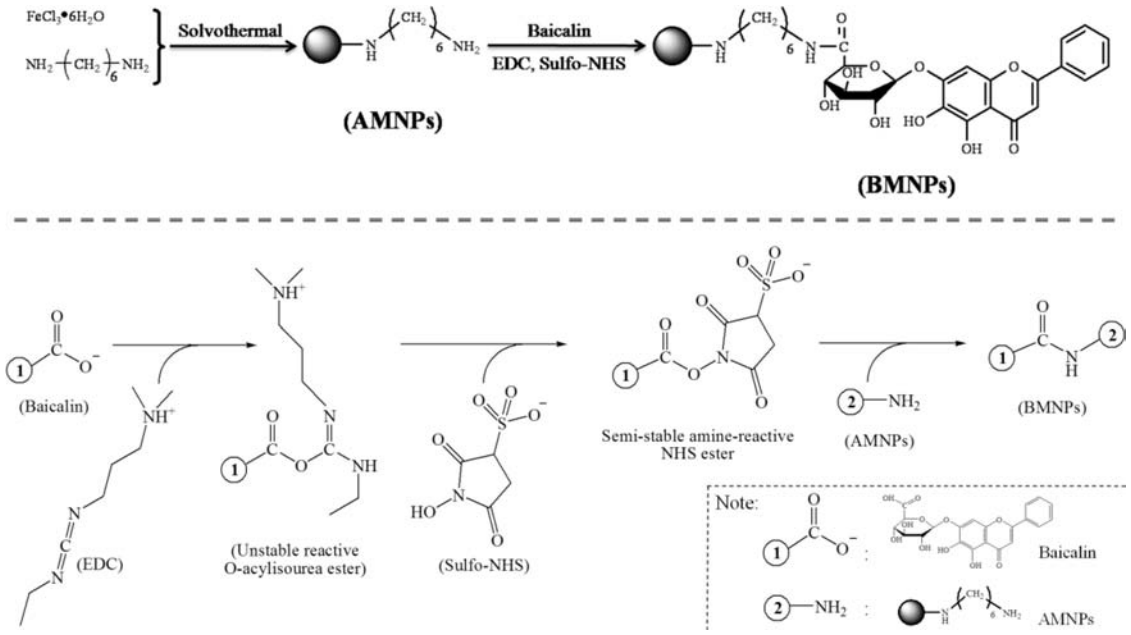



**Figure 2.** (upper) Illustration of the preparation of BMNPs; (lower) mechanism for EDC/sulfo-NHS cross-linking of baicalin with AMNPs.

