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ARTICLE

Isoflavones and Antioxidant Capacity of Commercial Soy-Based Beverages: Effect of Storage

Katia Rau De Almeida Callou, Shamil Sadigov, Franco Maria Lajolo, and Maria Ines Genovese*

Laboratório de Química, Bioquímica e Biologia Molecular de Alimentos, Departamento de Alimentos e Nutrição Experimental, FCF, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, Bloco 14, 05508-900 São Paulo, SP, Brazil

Samples of 11 different brands of commercially available soy-based beverages (n = 65), including products made from soy protein isolate (SPI) and soy milk, mixed with fruit juice and/or flavoring, were analyzed for their isoflavone content and in vitro antioxidant activity. There was a large variation in isoflavone and total phenolics contents ranging from 0.7 to 13 mg of isoflavones/200 mL and from 6 to 155 mg equivalents of catechin/200 mL, respectively. The antioxidant activity also varied significantly among products. Storage of the beverages at room temperature caused a significant decrease of antioxidant capacity, soluble phenolics, and isoflavone contents after 9 months. When soybeans used for beverage production were stored for up to 6 months in silos, the resulting products were not affected. However, a decrease of malonyl and a proportional increase of free glucosidic forms of isoflavones were observed after storage of both the raw material and the beverages.

KEYWORDS: Soy beverages; antioxidant capacity; isoflavones; storage

INTRODUCTION

Soybeans are the most important food source of isoflavones, which have been associated with beneficial health effects in humans, including prevention of cancer, cardiovascular diseases, osteoporosis, and relief of menopausal symptoms (*1*). There are 12 isoflavones in soybeans and soy products, 3 free aglycones (genistein, daidzein, and glycitein) and their respective glucosidic, malonyl, and acetylglucosidic conjugates (*2*). Isoflavone content and distribution in soy and soy-based products depend on climatic and environmental conditions, dilution with nonsoybean ingredients, and processing and storage conditions (*3*−*6*).

In 1999, the U.S. Food and Drug Administration (FDA) approved a health claim on food labels for products containing soy protein due to the relationship between soy protein and a reduced risk of coronary heart disease (*7*). Since then, many products have been developed to provide functional foods. Defatted soy flours, protein isolates, concentrates, and textured proteins are used mainly due to certain physicochemical properties such as gelation, water-absorbing, and emulsifying capacities (*8*). Among these products, soy milk has been highlighted due to its wide applicability in the food industry and is used directly as a beverage or as ingredient in soy milk products such as yogurt, infant formulas, creams, and desserts. In addition, low cost and high protein content are among factors that contributed to increasing soy milk use by food industries (*9*).

Soy-based beverage consumption has increased all over the world due to the development of new products based on water-soluble extract or soy protein isolate in combination with fruit juices and flavoring agents. These products have only traces of the undesirable grassy−beany flavor of soybeans, and their presence and expansion in the Brazilian market in recent years indicate that consumers were receptive to incorporating these products in their dietary habits (*10*). In recent years, the consumption of soy-based beverages grew gradually from almost 51 million liters in 2002 to 110.5 million liters in 2005. The line of soy beverages has achieved a more rapid expansion among soy-based foods of approximately 30% per year in Brazil and 25% in the United States (*11*).

Because soybean consumption in the Western is not as significant as in Asian countries, soy beverages represent a way to include these bioactive substances in the diet. However, there are no studies assessing the effects of the storage of the beverages, during the shelf-life period, and of storage of the grains used in their production on isoflavone content and in vitro antioxidant capacity, the aims of the present work.

MATERIALS AND METHODS

Materials. Samples (n = 65, three different batches of each) of 11 different commercially available brands of soy beverages in Brazil were purchased from local supermarkets in São Paulo in 2007−2009. These products were classified in four different groups according to the soy protein source (soy milk or soy protein isolate) and the addition of fruit juices or flavorings (**Table 1**). Samples for storage studies were provided by Yoki Alimentos S/A (Minas Gerais, Brazil). Samples consisted of four packages of each beverage obtained immediately after manufacture and analyzed at 0, 3, 6, and 9 months of storage at room temperature (25 ± 3 °C). Samples of the soybeans stored in the silo of the company were

*Corresponding author (telephone 55-11-30913647; e-mail genovese@usp.br).

