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Use of a Simplified HPLC–UV Analysis for Soyasaponin B Determination: Study of Saponin and Isoflavone Variability in Soybean Cultivars and Soy-Based Health Food Products

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Soyasaponins are phytochemicals of major interest for health. Their identification and quantification remain difficult owing to the large number of structural isomers in soybeans and the lack of stable standards. In this study, a rapid method using high performance liquid chromatography (HPLC) using a UV detector (205 nm) was developed to identify and quantify soyasaponins belonging to group B and compare them with isoflavones in different soy materials. 2,3-Dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)-conjugated soyasaponins were determined using external calibration or a molecular mass ratio after alkaline hydrolysis to cleave their DDMP moieties. The detection limit of soyasaponin I, used as a reference molecule to simplify the analysis, was 0.065 μmol/g. Soyasaponin contents in seven soybean varieties ranged from 13.20 to 42.40 μmol/g in the germ and from 2.76 to 6.43 μmol/g in the cotyledons. The within-day and between-days variation coefficients did not exceed 7.9 and 9.0%, respectively, for the major soyasaponins. Soyasaponin B quantification in different soy-based health supplements was reported along with measurements of their isoflavone content to provide information on the variability of these bioactive compounds among different types of soy food materials.

KEYWORDS: Soybean; soyasaponin; HPLC/UV

INTRODUCTION

Soybeans and soy-based food products are believed to have health-promoting properties. This was formally highlighted by the FDA Health Claim linking soy protein consumption with the prevention of cardiovascular disease (*1*). Thus, dietary supplements and soy foods have gained a considerable place on the market in recent years, with numerous claims linking their health-promoting effects to their isoflavone contents. Ongoing research has shown that isoflavones might be effective in reducing the risk of coronary heart diseases and several cancers (*2*−*5*). However, recent studies showed that these effects could be synergically working or mediated by other compounds in soy food products (*6*−*12*). Indeed, soy fractions also contain complex mixtures of potentially bioactive phytochemicals, such as lignans (*7*) or soyasaponins (*13*), which are less well-studied than isoflavones.

Soyasaponins may also present health protective effects including hypochosterolemic (*5*), anticarcinogenic (*14*, *15*), hepatoprotective (*16*), and antiviral activities (*17*). They are triterpenoid glycosides naturally occurring in native soybean seeds. According to their structure, soyasaponins are classified in two major groups, A and B (**Figure 1**). Soyasaponins A are supposed to be responsible for the undesirable bitter and astringent taste in soy food products (*18*). Group B saponins are monodesmosidic, having just one glycosylation site, and might be responsible for the health-contributing activities of soyasaponins (*13*, *19*). Kudou et al. (*20*) reported that the genuine soyasaponins (i.e., αg, βg, βa, γg, and γa) are the conjugations of 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) and predominate their corresponding non-DDMP structures (i.e., V, I, II, III, and IV, respectively), formed as artifacts during heat treatment (**Figure 1**). Dietary supplements of soybean and soy food vary widely in active compounds owing to variations in raw materials themselves used for their processing. They are likely obtained from different sources such as whole seed, soy meal, or dehulling byproducts containing up to 99% of germs (*9*, *21*−*23*). Depending on the variety, growing location, cultivation year, and degree of maturity, soybean seeds contain about 0.2% dry weight isoflavones (*24*, *25*) and from 0.6% to as much as 6.2% dry weight saponins (*19*, *21*, *26*). Moreover, the seed parts present a large variability in their content and composition: Among soyasaponins B, soyasaponins V and αg together are found exclusively in the germ, while soyasaponins II and βa are mainly located in the cotyledons (*19*, *21*). Soyasaponin I and its DDMP corresponding form βg

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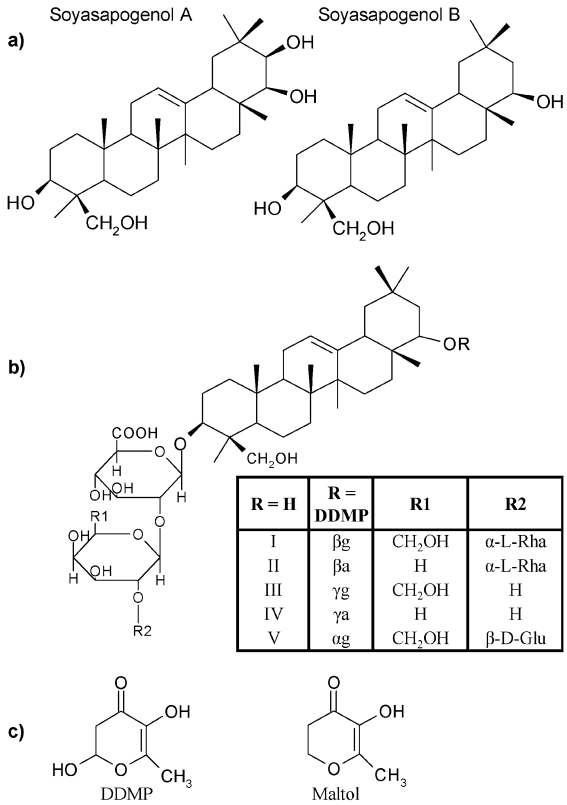

