Quantification of the Group B Soyasaponins by High-Performance Liquid Chromatography

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High-performance liquid chromatographic methods were developed for the isolation and quantitative determination of the group B soyasaponins, including 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)-conjugated soyasaponins αg, βg, and βa, and their non-DDMP counterparts, soyasaponins V, I, and II, respectively, with formononetin used as the internal standard. The limits of quantification for soy products were 0.11−4.86 μmol/g. The within-day and between-days assay coefficients of variation were <9.8 and < 14.3%, respectively. The group B soyasaponin concentrations in 46 soybean varieties ranged from 2.50 to 5.85 μmol/g. Soy ingredients (soybean flour, toasted soy hypocotyls, soy protein isolates, textured vegetable protein, soy protein concentrates, and Novasoy) and soy foods (commercial soy milk, tofu, and tempeh) contained the group B soyasaponins from 0.20 to 114.02 μmol/g. There was no apparent correlation between isoflavone and soyasaponin concentrations in the soy products examined.

KEYWORDS: Soyasaponin; isoflavone; soybean foods

INTRODUCTION

Saponins are triterpenoid or steroid glycosides naturally occurring in plants. Relatively high concentrations of saponins have been reported in soybeans and soy products (*1*, *2*). Group B soyasaponins were found as the primary soyasaponins present in soybeans (*Glycine max*) (*2*). Kudou et al. (*3*) reported that the 2,3-dihydro-2, 5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)-conjugated soyasaponins αg, βg, and βa were the genuine group B saponins present in soybeans and that their non-DDMP counterparts, soyasaponins V, I, and II, were products formed during heat treatment (**Figure 1**). There are two different nomenclature systems used with saponins, Kudou's (*11*), which we use here, and Shiraiwa's (*16*).

There has been much attention given to the health effects of soy consumption. Soybean saponins have been considered as major active components contributing to the cholesterol-lowering effect of soy products (*4*). Soybean saponins were reported to inhibit tumor development *in vivo* and *in vitro*, especially in colon cancer models (*5*, *6*). Soyasaponin I showed antihepatotoxic activity against carbon tetrachloride damage in primary cultured rat hepatocytes (*7*, *8*). To investigate the biological properties of soybean saponins, saponin contents and composition in soy products need to be characterized. However, there is very limited information available on the concentration and composition of soyasaponins in soybeans and soy products.

Soyasaponin quantification is a challenge due to difficulties in isolation of the authentic standards and the ability to detect triterpene saponins, which do not contain a prominent ultraviolet
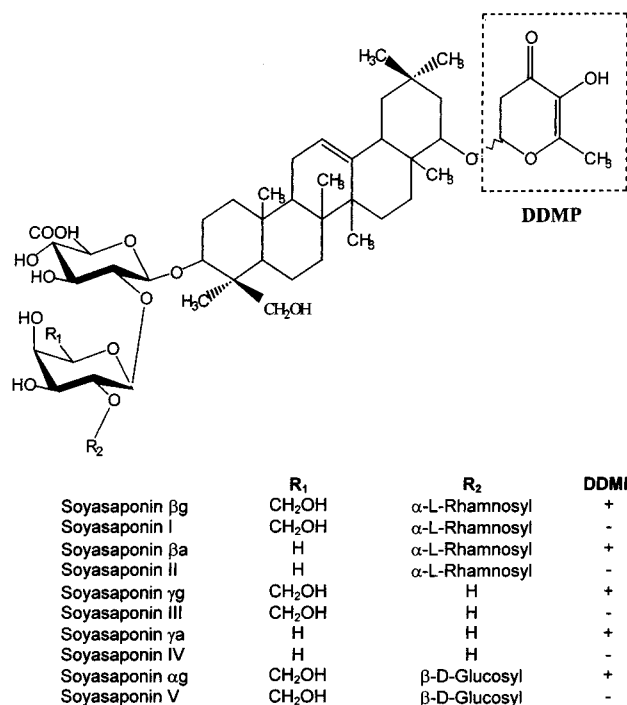
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	R_1	R_2	DDMP
Soyasaponin βg	CH_2OH	α-L-Rhamnosyl	+
Soyasaponin I	CH_2OH	α-L-Rhamnosyl	-
Soyasaponin βa	H	α-L-Rhamnosyl	+
Soyasaponin II	H	α-L-Rhamnosyl	-
Soyasaponin γg	CH_2OH	H	+
Soyasaponin III	CH_2OH	H	-
Soyasaponin γa	H	H	+
Soyasaponin IV	H	H	-
Soyasaponin αg	CH_2OH	β-D-Glucosyl	+
Soyasaponin V	CH_2OH	β-D-Glucosyl	-

Figure 1. Structures of group B soyasaponins: DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one.

chromophore. Ireland et al. (*9*) estimated soybean saponin contents by analyzing the soyasaponin aglycons with a high-performance liquid chromatography (HPLC)−mass detection

method. Oleszek et al. (*10*) quantified saponins by derivatizing the glucuronic acid group of saponins to generate an ultraviolet chromophore for detection. Tsukamoto et al. (*1*) quantified the DDMP-conjugated soyasaponins in soybeans with an HPLC method with ultraviolet (UV) absorbance detection. However, no method reported has been able to quantify the group B soyasaponins including all DDMP-conjugated forms and their non-DDMP counterparts with one chromatographic method. Thus, the aim of the present work was to isolate the group B soyasaponins as the authentic standards and to develop an HPLC method to quantify the DDMP-conjugated soyasaponins and non-DDMP soyasaponins in various soy products. The stability of DDMP-conjugated soyasaponins in methanol and in food matrices was investigated in the present study as well as the relationship between soyasaponin and isoflavone contents of soybeans and soy products. The information obtained will be valuable for the evaluation of the potential of dietary soya-saponins as health-enhancing agents. The data of soyasaponin concentrations will provide quantitative information on these potentially beneficial compounds in soy products.

