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ARTICLE

Effect of Protein Content and Denaturation on the Extractability and Stability of Isoflavones in Different Soy Systems

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The effect of protein on the thermal stability of isoflavones and the efficiency of their extraction from various soy matrixes was investigated. Isoflavones were extracted from soymilk, soybean. and soy protein isolate, varying in protein content and/or denaturation state, following nonenzyme-assisted solvent extraction or enzyme-assisted extraction. Selected samples were subjected to thermal treatment prior to and posthydrolysis of the protein. The extraction efficiency was a function of the enzyme used, sample to solvent ratio, protein content, and protein denaturation state. Proteolysis and reducing the sample to solvent ratio enhanced the isoflavone extractability, especially in samples with denatured protein. Results demonstrated clearly that the solvent extraction techniques currently used considerably underestimate isoflavone content in complex soy systems with high protein content. The stability of isoflavones was reduced upon protein hydrolysis, indicating that the protein moiety may have a protective effect against the thermal degradation of isoflavones.

KEYWORDS: Isoflavones; isoflavone−protein association; isoflavone extraction; isoflavone thermal stability

INTRODUCTION

Profiling and accurately quantifying isoflavones present in various soy products is crucial for commodity characterization, establishing economic value, adequate monitoring of processing effects, and consistency of clinical research. Not all soy foods deliver the same isoflavone-associated benefits. Inconsistency in isoflavone research has led the National Institutes of Health (NIH), particularly the National Center for Complementary and Alternative Medicine (NCCAM), to put a hold on soy isoflavone research. This inconsistency in isoflavone research is partly attributed to the inadequate profiling of isoflavones, lack of standardization of the source of isoflavones (different soy matrixes and supplements), and lack of standard analytical methods for profiling and quantifying isoflavones present in different soy matrixes (NIH Scientific Workshop, July 2009).

Numerous processing conditions are employed in the production of soy foods that meet the market's demand for improved, versatile, and safe soy products. The profile and content of isoflavones depend largely on the processing conditions, namely, pH, temperature, and time (*1*). Malonylglucosides, which are the most abundant and most thermally labile isoflavone forms, were found to contribute the most to the profile and content changes of isoflavones upon processing (*1*, *2*). On the basis of the processing conditions employed, interconversions can occur, namely, decarboxylation of malonylglucosides into acetylglucosides (*3*) and/or de-esterification of malonyl- and acetylglucosides into their respective nonconjugated glucosides (*4*, *5*). Loss in total isoflavone content has been observed upon thermal treatment, in both buffered (*6*, *7*) and complex systems (*8*). The change in profile and content of isoflavones upon processing can ultimately affect their physiological contributions. Therefore, it is crucial to accurately determine the isoflavone profile and content in different soy matrixes.

Owing to their phenolic nature, isoflavones interact with the hydrophobic interior of the globular soy proteins and thus are concealed from the aqueous phase (*9*). The isoflavone−protein association may have a considerable effect on the analytical extraction efficiency of isoflavones and their thermal stability. This association is believed to be a function of protein content as well as the protein denaturation state, which in turn are dependent on processing conditions. Raw soybeans and soy-based products vary considerably in their protein denaturation state (*10*) and protein content, the latter of which ranges from 3.5% in soymilk to 95% in soy protein isolate (SPI). Chiari et al. (*11*) reported a negative correlation between protein and isoflavone contents in various soy matrixes that could be the result of inefficient isoflavone extraction from samples with high protein content. When the protein content is high, isoflavones would likely have a greater potential to associate with the hydrophobic interior of the globular protein, thus making their extraction harder. Achouri et al. (*12*) followed sequential solvent extractions of isoflavones from samples high in protein content (such as SPI), demonstrating that protein−isoflavone interactions may complicate the analytical extraction. Processing conditions may cause denaturation and unfolding of proteins, which can also potentially affect the extraction efficiency of isoflavones. Exposure of isoflavones to the aqueous surroundings following protein

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unfolding may make them more prone to thermal degradation. Therefore, it is crucial to understand the thermal stability of isoflavones as affected by the protein moiety.

Several researchers have tried to optimize the extraction protocol of isoflavones by considering parameters such as solvent selection, solvent volume, extraction time, pH, and temperature (*1*, *12*−*15*). However, quantification of isoflavones still varies in accuracy, reproducibility, and sensitivity (*16*). These inconsistencies can be related to several factors such as the strength of the solvent, the duration of extraction, addition of water and/or acid to the extracting solvent, etc. (*16*). Variability can also result from disregarding the protein−isoflavone association when working with soy matrixes with different protein content and denaturation states.

Objectives of this work were to determine the effect of protein content and denaturation state on the extraction efficiency of isoflavones from different soy matrixes and to investigate the effect of the protein moiety on the thermal stability of isoflavones.

MATERIALS AND METHODS

Materials. Commercially available pasteurized soymilk samples were obtained from local grocery stores. The samples were lyophilized and stored in sealed containers at −20 °C until analyzed. Two soybean varieties (MN1412SP and MN1502SP) were obtained from the Department of Agronomy and Plant Genetics, University of Minnesota (St. Paul, MN). The soybean varieties were grown at the same time in 2007 at UMore Park (Rosemount Research and Outreach Center) under the same environmental conditions and were harvested at the same time. The soybean samples were finely ground and stored in sealed containers at −20 °C until analyzed. Commercially available soy protein isolate (SPI-C) was kindly provided by Solae, LLC (Memphis, TN). Minimally heated and defatted soy flakes (7B) were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL) and were used to prepare a soy protein isolate (SPI-P). Soy protein standards, glycinin, and β-conglycinin were kindly supplied by EPL Bio Analytical Services (Niantic, IL). High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Other analytical reagent grade chemicals were purchased from Fisher Scientific and Sigma Chemicals Co. (St. Louis, MO). Isoflavone standards: genistin, daidzin, malonylgenistin, acetyldiadzin, and acetylgenistin were purchased from LC Laboratories (Woburn, MA); malonyldiadzin and malonylglycitin were purchased from Wako Chemicals, USA via Fisher Scientific; and genistein, daidzein. and glycitein were purchased from Indofine Chemical Co. (Somerville, NJ). Standard solutions of 500 μg/mL were prepared using 80% aqueous methanol and stored at −20 °C. Lyophilized porcine trypsin (EC 3.4.21.4, T-0303, 13100 units/mg), lyophilized porcine pepsin (EC 3.4.23.1, P-6887, 3440 units/mg). and lyophilized papaya latex papain (EC 3.2.22.2, P-4762, 24 units/mg) were obtained from Sigma Aldrich. Lyophilized nattokinase (EC 3.4.21.14) from *Bacillus* sp. was obtained from Ace Biotech (Chungcheongbuk, Korea). Prestained broad-range molecular weight standard, Laemmli sample buffer, 10× Tris/glycine/SDS running buffer, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethyl-ethylenedimine, and 40% Acrylamide/Bis solution were purchased from BioRad (Hercules, CA).