R = H	R = DDMP	R1	R2
I	βg	CH_2OH	α-L-Rha
II	βa	H	α-L-Rha
III	γg	CH_2OH	H
IV	γa	H	H
V	αg	CH_2OH	β-D-Glu

Figure 1. Structures of (a) hydrophobic aglycone precursors of A and B soyasaponins; (b) DDMP- and non-DDMP soyasaponins B; and (c) structure of the DDMP group and of its corresponding maltol hydrolyzed form.

are detected in both parts. The ratio of soyasaponins B in the axis/cotyledon was found to be 9:1 (*27*).

Many analytical methods have been proposed for identification and quantification of soyasaponins. The major difficulties in their determination by high performance liquid chromatography (HPLC) are the lack of an efficient detection method appropriate for routine analysis and the laborious preparation of pure analytical standards owing to a large number of similar saponin glycosides. The early methods of quantification use UV detection (*18*, *20*, *28*). The maximum absorption wavelength of non-DDMP soyasaponins, which do not contain a prominent ultraviolet chromophore, is around 205 nm, and DDMP soyasaponins have their absorption maxima at 292 nm. An analytical method using UV detection of all the known group B soyasaponins at 205 nm was developed by Hu et al. (*29*). This analytical method requires the preparation of non-DDMP and DDMP soyasaponin purified standards. Other procedures use mass spectrometric detection to identify all DDMP and non-DDMP soyasaponins (*30*). The structural diversity was overcome by partial alkaline degradation followed by LC/ ESI-MS (*31*) or by complete acid hydrolysis to their principal aglycone forms, soyasapogenol A and B, followed by HPLC coupled with an evaporative light scattering detector instead of a UV detector (*27*, *32*). These analytical procedures often require an expensive apparatus, not appropriate for daily routine analysis. Thus, the first goal of this study was to develop and validate an economical, rapid, and accurate analytical method using HPLC/ UV to determine the composition of all soyasaponins belonging to group B, including the DDMP-conjugated (αg, βg, βa, γg, and γa) and their non-DDMP corresponding forms (V, I, II, III, and IV, respectively), with the same apparatus and the same type of analytical procedure as for isoflavone analysis. Since external calibration for all soyasaponins is expensive or impossible to obtain without isolation of preparative materials, the alternative of using the more common and stable soyasaponin I as a unique external standard was also investigated. This method was used to evaluate the composition and the variability of soyasaponin B in different parts of soybean seeds from seven varieties and to determine the content of isoflavones and saponins in different commercial dietary supplements, including soy isoflavone concentrates or soy extracts enriched in isoflavones, available in European and North American markets, mainly destinated for the use by menopausal women. We evaluated 15 different product capsules or tablets.

MATERIALS AND METHODS

Soybean Samples, Processed Soy Food Products, and Chemicals. Seven soybean varieties (Imari, Conrad, Savoy, Sponsor, Queen, Jack, and Loda) were obtained from field-grown experiments carried out in 2001 (Toulouse, France) and in 2002 (Champaign Urbana, USA). Germs were collected by hand from lyophilized seeds and ground with a mortar and pestle. Whole seed samples were finely ground (particle size < 200 μm) with a mill (IKA, Labortechnik, Germany). Dietary supplements were purchased locally. Duplicate samples of a combined mixture of 10 tablets or capsules of each supplement were ground and analyzed. A sample (2 kg) of soybean germs issued from an industrial load (unknown cultivar) was provided by Genibio (Saint Girons, France) and used as control sample. All samples were stored at −20 °C. HPLC-grade solvents and other chemical reagents with proper purity were used (SDS, Peypin, France). Purified standard soyasaponins I, II, and III were provided by Chromadex (Santa Ana, CA).

Identification and Quantification of Non-DDMP Soyasaponins I, II, and III by HPLC/UV Analysis. Each individual standard of soyasaponins I, II, and III (1.5 mg) was accurately weighted, dissolved in 0.5 mL of DMSO (SDS, Peypin, France), and adjusted to 10 mL with pure methanol. After a 20 min homogenization, each of the three stock solutions was submitted to serial dilutions (4, 10, 25, 50, 75, and 100 μg/mL) in pure methanol. The final concentrations were calculated with high accuracy by weighting the solutions (methanol density: 0.7915; DMSO: 1.1).