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Table 1. Classification of Soy-Based Beverages Available in the Brazilian Market in 2007−2009, Adopted in This Work

group	total no. of beverages	brand	juice type or flavor	version
group 1, beverages containing soy milk and fruit juice	30	A (n = 18)	apple, pineapple, guava, passion fruit, mango, peach, orange, strawberry, grape, watermelon, pineapple/mint, peach/tangerine, Swiss lemonade	normal
			grape, apple, mango, peach, orange	*light*
		B (n = 5)	pineapple, apple, passion fruit, strawberry, papaya/orange	normal
		C (n = 3)	apple, grape, strawberry	normal
		D (n = 4)	apple, grape, strawberry, passion fruit	normal
group 2, beverages containing SPI[a] and fruit juice	22	E (n = 5)	apple, orange, peach, grape, pineapple	normal
		F (n = 7)	apple, pear, passion fruit, orange/peach, tropical fruit, red fruits, grape	normal
		G (n = 4)	pineapple/mango/passion fruit, grape, peach, apple	normal
		H (n = 4)	pineapple/coconut, apple, orange, peach	normal
		I (n = 2)	apple, grape	normal
group 3, beverages containing soy milk and flavoring ingredients	8	A (n = 4)	original	normal
			original	*light*
			chocolate	normal
			chocolate	*light*
		I (n = 2)	original	normal
			strawberry	normal
		J (n = 1)	original	*light*
		G (n = 1)	original	normal
group 4, beverages containing SPI and flavoring ingredients	5	E (n = 1)	original	normal
		F (n = 2)	original	normal
			chocolate	normal
		G (n = 1)	chocolate	normal
		K (n = 1)	original	*light*
total	65			

[a] SPI, soy protein isolate.

analyzed over a 6 month period starting from the arrival of the 2008 harvest. All chemicals and solvents used were of reagent or HPLC grade.

Isoflavone Analysis. *Solid-Phase Extraction*. Aliquots (5, 10, or 20 mL) of soy beverages were centrifuged (10000*g* for 20 min) and applied directly to polyamide (CC 6, Macherey-Nagel, Germany) columns (1 g/6 mL), previously conditioned with 20 mL of methanol and 60 mL of distilled water. Impurities were washed out with 20 mL of distilled water, and retained isoflavones were eluted with 50 mL of 99.5:0.5 methanol/ammonia. The flow rate through the columns was controlled by means of a vacuum manifold Visiprep 24 DL (Supelco, Bellefonte, PA). The effluents were evaporated on a rotary evaporator until the volume of 0.2−0.4 mL, and then the volume was adjusted to 2 mL with HPLC grade methanol (*8*, *9*). Aliquots of the samples were filtered through a 0.22 μm PTFE filter unit [poly(tetrafluoroethylene), Millipore Ltd., Bedford, MA] and analyzed by HPLC.

HPLC Identification and Quantification of Isoflavones. Isoflavone separation and determination were performed according to the method of Genovese and Lajolo (*9*) with a C18 NovaPak (30 cm × 4.6 mm id) column (Waters, Milford, MA) and a Hewlett-Packard 1100 system equipped with a diode array detector and the software ChemStation (Palo Alto, CA). Identification was made on the basis of the spectra and retention time and quantification using external standard calibration curves of area versus concentration. The 12 isoflavone standards were from LC Laboratories (Woburn, MA). **Figure 1** shows a typical chromatogram of soy milk-based beverages and the retention times for the reference compounds. Total isoflavone contents were expressed as milligrams of aglycones equivalents per 200 mL (1 cup) of sample, after normalization of individual isoflavones to account for differences in molecular weights between glucoside derivatives. The mass of each isoflavone form (β-glucoside, malonylglucoside, and acetylglucoside) was multiplied by the ratio of its aglycone molecular weight to the molecular weight of the individual form before summing (*12*).

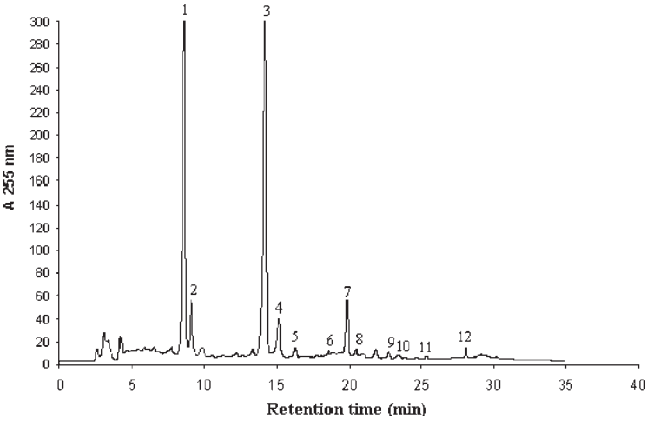

Figure 1. Typical HPLC chromatogram (255 nm) of soy milk-based beverages and retention times of the reference compounds: (1) daidzin, (2) glycitin, (3) genistin, (4) malonyldaidzin, (5) malonylglycitin, (6) acetyldaidzin, (7) malonylgenistin, (8) acetylglycitin, (9) daidzein, (10) acetylgenistin, (11) glycitein, and (12) genistein.