MATERIALS AND METHODS

Materials. Vinton 81 variety soybean seeds (*Glycine max*) from the 1994 crop year were provided by the Department of Agronomy, Iowa State University. Soybean hypocotyls were provided by Schouten USA, Inc. Reagent grade ethanol was purchased from Chemistry Stores, Iowa State University. HPLC grade acetonitrile, methanol, and trifluoroacetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all of the mobile phases for HPLC analysis. Formononetin was purchased from Indofine Chemical Co. Inc. (Somerville, NJ). The 46 varieties of soybeans analyzed in this study were grown in Iowa in 1999 and generously provided by Dr. C. R. Hurburgh, Department of Agriculture and Biosystems Engineering, Iowa State University. Soy protein concentrates and Novasoy ingredients were donated by Archer Daniels Midland Company (ADM) (Decatur, IL). Soy protein isolates were donated by Protein Technologies International (PTI) (St. Louis, MO). The commercial soy foods, tofu (firm, Mori-nu, Torrance, CA), tempeh (Quong Hop and Co., San Francisco, CA), and soy milk (plain, White Wave, Inc., Boulder, CO), were purchased locally.

Preparation and Isolation of the Group B Soyasaponins. One hundred grams of dried, finely ground Vinton 81 soybeans or soy hypocotyl flour was extracted with 1 L of 70% aqueous ethanol with stirring for 3 h at room temperature. The ethanol extract was condensed to 100 mL with a rotary evaporator (Büchner, Brinkman, R-114, Switzerland) at <30 °C under reduced pressure. The concentrated extract was loaded on a C_{18} Lobar column (Merck Lichroprep RP-18, 40–63 μm, 310 × 25 mm i.d., EM Science Co., Gibbstown, NJ) equilibrated with 10:90 (v/v) acetonitrile/water and then fractionated with a linear gradient of aqueous acetonitrile from 30 to 100% at a flow rate of 3 mL/min over a 3-h period. The fractions containing the DDMP-conjugated saponins were collected and evaporated to dryness at <30 °C. The residue was redissolved in 50 mL of 80% aqueous methanol to obtain a crude DDMP-conjugated soyasaponin solution. Purification of individual soyasaponins was conducted on a semi-preparative HPLC system composed of a Beckman model 110A pump, a model 163 UV detector, and an RP-18, 5 mm, 250 × 10 mm i.d. YMC-ODS-AM-303 column (YMC, Inc., Wilmington, NC) using an acetonitrile/water/trifluoroacetic acid mobile phase (40:59.95:0.05) at a flow rate of 2.5 mL/min with the absorbance monitored at 292 nm. The individual saponins were collected, and acetonitrile was evaporated. The residue was then freeze-dried to obtain dry soyasaponins.

To isolate soyasaponins I, II, and V, Vinton 81 soybean flour was extracted according to the same procedure as described above except the ethanol extraction was done by refluxing at 100 °C for 3 h. An acetonitrile/water/trifluoroacetic acid (36:63.95:0.05) mobile phase was used on the semipreparative HPLC system with the absorbance monitored at 205 nm.

Chemical Identification of Purified Soyasaponins. The identity of soyasaponins was confirmed by HPLC retention times, UV absorption spectra, and electrospray ionized (ESI) mass spectra. The DDMP-conjugated soyasaponins show maximal absorbance at 292 nm (*11*). The purified DDMP-conjugated saponins were identified according to their UV spectra at 190−350 nm and retention times. The mass spectrum of each soyasaponin was determined by high-resolution ESI mass spectrometry. Electrospray ionization was performed on a Finnigan TSQ 700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) fitted with a Finnigan ESI interface. The mass spectrometer was used in the positive ion Q1MS mode, and the scan range was maintained from m/z 100 to 2000 with a scan rate of 3 s per scan. The voltage on the ESI needle tip was 5 kV in all experiments.

The molar extinction coefficients (ϵ) of the soyasaponins were determined. The purified compound was dissolved in methanol, and the standard concentrations were prepared by serial dilution in volumetric glassware to give absorbances from 0.1 to 1.0 at 205 nm. The absorbance of the solutions was measured on the spectrophotometer (model 250, Gilford Instrument Laboratories Inc.) at 205 and 292 nm for DDMP saponins. The absorbance of soyasaponins I, II, III, IV, and V was measured at 205 nm. The molar extinction coefficient ($\epsilon_{\text{methanol}}$) was calculated according to the Beer−Lambert equation.

Determination of Thermostability of the Purified DDMP-Conjugated Soyasaponins. A 1.2 mg/mL soyasaponin βg stock solution was prepared in methanol. The solution was stored in a sealed flask at −20 °C for 22 days. A 1-mL aliquot was taken from the stock solution daily and analyzed by HPLC after the solution was warmed to room temperature. The soyasaponin βg peak areas were recorded, and the change of concentration was calculated.

The thermostability of soyasaponin βg was evaluated at 30 and 65 °C over 3 h. The solutions of soyasaponin βg in methanol contained in the sealed flasks were heated in a water bath (Isotemp water bath) at 30 and 65 °C for 3 h. An aliquot was taken every 30 min, cooled to room temperature, and then analyzed by HPLC. The percentage of change in soyasaponin βg concentration was calculated. These analyses were done in duplicate.

Determination of Stability of DDMP-Conjugated Soyasaponins in a Food Matrix. Vinton 81 soybean seeds were ground into fine flour. Four grams of soy flour was placed in 20 replicate flasks. Samples were heated at 50 or 80 °C in a convection oven (Fisher Scientific, Pittsburgh, PA). For each temperature, duplicate samples were removed from the oven at 0, 15, 30, 60, and 90 min and then cooled to room temperature in a desiccator. Each sample was analyzed by HPLC for soyasaponin composition and concentrations.

Duplicate 10.0 g samples of Vinton 81 soybean seeds were hydrated in 50.0 mL of distilled water at room temperature for 20 h. One sample of hydrated soybeans including the soaking water was freeze-dried. The other portion was ground into soy milk with the addition of 100 mL of distilled water. The soaking container and the blender (Waring Products Divisions, New Hartford, CT) were carefully rinsed with water so that all residue was retained in the soy milk. The raw soy milk was frozen immediately and then freeze-dried. The saponin composition and concentrations in freeze-dried hydrated beans and raw soy milk were analyzed by HPLC.

