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Complete Quantification of Group A and Group B Soyasaponins in Soybeans

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A combination of high-pressure extraction and preparative chromatography was used to purify the group A and group B soyasaponins from soy germ for use as analytical standards and for use in biological assays. A standardized sample preparation and extraction method was developed for the analysis of phytochemicals found in soy and processed soy products, which is reproducible in other laboratories. The extracts can be analyzed with standard liquid chromatography−mass spectrometry and high-performance liquid chromatography methods to identify and quantitate the group A and group B forms of the soy saponins, as well as the soy isoflavones. Complete saponin analysis of the extracts prepared from soy germ (hypocots), hulls, and cotyledons shows that a significant portion of the saponins is concentrated in the germ. The germ contains nearly all of the group A soyasaponins, while the group B soyasaponins are nearly equally distributed between the germ and the cotyledons. The hulls contain little of either isoflavones or saponins. Whole (full fat) soybeans grown on a tract in central Illinois in 2003 contain approximately 4−6% saponins on a weight basis, of which about one-fifth or less of the total saponin content are group A soyasaponins; the balance is group B soyasaponins.

KEYWORDS: Soy; *Glycine max*; germ; hull; cotyledons; purification; analysis; saponins; isoflavones; phytochemicals

INTRODUCTION

Saponins are naturally occurring triterpenoids found in many food materials derived from a wide variety of plant species (*1*). They are secondary plant metabolites containing a steroid or triterpenoid aglycone with a number of carbohydrate moieties linked through ether and ester linkages at one or more glycosylation sites. Seeds of the soybean (*Glycine max* L. Merrill) contain from 0.6% to as much as 6.5% dry weight triterpenoid saponins depending on the variety, cultivation year, location grown, and degree of maturity (*2*−*8*). The soybeans that we examined were composed of the hull (7 wt %), the germ or hypocot (3 wt %), and the cotyledons (90 wt %). Near-infrared (NIR) analysis showed that these soybeans had on average 40% protein, 20% oil, and 7.5% moisture. It is generally accepted that these components, as well as the phytochemical composition in the mature beans, can vary considerably and are influenced by the genetic variability in the various cultivars and the environmental factors (sunlight intensity, rain, disease and insect attack, etc.) in the locations where they are grown, which vary from year to year.

The saponins in the mature soybean have been divided into group A and group B soyasaponins on the basis of their aglycone structures (*9*−*13*). The group B soyasaponins appear to exist in the intact plant tissue as conjugates of 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) at the 22 hydroxyl position (*10*). The DDMP conjugates are relatively labile and are easily degraded, most likely resulting in the formation of the non-DDMP group B soyasaponins. The other various forms of the group B soyasaponins arise from alternate sugars in the oligosaccharide attached to the 3-hydroxyl position of the aglycone. The group A soyasaponins are didesmosidic with alternate sugar compositions in both sets of oligosaccharides attached to the aglycone at the 3- and 21-hydroxyl positions (*13*). Several other saponin forms, including the group E soyasaponins and a number of the shorter sugar chain saponins, are probably artifacts arising from the extraction and processing steps (*14*−*16*). This has resulted in published references to over 20 different saponin forms in soy and its processed products. The structures and the complicated naming nomenclature of the soy saponins are shown in **Figure 1**.

Soy and soy foods have been linked to many important biological activities in humans often attributed to their phytochemical composition that includes isoflavones, lignans, and saponins (*17*). The saponins have been shown to have a number

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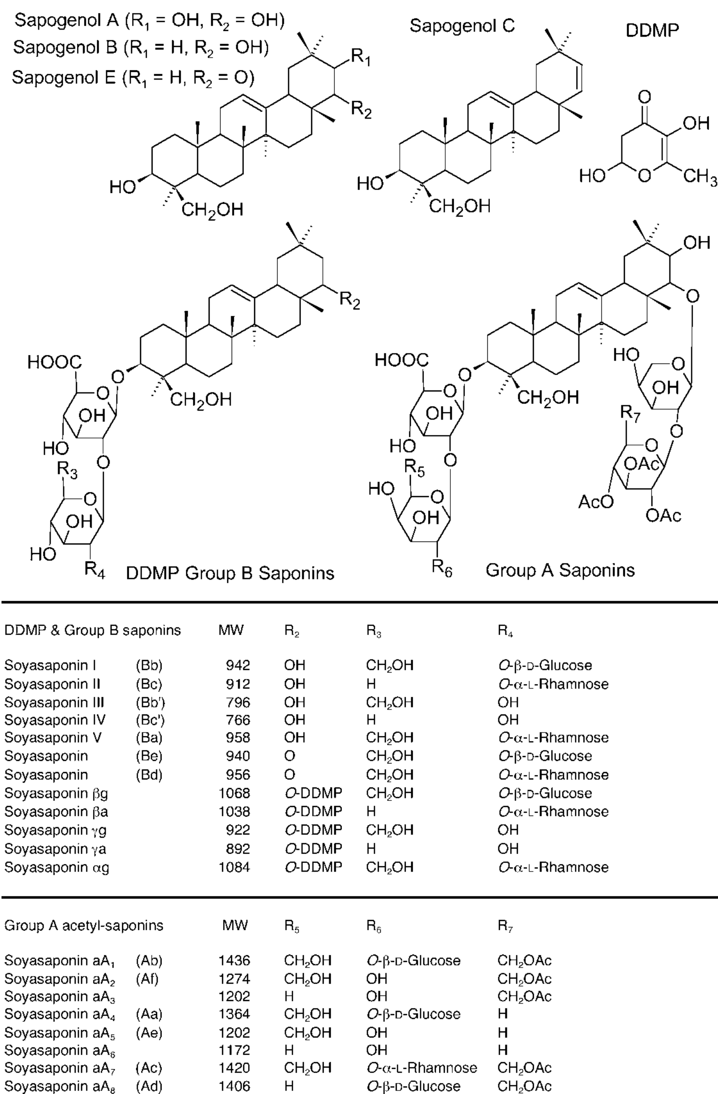

