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# Detection of the isoflavone aglycones genistein and daidzein in urine using solid-phase microextraction–high-performance liquid chromatography–electrospray ionization mass spectrometry

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**Abstract**

An improved method of detection of the isoflavone aglycones, genistein and daidzein, is reported using solid-phase microextraction–high-performance liquid chromatography–electrospray ionization mass spectrometry (SPME–HPLC–ESI-MS). Extraction of the isoflavonoids from urine using SPME with a Carbowax–templated resin fiber coating allows rapid preconcentration of the analytes without the usual sample preparation required by other methods. Detection of the analytes is accomplished by HPLC–ESI-MS. Analysis of spiked samples of urine resulted in a linear range of 0.25 to 250 ng/ml for daidzein and 0.27 to 27.0 ng/ml for genistein. Limits of detection of daidzein and genistein were measured at 25.4 pg/ml for daidzein and 2.70 pg/ml for genistein. Daidzein and genistein were detected in urine following consumption of a soy drink.


*Keywords:* Genistein; Daidzein

## 1. Introduction

As a class of flavonoids distinct in their structure, sources and activity, the isoflavones have come under increasing scrutiny due to their estrogen-like properties [1,2]. Isoflavones, with a polyphenolic structure in which the B ring is at carbon 3 instead of the more typical carbon 2 (see Fig. 1), are found in the greatest amounts in soybeans. They have been implicated in preventing the oxidation of low-density lipoprotein (LDL), thus reducing atherogenesis [3], decreasing bone reabsorption in a manner similar to the activity of estrogen [4], and lowering the prevalence of breast and prostate cancer in societies where consumption of soy is common [5–7]. The mode of action of the isoflavonoids as phytoestrogens is poorly understood but based on the similarity in structure of the isoflavone aglycones daidzein and genistein to estrogen, thus resulting in similar affinities for estrogen receptor β [8].

There has been active interest in recent years in

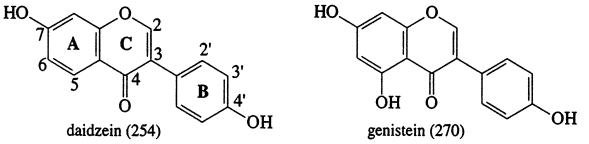


Fig. 1. Structures of isoflavones used. (Molecular mass).

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developing and optimizing analytical methods for detection of isoflavones in key foods and biological mixtures. Most of these methods have focused on detection of the isoflavones after hydrolysis of the glycoside, glucuronide or sulfate forms [9–12]. In some plant sources, flavonoids occur most frequently as glycosides, while after consumption the flavonoid glycosides are generally thought to be hydrolyzed to aglycones [13], although one recent study reported detection of the citrus flavanone disaccharide, naringin, in urine after consumption of citrus [14]. In the body, the isoflavone aglycones are metabolized to isoflavone glucuronides, sulfates, or undergo further metabolism to products such as equol, dihydrogenistein and dihydrodaidzein [15]. Although studies of the health effects of the isoflavones have concentrated on the aglycones [3,4,6,16], few analytical studies have detected the presence of the aglycones [17,18], presumably due to the low levels relative to the glycosides or conjugated isoflavones. For monitoring of the glycosides or conjugated isoflavones, the initial step is hydrolysis, carried out by lowering the pH of the samples when a variety of conjugate types are involved, or by enzymatic hydrolysis specific for glucuronides or sulfates, followed by detection of aglycone forms. In this report we present a method of detection of the isoflavone aglycones that is sensitive enough for direct detection of the intrinsic unconjugated aglycones, not the hydrolyzed glycosides or conjugates, in urine after consumption of isoflavone-containing products.