The calibration curves of individual soyasaponins I, II, and III were also established by serial dilution of the stock solutions in plant matrix. Finely ground soy germ (0.2 g) and whole seeds powders (0.5 g) were dissolved in 80% aqueous methanol (5 mL) and extracted for 2 h at room temperature. The residue was removed by centrifuging the extract at 12000*g* for 10 min and decanting the clear supernatant. The extracts were filtered through 0.45 μm filters (Acrodisc, Pall, NY) and separated into six aliquots (250 μL) in which 0, 50, 100, 150, 200, and 250 μL of the stock solution of purified standards were added, respectively. The solutions were made up to 500 μL with 80% aqueous methanol if needed.

The standard curves were established by plotting the peak area obtained from HPLC at 205 nm as a function of soyasaponin concentration expressed in micromoles per milliliter. The response factors were calculated by linear regression. The purity of each standard, specified by Chromadex on the certificate of analysis, was taken into account. Identities of soyasaponins were confirmed by HPLC retention times. The preparation of each serial dilution was replicated twice for each individual standard of soyasaponins I, II, and III. The filtered extracts and the standard preparations were analyzed by HPLC with a P4000 pump controller, AS3000 autosampler, and UV2000 detector (Spectra Physics Analytical Inc., Fremont, California, US). The analytical column, 250 × 4.6 mm i.d, 5 μm, Satisfaction RP-C_{18}-AB (Cluzeau, Sainte Foy La Grande, France) was kept at 30 °C in a thermal chamber. The mobile phases were 0.05% (v/v) trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as described by Hu et al. (*29*): solvent B increased from 37 to 40% in 12 min, then increased to 48% in 25 min, and finally 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 injection volume was 50 μL, and the flow rate was 1 mL/

min. UV absorbance was monitored at 205 nm. Chromatograms were recorded and integrated with the SpectraSystem PC1000 software.

Determination of DDMP-Conjugated Soyasaponins. As shown by Gu et al. (*31*), when the DDMP-conjugated soyasaponins are degraded into their non-DDMP corresponding forms under basic conditions, the total molar content of soyasaponins remains constant. Thus, the response factor of the DDMP-conjugated soyasaponins was determined using the equivalence between the molar content of soyasaponins extracted under mild conditions or under alkaline conditions. The alkaline degradation procedure was conducted as described by Gu et al. (*31*) with minor modifications. Briefly, soy germs (0.2 g) were extracted with 80% aqueous methanol (5 mL) for 2 h at room temperature. The residue was removed by centrifugation and decantation of the clear supernatant. Each extract was filtered and divided in two aliquots. The first was directly analyzed by HPLC as described above, whereas, prior to analysis, the second was submitted to mild alkaline degradation as follows: 2 mL was mixed with 20 μL of KOH (5% v:v) for 15 min at room temperature. Acetic acid (50 μL) was added to neutralize and maintain the solutions between pH 4 and pH 6. Both DDMP and non-DDMP were detected at 205 nm.

Evaluation of the Precision. Two control samples were chosen for their very different saponin contents and composition: isolated soy germs (Genibio, St Girons, France) and whole seeds (i.e., Imari; Monsanto Deklab seeds, France) lyophilized and stored at 4 °C. These two control samples were used for each new run to confirm that the HPLC system was operating correctly and to provide the expected concentrations of the two known samples. Ten replications of the extraction procedure followed by HPLC analysis were carried out on each matrix within 24 h to evaluate the within-day variation. The procedure was replicated three times over a month to determine the between-days variation. The means, standard deviations, and coefficients of variation of within-day and between-days assays were calculated for each soyasaponin.

Isoflavone Analysis. The extracts were analyzed with the same column and apparatus used for soyasaponin B determination. UV absorbance was monitored at 260 nm. The mobile phases were 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as reported by Murphy et al. (*25*) with minor modifications: solvent B increased from 0 to 15% in 2 min, then to 18% in 4 min, to 24.5% in 26 min, to 40% over 7 min, then to 50% in 1 min, and finally increased to 100% in 6 min. The gradient program recycled back to the initial state of 100% solvent A in 2 min. The column temperature was 30 °C. The injection volume was 10 μL, and the flow rate was 1.5 mL/min. Calibration curves were established with the six readily available standards daidzin, genistin, glycitin, daidzein, genistein, and glycitein (Chromadex, Santa Ana, CA). Their respective stock solutions and serial dilutions were prepared as described above for soyasaponins. Instead of using unstable conjugated-isoflavone standards, the response factors of the malonyl and acetyl forms were calculated from those of their corresponding glucoside forms, correcting them in a molecular mass ratio. We hypothesized that the absorption properties of a glucoside form at 260 nm should not be modified by a malonyl or an acetyl group and that their response factor was only dependent upon their molecular weight. This method is justified since a malonyl or an acetyl group does not contain an ultraviolet chromophore. This method can lead to a slight overestimation of the acetyl forms (*33*). However, the samples analyzed here generally did not contain high quantities of acetyl forms.