Quantification of Soyasaponin Content in Soybeans and Soy Products by HPLC. The calibration curves of soyasaponin standards V, I, II, αg, βg, and βa were prepared as follows for HPLC assay. The stock solutions of the individual soyasaponins were made by dissolving purified standards in methanol followed by serial dilutions. The standard curves were obtained by plotting the soyasaponin concentration as a function of peak area obtained from HPLC at 205 nm.

Four grams of dried, finely ground soy samples was extracted with 100 mL of 70% aqueous ethanol with stirring for 2.5 h at room temperature. A 0.2−1-g sample was used for the concentrated soy ingredients such as Novasoy and soybean hypocotyls. The filtered extract was evaporated to dryness at <30 °C. The residue was redissolved in 80% HPLC grade aqueous methanol and made up to 10.0 mL. An aliquot of the sample extract was filtered through a 0.45 μm poly(tetrafluoroethylene) filter (Alltech Associates Inc., Deerfield, IL) and analyzed by HPLC with an RP-18, 5 μm, 4.6 i.d. × 250 mm YMC-ODS-AM-303 column (YMC, Inc., Wilmington, NC). The

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mobile phases were 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as follows: solvent B increased from 37 to 40% in 12 min, then solvent B increased to 48% in 25 min, and finally solvent B increased to 100% in 1 min and remained at 100% for 2 min. The gradient program recycled back to the initial state of 37% solvent B in 5 min. The column temperature was 30 °C. The injection volume was 50 μL. The flow rate was 1 mL/min. The UV absorbance was monitored from 190 to 350 nm.

The HPLC system consisted of two Beckman model 110B pumps, a Beckman model 420 microcontroller, a Waters 991 photodiode array detector (PDA), and an NEC Power Mate SXP Plus computer (NEC, Boxborough, MA) equipped with Waters 990 PDA data processing software (Millipore Corp., 1990).

The retention factors, peak asymmetry factors, and resolution of analyte peaks on HPLC were determined to assess the quality of the separation according to the method of Snyder et al. (*12*). Calibration curves were used to calculate the soyasaponin concentrations in soybeans and soy products. The results were expressed as micromoles of soyasaponin per gram of soy sample with adjustment for recoveries and reported on an "as is" weight basis after the moisture contents had been adjusted. The moisture contents of soy ingredients were determined according to AOAC Method 945.39. The moisture contents of soy milk and tofu were determined by using Nielsen's (*13*) freeze-drying method.

Evaluation of Precision and Accuracy. Vinton 81 soybean seeds and textured vegetable protein (U-118, minced 180, ADM) were analyzed to determine the precision of the HPLC assay. Five replications of each sample were analyzed within 24 h to evaluate the within-day variation. The samples were analyzed in duplicate for five separate days over a monthlong period to determined the between-days variation of the assay. The means, standard deviations (SD), and coefficients of variation (CV) of within-day and between-days assays were calculated for the individual soyasaponins in the two types of samples.

To evaluate the accuracy of the HPLC analysis, a recovery study of the internal and external standards was carried out using soybean flour, textured vegetable protein, and dried tofu. Formononetin (7-hydroxy-4′-methoxyisoflavone) was used as internal standard to aid in peak identification and quantification. A 500-μL aliquot of a 1.50 μmol /mL methanol–formononetin solution, as internal standard, was added into 3.0 g of dry soy sample. Soyasaponin I, as the external standard, was spiked into the soy samples. The amount spiked represented 100% of the expected amount of saponin I in the original sample matrix. The samples were mixed thoroughly and stored at room temperature until the methanol had evaporated. Extraction and analysis were performed as described above. The percentage of recovery of spiked soyasaponin I was calculated.

Quantification of Isoflavone Content in Soybeans and Soy Products. The isoflavone concentrations of all the soybeans and soy products were analyzed in duplicate according to the method of Murphy (*14*).

Statistical Analysis. The statistical analysis of the data, including average, SD, and CV, were calculated for the accuracy and precision analyses. Linear regression was used to obtain the calibration curves for the soyasaponin standards. The data of stability of soyasaponins in methanol and food matrix and the comparison of percentage of soyasaponin I recovery among the three soy products were evaluated by ANOVA using the SAS system (version 6, SAS Institute Inc., Cary, NC). Comparison of soyasaponin concentrations in Round-up Ready soybeans and conventional soybeans was done using Student's *t* test.

RESULTS AND DISCUSSION

Isolation of Group B Soyasaponins. The DDMP-conjugated group B soyasaponins αg, βg, and βa and their non-DDMP counterparts, soyasaponins, V, II, and I, were isolated from soybeans and soybean hypocotyls. The yields of soyasaponins αg, V, βg, I, βa, and II, defined as the amount of individual soyasaponins isolated from 100 g of starting materials, were 29, 17, 63, 49, 28, and 22 mg, respectively. Compared with the original amount of soyasaponins in the starting materials, we
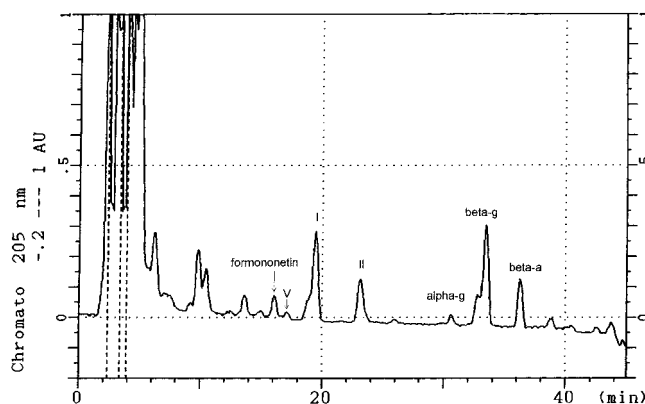

Figure 2. HPLC chromatogram of soyasaponins in textured vegetable protein with UV absorbance spectra at 205 nm.

recovered 5.7% of soyasaponin αg, 5.5% of soyasaponin V, 22.8% of soyasaponin βg, 31.2% of soyasaponin I, 40.2% of soyasaponin βa, and 52.7% of soyasaponin II; these percentages are reasonable considering the thermal lability of the DDMP-conjugated soyasaponins (*15*).

