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Functional Components in Soybean Cake and Their Effects on Antioxidant Activity

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The antioxidant activities of four fractions of isoflavones from soybean cake were evaluated and compared with those of ISO-1 and ISO-2 fractions, five isoflavone standards, and mixtures of two or four isoflavone standards, as well as four commercial antioxidants, using DPPH, TEAC, reducing power, metal ion chelating, conjugated diene, and TBARS assays. Both malonylglucoside and glucoside fractions were isolated using preparative chromatography with Diaion HP-20 as adsorbent, whereas acetylglucoside and aglycone fractions were separated with silica gel as adsorbent. The other two fractions, ISO-1 and ISO-2, were soybean cake extracts containing 12 isoflavones for the former and a combination of 4 fractions for the latter. Both acetylglucoside and ISO-1 fractions exhibited the highest efficiency in scavenging DPPH free radicals, whereas all six fractions were effective in inhibiting conjugated diene formation. However, a low reducing power was observed for all six fractions and isoflavone standards. The aglycone fraction and genistein standard showed a pronounced increase of TEAC value and a moderate decrease of TBARs value. For chelating metal ions, both ISO-1 and ISO-2 fractions were the most efficient. Overall, the isoflavone fractions showed a better antioxidant activity than the isoflavone standards, probably caused by the presence of some other functional components such as saponin, flavonoid, and phenolic compounds in soybean cake.

KEYWORDS: Soybean cake; antioxidant activity; isoflavone extract; functional components

INTRODUCTION

Epidemiological studies have demonstrated that consumption of soy foods is associated with a decreased risk of chronic diseases such as cancer (*1*, *2*), which may be due to the presence of high amounts of isoflavones in soybean. Twelve isoflavones are found in soybean and are present in four chemical forms (**Figure 1**): malonylglucoside (malonyldaidzin, malonylgenistin, and malonylglycitin), acetylglucoside (acetyldaidzin, acetylgenistin, and acetylglycitin), glucoside (daidzin, genistin, and glycitin), and aglycone (daidzein, genistein, and glycitein); however, the glucoside forms dominate (*3*, *4*). The potential health benefits of isoflavones on the prevention of atherosclerosis, osteoporosis, and postmenopausal syndrome have been well documented (*5*−*7*). In addition, the antioxidant activity of isoflavone has been shown to be correlated well with the reduction of heart disease (*2*).

Romero et al. (*8*) reported that isoflavones possess both antioxidant activity and metal ion chelating properties. Also, the antioxidant activity was closely related to the number of hydroxy groups in rings A and B, whereas metal ion chelation was facilitated by the presence of hydroxy groups in both rings A and B and by the presence of the keto group in the C ring as well. According to this statement, the antioxidant activity of aglycones such as genistein should be higher than that of its glucoside such as genistin. However, several studies have found no significant difference in antioxidant activity between glucosides and aglycones (*9*, *10*). Most studies are focused on the antioxidant activity of the isoflavone standards, that is, genistein, daidzein, genistin, and daidzin (*11*−*13*). The antioxidant activity of the isoflavone fractions, namely, malonylglucoside, acetylglucoside, glucoside, and aglycone, remains uncertain. The presence of functional components such as isoflavone, carotenoid, saponin, phenolic acid, ascorbic acid,

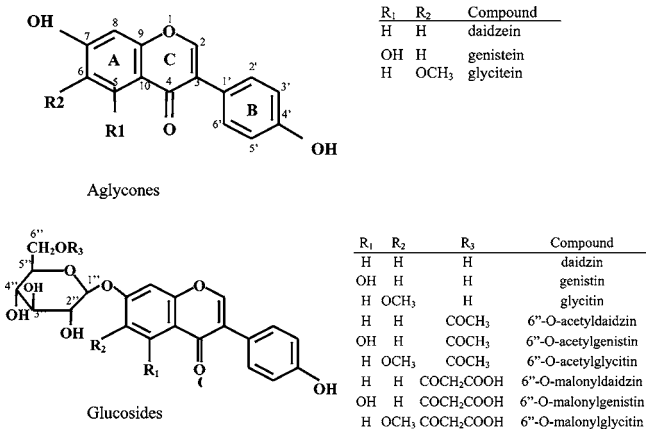

Figure 1. Chemical structures of isoflavones.

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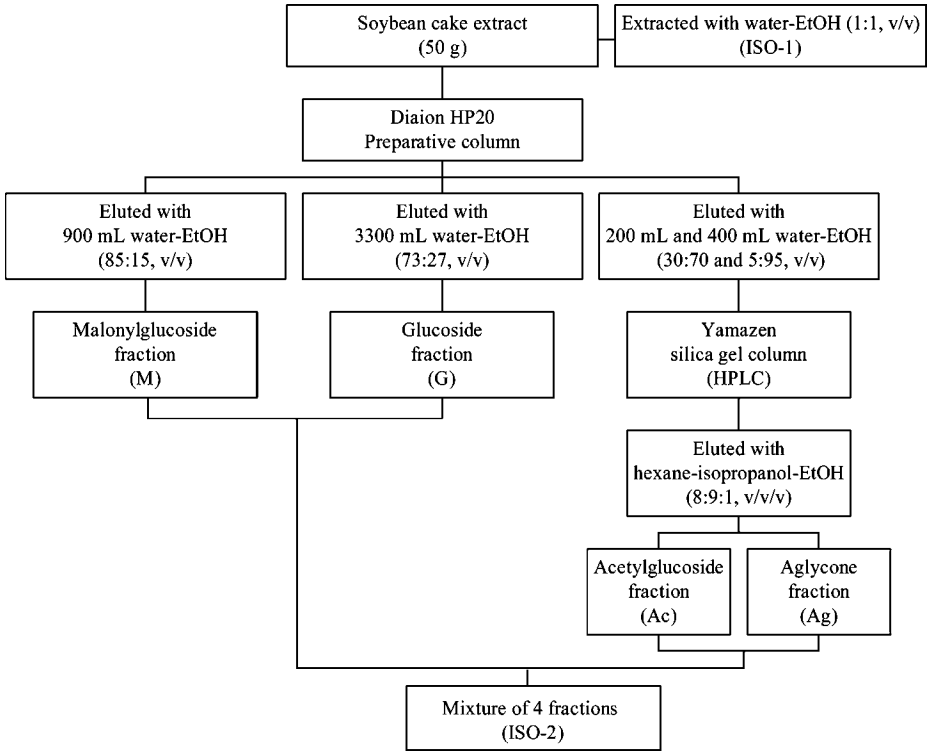

Figure 2. Flow diagram for preparation of isoflavone extracts for antioxidant activity test.

tocopherol, and riboflavin in soybean has been well documented (*2*, *14*, *15*). Soybean cake, a byproduct obtained during the processing of soybean oil, was found to be a rich source of isoflavones (*16*). However, the variety of other functional components present in soybean cake remains unknown. It would be an advantage to the health food industry if the various fractions of isoflavones in soybean cake could be separated and developed as functional products. The objectives of this study were to isolate four fractions of isoflavone extracts from soybean cake by preparative column chromatography, determine their functional components and antioxidant activities to elucidate the antioxidative mechanism, and compare them with ISO-1 and ISO-2 fractions, five isoflavone standards, and mixtures of two or four isoflavone standards, as well as four commercial antioxidants. In addition, we intended to develop a purification and separation method for the determination of saponin in soybean cake.

MATERIALS AND METHODS

Materials. Five isoflavone standards, with purities >98%, including malonylgenistin, genistin, acetylgenistin, genistein, and daidzein, were purchased from LC Laboratories (Woburn, MA) and were used without further purification. Soyasapogenol A and soyasapogenol B standards were obtained from Chromadex (Santa Ana, CA). Catechin, gallic acid, caffeic acid, and chlorogenic acid standards were from Sigma (St. Louis, MO). Reagents, including sodium dihydrogen phosphate, sodium hydrogen phosphate, manganese dioxide, ascorbic acid, butylated hydroxyanisole (BHA), ferrous sulfate, hexamethylene tetramine, ethylenediaminetetraacetic acid (EDTA), aluminum chloride hexahydrate, and sodium nitrite were obtained from Nacalai Tesque Co. (Kyoto, Japan). Iron(II) chloride, iron(III) chloride, and potassium ferricyanide were from Showa Chemical Co. (Tokyo, Japan). Ferrozin [5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4′,4″-disulfonic acid], DPPH (2,2-diphenyl-1-picrylhydrazyl), TCA (trichloroacetic acid), linoleic acid, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), TBA (2-thiobarbituric acid), α-tocopherol, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and PICB (1-pentane sulfuric acid sodium salt) were from Sigma. Folin−Ciocalteu reagent was from Merck (Darmstadt, Germany). Metaphosphoric acid was from Riedel-de Haen (Barcelona, Spain). Sylon BTZ was from Supelco (Bellefonte, PA). Ethanol (95%) was from Taiwan Tobacco and Liquor Co. (Tainan, Taiwan). Methanol was from Mallinckrodt Co. (Paris, KY). Lecithin was from Cheng-Fang Co. (Taipei, Taiwan). Deionized water was made using a Milli-Q water purification system (Bedford, MA). The HPLC C18 columns 201TP54 (250 × 4.6 mm i.d., 5 μm), HyPURITY (150 × 4.6 mm i.d., 5 μm), and Gemini (250 × 4.6 mm i.d., 5 μm) were from Vydac Co. (Hesperia, CA), Thermo Hypersil-Keystone Co. (Bellefonte, PA), and Phenomenex Co. (Torrance, CA), respectively. The glass column (375 × 45 mm i.d.) was from Zen-Mei Co. (Taipei, Taiwan). The silica gel column (170 × 48 mm i.d., 40 μm) was from Yamazen Co. (Osaka, Japan).