DDMP & Group B saponins		MW	R_2	R_3	R_4
Soyasaponin I	(Bb)	942	OH	CH_2OH	*O*-β-D-Glucose
Soyasaponin II	(Bc)	912	OH	H	*O*-α-L-Rhamnose
Soyasaponin III	(Bb')	796	OH	CH_2OH	OH
Soyasaponin IV	(Bc')	766	OH	H	OH
Soyasaponin V	(Ba)	958	OH	CH_2OH	*O*-α-L-Rhamnose
Soyasaponin	(Be)	940	O	CH_2OH	*O*-β-D-Glucose
Soyasaponin	(Bd)	956	O	CH_2OH	*O*-α-L-Rhamnose
Soyasaponin βg		1068	*O*-DDMP	CH_2OH	*O*-β-D-Glucose
Soyasaponin βa		1038	*O*-DDMP	H	*O*-α-L-Rhamnose
Soyasaponin γg		922	*O*-DDMP	CH_2OH	OH
Soyasaponin γa		892	*O*-DDMP	H	OH
Soyasaponin αg		1084	*O*-DDMP	CH_2OH	*O*-α-L-Rhamnose

Group A acetyl-saponins		MW	R_5	R_6	R_7
Soyasaponin aA_1	(Ab)	1436	CH_2OH	*O*-β-D-Glucose	CH_2OAc
Soyasaponin aA_2	(Af)	1274	CH_2OH	OH	CH_2OAc
Soyasaponin aA_3		1202	H	OH	CH_2OAc
Soyasaponin aA_4	(Aa)	1364	CH_2OH	*O*-β-D-Glucose	H
Soyasaponin aA_5	(Ae)	1202	CH_2OH	OH	H
Soyasaponin aA_6		1172	H	OH	H
Soyasaponin aA_7	(Ac)	1420	CH_2OH	*O*-α-L-Rhamnose	CH_2OAc
Soyasaponin aA_8	(Ad)	1406	H	*O*-β-D-Glucose	CH_2OAc

Figure 1. Structures and nomenclature of the soybean saponins. The nomenclature of the group B soyasaponins used in this paper is that of Kuduo et al. (*10*) and Yoshihiki et al. (*25*) with the nomenclature of Shiraiwa et al. (*12*) in parentheses. The nomenclature of the DDMP saponins used in this paper is that of Kuduo et al. (*10*). The nomenclature of the deacetylated group A saponins used in this paper is that of Kitagawa et al. (*53*), which adds the new designations A_7 and A_8 for two saponins identified by Shiraiwa et al. (*13*). We have added the initial "a" to the Kitagawa nomenclature indicating the presence of the fully acetylated form. The fully acetylated group A nomenclature of Shiraiwa et al. (*13*) is indicated in parentheses.

of biological activities in animal systems including chemoprotective, hypocholesterolemic, haemolytic, immunostimulatory, antiviral, and anticarcinogenic activities (*18*−*26*). These activities are only just beginning to be characterized in cell culture and animal feeding studies. Recent research has shown that these compounds induce apoptosis (*27*, *28*) and macroautophagy (*29*, *30*) in cultured cancer cells. Work is beginning on the evaluation of saponin effects on health in whole animal studies (*24*, *31*) and in humans. Despite a great deal of analytical research over the past 30 years, more detailed studies of the biological activities have been hampered by the lack of purified saponins in quantities required for research studies, as well as poor or outdated procedures for the extraction and quantification of these compounds in foods and food products.

Reproducible methodology for the accurate and efficient extraction of the phytochemical components from soybeans and processed soy products continues to be a problem that results in large interlaboratory variations. To achieve rapid, accurate, and reproducible measurements of any plant phytochemical, an efficient extraction method must be used. Once extracted, the analysis of both the isoflavones and the saponins is fairly straightforward, if analytical standards are available. Several analytical methods exist for the identification and quantitation of the isoflavones in soy (*32*−*38*). Several excellent high-performance liquid chromatography (HPLC) and liquid chromatography−mass spectrometry (LC-MS) saponin identification and quantitation methodologies have been published over the past few years mostly focused on the more abundant group B soyasaponins (*39*−*45*). Some work has been done on the characterization of the group A soyasaponins but often with only the aglycone form (*5*, *46*) or one or more characterized group A soyasaponins (*6*, *7*, *47*, *48*). The current analytical trend is for the complete isoflavone and saponin profiles in samples (*8*, *35*, *49*−*51*).

We have developed a reliable method for the preparation of soybeans for reproducible extraction of both isoflavones and saponins. Coupled with LC-MS analysis, we have used this method to identify and quantitate the major isoflavones glycosides and the group A and group B saponins extracted from soy. Previous work in our laboratory has elucidated methods for the isolation of the group B soyasaponins from extracts prepared from whole soy (*39*). We present here a methodology for the isolation of group A soyasaponins from extracts prepared from soy germ.

MATERIALS AND METHODS

Soybeans. A local grower from Tazewell County, Illinois, provided about 1 kg of harvested soybeans during the 2004 and 2005 growing seasons. The individual cultivars designated in this work as AG1, AG2, AG3, and AG4 were grown on a single farm field in McLean County, Illinois, in 2003, and 0.25 kg of each bean variety was provided by the Ag Guild of Illinois.

Soy Sample Preparation and Extraction. Whole soybeans from commercial growers were freeze-dried overnight to remove the remaining moisture. For whole bean analysis, the oven-dried beans were ground in a commercial coffee mill to a fine powder. For component analysis, freeze-dried beans were cracked in a Glen Mills S500 disk mill set at 4.1 mm, which gently broke apart the beans and did not cause any fragmentation of the cotyledons. The hulls were removed by aspiration; the germ and cotyledons were separated using a no. 5 and no. 18 screen. This resulted in four bean fractions, the hulls, the whole germ, the cracked or broken germ, and the cotyledons. Each fraction was ground to a fine powder in a commercial coffee mill. All powdered soy samples were defatted with hexane in a Soxhlet apparatus overnight, allowed to dry, and then ground again in a commercial coffee mill to form a fine powder.

For quantitative isoflavone and saponin analysis, defatted samples (typically 0.25 g) were placed in a vial and 3 mL of a dimethyl sulfoxide−methanol (1:1) solution was added. The vials were capped and wrapped with sealing tape and incubated in an oven for 72 h at 50 °C. Then, the samples were sonicated for 15 min at 50 °C and allowed to stand at room temperature for 1−2 h. An aliquot was removed from the vial and filtered through a 0.45 μM nylon 66 filter for HPLC analysis for both isoflavones and saponins. For multistep extractions, the liquid material was removed by glass pipet, and 2 mL of methanol was added. The samples were recapped, sonicated, and kept at least 1 h before the liquid was removed by pipet. This was repeated at least twice. The total extracts from each sample were either analyzed or pooled, and the total volume was determined. This basic methodology was altered for the various experimental conditions.

