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Effects of Solvent Polarity and Acidity on the Extraction Efficiency of Isoflavones from Soybeans (*Glycine max*)

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Soy isoflavones have been correlated with beneficial health effects. The predominant chemical forms of isoflavones present may affect their biological activities. Choosing the solvent system that can accurately quantify the amounts of individual isoflavones present in these products is paramount. Our objectives were to compare frequently used solvent systems and to evaluate the effects of polarity and acidity on the recovery of isoflavones from soybeans. Isoflavones were extracted from pulverized Manokin soybeans using six solvent systems, which are the combinations of three polarity levels (83% acetonitrile, 80% methanol, and 58% acetonitrile) and two acidity levels (nonacidified and acidified). The pulverized soybean was stirred for 2 h in each solvent system before filtration and concentration using rotary evaporation. The extract was resuspended in 16% acetonitrile and analyzed by high-performance liquid chromatography. Recoveries of pure standards were evaluated with all solvent systems. Solvents with a higher polarity extracted a significantly higher amount of total isoflavones. For individual isoflavones, 58% acetonitrile (highest polarity) extracted either the highest amounts or no less than other solvents, while 83% acetonitrile (lowest polarity) extracted either the lowest amounts or no more than other solvents except for the aglycone form. Acidification significantly reduced the recovery of the malonylglucoside form and the total isoflavones. The recovery study revealed that acidification favored the chemical transformations of isoflavones during the extraction. Among the six solvent systems examined, 58% acetonitrile aqueous solution without acid was the best for extraction of isoflavones from soybeans.

KEYWORDS: Isoflavones; soybean; extraction; solvent; polarity; acidification

INTRODUCTION

Isoflavones found in high concentrations in soybeans have received great attention due to their potential beneficial effects on human health (*1*). These physiological effects are closely related to the chemical structures of isoflavones, which are similar to those of mammalian estrogens. The weak estrogenic activity of isoflavones is one of many proposed mechanisms of their physiological effects (*2*).

There are three types of isoflavones existing in four possible chemical forms in soybeans and soyfoods. The three isoflavone aglycones are genistein, daidzein, and glycitein. Their conjugated forms include the β-glucoside form (genistin, daidzin, and glycitin), the acetylglucoside form (6″-*O*-acetylgenistein, 6″-*O*-acetyldaidzin, and 6″-*O*-acetylglycitin), and the malonylglucoside form (6″-*O*-malonylgenistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonlyglycitin) (*3*). The isoflavone aglycones are the ones with structures mimicking mammalian estrogens. The evidence in the literature suggests that the biological effects of soy isoflavones do not depend on the attached glucose or acid moiety (*4*). It is generally thought that the conjugated isoflavones are converted to their corresponding aglycones by gut microflora or gut glucosidases before absorption (*5*, *6*). However, some researchers have found that glucosides were different from their aglycones in the absorption, distribution, metabolism, or excretion (*7*, *8*). To correctly evaluate the biological effects of isoflavones, it is crucial that we know exactly how much of each form of isoflavones is present, which requires accurate quantitation of isoflavones in their original forms.

The quantitation of isoflavones in soybeans and soy-containing foods is usually done by extracting isoflavones from the food matrix using a certain solvent and then analyzing the extract by high-performance liquid chromatography (HPLC). The extraction procedure is as important as the HPLC analysis, since the extract should represent the original isoflavone composition as much as possible. The industrial processing of soyfoods has been reported to change the isoflavone profiles tremendously (*9*). These chemical changes may also happen during the extraction process for analyzing isoflavones, since mild heat and acid are frequently involved in the extraction, which could cause the degradation of malonyl isoflavones and the hydrolysis

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of glucosides. Therefore, when choosing the extraction solvent for analyzing isoflavones, we should not only consider the extraction efficiency of the solvent but also minimize the artificial transformations.