Instrumentation. The HPLC system is composed of two Jasco pumps (models PU 980 and PU 1980, Jasco Co., Tokyo, Japan), a Rheodyne injector (model 7161, Rheodyne Co., Cotati, CA), a Jasco UV-970 detector, a Jasco MD-915 photodiode array detector, and an Alltec ELSD 800 evaporative light scattering detector (Deerfield, IL). The GC instrument (model 6890) equipped with a mass spectrometer (model 5973) was from Agilent Technologies (Palo Alto, CA). The high-speed centrifuge was from DuPont Co. (Wilmington, DE). The N-1 rotary evaporator was from Eyela Co. (Tokyo, Japan).

Methods. *Preparation of Isoflavone Extracts.* A method similar to that described by Kao et al. (*16*) was used to prepare four fractions of isoflavone from soybean cake (**Figure 2**). Briefly, a 50-g soybean cake sample was mixed with 150 mL of ethanol/water (1:1 v/v) and shaken at room temperature for 2 h, after which the mixture was centrifuged at 6000 rpm for 20 min at 25 °C. Then 80 mL of soybean cake extract was poured into the top of a glass column (375 × 45 mm i.d.) containing 200 g of adsorbent Diaion HP-20, which was prewet with 1 L of ethanol and 1 L of deionized water. The water-soluble impurities were removed with 400 mL of deionized water, followed by elution of the malonylglucoside and glucoside fractions with 900 mL of water/ethanol (85:15, v/v) and 3300 mL of water/ethanol (73:27, v/v), respectively. The acetylglucoside and aglycone fractions were eluted with 200 mL of water/ethanol (30:70, v/v) and 400 mL of water/ethanol (5:95, v/v), respectively. Each fraction was injected into HPLC to monitor the composition of isoflavone. An HPLC gradient mobile phase developed by Hsieh et al. (*17*) was used to determine the various isoflavones in

Table 1. Contents (Micrograms per Milliliter) of Isoflavones in Each Fraction and Standard Solution for Antioxidant Activity Test[a]

	isoflavone fractions						isoflavone standard solutions						
isoflavone	M	G	Ac	Ag	ISO-1	ISO-2	Mgin	Gin	Agin	Gein	Dein	2 std	4 std
malonyldaidzin	48.8 ± 0.3				22.9 ± 0.0	12.0 ± 0.0							
malonylglycitin	16.0 ± 0.2				8.1 ± 0.2	4.0 ± 0.0							
malonylgenistin	35.1 ± 1.2				17.0 ± 0.1	8.8 ± 0.1	100.0						100.0
daidzin		25.3 ± 0.1			9.1 ± 0.1	6.3 ± 0.1							
glycitin		29.2 ± 0.2			9.0 ± 0.1	7.3 ± 0.1							
genistin		45.9 ± 1.6			26.3 ± 0.1	11.5 ± 0.2		100.0					100.0
acetyldaidzin			19.7 ± 0.9	-	1.3 ± 0.0	4.9 ± 0.1	-						
acetylglycitin			12.8 ± 0.7	-	1.0 ± 0.0	3.2 ± 0.2							
acetylgenistin			69.0 ± 1.6	-	2.5 ± 0.0	17.3 ± 0.3		-	100.0				100.0
daidzein				47.3 ± 0.5	1.4 ± 0.1	11.9 ± 0.1					100.0	100.0	
glycitein				16.3 ± 0.2	0.6 ± 0.0	4.1 ± 0.0							
genistein				36.4 ± 0.1	1.0 ± 0.0	9.2 ± 0.1				100.0		100.0	100.0
total	99.9 ± 1.7	100.4 ± 0.8	101.5 ± 2.1	100.0 ± 0.6	100.2 ± 0.0	100.5 ± 0.2	100.0	100.0	100.0	100.0	100.0	200.0	400.0

[a] M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycone fraction; ISO-1, soybean cake extract containing 12 isoflavones; ISO-2, a mixture of four fractions of isoflavone extracts; Mgin, malonylgenistin; Gin, genistin; Agin, acetylgenistin; Gein, genistein; Dein, daidzein; 2 std, a mixture of daidzein and genistein standards; 4 std, a mixture of malonylgenistin, genistin, acetylgenistin, and genistein standards.

soybean cake extract: 8% acetonitrile (A) and 92% water (B) in the beginning, increased to 10% A in 2 min, to 12% A in 4 min, to 22% A in 10 min, to 23% A in 11 min, to 35% A in 12 min, and to 50% A in 13 min, maintained for 3 min, and returned to 8% A in 20 min. The 12 isoflavones plus internal standard formononetin were resolved within 15 min by using a Vydac 201TP54 C18 column, with flow rate at 2.0 mL/min and column temperature at 35 °C as well as detection at 262 nm. The identification and quantification of each isoflavone were carried out using a method described by Kao et al. (*16*). On the basis of HPLC analysis, the malonylglucoside fraction was found to contain malonyldaidzin, malonylgenistin, and malonylglycitin, whereas the glucoside fraction contained daidzin, genistin, and glycitin. However, in addition to daidzein, genistein, and glycitein, the aglycone fraction was found to contain a high amount of acetylgenistin. To further separate the aglycone and acetylglucoside fractions, both were combined, and solvents were removed under vacuum and dissolved in 20 mL of isopropanol. Then 10 mL of eluate was injected into a preparative HPLC Yamazen Hi-Flash silica gel column (170 × 48 mm i.d.), and the acetylglucoside and aglycone fractions were separated with a mobile phase of hexane/isopropanol/ethanol (8:9:1, v/v/v) with a flow rate at 20 mL/min. Two large peaks were present on the HPLC chromatogram. After collecting the eluate of each peak and subjecting it to HPLC analysis, one peak was found indeed to be the aglycone fraction containing daidzein, genistein, and glycitein, whereas the other peak was the acetylglucoside fraction containing acetyldaidzin, acetylglycitin, and acetylgenistin. After quantification, the concentrations of malonylglucoside, glucoside, acetylglucoside, and aglycone fractions were 1343.4, 931.0, 123.2, and 134.8 μg/mL, respectively. For the preparation of working solutions, 2 mL of malonylglucoside, 3 mL of glucoside, 18 mL of acetylglucoside, and 18 mL of aglycone were mixed with 24.8, 24.9, 4.1, and 6.2 mL of water/ethanol (1:1, v/v) separately to obtain a concentration of 100 μg/mL each. For the other two fractions ISO-1 and ISO-2, the crude soybean cake extract containing 12 isoflavones with a total concentration of 1670.0 μg/mL was used for the preparation of ISO-1 at 100 μg/mL by mixing 2 mL of soybean cake extract and 31.4 mL of water/ethanol (1:1, v/v), whereas a mixture of four fractions of isoflavone (malonylglucoside, glucoside, acetylglucoside, and aglycone) with a total concentration of 100 μg/mL was prepared for ISO-2 by mixing 5 mL of each fraction containing 100 μg/mL isoflavone as shown above. For the other treatments, the standard solutions of malonylgenistin (Mgin), genistin (Gin), acetylgenistin (Agin), genistein (Gein), daidzein (Dein), α-tocopherol (Vit E), ascorbic acid (Vit C), EDTA, and BHA were each prepared with a concentration of 100 μg/mL. In addition, mixtures of 100 μg/mL each of diadzein and genistein (2 std), and 100 μg/mL each of malonylgenistin, genistin, acetylgenistin and genistein (4 std) were also prepared. **Table 1** shows the contents of various isoflavones in each fraction and standard solution. Also, for the reducing power assay, one more concentration of 50 μg/mL was used for all of the standards because a high absorbance (>1.2) occurring at 100 μg/mL would decrease the quantitation accuracy.