Preparation of SPI-P. Minimally heated and defatted soy flour was used to prepare SPI following the method outlined by Tsumura et al. (*17*). Soy flour and deionized distilled water (DDW) dispersion (1:10 w/w) was adjusted to pH 7.0 with 2 N HCl, stirred at room temperature for 1 h, and centrifuged at 5000*g* for 30 min to remove insoluble material. The supernatant was adjusted to pH 4.5 with 2 N HCl and centrifuged at 5000*g* for 10 min to precipitate the protein. The pellet was redispersed (1:4 w/w) in DDW, neutralized with 2 N NaOH, and freeze-dried. The lyophilized sample was kept at −80 °C until further analysis.

Experimental Design. Three independent experiments were carried out to test the effects of multiple variables on isoflavone extraction efficiency. The extraction variables included enzyme type, soy matrix, protein content, protein denaturation state, and/or sample to solvent ratio. Since we hypothesize that proteins limit isoflavone extraction, samples were subjected to protein hydrolysis prior to solvent extraction. The experimental design of each of the three independent experiments is outlined as follows: (1) a two-factor experimental design, completely crossed, to be performed in triplicate, to determine the effect of enzyme-assisted extraction on isoflavone yield from different soy matrixes, with enzyme type (2 levels, pepsin and trypsin) and soy matrix (4 levels, soymilk, 33.22% protein on a dry basis (db); soymilk, 24.16% protein (db); soybeans, 45.7% protein; and soybeans, 37.1% protein) as factors; (2) a two-factor experimental design, completely crossed, to be performed in triplicate, to measure the effect of sample to solvent ratio on the efficiency of isoflavone extraction from different soy matrixes, varying in their protein content and protein denaturation state, with soy matrix (4 levels, soybeans, 45.7% protein; soybeans, 37.1% protein; SPI-C, 86.7% protein; and SPI-P, 89.5% protein) and sample to solvent ratio (5 levels, 2:19; 1:19; 0.5:19; 0.1:19; and 0.05:19 g/mL) as factors; (3) a two-factor experimental design, completely crossed, to be performed in triplicate, to examine the effect of protein denaturation on enzyme-assisted extraction, with protein denaturation state (2 levels, SPI-C and SPI-P) and enzyme type (3 levels, papain; nattokinase; and trypsin) as factors.

To study the effect of the protein moiety on the stability of the associated isoflavones, the thermal stability of isoflavones was monitored prior to and posthydrolysis of SPI-C. A two-factor experimental design, completely crossed, performed in triplicate, was carried out, with enzyme hydrolysis (2 levels, no hydrolysis and papain hydrolysis) and thermal treatment (2 levels, no thermal treatment and thermal treatment at 95 °C for 1 h) as factors. Following the treatments, the samples were subjected to nonenzyme-assisted solvent extraction of isoflavones using a sample to solvent ratio of 0.05:19 (g/mL).

Analysis of Protein Content. Protein content of the samples was determined using a LECO TruSpec nitrogen analyzer (St Joseph, MI) following the Dumas AOAC method 992.15 (*18*). Ethylenediaminetetraacetic acid (EDTA) was used as a standard. The nitrogen percentage was converted into protein percentage using a conversion factor of 6.25.

Enzyme-Assisted Extraction of Isoflavones. Enzyme hydrolysis of the different soy samples was done prior to solvent extraction. Enzyme hydrolysis conditions (enzyme activity, sample to enzyme ratio, and incubation time) using the chosen enzymes were optimized in preliminary trials. Different samples were weighed (0.05, 0.1, 0.5, 1, or 2 g) in triplicate and mixed with 5 mL of DDW and 2 mL of either trypsin solution (131×10^4 U/g sample), papain solution (2400 U/g sample), or nattokinase solution (0.03 g enzyme/g sample). In the case of pepsin hydrolysis, samples were mixed with 5 mL of pH 2 solution (prepared using 0.01 N HCl) and 2 mL of pepsin solution at pH 2 (344×10^3 U/g sample). Enzyme solutions were prepared by dissolving the enzymes in DDW and adjusting the pH as needed. Sample mixtures were incubated at 37 °C for 1 h while stirring. An aliquot (10 μL for samples weighing >0.5 g, 50 μL for samples weighing <0.5 g) of each sample was taken after incubation and was stored at −20 °C prior to analysis by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Enzyme activity was terminated by adding 10 mL of acetonitrile and 2 mL of 0.1 N HCl. Samples then were stirred (400 rpm) at room temperature for 2 h. Extracts were centrifuged at 13,750*g* for 10 min at 15 °C, and the supernatant was filtered through Whatman no. 42 filter paper. Acetonitrile from the filtrates was evaporated using a rotary evaporator at 37 °C for 15 min. The concentrated extracts were redissolved in 80% methanol, placed in amber glass bottles, and stored at −20 °C until analyzed by HPLC. A control for each enzyme treatment was prepared by weighing the same amount of sample and subjecting it to nonenzyme-assisted solvent extraction, as outlined below, without the addition of enzyme.

Nonenzyme-Assisted Solvent Extraction. The nonenzyme-assisted solvent extraction protocol was based on the method outlined by Murphy et al. (*1*). Samples were weighed and mixed with 7 mL of DDW. After mixing well at room temperature, 10 mL of acetonitrile and 2 mL of 0.1 N HCl were added. Extraction continued as outlined above.