Extraction of isoflavones has been done in methanol, ethanol, acetone, and acetonitrile with water and/or diluted acid (*10*). Murphy (*11*) and Eldridge (*12*) compared several extraction solvents in the early 80s. The former found that acidified acetonitrile and the latter found that 80% methanol were the best extraction solvents among the ones they had examined. As a result of those studies, 80% methanol and acidified 83% acetonitrile (10 mL of acetonitrile plus 2 mL of 0.1 N HCl) have became the most commonly used extraction solvents in isoflavone analysis. However, those studies were both done before the acetylglucoside and malonylglucoside isoflavones were identified. Recently, Murphy et al. (*13*) reevaluated the acidified 83% acetonitrile and found that adding a certain amount of water could optimize the total extraction. Murphy et al. (*14*) further compared acetonitrile with acetone, ethanol, and methanol in a 53% aqueous solution and concluded that acetonitrile was superior to other solvents. It may be necessary to compare the extraction efficiency of different solvents based on their polarity. Also, the previously recommended use of acidified conditions has been reported as unnecessary. Our objective was to compare the solvent systems for isoflavone extraction that are most frequently reported in the literature, since most of the currently available data on the content of isoflavones in soybeans and soyfoods were obtained by using these two extraction solvent systems. Furthermore, we also evaluated the effects of polarity and acidity on the efficiency of extraction and the maintenance of the original isoflavone profile.

MATERIALS AND METHODS

Chemicals. Isoflavone standards (genistein, daidzein, glycitein, genistin, daidzin, glycitin, and acetylgenistin) were purchased from LC Laboratories (Woburn, MA). The internal standard flavone, hydrogen chloride, and HPLC grade acetonitrile, acetic acid, and methanol were reagents from Fisher Scientific (Fair Lawn, NJ).

Solvent Systems. Six solvent systems were examined. They were as follows: A, 83% acetonitrile (10 mL of acetonitrile + 2 mL of H_2O); B, acidified 83% acetonitrile (10 mL of acetonitrile + 2 mL of 0.1 N HCl); C, 80% methanol (12 mL of 80% methanol in water); D, acidified 80% methanol (9.6 mL of methanol + 2 mL of 0.1 N HCl + 0.4 mL of H_2O); E, 58% acetonitrile (7 mL of acetonitrile + 5 mL of H_2O); and F, acidified 58% acetonitrile (7 mL of acetonitrile + 2 mL of 0.1 N HCl + 3 mL of H_2O).

Extraction of Isoflavones from Soybeans. Manokin soybeans (provided by William Kenworthy from the Department of Natural Resources Sciences and Landscape Architecture, University of Maryland, College Park) were ground for 1.5 min at intervals of 15 s using a laboratory blender (Waring Commercial, New Hartford, CT). Two grams of the pulverized soybeans was mixed with 12 mL of one of the six solvents for 2 h at room temperature. The mixture was then vacuum filtered through Whatman no. 41 filter paper using a Buchner funnel. The filtrate was evaporated using a rotary evaporator with a 40 °C water bath (Buchi, Switzerland). The extract was redissolved in 5 mL of a 16% acetonitrile solution and refrigerated until analyzed by HPLC. Extractions using each of the six solvents were carried out in triplicate.

Isoflavone Standards Recovery. Known amounts of the standards genistein and daidzein (0.2 mg/mL in 80% methanol), genistin and daidzin (0.5 mg/mL in 80% methanol), or acetylgenistin (0.2 mg/mL in 80% methanol) were added to the pulverized soybean samples. The samples were extracted using each of the six solvent systems after the added methanol was dried. Known amounts of pure isoflavone standards or standard combinations (genistein + daidzein, genistin + daidzin, or acetylgenistin) also went through the same extraction process using each of the six solvent systems to determine the recovery rates and/or the changes of isoflavones during the extraction process.