Determination of Other Functional Components in Isoflavone Extracts. (a) Phenolic Acid. A method based on that of Shen (*18*) was modified to analyze phenolic acid. A 1-mL isoflavone extract was evaporated to dryness under nitrogen and dissolved in 0.9% acetic acid solution, which was filtered through a 0.2-μm membrane filter, and 20 μL was injected for HPLC analysis. The mobile phase was composed of 0.9% acetic acid solution (A) and acetonitrile (B) with the following gradient elution: 92% A and 8% B in the beginning, maintained for 2 min and decreased to 0% A in 8 min. Gallic acid, caffeic acid, and chlorogenic acid were separated within 7 min by using a Thermo Hypersil-Keystone HyPURITY C18 column with flow rate at 1 mL/min and detection at 280 nm. Various concentrations of gallic acid, caffeic acid, and chlorogenic acid were prepared to obtain standard curves, and the linear regression equation of each was used for quantitation.

(b) Total Flavonoids. A method based on that of Jia et al. (*19*) was modified to quantify total flavonoids in isoflavone extracts, which were based on catechin equivalents. Five concentrations of 1, 5, 10, 25, and 50 μg/mL were prepared by dissolving catechin standard in deionized water/ethanol (1:1, v/v). A 500-μL solution was collected and mixed with 75 μL of 5% sodium nitrite solution, and the mixture was allowed to stand for 5 min, followed by the addition of 150 μL of 10% aluminum chloride solution, another 5 min of standing, and then mixing with 500 μL of 1 M sodium hydroxide solution. The absorbance of the final solution was measured at 510 nm, and the standard curve was obtained by plotting standard concentration against absorbance. Likewise, a 500-μL isoflavone extract was collected and prepared using the same procedure shown above, and the absorbance was measured at 510 nm. Quantitation was carried out using the linear regression equation of the catechin standard curve.

(c) Total Phenolic Compounds. A method based on that of Soong and Barlow (*20*) was modified to quantify total phenolic compounds, which were based on gallic acid equivalents. Six gallic acid standard solutions of 50, 100, 200, 250, 300, and 350 μg/mL were prepared in deionized water, of which a 50-μL solution was collected and mixed with 200 μL of Folin−Ciocalteu reagent. After mixing and standing at room temperature for 5 min, a 1000-μL 15% sodium carbonate solution was added, and the mixture was allowed to stand at room temperature for 60 min. The absorbance of the solution was measured at 750 nm, and the standard curve was obtained by plotting concentration against absorbance. Similarly, a 50-μL isoflavone solution was collected and prepared using the same procedure shown above, and quantitation was performed using the linear regression equation of the gallic acid standard curve.

(d) Ascorbic Acid. A method based on that of Kacem et al. (*21*) was modified to quantify ascorbic acid in isoflavone extracts. A 1-mL

isoflavone extract was evaporated to dryness under nitrogen and dissolved in 5 mM PICB solution (pH 3.1), and the solution was filtered through a 0.2-μm membrane filter for HPLC analysis. A mobile phase of 5 mM aqueous PICB solution (pH 3.1) and a Thermo Hypersil-Keystone HyPURITY C18 column was used to separate ascorbic acid with a flow rate at 0.6 mL/min and detection at 254 nm. Quantitation was carried out using the standard curve of ascorbic acid, which was obtained by plotting concentration against area.

(e) α-Tocopherol. A method based on that of Anna and Ewa (*22*) was modified to quantify α-tocopherol in isoflavone extracts. A 1-mL isoflavone extract was filtered through a 0.2-μm membrane filter for HPLC analysis. A mobile phase of methanol/ethyl acetate (60:40, v/v) and a Thermo Hypersil-Keystone HyPURITY C18 column with a flow rate at 1 mL/min and detection at 290 nm was used to analyze α-tocopherol. Quantitation was accomplished using the standard curve of α-tocopherol, which was obtained by plotting concentration against area.

(f) Soyasaponins. A method based on that of Rupasinghe et al. (*14*) was modified to quantify soyasaponins in isoflavone extracts. A 20-mL isoflavone extract was evaporated to dryness and dissolved in 5 mL of 1 N methanolic hydrochloric acid solution, and the mixture was shaken at 75 °C (150 rpm) for 2.5 h for hydrolysis. The hydrolyzed solution was also evaporated to dryness, dissolved in 5 mL of methanol, and filtered through a 0.2-μm membrane filter. A 0.5-mL solution was collected and poured into a Phenomenex Strata C-18 cartridge, which was previously activated with 10 mL of methanol. The water-soluble impurities were first eluted with 3 mL of deionized water, followed by elution of soyasaponins with 3 mL of methanol. Six fractions of 0.5 mL each for deionized water and methanol were collected to monitor the presence of soyasaponins in each fraction by HPLC analysis (**Figure 3**). It was found that the most appropriate volumes for deionized water and methanol were 2 and 2 mL, respectively. A mobile phase of 100% acetonitrile and a Phenomenex Gemini C18 column (250 × 4.6 mm i.d., particle size = 5 μm) with a flow rate at 1 mL/min and detection by ELSD were used to separate soyasapogenol A, soyasapogenol B, and 20(*S*)-protopanaxadiol with a temperature of 70 °C, a pressure of 3 bar, and a gain value of 8.

For positive identification of soyasaponins in isoflavone extracts, a GC-MS technique was also used (**Figures 4** and **5**). A concentration of 100 μg/mL soyasapogenol A and soyasapogenol B standard in methanol was prepared separately, and 100 μL of each was collected and evaporated to dryness. One hundred microliters of Sylon BTA reagent was added to the dried residue and subjected to derivatization at 70 °C for 30 min, after which a 1-μL sample was injected for GC-MS analysis. The column temperature was programmed as follows: 280 °C initially, maintained for 1 min, raised to 300 °C at 5 °C/min, and maintained for 30 min. The injector temperature was 270 °C and the GC-MS interface temperature 280 °C. Both soyasapogenol A and soyasapogenol B were separated within 24 min by using an Agilent HP-5MS capillary column with helium as carrier gas, a flow rate at 0.6 mL/min, and a split ratio at 2:1. Similarly, a 100-μL purified isoflavone extract from the acetylglucoside fraction was derivatized using the same procedure shown above for GC-MS analysis. Both soyasapogenol A and soyasapogenol B were identified by comparing retention time and mass spectra of unknown peaks with those of reference standards, and a high match quality of 93% was attained.

Quantitation was performed by using an internal standard method for HPLC analysis. Seven concentrations of 10, 50, 100, 150, 200, 300, and 400 μg/mL of soyasapogenol A and soyasapogenol B in methanol were each prepared and mixed with internal standard for a final concentration of 100 μg/mL 20(*S*)-protopanaxadiol. A 20-μL sample of each concentration was injected for HPLC, and the standard curves were obtained by plotting concentration ratio against area ratio. The linear regression equations and correlation coefficient (r^2) obtained for each standard curve were used for quantitation. Recovery was carried out by adding 50 and 200 μg/mL of soyasapogenol A or soyasapogenol B to 20 mL of isoflavone extract (ISO-1) and using the same extraction and purification procedures shown above. After HPLC analysis, the recovery was calculated on the basis of the ratio of the amount of soyasapogenol standard after and before HPLC.

DPPH Assay. A method described by Lee et al. (*23*) was used. One milliliter of soybean cake extract of malonylglucoside, acetylglucoside, glucoside, aglycone, ISO-1, and ISO-2, as well as standard solutions of Mgin, Gin, Agin, Gein, Dein, 2 std, 4 std, Vit E, Vit C, EDTA, and BHA, were each mixed with 0.25 mL of methanolic DPPH solution (1 mM). After mixing thoroughly, the mixture was left in the dark for 30 min and the absorbance (ABS) was measured at 517 nm. The scavenging effect (percent) was calculated as follows:

$$\text{scavenging effect (\%)} = \frac{1 - \text{ABS of sample at 517 nm}}{\text{ABS of control treatment at 517 nm}} \times 100$$

TEAC (Trolox Equivalent Antioxidant Capacity) Assay. A method based on that of Miller et al. (*24*) was modified. A 20-mL $ABTS^{\bullet+}$ standard solution (1 mM) was prepared and filtered through a Whatman no. 5 filter paper containing 2 g of manganese dioxide, after which the filtrate was passed through a 0.2-μm PVDC syringe filter to remove excess manganese dioxide. Then the blue-green solution was diluted with 5 mM phosphate-buffered saline (pH 7.7) until the absorbance was 1.00 (± 0.02) when measured at 734 nm. The $ABTS^{\bullet+}$ solution was prepared fresh each day.