HPLC Analysis. Extracts from both enzyme-assisted extraction and nonenzyme-assisted solvent extraction, were passed through 0.45 μm membrane filters into HPLC vials. Extracts were diluted to fit the standard curve range using 80% methanol. To separate and quantify isoflavones, the HPLC method outlined by Ismail and Hayes (*19*) was followed, with modification only in the calibration approach. A Shimadzu HPLC system was used, equipped with an SIL-10AF auto injector, an SPD-M20A photo diode array detector (PDA), and a CTO-20A column oven. The column

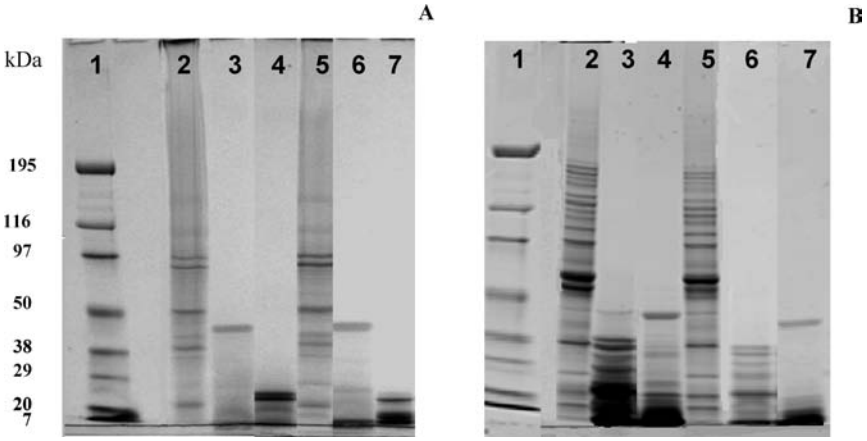

Figure 1. SDS−PAGE visualization of the protein hydrolysis pattern in (**A**) soymilk samples (HP, 33.22% protein; LP, 24.16% protein) and (**B**) Soybean samples (HP, 45.7% protein; LP, 37.1% protein). For both gels A and B, lane 1, molecular weight markers; lane 2, control HP; lane 3, pepsin-hydrolyzed HP; lane 4, trypsin-hydrolyzed HP; lane 5, control LP; lane 6, pepsin-hydrolyzed LP; lane 7, trypsin-hydrolyzed LP.

used was a 250 mm × 4.6 mm i.d., 5 μm, YMC pack ODS AM-303 RP-18 column, with a 20 mm × 4 mm guard column of the same material (YMC pack ODS AM). A linear HPLC binary gradient was used: solvent A was 0.1% acetic acid in distilled water, and solvent B was 0.1% acetic acid in acetonitrile. Following the injection of 20 μL of a sample, solvent B was increased from 17% to 25% over 25 min, held at 25% for 5 min, increased to 30% over the next 10 min, and then held at 30% for 10 min; finally, solvent B was decreased back to 17% over the next 2 min and held at 17% for 8 min, to return the baseline to its original level. The oven temperature was maintained at 35 °C, and the flow rate was held at 1.2 mL/min throughout the run time, which was a total of 60 min. Both solvents were filtered through 0.45 μm Millipore nylon membrane filters (HNWP, 47 mm) and degassed before use. Identification of the peaks was determined using a UV scan from 200 to 400 nm. External calibrations were calculated from standard solutions (0.1, 0.5, 1.0, 2.0, 4.0, 8.0, and 10.0 μg/mL) containing all 12 isoflavone standards in 80% methanol. Peak areas of isoflavone standards and isoflavones present in the extracts were measured using Shimadzu software (version 7.4) at a wavelength of 256 nm. Isoflavone concentration in μg/mL was converted to moles (μmoles) per gram dry weight of the sample for better comparison since different isoflavone forms have different molecular weights. When applicable, percent interconversions between isoflavone derivatives and percent loss in total isoflavones were calculated following the equations of Nufer et al. (*8*).

SDS−PAGE. SDS−PAGE was carried out to visualize the hydrolysis pattern of the soy protein following the various enzyme treatments. An aliquot of each sample was mixed with Laemmli buffer in either a 1:1 or 1:2 ratio, on the basis of protein concentration. Molecular weight standards (5 μL) and samples (5 μL) were loaded onto 18-well 4−15% gradient polyacrylamide gels, electrophoresed, stained, and destained as outlined by Nufer et al. (*8*).

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) was performed to determine the thermal denaturation of soy protein subunits (glycinin and β-conglycinin) of SPI-C and SPI-P using a DSC 7 instrument (Perkin-Elmer, Waltham, MA) according to the method outlined by Tang et al. (*20*), with slight modification. Duplicates of SPI-C, SPI-P, and soy protein standards were solubilized to a 15% solution (w/v) in a 0.05 M potassium phosphate buffer (pH 7) and allowed to equilibrate at room temperature for 12 h. Each solution was transferred into an aluminum solid pan, which was then hermetically sealed and placed in a DSC chamber. Samples were equilibrated at 20 °C for 5 min and then heated from 20 to 120 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference.

Thermal Treatment. SPI-C samples (1 g), in triplicate, were mixed with 5 mL of DDW and 2 mL of papain (2400 U/g sample). The sample mixtures were incubated at 37 °C for 1 h while stirring. A control was subjected to the same treatment but without the addition of enzyme. Papain-hydrolyzed and control SPI-C samples were lyopholized and mixed with 32 mL of DDW (to achieve a consistency similar to that of soymilk with no precipitation) in 50 mL glass test tubes, enclosed with screw caps, then either left with no further treatment or subjected to thermal treatment at 95 °C (±2 °C) for 1 h in a water bath. Time to reach 95 °C was monitored using a thermocouple (up to 5 min). All samples were lyopholized and stored at −20 °C until analyzed. Isoflavone extraction was carried out following nonenzyme-assisted solvent extraction as outlined above with a sample to solvent ratio of 0.05:19 (g/mL).

Statistical Analysis. Analysis of variance (ANOVA) was carried out utilizing SPSS 15 for Windows (*21*). When a factor or an interaction effect was found significant ($P \leq 0.05$), differences between the respective means (if more than two means) were determined using the Tukey−Kramer multiple means comparison test.

