JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simultaneous Identification of Soyasaponins and Isoflavones and Quantification of Soyasaponin B_b in Soy Products, Using Liquid Chromatography/Electrospray Ionization-Mass Spectrometry

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A method has been developed for simultaneous identification of soyasaponins and soy isoflavones in soy products, based on liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS). Soy-based nutraceutical products were analyzed by LC/ESI-MS with detection of protonated and sodiated molecular ions, as well as characteristic fragment ions for these compounds. Soy isoflavones were characterized by a strong protonated molecular ion in addition to corresponding $[\text{aglycone} + H]^+$ ions. Monitoring the soyasaponin-specific protonated aglycone and dehydrated aglycone ions throughout the chromatogram provided a unique fingerprint for soyasaponin content in the samples. This mass spectrometric fingerprint also allowed immediate classification of the soyasaponin analytes as group A or B soyasaponins, based on the unique masses of aglycone ions observed for each class. Quantification of soyasaponin B_b in soy-derived materials, based on the use of a purified soyasaponin B_b standard and a glycyrrhizin internal standard, has been accomplished.

KEYWORDS: Bioactive; diet; *Glycine max*; isoflavones; natural product; nutrition; saponins

INTRODUCTION

In the last 10 years, the relationship of diet and health has become a very active area of research and debate (*1*, *2*). Identification and characterization of specific diet-derived chemicals, and an increased understanding of their biological activity have provided support for a role for natural products and micronutrients in disease prevention and treatment. Subsequent development of these compounds as nutraceuticals will necessitate new analytical methods for their identification and measurement in quality assessment and regulation procedures.

Saponins are naturally occurring amphiphilic triterpene glycosides found in a variety of foods of plant origin. Soybeans (*Glycine max*) contain approximately 5−6% soyasaponins (*3*). The majority of saponins found in soy can be divided into two classes (Class A and Class B), based on their aglycone structures (**Figure 1**). Reports on the chemistry and health effects of soyasaponins are numerous and suggest a number of important biological properties, especially in B-class soyasaponins, including hypocholesterolemic, immunostimulatory, antitumorogenic, and anti-mutagenic activities (*4*, *5*). Methods published for the separation and identification of soyasaponins include liquid chromatography combined with ultraviolet absorbance (UV) (*6*−*8*), thermospray mass spectrometric (*9*), and electrospray ionization mass spectrometric (*10*) detection. Reported LC/UV methods are confounded by a lack of sensitivity, because soyasaponins do not possess a significant chromophore, and selectivity, due to the necessity for detection at a nonselective wavelength (205 nm). LC/MS analysis overcomes these limitations and allows identification of soyasaponins without the need for purified standards. Although the reversed-phase LC/UV and LC/MS methods referenced above all demonstrate separation and detection of select soyasaponins with varying degrees of success, they all have notable limitations with regard to simple, accurate, and comprehensive qualitative and quantitative measurements of soy-borne analytes. These include analysis of only one class of soy-borne analytes at a time, failure to provide complete separation of the analytes from each other and/or from other compounds in the mixture, lack of diagnostic markers for unambiguous classification of analytes as A- or B-class soyasaponins, a requirement for extensive preanalytical sample preparation, the inability to perform simultaneous detection of soyasaponins and soy isoflavones, and in some cases, the need for time-consuming degradation or hydrolysis procedures prior to quantification. While several investigators have reported on determination of soy isoflavones using mass spectrometry (*11*−*14*), the present report describes the first use of liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) for the simultaneous identification of soyasaponins and soy isoflavones in soy preparations. Direct mass spectrometric analysis yields molecular mass and structural information characteristic of each analyte. In addition, the method is capable

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10.1021/jf030036l CCC: $25.00
Published on Web 05/10/2003