Monitoring of conjugated isoflavones via conversion to unconjugated counterparts after enzymatic hydrolysis is commonly performed by a multi-step method involving solid-phase extraction, followed by adsorption chromatography (Sephadex), derivatization with a silylating agent and then isotope dilution gas chromatography–mass spectrometry (GC–MS) in a method developed by Aldercreutz et al. [9]. This method has been used extensively for detection of isoflavones in raw soybeans, soybean baby food, tofu and other processed soy products [12,19,20], as well as for detection of isoflavones in urine after soy consumption [10,11,15,21]. While widely accepted, the isotope dilution method is time consuming and demanding, requiring careful attention at all the multiple steps involved. Other methods of detection of isoflavones after hydrolysis have been developed using electrospray ionization mass spectrometry (ESI-MS) in the positive mode for detection of the protonated analytes in conjunction with separation by high-performance liquid chromatography (HPLC) [22,23].

Few researchers have investigated the intrinsic levels of isoflavone aglycones, that is, not involving the conjugated forms or glycosides. Tekel et al. developed a GC–MS method for detection of the derivatized isoflavone aglycones using solid-phase extraction (SPE) and on-column methods for purification, but only applied the method to spiked samples [18]. A study using liquid–liquid extraction with HPLC–ESI-MS detection that measured isoflavonoids in rat urine following a soy protein diet reported that 40–50% of the daidzein and genistein were excreted as the aglycones, compared to less than 1% excreted in the urine of the one human test subject [17]. Detection limits for genistein and daidzein in urine range from 1 to 3 ng/ml [18] to 40 ng/ml [17,23].

An alternative to liquid–liquid and solid-phase extractions is the use of solid-phase microextraction (SPME) in which a thin polymeric coating of stationary phases such as polyacrylate or polydimethylsiloxane on a fused-silica fiber is used to extract analytes from a liquid or gas phase solution, or from the headspace above a liquid solution [24–26]. Relative to other methods of extraction such as liquid–liquid extraction and SPE, SPME offers the benefits of analyte concentration, elimination of organic solvents, and increased throughput due to the lack of sample pretreatment necessary. For SPME–HPLC applications, the SPME fiber is placed in the mobile phase of the HPLC instrument and desorption of analytes occurs via flushing with the mobile phase and detection by UV absorption [27–29] or ESI-MS [30–32].

In an effort to find a simpler, less complex method but with comparative effectiveness as previous techniques for detection of the isoflavone aglycones genistein and daidzein (see Fig. 1), we have developed an SPME–HPLC–ESI-MS method for detection in urine. SPME–LC–MS was targeted in an effort to avoid derivatization of the analytes and thus improve detection and throughput by decreasing sample manipulation. Use of SPME allows direct extraction of the isoflavones from urine with no prior treatment, then subsequent desorption into the LC mobile phase enables efficient separation and de-

tection by ESI-MS. ESI-MS is undertaken in the positive mode with detection of the protonated analytes. Analysis of this specialized class of flavonoids, isoflavones, is possible with this simplified method and results in limits of detection more than an order of magnitude below literature reports using the isotope dilution method or HPLC–ESI-MS, but with minimal sample preparation required. With reduced limits of detection, direct detection of the aglycones in urine (i.e., without requiring hydrolysis of the glycosides or conjugates to increases levels) becomes possible.

Development of SPME–HPLC–ESI-MS for detection of isoflavones in urine involves optimization of extraction conditions such as fiber type, temperature, salt content, pH, and extraction time, and optimization of desorption conditions such as dynamic vs. static desorption, carryover, composition of mobile phase and desorption time. Chromatographic and mass spectrometric detection parameters were also optimized. After validation of the method, daidzein and genistein were detected in human urine after consumption of an isoflavone-containing beverage, Genisoy.

## 2. Experimental

### *2.1. Chemicals and reagents*

Genistein and daidzein were purchased from Aldrich (Milwaukee, WI, USA) and used without further purification. The HPLC-grade methanol came from EM Science (Gibbstown, NJ, USA). For experiments involving detection of genistein and daidzein in urine, GeniSoy (GeniSoy Products, Fairfield, CA, USA), a soy protein powder was used after purchase from a local grocery store. A scoop of 35 g was added to 200 ml of skim milk and consumed daily for 4 days. According to the Genisoy label, 35 g contains 9.0–13.4 mg of daidzein and 16.8–25.2 mg of genistein.