For the extraction of soy saponins, an ASE 300 automated solvent extractor system (Dionex Corp., Hercules, CA) was used. Powdered defatted soy samples were mixed with Celite 545 (Baker, Phillipsburg, NJ) and acid-washed sand (Sigma-Aldrich, St. Louis, MO) in a ratio of four parts sample, one part Celite, and one part sand. Aliquots of this mixture were loaded into 34 mm ASE 300 cells (filled to approximately four-fifths the total cell volume) for extraction using methanol as the solvent. The extraction conditions were as follows: cells were loaded with solvent, then pressurized to 1500 PSI, held at static at 100 °C for 5 min, and then flushed at 60% capacity. This was repeated nine times for each cell. After cooling and standing in the hood for 1−2 h, the pooled methanol extracts were filtered through #2 filter paper to remove solids and allowed to evaporate to dryness and then resuspended in a minimum volume of hot (45 °C) methanol. After cooling, this material was refiltered for preparative HPLC.

Preparative Chromatography for Purification of the Soy Saponins. For the general separation of the saponins from the isoflavones, a Buchi (Newcastle, DE) Sepacore flash chromatography system with dual C-605 pump modules, a C-615 pump manager, a C-660 fraction collector, and a C-635 UV photometer with SepacoreRecord chromatography software were used. A Buchi C-670 Cartridger system was used to load 40 mm × 150 mm flash columns with approximately 90 g of preparative C18 reverse phase, 125 Å, 55−105 μm, bulk packing material (Waters Corp., Milford, MA). The column was installed in the flash chromatography system and equilibrated with 30% acetonitrile and 0.5% acetic acid in water for 5 min at a flow rate of 30 mL/min. After samples (10−15 mL) were injected, the column was developed with a binary gradient to 80% acetonitrile over 30 min. The effluent was monitored at 210 nm, and fractions based on absorbance were collected in the fraction collector by the software program. Fractions containing saponins were determined by analytical chromatography and LC-MS. The procedure was repeated to obtain sufficient purified material. After evaporation to remove the organic solvent, saponins were recovered as white solids by either by precipitation/crystallization and filtration or by freeze drying.

For the final separation of the individual soy saponins, a Shimadzu (Columbia, MD) preparative HPLC system was used with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, and SCL 10Avp system controller all operating under the Shimadzu Class VP operating system. Freeze-dried saponin fractions from the flash chromatography system were dissolved in methanol, and aliquots (1−10 mL) were injected on a Luna C18(2) 10 μm, 100 Å, 250 cm × 50 cm i.d., semipreparative reverse phase column (Phenominex, Torrance, CA). The column was preequilibrated with 1% acetic acid, 30% acetonitrile, and 69% water at a flow rate of 50 mL/min, and the effluent was monitored at 210 nm. The column was developed to 70% acetonitrile over 45 min. UV absorbing peaks were collected by hand. Pooled material was allowed to evaporate to remove acetonitrile, and in the case of the B group saponins, the crystallized material was collected by filtration. Other saponins were recovered by freeze drying after all of the organic solvent was evaporated.

Soy Phytochemical Analytical Methodology. HPLC analysis was conducted on a Hewlett-Packard Series 1100 HPLC system (G1311A pump, G1322A degasser, G1313A autoinjector, G1314A variable wavelength detector, and G1316A column oven, running under HPChem Station version A.06.01). The column used was an Inertsil ODS-3 reverse phase C-18, 5 μm, 250 mm × 4.6 mm i.d., with a guard column (Varian, Torrance, CA).

For saponin analysis, the initial conditions were 30% acetonitrile and 0.025% trifluoroacetic acid (TFA) in water at a flow rate of 1 mL/min. The effluent was monitored at 210 nm on the variable wavelength detector. After injection (typically 20 μL), the column was developed to 50% acetonitrile and 0.025% TFA in a linear gradient over 45 min. Before the samples were run, an extinction coefficient for the saponins was determined from a linear standard curve based on mAbs units vs nanomoles injected. This curve was prepared from a dilution series of pure soyasaponin I standard that had been purified in this laboratory (**Figure 2**). The nanomolar extinction coefficient for soyasaponin I was also used to quantitate the group A soyasaponins and the DDMP group B soyasaponins, as pure standards for those compounds were not available, with the appropriate molecular weight factor in the calculation. All samples were run in triplicate, and an average and standard deviation were determined.

For isoflavone analysis, the initial conditions were 20% methanol and 0.025% TFA in water at a flow rate of 1 mL/min. The effluent was monitored at 285 nm on the variable wavelength detector. After injection (typically 15 μL), the column was held at the initial conditions for 2 min and then developed to 100% methanol and 0.025% TFA in a linear gradient over 53 min. Standard dilution sets of pure daidzin, glycitin, and genistin were freshly prepared and run before the samples to determine a nanomolar extinction coefficient for each class of isoflavone (*33*). All samples were run in triplicate, and an average and standard deviation were determined.

LC-Electrospray Ionization (ESI)-MS Analysis for Confirmation of Saponins. Samples were run on a ThermoFinnigan LCQ DECA XP Plus LC-MS system with a Surveyor HPLC system [autoinjector, pump, degasser, and photodiode array (PDA) detector] and a nitrogen generator all running under the Xcaliber 1.3 software system. The MS was run with the ESI probe in the positive mode. The column was a 3 mm × 150 mm Inertsil reverse phase C-18, ODS 3, 3 μm, column (Varian) with a guard column. The source inlet temperature was set at 250 °C, the sheath gas rate was set at 70 arbitrary units, and the sweep (auxiliary) gas rate was set at 20 arbitrary units. The MS was optimized for the detection of the soy saponins by using the autotune feature of the software while infusing a solution of soyasaponin I in with the effluent of the column and tuning on an atomic mass unit of 943 $[M + H]^+$ for soyasaponin I. For saponin HPLC separation, the initial conditions were 50% acetonitrile and 0.25% acetic acid in water at a flow rate of 0.3 mL/min. The column was developed to 80% acetonitrile and 0.25% acetic acid in water over 55 min. The effluent was monitored at 210 nm on the PDA, and MS data were collected from 250 to 2000 *m/z*.