Data Analysis. ANCOVA with separate slope design, test for the homogeneity of slopes, estimation of variance components, and post hoc comparisons (Newman and Keuls) were made using the GLM procedure of the Statistica v.6.1 for Windows (StatSoft France, 2004).

RESULTS AND DISCUSSION

External Calibration of Non-DDMP Soyasaponins I, II, and III Contents by HPLC/UV Analysis of the Purified Corresponding Standard. To ensure independent error determinations, separate stock solutions were prepared for each of the three commercially available soyasaponins (I, II, and III); they were then used to obtain calibration curves either in pure methanol or in germ or seed extracts, using the standard addition method. The latter method was applied to check the absence of interference with other compounds of the analyzed samples. All the serial dilutions were duplicated, and the complete trial (18 curves, with new stock solutions) was repeated one week later using new solvents. The linearity was very high, with $r^2 > 0.999$ for each of the 36 regression curves. The data analysis with the general MANCOVA model (separate slopes) did not show any significant difference between the slopes. Neither the matrix in which the calibrations were conducted (pure methanol or soybean extract) nor the structural differences of soyasaponins had an effect on the slope values. The mean response factors for soyasaponin I, II, and III were 18.8 ± 1.5, 18.9 ± 1.5, and 17.7 ± 1.5 area units/μmol, respectively. The intercepts in pure methanol were not different from zero ($p > 0.15$). The soyasaponin B response factors obtained at 205 nm in different matrixes ranged from 17.3 ± 1.5 to 19.2 ± 1.5 area units/μmol. These results corroborated with the hypothesis that all the non-DDMP soyasaponins could be determined using a unique calibration curve. This hypothesis is reinforced by a previous observation reported by Hu et al. (*29*), who found that the molar extinction coefficients at 205 nm for soyasaponins I, II, and V were very close. Similar conclusions between soyasaponins I and V were made using electrospray ionization (*31*) and between all the soyasaponins B using ELS detection (*32*).

The detection limit (LOD), defined as 3 times the signal (S) to noise (N) ratio (S/N) and the quantification limit (LOQ), defined as 10 times S/N, were 0.13 and 0.42 nmol, respectively, for soyasaponin I, which corresponds to 0.065 and 0.210 μmol/g in our dilution conditions. The noise was the signal width in blank injections realized between the sample injections. Noise peaks were integrated at the same time of elution as the soyasaponins, and a mean noise area was calculated. Recently, the LOD for soyasaponins I published with ELS detection were 0.04 nmol (*32*) and 12.3 μg/mL with 10 μL injection (i.e., 0.13 nmol) (*34*); thus the sensitivity of UV detection, even at the very low wavelength used here, is comparable to ELS detection.

The contribution of the calibration to the analytical error is generally estimated by the slope error σ_b. The variation coefficient of the slopes was <0.9% ($n = 6$), which indicates a good regression coefficient. Serial dilutions with low volumes can introduce an important variability, even if the accuracy of the dilution is reinforced by weighting the vials.

DDMP-Conjugated Soyasaponin Analysis through Alkaline Degradation. Owing to its chemical structure, the DDMP group can modify the absorbance properties of the soyasaponins. This experiment was carried out either on soy germs, since they have higher soyasaponin content, and significant levels of soyasaponins V and αg, or on whole seed extracts, for their higher relative soyasaponin βa content. Because of their very low level in our materials, the response factors of soyasaponin γa has not been determined. Ten separate replications of the extraction and alkaline degradation were done on the same preparation of ground soy germs or seeds, and the experiment was repeated a week later.

The alkaline hydrolysis was carried out to cleave the DDMP moieties of soyasaponins B. During this degradation, the total molar content of each pair of soyasaponins {I + βg}, {II + βa}, {III + γg}, {IV + γa}, and {V + αg} remain constant. The molar content (n) of a compound is obtained from the HPLC chromatogram with the following relationship: $n = A/(FD)$, where A is the area of the soyasaponin peak integrated from the chromatogram, F is the response factor of the compound,