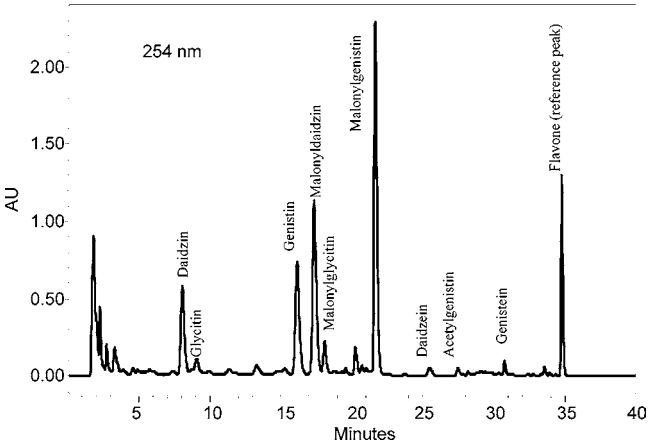

Figure 1. HPLC chromatogram of the manokin soybean sample. Column: Waters, Symmetry C18, 150 mm × 4.6 mm. Solvents: A, 0.1% acetic acid and 5% acetonitrile in water; B, 0.1% acetic acid in acetonitrile. Linear gradient from 10 to 70% B (90 to 30% A) in 30 min.

HPLC Analysis. Analysis of isoflavones was carried out using reverse phase separation of the compounds on a C18 column and using a gradient of acidified water and acidified acetonitrile (A, 0.1% acetic acid and 5% acetonitrile in water; B, 0.1% acetic acid in acetonitrile). A high-pressure liquid chromatography (Waters Delta 600 system) equipped with a photodiode array detector (Waters 996), autosampler (Waters 717plus), and Millennium^{32} software (Waters Corp., Milford, MA) was used. Elution was monitored at 254 nm, and spectral data from 200 to 450 nm were recorded and stored over the time of the run on all samples. After the injection of 50 μL of the extract sample, the linear gradient started from 10 to 14% B over 10 min, then increased to 20% over 2 min, maintained at 20% for 8 min, continued to increase to 70% over 10 min, maintained at 70% for 3 min, and returned to 10% at the end of the 34 min running time. The flow rate was maintained at 1 mL/min throughout the running of the sample. Isoflavones were identified by comparing spectral data and retention times to those of standard references. Calibration curves, prepared by using different concentrations of pure isoflavone standards (genistein, daidzein, genistin, daidzin, and acetylgenistin), were used for quantitation of the isoflavones in the samples. Because the molar extinction coefficients of the malonylglucoside conjugates approximate those of the β-glucoside conjugates, malonylglucosides were calculated from the β-glucoside standards.

Statistical Analysis. The comparison of the six solvent systems was carried out as a randomized complete block design (RCBD) with a 3 × 2 factorial treatment structure. Each block was processed as a batch, which contained one replication of each of the six solvent systems. In each batch, the treatments were completely randomized, and all of the conditions were maintained the same for each sample, to minimize the within block variance. Two factors were examined as follows: polarity of the solvent system with three different polarity levels (80% methanol, 83% acetonitrile, and 58% acetonitrile) and acidification with two acidification levels (nonacidified and acidified). The six solvent systems were the combination of the two factors. For the recovery study, regression analysis was performed to generate the relationship between total isoflavones recovered and added isoflavone standards to determine the recovery rates. Statistical analysis was done by using SAS/STAT package (version 8.1, 1999), developed by the SAS Institute Inc. (Cary, NC). Analysis of Variance (ANOVA) and regression analyses were conducted using PROC MIXED and PROC REG procedures in SAS.

RESULTS AND DISCUSSION

From the 12 soy isoflavones, all but acetyldaidzin, acetylglycitin, and glycitein were detected in the Manokin soybean (**Figure 1**). The contents of individual isoflavones from Manokin soybeans extracted by the six solvent systems are shown in

