Generation of Group B Soyasaponins I and III by Hydrolysis

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Soyasaponins are a group of oleanane triterpenoids found in soy and other legumes that have been associated with some of the benefits achieved by consuming plant-based diets. However, these groups of compounds are diverse and structurally complicated to chemically characterize, separate from the isoflavones, and isolate in sufficient quantities for bioactive testing. Therefore, the aim of this study was to maximize the extraction of soyasaponins from soy flour, remove isoflavones, separate group B soyasaponins from group A, and produce an extract that contained a majority of non-DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one)-conjugated group B soyasaponins I and III. Room temperature extraction in methanol for 24 or 48 h resulted in the maximum recovery of soyasaponins, and Soxhlet extraction resulted in the least. A solid-phase extraction using methanol (45%) was found to virtually eliminate the interfering isoflavones as compared to butanol−water liquid−liquid extraction and ammonium sulfate precipitation, while maximizing saponin recovery. Alkaline hydrolysis in anhydrous methanol produced the maximum amount of soyasaponins I and III as compared to aqueous methanol and acid hydrolysis in both aqueous and anhydrous methanol. The soyasaponin I amount was increased by 175%, and soyasaponin III was increased by 211% after alkaline hydrolysis. Furthermore, after alkaline hydrolysis, a majority of DDMP-conjugated group B soyasaponins such as βg, βa, γg, and γa transformed into the non-DDMP-conjugated soyasaponins I and III without affecting the glycosidic bond at position C-3 of the ring structure. Therefore, we have developed a method that maximizes the recovery of DDMP-conjugated saponins and uses alkaline hydrolysis to produce an extract containing mainly soyasaponins I and III.

KEYWORDS: Soyasaponin I; soyasaponin III; DDMP; soyasaponins; SPE; soy

INTRODUCTION

Soyasaponins have garnered a great deal of attention due to their potential health-promoting functions, particularly related to the chemopreventative (*1, 2*) and cholesterol-lowering properties (*3*). Specific soyasaponins have been reported to possess bioactive properties in cell culture experiments inducing apoptosis and altering cell membrane characteristics (*4−6*). Soyasaponins are oleanane triterpenes that are classified based on two main soyasapogenol (aglycone) structures and are found in various glycoside forms in soya products and other legumes such as green peas and lentils (*7−9*). Group B soyasaponins have been reported to be the most abundant group of soyasaponins (*10*), and the total saponin content is generally between 1.8 and 4.4%, depending on the variety and cultivation conditions (*11*). Some group B soyasaponins also have a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) moiety conjugated at position 22 of the ring structure, resulting in soyasaponins β_g, β_a, γ_g, and γ_a (**Figure 1**). Structurally related non-DDMP-conjugated soyasaponins such as I, II, III, IV, and V may be artifacts of extraction, which have been reported to be dependent on solvent, temperature, and storage time (*7, 11*). Group B

soyasaponins from lentils and chickpeas have been reported to be partially degraded into soyasaponin I during cooking (*8*). Soyasaponin β_g is likely heat labile and was found to significantly decrease when heated at 65 °C, while the corresponding concentration of non-DDMP soyasaponin I increased proportionally (*11*). When the alcoholic extract of soy hypocotyls was heated at 80 °C for 5 h, Kudou (*12*) observed that soyasaponins α_g and β_g converted into soyasaponins V and I, respectively. Soyasaponin β_g has been reported to be stable at acidic pH but rapidly degraded into soyasaponin I at basic pH or in the presence of ferric chloride ($FeCl_3$) (*13*). These reports suggest that to produce specific soyasaponins, either DDMP or non-DDMP counterparts require specific control of extraction pH, temperature, and solvent conditions. Non-DDMP group B soyasaponins such as soyasaponin I have been reported to be bioactive (*4, 14*). However, group B soyasaponins' chemistry and bioactivity have not been fully characterized due to the complex structure, artifacts (*15*), and labor-intensive purification and isolation of these compounds.