### *2.2. Equipment*

The SPME equipment was obtained from Supelco (Bellefonte, PA, USA) and included four types of fibers designed for use with the HPLC system: a 100 μm polydimethylsiloxane (PDMS), a 65 μm polydimethylsiloxane–divinylbenzene (PDMS–DVB), a 85 μm polyacrylate (PA) and a 50 μm Carbowax–templated poly(divinylbenzene) resin (CW–TPR). Also obtained from Supelco were the SPME fiber holder designed for use with the HPLC and the SPME–HPLC interface.

For separations, a Waters Alliance 2690 HPLC system (Milford, MA, USA) was used. Ultraviolet detection was monitored at 280 nm using a Waters 486 tunable absorbance detector. A Thermoquest LCQ Duo quadrupole ion trap mass spectrometer equipped with an electrospray interface was used for mass spectrometric detection and analysis.

### *2.3. Sample preparation*

For extraction, 4-ml glass vials with PTFE caps were used. A vial containing 3.0 ml of urine was placed in a warm water bath for extraction at 35°C on a heated stir plate for an extraction time of 10 min. No buffer was added to the urine solution. The solution was stirred during extraction at a constant rate through the use of a PTFE coated magnetic stir bar in the 4-ml vial. To ensure constant exposure of the SPME fiber to the urine solution, the fiber was retracted into the SPME holder and re-submerged into the solution several times during extraction to dislodge air bubbles.

SPE was used for extraction of the isoflavones from urine for comparison with SPME. Sep-Pak Vac-RC $C_{18}$ cartridges from Waters with 100 mg of sorbent were used. The cartridge was conditioned with 1.5 ml of methanol followed by 1.5 ml of distilled water. Without allowing the sorbent to dry, 3.0 ml of the spiked urine was poured through. The column was washed with 1 ml of distilled water–methanol (85:15), and the flavonoids were eluted with 2 ml of methanol. After elution, the methanol solution was dried under a stream of nitrogen and brought up to 150 μl with the addition of methanol and water in the same proportion as the mobile phase (methanol–water, 55:45). Auto injections of 10 μl were made onto the 50×2.1 mm $C_{18}$ column using identical conditions for detection after SPME.

### *2.4. Chromatographic conditions*

After extraction, the SPME fiber was placed in the SPME–HPLC interface designed to replace the

injection loop. Dynamic desorption was used with an initial flow-rate of 0.02 ml/min of methanol–water (55:45) for the 6 min of desorption, followed by an increase in flow-rate to 0.1 ml/min after the SPME fiber was removed. The use of static desorption was also investigated, in which the SPME fiber was soaked in 200 μl of the mobile phase prior to injection into the mobile phase flow at 0.1 ml/min. The analyte was transferred from the SPME fiber to the mobile phase and then to a Waters Symmetry $C_{18}$ (50×2.1 mm, 3.5 μm) column from Waters at room temperature with an isocratic mobile phase of methanol–water (55:45) at 0.1 ml/min for separation. The entire flow of the mobile phase was sent to the mass spectrometer without splitting.

### 2.5. Mass spectrometric conditions

For detection and analysis of protonated daidzein and genistein, a Thermoquest LCQ Duo mass spectrometer was used. The needle, lens and capillary voltages were optimized for maximum abundance of $[genistein+H]^+$ and $[daidzein+H]^+$, and set at 5.00, −20.0 and 33.0 V, respectively. The sheath gas flow-rate was 40 units and the auxiliary gas flow was 20 units. The capillary temperature was kept at 200°C. The base pressure in the ion trap with helium added was nominally $1.4 \cdot 10^{-5}$ Torr, as measured with an ionization gauge (1 Torr=133.322 Pa). Selected ion monitoring of $[daidzein+H]^+$ ($m/z$ 255) and $[genistein+H]^+$ ($m/z$ 271) was carried out with ion injection times of 100 ms and an average of 10 μscans.