Confirmation of Sapogenol Aglycone Structure. Purified saponin fractions (20−100 mg) were resuspended in about 50 mL of methanol containing 2 M HCl. The liquid was kept in the hood in a mantle-heated round-bottom flask attached to a condenser under refluxing

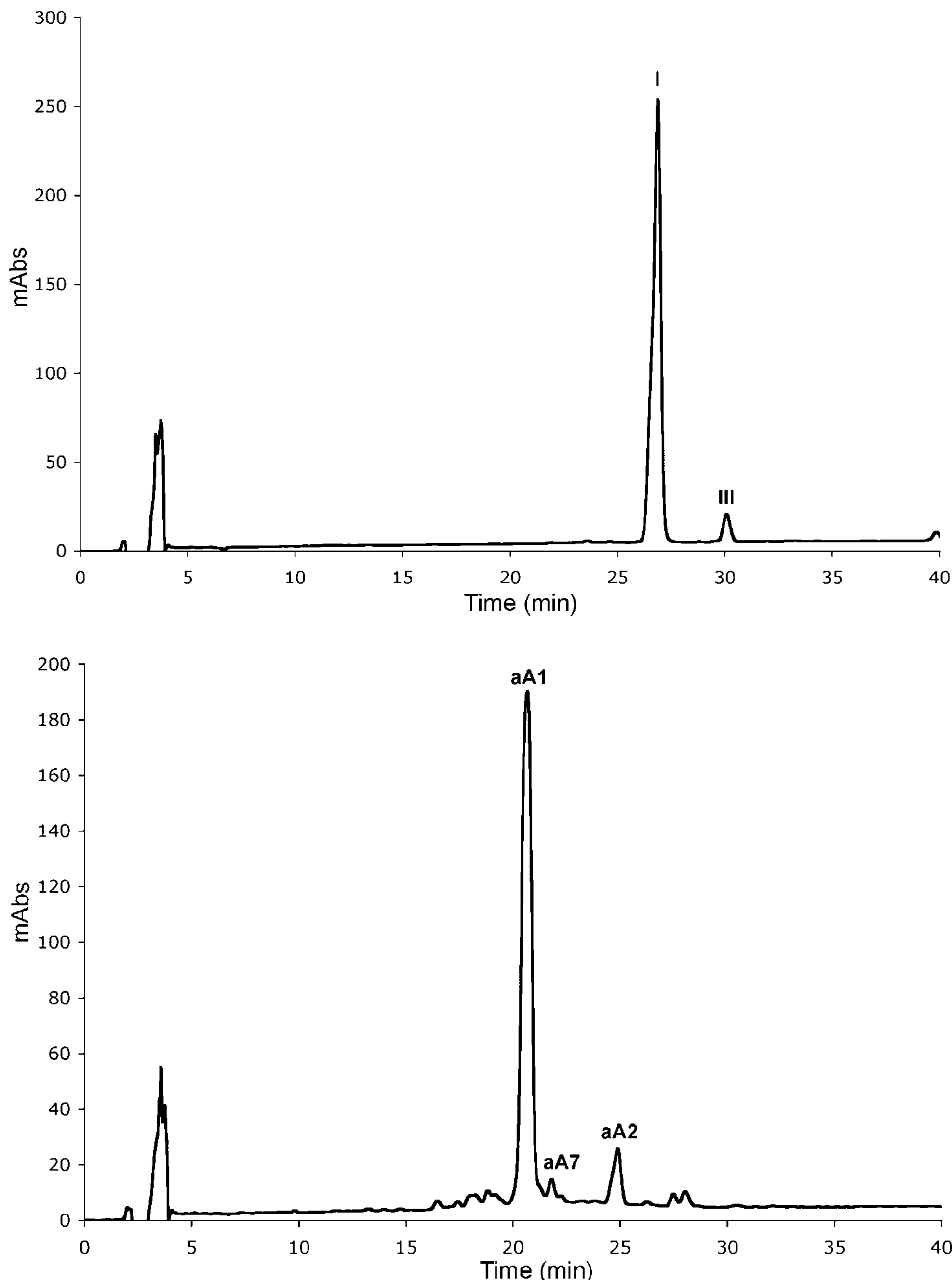

Figure 2. UV trace (210 nm) from HPLC chromatograms of purified soyasaponin I (top) and the group A soyasaponin fraction (bottom) prepared from defatted soy germ. The soyasaponin I sample contains a small amount of degradation product soyasaponin III.

conditions for at least 3 days to obtain total hydrolysis. Upon completion of the hydrolysis, an equal volume of water was added to the solution. The resulting white precipitate was collected by filtration, washed repeatedly with water, and then allowed to dry. The white powder was resuspended in a mixture of methanol and dichloromethane and spotted on a 250 μm thick silica gel 60 F254 thin-layer plate (EMD Chemical Inc., Darmstadt, Germany). The plates were developed with methanol: dichloromethane (5:95). Developed and dried plates were either sprayed with a solution of potassium dichromate in sulfuric acid (Alltech, Deerfield, Illinois) and heated in an oven at 70 °C for 5 min, which visualized the sapogenols as pink/purple spots, or the bands were identified by visual inspection, scraped, and extracted with a mix of methanol and dichloromethane to recover the purified sapogenols. Purified sapogenols were examined by NMR for structure confirmation. ^{1}H, ^{13}C, COSY, DEPT, and HSQC NMR spectra were obtained with a Bruker Avance 500 spectrometer equipped with a 5 mm inverse broadband Z-gradient probe (Billerica, MA). NMR spectra were recorded in either chloroform-*d* or methanol-d_4 using residual solvent peaks as the internal reference. The data were analyzed using the Advanced Chemistry Development, Inc., SpecManager 1D Processor and the HNMR and CNMR Predictor software suite (Toronto, Ontario).

NIR Spectroscopic Analysis of Soybean Components. Dry weight percentages were obtained with a Foss American model 1255 Infratec NIR Food and Feed analyzer. Two aliquots of 10−12 g each of whole beans were analyzed, with the results automatically averaged and stored. Calibrations were developed using Uniform and Regional test samples from the U.S. Department of Agriculture and State agricultural scientists.