The objectives of this research are to compare extraction conditions and remove interfering isoflavones from soyasaponins found in soy flour using a variety of extraction techniques, solid-phase extraction (SPE), high-performance liquid chromatography (HPLC), mass spectrometry (MS), and acid and base

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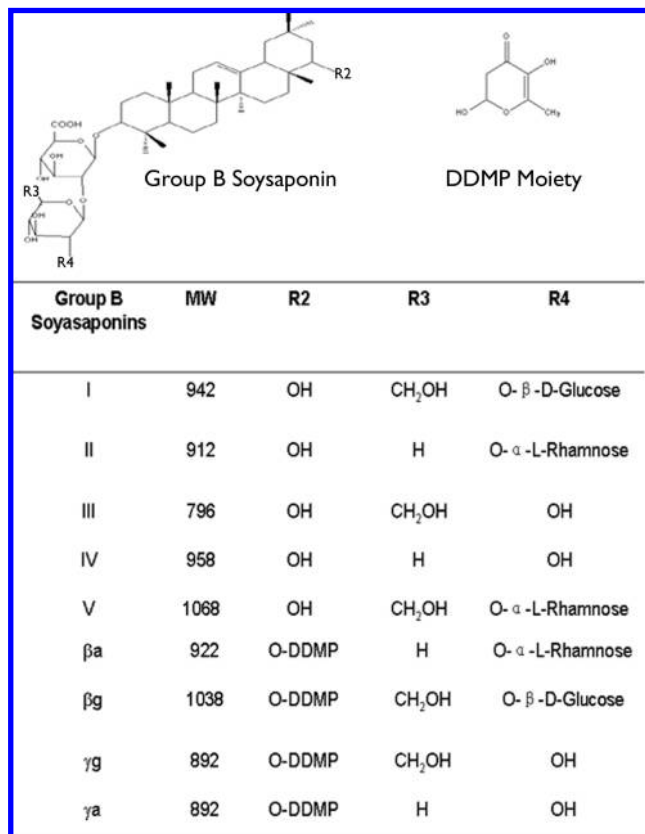
Figure 1. Structural characteristics and molecular weight of DDMP and non-DDMP group B soyasaponins. The DDMP moiety is conjugated at position R2 or carbon 22 of the structure. Be is a group E soyasaponin that has been reported to be an artifact of group B soyasaponin formed during extraction (*10*).

hydrolysis, to determine which conditions are optimal for obtaining an extract primarily containing soyasaponins I and III from soya flour.

MATERIALS AND METHODS

Commercial standards of soyasaponins I and III and soyasapogenol B were obtained from Apin Chemicals (Oxfordshire, United Kingdom). Isoflavones daidzin, glycitin, and genistin standards were obtained from Sigma (St. Louis, MO). Soyasaponins Be, β_g, β_a, γ_g, γ_a, and acetyl-daidzin and acetyl-genistin were identified as reported in the literature (*24–26*).

Soy Flour Extraction Methods Comparison. Defatted soy flour (10 g) was obtained from Archer Daniels Midland Co. (Decatur, IL) and refluxed in methanol (50 mL) for 4 h at 60 °C (*16*). After it was refluxed, the mixture was filtered, and the methanol extract was evaporated to 10 mL. An aliquot (250 μL) was collected and passed through 0.45 μm pore size nylon membrane filter (Millipore, Billerica, MA) before it was diluted with methanol to 2 mL for chromatographic analysis.

Ultrasonic extraction was carried out in Elmasonic D-78224 S60H sonicator (Elma, Germany) with a frequency of 50 Hz and a mean power of 550 W. Soy flour (10 g) was ultrasonically extracted in methanol (50 mL) twice, each time for 2 h (*17*). After extraction, the mixture was filtered, and the methanol extract was evaporated to 10 mL. An aliquot (250 μL) was collected as described above.

Soxhlet extraction was carried out in an automatic 2050 Soxtec Soxhlet (Foss, Sweden) extractor with a 2050 Soxtec Avanti Extract unit, 2050 Soxtec Avanti Control unit, and 2050 Avanti Drive unit. Soy flour (10 g) was boiled in methanol for 1.5 h before beginning the Soxhlet extraction (4 h). After extraction, the methanol extract was evaporated to 10 mL in a volumetric flask, and an aliquot (250 μL) was collected as described above.