For limit of detection studies, selected reaction monitoring was performed based on collisional activated dissociation of specific precursor ions. Detection of genistein was accomplished by monitoring $m/z$ 153, 215 and 243 as fragments of the protonated precursor at $m/z$ 271 with application of 1.95 V of collision energy. For detection of daidzein, the precursor at $m/z$ 255 was fragmented with 2.00 V of collision energy, and $m/z$ 199, 137 and 227 were monitored as fragments. During selected reaction monitoring, the injection time was 100 ms and 10 μscans were averaged for each spectrum.

These studies have been approved by the University of Texas at Austin Institutional Review Board for Protection of Human Subjects in Research.

## 3. Results and discussion

### 3.1. Optimization of extraction parameters

In order to successfully apply SPME–HPLC for detection of isoflavonoids in urine, many parameters dealing with the SPME process were initially optimized in aqueous solutions, including fiber type, the amount of carryover, extraction temperature, salt content and pH of the extraction solution, and extraction and desorption times. To best utilize the SPME interface to the HPLC system, the choice of mobile phase and the use of static and dynamic desorption were parameters also optimized.

Several types of fiber coatings of the SPME fibers are commercially available for use with HPLC, including PDMS, PDMS–DVB, PA and CW–TPR. For this study the relative extraction efficiency of all four fiber types for the extraction of genistein and daidzein in water was investigated. The aqueous samples were spiked with 270 ng/ml genistein and 250 ng/ml daidzein, extracted for 6 min and desorbed for 5 min in the mobile phase of the HPLC system using dynamic desorption. The PDMS–DVB and PA fibers produced similar results, with about half the relative extraction efficiency of the CW–TPR fiber coating. Use of the PDMS fiber for extraction of genistein and daidzein resulted in no observable signal so was not evaluated further. Since the signal intensity was so much greater for the CW–TPR fiber, use of other fiber choices was not considered after the initial tests. Submersion of the fiber in methanol with sonication was used to clear the fiber between experiments.

A variety of extraction temperatures were studied in order to maximize extraction: 25, 35, 45, 55 and 65°C (See Fig. 2). The difference in extraction efficiency of genistein and daidzein was greatest at the optimal temperature of 35°C, and decreased as the extraction temperature increased. The difference in extraction efficiency may be related to the increased polarity of genistein relative to daidzein. Based on the greatest extraction efficiency, 35°C was chosen for use throughout the study as the extraction temperature.

Salt content and pH levels were modified to determine their effect on the extraction efficiency of daidzein and genistein from spiked water solutions.

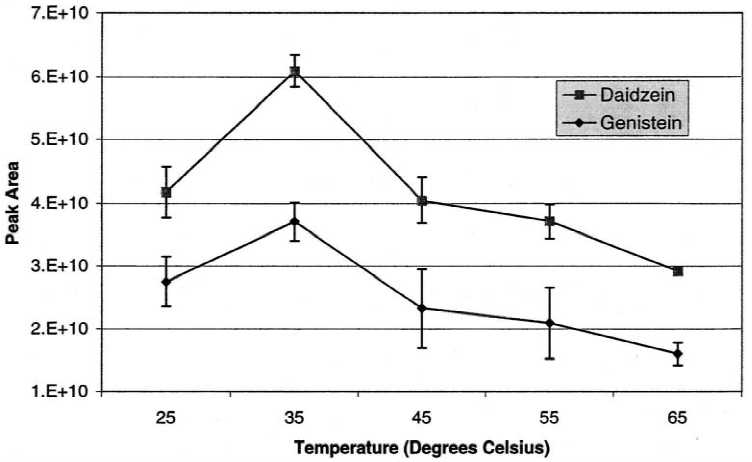


Fig. 2. Peak areas of daidzein and genistein $(L+H)^+$ using a Carbowax–templated resin (CW–TPR) SPME fiber as a function of extraction temperature. Errors bars represent $\pm 1$ standard deviation for $n=3$.