Seven concentrations (50, 100, 200, 400, 600, 650, and 700 μM) of Trolox standard solutions were prepared in 5 mM phosphate-buffered saline (pH 7.7), after which 0.1 mL of Trolox solution each was mixed with 1 mL of $ABTS^{\bullet+}$ solution separately. The solution was mixed thoroughly for 30 s and allowed to stand for another 30 s, and then the absorbance was measured at 734 nm. The standard solution of Trolox solution was obtained by plotting concentration against absorbance and the regression equation was calculated. Likewise, 0.1 mL of all six fractions of isoflavone extracts and isoflavone standards as well as Vit C, Vit E, EDTA, BHA, deionized water, and water/ethanol (1:1, v/v) were each mixed with 1 mL of $ABTS^{\bullet+}$ solution separately, and the absorbance at 734 nm was measured after each solution was mixed thoroughly for 30 s and allowed to stand for another 30 s. Both deionized water and water/ethanol (1:1, v/v) were used as control treatments. The relative Trolox concentration was calculated on the basis of the regression equation. A higher relative Trolox concentration indicated a better antioxidant activity.

Ferrous Iron Chelating Assay. A method based on that of Dinis et al. (*25*) was modified. One-milliliter samples of all six fractions of isoflavone extracts and isoflavone standards, as well as Vit E, Vit C, EDTA, BHA, and water/ethanol (1:1, v/v), were each mixed with 3.7 mL of methanol and 0.1 mL of iron(II) chloride (2 mM) separately. After thorough mixing for 30 s, 0.2 mL of ferrozine solution (5 mM) was added to each sample, and the reaction proceeded at room temperature for 10 min for measurement of absorbance at 562 nm. The chelating effect (percent) was calculated as follows:

$$\text{chelating effect (\%)} = \frac{1 - \text{ABS of sample at 562 nm}}{\text{ABS of control treatment at 562 nm}} \times 100$$

Reducing Power Assay. A method based on that of Romero et al. (*8*) was modified. One-milliliter samples of all six fractions of isoflavone extracts and isoflavone standards, as well as Vit E, Vit C, BHA, EDTA, deionized water, and water/ethanol (1:1, v/v), were each mixed with 0.5 mL of potassium ferricyanide (1%) separately. Each solution was heated in a water bath at 50 °C for 20 min and then cooled immediately, after which 0.5 mL of trifluoroacetic acid solution (10%) was added. After mixing, 1 mL of supernatant solution was collected and mixed with 1 mL of deionized water and 1 mL of iron(II) chloride solution (0.1%, w/w). The absorbance was measured at 700 nm after the reaction proceeded for 10 min. A higher absorbance indicated a better reducing power of the sample.

Conjugated Diene Assay. A method based on that of Romero et al. (*8*) was used. The standard solution of linoleic acid emulsion was prepared fresh by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20, and 50 mL of 0.2 M phosphate-buffered saline (pH 6.6), after which the mixture was homogenized. Half-milliliter samples of all six fractions of isoflavone extracts and isoflavone standards, as well as Vit E, Vit

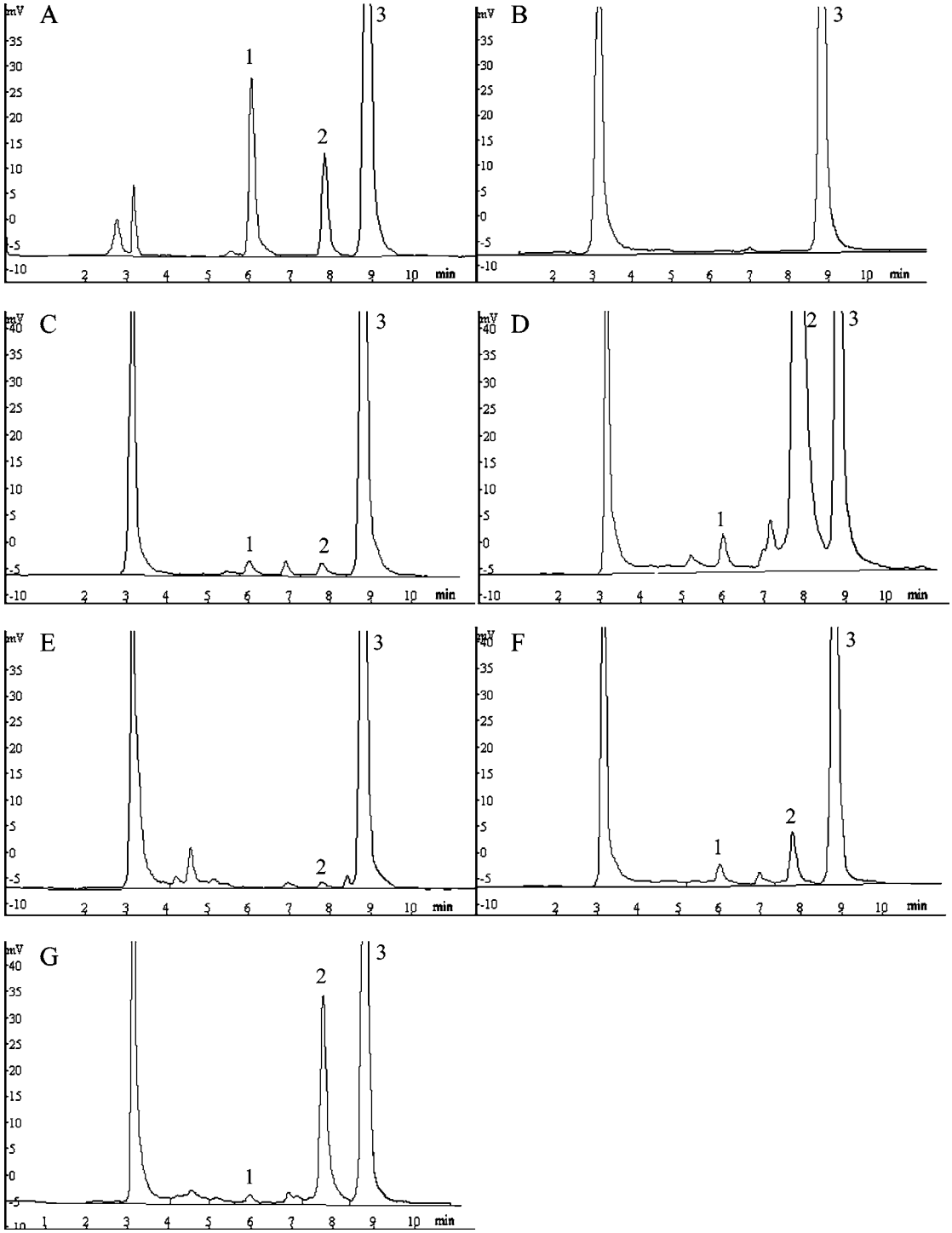

Figure 3. HPLC chromatogram of soyasaponin in isoflavone extracts from soybean cake: (**A**) soyasaponin standards; (**B**) malonylglucoside fraction; (**C**) glucoside fraction; (**D**) acetylglucoside fraction; (**E**) aglycone fraction; (**F**) soybean cake extract containing 12 isoflavones (ISO-1); (**G**) a mixture of 4 fractions of isoflavone extracts (ISO-2). Peaks: 1, soyasapogenol A; 2, soyasapogenol B; 3, 20(*S*)-protopanaxadiol (I.S.).

C, EDTA, BHA, and water/ethanol (1:1, v/v), were each mixed with 2 mL of linoleic acid emulsion separately. After thorough mixing, each solution was heated in a water bath at 37 °C for 15 h and then cooled immediately. A 0.1-mL sample was collected at 0 and 15 h separately, then mixed with 3 mL of methanol (80%), and the absorbance was measured at 234 nm. A lower absorbance indicated a higher antioxidant activity of the sample. The inhibition effect (percent) can be calculated as follows:

$$\text{inhibition effect (\%)} = 1 - \frac{\text{ABS of sample at 15 h} - \text{ABS of sample at 0 h}}{\text{ABS of control treatment at 15 h} - \text{ABS of control treatment at 0 h}}$$

TBARS Assay. A method based on that of Yin et al. (*26*) was used. The standard liposome solution was prepared fresh by mixing 300 mg of lecithin and 30 mL of 20 mM phosphate-buffered saline (pH 7.4), and then the mixture was homogenized. Half-milliliter samples of all six fractions of isoflavone extracts and isoflavone standards, as well as Vit E, Vit C, EDTA, BHA and water−ethanol (1:1, v/v), were each mixed with 2 mL of liposome solution, 0.1 mL of iron(II) chloride solution (25 mM), 0.1 mL of ascorbic acid solution (25 mM), and 1.2 mL of 20 mM phosphate-buffered saline (pH 7.4) separately, after which each solution was heated in a water bath at 37 °C for 15 h and then cooled immediately. Then 1-mL ethanolic BHT solution (20 mg/mL), 2 mL of TBA solution (1% in 1 M sodium hydroxide), and 1 mL of