Soy flour (10 g) was extracted in methanol (50 mL) with stirring for 24 and 48 h at room temperature. After extraction, the mixture was filtered and evaporated to 10 mL and 250 μL of the aliquot as described above.

HPLC Analysis of Soyasaponins and Soy Isoflavones. A Waters (Milford, MA) 2695 Separation Module with Waters 2996 Photodiode Array (PDA) detector HPLC system was used. The column used was a Shimadzu reversed phase C-18, 5 μm, 250 mm × 4.6 mm internal diameter (Shimazu, Shim-pack XR-ODS, United States). The mobile phases were 0.025% acetic acid (glacial, ACS certified, Fischer Scientific, Pittsburgh, PA) in water (solvent A) and 0.025% acetic acid in acetonitrile (solvent B) (Merck, Germany). The flow rate of the mobile phase was 1 mL/min with the column temperature set at 25 °C, and the detection wavelength was set at 210 nm.

In addition, external primary standard soyasapogenol B (Apin Chemical) concentrations of 10–50 μg/mL were utilized to obtain the calibration curve for the quantification of total soyasaponins present in the extracts. A stock solution of 1000 μg/mL of the standard was prepared and serially diluted. The elution gradient program of the analysis of total soyasaponins has been previously described (*6*).

LC/MS Analysis of Total Soyasaponins and Soy Isoflavones. Samples were analyzed on a Thermo Finnigan LCQ-ESI quadrapole ion trap LC-MS (Thermo Fisher Scientific, Waltham, MA) system with a Surveyor HPLC system coupled to a Finnigan AS3000 autosampler and using the Xcaliber 2.0 software system. The MS was run with the ESI probe in both the positive and the negative modes. The column used was a Waters reversed phase C-18, 5 μm, 250 mm × 4.6 mm internal diameter. The voltage applied for the ionization of sample was 4.5 kV. 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 (*18*), and a full scan mass spectrum was obtained over the range of 100–1500 m/z. The mobile phases and LC program were identical to the HPLC analysis and are described above.

Comparison of Soyasaponin Concentration Methods. Soy flour was extracted in methanol for 24 h at room temperature as described above and further subjected to three extraction methods described below. Each 100 mL of the aliquot of the total soyasaponin extract in methanol was evaporated to dryness under reduced pressure and dispersed in butanol–water in a ratio of 1:1 by volume. The mixture was shaken and allowed to stand overnight. The butanol organic layer was collected and evaporated to dryness under reduced pressure before dispersing it in methanol (*18*).

Aliquots (100 mL) of total soyasaponins extract were mixed in a ratio of 1:1 with 0.4 M ammonium sulfate and allowed to stand overnight at 4 °C (*16*). The mixture was then centrifuged at 450g for 5 min at 4 °C to remove any precipitate. The supernatant was then evaporated to 50%, and the total volume of the sample was made up to 200 mL with deionized water. Ammonium sulfate was added to bring the solution to a concentration of 3 M. The solution was then allowed to stand overnight at 4 °C. The precipitate was collected by centrifuging the mixture at 450g for 5 min at 4 °C. The residue was then dissolved in methanol and centrifuged as described above to remove the precipitate.

The total soyasaponin extract (100 mL) in methanol was diluted with water to a concentration of 40% methanol. A 10 g C-18 Sep-Pak (Phenomenex, Torrance, CA) cartridge was first washed with methanol before conditioning with water, and the samples were applied. The cartridge was washed with water (150 mL) to remove any traces of carbohydrates and washed using 45% methanol (200 mL) to remove any isoflavones (*16*). Soyasaponins were eluted by 70% methanol. Soyasaponins (70% methanol) were diluted to 30% methanol and reloaded on the SPE column. Absolute methanol was used to elute total soyasaponins.

Comparison of Hydrolysis Conditions to Generate Soyasaponins I and III. Total soyasaponins from 100 g of soy flour were obtained from room temperature extraction with SPE concentration as described above. Samples were loaded onto a SPE column described above, the isoflavones were removed with methanol (45%), and group A soyasaponins were removed with a 50% methanol wash. To elute group B soyasaponins, 100 mL of 80% methanol was used. The cartridge was then washed with absolute methanol to remove any nonpolar compound. Group B soyasaponins in 80% methanol were diluted

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to 50% and reloaded to SPE column and then eluted by 50 mL of absolute methanol.