RESULTS AND DISCUSSION

Effect of the Soy Matrix on Enzyme-Assisted Extraction of Isoflavones. *Protein Content and Selection of Samples*. Protein content, on dry basis, of five commercially available pasteurized soymilk samples, varied between 24.16% and 33.22%. Differences in the protein content of the various soymilk samples are most likely due to the variability in soybean varieties used, raw material used, and/or processing conditions employed. The samples with the highest and lowest protein content were chosen for isoflavone extraction and were designated as high-protein (HP) and low-protein (LP) soymilk, respectively. Protein content of the soybean varieties MN1412SP (designated as HP soybeans) and MN1502SP (designated as LP soybeans) was 45.7% and 37.1%, respectively. The difference in protein content is mainly attributed to varietal differences.

Enzyme Hydrolysis of Soy Protein. In soymilk samples, peptides with molecular weight between 38 and 50 kDa were present after pepsin hydrolysis (**Figure 1A**, lanes 3 and 6), whereas the remaining peptides after trypsin hydrolysis were close to 25 kDa and less (**Figure 1A**, lanes 4 and 7). However, in soybean samples, peptides of molecular weight between 38 and 50 kDa were present after the hydrolysis by either pepsin or trypsin (**Figure 1B**). The limited hydrolysis of the soybean protein and the consequent release of large peptides can be attributed mainly to the native state of the protein. Native and globular soy proteins are known to resist enzymatic hydrolysis (*22*). However, upon thermal processing, as in the case of the processed soymilk samples, the protein unfolds, which ultimately leads to easier access of the enzyme to the recognition sites. DSC measurements confirmed the denaturation state of the proteins in the soymilk samples, as endothermic peaks that correspond to the denaturation onset of β-conglycinin and glycinin, the major proteins in soy, were not apparent (data not shown). Furthermore, raw soybeans have a significant amount of trypsin inhibitor, which most likely may have limited the trypsin activity.

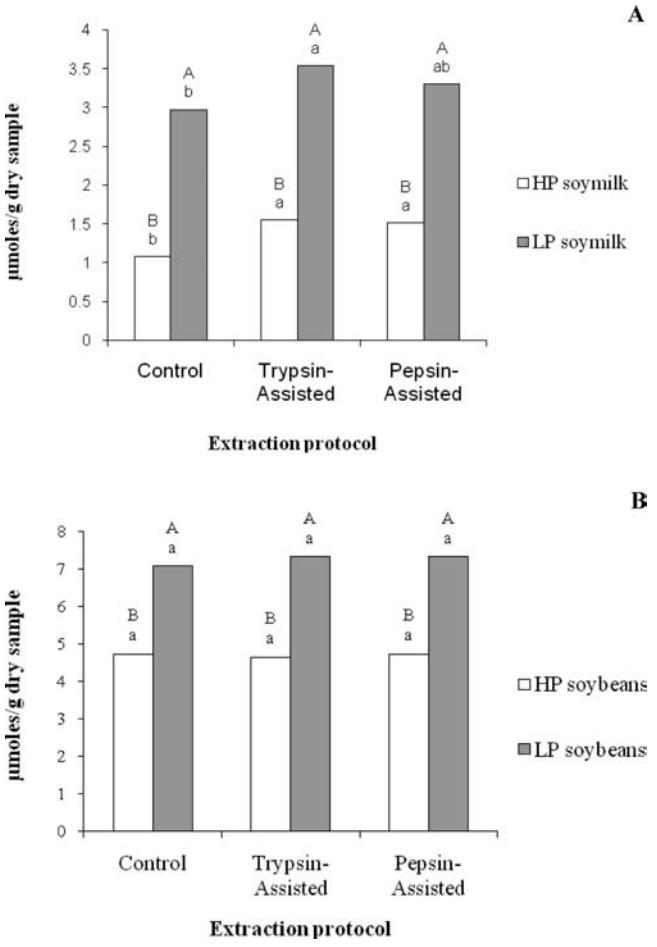

Figure 2. Total isoflavones extracted from (**A**) soymilk samples (HP, 33.22% protein and LP, 24.16% protein) and (**B**) raw soybean samples (HP, 45.7% protein and LP, 37.1% protein), following nonenzyme-assisted solvent extraction (control), trypsin-assisted extraction, and pepsin-assisted extraction. Different upper case letters indicate significant differences ($P \leq 0.05$) between HP and LP, within each extraction protocol and not across. Different lower case letters indicate significant differences between the three extraction methods, within each sample, according to the Tukey−Kramer multiple means comparison test ($P \leq 0.05$); $n = 3$.