The isolated soyasaponins were identified on reversed-phase HPLC with the absorbance monitored over 190–350 nm. The DDMP-conjugated saponins revealed the maximal absorbance at 292 nm from the DDMP moiety (**Figure 2**) as reported by Kudou (*3*). Soyasaponins I, II, and V showed nonspecific UV spectra with the maximum absorbance at 205 nm (**Figure 2**).

To detect both DDMP-conjugated soyasaponins and non-DDMP soyasaponins on HPLC, we chose 205 nm as the detection wavelength. Because the intensity of the detection signal at a wavelength is proportional to the molar extinction coefficient, ϵ, we measured the molar extinction coefficients ($\epsilon_{methanol}$) of isolated soyasaponins at 205 and 292 nm for DDPM soyasaponins and at 205 nm for non-DDMP soyasaponins. The ϵ values of soyasaponins were as follows: αg $\epsilon_{methanol}^{292} = 4838$ M^{-1} cm^{-1}, $\epsilon_{methanol}^{205} = 5946$ M^{-1} cm^{-1}; βg $\epsilon_{methanol}^{292} = 4504$ M^{-1} cm^{-1}, $\epsilon_{methanol}^{205} = 6267$ M^{-1} cm^{-1}; βa $\epsilon_{methanol}^{292} = 4795$ M^{-1} cm^{-1}, $\epsilon_{methanol}^{205} = 5645$ M^{-1} cm^{-1}; V $\epsilon_{methanol}^{205} = 4971$ M^{-1} cm^{-1}; I $\epsilon_{methanol}^{205} = 5278$ M^{-1} cm^{-1}; II $\epsilon_{methanol}^{205} = 5078$ M^{-1} cm^{-1}. Kudou et al. (*15*) reported the extinction coefficient $\epsilon_{methanol}^{292}$ of βg as 3700 M^{-1} cm^{-1}, which is close to the values we obtained. The relatively small molar extinction coefficients of these soyasaponins as well as a low wavelength for detection resulted in low detection sensitivity in the HPLC assay.

The identity of isolated soyasaponins was confirmed by the high-resolution electrospray ionized (ESI) mass spectra. ESI-MS (positive mode) of DDMP-conjugated soyasaponins αg ($C_{54}H_{84}O_{22}$, MW 1085.2), βg ($C_{54}H_{84}O_{21}$, MW 1069.2), and βa ($C_{53}H_{82}O_{20}$, MW 1039.2) showed molecular ions at m/z 1086 [M + H]$^+$, 1069 [M]$^+$, and 1039 [M]$^+$, respectively. The fragment ions resulting from loss of the DDMP group were observed in the mass spectra of these soyasaponins, as m/z 959 [M − DDMP]$^+$ for αg, 943 [M − DDMP]$^+$ for βg, and 913 [M − DDMP]$^+$ for βa. The ESI mass spectra of soyasaponins V ($C_{48}H_{78}O_{19}$, MW 959.1), I ($C_{48}H_{79}O_{18}$, MW 944.1), and II ($C_{47}H_{76}O_{17}$, MW 913.1) were characterized by the molecular ions at m/z 959 [M]$^+$, m/z 944 [M + H]$^+$, and m/z 913 [M]$^+$, respectively. The sodium adducts of the molecular ions and the fragment ions were observed in the spectra as well. These data were consistent with those reported in the literature (*11*, *16*, *17*).

Thermostability of Purified DDMP-Conjugated Soyasaponin. Purified soyasaponin βg was examined as a representa-

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tive compound to evaluate the stability of the isolated standards at different temperatures. Soyasaponin βg was relatively stable in methanol at -20 °C. There was not a significant change in βg concentration over 15 days. No significant change in βg concentration of the solution was observed over 3 h at 30 °C. However, the βg concentration significantly decreased when the solution was heated at 65 °C, and the concentration of soya-saponin I increased proportionally (P < 0.05). The results on the stability of DDMP-conjugated soyasaponins were not consistent in the literature. Kudou (*15*) observed that soyasaponins αg and βg converted into soyasaponins V and I, respectively, when the alcoholic extract of soy hypocotyls was heated at 80 °C for 5 h. Massiot (*18*) reported that soyasaponin βg converted into soyasaponin I in acidic or basic solutions or standing in alcoholic solution at ambient temperature. Okubo (*19*) found that the isolated soyasaponin βg was stable at acidic pH but rapidly degraded into soyasaponin I at basic pH solution or in the presence of FeCl$_3$. These results indicate that the temperature and pH should be carefully controlled during the sample preparation for soyasaponin isolation or analysis. The results from our study suggested the purified DDMP-conjugated saponins could be stored at -20 °C without significant change for 15 days, possibly longer. The samples containing DDMP-conjugated saponins can be processed in the alcoholic solution at room temperature for 3 h without significant loss of DDMP moiety.

Quantitative Analysis of Group B Soyasaponins by HPLC. Chromatography revealed well-resolved individual group B soyasaponins. **Figure 2** shows a chromatogram of these soya-saponins in textured vegetable protein. The retention times of soyasaponins V, I, II, αg, βg, βa, γg, and γa were 17.2 ± 0.9, 20.8 ± 1.2, 23.7 ± 0.8, 31.5 ± 1.1, 33.8 ± 1.2, 36.4 ± 0.8, 37.9 ± 0.9, and 39.4 ± 0.7 min, respectively. The retention factors (k) were 6.5 for soyasaponin V and 18.9 for soyasaponin γa. With k values of 2 < k < 20, soyasaponins were well dispersed, and analysis time was not excessive. The peak asymmetry factors (A) for the soyasaponins ranged from 0.6 to 1.2. Although the ideal peak shape is symmetrical with A of ~1, some of the peaks tailed or fronted slightly, indicating that more than one retention mechanism might be involved in chromatographic separation (*20*). The resolution of the two least separated soyasaponins, V and I, was >3.0, which satisfied the recommended separation quality with the minimum resolution at 1.7−2.0 (*12*).