Concentrated hydrochloric acid [5% (v/v)] was added to 10 mL of the aliquot containing group B soyasaponins, which was then suspended in aqueous methanol. The mixture was hydrolyzed for 30 min at temperatures of 80 and 100 °C in a temperature-controlled water bath and 120 °C in an oven in sealed tubes. After hydrolysis, the solution was diluted with water until the concentration of methanol was 50% and applied to Phenomenex 10 g C-18 Sep-Pak cartridge, which was preconditioned with deionized water. The cartridge was washed with water (200 mL), and the hydrolyzed solution was eluted with methanol (*16*). The hydrolysis was repeated again in anhydrous methanol as described above. The base hydrolysis of group B soyasaponins was similar to the method described above for the acid hydrolysis except that the acid was replaced by sodium hydroxide [5% (v/v)].

HPLC and ESI-MS Analysis of Hydrolyzed Group B Soyasa-ponins. The HPLC system was described above, and the mobile phase consisted of 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The flow rate of the mobile phase was 1 mL/min with the column temperature set at 35 °C. The elution gradient conditions of the separation of soyasaponins were as follows: Solvent A was decreased from 60 to 52% over 60 min, and the UV absorbance was set at 210 nm (*19*).

The LC/MS system and conditions are described above for the soyasaponins analysis with the following changes: The column used was a Shimadzu reversed phase C-18, 5 μm, 250 mm × 4.6 mm internal diameter, and the source inlet temperature was set at 200 °C. The mobile phase was 2.5% acetic acid in water (solvent A) and acetonitrile (solvent B), and the condition of elution was the same for the HPLC analysis of the hydrolyzed sample described above.

The external standard calibration curves using soyasapogenol B authentic standard resulted in two equations corresponding to two different HPLC programs described above; they are $y = 221451x - 700899$ and $y = 237234x + 89086$ for total soyasaponins and soyasaponins I and III, respectively. The curves were linear over the concentration tested with square correlation coefficients of $r^2 = 0.991$ for total soyasaponins and $r^2 = 0.994$ for soyasaponins I and III. Both calibration plots of standards and samples were constructed by HPLC areas from triplicate injections. To investigate the accuracy of the method, three repeated analyses of samples were performed to evaluate within-day variation, and two repeated analyses of samples including an extraction procedure at two different days by two different laboratory personnel were performed to evaluate the day-to-day and person-to-person variation. Statistical analysis used in these experiments consisted of analysis of variance testing with Tukey's posthoc comparison of means and a Student's paired *t* test with significance judged at *P* < 0.05.

RESULTS AND DISCUSSION

Extraction Methods Comparison. All four extraction techniques tested were able to extract crude soyasaponins and isoflavones found in soya flour. The main compounds extracted were identified as isoflavones such as daidzin, glycitin, genistin, and soyasaponins I, III, Be, β_g, β_a, γ_g, and γ_a. Interestingly, when crude or total soyasaponins amounts were measured after Soxhlet extraction in methanol, they yielded significantly (*P* < 0.05) lower amounts of soyasaponins as compared to all other extraction techniques, while room temperature stirring in methanol yielded significantly (*P* < 0.05) higher amounts. Although room temperature extraction for 48 h yielded the greatest amount, the 48 h extraction was not found to be significantly different from the 24 h extraction. The amounts of soyasaponins extract from lowest to highest were found to be Soxhlet < reflux < sonication < 24 h of stirring at room temperature < 48 h of stirring (**Figure 2**). A difference of slightly greater than 70 mg of soyasaponins per 100 g soy flour was obtained from room temperature as compared to Soxhlet extraction. The differences in the amount of total soyas-aponins obtained from the different extraction techniques
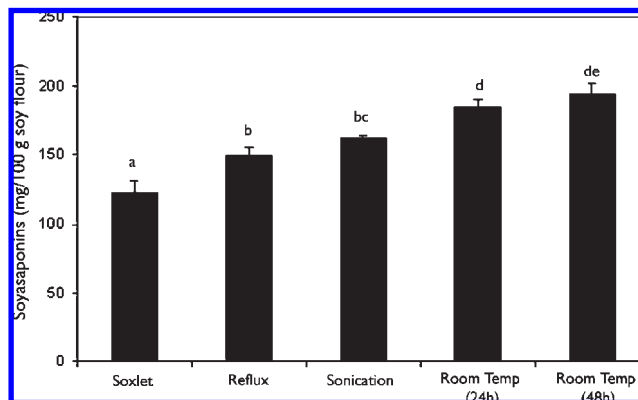
Figure 2. Effect of three different extraction techniques on the total amount of soyasaponins recovered. Values are expressed as means ± SDs, and bars with different subscripts letters are significantly different (*P* < 0.05) from each other. Samples were extracted on three separate occasions and analyzed three times. Total soyasaponins were calculated based on the external soyasapogenol B standard described in the Materials and Methods.