Protein Content. The protein content (N × 6.25) of samples was determined in triplicate according to the micro-Kjeldahl method (*13*).

Total Phenolic Content. The total phenolic content was determined according to the method of Singleton et al. (*14*) with some modifications (*15*): 0.25 mL of adequately diluted soy beverage was added to 2 mL of distilled water and 0.25 mL of the Folin−Ciocalteu reagent. After 3 min at room temperature, 0.25 mL of saturated sodium carbonate was added, and the tubes kept at 37 °C during 30 min for color development. The absorbance readings were performed at 750 nm in a Ultrospec 2000 UV−visible spectrophotometer (Amersham Biosciences, Cambridge, U.K.) and the results expressed

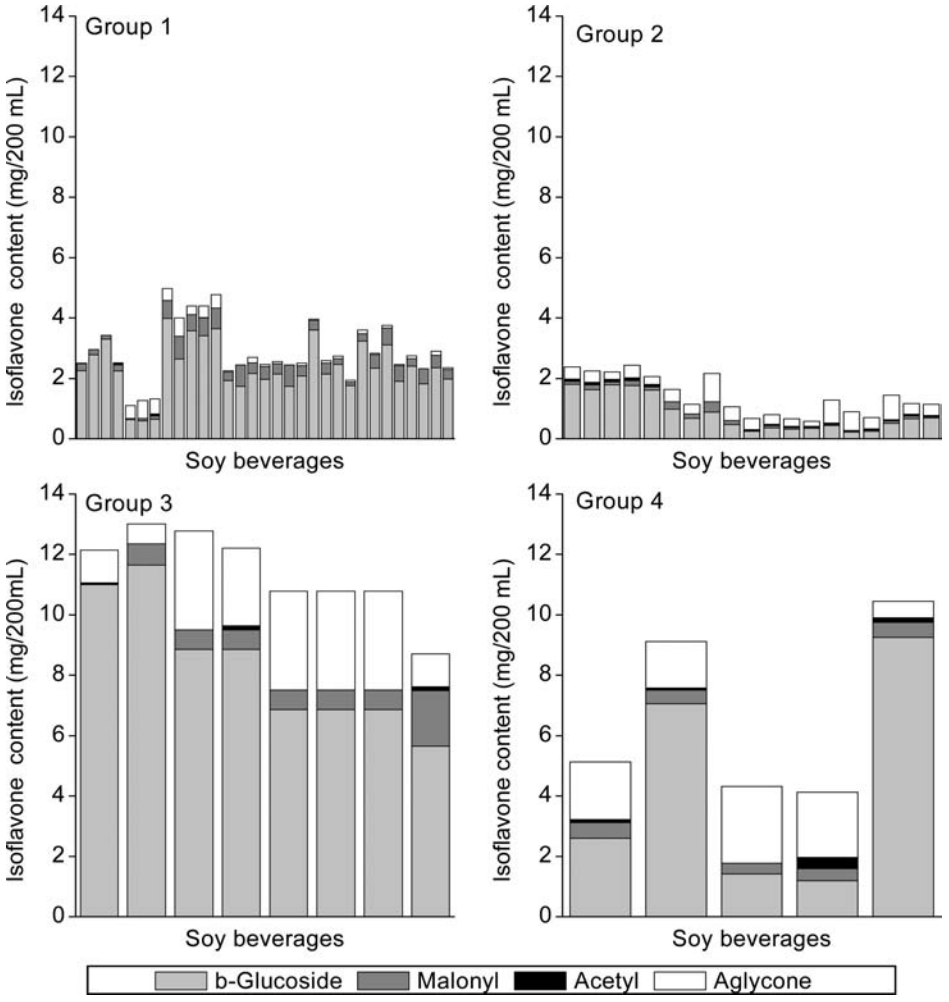

Figure 2. Isoflavone content (mg/200 mL) and distribution in commercial soy-based beverages grouped according to **Table 1** (65 samples from 11 different brands). Each bar represents the mean value for three different batches.

as milligram equivalents of catechin per 200 mL of soy beverage. The analyses were made in triplicate.

