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Determination of Soyasaponins in Soy with LC-MS Following Structural Unification by Partial Alkaline Degradation

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High-performance liquid chromatography coupled with electrospray ionization mass spectrometer was used to study the soyasaponins in soy. It was found that each soyasaponin belonging to group A existed mainly in their genuine acetylated forms. The partially to fully deacetylated structures coexisted in various proportions. Likewise, the soyasaponins belonging to group B in soy were detected as both 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) conjugated forms and non-DDMP forms. The structural diversity of soyasaponins hinders the separation and determination of the individual compounds in soy. In the present studies, the soyasaponins extracted from soy were treated with sodium hydroxide under mild conditions to cleave the acetyl groups from soyasaponins in group A as well as the DDMP from soyasaponins in group B, while the glycoside structures remained unaffected. By doing so, all soyasaponins originating from the same initial structures were unified into well-defined structures and then quantified individually using the selective ion recording of their $[M-H]^-$ ions. The pure deacetyl and non-DDMP soyasaponins were used as the external standards. The quantification limits of soyasaponins in group A and group B were 1.74 and 1.89 ng injected on column with recovery rates of 94.1% ± 4.2% and 96.9% ± 2.9%, respectively.

KEYWORDS: LC-MS; saponin; soyasaponin; soy; *Glycine max* L.

INTRODUCTION

Soyasaponins in legumes, especially soy, are the primary dietary sources of saponins from foods (*1*). Soyasaponins have been demonstrated to possess multiple health-promoting properties, such as lowering of cholesterol level by inhibiting its absorption, being anti-carcinogenic (*1*) and antihepatotoxic (*2*, *3*), and promoting the anti-infectivity of HIV (*4*). According to their structures, the soyasaponins can be divided into two groups: A and B (**Scheme 1**). Soyasaponin in "group A" are bidesmosidic saponins, having two glycosylation sites on their aglycone moiety (soyasapogenol A)(*5*). Soyasaponins in "group B" are monodesmosidic saponins, having one glycosylation site on two different aglycone moieties (soyasapogenol B and E) (*6*). The predominant and genuine soyasaponins in group B in legume are conjugated with 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) (*7*−*9*).

So far, many methods had been tried for the quantification of the soyasaponins. The values determined by different methods showed enormous discrepancy (*10*−*12*). We have compared the performance of staining reagents used for the colorimetric method toward soyasaponins. None of them were found to be sensitive and specific (*13*). The major hindrance in determining soyasaponins by HPLC is the lack of appropriate detection methods. The maximum absorption wavelength of soyasaponins is around 205 nm. UV detection at this wavelength suffers from enormous interference. The baseline shift caused by the gradient of solvent is significant. In the previous studies, soyasaponins had to be purified into group A and group B before they were separated on HPLC (*14*, *15*). Otherwise, it was difficult to distinguish the peaks. Postcolumn reaction methods have been tried to derivatize soyasaponins into fluorescence or UV active compounds. However, because of the lack of active chemical groups on soyasaponins, the derivatization reaction turned out to be difficult (*16*, *17*).

It has been reported that the length of oligosaccharide attached to the soyasaponin and the type of constituent monosaccharides differentiated the antihepatotoxic activities of soyasaponins (*2*, *3*, *18*) as well as their organoleptic properties in foods (*19*). This necessitates the determination of the individual soyasaponins with accuracy, which has not been accomplished by any existing methods so far. Using the HPLC coupled with electrospray ionization mass spectrometer, we have identified three novel malonyl isoflavone glycosides in soy germs and cotyledons (*20*). In the present studies, we extended our work to soyasaponins. The soyasaponins were quantified individually using the selective ion monitoring (SIR) of the mass spectrom-

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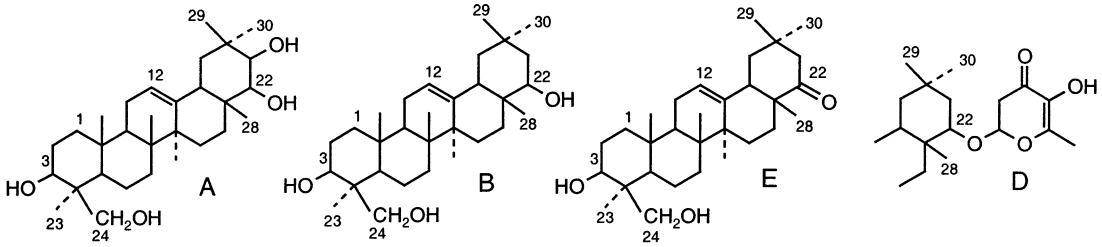
Scheme 1. Structures of Soyasaponins in Soy[a]



A, soyasapogenol A; B, soyasapogenol B; E, soyasapogenol E; D, DDMP conjugated to soyasapogenol B