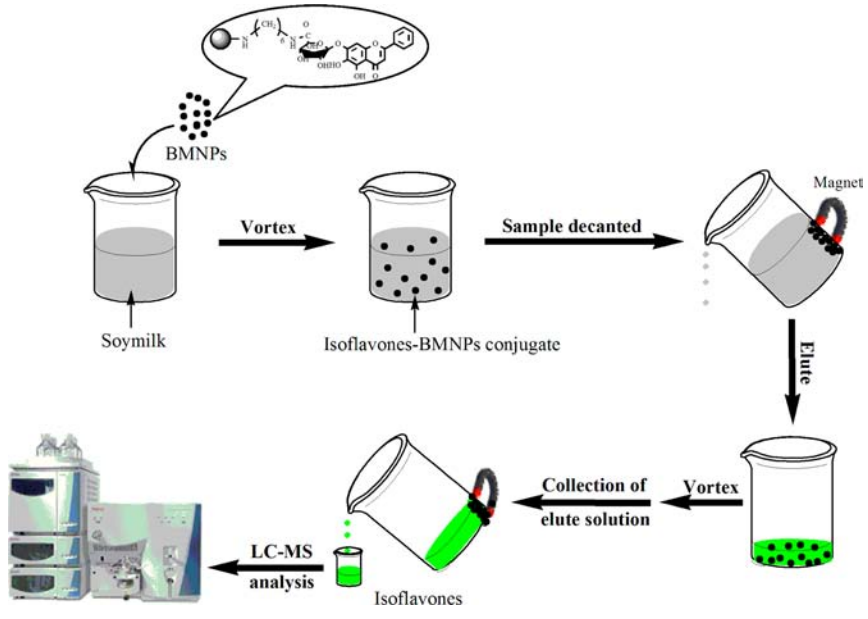



**Figure 3.** Illustration of the proposed MSPE procedure.

carbon came from baicalin and AMNP, and the ratio of carbon to nitrogen in the AMNPs part was constant (3:1). Thus, the mass percentage of loaded baicalin in BMNPs was calculated as 5.13 ± 0.06% that 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; $A_r$ (C), $A_r$ (N), and $M_r$ ($C_{21}H_{18}O_{11}$) are the relative atomic mass of carbon and nitrogen and the relative molecular mass of baicalin, respectively.

$$\text{baicilin}\,\% = \left(\frac{\text{C}\%}{A_r(\text{C})} - \frac{\text{N}\%}{A_r(\text{N})} \times 3\right) \times A_r(\text{C}) \Big/ \left(\frac{A_r(\text{C}) \times 21}{M_r(C_{21}H_{18}O_{11})}\right) \quad (1)$$

**MSPE Procedure.** BMNPs may serve as effective magnetic SPE sorbents for selective extraction of isoflavones. The affinity