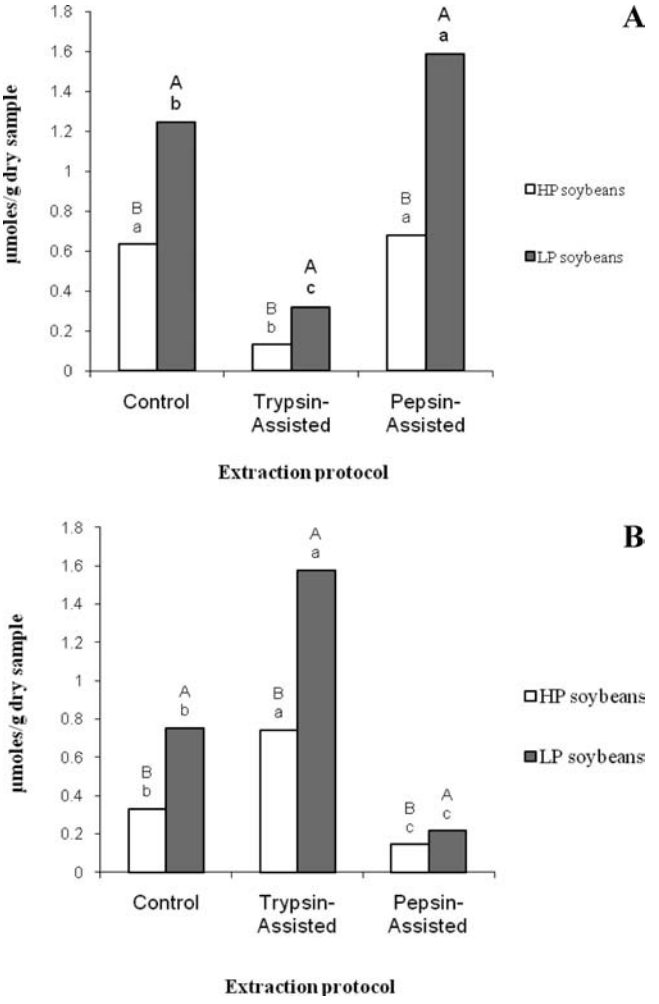

Figure 3. Total glucosides (**A**) and total aglycones (**B**) extracted from soybean samples (HP, 45.7% protein and LP, 37.1% protein), following nonenzyme-assisted solvent extraction (control), trypsin-assisted extraction, and pepsin-assisted extraction protocols. Different upper case letters indicate significant differences ($P \leq 0.05$) between HP and LP, within each extraction protocol. Different lower case letters indicate significant differences between the three extraction methods, within each sample, according to the Tukey−Kramer multiple means comparison test ($P \leq 0.05$); $n = 3$.

Enzyme-Assisted Extraction of Isoflavones. Following nonenzyme-assisted, trypsin-assisted, or pepsin-assisted extraction, the amount of total isoflavones extracted from LP soymilk and soybean samples was significantly higher than that extracted from HP soymilk and soybean samples, respectively (**Figure 2A** and **B**). This observation can be attributed to factors such as varietal differences, raw material used, processing conditions, and/or interference of the protein with isoflavone extraction. To best test the effect of protein content on extraction efficiency, it would have been ideal if both soymilk and soybeans samples had the same isoflavone content yet different protein contents. However, obtaining or formulating such samples is normally hard to achieve.

The efficiency of isoflavone extraction from LP soymilk samples was enhanced by 18.98% following trypsin-assisted extraction but was not enhanced by pepsin-assisted extraction (**Figure 2A**). Interestingly, the efficiency of isoflavone extraction from HP soymilk samples was enhanced by 44.08% and 40.69%, following trypsin- and pepsin-assisted extractions, respectively. This observation indicates that higher protein contents increase the potential for isoflavone entrapment, making nonenzyme-assisted solvent extraction less efficient. The higher efficiency of isoflavone extraction from soymilk samples, mainly the LP samples, subjected to trypsin hydrolysis as compared to samples subjected to pepsin hydrolysis can be attributed to differences in the proteolysis patterns (**Figure 1A**). The release of the relatively small molecular weight peptides most likely contributed to easier isoflavone extraction.

As compared to nonenzyme-assisted solvent extraction, enzyme-assisted extraction did not lead to a significant increase in extracted isoflavones from soybean samples (**Figure 2B**), in contrast to what was observed for soymilk samples. This observation could be attributed to the limited enzymatic hydrolysis of the native soybean proteins and the consequent release of relatively high molecular weight peptides (**Figure 1B**). Complete extraction of isoflavones associated with the remaining high molecular weight peptides was, therefore, assumed hard to achieve.

Following trypsin-assisted extraction of isoflavones from soybean samples, a significant increase in measured aglycones, accompanied by a significant decrease in measured glucosides, was observed (**Figure 3A** and **B**). This observation was attributed to the presence of native glucosidase in raw soybeans, which are capable of hydrolyzing glucosides into aglycones (*19*, *23*), upon

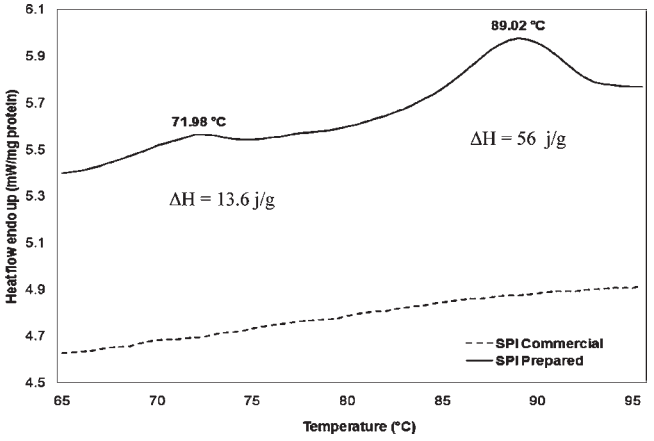

Figure 4. DSC thermograms showing different denaturation profiles of SPI-C and SPI-P. The scanning rate was 5 °C/min, and the sample concentration was about 15%. ΔH: enthalpy of denaturation.

incubation of the samples at 37 °C for 1 h at pH 7. Conversions of glucosides to aglycones were not observed following the pepsin-assisted hydrolysis. The acidic pH employed for pepsin hydrolysis was not optimal for glucosidase activity. Conversions of glucosides to aglycones were also not apparent in soymilk samples subjected to enzyme-assisted extractions (data not shown), most probably due to glucosidase enzyme inhibition upon the processing of the soymilk. However, extraction of conjugated isoflavones from both soymilk and soybean samples were significantly enhanced following enzyme-assisted extraction as compared to nonenzyme-assisted solvent extraction, except for the HP soybean sample (data not shown).

Extraction conditions that alter the isoflavone profile and content will lead to false conclusions and comparisons across various soy samples. Therefore, the choice of enzyme and the hydrolysis conditions has to be customized to the soy matrix. To enhance the extraction of isoflavones from complex matrixes such as soybeans, with native globular protein at relatively high concentrations, different approaches need to be employed, such as adjusting the sample to solvent ratio.

Effect of Sample to Solvent Ratio on Isoflavone Extraction Efficiency. *Protein Content and Denaturation State of the Chosen Samples*. The same raw soybean samples used in the previous section were chosen along with two SPI samples, SPI-P and SPI-C, which have similar protein contents, 89.45% and 86.67%, respectively. DSC measurements confirmed that SPI-C constitutes completely denatured proteins, as endothermic peaks that correspond to the denaturation onset of β-conglycinin and glycinin, the major protein components of SPI, were not apparent (**Figure 4**). Both glycinin and β-conglycinin proteins of the SPI-C have been denatured by prior heat processing. In SPI-P, two endothermic peaks attributed to the denaturation temperature onset of β-conglycinin and glycinin were observed at 71.98 and 89.02 °C, respectively, indicating that the SPI-P major proteins are present mostly in their native state.