Structures	Name	Formula	MW
glc(1→2)gal(1→2)glcUA(1→3)B	Ba	$C_{48}H_{78}O_{19}$	959.1
rha(1→2)gal(1→2)glcUA(1→3)B	Bb	$C_{48}H_{78}O_{18}$	943.1
rha(1→2)ara(1→2)glcUA(1→3)B	Bc	$C_{47}H_{76}O_{17}$	913.1
gal(1→2)glcUA(1→3)B	Bb'	$C_{42}H_{68}O_{14}$	797.0
ara(1→2)glcUA(1→2)B	Bc'	$C_{41}H_{66}O_{13}$	767.0
glc(1→2)gal(1→2)glcUA(1→3)E	Bd	$C_{48}H_{76}O_{19}$	957.1
rha(1→2)gal(1→2)glcUA(1→3)E	Be	$C_{48}H_{76}O_{18}$	941.1
glc(1→2)gal(1→2)glcUA(1→3)A(22←1)ara(3←1)xyl(2,3,4-tri-*O*-Acetyl)	Aa	$C_{64}H_{100}O_{31}$	1365.5
glc(1→2)gal(1→2)glcUA(1→3)A(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-Acetyl)	Ab	$C_{67}H_{104}O_{33}$	1437.5
rha(1→2)gal(1→2)glcUA(1→3)A(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-Acetyl)	Ac	$C_{67}H_{104}O_{32}$	1421.5
glc(1→2)ara(1→2)glcUA(1→3)A(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-Acetyl)	Ad	$C_{66}H_{102}O_{31}$	1391.5
gal(1→2)glcUA(1→3)A(22←1)ara(3←1)xyl(2,3,4-tri-*O*-Acetyl)	Ae	$C_{58}H_{90}O_{26}$	1203.3
gal(1→2)glcUA(1→3)A(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-Acetyl)	Af	$C_{61}H_{94}O_{28}$	1275.4
ara(1→2)glcUA(1→3)A(22←1)ara(3←1)xyl(2,3,4-tri-*O*-Acetyl)	Ag	$C_{57}H_{88}O_{25}$	1173.3
ara(1→2)glcUA(1→3)A(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-Acetyl)	Ah	$C_{60}H_{92}O_{27}$	1245.4

[a] glc, β-D-glucopyranosyl; gal, β-D-galactopyranosyl; glcUA, β-D-glucuronopyranosyl; ara, α-L-arabinopyranosyl; rha, α-L-rhamnopyranosyl; xyl, β-D-xylopyranosyl; MW, molecular weight.

eter after their diverse structures were unified with the partial alkaline degradation method.

MATERIALS AND METHODS

Reagents and Samples. Two varieties of soy samples were harvested in the fall of 1997 in Northeast China and supplied by Weiwei Food Group Inc. (Xuzhou, Jiangsu, China). One variety (Kennong 16) was separated into germs, cotyledons, and hulls using a dry dehulling procedure. Another variety (Jilin 3) was supplied in the form of defatted soy meal. Soy molasses (a byproduct from soy protein concentrate production; 59.5% ± 4.1% dry matter) was sampled from the Shanghai Liantang Food Factory (Shanghai, China). All samples were stored at −32 °C in a freezer in the laboratory before analysis. HPLC-grade solvents and other reagents with proper purity were purchased from Shanghai Pharmaceutical and Chemical Inc. (Shanghai, China).

Purification of Soyasaponin Standards. Soy germ (500 g) was ground and refluxed in 5 L 70% (v/v) aqueous ethanol. The extract was evaporated on a rotary evaporator to remove ethanol under vacuum at 40 °C until approximately 500 mL brown syrup was left. This syrup was extracted with an equal volume of hexane twice to eliminate the lipophilic substances, followed by extracting twice with an equal volume of *n*-butanol. The *n*-butanol extracts were evaporated to dryness and redissolved in 350 mL of 80% (v/v) aqueous ethanol. A white precipitate formed after the ethanol solution was kept at 4 °C in a refrigerator overnight. The major components in this precipitate proved to be isoflavone glycosides by LC-MS assay. The supernatant was evaporated to obtain 16.1 g of dry substance. It was redissolved into 100 mL methanol and mixed with 50 g silica gel (80−100 mesh, Qingdao Ocean Chemicals Inc., Qingdao, China). The mixture was evaporated to dryness and ground into powder and loaded onto an 80 × 5 cm column packed with the same silica gel in dry form. The column was washed with a mixture of chloroform, methanol, water, and acetic acid (65:35:9.8:0.2, v/v, the low layer in the funnel after vigorous shaking). The eluates were collected in 100 mL aliquots. Monitoring with LC-MS showed that the acetyl isoflavone glycosides eluted from the column in the first 500 mL solvent. Then the isoflavone glycosides, soyasaponins in group B, and soyasaponins in group A were washed out of the column subsequently with additional 500, 1000, and 600 mL of solvent. The eluting fractions containing the soyasaponins of group B and group A were combined and evaporated to dryness separately. The soyasaponins of group A (100 mg) were dissolved in 10 mL methanol containing 3% (w/v) sodium hydroxide and kept at 25 °C for 2 h. The solution was neutralizeded with 1 mol/L hydrochloric acid in water. The soyasaponins in group A were deacetylated with this method.

Purification of pure soyasaponins was performed on a semipreparative LC system consisting of two Waters 600 pumps, a gradient control module and a Waters 2478 dual wavelength UV detector (Bedford, MA). The separation was carried out on a Waters μBondapak C18 column (250 × 7.8 mm i.d.; 10 μm) with a flow rate 3 mL/min and 210 nm UV detection. The isocratic solvent system which consisted of methanol, 2-propanol, water, and formic acid (55:5:40:0.1, v/v) was used for separation of soyasaponins in group B. The proportion of solvent system was adjusted to 45:5:50:0.1 (v/v) for the separation of deacetyl soyasaponins in group A. Two predominant peaks in the chromatogram of soyasaponins in group B were collected, and the solvent was evaporated under vacuum to give rise to white needlelike crystals. They were identified to be non-DDMP soyasaponin Ba and non-DDMP soyasaponin Bb using ES-MS and confirmed with IR, UV, and NMR (^{1}H, ^{13}C, ^{1}H-^{13}C COSY) (*21*). The three peaks collected from the chromatography of soyasaponins in group A were identified to be deacetyl soyasaponin Aa, deacetyl soyasaponin Ab, and deacetyl soyasaponin Ae using ES-MS (*22*). The purity of all these compounds was found to be higher than 96% on the HPLC.