Typically, addition of salt may increase the extraction of analytes from urine solutions due to the ''salting out'' effect in which addition of salt decreases the solubility of the analyte of interest, thus pushing the equilibrium towards absorption of the analyte onto the SPME fiber. Addition of a sodium chloride solution to the spiked water samples was carried out to produce final concentrations of samples of 5, 10, 15 and 20% salt. A blank with no added sodium chloride was also tested. As the concentration of salt increased, the amount of genistein or daidzein extracted decreased. The largest drop in signal occurred upon the initial increase of the salt concentration from 0 to 5%; the detected amounts of daidzein and genistein both dropped about 50% upon addition of sodium chloride.

As seen in Fig. 3, the production of sodium cationized complexes is favored for the isoflavones, as commonly observed in positive ion mass spectra of the flavonoids even without addition of any salts due to the ubiquitous presence of alkali metals in solvents, glassware, and the electrospray interface. Although the sodium complexes are frequently more intense than the protonated flavonoids in the mass spectra, the sodium complexes do not give informative fragmentation patterns, prohibiting the use of selected reaction monitoring for quantitative purposes. Thus, our method relied on the detection of the protonated isoflavones, despite their apparent low intensity in the mass spectra, and the fragmentation patterns resulting from collisionally activated dissociation of the protonated isoflavones have excellent signal-to-noise ratios.

Alteration of the pH of the extraction solution was carried out by monitoring the extraction efficiency of genistein and daidzein in buffered aqueous solutions at pH 4, pH 7 and pH 10. Only extraction of neutral molecules, not ions, is favored by the SPME fiber coatings, thus increased production of ions in solution lowers extraction efficiencies. Because the flavonoids as a class are slightly acidic due to their multiple hydroxyl groups, a decrease in pH with a subsequent increase in available protons was proposed to discourage deprotonation and increase the analyte signal. Upon alteration of the pH of the extraction solution from pH 7 to pH 4, the extraction efficiency of daidzein increased slightly and that of genistein decreased by a greater amount. The mass spectrometric signal of daidzein increased by 6% from pH 7 to pH 4; a statistically insignificant change based on three trials at each pH. The genistein signal decreased by 12% from pH 7 to pH 4. This apparent lack of effect of increased acidity on the detected signal of genistein and daidzein reflects the relative $pK_a$ values of these flavonoids. Compared to some of the other flavonoids, daidzein and genistein appear to have higher $pK_a$ values than expected perhaps due to the presence of the B ring at