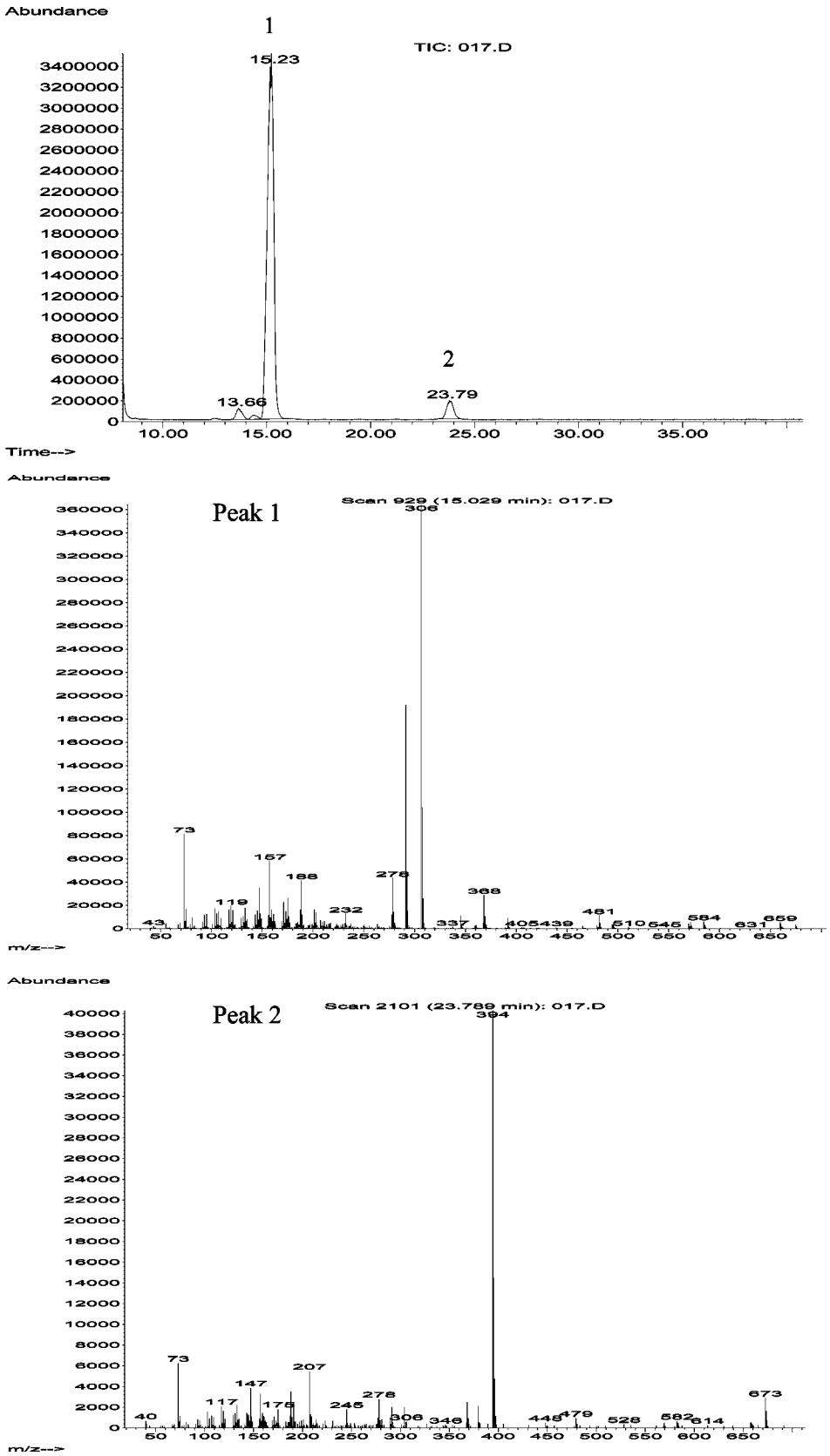

Figure 4. GC chromatogram and mass spectrum of soyasaponin in acetylglucoside fraction from soybean cake. Peaks: 1, soyasapogenol B; 2, soyasapogenol A.

trifluoroacetic acid solution (2.8%) were added to each solution, after which the mixture was heated at 100 °C for 20 min and the absorbance measured at 532 nm. A lower absorbance indicated a better antioxidant activity of the sample. The inhibition effect (percent) was calculated as follows:

$$\text{inhibition effect (\%)} = 1 - \frac{\text{ABS of sample at 532 nm}}{\text{ABS of control treatment at 532 nm}} \times 100$$

Statistical Analysis. All of the experiments were conducted in duplicate, and the data were analyzed using SAS software system (*27*).

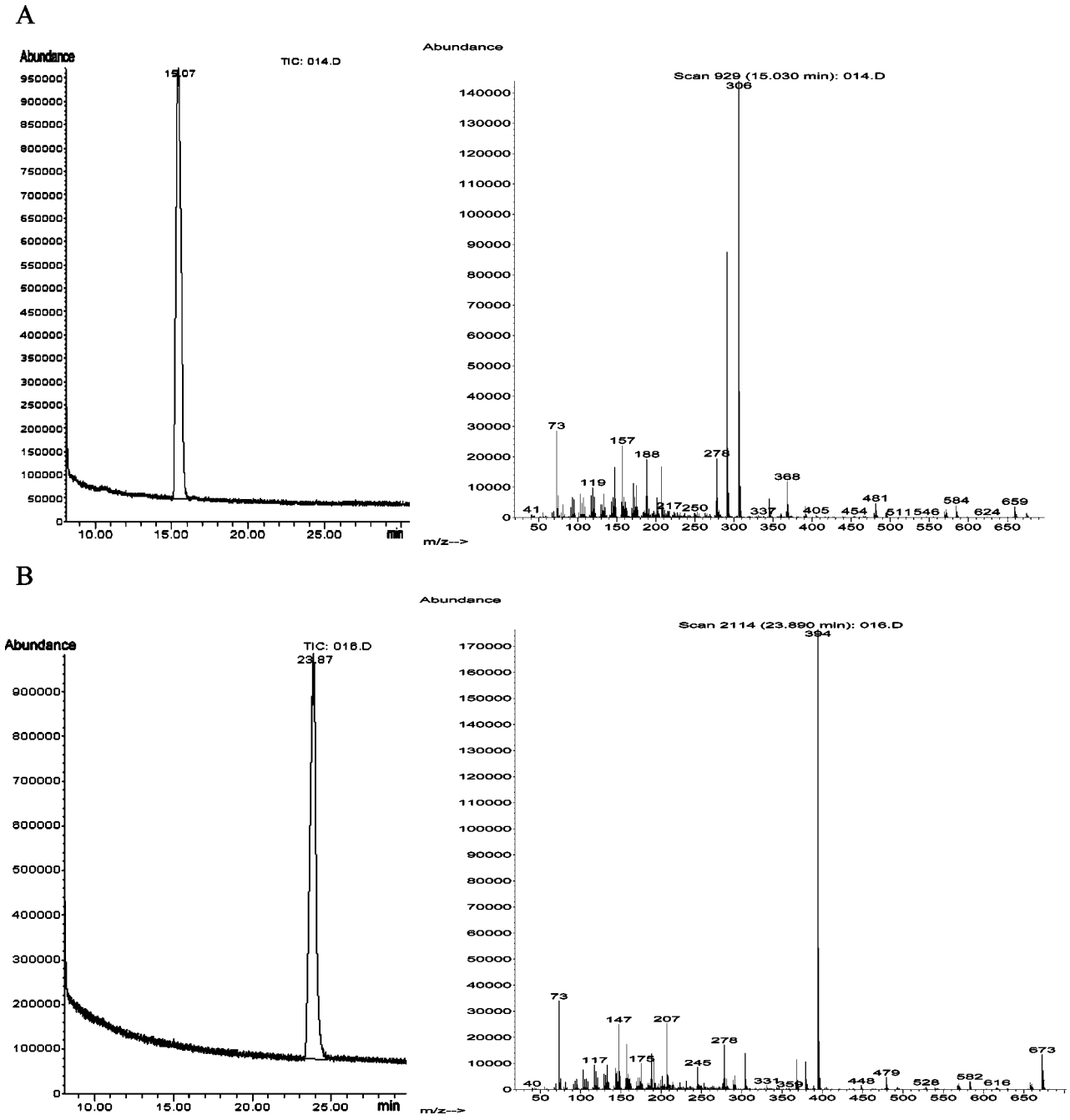

Figure 5. GC chromatogram and mass spectrum of soyasaponin standards: (**A**) soyasapogenol B; (**B**) soyasapogenol A.