Kudou (*11*) reported that soyasaponins γg and γa were minor saponin constituents in soybeans. Our results confirmed their findings. Therefore, in our study, only soyasaponins V, I, II, αg, βg, and βa were isolated for quantitative analysis. The linear detection range of the standards was 0.04−1.70 μmol/mL, which was carefully chosen to cover the saponin concentration of most of the soy products. The detection range of this analysis in soy products was 0.11−4.86 μmol/g of sample. Three to five grams of regular soy food samples was required to detect the analytes on the HPLC profile precisely. Smaller amounts could be used for the concentrated soy ingredients, such as Novasoy and soy hypocotyls.

A reference peak is useful to aid in peak identification during chromatography, but few studies have used them in saponin quantification. α-Hederin was used as reference compound and internal standard in an HPLC method for soyasaponins βg and I in lupine seeds (*21*). In that method, α-hederin eluted later than the latest analyte, soyasaponin βg, in the chromatogram and resulted in prolonged running time. Formononetin was chosen as the reference compound in our method. Formononetin

Table 1. Precision of HPLC Analysis of Soyasaponins in Soy Products

product	soyasaponin	content (μmol/g; mean ± SD)	CV (%) within day[a]	CV (%) between days[b]
soybean flour[c]	αg	0.14 ± 0.02	7.1	14.3
	V	0		
	βg	2.23 ± 0.16	3.6	7.3
	I	0		
	βa	0.48 ± 0.04	4.8	8.3
	II	0		
textured vegetable protein[d]	αg	0		
	V	0		
	βg	1.45 ± 0.16	6.9	11.1
	I	2.03 ± 0.14	4.4	6.8
	βa	0.42 ± 0.04	9.8	10.2
	II	1.03 ± 0.13	6.7	12.2

[a] The CV for within-day assay were calculated from five replications of each sample. [b] The CV for between-days assay were calculated from two replications of each sample over 5 days. [c] Vinton 81, 1994 crop. [d] Archer Daniels Midland Co.

Table 2. Recovery of Soyasaponin I and Formononetin in Soy Products

	external standard (saponin I) recovery[a] (%, mean ± SD)	CV (%)	internal standard (formononetin) recovery[a] (%, mean ± SD)	CV (%)
soybean flour[b]	120.9 ± 3.63[a]	13.0	98.4 ± 2.54[c]	2.6
textured vegetable protein[c]	103.2 ± 9.71[b]	9.4	99.5 ± 2.73[c]	2.7
tofu[d]	93.9 ± 8.99[b]	9.6	97.5 ± 1.86[c]	1.9

[a] Values followed by the same letter in the same column are not significantly different (P > 0.05). [b] Vinton 81, 1994 crop. [c] Archer Daniels Midland Co. [d] Dried powder, made at Iowa State University.

is stable, not present in soybeans or soy products, and commercially available. The retention time of formononetin was 16.2 ± 0.4 min under our HPLC conditions. The peak did not overlap with any soyasaponins and eluted earlier than soya-saponins. Using formononetin as the reference, soyasaponins can be easily identified according to their retention times relative to the retention time of formononetin.

Formononetin was used as the internal standard in our soyasaponin assay to evaluate the losses of the analytes during analysis. A linear relationship was obtained with a regression equation of $Y = 0.0752x - 0.013$ ($R = 0.9966$) for a variety of soy products including soybean flour, dried tofu, dried soy milk, tempeh, soy protein isolates, and textured vegetable protein. This linearity indicates that the internal standard interacted with the different sample matrices in a manner similar to soyasaponins and that the fraction of internal standard lost during extraction was proportional to the fraction of soyasaponins lost.

The analytical precision was determined by repeated measurements of soyasaponin concentrations in Vinton 81 soybeans and textured vegetable protein (**Table 1**). The within-day variation of the assay was <9.8%, and the between-days variation of the assay was <14.3%. This result indicates good precision of the analytical procedure for the soy samples. The external recovery study using soyasaponin I and formononetin was carried out to evaluate the accuracy. Soyasaponin I was used as the external standard because it is stable compared with the DDMP-conjugated soyasaponins. The data in **Table 2** show that the recovery of soyasaponin I was >93% in soybean flour, dry tofu, and textured vegetable protein. The recovery of formo-

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Table 3. Group B Soyasaponin Contents in Soybeans[a]