Solvent Extraction of Isoflavones Using Different Sample to Solvent Ratios. More isoflavones were extracted from both HP and LP soybeans and both SPI-C and SPI-P samples as the sample to solvent ratio decreased from 2:19 to 0.05:19 (g/mL) (**Figure 5A** and **B**). The percent increase in extracted isoflavones when the sample to solvent ratio dropped from 2:19 to 0.05:19 was 30.17% and 33.19% for HP and LP soybean samples, respectively. However, the percent increase in extracted isoflavones as the sample to solvent ratio dropped from 2:19 to 0.05:19 was 72.7% and 15.6% for SPI-C and SPI-P, respectively. Interestingly, the enhancement of isoflavone extraction from SPI-C was more than double that of the soybean samples, which had almost half the protein content of SPI-C. The results are consistent with the improved liberation of protein-entrapped isoflavones upon reducing the sample to solvent ratio as well as the enhancement of this phenomenon in the denatured SPI-C samples. The considerable difference in the percent increase of extracted isoflavones from SPI-C and SPI-P can be attributed to differences in protein denaturation. Because protein was denatured and thus unfolded in the SPI-C samples, isoflavones associated with the hydrophobic moiety most likely got exposed to the aqueous phase (and possibly their association with the protein weakened) and thus were extracted more readily.

The overall enhancement in isoflavone extraction cannot be attributed to saturation of the solvent when the isoflavone content is high in a sample. Isoflavone amount extracted from SPI-P samples (**Figure 5B**) was considerably higher than that extracted from soybeans samples (**Figure 5A**) using the same high sample to solvent ratio (2:19 or 1:19 g/mL), indicating that the extracts were not saturated at high sample to solvent ratio. Therefore, the markedly enhanced isoflavone extraction as the sample to solvent ratio was reduced is due most likely to lowering the strength of the protein−isoflavone interaction.

Regardless of the sample to solvent ratio, the total isoflavones extracted from SPI-P was higher than that extracted from SPI-C (**Figure 5B**). This observation can be attributed to varietal differences in the sources of the soybeans used to produce the different SPI samples or to the loss of isoflavones during the heat processing of SPI-C, as indicated by the presence of thermally denatured proteins.

Effect of Protein Denaturation on Enzyme-Assisted Extraction. *Protein Content and Denaturation State of the Chosen Samples*. SPI-P and SPI-C, with similar protein contents yet different denaturation states, were chosen to study the effect of protein denaturation state on the efficiency of enzyme-assisted extraction of isoflavones.

Enzyme Hydrolysis Pattern of SPI-P and SPI-C Samples. In addition to trypsin, we used papain and nattokinase instead of pepsin since the latter resulted in a limited hydrolysis of soy protein. Peptides with molecular weight up to 36 kDa were present after trypsin hydrolysis (**Figure 6**, lane 5) of SPI-P, whereas the remaining peptides after papain hydrolysis were ≤25 kDa (**Figure 6**, lane 3). Regardless of the enzyme used, hydrolysis of SPI-C (**Figure 6**, lane 6−9) resulted in the release of smaller peptides as compared to that of SPI-P (**Figure 6**, lane 2−5). This observation is attributed to the native state of the SPI-P proteins and the presence of trypsin inhibitor in its active form which would limit tryptic proteolysis (**Figure 6**, lane 5).

Enzyme-Assisted Extraction of Isoflavones from SPI-C and SPI-P. Since a low sample to solvent ratio resulted in enhanced isoflavone extraction efficiency, a 0.05:19 (g/mL) sample to solvent ratio was chosen for all extraction protocols. Compared to nonenzyme-assisted solvent extraction, more isoflavones were extracted from SPI-P and SPI-C following papain-assisted extraction (**Table 1**). Conversely, trypsin-assisted extraction did not result in enhanced isoflavone extraction from either SPI-P or SPI-C. This observation is partially attributed to differences in the proteolysis patterns observed for each enzyme. Following papain-assisted extraction, the efficiency of isoflavone extraction from SPI-C and SPI-P was further enhanced by 15.3% and 9.8%, respectively. The higher percent increase in extracted isoflavones from SPI-C is partially attributed to the hydrolysis pattern and the molecular weight of the peptides released (**Figure 6**, lanes 3 and 7). These observations confirmed that the efficiency of enzyme-assisted extraction is not only a function of the enzyme