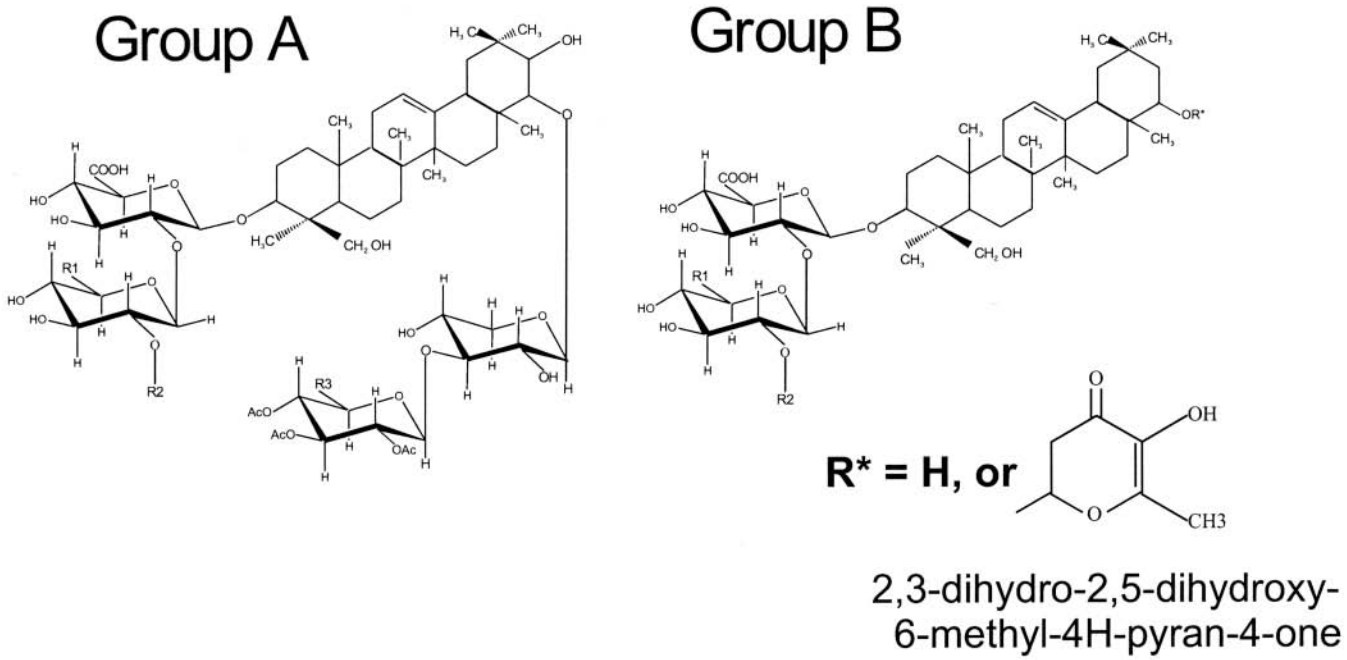

Figure 1. General structures of group A and group B soyasaponins. Detailed chemical compositions for R1, R2, and R3 groups can be found in ref 10, and are not reproduced here.

of direct quantification of intact, nondegraded, B-class soyasaponins, with the use of an appropriate internal standard.

MATERIALS AND METHODS

Preparation of Soyasaponin/Isoflavone Samples. For analysis of soyasaponins and isoflavones in commercially available soy nutraceutical tablets, 10 mg of the soy material and 1 mg of internal standard glycyrrhizin (Indofine Chemical Co., Belle Mead, NJ) were taken up in 0.5 mL of 95% (v/v) aqueous MeOH. The solutions were extracted with shaking for 3 h, and the supernatant was collected, followed by re-extraction and combination of supernatants. Solutions were then centrifuged and analyzed directly by LC/MS.

LC/MS. Analyses of soy preparations were carried out using a Waters/Micromass Ultima LC/MS instrument, consisting of a Waters 2690 liquid chromatograph with a Waters 996 photodiode array (PDA) absorbance monitor placed in series between the chromatograph and the triple quadrupole mass spectrometer. LC separations were made using a 250 mm × 4.6 mm i.d. Zorbax Eclipse XDB−C_{18} reversed-phase chromatography column at room temperature. An elution system modified from a simple linear gradient to maximize simultaneous separation of isoflavones and soyasaponins was employed in this study. The LC mobile phase consisted of (A) water containing 0.05% (v/v) trifluoroacetic acid, and (B) acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The gradient elution was linear from 13 to 30% B, 0−25 min; linear from 30 to 40% B, 25−35 min; isocratic at 40% B, 35−47 min; linear from 40 to 90% B, 47−55 min; linear from 90 to 100% B, 55−65 min; isocratic at 100% B, 65−70 min; then linear from 100 to 13% B, 70−72 min. The injection volume was 25 μL for the samples. The flow rate was 0.5 mL/min. All parameters of the ESI-MS system were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest and production of characteristic fragment ions. The following instrumental parameters were used for ESI-MS detection of isoflavones and saponins in the positive ion mode: capillary, 3.5 kV; cone, 20 V; hex 1, 20V; aperture, 0 V; hex 2: 0 V; source temperature, 100 °C; desolvation temperature, 350 °C; desolvation gas, 500 L/h; cone gas, 30 L/h; low mass resolution, 15.0; high mass resolution, 15.0; ion energy, 0.5; multiplier, 650.