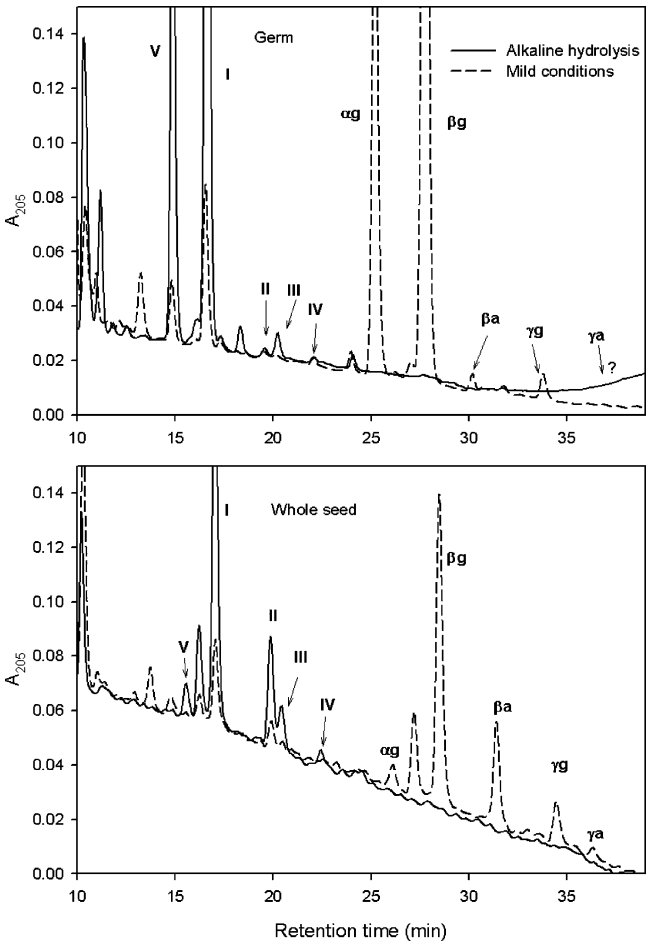

Figure 2. HPLC chromatogram (UV 205 nm) of the group B soyasaponins of soybean germ and whole seed extracted under mild conditions, before (dotted line) and after (continuous line) alkaline hydrolysis.

and D is the dilution factor. Thus, during the alkaline hydrolysis, the initial (n) and final (n') molar contents of each pair are linked by the following equation: $n_{DDMP} + n_{nDDMP} = n'_{DDMP} + n'_{nDDMP}$, from which the response factors (F_{DDMP}) of each DDMP soyasaponin can be expressed as a function of the corresponding non-DDMP response factor (F_{nDDMP}) and of an area depending ratio:

$$F_{DDMP} = F_{nDDMP}\,(DA'_{DDMP} - A_{DDMP})/(A_{nDDMP} - DA'_{DDMP})$$

where (A) and (A') correspond to the peak area before and after the alkaline degradation, and D is the dilution factor due to KOH and acetic acid addition. The area depending ratios ranged from 1.05 (CV = 9.8%, n = 10) to 1.20 (CV = 5.5%, n = 10) for the pairs {II + βa} and {I + βg}, respectively. The DDMP group slightly increases the response factor of the corresponding soyasaponin. These area ratios are similar to the ratios of the molar extinction coefficients given by Hu et al. (*29*). Consequently, a unique standard calibration curve was made for the five non-DDMP soyasaponins, and the DDMP soyasaponins were calculated using the mean calculated area ratios.

Quantification of Soyasaponins: Evaluation of Repeatability and Reproducibility. Considering that a unique calibration curve could be applied at 205 nm for the quantification of all the soyasaponins B, in agreement with Lin et al. (*32*), owing to its cost, stability, and sufficient concentration in soybean, soyasaponin I was selected as reference for quantification. All soyasaponins B were identified through calculation of their relative retention time. As shown on HPLC chromatograms in **Figure 2**, the group B soyasaponins were well resolved. The retention factor (k) of a molecule in RP-HPLC is determined with the equation $k = (t_r - t_m)/t_m$, where t_r is the retention time of the analyte, and t_m is the dead time or the retention time of a substance that is unretained on the column. The mean retention factors (k) of soyasaponins V, I, II, III, IV, αg, βg, βa, γg, and γa in germ or whole seed powder were 6, 6.6, 7.9, 8.3, 8.7, 10.6, 11.4, 12.7, 14.3, and 15.4, respectively.

The repeatability (within-day) and reproducibility (between days) were estimated by the replication of 10 analyses of two control samples, extracted from Imari seeds and isolated germs, 3 times over a month. First, as expected, the analysis of the two control samples shows (**Table 1**) that on an equal weight basis, the content of soyasaponins B in the germ is almost 10 times higher than in the whole seed. Like isoflavones, soyasaponins are particularly concentrated in soy germ (*21*, *35*). As it was expected, the soyasaponin V and its DDMP counterpart are mainly detected in the germ, whereas soyasaponins I and βg represent more than half of the total soyasaponins B either in the seed or in the germ fraction.