	soyasaponin concn (μmol/g)						
variety	V	I	II	αg	βg	βa	total
Prairie Brand 2630 RR	0	0.34	0.10	0.09	1.69	0.28	2.50
AgriPro 3083 R	0	0.62	0.26	0.06	1.24	0.35	2.53
FS2786RT RR	0	0.57	0.25	0.06	1.41	0.32	2.61
Producers 237 RR	0	0.35	0.19	0.13	1.75	0.31	2.73
Garst 261 RR	0	0.34	0.17	0.09	1.80	0.34	2.74
Trelay 227STS	0	0.58	0.30	0.09	1.41	0.41	2.78
Fontanelle F8933 RR	0	0.44	0.13	0.14	1.85	0.29	2.85
Stine 2698-4A RR	0	0.33	0.12	0.12	2.04	0.36	2.97
Dynagro 3281W	0	0.05	0.07	0.17	2.17	0.57	3.03
Latham 656 RR	0	0.42	0.21	0.10	1.96	0.37	3.06
Midwest 1985	0	0.58	0.14	0.12	1.94	0.33	3.11
Thompson seeds Ex8704	0	0.27	0.10	0.18	2.23	0.37	3.14
Mycogen 5242 RR	0	0.26	0.15	0.17	2.25	0.38	3.21
Pioneer 92B05 RR	0	0.08	0.04	0.13	2.47	0.54	3.26
Dekalb CX 285 RR	0	0.34	0.04	0.18	2.58	0.47	3.60
Excel8278 RR	0	0.17	0.09	0.19	2.54	0.64	3.63
Trelay268	0	0.08	0.03	0.12	2.39	0.55	3.64
AgriPro 2329	0	0.30	0.28	0.17	2.36	0.57	3.69
Garst 198 RR	0	0.30	0.13	0.20	2.68	0.44	3.76
Latham696 RR	0	0.22	0.04	0.21	2.91	0.43	3.82
Oekalb cx285 RR	0	0.09	0.06	0.18	2.80	0.70	3.83
Gold county Kardi	0	0.11	0.09	0.20	2.92	0.53	3.86
Jacobsen Hybrid 774	0	0.08	0.06	0.19	2.81	0.77	3.91
Trelay 268	0	0.16	0.00	0.20	3.05	0.53	3.94
AgriPro 2329	0	0.09	0.03	0.17	2.53	0.62	4.17
Latham L437 RR	0	0.08	0.00	0.22	3.35	0.54	4.20
Excel 8261 RR	0	0.29	0.29	0.21	2.75	0.71	4.26
MWSeed Genetic 2210 RR	0	0.29	0.10	0.23	3.03	0.73	4.38
LG6288	0	0.11	0.03	0.25	3.37	0.73	4.48
Croplan RT 2856 RR	0	0.20	0.00	0.21	3.54	0.57	4.52
AgriPro 2329	0	0.32	0.13	0.22	3.20	0.74	4.61
Mycogen 5261	0	0.09	0.12	0.29	3.33	0.84	4.67
NC+ 2A39 RR	0	0.09	0.07	0.29	3.41	0.82	4.68
LG Seeds 6245	0	0.12	0.03	0.27	3.60	0.75	4.76
Wilson 2630	0	0.12	0.10	0.31	3.41	0.87	4.80
HY-Vigor 2375	0	0.37	0.07	0.29	3.45	0.65	4.82
Garst D330	0	0.10	0.05	0.31	3.59	0.78	4.83
NK-X99223	0	0.10	0.04	0.31	3.66	0.76	4.87
Stine 2788	0	0.14	0.04	0.24	3.74	0.77	4.92
K2727A	0	0.29	0.08	0.32	3.52	0.73	4.94
Mycogen 5261	0	0.32	0.05	0.35	3.58	0.69	4.99
Jacobsen Hybrid J-7SO	0	0.31	0.07	0.26	3.68	0.72	5.04
Novartis S21A1	0	0.08	0.04	0.35	4.01	0.76	5.24
Wilson 2832 RR	0	0.19	0.06	0.32	4.19	0.87	5.63
Wilson 2630	0	0.14	0.05	0.32	4.31	0.87	5.69
AgriPro 2761 SCN	0	0.14	0.04	0.38	4.39	0.91	5.85
mean $\pm$ SD	0	0.24 $\pm$ 0.1	0.10 $\pm$ 0.1	0.21 $\pm$ 0.1	2.87 $\pm$ 0.8	0.60 $\pm$ 0.2	4.04 $\pm$ 0.9

[a] All of the soybeans were analyzed in duplicate. The data are reported on an "as is" weight basis and are corrected for recovery. RR = Round-up Ready varieties.

nonetin was >98% in the soy samples. The recovery of soyasaponin I in the raw soybeans was significantly higher than the recoveries in tofu and textured vegetable protein and had a relatively larger variation. This difference was probably because there was virtually no soyasaponin I in raw soybean flour (*11*). Usually the amount of analyte naturally present in the sample should be high enough to provide a reasonable signal to noise ratio to ensure a precise measurement (*12*). The recovered soyasaponin I might partially come from decomposition of soyasaponin βg in the soybean flour during the sample preparation. Overall, >93% of soyasaponin I could be recovered from the soy samples with our analytical method, indicating reasonable accuracy.

Soyasaponin Contents in Soybeans and Soy Products. Forty-six varieties of soybeans, grown in Iowa in 1999, were measured for the group B soyasaponin concentrations (**Table 3**). The total group B soyasaponins in 46 varieties of soybeans were on average 4.04 $\pm$ 0.91 μmol/g with a range of 2.50–5.85 μmol/g. The DDMP-conjugated soyasaponins represented 85–94% of the group B soyasaponins in soybeans. The minor amounts of non-DDMP soyasaponins observed in the raw soybeans may be the result of the original DDMP soyasaponin decomposition during sample preparation. The total soyasaponin contents varied among different soybean varieties. Tsukamoto et al. (*1*) reported the DDMP-conjugated soyasaponin contents in 13 Japanese soybean varieties. Their data showed the total concentration of soyasaponins αg, βg, and βa was in a range of 1.39–3.25 μmol/g. The level and composition of soyasaponins in our Iowa soybeans were similar to their results.

Twenty of these soybean varieties were genetically modified to resist the herbicide Round-up, known as "Round-up Ready" soybeans. The total and individual group B soyasaponin concentrations in these varieties were significantly lower than in the conventional varieties ($P < 0.05$). It is possible that these soybeans produced less soyasaponins because they experienced less environmental stress from other plants (*22*). Tsukamoto et al. (*23*) showed that soyasaponin concentrations in the cultivated soybeans were 2-fold lower than in the wild soybeans.

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Table 4. Soyasaponin Content and Composition in Commercial Soy Products

	moisture (%)	group B soyasaponin content[a] (μmol/g)						
		V	I	II	αg	βg	βa	total
soybean flour[b]	6.43	0.00	0.28	0.21	0.17	2.19	0.47	3.31
tofu[c]	86.87	0.00	0.31	0.13	0.01	0.11	0.03	0.59
tempeh[d]	59.65[e]	0.00	0.76	0.39	0.01	0.28	0.09	1.53
soy milk[f]	90.75	0.00	0.22	0.12	0.00	0.09	0.04	0.47
acid-washed soy concentrates[g]	5.80[e]	0.00	2.41	1.05	0.19	4.90	0.86	9.41
ethanol-washed soy concentrates[g]	5.80[e]	0.00	0.08	0.12	0.00	0.00	0.00	0.20
isolated soy protein 500E[h]	4.87	0.87	5.73	2.39	0.10	1.20	0.31	10.60
isolated soy protein Supro 670[h]	4.56	0.00	5.59	2.50	0.07	1.01	0.33	9.51
textured vegetable protein[g]	5.66	0.00	1.89	0.87	0.11	1.26	0.38	4.51
soy hypocotyl[i]	3.55	4.41	5.80	0.00	4.71	12.53	0.00	27.46
Novasoy[g]	3.75	0.00	77.55	36.48	0.00	0.00	0.00	114.02

[a] All samples were analyzed in duplicate. Saponin contents are reported on an "as is" weight basis and are corrected according to the internal standard. [b] Vinton 81, 1994 crop. [c] Mori-nu, firm. [d] Quong Hop and Co. [e] Obtained from USDA food composition database. [f] White Wave, Inc. [g] Archer Daniels Midland Co. [h] Protein Technologies International. [i] Schouten USA Inc., toasted.