may be attributed to the presence of heat during the extraction process. Yang et al. reported that increasing the temperature from 10 to 60 °C increased the extraction yield of ultrasound-assisted extraction of soy hypocotyls (*20*). Our results indicate that applying heat to the extraction of soya flour during Soxhlet and reflux extraction likely caused deglycosylation of saponin glycosides as compared to room temperature extraction. A similar finding was reported for ginseng saponins extracted in boiling water (*21*). The oleanane structure of soyasaponins is also prone to fragmentation and breaking during electron ionization MS analysis at high temperature (*22*). Additional thermal energy would also transform the DDMP-conjugated group B soyasaponins such as β_g into in the corresponding non-DDMP soyasaponin I (*12*). Alternatively, room temperature extraction maximized the amount of DDMP soyasaponins recovered, which can be useful for experiments to generate soyasaponin I or III by hydrolysis or heat.

Comparison of Soyasaponin Concentration Methods. Soy iso-flavones are the main interfering compounds during soyasapo-nin separation and analysis. In this study, three methods, liquid−liquid (butanol−water), ammonium sulfate (3 M) precipitation, and SPE (45% methanol), were tested to remove isoflavones with the aim of maximizing the amount of soyasaponins. **Figure 3** shows the HPLC analysis of both the isoflavones and the soyasaponins present in three extraction methods. In this study, we have utilized two HPLC gradient programs: one to maximally resolve interfering isoflavones and one designed to maximally resolve soyasaponins. Isoflavones daidzin, glycitin, genistin, acetyl-daidzin, and acetyl-genistin were identified in this extract (**Figure 3a**), and the molecular weights were confirmed by MS. The use of ammonium sulfate (3 M) has been reported to help precipitate the soyasaponins (*16*) from the interfering compounds. However, as the HPLC chromatograph in **Figure 3b** indicates, there was no major difference in the overall profile of the chromatograph of the isoflavones with the exception of acetyl-daidzin, which was not detected, whereas a reduction of the peak area associated with soyasaponins was noted. We utilized a gradient SPE separation method directly from a crude methanol extract to maximize the soyasaponins and remove the isoflavones. A SPE column wash with methanol (45%) was sufficient to virtually eliminate most of the competing isoflavones and resulted in the greatest amount of soyasaponins out of the three concentration