The repeatability and reproducibility results presented in **Table 1** indicate a good precision of the analytical procedure for the two types of control sample matrixes: the within-day variation was <7.9% for the four major soyasaponins in the germ (I, V, αg, and βg accounted for >96% of the total soyasaponins B) and <6.8% for the major soyasaponins in whole seed (I, αg, βg, and βa accounted for >84% of the total). Hu et al. (*29*) reported a within-day variation <9.8% and a between-days variation <14.3%, which is quite similar to our

Table 1. Between-Days (Reproductibility) and Within-Day (Repeatability) Variability in Germ and Whole Seed Analysis of Group B Soyasaponins[a]

	germ			whole seed		
soyasaponin B	means ± SD (μmol/g DW)	CV (%) between-days	CV (%) within-day	means ± SD (μmol/g DW)	CV (%) between-days	CV (%) within-day
I	1.70 ± 0.04[c]	4.6	2.1	0.27 ± 0.02[c]	3.8	5.7
II	0.06 ± 0.02			0.06 ± 0.03[b]	14.4	14.1
III	0.06 ± 0.02	20.0	33.7	0.02 ± 0.01	17.3	29.9
IV	0.08 ± 0.03[b]	30.0	70.6	0.01 ± 0.01		
V	0.64 ± 0.05[c]	3.0	7.9	0.06 ± 0.01[b]	2.0	18.7
βg	12.70 ± 0.32[c]	8.4	2.7	1.14 ± 0.05[c]	0.4	3.7
βa	0.16 ± 0.02[b]	6.0	12.8	0.40 ± 0.02[c]	3.7	4.1
γg	0.30 ± 0.03[c]	3.6	10.6	0.17 ± 0.02[c]	1.2	11.0
γa	ND			0.05 ± 0.01[b]	29.8	27.1
αg	5.42 ± 0.12[c]	9.0	2.4	0.27 ± 0.02[c]	5.2	6.8
total	21.13 ± 0.46	6.7	2.3	2.46 ± 0.11	0.7	4.4

[a] Analysis of 3 times 10 extractions; ND: not determined. [b] Above detection threshold (LOD). [c] Above quantification threshold (LOQ).

Table 2. Comparison of Isoflavones and Soyasaponins B Contents and Compositions in 7 Soybean Cultivars and 15 Dietary Supplements

		isoflavones								soyasaponines B							ratios	
		aglycone profile				conjugation profile					triglucosidic α-L-Rha		diglucosidic α-L-Rha		trigl β-D-Glu			
matrix		total (μmol g^{-1})	daidzein (%)	genistein (%)	glycitein (%)	malonyl (%)	acetyl (%)	glucosyl (%)	aglycone (%)	total (μmol g^{-1})	I + βg (%)	II + βa (%)	III + γg (%)	IV + γa (%)	V + αg (%)	DDMP (%)	triglucosid/ diglucosid (α-L-Rha)	isof/ sapo
soy germs	Imari	39.3	62.3	12.2	25.5	47.2	1.1	48.6	3.1	13.20	72.77	1.54	2.17	1.70	21.82	81.65	19.22	2.98
	Dwight	48.2	38.6	11.4	50.0	44.6	1.0	51.8	2.6	14.53	65.19	0.83	1.75	0.99	31.23	78.81	24.03	3.32
	Savoy	48.7	62.1	12.4	25.5	53.8	1.4	42.9	1.9	22.69	66.86	0.88	1.39	1.44	29.42	85.48	23.89	2.14
	Sapporo	49.2	62.2	13.1	24.7	55.5	1.0	41.6	2.0	28.29	68.08	1.40	1.78	1.47	27.27	87.73	21.38	1.74