Antioxidant Capacity. *DPPH• Scavenging Activity*. DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) scavenging activity of soy beverage phenolics was assessed according to the method of Brand-Williams et al. (*16*), with some modifications (*15*). Briefly, a 50 μL aliquot of the extract previously diluted and 250 μL of a methanol solution of DPPH• (0.5 mM) were shaken, and after 20 min, the absorbance was measured at 517 nm using the microplate spectrophotometer (Benchmark Plus, Bio-Rad). Results were expressed as micromole equivalents of Trolox per 200 mL of sample.

Statistical Analysis. Statistical analysis was done by using the SAS statistical software package version 9.2 (SAS Institute Inc., Cary, NC). A repeated-measures ANOVA and Tukey−Kramer adjustment for the multiple comparisons of the means ($\alpha = 0.05$) were applied to results. Pearson's correlation coefficients were used to investigate pairwise association between continuous variables.

RESULTS AND DISCUSSION

A survey of all soy beverages available on the Brazilian market in their several versions, including regular or light types, was carried out. Eleven soy beverage brands of 65 different products were identified and the beverages classified into four groups (**Table 1**). As can be seen, soy protein is incorporated into the product in the form of soy milk or soy protein isolate (SPI). Besides, concentrated fruit juices or flavorings such as vanilla, chocolate, and strawberry can be added.

Table 2. Protein Content of Soy-Based Beverages Available in the Brazilian Market in 2007−2009

	protein content[a] (g/200 mL)	
beverage group	determined	label
1	0.9−2.9	1.0−3.0
2	0.8−2.2	1.0−2.1
3	4.8−5.9	5.0−5.2
4	5.4−6.0	5.0−5.2

[a] N total × 6.25.

The average protein contents (three different batches of each product) were determined and compared to the corresponding values on the product labels (**Table 2**). In general, the protein content was in accordance with the information provided by suppliers, except for beverages of brand C, which presented lower values than specified. In group 1 the protein content was in the range of 0.91−2.95, whereas group 2 presented 0.8−2.2 g/200 mL of beverage. Higher protein content was found in groups 3 and 4, ranging from 4.8 to 5.9 g and from 5.4 to 6.0 g per 200 mL serving, respectively. These last groups are characterized by the absence of fruit juices, which are not protein sources and in this way cause soy protein dilution. Indeed, beverages of original (vanilla) or chocolate flavor had the highest protein contents.

In general, consumption of 200 mL of soy beverages from groups 3 and 4 corresponds to approximately 33 g of cooked (boiled) soybeans, which is equivalent to 5.5 g of protein (*17*).

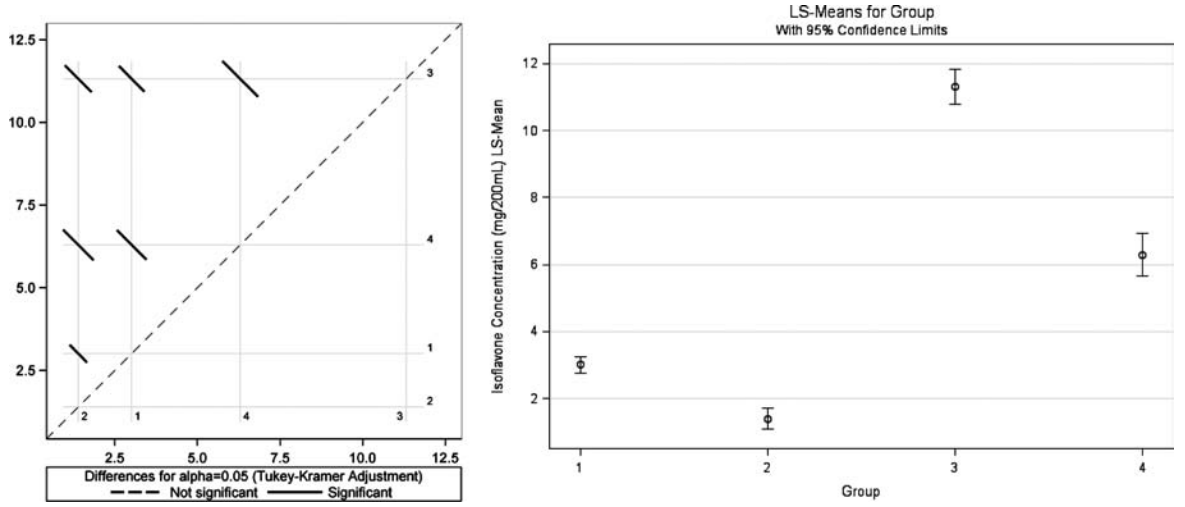

Figure 3. Least-squares means and pairwise comparison plots for group isoflavone means as determined by the HPLC method.