The data were also subjected to analysis of variance (ANOVA) and Duncan's multiple-range test for comparison of significant difference ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Determination of Other Functional Components in Soybean Cake. Several studies have demonstrated that in addition to isoflavones, there are some other functional components such as phenolic acid, saponins, carotenoids, riboflavin, ascorbic acid, and tocopherol present in soybean (*2*, *14*, *15*). Because most of these components have been shown to possess high antioxidant activity, they were determined in our experiment to elucidate the antioxidative mechanism of isoflavone extracts from soybean cake. Vedavanam et al. (*15*) reported that phenolic acid may be present in soybean extract, but the identity remains unknown. In our study, three phenolic acids, that is, gallic acid, caffeic acid, and chlorogenic acid, were investigated; however, none were detected by HPLC analysis (**Table 2**). Likewise, no ascorbic acid or α-tocopherol was detected in soybean cake extract, both of which might be removed during the oil extraction process. The contents of total flavonoids in each fraction ranged from 1.82 to 26.44 μg/mL (catechin equivalents) (**Table 2**), which were much lower when compared to isoflavones in soybean cake. The aglycone fraction showed the highest level of total phenolic compounds, which were equivalent to 319.2 μg/mL gallic acid (**Table 2**), followed by ISO-2 (258.9 μg/mL), ISO-1 (203.6 μg/mL), acetylglucoside fraction (201.9 μg/mL), malonylglucoside fraction (179.2 μg/mL), and glucoside fraction (142.3 μg/mL). This result indicated that the presence of phenolic compounds in soybean cake extract may also be responsible for antioxidant activity.

Soyasaponins belong to a class of triteroenois glycosides, which are divided into three groups, that is, soyasapogenol A, soyasapogenol B, and soyasapogenol E in combination with

Table 2. Contents (Micrograms per Milliliter)[a] of Functional Components in Various Isoflavone Fractions and Extracts[b]

	phenolic acids							saponins	
isoflavone extract	gallic acid	caffeic acid	chlorogenic acid	total flavonoid[c]	total phenolic compound[d]	α-tocopherol	ascorbic acid	soyasapogenol A	soyasapogenol B
M	nd[e]	nd	nd	5.62 ± 0.02e	179.2 ± 4.4d	nd	nd	nd	nd
G	nd	nd	nd	1.82 ± 0.01f	142.3 ± 7.9e	nd	nd	16.41 ± 0.07c	16.33 ± 0.04d
Ac	nd	nd	nd	22.66 ± 1.43b	201.9 ± 9.1c	nd	nd	40.03 ± 0.40a	332.2 ± 11.1a
Ag	nd	nd	nd	26.44 ± 0.19a	319.2 ± 9.5a	nd	nd	nd	14.75 ± 0.13e
ISO-1	nd	nd	nd	17.63 ± 0.81d	203.6 ± 9.5c	nd	nd	35.46 ± 0.59b	41.57 ± 1.50c
ISO-2	nd	nd	nd	19.36 ± 0.12c	258.9 ± 2.0b	nd	nd	15.84 ± 0.21d	92.49 ± 2.36b

[a] Average of duplicate analyses ± standard deviation. [b] Entries bearing different letters (a–f) in the same column are significantly different ($P < 0.05$). M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycone fraction; ISO-1, soybean cake extract containing 12 isoflavones; ISO-2, a mixture of 4 fractions of isoflavone extracts. [c] Data expressed as μg/mL of catechin equivalents. [d] Data expressed as μg/mL of gallic acid equivalents [e] Not detected.

sugar moiety. Of the three soysaponins, soyasapogenol E with a sugar moiety was present in lowest amount as it is a photooxidation product of soyasapogenol B with a sugar moiety (*28*). In addition, soyasapogenol E has been reported to be susceptible to heat and acid losses, as evidenced by a conversion to soyasapogenol B during acid hydrolysis (*29*). Thus, Rupasinghe et al. (*14*) used 1 N methanolic hydrochloric acid to hydrolyze soyasaponins to soyasapogenol A and soyasapogenol B, which were analyzed by HPLC with ELSD for quantitation of soyasaponins. Following the same approach in our study (**Table 2** and **Figure 3**), the acetylglucoside fraction was found to contain the highest amounts of soyasapogenol A (40.03 μg/mL) and soyasapogenol B (332.2 μg/mL), followed by ISO-2 (15.84 and 92.49 μg/mL), ISO-1 (35.46 and 41.57 μg/mL), and the glucoside fraction (16.41 and 16.33 μg/mL), respectively, whereas the aglycone fraction contained only soyaspogenol B (14.75 μg/mL), and no soyasaponins were detected in the malonylglucoside fraction.

DPPH Assay. Table 3 shows the DPPH free radical scavenging activity of isoflavones, Vit C, Vit E, EDTA, and BHA. The highest scavenging effect was shown for ascorbic acid (95.8%), followed by BHA (84.8%) and α-tocopherol (84.6%). However, no or minor scavenging effect (0−4.0%) was found for the various isoflavone standards, implying that the scavenging activity was not affected by the presence or absence of the sugar moiety. Mixtures of two and four isoflavone standards were more effective in scavenging DPPH free radicals, which equaled 5.6 and 10.5%, respectively, indicating that a slight synergistic effect did occur. A similar phenomenon was reported by Lee et al. (*23*), who found that most isoflavones possessed low scavenging potency for DPPH free radicals, with the scavenging effect being only half that of α-tocopherol and one-third that of epicatechin. The weak scavenging activity of isoflavone standards is probably because of the steric effect of the hydroxy groups and sugar moieties of isoflavone, and its glucosides may behave differently in the reaction media (*23*). For the soybean cake extract, both ISO-1 and acetylglucoside fractions showed a high scavenging effect, which amounted to 70.0 and 68.1%, respectively, whereas the fractions of ISO-2, aglycone, glucoside, and malonylglucoside showed lower scavenging effects of 46.9, 44.9, 7.1, and 39.3%, respectively. This result clearly demonstrated that the isoflavone extracts exhibited a greater scavenging activity than the isoflavone standards. In addition to the synergistic effect contributed by the various isoflavones in the soybean cake extract, the presence of some other functional components cannot be ignored. Several reports have revealed that soyasapogenol B was effective in scavenging reactive oxygen (*30*, *31*) and inhibiting oxidative damage caused by reactive oxygen when administered to rats (*31*). Thus, the antioxidant activity of the acetylglucoside fraction can be mainly attributed to the presence of a large amount of soyasapogenol B (**Table 2**). Although the aglycone fraction contained a lower level of saponin (14.75 μg/mL), it was still efficient in scavenging DPPH free radicals, which may be due to the presence of high contents of total phenolic compounds (319.2 μg/mL) and flavonoids (26.44 μg/mL). A similar outcome was observed for ISO-1 and ISO-2 fractions, which also contained phenolic components and flavonoids, which equaled 203.6 and 258.9 μg/mL and 17.63 and 19.36 μg/mL, respectively (**Table 2**). Conversely, both malonylglucoside and glucoside fractions were less effective in scavenging DPPH free radicals, probably because they contained a lesser amount of phenolic compounds and flavonoids (**Table 2**). Vedavanam et al. (*15*) studied the effect of ethanol extract of soybean protein and isoflavone standards (daidzein and genistein) on the inhibition of low-density lipoprotein (LDL) oxidation, and a synergistic effect was shown for the extract. The authors pointed out that the presence of some other antioxidants such as phenolic acid in the extract may generate a higher free radical scavenging activity than genistein or daidzein. In a similar study, Lee et al. (*32*) evaluated the antioxidant activity of 17 soybean cultivars and reported that the low scavenging potency of DPPH free radicals of soybean extract may be caused by the presence of a small amount of isoflavone in aglycone form. However, in our study both daidzein (47.3 μg/mL) and genistein (36.4 μg/mL) in the aglycone fraction may play a more important role in scavenging DPPH free radicals.

TEAC Assay. Table 3 shows the TEAC values of isoflavones, Vit E, Vit C, EDTA, and BHA. BHA showed the most pronounced effect, followed by four isoflavone standards, two isoflavone standards, genistein, and daidzein, which were equivalent to 721, 695, 688, 661, and 566 μM Trolox, respectively. For isoflavone extracts, the aglycone fraction showed the highest TEAC value (615 μM), followed by ISO-2 (487 μM), acetylglucoside fraction (446 μM), ISO-1 fraction (437 μM), glucoside fraction (304 μM), and malonylglucoside fraction (237 μM). It was also observed that the greater the contents of total flavonoids and phenolic compounds, the higher the TEAC value. Both isoflavone extracts and standards showed a similar tendency; that is, the aglycone extract or standard was more effective than the glucoside extract or standard, which may be due to the presence of one more hydroxy group on genistein when compared to the glucose-containing isoflavone. This phenomenon was also observed by Ruiz-Larrea et al. (*12*) and Mitchell et al. (*33*). Following the same principle, genistein should be more effective in scavenging $ABTS^{\bullet+}$ radical cation than the other aglycone standard daidzein, because the former contains two hydroxy groups and the latter contains one. However, no significant difference in antioxidant activity between daidzein and daidzin was reported by Ruiz-Larrea (*12*).