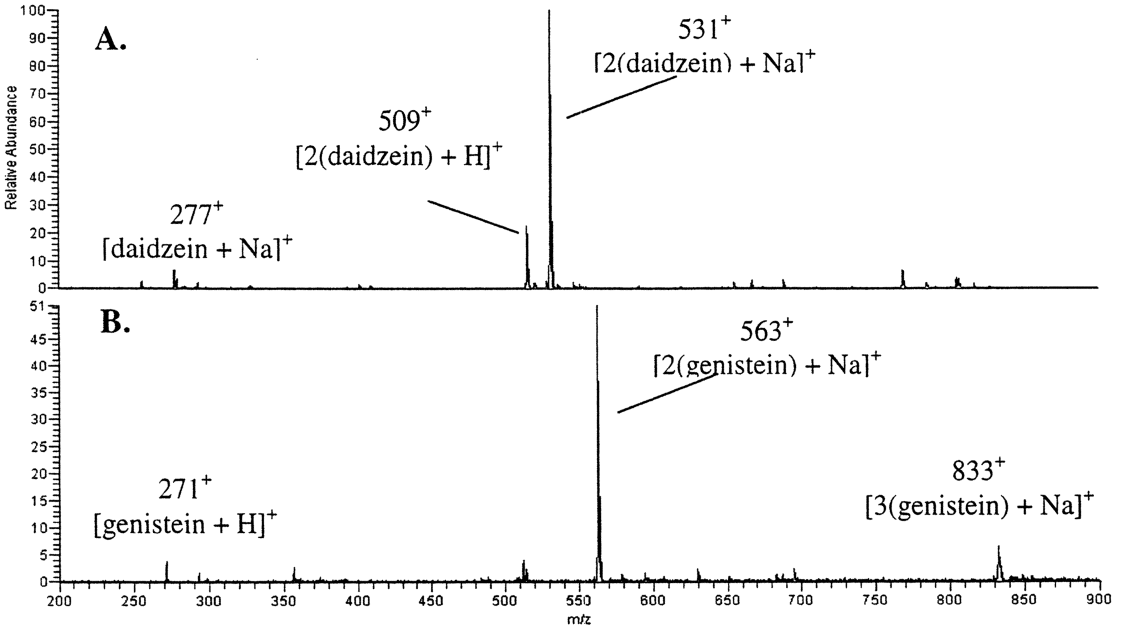


Fig. 3. Fullscan mass spectra of (A) daidzein and (B) genistein in methanol illustrating the sodium cationized ions due to inherent sodium.

carbon 3, instead of the typical 2 position, as shown in Fig. 1. Spectrophotometric studies from which the p$K_a$ values of monohydroxyflavones with a single hydroxyl group at carbon 3, carbon 5, carbon 7 or carbon 4′ were calculated indicate that the hydroxyl group at carbon 7 is the most acidic with a p$K_a$ of 7.39. 3-Hydroxyflavone, 5-hydroxyflavone and 4′-hydroxyflavone have p$K_a$ values of 9.6, 11.56 and 8.28, respectively [33]. Replacement of the carbon 3 hydroxyl group with a phenolic ring, decreasing the resonance possibilities of the B ring, may have moderate electron donating effects, affecting the acidity of the hydroxyl groups at the 4′, 5 and 7 positions. When the pH of the extraction solution was raised to pH 10, no discernable extraction of genistein and daidzein occurred. The total lack of signal is undoubtedly due to the deprotonation of genistein and daidzein encouraged by the addition of base. As mentioned previously, absorption of ions into the SPME fiber is not favored.

In order to maximize the amount of daidzein and genistein detected, the extraction and desorption times were optimized. To investigate the effect of extraction time on extraction efficiency, extraction times of 5, 10, 20, 40 and 60 min were used for spiked samples containing $1.0 \cdot 10^{-6}$ *M* genistein and daidzein (see Table 1). Comparison of the amounts extracted at 5 min to the amount extracted at 40 min indicates an increase of more than 100% for daidzein and of about 70% for genistein. Based on the adequate extraction levels seen at 5 min, an extraction time of 5 min was chosen. Even though equilibrium was not reached, 5 min extraction was a compromise between amount extracted and time; extraction for a longer time results in greater amounts extracted and presumably greater precision, but also decreases throughput.

Various desorption times were also investigated in conjunction with use of the two types of SPME desorption modes, static vs. dynamic. The static mode involves isolation of 200 μl of mobile phase in a loop into which the SPME fiber is placed. Desorption is allowed to occur for a set time after which the chamber is opened and the mobile phase with analyte allowed to flow to the chromatographic system. Because of the small volume of the static desorption loop, as compared to the 3.0 ml of the extraction vial, desorption of the analytes in this study was not favored; indeed retention of the analytes is favored, leading to poor detection levels and high carryover.

Table 1
Peak areas of daidzein and genistein with varying extraction times

| | Extraction time (min) | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 20 | 40 | 60 |
| Daidzein | $7.33 \cdot 10^{10}$ (6.6%) | $9.35 \cdot 10^{10}$ (6.8%) | $1.37 \cdot 10^{11}$ (8.4%) | $1.95 \cdot 10^{11}$ (1.4%) | $1.77 \cdot 10^{11}$ (14.0%) |
| Genistein | $1.39 \cdot 10^{11}$ (2.3%) | $1.70 \cdot 10^{11}$ (6.5%) | $1.73 \cdot 10^{11}$ (2.5%) | $2.38 \cdot 10^{11}$ (0.3%) | $1.94 \cdot 10^{11}$ (10.5%) |

Relative standard deviation in parentheses.