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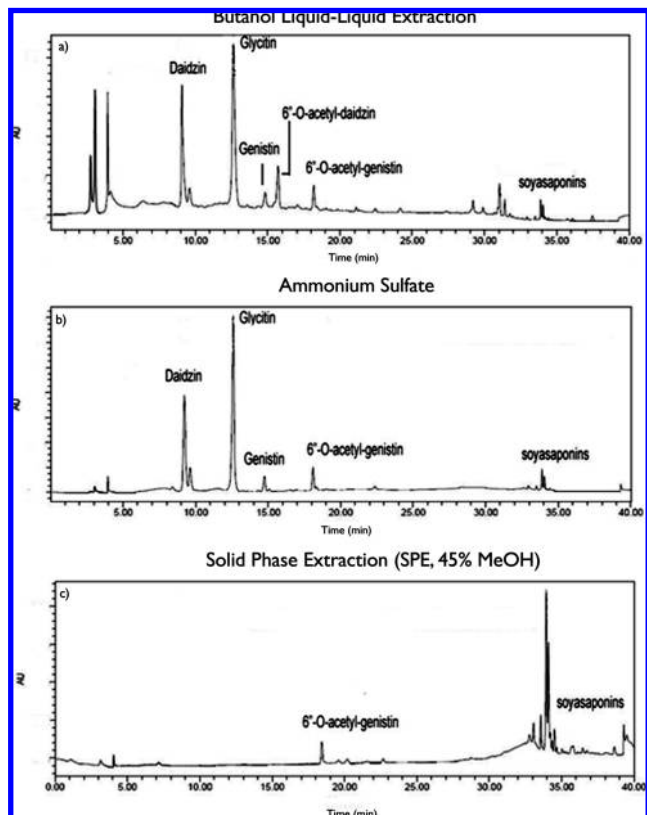
Figure 3. HPLC chromatographs of three different procedures to remove interfering isoflavones. Panel **a** utilized a butanol—water liquid—liquid extraction, panel **b** shows the effect of ammonium sulfate (3 M) precipitation, and panel **c** employed a SPE as described in the Materials and Methods.


Figure 4. HPLC chromatograph of the group B soyasaponins (**a**) obtained from SPE (refer to **Figure 3c**), followed by the removal of group A soyasaponins (refer to the Materials and Methods). Panels **b** and **c** represent saponins following acid or alkaline hydrolysis as described in the Materials and Methods.

methods tested. **Figure 3c** shows only one small peak corresponding to acetly genistin ($<1\%$), and an increase in peak area corresponding to soyasaponins was observed. SPE concentration was employed to further separate the soyasaponins in further experiments reported herein.

Hydrolysis of Group B Soyasaponins. By employing a gradient for the SPE concentration described above, isoflavones were successfully removed from soyasaponins. One additional methanol (50%) wash was found to eliminate all group A soyasaponins, which resulted in a concentrated extract of group B soyasaponins containing soyasaponins I, III, Be, β_g, β_a, γ_g, and γ_a (**Figure 4a**). Individual molecular weights were confirmed by LC/MS. The most abundant molecular ions of all six group B soyasaponins and one group E soyasaponin (Be) were observed as $[M + H]^+$. The concentrated group B soyasaponins were further subjected to both acid and base hydrolysis at different environmental temperature conditions in both methanol and anhydrous methanol to determine the optimal conditions to produce soyasaponins I and III.

We hoped to increase soyasaponins I and III by targeting the removal of the DDMP moiety from the structure without interfering with the glycosidic bond at position C3 of the structure. Soyasaponin I has been reported to be bioactive in cell culture experiments by possibly modifying invasive behavior (*4, 5*), and soyasaponin III, structurally related to soyasaponin III, may possess similar properties. **Figure 4b,c** shows that base hydrolysis provides a better control of the hydrolysis process, while producing more soyasaponins I and III as compared to acid hydrolysis. Partial alkaline hydrolysis (NaOH) to remove the DDMP moiety has been reported to quantify non DDMP soyasaponins (*23*). Especially, **Figure 5** shows that base
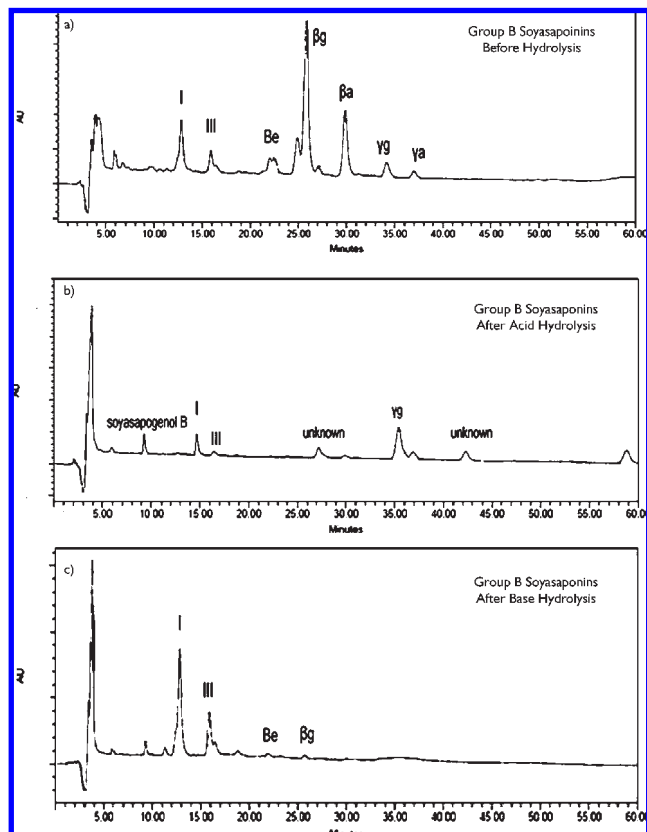
hydrolysis in anhydrous methanol produced the greatest amounts of both soyasaponins I and III and showed a maximal amount at 80 °C as compared to acid hydrolysis. Anhydrous conditions have been reported to produce the maximum amount of authentic soyasapogenols A and B during complete hydrolysis (*10*) but to our knowledge has not specifically been used to produce soyasaponins I and III.