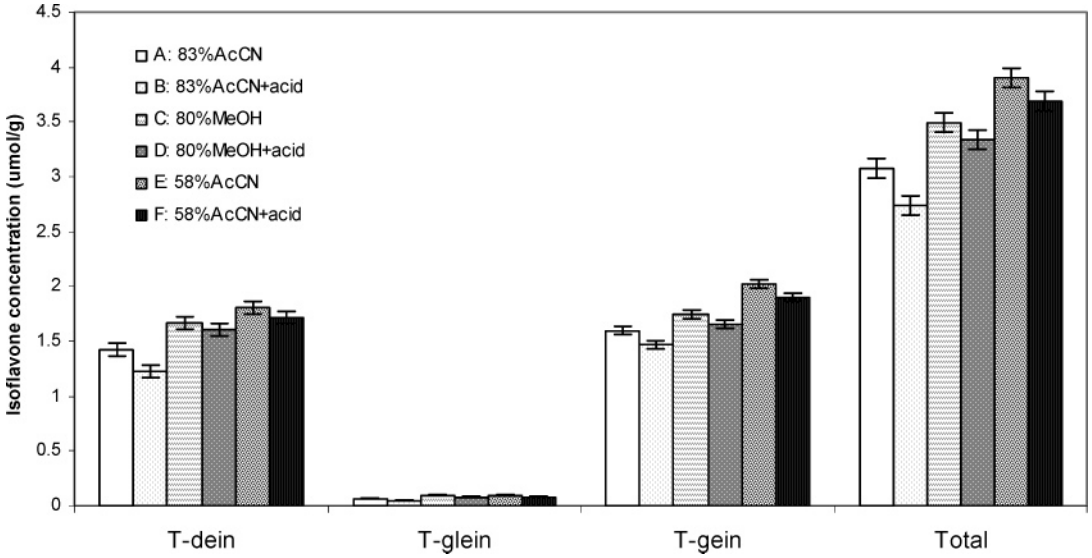

Figure 2. Total daidzein, total glycitein, total genistein, and total isoflavone concentrations extracted by the different solvent systems. T-daidzein, total daidzein and its derivatives; T-glein, total glycitein and its derivatives; T-gein, total genistein and its derivatives; and total, total isoflavones. Solvent abbreviations: See **Table 1**.

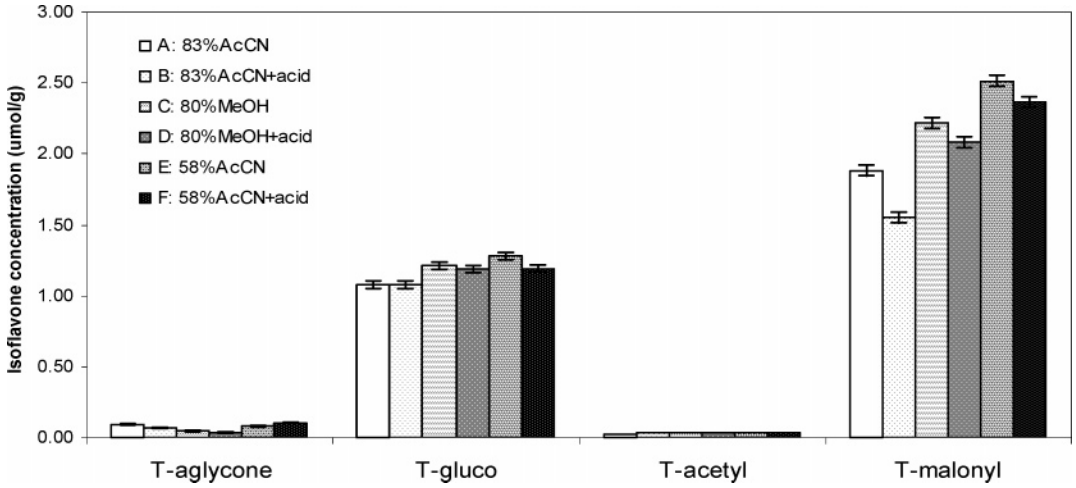

Figure 3. Total isoflavone concentrations in the four chemical forms extracted by the different solvent systems. T-aglycone, total aglycone isoflavones; T-gluco, total β-glucoside isoflavones; T-acetyl, total acetylglucoside isoflavones; and T-malonyl, total malonylglucoside isoflavones. Solvent abbreviations: See **Table 1**.