Use of the dynamic desorption capabilities of the SPME–HPLC interface permits continuous exposure of the SPME fiber to the mobile phase for a set period of time, but at a reduced flow-rate to discourage diffusion of the analyte. For the detection of daidzein and genistein in the urine study, desorption times of 2, 3, 4, 5, 6, 7 and 10 min were tested. When aqueous samples were spiked with 250 ng/ml daidzein and 270 ng/ml genistein, the average peak areas almost doubled when desorption times were increased from 2 to 6 min. Further increases in desorption times of 7 and 10 min were investigated, with modest increases in signal intensities, however, the increases were not great enough to compensate for the increase in analysis time and increase in diffusion of the analyte, which led to a decreased signal/noise ratio. After the dynamic desorption period, the SPME fiber is removed from the SPME–HPLC interface, and the flow-rate of the mobile phase increased to 0.1 ml/min. Increasing the desorption time with its resultant decreased flow-rate can substantially increase the analysis time, which is in part why a desorption time of 6 min was chosen for use.

### 3.2. Comparison of SPME to SPE for detection

In order to compare the efficiency of detection and ease of use of the SPME–HPLC–ESI-MS method to a method of detection already in use [18,34], SPE of a spiked urine sample was performed. A sample of urine was spiked with daidzein and genistein, subjected to a SPE method designed for extraction of flavonoids in urine (developed in a separate study [35]), and used for injection into the HPLC system and detection by ESI-MS of the protonated analytes in the positive mode. Starting with a concentration of 13.5 ng/ml genistein and 12.7 ng/ml daidzein, daidzein was detected in the urine sample, as verified by selected reaction monitoring. Genistein was not observed in the spiked urine sample by detection of its protonated molecule based on retention time or selected reaction monitoring. These results highlight the ease and efficiency of extraction of daidzein and genistein using SPME in comparison to SPE.

### 3.3. Detection of daidzein and genistein in urine

After optimization of the SPME method in water, the method was applied to urine. No isoflavones were detected for 3.0 ml each of several blank urine samples from a subject who had not consumed any soy products within the past 2 months. To determine the impact of the urine on analyte extraction, samples of urine and water were spiked with 25 ng/ml daidzein and 27 ng/ml genistein. The signal intensities obtained in urine were about 56% for daidzein and 86% for genistein compared to those obtained in water (i.e., normalized to 100%). The linear range of detection of daidzein and genistein were determined based on a calibration curve. Daidzein was detected using SPME–LC–MS at concentrations from 0.25 to 250 ng/ml in a linear fashion with a correlation coefficient of 0.995 ($n=3$ for each of four concentrations). A linear relationship between the amount detected and spiked urine amounts was determined for genistein with a correlation coefficient of 0.999 and a linear range of 0.270 to 27 ng/ml ($n=3$ for each of three concentrations). Based on a signal/noise level of three and validation by selected reaction monitoring, the limits of detection of daidzein and genistein were 25.4 and 2.70 pg/ml, respectively (see Fig. 4). These values are one- to