Extraction and Partial Alkaline Degradation. The sample extraction method in an earlier paper was followed with minor modification (*20*). In brief, the solid samples were milled to pass through a 30-mesh sieve prior to extraction. Soy cotyledons, hulls, or defatted soy meals (1 g) were extracted with 70% (v/v) aqueous ethanol (10 mL) in a screw-top flask in a 25 °C ultrasonic water bath for 1 h. Soy germ

(250 mg), which had been reported to contain more soyasaponins than the cotyledons, was extracted with 10 mL aqueous ethanol as described for soy cotyledons to ensure a complete extraction. Soy molasses was dissolved directly in 70% (v/v) aqueous ethanol. The extraction mixtures were centrifuged at 3000 rpm for 10 min. Partial alkaline degradation was carried out in a 1.5 mL centrifuge tube. Supernatant of the extracts (400 μL) were mixed with 200 μL of 600 mmol/L sodium hydroxide in 70% (v/v) aqueous methanol. Formic acid [200 μL 4% (v/v)] in 70% (v/v) aqueous methanol was added 2 h later to neutralize the alkaline and maintain the pH of the solution between 4 and 6. When sodium hydroxide with different concentrations was used, the concentration of formic acid was adjusted proportionally. All the reactions were performed at room temperature (25 °C). The reaction media was centrifuged at 14 000 rpm for 15 min before 20 μL was injected for LC-MS analyses.

HPLC-ES-MS Assay. Analyses were conducted on a Waters 2690 Alliance HPLC system (Bedford, MA) coupled with a Micromass mass spectrometer (Micromass ZMD, Manchester, U.K.). Sample separation was carried out using a Supelcosil LC-18-DB column (250 × 4.6 mm i.d.; 5 μm, Supelco, Bellefonte, PA). The solvent system consisted of (A) water:formic acid (100:0.2, v/v) and (B) methanol:formic acid (100:0.2, v/v). The linear gradient was as follows: 0−40 min, 30−60% B; 40−60 min, 60−80% B; 60−80 min, 80−90% B. The flow rate (1 mL/min) eluting out of the column was split 4:1 so that in 1 min, 200 μL effluent was delivered to the electrospray ionization source and 800 μL passed through a Waters 996 PAD detector which scanned over the range of 200−400 nm. Nitrogen gas was used both as drying and auxiliary gas for the mass spectrometer. The parameters applied to MS on negative mode included capillary voltage 3700 V, cone voltage 80 V, extractor voltage 5 V, source block temperature 120 °C, and drying gas temperature 200 °C. The conditions employed in the positive mode were the same as those for the negative mode except that the capillary voltage was 4000 V and the cone potential was 100 V. For the identification of soyasaponins eluting from the column, the full scan mass spectra over the *m*/*z* range 200−1500 amu in both negative and positive mode were acquired in the same HPLC separation by switching the electric polarity of the capillary based on a time-cutting mechanism. The selected ion recording (SIR) method on negative mode was applied for the purpose of soyasaponin quantification. The chromatographic and spectral data were recorded using Masslynx software (version 3.1, Micromass, Manchester, U.K.).

For the direct injection of standard compounds and for the optimization of the electrospray parameters, the pure soyasaponin compounds were dissolved in mobile phase used for LC-MS and injected using a Harvard syringe pump (model II; South Natick, MA) at a flow rate of 10 μL/min.

Statistical Analysis. Microsoft excel 97 was used for the statistical analysis. Evaluation, and comparison of linear standard curves was described by Deng (*23*).

RESULTS AND DISCUSSION

Characterization of Soyasaponin on ES-MS. The mass spectra of deacetyl soyasaponin Aa and non-DDMP soyasaponin Ba obtained in both negative and positive mode are illustrated in **Figure 1**. The mass spectral data are elucidated in **Table 1**. The soyasaponins formed three types of quasimolecular ions in the positive mode, including $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$. The relative intensities of these ions were found to depend on the pH and composition of the solvents (*20*). The $[M+Na]^+$ of deacetyl soyasaponin Aa was enhanced by the sodium ions introduced in the alkaline deacetylation procedure. The fragmentation of soyasaponins in positive mode was extensive and characteristic, providing the information of the monosaccharidic sequence of the oligosaccharide chain attached to the soyasapogenols (*24*). No fragmentation of soyasaponins was observed in negative mode over a large range of capillary voltages (3000−4000 V) and cone potentials (50−150 V). The $[M-H]^-$ ions were observed as the primary signal. The negative mode was selected for the quantitative analysis of the soyasaponins due to the simplicity and stability of mass spectra as well as the lower background noise.

Table 1. Fragmentation of Two Soyasaponins on Electrospray Ionization Mass Spectrometer[a]

non-DDMP soyasaponin Ba		deacetyl soyasaponin Aa	
m/*z*	ions	*m*/*z*	ions
998.0	$[M+K]^+$	1276.2	$[M+K]^+$
982.2	$[M+Na]^+$	1262.2	$[M+Na]^+$
960.0	$[M+H]^+$	1130.2	$[M\text{-xyl}+Na]^+$
797.9	$[M\text{-glc}+H]^+$	997.8	$[M\text{-xyl-ara}+Na]^+$
635.7	$[M\text{-glc-gal}+H]^+$	761.7	$[M\text{-glc-gal-glcUA}+Na]^+$
617.7	$[M\text{-glc-gal-}H_2O+H]^+$	615.6	$[M\text{-xyl-glc-gal-glcUA}+Na]^+$
599.8	$[M\text{-glc-gal-}2H_2O+H]^+$	539.4	$[glc\rightarrow gal\rightarrow glcUA+K]^+$
523.2	$[glc\rightarrow gal\rightarrow glcUA+Na]^+$	523.4	$[glc\rightarrow gal\rightarrow glcUA+Na]^+$
441.6	$[aglycone\text{-}OH]^+$	439.6	$[aglycone\text{-}2H_2O+H]^+$
423.4	$[aglycone\text{-}OH\text{–}H_2O]^+$	421.1	$[aglycone\text{-}3H_2O+H]^+$
347.2	$[glc\rightarrow gal+Na]^+$	347.2	$[glc\rightarrow gal+Na]^+$
958.2	$[M-H]^-$	1238.2	$[M-H]^-$

[a] M, the molecule of the soyasaponins; + and –, gain or loss of specific ions or a neutral moiety; →, bond between monosaccharide units.