Table 5. Stability of DDMP-Conjugated Soyasaponins in Food Matrices

treatment		soyasaponin concn[a] (μmol/g)						
		V	I	II	αg	βg	βa	total[b]
soybean flour								
unheated		nd[c]	0.40 ± 0.13	0.19 ± 0.06	0.14 ± 0.02	2.01 ± 0.11	0.49 ± 0.03	3.13 ± 0.06^a
50 °C	15 min	nd	0.33 ± 0.03	0.16 ± 0.01	0.15 ± 0.01	2.03 ± 0.02	0.53 ± 0.01	3.20 ± 0.03^a
	30 min	nd	0.33 ± 0.02	0.17 ± 0.05	0.14 ± 0.01	2.02 ± 0.06	0.51 ± 0.02	3.19 ± 0.09^a
	60 min	nd	0.35 ± 0.08	0.17 ± 0.05	0.15 ± 0.02	2.01 ± 0.05	0.51 ± 0.01	3.18 ± 0.07^a
	90 min	nd	0.39 ± 0.05	0.18 ± 0.02	0.15 ± 0.01	1.97 ± 0.10	0.50 ± 0.03	3.19 ± 0.08^a
80 °C	15 min	nd	0.33 ± 0.03	0.20 ± 0.05	0.16 ± 0.01	2.00 ± 0.06	0.55 ± 0.01	3.07 ± 0.10^a
	30 min	nd	0.40 ± 0.06	0.17 ± 0.02	0.15 ± 0.02	1.99 ± 0.05	0.51 ± 0.01	3.04 ± 0.04^a
	60 min	nd	0.38 ± 0.02	0.18 ± 0.03	0.16 ± 0.01	1.96 ± 0.04	0.55 ± 0.01	3.23 ± 0.07^a
	90 min	nd	0.35 ± 0.03	0.16 ± 0.01	0.15 ± 0.01	2.01 ± 0.01	0.54 ± 0.01	3.20 ± 0.03^a
soaked soybeans[d]		nd	0.25 ± 0.05	0.07 ± 0.01	0.11 ± 0.03	1.93 ± 0.03	0.62 ± 0.01	2.99 ± 0.09^a
raw soy milk[d]		0.20 ± 0.01	1.33 ± 0.03	0.71 ± 0.03	0.01 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	2.28 ± 0.06^b

[a] Values are the mean of two duplicate determinations ± standard deviation. [b] Values followed by the same letter in the same column are not significantly different (P >0.05). [c] nd = not detected. [d] Concentrations were calculated as μmol/g of original seeds used.

The soyasaponin contents of several commercial soy products were examined (**Table 4**). These products included the soy ingredients soybean flour, soy protein isolates, soy protein concentrates, textured vegetable protein, Novasoy, and toasted soy hypocotyls and the soy foods soy milk, tofu, and tempeh. The soyasaponin concentrations were adjusted for recoveries and reported on an "as is" weight basis. The total group B soyasaponin concentrations in the soy ingredients ranged from 0.20 to 114.02 μmol/g. The soyasaponin concentrations in the soy foods ranged from 0.47 to 1.53 μmol/g. The DDMP-conjugated soyasaponins were the primary group B soyasaponins detected in the raw soybean flour, whereas the non-DDMP soyasaponins were the major forms detected in the processed soy products. High concentrations of soyasaponins αg and βg and their non-DDMP products soyasaponins V and I were detected in the toasted soy hypocotyls, as we expected. The ratio of soyasaponin αg to βg was approximately 1:2 in soy hypocotyls and in good agreement with Shiraiwa's data (16). The concentrations of soyasaponins in the soy protein isolates were significantly higher than in the raw soybean flour. The group B soyasaponins were undetectable in ethanol-washed soy protein concentrates but were high in the acid-washed soy protein concentrates, the soyasaponin concentrations of which were similar to those of soy protein isolates. Soy protein isolate is usually prepared by the isoelectric precipitation of soy proteins from aqueous extract of defatted soy flakes. The protein concentrate is usually made by washing defatted soybean flakes with aqueous alcohol or water to remove the soluble carbohy-

drates. The alcohol wash would result in the loss of soyasaponins in soy protein concentrates. Novasoy is produced by drum-drying the alcoholic extracts from soy protein concentrate production and is a very concentrated source of non-DDMP soyasaponins. Soy milk, tempeh, and tofu appeared to be low in soyasaponin content compared to the raw soybeans on an "as is" weight basis. However, the soyasaponin concentrations on a dry weight basis in these soy foods were 3.79 μmol/g in tempeh, 4.49 μmol/g in tofu, and 5.06 μmol/g in soy milk, which were close to or greater than those in the raw soybean flour. Although direct comparisons of saponin concentrations might not be proper among the soy products reported here because the soybean varieties used to produce each product were unknown, it was evident that the composition and concentration of soyasaponins were different among the soy products depending on the processing conditions.

Stability of DDMP-Conjugated Soyasaponins in Food Matrix. The effect of heating on the soyasaponin content and composition of soybean flour is shown in **Table 5**. The DDMP-conjugated soyasaponins were the primary saponins detected in all of the unheated and heated soy flour. No significant differences in soyasaponin composition and concentration were found between heated flour and unheated flour at either 50 or 80 °C regardless of the heating time. The DDMP-conjugated soyasaponins in the solid soy matrix was stable under the heating conditions described above.

To understand the stability of DDMP-conjugated soyasaponins in the liquid matrix, we measured soyasaponin concen-

tration and composition in soaked soybean seeds and raw soy milk made from soaked beans. After soaking for 20 h, the soybean seeds became imbibed. The composition and concentration of soyasaponins in soaked seeds were significantly different from those in the raw soy milk, although the soy milk was frozen immediately after grinding (**Table 5**). In soaked seeds, the soyasaponin profile and concentration were similar to those of the original dry seeds. However, in the raw soy milk made from the soaked seeds, the total amount of soyasaponins was only 76% of total soyasaponins of the soaked seeds used and the non-DDMP saponins were major saponins present even though no external heating occurred. These results were unexpected. The data indicate that the DDMP-conjugated soyasaponins converted into non-DDMP saponins during the grinding process in making soy milk.