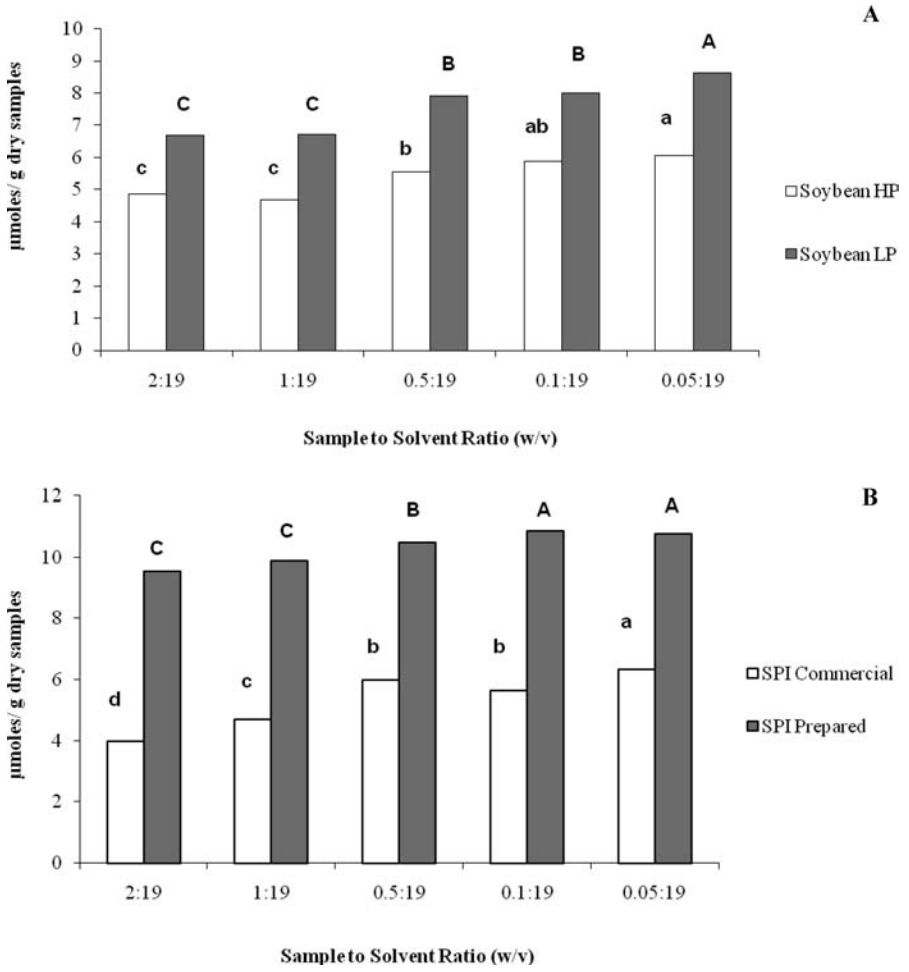

Figure 5. Total isoflavones extracted from (**A**) soybean samples (HP, 45.7% protein and LP, 31.7% protein) and (**B**) the soy protein isolate sample (SPI-C 86.67% protein; SPI-P 89.45% protein) upon varying solvent to sample ratios. Different lower case letters and upper case letters indicate significant differences within each sample type, according to the Turkey−Kramer multiple means comparison test ($P \leq 0.05$); $n = 3$.

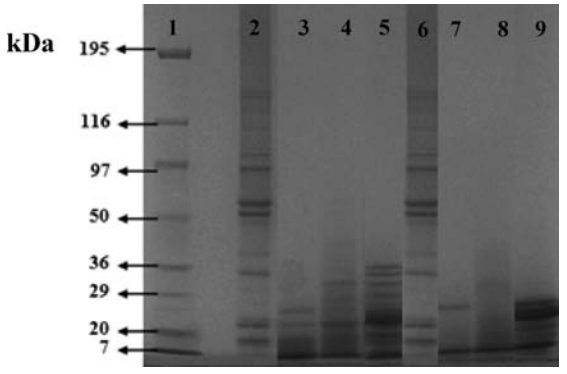

Figure 6. SDS−PAGE visualization of the protein hydrolysis pattern in SPI-P and SPI-C. Lane 1, molecular weight markers; lane 2, control SPI-P; lane 3, papain-hydrolyzed SPI-P; lane 4, nattokinase-hydrolyzed SPI-P; lanes 5, trypsin-hydrolyzed SPI-P; lanes 6, control SPI-C; lane 7, papain-hydrolyzed SPI-C; lane 8, nattokinase-hydrolyzed SPI-C; lane 9, trypsin-hydrolyzed SPI-C.

used but also is a function of the denaturation state of the protein substrate.

Results of the three experiments combined revealed that the choice of the extraction protocol has to be tailored on the basis of protein content, protein denaturation state, and soy matrix. Out of all the tested variables, the use of the papain enzyme in conjuction with the reducing sample to solvent ratio can result in a more precise quantification of isoflavones. Further optimization and validation of isoflavone extraction are recommended to ensure the maximal release of the protein-bonded isoflavones and therefore accurate quantification. Enzyme hydrolysis conditions can be further modified to ensure maximal hydrolysis of the globular proteins through the use of multienzyme systems at various levels, incubation temperatures, and times. Also, the combination of solvents can be investigated, such as DMSO and acetone. Care should be given, though, to isoflavone stability and change in profile during the extraction scheme.

Table 1. Total Isoflavones Extracted from Samples with Different Protein Denaturation States Following Solvent and Enzyme-Assisted Extraction Protocols

	isoflavone content[a]	
extraction protocol	SPI-C[b]	SPI-P[c]
nonenzyme-assisted solvent extraction	6.20 b[d]	9.93 bc
papain-assisted extraction	7.15 a	10.89 a
nattokinase-assisted extraction	6.26 b	10.49 ab
trypsin-assisted extraction	6.75 ab	9.54 c

[a] Total isoflavones expressed as μmol/gram dry weight, obtained by summing all 12 isoflavone forms. [b] SPI-C: commercially available soy protein isolate. [c] SPI-P: prepared soy protein isolate. [d] Means in each column followed by the same letter are not significantly different according to the Tukey−Kramer multiple mean comparison test ($P \leq 0.05$).

Additionally, observed results can partially explain the conflicting reports with regard to isoflavone degradation and change in profile during the processing of different soy systems reported in the literature (*1*,*6*−*8*,*24*). Heating soymilk in a closed system at 80 °C for 3 h showed significant interconversion of conjugated glucosides to their respective nonconjugates without any observed loss or degradation (*1*). However, in closed protein-free buffered systems, heating conjugated isoflavones at 80 and 100 °C for 2 h, resulted not only in interconversions but also in the loss (on molar basis) of isoflavones up to 30% (*7*). Differences in the recorded loss between model systems (*7*) and complex systems (*1*) can be attributed partially to protein−isoflavone association. In complex systems such as soymilk, heat causes unfolding of the protein, thus exposing isoflavones to the solvent, resulting in enhanced extraction and, therefore, masking any loss that might have incurred during the processing. In fact, Nufer et al. (*8*) reported that heating soymilk at 95 °C for 1 h showed a significant increase in extracted isoflavones. However, when the researchers interrupted protein−isoflavone association via proteolysis using trypsin, the extraction efficiency of isoflavones from raw soymilk was improved. The researchers were then able to detect up to 15% loss in total isoflavones of the thermally processed soymilk as compared to raw soymilk (*8*). Therefore, the discrepancy in the reported isoflavone loss with processed soymilk systems can be attributed to the isoflavone extraction protocols employed by the different researchers (*1*, *8*), i.e., enzyme-assisted versus none-nzyme-assisted solvent extraction, as our results illustrate.