The amounts of soyasaponin I produced by anhydrous methanol alkaline hydrolysis increased the level of soyasaponin I by approximately 32 mg/100 g soy flour or a percentage increase of 175%. Soyasaponin III showed a similar increase of 16 mg increase corresponding to a 211% change. Interestingly, increasing the environmental temperature had different effects on soyasaponins I and III during acid hydrolysis. Soyasaponin I significantly ($P < 0.05$) decreased as the environmental temperature increased in both methanol and anhydrous methanol. An increased temperature significantly ($P < 0.05$) increased soyasaponin III in methanol but reached a maximum at 100 °C. Analysis of the transition from DDMP-conjugated group B soyasaponins to non-DDMP soyasaponins I and III is shown in **Figure 6**.
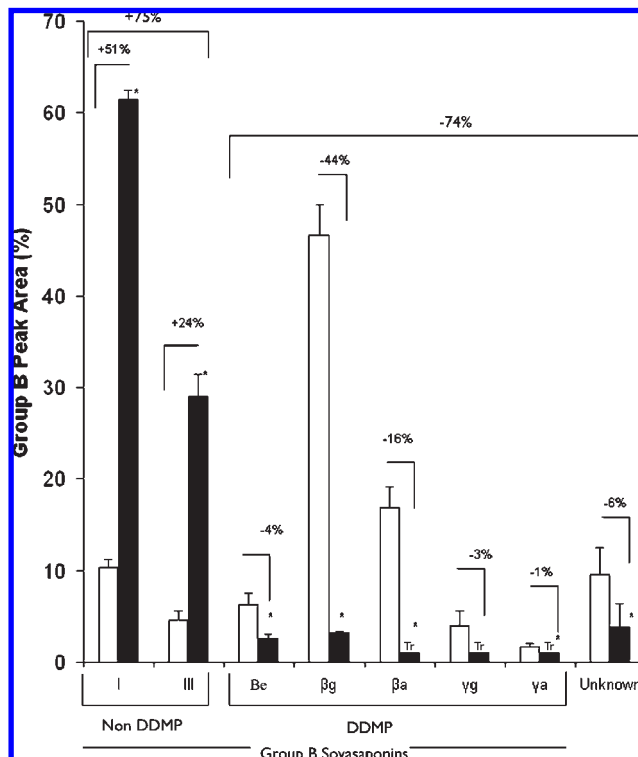
Four DDMP group B soyasaponins, β_g, β_a, γ_g, and γ_a, unidentified peaks, and soyasaponins I and III were monitored before and after base hydrolysis in anhydrous methanol. Soyasaponins I and III both significantly ($P < 0.05$) increased as compared to before hydrolysis, while Be, β_g, β_a, γ_a, and the unidentified peaks significantly ($P < 0.05$) decreased. The exception was soyasaponin γ_g, which was not found to be significant but was also reduced. It is noteworthy that β_a, γ_a, and γ_g were reduced to trace amounts or below 1% of the peak