Table 1. Concentrations of Individual Isoflavones from Manokin Soybeans Extracted by the Different Solvent Systems (μmol/g)[a]

	solvent						
isoflavone	83% AcCN	83% AcCN + acid	80% MeOH	80% MeOH + acid	58% AcCN	58% AcCN + acid	LSD
Din	0.519^{b}	0.528^{b}	0.603^{a}	0.593^{a}	0.593^{a}	0.575^{a}	0.19
Glin	0.022^{c}	0.019^{c}	0.035^{ba}	0.030^{b}	0.038^{a}	0.030^{b}	0.08
Gin	0.536^{c}	0.532^{c}	0.567^{bc}	0.566^{bc}	0.648^{a}	0.584^{b}	0.20
Mdin	0.840^{c}	0.656^{d}	1.048^{b}	0.997^{b}	1.159^{a}	1.072^{ab}	0.29
Mglin	0.033^{a}	0.022^{c}	0.048^{a}	0.045^{a}	0.052^{a}	0.049^{a}	0.08
Mgin	1.002^{c}	0.876^{d}	1.124^{b}	1.040^{c}	1.308^{a}	1.244^{a}	0.21
Agin	0.027^{b}	0.029^{b}	0.029^{b}	0.029^{b}	0.033^{a}	0.033^{a}	0.08
Dein	0.063^{a}	0.048^{b}	0.023^{c}	0.020^{c}	0.047^{b}	0.065^{a}	0.08
Gein	0.032^{a}	0.025^{b}	0.018^{c}	0.015^{c}	0.027^{b}	0.0326^{a}	0.06

[a] The means of isoflavone concentrations with any identical letter are not significantly different with each other at the $\alpha = 0.05$ level by least significant difference (LSD) test; $n = 3$. AcCN, acetonitrile; MeOH, methanol; Din, daidzin; Gin, genistin; Glin, glycitin; Mdin, malonyldaidzin; Mgin, malonylgenistin; Mglin, malonylglycitin; Agin, acetylgenistin; Dein, daidzein; and Gein, genistein.

Table 1. **Figure 2** shows the concentrations of total daidzein, total glycitein, total genistein, and total isoflavones extracted by the six solvent systems, while **Figure 3** shows the concentrations of isoflavones in the four different chemical forms (aglycone, β-glucoside, acetylglucoside, and malonylglucoside forms). The extraction by the six solvent systems resulted in significant differences ($P < 0.0001$) among the concentrations of each detectable isoflavone as well as the total isoflavones. In the present study, flavone was used only as a reference peak in the HPLC analysis, not as an internal standard for quantitation purpose, since the recovery of the internal standard by the six solvent systems may also differ from each other, which would add a confounding factor to the comparison of the extraction. However, for other studies where the purpose is to quantify isoflavone concentrations, it is recommend the use of the internal standard to account for losses during the extraction (*16*).

The six solvent systems were the combinations of two factors: polarity with three levels being evaluated (83% acetonitrile, 80% methanol, and 58% acetonitrile) and acidity with two levels evaluated (no acid and 2 mL of 0.1 N HCl/12 mL solvent). Solvents B (acidified 83% acetonitrile) and C (80% methanol) were the two most frequently reported solvent systems for isoflavone extraction from soybeans and soyfoods. Song et al. (*15*) reported the modification of solvent B by adding water

(10 mL of acetonitrile + 2 mL of 0.1 N HCl + 5 mL of H_2O). The percentage of acetonitrile in solvent F was established according to the solvent described by Song et al. but maintained the same concentration of acid as in solvent B.

Effects of the Solvent Polarity. Polarity represents the sum of all of the molecular properties responsible for all of the interaction forces between solvent and solute molecules (*17*). It is the intermolecular interaction between solvent and solute molecules that determines the mutual solubility (*18*). Murphy et al. (*13*, *14*) found that adding a certain amount of water (5−10 mL depending on the matrix) to different organic solvents optimized the total extraction efficiency. However, with the same percentage of water, the polarities of those organic solutions were actually different.