Quantification of Soyasaponin B_b in Soy. To quantify soyasaponin B_b in soy-based nutraceutical products, a calibration curve was constructed, using a primary soyasaponin B_b standard (>99% purity, Chromadex, Inc., Laguna Hills, CA). For this standardization, four calibration mixtures were prepared for soyasaponin B_b by mixing known amounts of B_b and the internal standard glycyrrhizin to achieve four different mass ratios in the mixtures (B_b concentration range, 0−2 mg/mL). These solutions were then analyzed by LC/MS in selected ion monitoring mode for B_b and glycyrrhizin, and the data were subjected to a linear least-squares analysis. Soy samples were prepared as described above, containing a precisely known amount of soy material, and internal standard. The peak area ratios (soyasaponin B_b/glycyrrhizin) were then used in conjunction with the calibration curve to derive the mass percent soyasaponin B_b in the starting material.

RESULTS AND DISCUSSION

The structures of the two general classes of soyasaponins are shown in **Figure 1**. For these compounds, variations in the quantity and type of sugars attached to the parent structure (*10*), as well as in the extent of chemical modification (acetylation, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one-coupling, etc.) distinguish the different forms of each present in a given mixture. These modifications change the mass of the parent structure, making MS a powerful and accurate means of identifying multiple forms of these analytes in mixtures. **Figure 2** illustrates the utility of LC/ESI-MS for separation and detection of soyasaponins and simultaneous determination of soy isoflavones in a commercially available soy nutraceutical product. Identities of 13 soyasaponins and 10 isoflavones were established in this product, based on continuously recorded mass spectra. As an example, tetra-deacetyl-soyasaponin A_b in **Figure 2** was assigned as such, based on the characteristic mass spectrum illustrated in **Figure 3A**. This assignment is based on detection of the $[M + H]^+$ ion at *m*/*z* 1270 ($[M + Na]^+$ 1292), the soyasaponin-specific sequentially dehydrated aglycone (*m*/*z* 475) peaks at *m*/*z* 457, *m*/*z* 439, and *m*/*z* 421, and the [aglycone + glucuronic acid − $2H_2O$ + $H]^+$ peak at *m*/*z* 615. These assignments are corroborated by previous reports documenting the fragmentation behavior of soyasaponins (*7*, *9*). A-class soyasaponins identified to date in soy-based nutraceutical products are summarized in **Table 1**. In addition to tetra-deacetyl-soyasaponin A_b (**Figure 3A**) and tetra-deacetyl-soyasaponin A_f ($[M + H]^+$ 1108), differentially acetylated forms

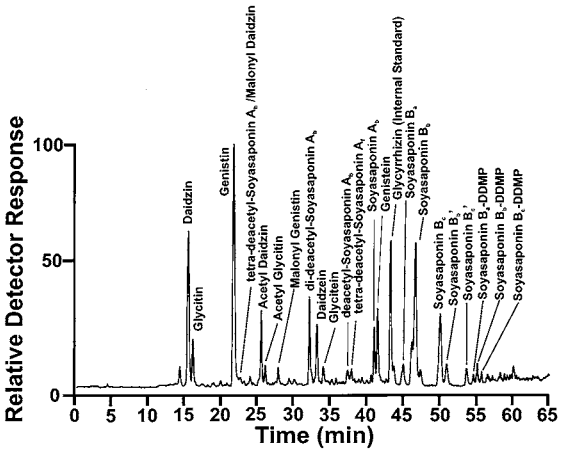

Figure 2. LC/ESI-MS total ion chromatogram (*m/z* 200–1500) analysis of a soy-based nutraceutical product. Peak identities indicated were established based on continuously recorded mass spectra. DDMP = 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one.