	Sponsor	53.5	58.5	12.8	28.7	51.5	1.3	45.2	2.0	28.62	66.77	0.89	1.70	1.30	29.34	85.76	22.61	1.87
	Conrad	71.0	66.6	11.0	22.4	67.0	1.0	31.0	1.1	23.23	71.67	1.24	1.14	1.10	24.85	90.44	32.57	3.06
	Loda	75.1	64.3	9.4	26.3	65.7	0.9	32.0	1.4	42.40	68.81	1.24	1.15	1.24	27.56	88.78	29.36	1.77
soy cotyledons	Imari	6.1	34.9	62.8	2.3	41.7	2.8	51.3	4.3	2.76	57.75	9.34	10.78	6.87	5.26	82.80	4.37	2.22
	Dwight	6.9	48.5	49.5	2.0	43.9	2.5	48.4	5.2	2.99	57.14	6.95	10.03	4.18	1.70	84.52	5.92	2.32
	Savoy	7.7	40.0	58.3	1.7	48.3	2.6	46.2	2.8	3.17	53.90	2.56	8.48	5.88	9.18	87.73	5.32	2.43
	Sapporo	4.5	34.4	62.7	2.9	52.4	2.8	41.3	3.5	3.63	59.89	4.29	8.33	5.30	2.19	87.49	6.18	1.25
	Sponsor	7.6	32.9	65.0	2.1	47.8	3.0	45.8	3.4	3.34	54.91	1.40	12.39	9.14	2.17	84.42	3.54	2.28
	Conrad	9.2	37.8	60.3	1.9	64.0	2.4	32.3	1.3	4.14	54.72	0.43	7.70	5.58	1.57	94.80	6.41	2.22
	Loda	10.1	35.3	61.4	3.3	64.8	2.8	30.7	1.7	6.43	59.36	7.65	6.54	6.45	0.00	95.39	6.70	1.56
germ-based products[b]	P5	17.8[a]	60.0	11.3	28.7	3.6	41.7[a]	50.5	4.1	8.32	68.36	0.56	0.94	1.41	28.73	11.30	29.31	2.14
	P2	61.9	51.0	13.7	35.3	1.2	2.8	93.1	2.8	6.62	63.62	0.44	3.23	4.92	27.79	10.26	7.86	9.35
	P4	101.0[a]	54.1	15.6	30.3	1.8	12.4[a]	82.9	3.0	19.12	67.96	0.48	0.92	1.71	28.92	5.45	25.98	5.28
	P12	103.1	53.8	16.0	30.2	19.7	5.4	71.2	3.6	31.77	71.68	0.62	0.89	1.30	25.52	30.68	33.09	3.25
	P7	104.1	49.5	17.7	32.8	1.5	3.4	93.5	1.6	17.49	67.02	0.54	2.09	1.74	28.61	6.41	17.64	5.95
	P14	106.0	51.4	14.8	33.8	1.2	3.1	93.6	2.1	12.69	66.93	0.45	0.90	1.86	29.86	2.00	24.45	8.35
	P15	113.3	57.3	14.8	27.9	29.5	1.7	66.0	2.7	55.74	70.65	0.65	0.97	0.61	27.12	50.11	45.01	2.03
	P3	116.7[a]	56.5	16.1	27.4	1.5	10.4[a]	80.9	7.2	41.36	67.51	0.48	1.36	1.50	29.15	6.67	23.75	2.82
	P11	140.1	47.7	10.9	41.3	48.3	3.5	46.2	2.0	43.46	67.80	0.83	1.05	1.78	28.54	51.11	24.25	3.22
seed-based products[b]	P8	51.8	35.4	61.5	3.1	42.1	6.8	43.4	7.7	3.47	59.11	0.38	22.02	4.60	3.88	17.73	2.61	4.91
	P9	55.3	33.9	63.2	2.9	40.4	6.7	47.4	5.5	3.70	53.61	6.18	22.64	2.77	14.81	24.14	2.35	14.93
	P10	61.1	52.5	38.0	9.5	1.6	1.9	91.5	5.1	7.82	55.05	1.03	29.3	8.28	6.31	0.00	1.49	7.81
	P1	117.1	38.2	61.8	0.1	0.0	0.0	0.4	99.6	6.73	7.77	1.17	71.17	2.82	17.07	6.25	0.12	17.39
	P13	123.2	17.5	79.6	2.9	2.3	0.9	82.8	14.0	40.70	58.71	5.23	27.78	6.04	2.24	11.79	1.89	3.03
	P6	133.1[a]	38.2	52.1	9.7	1.2	10.0[a]	82.9	5.9	25.72	49.07	7.99	24.39	14.96	3.59	8.37	1.45	5.17