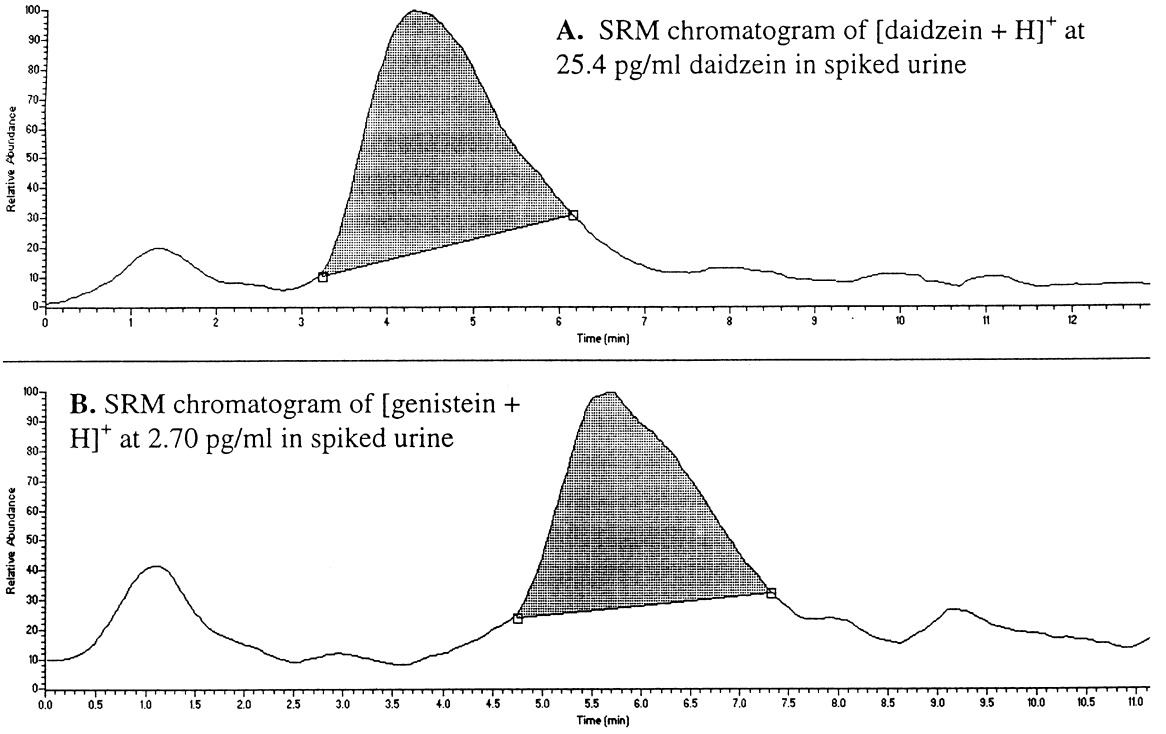


Fig. 4. Chromatograms of selected reaction monitoring of the collisionally-activated dissociation of (A) $[daidzein+H]^+$ and (B) $[genistein+H]^+$ at the limits of detection. The signal/noise ratios were 10 and 4, respectively.

two-orders of magnitude lower than literature values [17,18,23].

To test the validity of this method, blank urine samples were spiked with daidzein and genistein in amounts unknown to the laboratory technician. The amounts of the isoflavones were measured using the optimized conditions and quantitation based on a calibration curve. When the urine sample was spiked with 25 ng/ml daidzein and 27 ng/ml genistein, the amounts detected were 22 ng/ml daidzein (RSD= 9.6%, $n=3$) and 29 ng/ml genistein (RSD=26.7%, $n=3$), respectively.

As an application of this method to real samples, detection of genistein and daidzein was accomplished in urine after consumption of a soy protein powder, Genisoy. After several weeks of a diet void of soybean products, a volunteer began daily consumption of approximately 35 g of the soy protein powder mixed with 200 ml cows' milk per day. Daidzein and genistein were detected in the urine of the participant approximately 3 h after consumption. Based on three replicates using 3.0 ml of urine, daidzein was detected at a level of 16 ng/ml (RSD= 20.1%, $n=3$), and genistein was detected at a level 16 ng/ml (RSD=17.1%, $n=3$) using standard addition SPME–HPLC–ESI-MS.

## 4. Conclusion

SPME–HPLC–ESI-MS provides excellent detection of very small amounts of the isoflavone aglycones daidzein and genistein in urine following soy consumption. Not only are the limits of detection substantially lower than other methods, but the ease of analysis with the lack of sample clean up and swift throughput makes this method superior. Although further manipulation of the sample in order to free the conjugated isoflavones for detection increases analysis time, the use of SPME should find a place as a screening device for unconjugated iso-

flavones prior to analysis with more complicated and time-consuming methods, if necessary.

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