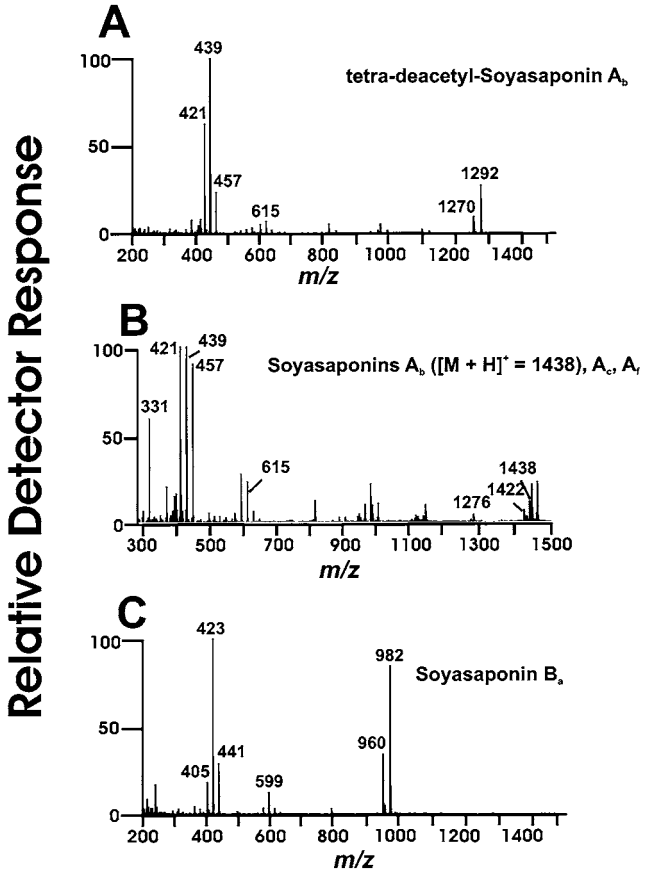

Figure 3. Characteristic positive ion ESI mass spectra of group A and group B soyasaponins. (A) mass spectrum of tetra-deacetyl soyasaponin A_b; (B) mass spectrum of soyasaponins A_b, A_c, and A_f; (C) mass spectrum of soyasaponin B_a. Ions characteristic of each class of soyasaponin are noted.

of soyasaponins A_b, A_c, A_d, and A_f are also observed for the mixture shown in **Figure 2**. A mass spectrum of fully acetylated soyasaponins Ab, Ac, and Af, that coelute under the separation conditions employed, is shown in **Figure 3B**. The assignments in **Figure 3B** are based on the observed $[M + H]^+$ ions at *m/z* 1276, *m/z* 1422, and *m/z* 1438, respectively, as well as the soyasaponin-specific fragmentation characteristics of these compounds (*7*, *9*). The assignment of acetylated forms of A-class soyasaponins such as these is also corroborated by the presence

Table 1. Protonated Molecular Ion Mass Values of Common Soyasaponins and Soy Isoflavones Observed in Soy-Based Nutraceutical Products

analyte	observed $[M + H]^+$
daidzin	417
glycitin	447
genistin	433
malonyl daidzin	503
malonyl glycitin	533
malonyl genistin	519
acetyl daidzin	459
acetyl glycitin	489
acetyl genistin	475
daidzein	255
glycitein	285
genistein	271
soyasaponin A_b	1438
deacetyl-soyasaponin A_b	1396
di-deacetyl-soyasaponin A_b	1354
tetra-deacetyl-soyasaponin A_b	1270
soyasaponin A_c	1422
deacetyl-soyasaponin A_c	1380
tri-deacetyl-soyasaponin A_d	1266
soyasaponin A_f	1276
deacetyl-soyasaponin A_f	1234
tetra-deacetyl-soyasaponin A_f	1108
soyasaponin B_a	960
soyasaponin B_b	944
soyasaponin B_b'	798
soyasaponin B_c	914
soyasaponin B_c'	768
soyasaponin B_b–DDMP	1070
soyasaponin B_c–DDMP	1040
soyasaponin B_a–DDMP	1086

of the glucose-2,3,4,6-tetraacetate fragment ion at *m/z* 331 (*7*). The presence of this fragment ion, and the absence of mass shifts in the aglycone ions at *m/z* 615, *m/z* 457, *m/z* 439, and *m/z* 421 verifies the site of acetylation on A-class saponins as the glucose moiety. In-source (MS1) fragmentation patterns for selected A- and B-class soyasaponins have been verified using MS/MS.