Table 3. Antioxidant Activities[a] of Isoflavone Extracts and Isoflavone Standards as well as Commercial Antioxidants[b]

	isoflavone extracts						isoflavone standards							commercial antioxidants			
method	M	G	Ac	Ag	ISO-1	ISO-2	Mgin	Gin	Agin	Gein	Dein	2 std	4 std	Vit E	Vit C	EDTA	BHA
DPPH[c]	39.3 ± 0.2g	7.1 ± 0.6i	68.1 ± 0.5d	44.9 ± 0.0f	70.0 ± 0.2c	46.9 ± 0.0e	4.0 ± 0.7k	0.0 ± 0.0m	2.3 ± 0.0l	3.7 ± 0.0k	0.0 ± 0.0m	5.6 ± 0.3i	10.5 ± 0.5h	84.6 ± 0.0b	95.8 ± 0.1a	3.8 ± 0.7k	84.8 ± 0.1b
TEAC[d]	237 ± 2m	304 ± 3k	446 ± 3h	615 ± 2d	437 ± 0hi	487 ± 8g	247 ± 7l	214 ± 3n	327 ± 10j	661 ± 4c	566 ± 1e	688 ± 3b	695 ± 0b	427 ± 9i	501 ± 4f	0 ± 0o	721 ± 2a
metal ion chelating[e]	43.6 ± 3.3d	46.0 ± 1.8d	8.4 ± 2.3fg	3.3 ± 1.0h	67.6 ± 0.8b	64.3 ± 0.4c	2.8 ± 0.4hi	0.0 ± 0.0i	1.0 ± 0.8hi	6.9 ± 0.7fg	6.8 ± 0.7g	9.8 ± 0.7ef	11.6 ± 0.7e	1.8 ± 1.4hi	0.5 ± 0.0hi	98.8 ± 0.0a	8.9 ± 0.7efg
reducing power[f] (50 μg/mL)	0.1 ± 0.0f	0.1 ± 0.0g	0.2 ± 0.0d	0.2 ± 0.0c	0.2 ± 0.0e	0.2 ± 0.0e	0.0 ± 0.0hi	0.0 ± 0.0hi	0.0 ± 0.0hi	0.1 ± 0.0gh	0.0 ± 0.0i	0.0 ± 0.0i	0.1 ± 0.0g	0.2 ± 0.0c	0.8 ± 0.0a	0.0 ± 0.0i	0.7 ± 0.0b
reducing power[g] (100 μg/mL)	0.2 ± 0.0g	0.1 ± 0.0h	0.4 ± 0.0d	0.5 ± 0.0d	0.4 ± 0.0e	0.3 ± 0.0f	0.1 ± 0.0i	0.1 ± 0.0i	0.1 ± 0.0i	0.1 ± 0.0h	0.1 ± 0.0i	0.1 ± 0.0i	0.1 ± 0.0hi	0.7 ± 0.0c	2.6 ± 0.0a	0.1 ± 0.0i	1.3 ± 0.0b
conjugated diene[h]	90.7 ± 0.0c	86.9 ± 0.8d	100.0 ± 0.4ab	98.4 ± 0.6b	98.7 ± 0.2b	101.9 ± 0.1a	14.3 ± 1.7i	11.8 ± 1.5j	14.8 ± 0.4i	51.0 ± 1.8g	39.2 ± 0.6h	55.2 ± 0.2f	60.5 ± 0.2e	99.9 ± 1.1ab	1.4 ± 0.2k	53.6 ± 3.5f	101.9 ± 0.7a
TBARs[i]	17.4 ± 0.5h	12.0 ± 1.0j	11.6 ± 1.2j	20.2 ± 0.7g	14.7 ± 1.1i	21.2 ± 0.5fg	14.5 ± 0.9i	15.1 ± 1.1i	18.0 ± 0.5h	28.6 ± 1.8e	23.1 ± 0.5f	31.4 ± 2.1d	42.7 ± 0.5b	34.8 ± 1.5c	31.6 ± 0.6d	20.5 ± 0.3g	50.0 ± 0.3a

[a] Average of duplicate analyses ± standard deviation. [b] Values within a row with different letters are significantly different ($P < 0.05$). M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycone fraction; ISO-1, soybean cake extract containing 12 isoflavones; SO-2, a mixture of 4 fractions of isoflavone extracts; Mgin, malonylgenistin; Gin, genistin; Agin, acetylgenistin; Gein, genistein; Dein, daidzein; 2 std, a mixture of daidzein and genistein standards; 4 std, a mixture of malonylgenistin, genistin, acetylgenistin, and genistein standards; Vit E, α-tocopherol; Vit C, ascorbic acid; EDTA, ethylenediaminetetraacetic acid; BHA, butylated hydroxyanisole. [c] DPPH free radical scavenging effect (%). [d] Trolox equivalent antioxidant capacity (expressed as Trolox concentration). [e] Metal ion chelating effect (%). [f] Absorbance measured at 700 nm for 50 μg/mL of each sample. [g] Absorbance measured at 700 nm for 100 μg/mL of each sample. [h] Conjugated diene inhibition effect (%). [i] Liposome oxidation inhibition effect (%).

This result revealed that the sugar moiety in ring A may behave insignificantly in scavenging the $ABTS^{\bullet+}$ radical cation. Instead, the hydroxy groups at C-5 of the A ring and at C-4′ of the B ring could have a great impact on antioxidant activity because of their hydrogen-donating ability. Unlike the DPPH assay, the synergistic effect was not observed for the fractions, probably because the $ABTS^{\bullet+}$ radical's scavenging ability of isoflavone may be stronger than that of the other phenolic compounds in the fractions. Mitchell et al. (*33*) demonstrated that the assessment of antioxidant activity can be affected by many factors, such as pH value and varieties of solvents and free radicals as well as metal ions in the system analyzed. In our study we further proved that the various isoflavones do possess different antioxidant activity in terms of the DPPH and TEAC assays.

Chelating of Ferrous Ion. Table 3 shows the metal ion chelating efficiency of isoflavones, Vit E, Vit C, EDTA, and BHA. The highest chelating effect was found for EDTA (98.8%), followed by ISO-1 (67.6%), ISO-2 (64.3%), glucoside fraction (46.0%), and malonylglucoside fraction (43.6%). A low chelating effect was shown for the other isoflavone fractions and standards. It has been well-established that for flavonoids both hydroxy groups at C-3′ and C-4′of the B ring possess the ability to chelate metal ions (*34*). Likewise, the keto group at C-4 and the hydroxy group at C-3 of the C ring, or the keto group at C-4 of the C ring and the hydroxy group at C-5 of the A ring, both were able to chelate metal ions (*34*). However, for isoflavones, no hydroxy group is present at C-3 of the C ring, which would decrease the chelating ability substantially. Furthermore, the hydroxy group of the phenol-type compound is slightly acidic, which should be more prone to chelating metal ions when ionized under alkaline condition. However, in our experiment, the neutral system should make the ionization of hydroxy groups much more difficult. All five isoflavone standards when used alone or in combination were less effective in chelating metal ion. In some other studies dealing with chelating efficiency of metal ions by isoflavones, Record et al. (*11*) depicted that genistein failed to chelate ferrous ion at a concentration of 2×10^{-4} M. Similarly, Ruiz-Larrea (*12*) reported that isoflavones such as genistein, genistin, daidzein, and daidzin were unable to chelate cupric ion at concentrations of 30 and 50 μM. Moran et al. (*35*) compared the chelating effect of phenolic acid, cinnamic acid, and flavonoids from soybean nodules and attributed the chelating ability to the presence of catechol or pyrogallol group, as well as the keto group at C-4 and the hydroxy group at C-3 of the C ring. Van Acker et al. (*36*) studied the iron chelation ability of 24 flavonoids and demonstrated that the hydroxy group at 3-C and catechol appeared to be more important than the hydroxy group at 5-C, and compounds chelating through 5-OH of the A ring and 4-keto of the C ring are weak iron chelators. The lower iron-chelating ability of isoflavones may be accounted for by this phenomenon. Surprisingly, ascorbic acid did not show any chelating effect, which may be due to the presence of iron(II) chloride in the system to induce ascorbic acid degradation. The same outcome was also reported by Satoh and Sakagami (*37*) and Yen et al. (*38*).

Of the various isoflavone extracts, both acetylglucoside and aglycone fractions showed a lower chelating effect, which amounted to 8.4 and 3.3%, respectively. This outcome revealed that the presence of high levels of flavonoids, phenolic compounds, and saponins in the acetylglucoside fraction may play a minor role for ferrous ion chelating. Conversely, both malonylglucoside and glucoside fractions showed a higher chelating effect, which equaled 43.6 and 46.0%, respectively,

which may be caused by the presence of a carboxyl group for the former and some other unidentified components for the latter. Hong et al. (*39*) studied the effect of glucuronic acid containing saponin and EDTA on the sorption of metal in incinerator fly ash and found that saponin was effective in adsorbing zinc, copper, cadmium, and lead at low pH. However, the sorption was ineffective for iron and silica at low or high pH. This result further demonstrated that the saponin-containing acetylglucoside fraction was less efficient for chelating ferrous ion.