RESULTS AND DISCUSSION

The commercially grown soybeans obtained from local growers were composed of the hull (7 wt %), the germ or hypocot (3 wt %), and the cotyledons (90 wt %). NIR analysis (**Table 1**) showed that these beans had on average 40% protein, 20% oil, and 7.5% moisture. There is considerable variation in

Table 1. NIR Analysis and Hexane Extract Loss Analysis of Four 2003 Soy Samples

NIR	AG 1 (%)	AG 2 (%)	AG 3 (%)	AG 4 (%)
protein	39.51	39.49	41.24	40.16
oil	20.58	20.61	18.79	19.57
moisture	7.41	7.66	7.93	7.52
% hexane extractables	23.9	24.0	22.8	24.5

soy phytochemical composition between the various bean varieties, the location grown, and the harvest year, due to both genetic and environmental factors (*4*, *5*, *7*, *8*, *47*, *48*, *52*). Accurate measurement is vital for the evaluation of the levels of these compounds in the beans and in products prepared from soy. Reproducible methodology for the accurate and efficient extraction of the phytochemical components from soybeans and processed soy products continues to be a problem that results in large interlaboratory variations in results (*52*). Efficient extraction requires efficient drying processes, grinding methodology to form a fine powder, quantifiable defatting methodology, and exhaustive extraction with the proper solvents over sufficient time.

A key requirement for both the analysis and the assessment of the biological activity of plant phytochemicals is the availability of pure compounds. While most forms of the soy isoflavones are now generally available at least in analytical standard quantities, pure soy saponins have generally not been available. Recent work with a soy processing company has made the more abundant group B soyasaponins commercially available both as a bulk mixture and as purified compounds. The group A soyasaponins are currently not available commercially. It has been observed that group A soyasaponin levels are generally much lower than group B soyasaponin levels in extracts and products prepared from whole soy and that the soy germ was enriched in A group saponins (*5*, *7*, *8*, *47*, *48*).

Generally, most processing results in the damage or fragmentation of the cotyledons contaminating the germ fraction. Freeze drying the soybean results in a dry brittle bean that gentle pressure can fragment into its component parts. The hulls were removed by aeration; the germ and the intact cotyledons were easily separated by sieving through a set of screens. The saponin composition in each fraction was then determined by a modification of the analytical methodology that we have published previously (*39*). Using a shallow gradient of water and acetonitrile with formic acid, we were able to separate and identify the various saponin compounds in prepared extracts from soy germ using ESI-LC-MS in the positive mode. From a table of molecular weights constructed from the literature (**Figure 1**), successful identification of the various A, B, and DDMP group saponins was achieved using their characteristic molecular ions $[M + H]^+$. This methodology was transferred to an analytical HPLC system, and a representative chromatogram is shown in **Figure 3**. Purified soyasaponin I standard (**Figure 2**) was used to prepare a standard curve for the quantitation of the saponins, and a set of group B soyasaponin standards were used to determine retention times (*39*).

Isolation of a purified group A soyasaponin fraction was achieved by extraction of 1 kg of a pure defatted soy germ fraction prepared from freeze-dried soybeans. The methanol extracts obtained by the automated solvent extraction system were clarified by filtration during the cooling and desolvation process to remove protein and carbohydrate contaminants. The dried extracts were resuspended in a methanol/water mixture with sonication, and any remaining solid material was removed by filtration. A 5−10 mL amount of this concentrate was injected on the flash chromatography system, which was monitored at 210 nm while being developed. After the elution of the isoflavones, three distinct saponin-rich peaks were collected. Evaporation of the organic solvent resulted in the crystallization of most of the group B saponins in all three fractions. After freeze-drying to remove the remaining water, the pooled fluffy solid material obtained from each was examined by LC-MS and shown to contain predominately the group A soyasaponins, the group B soyasaponins, and the DDMP group B soyasaponins, respectively. The dried material from the second and third flash column peaks was resuspended in a small volume of methanol, and 5−10 mL of this solution was injected on the preparative HPLC system. Fractions were collected by hand, monitoring the UV at 210 nm. Preparative HPLC afforded several major peaks, which were evaluated by LC-MS. Eight major peaks (A−H), eluting between 18 and 40 min, were shown to contain the soy saponins. Peaks C−H formed white crystals as the acetonitrile evaporated off overnight and were shown by LC-MS to be various group B soyasaponins, roughly in the order soyasaponin V, soyasaponin I, soyasaponins II and III, soyasaponin αg, soyasaponin βg, and soyasaponins βa, γg, and γa. Fractions A and B had to be carefully freeze-dried (in a loosely capped jar to prevent foaming) to remove the water and were shown to contain the A group soyasaponins, mostly soyasaponin aA_1 (**Figure 2**) and an enriched fraction containing soyasaponins aA_7 and aA_2. Soyasaponin aA_4 was detectable but at very low levels in these samples. We were able to obtain about 1 g of light-fluffy white crystals of the group A soyasaponins from about 1 kg of defatted soy germ. This methodology can be scaled up to produce larger quantities for biological studies.

To confirm the identity of the sapogenols, complete acid hydrolysis was performed on a sample of our group A and group B soyasaponins. Monitoring by thin-layer chromatography (TLC) during the process showed that 3 days were required for complete hydrolysis. The sapogenols were recovered by precipitation and analyzed by TLC. The hydrolyzed group A soyasaponins provided a single sapogenol spot with an R_f of 0.4, while the hydrolyzed group B soyasaponins provided two sets of double spots with R_f values of 0.5 and 0.57, respectively. The two sets of B group sapogenols were reisolated by preparative TLC, and all three sapogenol fractions were examined by NMR.

The proton and DEPT spectra obtained matched those of previously published spectra and confirmed the identity of sapogenol A (R_f 0.4) and sapogenol B (R_f 0.5) (*12*, *13*). Examination of the ^{13}C NMR data for the R_f 0.57 fraction showed one peak with a chemical shift of 217.0, corresponding to a carbonyl group (at C-22) and shifts at 136.1 and 134.4 ppm corresponding to two olefinic carbons (at C-21 and C-22), in addition to the olefinic carbon shift at 122.3 ppm from the C-12 carbon in the general saponin structure. This shows this fraction to be a mixture of sapogenol C and sapogenol E. This is in agreement with published work on the DDMP-conjugated saponins, which indicates that the hydrolysis of DDMP results in the formation of the group B, and to a lesser extent the group E, and group C soyasaponins depending on the processing and isolation conditions (*9*, *15*, *16*, *42*, *53*). As the loss of DDMP to form the hydroxyl at the 22 position on sapogenol structure leads to the formation of an asymmetric center, it is probable that the two TLC spots at R_f 0.5 in the hydrolyzed group B soyasaponins are the two isomers of sapogenol B. It is interesting to note that in our previous publication (*39*) we noticed an