Structural Diversities of Soyasaponins in Soy. A chromatogram of soy germ extracts before alkaline treatment detected at 205 nm is shown in **Figure 2A**. The peaks eluting before 20 min have been identified as isoflavones in glycoside or malonyl glycoside forms (*20*). Because the molecular extinction coefficients of soyasaponins are very low (5278 $mol^{-1}cm^{-1}$ reported for non-DDMP soyasaponin Bb) (*25*), no distinguishable peak was observed on the UV trace. The peaks in the total ion chromatogram (TIC) showed more peaks other than the isoflavones [**Figure 2B**]. The $[M-H]^{-1}$ ions of DDMP soyasaponin Ba (1084.2 *m*/*z*) and the non-DDMP soyasaponin Ba (958.2 *m*/*z*) were used to construct the selected ion chromatogram from the TIC. Two peaks were observed in **Figure 2C**, with non-DDMP soyasaponin Ba presenting at 54.23 min and DDMP soyasaponin Ba at 68.37 min. The early reports concluded that the DDMP soyasaponins in group B represented the genuine structures in the native legumes (*7*, *8*). It was reported that approximately 94.1% of the soyasaponins in group B existed as the DDMP conjugated forms in newly harvested peas (*26*). However, the DDMP soyasaponins are extremely unstable. They can degrade gradually to lose the DDMP moiety during the storage of soy, and more quickly during the process of extraction and purification in aqueous solvents (*9*). The proportion of non-DDMP and DDMP soyasaponin Ba in our soy germ samples were found to be 23.3:76.7, calculated on the basis of their areas on the selected ion chromatogram. Similar proportions were observed for soyasaponins Bb, Bc, Bb′, and Bc′. These values were in agreement with those of Daveby et al. (*26*). According to their reports, the ratio of non-DDMP soyasaponin to the total soyasaponin in group B increased to 25.4% and 43.2% after 9 months and 7 years of storage for peas. Kudou et al. (*9*) reported that heating the extracts at 100 °C for 1 h in aqueous methanol led to degradation of virtually all the DDMP soyasaponins into non-DDMP forms. The half time of DDMP soyasaponins was 150 h in 80% (v/v) aqueous ethanol at the room temperature (*26*). Our experiments indicated that 16 h heating at 80 °C in 70% (v/v) aqueous ethanol rendered all the DDMP soyasaponins undetectable on LC-MS. The cleavage of DDMP moiety followed a zero order kinetic with respect to time.

Single peaks were observed on the selected ion chromatograms of soyasaponin Aa (1364.4 *m*/*z*) and soyasaponin Aa which had lost all its three acetyl groups (1238.3 *m*/*z*) [**Figure**