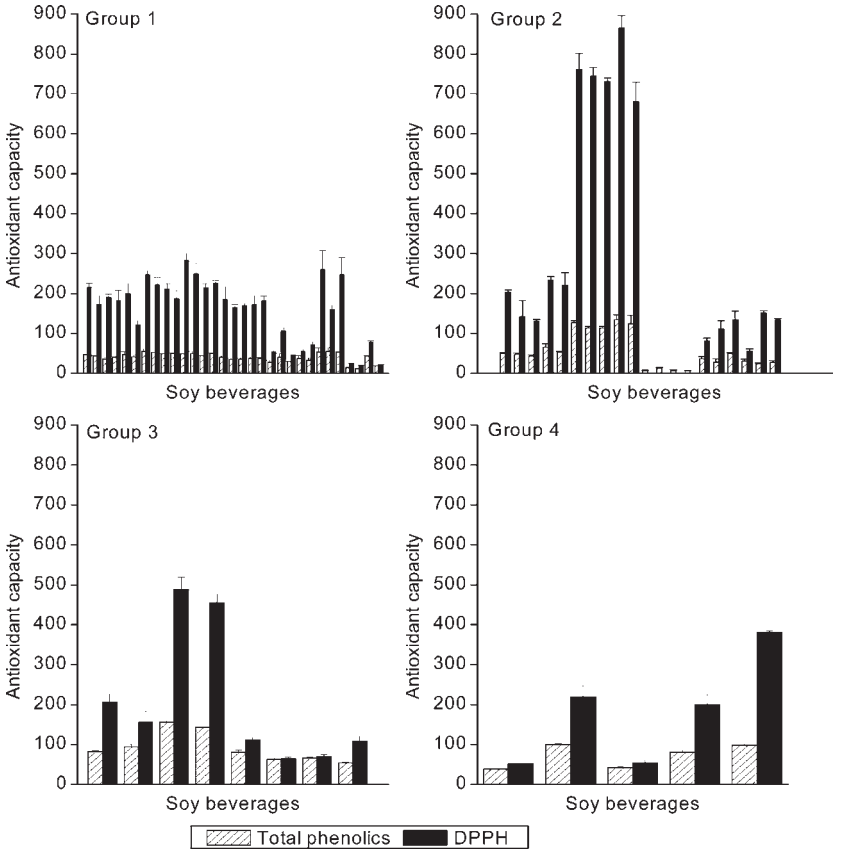

Figure 4. Total phenolics content (mg equiv of catechin/200 mL) and DPPH radical scavenging capacity (μmol equiv of Trolox/200 mL) of soy-based beverages grouped according to **Table 1** (65 samples from 11 different brands). Each bar represents the mean value for three different batches, and errors bars denote the relative standard deviation.

Although the FDA recommends a daily intake of 25 g of soy protein a day, epidemiological data show that consumption of approximately 10−11 g could also promote health benefits (*18*). Therefore, soy beverages could contribute to reducing the risk of chronic diseases and getting a more balanced diet.

The isoflavone content and distribution (malonyl, acetyl, β-glucosides, and aglycones) of soy-based beverages are shown in **Figure 2**. Products containing soy milk and fruit juice (group 1) showed large variability in isoflavone levels, ranging from 1 to 5 mg per serving of 200 mL. In all beverages blended with fruit juice, lower isoflavone content was present: for those containing SPI (group 2), it varied from 0.6 to 2.4 mg/200 mL. The highest mean concentration of isoflavones in all samples analyzed in the present study was presented by group 3. This group contains beverages based on soy milk and flavoring ingredients and does not contain any fruit juice. These values were in the range of 9−13 mg of isoflavones/200 mL, whereas group 4 (SPI-based beverages) presented lower isoflavone contents ranging from 4 to 10 mg/200 mL. All isoflavone concentration group means were significantly different after adjustment for multiple comparisons (**Figure 3**). A repeated-measures analysis revealed no significant difference in isoflavone content between batches (p value = 0.55).