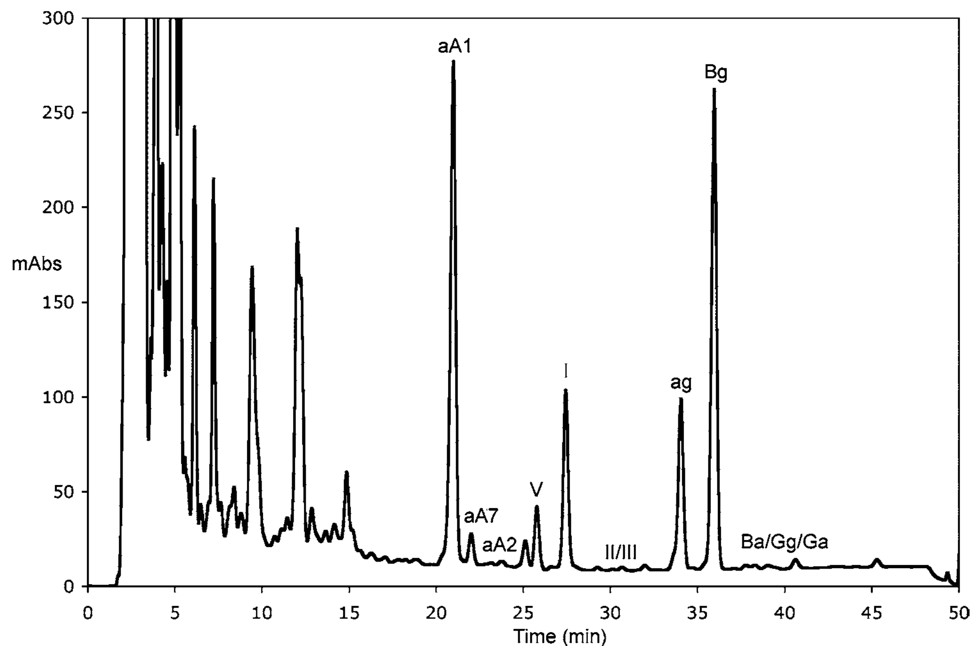

Figure 3. UV trace (210 nm) from a HPLC chromatogram of an ASE extract prepared from defatted soy germ. Identified peaks are (from left to right) soyasaponin acetyl-A_1, soyasaponin acetyl-A_7, soyasaponin acetyl-A_2, soyasaponin V, soyasaponin I, soyasaponin II/III (unresolved), soyasaponin αg, soyasaponin βa, and an unresolved series of peaks containing soyasaponins βa, γg, and γa.

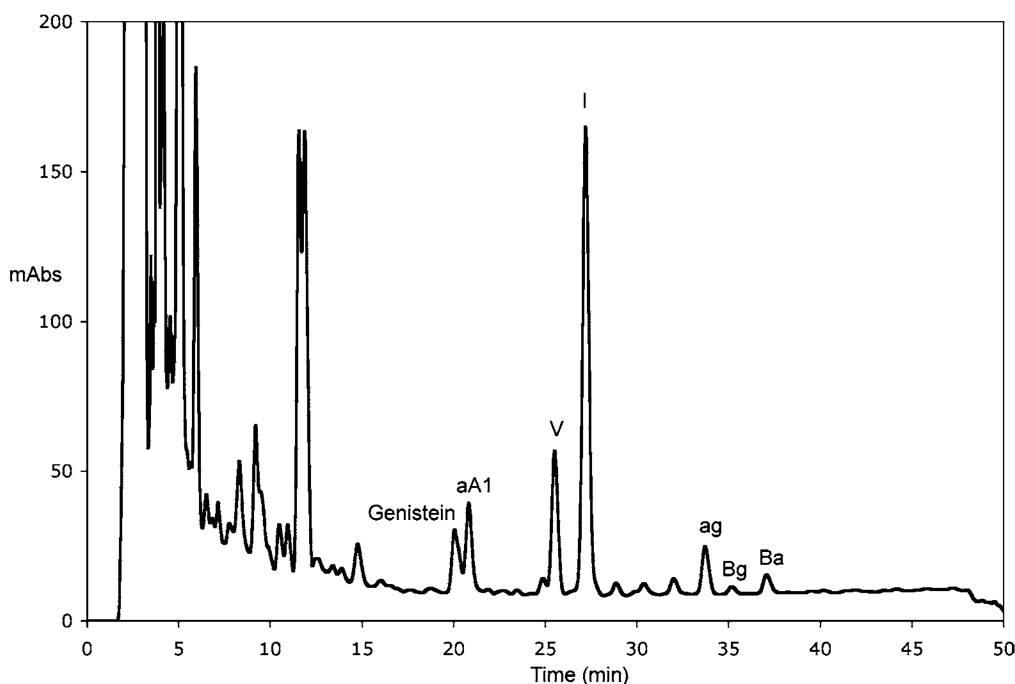

Figure 4. UV trace (210 nm) from a HPLC chromatogram of an extract prepared from ground, defatted whole soy, which had been stored at room temperature for 6 months. Identified peaks are (from left to right) genistein, soyasaponin acetyl-A_1, soyasaponin V, soyasaponin I, soyasaponin αg, soyasaponin βg, and soyasaponin βa.

asymmetric soyasaponin I peak, which gives a uniform mass spectrum, in the analysis of the intact group B saponins, which has been observed by other researchers (*24*).

We then examined the saponin and isoflavone compositions in both whole soybeans and the soybean components. Care must be taken to correctly identify the saponin peaks in the HPLC chromatogram as there are a few contaminating peaks in the area at which the group A saponins elute. As shown in **Figure 4**, a peak sometimes appears and elutes just before soyasaponin aA_1. This was shown to be the isoflavone aglycone genistein by spiking a sample with pure genistein. Genistein is often found in heavily processed soy products that have been exposed to high temperatures and strong acidic or basic conditions.