The Snyder polarity index (P') has been widely used for the characterization of solvent polarity in liquid−liquid and liquid−solid chromatography (*19*). On the basis of the Snyder polarity index (*20*), the order of the polarities of the three level is 58% acetonitrile ($P' = 7.4$) > 80% methanol ($P' = 7.1$) > 83% acetonitrile ($P' = 6.7$). The extraction results indicated that for all of the glycosylated isoflavones, the solvent with a higher polarity either extracted significantly ($P < 0.05$) higher amounts of isoflavones or did not significantly differ from the solvent with a lower polarity (**Figure 3**). The differences between 58% acetonitrile (most polar) and 83% acetonitrile (least polar) were very significant ($P < 0.001$). Comparisons made between 58% acetonitrile and 80% methanol (acetylgenistin) or between 80% methanol and 83% acetonitrile (daidzin, glycitin) were not always significant. We also noted the remarkable differences among the solvents with different polarities in extracting the malonylglucoside isoflavones. On average, 58% acetonitrile obtained 18 and 36% more malonylgenistin, 9 and 49% more malonyldaidzin, and 2 and 79% more malonylglycitin than 80% methanol and 83% acetonitrile. Recoveries of aglycones, genistein, and daidzein with 80% methanol (intermediate polarity) resulted in significantly ($P < 0.0001$) lower amounts than 83% acetonitrile or 58% acetonitrile, while no significant difference was found between the most and the least polar solvents (**Figure 3**).

As for the total isoflavones obtained, the differences between the solvents with various polarities mainly reflected the differences in malonylglucosides (**Figures 2** and **3**), because malonylglucoside was the major form of isoflavones in the soybeans and it was also the form that demonstrated the largest differences caused by the solvent polarity. On average, 58% acetonitrile obtained 11 and 31% more total isoflavones than 80% methanol and 83% acetonitrile, respectively.

Effect of Solvent Acidity. A 1981 study recommended the use of acidic conditions for isoflavone extraction (*11*). However, because the study was done before acetylglucoside and malonylglucoside isomers were identified, the results needed to be revised for the analysis of the 12 soy isoflavone isomers. Recently, Murphy et al. (*14*) found no systematic impact pattern of the use of acidic conditions during extraction and recommended not to use acid in order to simplify the extraction protocol. In the present study, we have found that acidified solvents either extracted significantly ($P < 0.05$) lower amounts of isoflavones or did not significantly differ from the solvent without acid. Solvents with and without acidification showed very significant differences ($P < 0.005$) in extracting malonylglucoside isoflavones. On average, nonacidified solvents obtained 12% more malonyldaidzin, 9% more malonylgenistin, and 15% more malonylglycitin than acidified solvent. For the β-glucoside isoflavones, the acidification of the solvent showed a less significant effect (P value ranged from 0.01 to 0.05) on genistin and glycitin and no significant effect on daidzin. No significant effects of the acidification ($P > 0.05$) were found in the extraction of acetylgenistin and the aglycones, genistein and daidzein. Again, the differences in the total isoflavones obtained between acidified and nonacidified solvents mainly reflected the differences in malonylglucoside isoflavones. Nonacidified solvents obtained on average 7% more isoflavones than acidified solvents. A significant polarity−acidity interaction was found for aglycone extraction ($P = 0.0014$ for genistein, $P < 0.0001$ for daidzein), which suggests that the effect of the acid was not the same in solvents with different polarities.