The absence of a ring hydroxyl group on group B saponins (**Figure 1**) decreases the mass of the aglycone by 16 Da relative to group A saponins, allowing one to corroborate assignment of soyasaponins as group A or group B based on the series of aglycone peaks in the corresponding mass spectra. This distinguishing feature can be seen in the mass spectrum of soyasaponin B_a illustrated in **Figure 3C**. Note that the [aglycone + glucuronic acid − $2H_2O$ + H]$^+$ for B_a has shifted to *m/z* 599, and that the series of dehydrated aglycone peaks have shifted to *m/z* 441, *m/z* 423, and *m/z* 405, respectively (*9*). In addition to the five B-class soyasaponins known to occur in soy that have been identified in this study, chemically modified versions of these saponins containing a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one moiety attached via an ether linkage at C22 (**Figure 1**) can also be identified by their corresponding mass increase of 126 Da. Three such saponins, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one -soyasaponin B_b, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one -soyasaponin B_c, and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one -soyasaponin B_a, have been identified here.

In addition to providing separation and detection of soyasaponins in natural product mixtures, the LC/MS method described herein provides concomitant identification of soy isoflavones. Twelve different soy isoflavones have been identified using this method in the analysis of various soy-based products, the identities of which are summarized in **Table 1**. **Figure 2** shows a total ion chromatogram for the analysis of one such product,

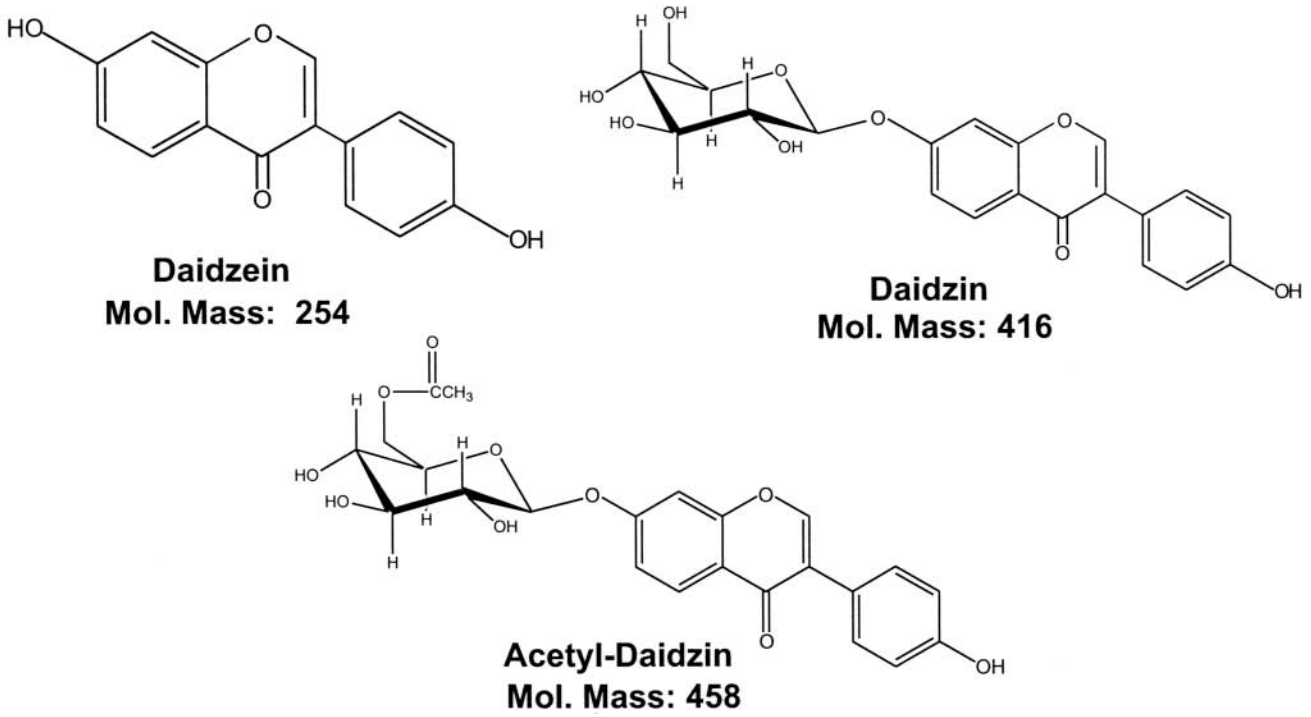

Figure 4. Structures and molecular masses of three daidzein-related isoflavones.