As anticipated, the soy germ had the predominant concentrations of the group A soyasaponins but also a large proportion of the group B soyasaponins, as shown in **Table 2**. The phytochemical concentrations can vary considerably as shown by the comparison of the analysis of germ and cotyledon prepared from soybeans harvested by the same farmer but batches from two different years (summarized in **Table 4**). The soybeans from the year 2004 harvest had nearly double the phytochemical concentrations than those from the year 2005

Table 2. Saponin Concentrations (mg/g Dry Weight) in Defatted Soy Hulls, Germ, and Cotyledons from Commercial Soybeans Grown and Harvested in 2004 and 2005 in Tazewell County, IL[a]

saponins (mg/g)	2004 hulls	2004 cotyledon	2004 germ	2005 hulls	2005 cotyledon	2005 germ
soyasaponin I		0.79 ± 0.03	5.86 ± 0.04	0.23 ± 0.05	1.50 ± 0.07	4.76 ± 0.18
soyasaponin II		0.22 ± 0.02	0.13 ± 0.01		0.48 ± 0.01	0.07 ± 0.02
soyasaponin III		0.04 ± 0.04			0.08 ± 0.01	0.07 ± 0.01
soyasaponin IV		0.15 ± 0.02	0.08 ± 0.01		0.10 ± 0.01	0.30 ± 0.01
soyasaponin V		0.17 ± 0.01	2.24 ± 0.02		0.09 ± 0.02	1.59 ± 0.09
soyasaponin βg		2.94 ± 0.06	17.16 ± 0.06		1.53 ± 0.09	2.98 ± 0.20
soyasaponin βa		1.21 ± 0.06	0.21 ± 0.01		0.55 ± 0.03	
soyasaponin γg			0.23 ± 0.02			
soyasaponin γa						
soyasaponin αg		0.67 ± 0.01	8.78 ± 0.06		0.31 ± 0.01	0.06 ± 0.03
soyasaponin aA_1	0.23 ± 0.01	0.32 ± 0.03	27.87 ± 0.19	0.26 ± 0.15	0.40 ± 0.01	12.98 ± 0.62
soyasaponin aA_2				0.17 ± 0.02	0.13 ± 0.03	0.95 ± 0.08
soyasaponin aA_4						0.93 ± 0.05
soyasaponin aA_7	0.02 ± 0.04	0.24 ± 0.08	6.24 ± 0.38		0.24 ± 0.02	1.21 ± 0.08
total in defatted (mg/g)	0.27 ± 0.01	4.44 ± 0.07	68.60 ± 0.42	1.71 ± 0.24	5.53 ± 0.23	28.10 ± 0.99
total in whole meal (mg/g)	0.25	4.05	54.19	1.61	4.40	24.59
% by weight in whole meal	0.03%	0.40%	5.42%	0.16%	0.44%	2.46%

[a] Samples were run in triplicate, and the average values are shown with standard deviations. A blank cell indicates that levels were below detectable limits.

Table 3. Isoflavone Concentrations (mg/g Dry Weight) in Defatted Soy Hulls, Germ, and Cotyledons from Commercial Soybeans Grown and Harvested in 2004 and 2005 in Tazewell County, IL[a]

isoflavones (mg/g)	2004 hulls	2004 cotyledon	2004 germ	2005 hulls	2005 cotyledon	2005 germ
daidzin		1.08 ± 0.01	6.53 ± 0.08	trace	0.62 ± 0.02	3.10 ± 0.08
glycitin			6.79 ± 0.21		trace	4.64 ± 0.15
genistin	0.05 ± 0.04	1.65 ± 0.04	2.53 ± 0.02	trace	0.97 ± 0.03	1.66 ± 0.01
6′′-*O*-acetyl-daidzin		0.20 ± 0.01	0.51 ± 0.24		trace	trace
6′′-*O*-acetyl-glycitin			0.86 ± 0.01			trace
6′′-*O*-acetyl-genistin		0.29 ± 0.01	–		trace	trace
6′′-*O*-malonyl-daidzin		0.60 ± 0.03	2.25 ± 0.04		0.01 ± 0.02	0.48 ± 0.01
6′′-*O*-malonyl-glycitin			2.47 ± 0.05			0.92 ± 0.02
6′′-*O*-malonyl-genistin		0.54 ± 0.01	0.60 ± 0.02		0.01 ± 0.01	0.26 ± 0.02
daidzein		0.08 ± 0.01	0.54 ± 0.01			
glycitein			0.55 ± 0.04			
genistein			0.15 ± 0.01			
total defatted meal (mg/g)	0.05 ± 0.04	4.44 ± 0.07	23.79 ± 0.60	trace	1.61 ± 0.05	11.06 ± 0.25
total in whole meal (mg/g)	0.05	3.51	20.70		1.28	9.67
% by weight in whole meal	trace	0.35%	2.07%		0.13%	0.97%

[a] Samples were run in triplicate, and the average values are shown with standard deviations. A blank cell indicates that levels were below detectable limits.

Table 4. Percent Contribution of Each Bean Component to the Phytochemical Concentration in Whole Beans Harvested in Two Different Growing Seasons

	2004 isoflavones in whole (%)	saponins in whole (%)	2005 isoflavones in whole (%)	saponins in whole (%)
hulls	trace	trace	trace	0.01
germ	0.06	0.11	0.03	0.08
cotyledon	0.28	0.32	0.12	0.40
total bean	0.33	0.44	0.15	0.49

harvest, which may have been due in part to the variation in the severity of drought conditions in central Illinois during those years. Analysis of four varieties of soybeans grown on the same farm in central Illinois in 2003 showed a lesser degree of saponin concentration variation (**Table 5**).

The extraction methodology presented here works equally well for the soy isoflavones. Complete analysis of the isoflavone contents of the samples examined is presented in **Tables 3** and **6** for comparison. The extraction at 50 °C for 3 days promotes the degradation of the malonyl forms of the isoflavonoids, as well as the DDMP forms of the group B saponins. This degradation is a necessary trade off to get more exaustive extraction of the isoflavone and saponin contents from the plant marc. The distribution of isoflavone forms seen by this extraction method is an artifact of the conditions chosen and is not exactly what was present in the intact beans. Given the severe degradation of the isoflavone malonates in these extracts, it is significant to note that the group A saponins did not seem to be degraded by the extraction, as the acetylated groups of soyasaponin aA_1 were apparently not hydrolyzed to a large degree, although deacetylated forms of soyasaponin aA_1 could be detected in the LC-MS (data not shown).