[a] Molar extinction coefficients of acetyl-glucosides are larger than other forms (*33*, *43*). Products containing significant amounts of acetyl-isoflavones are slightly overestimated. [b] Commercial products are enriched isoflavone dietary supplements designed for menopausal women (European and North American markets).

results. Moreover, in the germ, the within-day variation did not exceed 3% for the soyasaponin I and its DDMP form. The between-days variation was <9 and <5.2% for the major components of the germ or the seed, respectively. This indicates a very good precision of the analytical procedure, considering the very low wavelength detection used. As demonstrated above, one of the major sources of between-days variability could be provided by the calibration error. The low amount of γa, saponins III, IV, and V in the seed explains the lack of accuracy concerning these molecules. These results are slightly better than the intraday and interday variability found recently with an HPLC/ELS detection (*32*), but the nonlinear response with ELS detection makes generally the quantification more difficult.

Soyasaponin and Isoflavone Contents in Different Soybean Varieties. The correlations between soyasaponin and isoflavone content and composition were investigated in seven different soybeans cultivars. The nine distinctive forms of soyasaponins (I, II, III, IV, V, αg, βg, βa, and γg) and the 12 soybean isoflavones (three aglycons and their conjugated forms) were analyzed as described above.

As shown in **Table 2**, depending of the cultivar, the total amount of soyasaponins B varied from 13.20 to 42.40 μmol/g DW and from 2.76 to 6.43 μmol/g DW in germs and cotyledons, respectively. The range of variation is the same for isoflavones content at the whole seed level, in agreement with previous works (*27*, *29*). The values of soyasaponins contents (*21*, *27*, *29*, *31*, *32*) as well as the isoflavone contents (*34*, *36*−*38*) reported here are in agreement with the results presented in the literature. In all the cultivars studied, soyasaponin I and its DDMP counterpart βg had the highest concentration, from 54 to 73% in soy germ or in cotyledons. Soyasaponin V was not detected in the cotyledons, whereas its concentration in germ represents from 22 to 31% of the total soyasaponins B. On the other hand, the trisaccharidic soyasaponin II (**Figure 1**) and its DDMP counterpart βa are more characteristic of the cotyledons. There is no significant correlation between soyasaponin and isoflavone concentrations in the germs ($r = 0.733$; $p = 0.06$) and in the cotyledons ($r = 0.686$; $p = 0.09$). This was in agreement with the previous works which did not find correlations at the whole seed level (*27*, *29*). The isoflavone-to-soyasaponin molar ratio varied from 1.3 to 3.3, whatever the seed part was considered and without any correlation between germ and cotyledons. This suggests some independence between the metabolic and the genetic control of secondary metabolism in these two seed fractions.

The malonyl-conjugated isoflavone forms are predominant in the seed, whatever seed part was analyzed. As reported in the literature, the malonyl forms represent 70−90% (w:w) of the total isoflavones, and the glucosides represent 10−25% (w:w) (*21*, *37*−*39*). The samples used for these assays corresponded to 2-year-old seeds stored at 20 °C. Consequently, the malonyl isoflavone contents are rather low and the glucoside and aglucon forms are rather high, as already described in other studies (*9*, *40*). It is generally considered that almost all the soyasaponins B are DDMP-conjugated in the seed (*21*, *29*). The seeds analyzed in this study presented at least 78.8% to more than 95% of DDMP soyasaponins, which indicates a good stability of these forms, compared with the malonyl isoflavones. Tsukamoto et al. (*21*) already stated that DDMP conjugates were not altered by high temperature during seed development. In contrast, significant conversion of DDMP soyasaponins βg to soyasaponins I was observed in peas during 9 months of storage (*41*).

Isoflavone versus Soyasaponins B Contents in 15 Soy-Based Health Supplements. Comparison of isoflavone and soyasaponin B contents and compositions in 15 soy-based health supplements are presented in **Table 2**. On the basis of their percentage of glycitein or soyasaponins V and αg contents, we can recognize the difference between the germ- and seed-based products. The range of soyasaponin B contents in commercial products is roughly the same as in the soybean cultivars but is considerably higher for the isoflavones content, which is expected, as these products are supposed to be isoflavone concentrates. We can observe higher soyasaponin concentration in the seed-based products. This indicates that the extraction process can greatly modify the isoflavone-to-saponin ratio, from 2.03 to 9.35, and from 3.03 to 17.39 in germ- or seed-based products, respectively (**Table 2**). Alcohol washing is known to result in the loss of the saponins in protein isolates, but other processes, like acid washes are not (*29*, *42*).

When soy is processed under mild conditions, malonylglucosides are converted into their β-glucoside forms, which are then converted into aglucones in the presence of β-glucosidase. In contrast, DDMP soyasaponins are quite stable under heat in solid matrixes, but they are converted into their non-DDMP form in soaked conditions (*29*). In the products analyzed here, higher malonyl contents generally correlate with higher DDMP contents. This could be explained by differences in the range of pH used or extracting temperature.

Some products showed a higher level of disaccharidic soyasaponins III and IV. These soyasaponins are produced by the loss of the third sugar (α-L-rhamnose) of soyasaponins I and II, respectively (**Figure 1**). Such degradations were observed under particular alkaline conditions or by enzymatic degradation (*18*, *20*, *31*, *43*). The trisaccharides (soyasaponins I and II) to disaccharides (soyasaponins III and IV) ratio was higher in the germs (from 19.22 to 32.57) than in the cotyledons (from 3.54 to 6.70); however, seed-based product P1 and germ-based product P2 had very low ratios, which could indicate that these products are processed under more drastic conditions.

In conclusion, we proposed a rapid, economical, and accurate HPLC/UV method for routine analysis of all group B soyasaponins, which allows the quantification of all isoflavones and soyasaponins B on the same extract, using the same apparatus. This work presents another identification method suitable not only for non-DDMP soyasaponins but also for the αg, βg, βa, and γg DDMP soyasaponins. The described method demonstrates an efficient separation and detection of the 10 group B soyasaponins and provides several advantages, including direct quantification of non-hydrolyzed soyasaponins, eliminating the use of unstable purified standards and avoiding purification steps. Soyasaponin concentration and composition depend on soybean variety and vary among commercial products. Soybean processing leads to changes in the composition of secondary metabolites compared with whole soybeans seeds. Many studies evaluating biological properties have been conducted using isolated fractions from soy materials, but attribution of functional effects or mechanism of action cannot be determined unless each phytochemical is evaluated individually. Soyasaponin content of a soy commercial product should be taken into consideration when discussing and evaluating the possible health effects of a soy-based product.

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