Reducing Power. Table 3 shows the reducing power of isoflavones, Vit E, Vit C, EDTA, and BHA. With concentrations at 50 and 100 μg/mL, the highest reducing power was shown for ascorbic acid, followed by BHA, α-tocopherol, and EDTA. However, a low reducing power was found for both isoflavone extracts and standards, especially for the latter with a low absorbance ranging from 0 to 0.1. Compared to the other isoflavone fractions, both malonylglucoside and glucoside fractions showed poor reducing power at a concentration of 50 or 100 μg/mL. In contrast, both acetylglucoside and aglycone fractions produced a higher reducing power for the level of 100 μg/mL, and the presence of high contents of the flavonoids and phenolic compounds in the isoflavone extracts may also be responsible this effect (**Tables 2** and **3**). This outcome further implied the reducing power of isoflavone extracts did not correlate well with the level of saponins. The low reducing power of isoflavones was also reported by Mitchell et al. (*33*) and Lee et al. (*23*), which may be postulated by the lack of an enediol group in the isoflavone structure. Moran et al. (*35*) compared the reducing power of several phenolic compounds from the soybean nodule; both phenolic acid and cinnamic acid possessing a catechol or pyrogallol group were shown to reduce ferric ion rapidly at pH 5.5, but slowly at pH 7.0. As for flavonoids, the catechol group in the B ring was the most efficient for iron reduction, whereas the hydroxy group at C-3 and the keto group at C-4 were less effective, with the hydroxy group at C-5 and the keto group at C-4 being inactive (*35*). The lower reducing ability of isoflavones may be explained by this phenomenon.

Conjugated Diene Assay. Table 3 shows the inhibition effect of conjugated diene by isoflavones, α-tocopherol, ascorbic acid, EDTA, and BHA. With the exception of the glucoside fraction, a pronounced inhibition effect (90.7−101.9%) was found for the other isoflavone fractions, which can be comparable to that of α-tocopherol (99.9%) or BHA (101.9%). This result clearly revealed that the isoflavone extract was effective in inhibiting the initial lipid oxidation. The presence of high levels of flavonoids and phenolic compounds in isoflavone extracts may contribute to this effect. On the contrary, a poor inhibition effect was shown for the isoflavone standards, which equaled 51.0, 39.2, 14.8, 14.3, and 11.8% for genistein, daidzein, acetylgenistin, malonylgenistin, and genistin, respectively. A synergistic effect occurred for two and four isoflavone standards, with high inhibition percentages of 55.2 and 60.5%, respectively. However, the difference was minor when compared to genistein standard. Ruiz-Larrea et al. (*12*) studied the effect of isoflavone on the metal ion induced LDL oxidation and reported that genistein was more efficient in inhibiting conjugated diene formation than genistin; the concentrations required for a 50% inhibition were 13 μM for the former and 29 μM for the latter. In another study dealing with the effect of various concentrations of fermented soybean isoflavone extract on the inhibition of conjugated diene, a profound inhibition effect (75−96%) also occurred for the isoflavone extract (*8*).

TBARS Assay. Table 3 shows the inhibition effect of liposome oxidation (TBARS) by isoflavones, α-tocopherol, ascorbic acid, EDTA, and BHA. Unlike conjugated diene formation, the TBARS assay was often used for assessing the extent of hydroperoxide degradation after drastic lipid oxidation. Among all of the treatments, BHA showed the highest inhibition efficiency (50.0%), followed by α-tocopherol (34.8%), ascorbic acid (31.6%), EDTA (20.5%), isoflavone standards (14.5−42.7%), and isoflavone fractions (11.6−21.2%). The inhibition effects for the various isoflavone fractions, namely, ISO-2, aglycone, malonylglucoside, ISO-1, glucoside, and acetylglucoside, were 21.2, 20.2, 17.4, 14.7, 12.0, and 11.6%, respectively. The presence of high levels of flavonoids and phenolic compounds may contribute to the inhibition effect of TBARS. Also, the mixture of isoflavone standards showed a synergistic effect, as evidenced by higher inhibition percentages for 4 std (42.7%) and 2 std (31.4%) when compared to genistein (28.6%) and daidzein (23.1%). In addition, the inhibition efficiency may also depend on the concentration of the individual isoflavones in the extract. Romero et al. (*8*) proved that a high concentration of fermented soybean isoflavone extract may provide a better inhibition effect of TBARs than a low concentration of isoflavone extract. Interestingly, the isoflavone extracts did not show better inhibition effects than the isoflavone standards, implying that the isoflavone itself may play a more important role than flavonoids or phenolic compounds in inhibiting TBARS formation. Also, the presence of a much lower level of an isoflavone standard such as genistein (36.4 μg/mL) in the aglycone fraction may account for this effect.

By comparison of the results shown above, it can be concluded that the antioxidant activity of the various isoflavone standards and extracts may be varied depending on the assay used for determination. Theoretically, the low-polar antioxidants should perform better than the high-polar antioxidants in an oil/water emulsion system because the former tend to be oriented in the oil phase (*40*). This is why the aglycone fraction is more effective in inhibiting the formation of conjugated diene and TBARS than the other isoflavones containing a sugar moiety. Moreover, genistein contains one more hydroxy group when compared to the other isoflavone standards, which should also be responsible for this effect. Nevertheless, in our experiment we also observed a better inhibition effect of conjugated diene than TBARS, indicating that the incorporation of the various isoflavone extracts to the oil-based food system may be beneficial in retarding the initial lipid oxidation. Although the aglycone fraction contained less saponin than the acetylglucoside fraction, the former still showed high antioxidant activity, probably because of the presence of a high amount of total flavonoids and phenolic compounds. The measurement of saponin content is meaningful as the acetylglucoside fraction contains a high amount of saponin. Thus, it may be inferred that both isoflavones and saponins may contribute to the antioxidant activity of the acetylglucoside fraction, whereas flavonoids and phenolic compounds as well as isoflavones contribute to the antioxidant activity of the aglycone fraction. All in all, the effect of isoflavones in scavenging DPPH free radicals followed the order ascorbic acid > BHA ≅ α-tocopherol > ISO-1 > acetylglucoside fraction > ISO-2 > aglycone fraction > malonylglucoside fraction ≫ 4 standards > glucoside fraction > 2 standards > Mgin ≅ EDTA ≅ Gein > Agin > Gin ≅ Dein. TEAC values followed the order BHA > 4 standards ≅ 2 standards > Gein > aglycone fraction > Dein > ascorbic acid > ISO-2 > acetylglucoside fraction ≥ ISO-1 ≥ α-tocopherol > Agin > glucoside fraction > Mgin >

malonylglucoside fraction > Gin ≫ EDTA. Metal ion chelating followed the order EDTA ≫ ISO-1 > ISO-2 > glucoside fraction ≅ malonylglucoside fraction ≫ 4 standards ≥ 2 standards ≥ BHA ≥ acetylglucoside fraction ≅ Gein ≥ Dein > aglycone fraction ≥ Mgin ≅ α-tocopherol ≅ Agin ≅ ascorbic acid ≥ Gin. Reducing power followed the order ascorbic acid > BHA > α-tocopherol > aglycone fraction ≅ acetylglucoside fraction > ISO-1 > ISO-2 > malonylglucoside fraction > glucoside fraction ≅ Gein ≥ 4 standards ≥ Mgin ≅ Gin ≅ Agin ≅ 2 standards ≅ Dein ≅ EDTA. Inhibition of conjugagted diene followed the order BHA ≅ ISO-2 ≥ acetylglucoside fraction ≅ α-tocopherol ≥ ISO-1 ≅ aglycone fraction > malonylglucoside fraction > glucoside fraction > 4 standards > 2 standards ≅ EDTA > Gein > Dein ≫ Agin ≅ Mgin > Gin ≫ ascorbic acid. Inhibition of TBARS followed the order BHA > 4 standards > α-tocopherol > ascorbic acid ≅ 2 standards > Gein > Dein ≥ ISO-2 ≥ EDTA ≅ aglycone fraction > Agin ≅ malonylglucoside fraction > Gin ≅ ISO-1 ≅ Mgin > glucoside fraction ≅ acetylglucoside fraction. In conclusion, soybean cake was indeed found to contain various functional constituents such as isoflavones, flavonoids, phenolic compounds, and saponins, all of which were present in high amount in both acetylglucoside and aglycone fractions. The developed HPLC or GC-MS method was effective in determining saponins in soybean cake as well. Further research is necessary to study the biological activities of the most active aglycone and acetylglucoside fractions. Finally, the aglycone and acetylglucoside fractions of soybean cake extract obtained in this experiment may be used as a basis for possible commercial production of functional foods in the future.

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