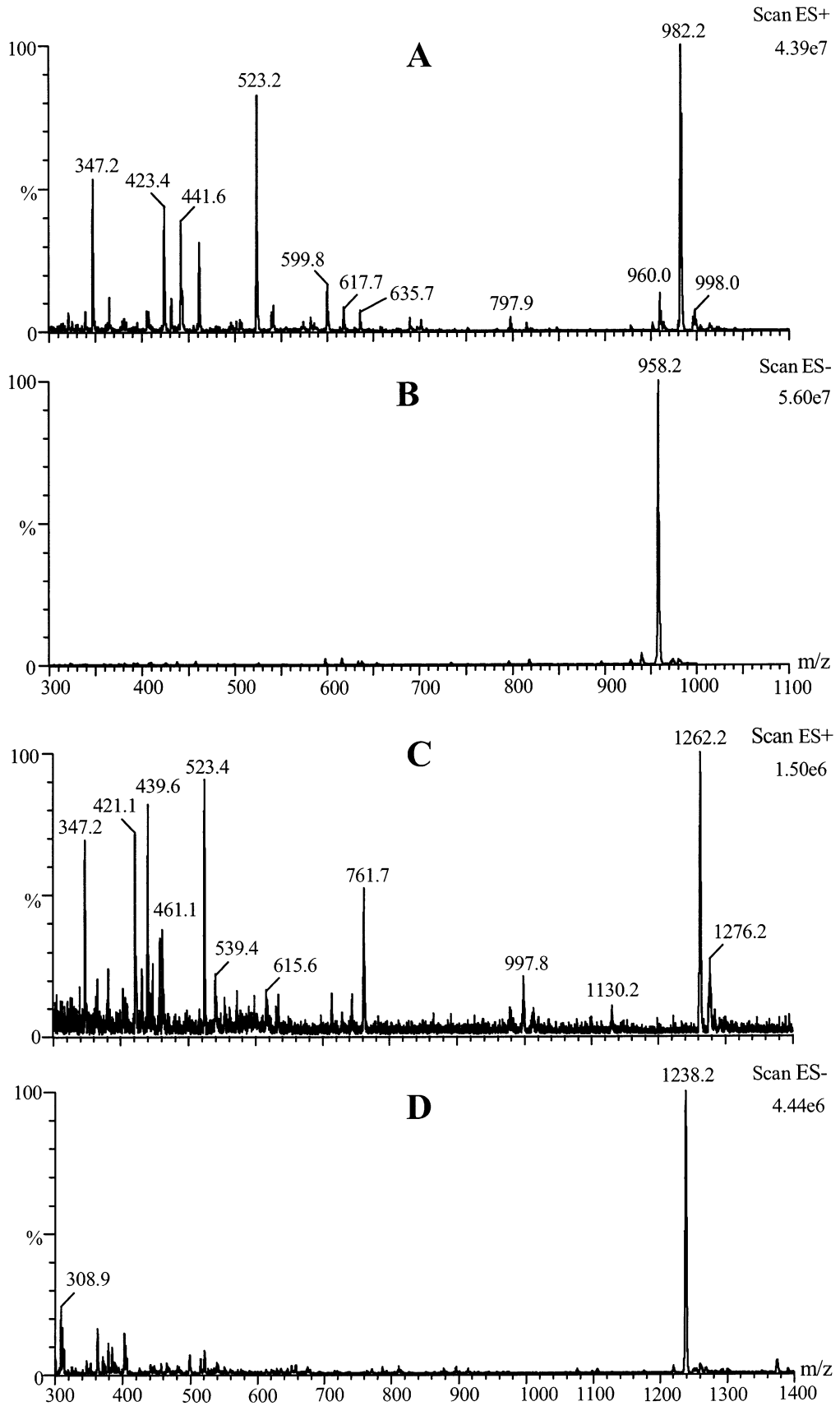


Figure 1. Mass spectra of non-DDMP soyasaponin Ba (A, positive mode; B, negative mode) and deacetyl soyasaponin Aa (C, positive mode; D, negative mode) obtained by direct injection of pure compounds.

2D,**G**]. However, for soyasaponin Aa which had lost one or two acetyl groups (1322.4 *m*/*z* or 1280.3 *m*/*z*), three peaks were detected in their selected ion chromatograms, reflecting the existence of three possible position isomers [**Figure 2E**,**F**]. The peak areas of soyasaponin Aa having three, two, one, or no acetyl groups in soy germ were integrated from their selected

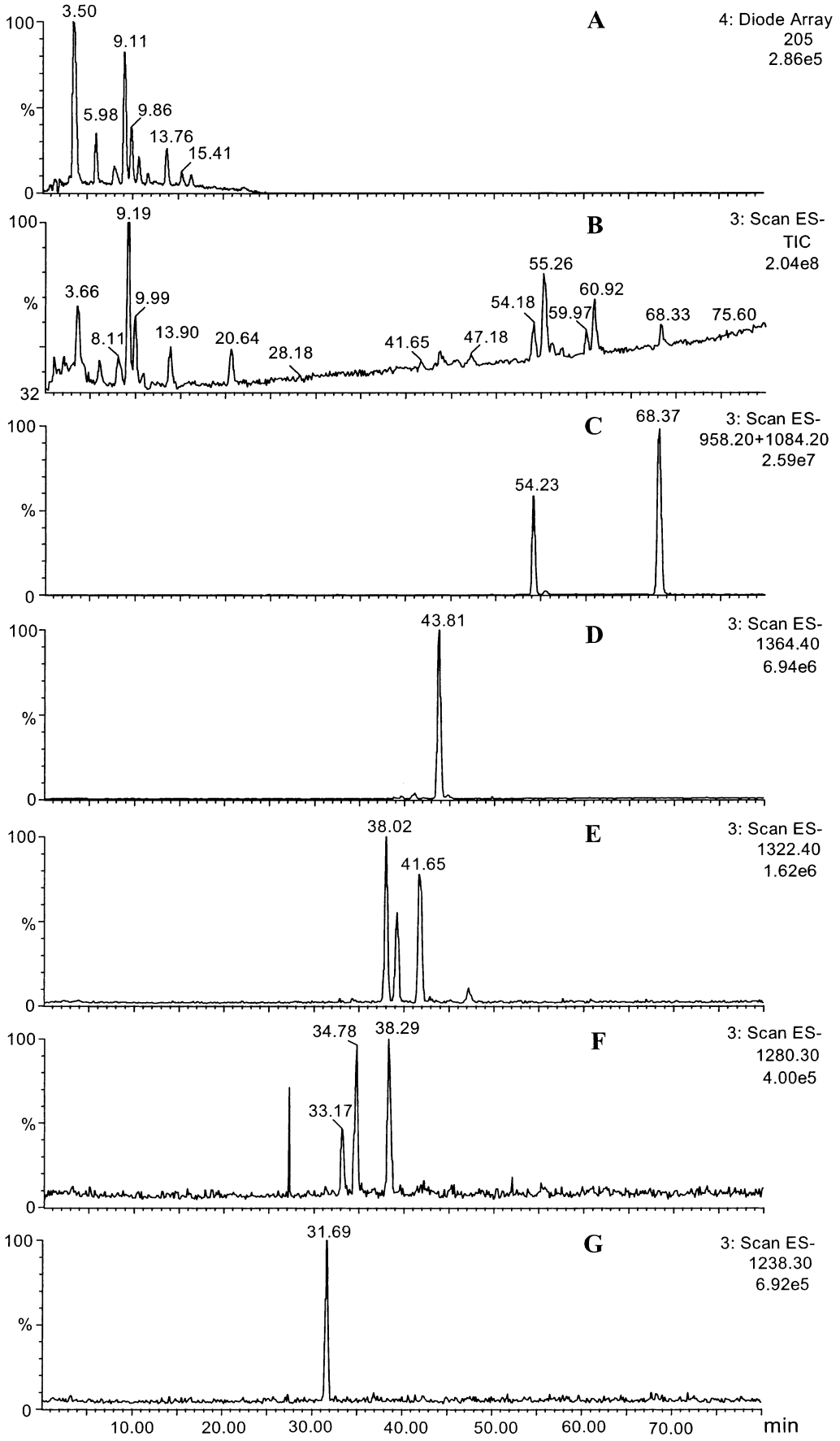


Figure 2. Chromatogram of soy germ (Kennong 16) extract without alkaline treatment detected at UV 205 nm (A) and the total ion chromatogram of negative mode ES-MS detection (B). The selected ion chromatogram of the non-DDMP soyasaponin Ba and DDMP soyasaponin Ba is showed in panel C. The selected ion chromatogram of soyasaponin Aa, soyasaponin Aa losing one acetyl, soyasaponin Aa losing two acetyls, and soyasaponin Aa losing three acetyls are showed in panels D–G, respectively.