in which 10 soy isoflavones were identified. Continuously recorded mass spectra allow unambiguous determination of the three major soy isoflavones, genistein, daidzein, and glycitein, as well as nine other structurally distinct (chemically modified) isoflavone analytes within the same families. For the major isoflavones, assignments were corroborated based on retention times and mass spectra of commercially available standards (not shown). **Figure 4** illustrates three analytes within the daidzein family, namely, daidzein, daidzin (daidzein glucoside), and acetyl daidzin. The corresponding mass spectra for each of these compounds are shown in **Figure 5**. The mass spectrum of daidzein shown in **Figure 5A** is a simple spectrum consisting of a protonated molecular ion ($[M + H]^+$) at *m*/*z* 255. The mass spectrum of daidzin (**Figure 5B**) is characterized by an $[M + H]^+$ ion 162 Da (replacement mass of a glucose moiety) higher than daidzein, in addition to the daidzein-specific fragment ion at *m*/*z* 255 that corresponds to the deglycosylated parent. The mass spectrum of acetyl-daidzin (**Figure 5C**) is characterized by a protonated molecular ion with an additional mass shift of 42 Da (replacement mass of an acetyl moiety) relative to daidzin, that is deacetylated and deglycosylated to form the protonated daidzein ion at *m*/*z* 255. Malonylated isoflavone-glucosides (**Figure 1**, **Table 1**) are also detected in mixtures, based on their distinctive mass spectra, consisting of an $[M + H]^+$ ion 86 Da higher than the corresponding isoflavone-glucoside (e.g., daidzin, glycitin, genistin), as well as the demalonylated, deglycosylated parent isoflavone ion (not shown).

A calibration curve using the internal standard glycyrrhizin was constructed for soyasaponin B_b ($y = 0.855x + 0.039$, $R^2 = 0.998$) in the concentration range 0−2 mg/mL, and used to quantify this analyte in four soy-based nutraceutical products. Three replicate measurements were made for soyasaponin B_b in extracts of each product. Mean values and relative standard deviations were calculated and are shown in **Table 2**. At the time of this study, soyasaponin B_b was the only commercially available soyasaponin standard. Future availability of other standards for this family of compounds will allow extension of the presently described method to more accurate and comprehensive soyasaponin determination.

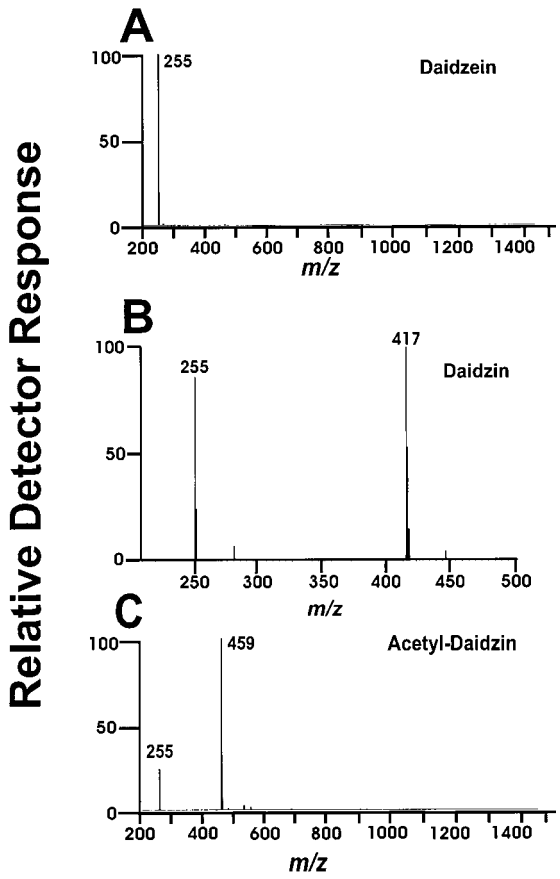

Figure 5. Characteristic positive ion ESI mass spectra of three daidzein-related soy isoflavones.

In conclusion, the goal of the present work was to develop a robust LC/MS method for simultaneous identification of soyasaponins and soy isoflavones that can also be employed for

Table 2. Quantification of Soyasaponin B_b in Four Commercially Available (tablet form) Soy-Nutraceutical Products

product	mass % B_b (% RSD, $n = 3$)
1	12.9 (0.80)
2	14.2 (1.2)
3	1.4 (1.6)
4	2.6 (1.0)

quantification of intact, non-hydrolyzed soyasaponins. The described method demonstrates the efficient and selective separation and detection of analytes characteristic of LC/MS analysis, while providing several advantages over previously described methods, including improved chromatographic separation, simultaneous separation of 30 soy-borne analytes, and direct quantitation of native saponin analytes, eliminating the need for time-consuming, labor-intensive preanalyzis purification or degradation procedures.

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Received for review January 16, 2003. Revised manuscript received April 1, 2003. Accepted April 7, 2003.

JF030036L