There are very few studies on the effect of heating and food processing on DDMP-conjugated soyasaponins in food matrices. Ruiz et al. (*24*, *25*) found neither soaking nor germination changed saponin content or composition in chickpeas and lentils regardless of the pH of the soaking solution, but boiling resulted in the partial conversion of soyasaponin βg into soyasaponin I in these soaked peas and lentils. Our data suggested that DDMP-conjugated soyasaponins are stable in the solid matrix but may convert into non-DDMP saponins by simple mechanical disruption of the soaked soybean seeds in water solution. One possible explanation for the rapid conversion of soyasaponins in this case may be that the soaked beans as part of germination, produce certain enzymes that are released during cellular disruption of soy milk production and hydrolyze DDMP off the native soyasaponins. Further study will be needed to investigate the mechanisms of this phenomenon.

Previous literature provided very limited information on the soyasaponin contents in soy products. Ireland et al. (*2*) determined saponin contents in soy protein isolate, soy protein concentrate, and soy milk by measuring the concentration of soyasapogenols, the aglycons of saponins. They reported that the soyasaponin levels were undetectable in soy protein concentrate and were 0.026 g/100 g in soy milk and 0.76 g/100 g in soy protein isolate on an "as is" weight basis. The soyasaponin concentrations from our study were expected to be lower because only the group B soyasaponins were measured, whereas Ireland et al. measured the total group A and B saponins. However, the saponin levels found in our study were 1.04 g/100 g for soy protein isolate and 0.048 g/100 g for soy milk, both of which were higher than Ireland's data. However, the variation from different soy sources used in the two studies must be considered. Kitagawa et al. (*26*) reported that the total saponin contents were 0.3 g/100 g in tofu on a dry weight basis. We observed similar soyasaponin levels in the commercial tofu analyzed.

Isoflavones are another important group of secondary metabolites found in soybeans. Many studies have attempted to correlate the consumption of soy isoflavones with the inhibition of bone resorption in postmenopausal women (*27*), the prevention of hormone-related cancers such as breast and prostate cancers (*28*), and the ability to lower cholesterol concentration and decrease the risk of cardiovascular diseases (*29*). In the present study, the isoflavone concentrations in these soy products were compared to the soyasaponin concentrations to determine whether there was any correlation. The ratio of total isoflavone to total soyasaponins in the 46 varieties of soybeans ranged from 0.9 to 2.9 on a mole basis. There was no statistically significant correlation between isoflavone and soyasaponin concentrations among the soybean varieties we examined ($R = 0.1285$, $P >$
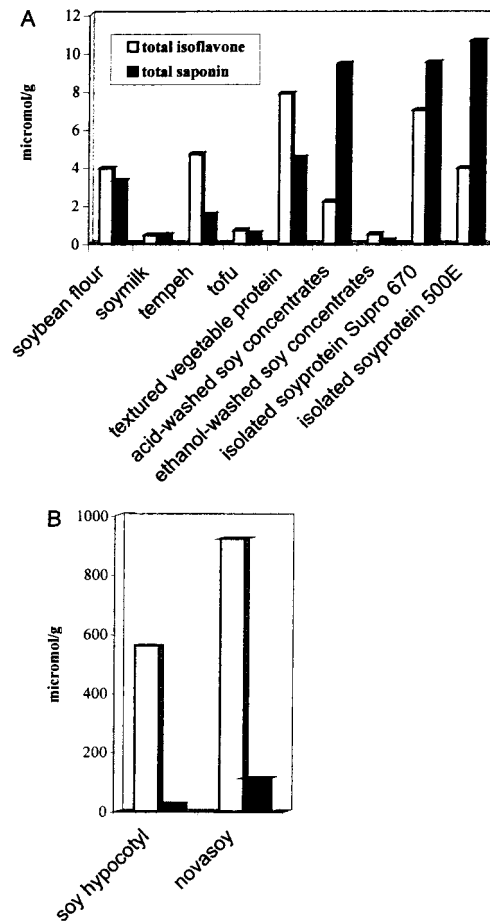

Figure 3. Total isoflavone and soyasaponin concentrations in commercial soy products: (A) soy foods; (B) soy supplements.

0.05). The ratio of total isoflavone to soyasaponin in the commercial soy products ranged from 0.2 to 20.4 on a mole basis in the soy products (**Figure 3**). The production process for each type of soy product apparently did affect the ratio of isoflavones to soyasaponins, which indicates the changes of isoflavone contents and soyasaponin contents are independent from each other. The isoflavone concentration was slightly higher than soyasaponin concentrations in raw soybean seeds. Higher isoflavone to soyasaponin ratios were observed for the tofu and textured vegetable protein. The isoflavone concentration was similar to saponin concentration in the soy milk. In contrast, higher concentrations of soyasaponins relative to isoflavones were detected in soy protein isolates and acid-washed soy protein concentrates. The concentrations of both isoflavones and soyasaponins in ethanol-washed soy protein concentrates were very low compared to the other soy ingredients. This difference among soy ingredients may be because soyasaponins are more hydrophobic and, therefore, might bind soy protein more tightly in these high-protein products. In contrast, the less hydrophobic isoflavones might be leached out during the acidic or alkaline washing process. Soy protein isolates have been used in a number of studies to examine the cholesterol-lowering effects of soy protein. Significant amounts of soyasaponins are present in these protein products; therefore, the conclusions from previous studies might need to be re-evaluated given the potential hypocholesterolemic activity of soyasaponins (*30*).

In summary, our soyasaponin quantification method is rapid, practical, and accurate in analyzing the three major group B DDMP-conjugated soyasaponins and their non-DDMP counterparts in a variety of soybean products. The soyasaponin

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concentration and composition varied in soybeans, soy foods, and soy ingredients an may depend on the variety of soybeans and the processing conditions used to produce a particular product. A database of soyasaponin contents in soy products will be valuable for evaluating the biological activities of these compounds and clarifying the health-promoting effects of soy products.

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