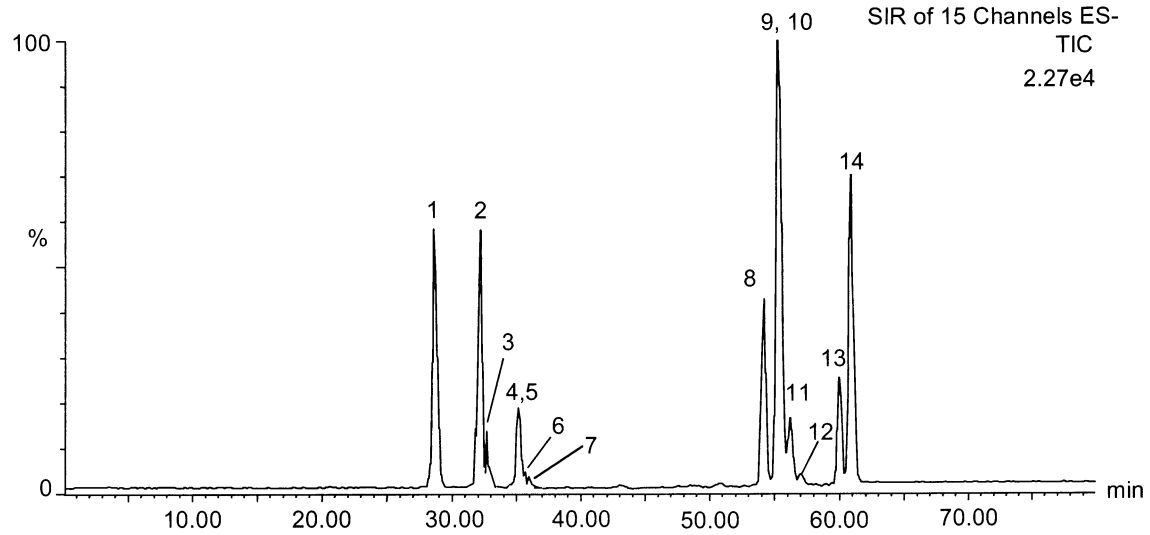


Figure 3. Selected ion recording (SIR) of $[M–H]^-$ of the soyasaponins in soy germ (Kennong 16) extract after alkaline treatment. Peaks 1–7 are deacetyl soyasaponin Ab, Aa, Ac, Ae, Af, Ag, and Ah, respectively. Peaks 8–14 are non-DDMP soyasaponin Ba, Bb, Bc, Bb′, Bc′, Bd, and Be, respectively.

ion chromatograms, and the proportion was 54.1:32.6:8.4:4.8. We have found that the soyasaponins with similar structure have the same ionization efficiency; thus, the same response factors on mass detection (area/ng). This area proportion could reflect their actual distribution in soy. Likewise, the proportion of Ab having four, three, two, one, and no acetyl groups was calculated to be 56.7:27.3:9.8:1.6:4.5 using the same method. The soyasaponins in group A were initially identified in deacetylated forms when the chemical degradation method was applied to study the monosaccharidic sequence of the glycoside (*27*, *28*). However, modern spectroscopic techniques indicated that the genuine structures of soyasaponins in group A in native soy had three or four acetyl groups in the far end of the oligosaccharide connected to the C22 on soyasapogenol A (*9*, *29*). The deacetyl soyasaponins found earlier were the artifacts of chemical degradation. There is no report concerning the intrinsic spontaneous deacetylation of soyasaponins in group A in soy or the coexistence of soyasaponin in group A having varied number of acetyl groups. It can be deduced that the deacetylation of soyasaponin A took place during the storage of the soy. Besides the possible spontaneous deacetylation induced by the deacetylase which exists in all plants (*30*), the chemical and biological deacetylation could happen during soy food processing and cooking. Over 40 structures of soyasaponins were detected by us in soy using the LC-MS. They existed in various proportions and their peaks overlapped severely on the HPLC chromatograms. To distinguish all these peaks would be impractical using UV detection or evaporative light scattering detection.