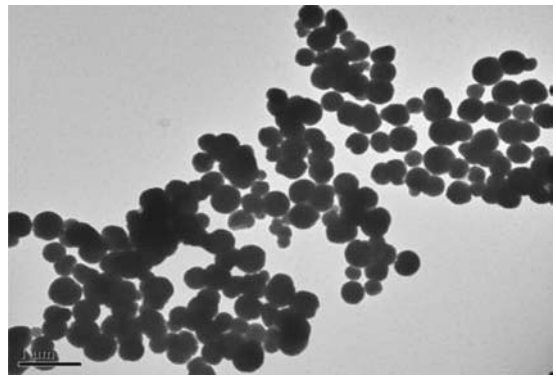


**Figure 4.** TEM image of BMNPs prepared with ~400 nm in diameter.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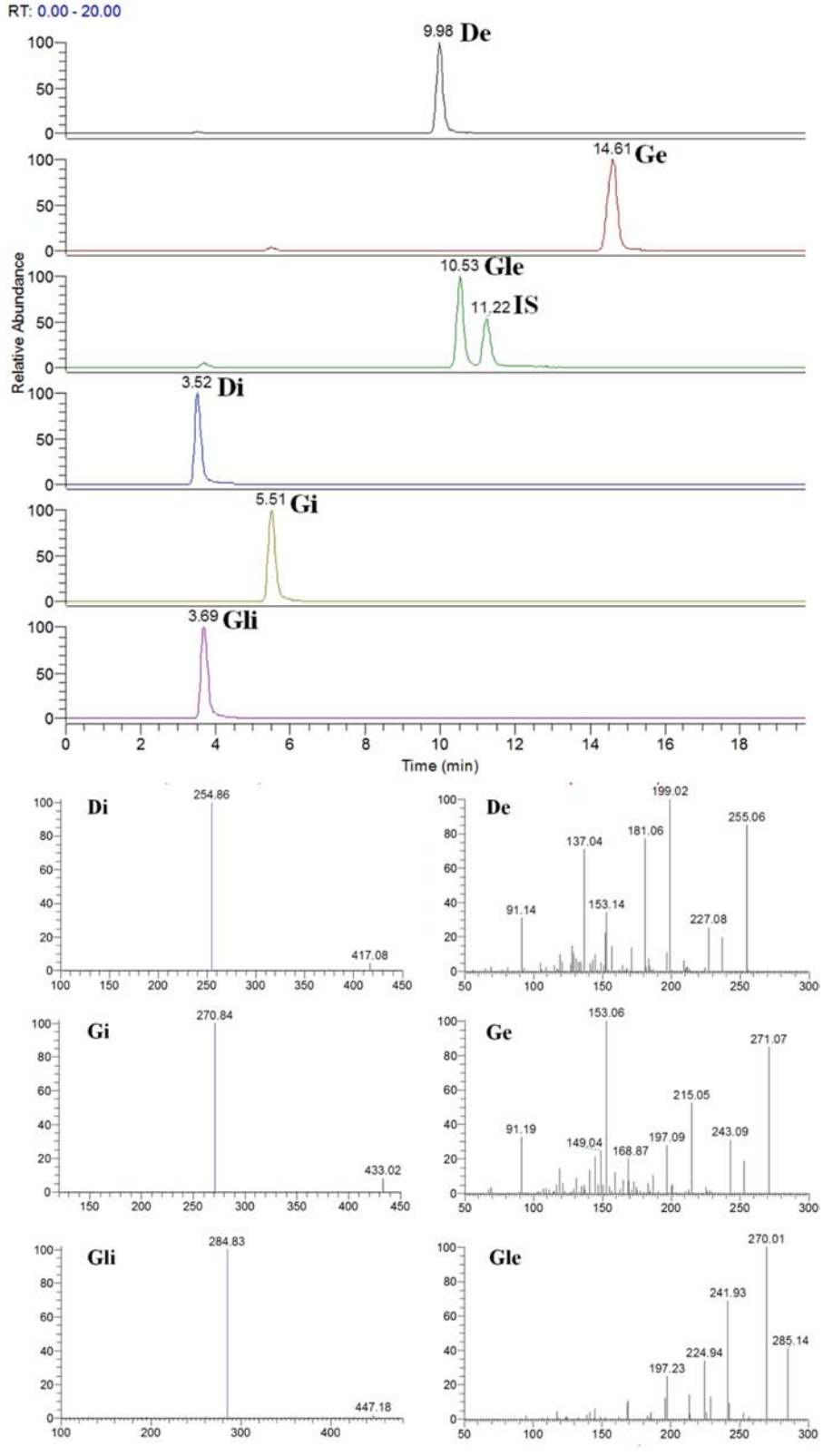


**Figure 5.** HPLC-MS total ion count chromatograms and $MS^2$ spectra of authentic isoflavones (De, daidzein; Di, daidzin; Ge, genistein; Gi, genistin; Gle, glycitein; Gli, glycitin; IS, internal standard).

may be attributed to the following factors: the polar functional groups in the baicalin molecule, such as −OH and −CHO−, can form hydrogen bonds with isoflavones; the planar structure of the baicalin molecular skeleton ($C_6$−$C_3$−$C_6$, 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 $\pi$−$\pi$ system in the 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


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Table 1. Concentrations of Isoflavones in Commercial Soymilk Samples (mg/L)

| sample | Di | Gli | Gi | De | Gle | Ge |
|---|---|---|---|---|---|---|
| soymilk 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 |
| soymilk 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 |
| soymilk 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 |

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 that 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 ± 0.2% to 89.5 ± 0.3%), while the eluting efficiencies remained almost the same for elution times of 1, 3, and 5 min. Therefore, 5 and 1 min were selected for extraction and eluting times, respectively. Isoflavones are soluble in methanol, ethyl acetate, and 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 the 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, an isomer of one of the isoflavones targeted (i.e., glycitein) was used as the IS. 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 the IS. LC-MS analysis of authentic daidzein, genisetin, glycitein, daidzin, genistin, glycitin, and IS 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 (IS), $m/z$ 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 IS. Five-point calibration curves were prepared with authentic isoflavones solutions at concentrations ranging from 0.3 to 80 mg/L while keeping the IS 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 $y = 0.8505x + 0.0366$ $r^2 = 0.993$