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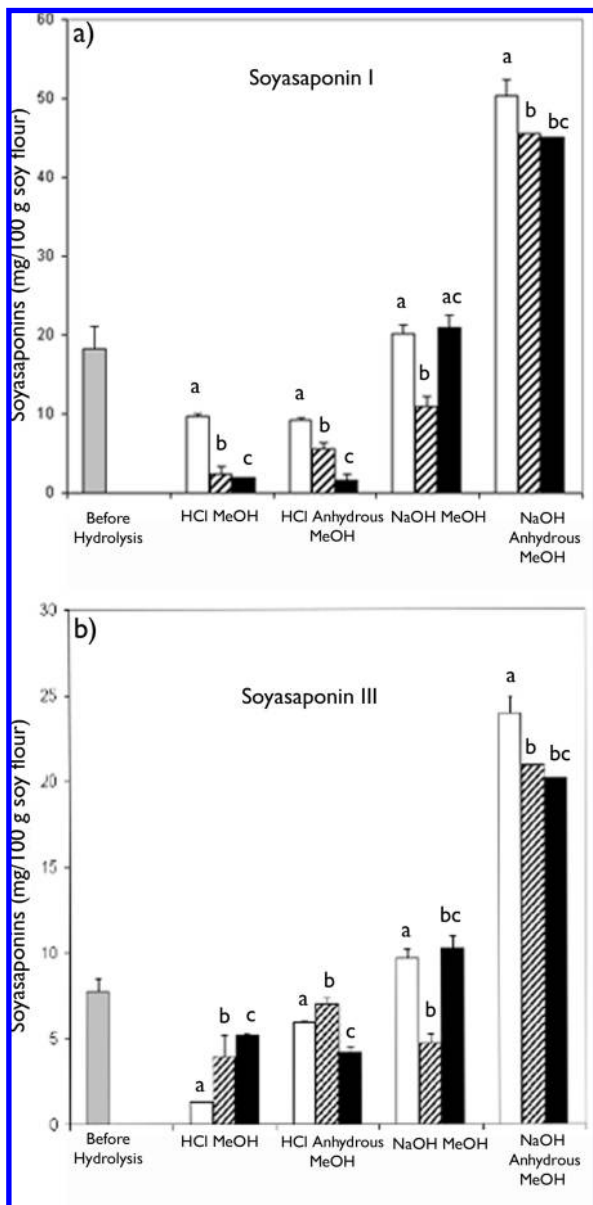
Figure 5. Effect of acid and base hydrolysis, environmental temperature, and both methanol and anhydrous methanol hydrolysis on the generation of soyasaponins I and III. The gray bar represents the amount of soyasaponin I (**a**) and soyasaponin III (**b**) before hydrolysis. The open bar represents hydrolysis in a closed container in a 80 °C water bath, hatched bars represent hydrolysis at 100 °C, and solid bars represent hydrolysis in a 120 °C dry oven. Values are expressed as means ± SDs, and bars with different letters are significantly different ($P < 0.05$) from each other of the same solvent group.

Figure 6. Appearance and disappearance of group B soyasaponins before (open bar) and after (solid bar) anhydrous alkaline hydrolysis outlined in the Materials and Methods. An asterisk (*) refers to a significant ($P < 0.05$) difference as compared to before and after hydrolysis.

saponins. Anhydrous alkaline hydrolysis conditions following a gradient SPE elution were effective to produce a concentrated extract consisting of primarily soyasaponins I and III. This is an important finding to produce adequate amounts of the compounds that are required for bioactive testing.

area. The increase in the HPLC peak area of soyasaponins I (51%) and III (24%) resulted in a net gain of 75%, which nearly matches the total reduction of Be (−4%), β_g (−44%), β_a (−16%), γ_g (−3%), γ_a (−1%), and unidentified peaks (−6%), resulting in a loss of 74% of peak area. Thus, alkaline anhydrous hydrolysis can increase both soyasaponins I and III from DDMP-conjugated group B soyasaponins without hydrolyzing the glycosidic bond at position C-3.

We have effectively shown that maximizing the extraction of soyasaponins utilizing room temperature extraction in methanol for 24 or 48 h produced the greatest amount of total saponins. The use of SPE effectively removed virtually all interfering isoflavones and increased the retention of soya-

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