Partial Alkaline Degradation of the Soyasaponins. To overcome the structure diversities of soyasaponins, a partial alkaline degradation method was tried to unify all the existing forms of each soyasaponins into defined deacetylated or non-DDMP structures. Various concentrations of sodium hydroxide in the solution were tried (500, 300, 200, 100, 50, 25, and 10 mmol/L after the mixing) with a fixed reaction time of 2 h at 25 °C. The neutralized solution was analyzed on HPLC with full scan MS detection at both negative and positive mode. No peaks of DDMP soyasaponins (defined as signal-to-noise ratio > 3) could be observed on their selected ion chromatograms after being treated with sodium hydroxide at a concentration as low as 25 mmol/L. There was still 51.8% of soyasaponins in group B, which existed in the DDMP conjugated forms after being treated with 10 mmol/L sodium hydroxide. The lowest effective concentration for alkaline degradation in which all the acetylated soyasaponins were undetectable was 50 mmol/L. The glycosidic linkages in the oligosaccharide were generally regarded to be stable under a mild alkaline environment (*29*). However, hydrolysis was still feasible at higher alkaline concentrations. The stability of pure soyasaponins was tested in the alkaline solutions for a 2 h period at 25 °C. The deacetyl soyasaponins Aa and non-DDMP soyasaponin Ba did not show detectable degradation after being treated with sodium hydroxide solution at any concentration less than 300 mmol/L. Deacetyl soyasaponins Aa degraded slightly in 300 mmol/L and significantly in 500 mmol/L sodium hydroxide. A marginally significant decrease was observed for non-DDMP soyasaponin Ba after treatment with 500 mmol/L sodium hydroxide. The glycosidic structures in group B soyasaponins appeared to be more stable than those in the group A soyasaponins. This was probably caused by the difference in glycoside structures. The glycoside in soyasaponins of group B is through 1→2 linkages, while both 1→2 and 1→3 linkages are present in soyasaponins of group A. The 1→2 linkage had found to be the most resistant structure to alkaline degradation among all the possible linkages. The 1→3 linkage is much more vulnerable to alkaline hydrolysis (*31*).

The instability of the DDMP soyasaponins in basic pH was observed by Okubo et al. (*32*). In this study, we found that the DDMP could be cleaved from the soyasaponin more easily than the acetyl groups in alkaline solution. Partial alkaline degradations in 200 mmol/L sodium hydroxide gave rise to consistent results; thus, this concentration was used for quantitative analyses.

Standard Curves and Quantification Limits. The selected ion recording function on negative mode was applied to obtain the chromatogram of the soyasaponins. The sensitivity of SIR is much higher than the full spectrum scan. In such a method, only the $[M-H]^{-1}$ ions of 15 known soyasaponins (in non-DDMP and deacetyl forms) were scanned by the mass detector, and all other ions were ignored. All the peaks presenting on the total ion chromatogram of SIR were identified to be soyasaponins (**Figure 3**). The peak of each individual soyasaponin was distinguished by constructing its selected ion chromatogram, and the peak area was integrated based on it. The solutions of pure soyasaponins were diluted into a series of concentrations and analyzed under the same conditions. The standard curves had correlation coefficients higher than 0.99

Table 2. Contents of Soyasaponins in Soy Samples (mg/100 g, Mean ± SD, $n = 2$)[a]

components	soy germs	soy cotyledons	defatted soy meal	molasses
soyasponin Ba	468.6 ± 25.8 (4.886)	24.6 ± 1.3 (0.256)	41.5 ± 1.4 (0.433)	725.0 ± 34.8 (7.559)
soyasponin Bb	1346.7 ± 57.5 (14.28)	581.5 ± 30.0 (6.166)	464.7 ± 27.4 (4.927)	1689.3 ± 67.9 (17.91)
soyasponin Bc	106.0 ± 4.0 (1.661)	183.5 ± 5.5 (2.010)	333.9 ± 18.3 (3.657)	8.3 ± 1.1 (0.091)
soyasponin Bb′	72.7 ± 2.7 (0.912)	72.6 ± 2.4 (0.911)	45.9 ± 2.2 (0.576)	76.9 ± 4.0 (0.965)
soyasponin Bc′	10.4 ± 1.6 (0.136)	19.8 ± 1.2 (0.258)	20.2 ± 1.0 (0.263)	80.8 ± 2.3 (1.053)
soyasponin Bd	254.6 ± 12.0 (2.660)	6.0 ± 0.2 (0.063)	–	406.5 ± 10.4 (4.247)
soyasponin Be	781.8 ± 36.2 (8.307)	47.5 ± 2.0 (0.505)	14.9 ± 0.7 (0.158)	941.2 ± 29.4 (10.00)
soyasponin Aa	640.0 ± 44.3 (5.164)	–	–	1038.9 ± 57.7 (8.382)
soyasponin Ab	590.5 ± 30.2 (4.652)	–	93.9 ± 3.9 (0.740)	1033.0 ± 40.8 (8.138)
soyasponin Ac	131.3 ± 4.1 (1.084)	–	29.4 ± 1.6 (0.253)	152.5 ± 6.1 (1.217)
soyasponin Ad	-	–	23.1 ± 1.3 (0.189)	-
soyasponin Ae	194.1 ± 7.0 (1.802)	–	–	292.0 ± 13.3 (2.711)
soyasponin Af	125.9 ± 7.8 (1.137)	–	33.3 ± 1.0 (0.301)	113.2 ± 4.6 (1.022)
soyasponin Ag	17.3 ± 2.2 (0.165)	–	–	25.6 ± 0.9 (0.244)
soyasponin Ah	8.7 ± 0.5 (0.081)	–	7.9 ± 0.5 (0.073)	-
total	4748.5 ± 182.1 (46.39)	935.4 ± 50.7 (10.17)	1108.7 ± 35.8 (11.55)	6583.2 ± 250.5 (63.54)

[a] The mean content of each soyasaponins expressed as μmol/g is presented in parentheses. All the soyasaponins are in the non-DDMP form in group B and deacetyl form in group A; –, not detected.