Table 2. Polarity Index of Different Solvent Systems and Isoflavones Transformations during Extraction

extraction solvent	Snyder polarity index	% of genistin being transformed to genistein	% of daidzin being transformed to daidzein
83% acetonitrile	6.7	11	7
acidified 83% acetonitrile	6.7	14	12
80% methanol	7.1	7	5
acidified 80% methanol	7.1	13	9
58% acetonitrile	7.4	6	4
acidified 58% acetonitrile	7.4	9	7

Possible Transformations during Extraction. Chemical changes on isoflavone structures have been reported to occur during the processing of soybeans and soy products (*9*, *14*, *21*). The most frequently observed chemical changes of isoflavones during the processing are the cleavages of the side chains, including acylations and/or glycosylation. Malonylglucosides were changed to acetylglucosides under dry heat and to β-glucosides under moist heat (*14*). Glucosidase that naturally occurred in soybeans or from fermentation culture led to hydrolysis of isoflavone glucosides to aglycones (*9*). It is possible for acetylglucosides to change into β-glucosides, and it is also possible for all different conjugated forms to change into aglycones by cleaving of the glycosidic bond. Such isoflavones transformations could also occur during the extraction process. Breakdown of the main structure is unlikely, since the extraction process involves only mild conditions. To test the effects of extraction conditions on chemical transformations of isoflavones, known amounts of isoflavone standards were spiked into soybean samples. Ideally, the extraction process should preserve the chemical form of the compounds and generate a linear relationship between the added amount and the total amount (added plus in the sample).

Addition of known amounts of β-glucosides (genistin or daidzin) to the soybean resulted in higher recoveries of the β-glucosides as well as higher recoveries of their corresponding aglycone (genistein or daidzein). The recoveries of aglycones following adition of glucoside forms were even higher when samples were extracted with acidified solvents. This suggested that the β-glucoside form was being transformed to the aglycone form during the extraction procedure. **Table 2** shows the percentages of added β-glucosides that were transformed to the corresponding aglycones.

Isoflavone chemical transformations were also evident when a pure standard of acetylgenistin was subjected to the extraction procedure, without the use of the soybean matrix. Although only one isoflavone was used, genistin was detected in all of the samples extracted by acidified solvent systems (**Figure 4**) after the typical conditions of β-glucoside extraction.