genistein $y = 1.0713x + 0.0323$ $r^2 = 0.995$

glycitein $y = 0.6809x + 0.0230$ $r^2 = 0.993$

daidzin $y = 5.0499x + 0.0650$ $r^2 = 0.998$

genistin $y = 5.1745x + 0.0560$ $r^2 = 0.994$

glycitin $y = 4.9001x + 0.0183$ $r^2 = 0.997$

where $y$ is the peak area ratio of the analyte to the IS, $x$ is the concentration of the analyte in milligrams per liter, 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. By using the present HPLC-ESI-MS/MS method, six isoflavones (daidzin, genistin, glycitin, daidzein, genistein, and glycitein) 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 the same MS and $MS^2$ behavior to genistein was observed at a retention time of 5.2 min (Figure 6). Because it was well separated from genistein by HPLC, it did not 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 milligram per liter level that 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 from 97.5 ± 0.7% to 102.0 ± 1.2%.

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 |

Baicalin-functionalized core−shell superparamagnetic nanoparticles 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 the previous one.[33] By

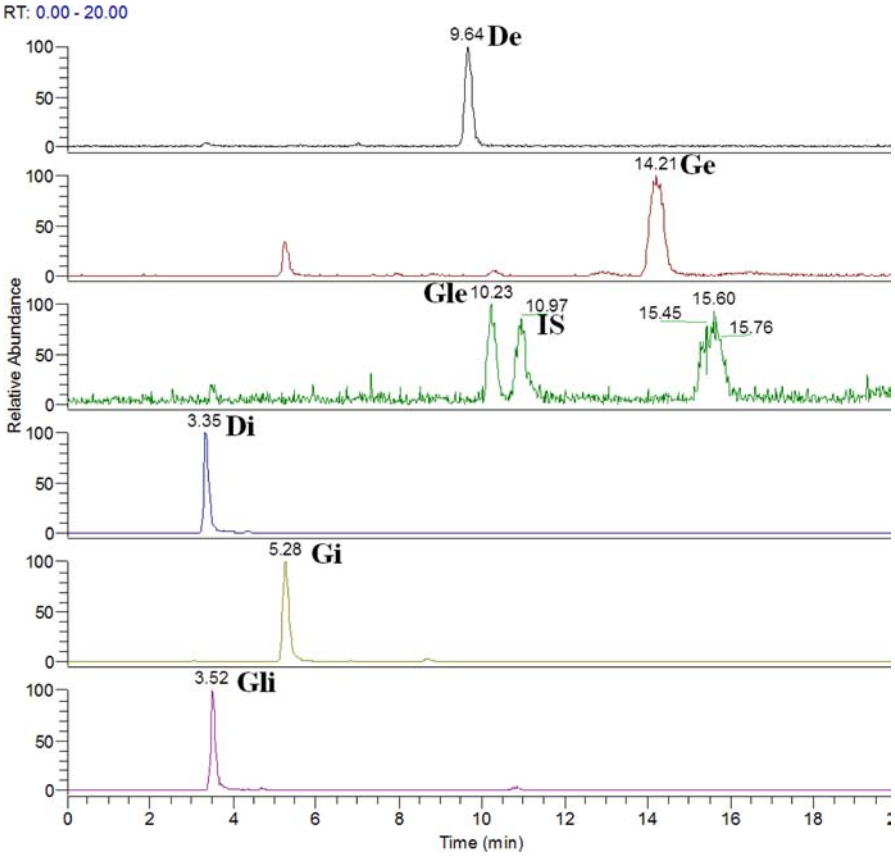


**Figure 6.** HPLC-ESI-MS/MS determination of isoflavones in a commercial soymilk sample (De, daidzein; Di, daidzin; Ge, genistein; Gi, genistin; Gle, glycitein; Gli, glycitin).

using BMNPs as a 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 were 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.

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### Notes

The authors declare no competing financial interest.

## 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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