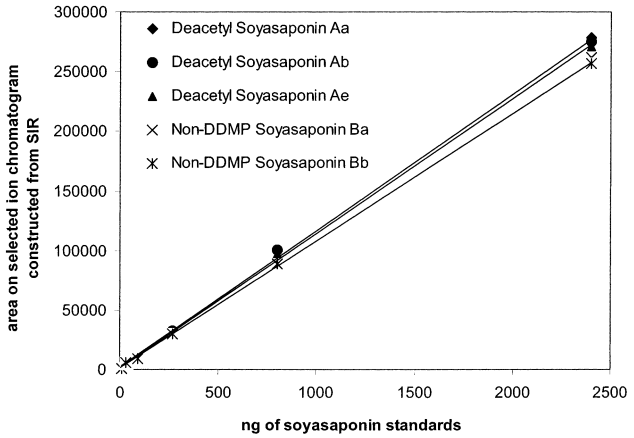


Figure 4. Standard curves of five pure soyasaponins.

and intercepts close to origin (**Figure 4**). The pure non-DDMP soyasaponin Ba and Bb with same concentration gave rise to the same response area on mass detection due to their structural similarity. Their standard curves did not show any significant difference ($p < 0.05$) in slope and intercept. This implied that the quantity of non-DDMP soyasaponin Bb could be calculated using the standard curve of non-DDMP soyasaponin Ba without a significant bias. A similar observation was made for the deacetyl soyasaponin Aa and Ab. Because the aglycones of soyasaponins were similar, the number of sugar units connected on them seemed to be the main factors influencing their MS detection response. The deacetyl soyasaponin Aa contained one more sugar unit than the deacetyl soyasaponin Ae and two more sugar units than non-DDMP soyasaponin Ba. The average response factor (area/ng) of deacetyl soyasaponin Aa calculated from the standard curve was 2.9% higher than that of deacetyl soyasaponin Ae and 7.7% higher than that of non-DDMP soyasaponin Ba, respectively. Solvent composition was another factor affecting the ionization efficiency of ES-MS detection. Soyasaponins in group A were eluted from the column within a narrow range of methanol gradient (50−60%). No difference in MS detection response was observed for pure soyasaponins within this gradient range. The same observation was made on soyasaponins in group B.

The quantification limits (defined as signal-to-noise ratio ≤ 10) were determined to be 1.74 and 1.89 ng for the deacetyl soyasaponin Aa and non-DDMP soyasaponin Ba, respectively. The pure soyasaponins (0.5 mL of 1 mg/mL solution) were spiked into defatted soy meal (1 g), then extracted and analyzed. The recovery rates of deacetyl soyasaponin Aa and non-DDMP soyasaponin Ba were 94.1% ± 4.2% and 96.9% ± 2.9% ($n = 4$), respectively.

Determination of Soyasaponins in Various Soy Samples. The high sensitivity of LC-MS allowed the quantification of all the soyasaponins in soy, including the very minor components. However, obtaining all the pure soyasaponins in enough quantities to use as external standards was not easy. Some compromise had to be made at this point. On the basis of our observation that soyasaponins with similar structures had the same MS detection response, the deacetyl soyasaponin Ac and Ad were quantified using the standard curve of deacetyl soyasaponin Aa. Likewise, the deacetyl soyasaponin Ae, Af, Ag, and Ah were quantified using the standard curve of deacetyl soyasaponin Ae. The soyasaponins in group B without a standard were quantified using the standard curve of the non-DDMP soyasaponin Ba. The minor components in group B (soyasaponin Bb′ and Bc′) contain one less sugar unit than the non-DDMP soyasaponin Ba. The quantification deviation caused by this structural difference was estimated in a range of 3−7% on the basis of the response factor difference observed for soyasaponin Aa, Ae, and Ba. Because these two minor soyasaponins contributed to less than 10% of total soyasaponins in soy, the deviation would not affect the total soyasaponin contents in soy significantly. The lower quantification deviation of soyasaponin Bb′ and Bc′ was due to the closeness of slopes (area/ng) of the standard curves of pure soyasaponins (**Figure 4**). A much higher deviation of these two components (>17%) could be expected if the slopes or response factors of soyasaponins were based on mole (area/μmol), thus it was not used.

The contents of each soyasaponins having the unified structures in four representative soy samples are listed in **Table 2**. Tsukamoto et al. (*33*) reported that the total content of DDMP soyasaponin Ba, Bb, and Bc in soy seeds of 17 varieties was 140.9−344.8 mg/100 g. These contents were lower than our data because the authors did not include the contents of non-DDMP soyasaponins. Hu et al. (*25*) reported that the total content of soyasaponin Ba, Bb, and Bc in soy seeds was 2.50−5.85 and 27.46 μmol/g in soy germ. The level and composition of soyasaponins in our samples were similar to their results. Shiraiwa et al. (*15*) analyzed soy germ in 154 varieties and concluded that the proportions of soyasaponins Aa and Ab was around 1:1 and the proportion of Ba and Bb was 1:2. Similar

results were found in our determinations. The proportion of soyasaponins in group A and group B in our soy germ was 36.0:64.0 (w/w). A different proportion [5:4 (w/w)] had been reported by Shimoyamada et al. (*14*). Soyasaponin Ba, which had been reported to exist only in soy germs (*14*, *16*), was detected in our soy cotyledons. This might be explained by the difference in varieties, or the small amount of soyasaponin Ba was not detected by the UV detection method used previously. The soy hulls contained no detectable soyasaponins. This was in agreement with the early distribution studies of soyasaponins in soy (*14*, *16*).

The existence of novel soyasaponins other than those listed in **Scheme 1** may exist according to the report of Hosny et al. (*34*). They isolated and identified a novel hexaglycosidic soyasaponin in soy molasses. The existence of this novel soyasaponin as well as the other three novel isoflavones was not confirmed in our LC-MS studies of native soy (*20*). Again, we postulated that these structures might be artifacts formed during the soy storage and processing.

In summary, this paper revealed the structural diversity of soyasaponins in soy. The diverse structures of soyasaponins were unified and quantified using LC-MS. Quantification data of soyasaponins obtained with this method would be more complete, and comparable among different soy or soy foods. This method is also applicable for soyasaponins in other legume plants.

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