Effect of the Protein Moiety on the Thermal Stability of the Associated Isoflavones. To study the thermal stability of isoflavones as affected by the protein−isoflavone interactions, SPI-C was chosen as the soy matrix. SPI-C is high in protein and consists of denatured proteins, which allows for a more efficient hydrolysis by papain. A sample high in protein content was desired to maximize a potential protein effect.

Heating the nonhydrolyzed SPI-C control actually resulted in a significant increase in isoflavones as compared to that in nonhydrolyzed nonheated SPI-C control (**Table 2**). This observation is attributed to the enhanced solvent extraction upon heating (*8*). Even when enzyme-assisted extraction was employed for the extraction of isoflavones from nonhydrolyzed control and heated samples, an increase in extracted isoflavones upon thermal treatment was observed (data not shown). This observed increase in extracted isoflavones is attributed to the combined effect of heating and enzyme-assisted extraction on the overall efficiency of isoflavone extraction.

However, upon heating the hydrolyzed SPI-C sample, total isoflavones dropped significantly (**Table 2**), resulting in a 9.6% loss (% loss was calculated on the basis of the isoflavone content in the hydrolyzed nonheated sample). The hydrolysis of SPI-C proteins, which resulted in the release of smaller peptides, most likely caused the exposure of the isoflavones to the aqueous surrounding. This exposure could partially explain the reduced isoflavone thermal stability as compared to that observed in the SPI-C nonhydrolyzed control samples. It is, therefore assumed that the isoflavones associated with the intact protein were protected against the aqueous thermal degradation. These results can partially explain the discrepancies in the reported isoflavone loss (up to 30%) incurred upon heating of a protein-free buffer system (*7*) and that incurred (up to 15%) upon heating of a soymilk system (*1*,*8*). The observed loss in isoflavones, however, was less than that observed when heating under similar conditions a soy system with relatively low protein content (*8*). It is assumed that the higher the protein content, the higher is the isoflavones association potential, thus the lower is the susceptibility to thermal degradation. Soy matrixes with low protein content and high level of protein denaturation may, therefore, jeopardize the thermal stability of isoflavones. Understanding the protective effect of the protein against isoflavone degradation and interconversion among various forms will allow soy processors to minimize loss through modifying processing conditions on the basis of the protein content and denaturation state. To validate this conclusion, further work has to be done using systems with lower protein content.

Table 2. Isoflavones Extracted from Nonhydrolyzed and Papain-Hydrolyzed SPI-C Samples That Were Either Left Unheated or Were Heated at 95 °C for 1 h

		Isoflavone Content[a]		
		daidzein derivatives		
enzyme treatment	heat treatment	daidzin	malonyldaidzin	total daidzein glucosides
nonhydrolyzed control	nonheated	0.35 c[b]	0.94 a	1.29 b
	heated	0.97 a	0.70 b	1.68 a
papain-hydrolyzed	nonheated	0.40 c	0.93 a	1.34 b
	heated	0.71 b	0.62 b	1.34 b
		genistein derivatives		
		genistin	malonylgenistin	total genistein glucosides
nonhydrolyzed control	nonheated	0.72 d	2.20 a	2.92 b
	heated	2.26 a	0.93 c	3.19 a
papain-hydrolyzed	nonheated	0.86 c	2.32 a	3.19 a
	heated	1.79 b	1.11 b	2.91 b
		Total Isoflavones[c]		
nonhydrolyzed control	nonheated	6.79 b		
	heated	7.22 a		
papain-hydrolyzed	nonheated	7.43 a		
	heated	6.72 b		

[a] Isoflavone content expressed as μmol/g dry weight. [b] Means in each column of each subsection followed by the same letter are not significantly different according to Tukey−Kramer multiple means comparison test ($P \leq 0.05$). [c] Total detected isoflavones obtained by summing all 12 isoflavone forms. Acetyl glucosides and glycitin glucosidic forms were present in trace amounts.

Apart from the observed change in total isoflavones upon heating, a change in the isoflavone profile was observed. A significant decrease in malonylgenistin and malonyldaidzin, with a significant increase in their respective nonconjugated glucosides, i.e., daidzin and genistin, was observed upon heating. This profile change is attributed to the de-esterification of malonylglucosides into their respective nonconjugated glucoside forms during moist heating (*7*). Since heating of the nonhydrolyzed control actually increased the extracted isoflavones, the stability of individual isoflavone forms could not be deduced. However, the stability of the isoflavone forms in the hydrolyzed samples was determined. Overall, conversion and loss in the genistein derivatives were 38% and 15.6%, respectively; while conversion and loss in the daidzein derivatives were 30% and 4.6%, respectively, indicating higher thermal stability. In protein-free buffered systems, thermal stability of isoflavone derivatives was significantly lower for daidzein than for genistein glycosidic forms (*7*). This discrepancy is attributed to stronger association of the daidzein derivatives with the protein than the genistein derivatives. The difference in the association strength is attributed to the variation in the chemical structure of isoflavones. For example, genistein has an additional hydroxyl group in its basic phenolic structure,

whereas diadzein does not. The association of individual isoflavones with proteins under various processing conditions needs to be researched.

In conclusion, our results confirmed that the association of the isoflavones with the interior moiety of the soy protein affects to a great extent their extractability and thermal stability. Isoflavone extraction efficiency and their thermal stability can be influenced not only by the protein content but also by protein denaturation state. Our work demonstrates clearly that the currently used solvent extraction techniques considerably underestimate the isoflavone content in complex soy systems. For accurate profiling of isoflavones, extraction should be maximized, while minimizing any change in the profile of the different isoflavone forms. Accurate profiling of isoflavones in different soy matrixes result in more accurate conclusions regarding the thermal stability of isoflavones. The thermoprotective character and extraction inhibitory effect of the endogenous proteins on isoflavones contribute to false results when the extraction method used for isoflavone profiling does not take into account the protein content and denaturation state. Inaccurate quantification and profiling most certainly lead to misleading conclusions and more controversy, especially in clinical studies.

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