There are a number of reports in the literature of simultaneous determination of isoflavone and saponin levels in soy samples (*8*, *35*, *49*−*51*). We feel that our methodology is rigorous enough to accurately measure both classes of compounds in a single extract but as separate HPLC runs. This methodology is a variation of our standard plant phenolic analytical procedures developed for the analysis of flavonoids in citrus (*54*), which can be applied to the analysis of isoflavonoids (*33*). We used a detection wavelength of 280 nm as part of our general routine and have no trouble with quantitation, but to optimize and detect lower levels, it may be best to shift the detection wavelength to 260 nm, which is closer to the absorption maximum of the soy isoflavones. Recently, this method of extraction and isoflavone analysis was performed on the same sample in two Brazilian EMBRAPA labs in Londrina and Rio DeJanerio, as

Table 5. Saponin Concentrations in Defatted Samples of Four Soybean Cultivars Grown on the Same Farm Field in Central IL in 2003[a]

saponins	AG 1 (mg/g)	AG 2 (mg/g)	AG3 (mg/g)	AG4 (mg/g)
		B group		
soyasaponin I	1.69 ± 0.07	1.75 ± 0.05	1.92 ± 0.15	1.77 ± 0.03
soyasaponin II	0.39 ± 0.02	0.44 ± 0.01	0.42 ± 0.03	0.44 ± 0.01
soyasaponin III	0.08 ± 0.01	0.12 ± 0.01	0.08 ± 0.01	0.11 ± 0.01
soyasaponin IV	0.09 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.14 ± 0.01
soyasaponin V	0.09 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.18 ± 0.23
soyasaponin βg	1.62 ± 0.06	1.49 ± 0.05	1.74 ± 0.11	0.51 ± 0.03
soyasaponin βa	0.43 ± 0.02	0.44 ± 0.02	0.46 ± 0.01	0.10 ± 0.03
soyasaponin γg				
soyasaponin γa				
soyasaponin αg	0.038 ± 0.02	0.13 ± 0.03	0.43 ± 0.02	1.13 ± 0.10
		A group		
soyasaponin aA_1	0.42 ± 0.03	0.53 ± 0.02	0.71 ± 0.43	1.13 ± 0.10
soyasaponin aA_2	0.20 ± 0.04	0.27 ± 0.12	0.12 ± 0.21	0.29 ± 0.25
soyasaponin aA_7	0.45 ± 0.05	0.47 ± 0.21	0.47 ± 0.03	
total saponins in defatted meal	5.86 ± 0.29	6.02 ± 0.15	6.55 ± 0.63	6.58 ± 0.05
total saponins in whole bean	4.73	4.85	5.33	5.75
% saponins in whole bean	0.47%	0.48%	0.53%	0.57%

[a] Samples were run in triplicate, and the average values are shown with standard deviations. A blank cell indicates that levels were below detectable limits.

Table 6. Isoflavone Concentrations in Defatted Samples of Four Soybean Cultivars Grown on the Same Farm Field in Central IL in 2003[a]

isoflavones	AG1 (mg/g)	AG2 (mg/g)	AG3 (mg/g)	AG4 (mg/g)
daidzin	1.45 ± 0.13	1.63 ± 0.05	1.99 ± 0.19	1.70 ± 0.03
glycitin	0.04 ± 0.06	0.06 ± 0.04		0.01 ± 0.01
genistin	1.85 ± 0.06	2.07 ± 0.04	1.56 ± 0.07	1.63 ± 0.03
6′′-*O*-acetyl-daidzin	trace	trace	trace	trace
6′′-*O*-acetyl-glycitin				
6′′-*O*-acetyl-genistin	trace	trace	trace	trace
6′′-*O*-malonyl-daidzin	0.12 ± 0.04	0.17 ± 0.04	0.20 ± 0.01	0.24 ± 0.02
6′′-*O*-malonyl-glycitin				
6′′-*O*-malonyl-genistin	0.17 ± 0.02	0.26 ± 0.04	0.11 ± 0.02	0.16 ± 0.02
total isoflavones in defatted meal	3.65 ± 0.27	4.20 ± 0.19	3.86 ± 0.27	3.74 ± 0.03
total isoflavones in whole meal	2.94	3.38	3.14	3.27
% isoflavones in whole bean	0.29%	0.34%	0.31%	0.33%

[a] Samples were run in triplicate, and the average values are shown with standard deviations. A blank cell indicates that levels were below detectable limits.

well as here in Peoria. The total isoflavone levels obtained agreed within a standard error of ±0.05%.

The methodology presented here is an accurate and reproducible one-step extraction procedure for the accurate analysis of total isoflavones and total saponins in soybean samples by HPLC. Less rigorous extraction methods can be used for the analysis of processed products from soy. Any accurate HPLC methodology can be used for quantitation of the soy isoflavones as long as the various malonyl and acetyl forms are eliminated by hydrolysis or accounted for in the chromatogram. Our saponin analysis has been expanded to include the A group soyasaponins, giving a more accurate picture of the total saponin content in soybeans and soy products. The data presented here show that soy germ contains a large percentage of the total soy saponin content. In general, our standard deviations run between 1 and 5% for samples containing 10 mg/g or more of any given phytochemical species.

The current literature points to the accumulation of the DDMP group B soyasaponins and the group A soyasaponins as the final natural product in the intact soybean seed. Variation of saponin composition occurs from the substitution of sugars in the group A and B soyasaponins as well as the possible incomplete addition of all of the sugar moieties to the oligosaccharide chains as the bean matures and the biosynthetic processes come to a halt in the mature seed. The location of storage (in either the vacuole, the cytoplasm, or the cell wall), coupled with pH factors and variations in temperature during storage, may contribute to low rates of hydrolysis during seed storage, possibly increasing the number of saponin forms in older soybeans. This degradation is enhanced during the process of extraction and sample preparation. Loss of the DDMP group, which is relatively easy depending on the heat and pH conditions of the extraction and possibly some of the other sugar conjugates, results in the formation of the group B soyasaponins, the most common form found in processed soy products. The group A soyasaponins reside predominately in the soy germ, and as the soy germ only contributes 3 wt % of the total bean, the levels of A group soyasaponins are relatively low in extracts and products prepared from whole beans, about one-fifth the levels of the B group soyasaponins.

The preparation of pure analytical standards is laborious and difficult due to the large numbers of very similar saponin glycosides. We have developed a reliable method for the preparation of soybeans for reproducible extraction of both isoflavones and saponins, which can be scaled up to produce useful quantities of pure standards for analysis and biological assay.

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