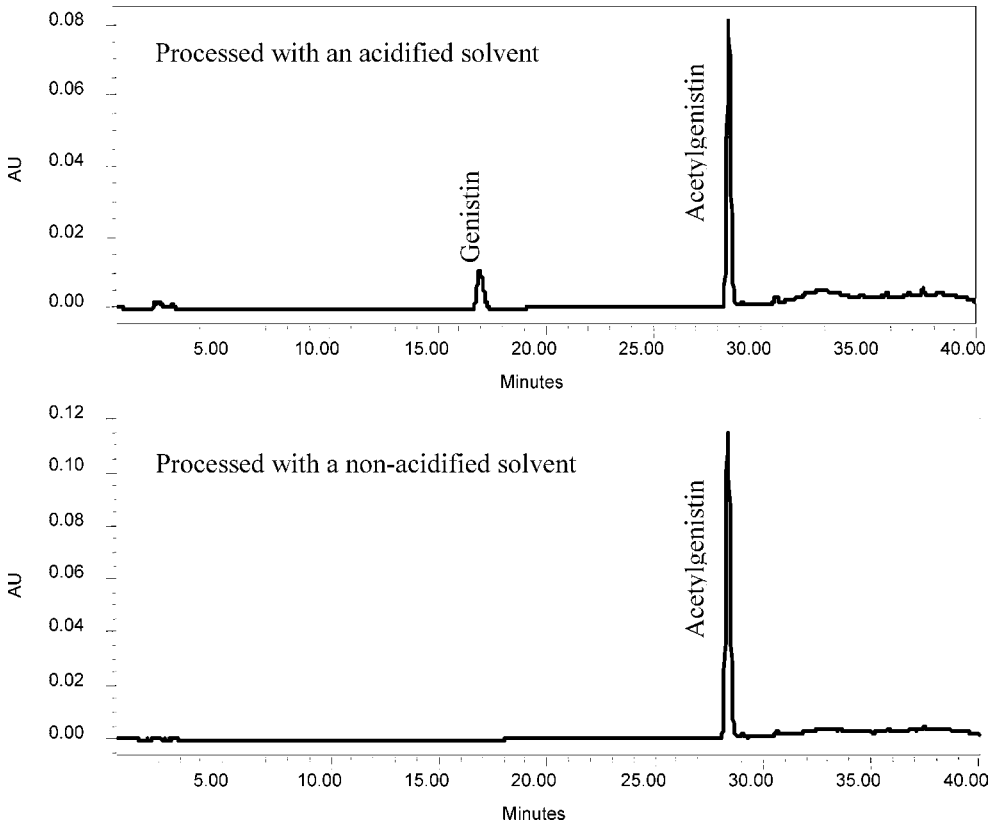

Figure 4. HPLC chromatograms of the acetylgenistin after the extraction processes with acidified solvent and nonacidified solvent. Column: Waters, Symmetry C18, 150 mm × 4.6 mm. Solvents: A, 0.1% acetic acid and 5% acetonitrile in water; B, 0.1% acetic acid in acetonitrile. Linear gradient from 10 to 70% B (90 to 30% A) in 30 min.

These results showed that β-glucoside isoflavones were transformed to their corresponding aglycones and that acetylglucoside isoflavones were transformed into their corresponding β-glucoside forms when subjected to the typical isoflavones extraction conditions and that acidified conditions significantly favored such changes.

Optimizing Conditions for Isoflavone Extraction. In view of the fact that isoflavones chemical transformations may occur during the extraction process, two issues need to be considered when comparing the extraction solvents: (i) isoflavone extraction efficiency for the particular solvent and (ii) preservation of original isoflavone composition during the extraction, minimizing the chemical transformations. We have shown that malonylglucoside isoflavones could be affected by such transformations during extraction. Therefore, the higher amount of malonylglucosides present in the extracted material indicates either higher extraction efficiency of the solvent, better protection from the chemical transformations, or both. In this study, solvents with a higher polarity extracted more malonyl isoflavones, and solvents without acid yielded more malonyl forms than solvents with acid. It was clear that among the examined six solvent systems, 58% acetonitrile without acid was the solvent that yielded the highest malonyl isoflavones concentrations. As for the two most widely used solvent systems, 80% methanol yielded more malonylglucosides than acidified 83% acetonitrile.

Concentrations of β-glucoside and acetylglucoside forms could be increased or decreased during the extraction procedure, and aglycones could be increased as a consequence of chemical transformations. Therefore, higher amounts obtained did not necessarily mean higher extraction efficiency since it could be the result of the transformations. Therefore, it was more difficult to determine which solvent was better than others in extracting β-glucoside, acetylglucoside, or aglycone isoflavones by simply comparing the amounts obtained. Several contrasts between different polarity levels and between acidified solvents and nonacidified solvents failed to find significant differences (**Table 1** and **Figure 3**). The significantly higher amounts of aglycone isoflavones extracted by acidified or nonacidified 83% acetonitrile may also be due to the transformation from malonylglucoside or β-glucoside isoflavones.

When comparing the extraction solvents in terms of the total isoflavones extracted (**Figure 2**), it was also clear that solvents with relatively higher polarity and no acid showed improved isoflavones extraction efficiency. Because isoflavones transformations would not affect the total molar amount of isoflavones extracted, the results indicated that solvents with relatively higher polarity and no acid were more efficient in general for extracting isoflavones from the matrix.

In conclusion, a relatively higher polarity of the solvent (in the range of Snyder polarity index $P' = 6.7-7.4$) was desirable for isoflavone extraction from soybeans. Acidification of the extraction solvent favored isoflavone transformations during the extraction and should be avoided for quantification of intact isoflavones. Among the six solvent systems examined in this study, 58% acetonitrile aqueous solution ($P' = 7.4$) without acidification was the best solvent for extraction of isoflavones from soybeans, since it yielded the highest total amounts and best maintained the intact structures. With regard to the two most widely used solvent systems, 80% methanol had a higher extraction efficiency and better protection against chemical transformations than acidified 83% acetonitrile.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; RCBD, andomized complete block design; ANOVA, analysis of variance.

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