The great variability in isoflavone content possibly resulted from the addition of various amounts of soy protein ingredients and whether or not fruit juices were added. Soy milk and SPI are sources of isoflavones, but their contents can be highly variable (*19*, *20*), and thus appear to be partially responsible for the large variability of isoflavone contents in soy-based beverages. In addition, a high positive correlation ($r = 0.81$, $p < 0.05$) was observed between protein and isoflavone content. These results show that the isoflavone content of these products is strongly associated with their protein content, as much of the protein present is derived from soybeans. Our previous results show that there are also physicochemical interactions between the main storage soy proteins, glycinin and β-conglycinin, and isoflavones, through electrostatic binding or hydrophobic interaction (*19*).

The isoflavone contents of beverages containing soy milk and flavorings were similar to those reported by Chan et al. (*20*) for soy beverages from China (12.2 mg/200 mL), but lower than those reported by Genovese and Lajolo (*9*) (16.6 mg/200 mL) and found in the USDA database (*17*) (15.6−21.46 mg/200 mL). The large variability in isoflavone content found in soy beverages containing either soy milk or SPI could result from different concentrations of these compounds in the raw material because the amount of isoflavones in soybeans varies according to genetics, crop years, and growth location (*21*, *22*). For example, the isoflavone content reported for Brazilian soybean varieties was in the range of 57−188 mg/100 g (*23*), and commercial soy protein isolates presented isoflavone contents from 88 to 164 mg/100 g (*24*). Coward et al. (*3*) reported that the concentration and conjugation of isoflavones in soybean protein products depend on processing conditions, mainly temperature. Furthermore, Izumi et al (*25*) suggested that chemical forms of isoflavones could have significant impact on the bioavailability and thus on the biological effects attributed to these compounds. However, other studies have not supported this conclusion (*26*, *27*).

The percentage of aglycones found in SPI beverages was similar to that found by Barbosa et al. (*28*) in pure SPI (37%). The high content of these compounds is the result of the endogenous β-glucosidase activity present in soybeans during the extraction process, leading to hydrolysis of β-glucosides into aglycones. It is important to note that the low acetyl-glucosides content of these beverages indicates that high temperatures during spray-drying and toasting of soybean flour used as raw material to obtain the SPI were probably not used (*6*).

Products blended with fruit juice showed a wide variability in the antioxidant capacity determined by the DPPH radical scavenging and in the total phenolics content (**Figure 4**), ranging from 44 to 280 μmol of Trolox equiv/200 mL and from 28 to 56 mg of catechin equiv/200 mL, respectively, for soy milk-based beverages (group 1) and from 0 to 865 μmol of Trolox equiv/200 mL and from 6 to 146 mg of catechin equiv/200 mL, respectively, for SPI-based beverages (group 2). For beverages without fruit juices, phenolic contents varied in the ranges of 54−156 mg of catechin equiv/200 mL (group 3) and 38−100 mg of catechin equiv/200 mL (group 4), whereas the antioxidant capacity ranged from 64 to 488 μmol equiv of Trolox/200 mL (group 3) and from 51 to 381 (group 4) μmol equiv of Trolox/200 mL.

There was a positive correlation between phenolic content and DPPH scavenger capacity ($r = 0.83$, $p < 0.05$). However, there was no correlation between antioxidant capacity and isoflavone contents for both groups of soy beverages, with or without fruit juices (**Table 3**). These results suggest that isoflavones do not represent the only source of antioxidant compounds in soy beverages. In fact, vitamin C (naturally present in fruits or added to beverages) and synthetic antioxidants such as TBHQ can be found in their composition. Similarly, phenolics can also be derived from fruit juices. Among all beverages blended with fruit juices, those with strawberry or grape juice presented the highest antioxidant capacity. These results are in agreement with Harvolsen et al. (*29*), who found higher antioxidant capacity in red fruits, mainly due to the contents of phenolic acids and flavonoids such as anthocyanins. Kuskoski et al. (*30*) reported high DPPH scavenging capacities for grape pulp, followed by strawberry and guava pulps, which were positively correlated with levels of total phenolic compounds, as well as levels of anthocyanins present. In this way, the great variability in the antioxidant capacity of soy beverages seems to depend on concentration and type of phenolic compounds present.

Table 3. Pearson Correlation between Isoflavone Contents and Antioxidant Capacity

	Pearson correlation coefficient (r)	
product	phenolics × isoflavone	DPPH × isoflavone
soy beverages containing fruit juice (groups 1 and 2)	−0.17	−0.24
soy beverages without fruit juice (groups 3 and 4)	0.54	0.37

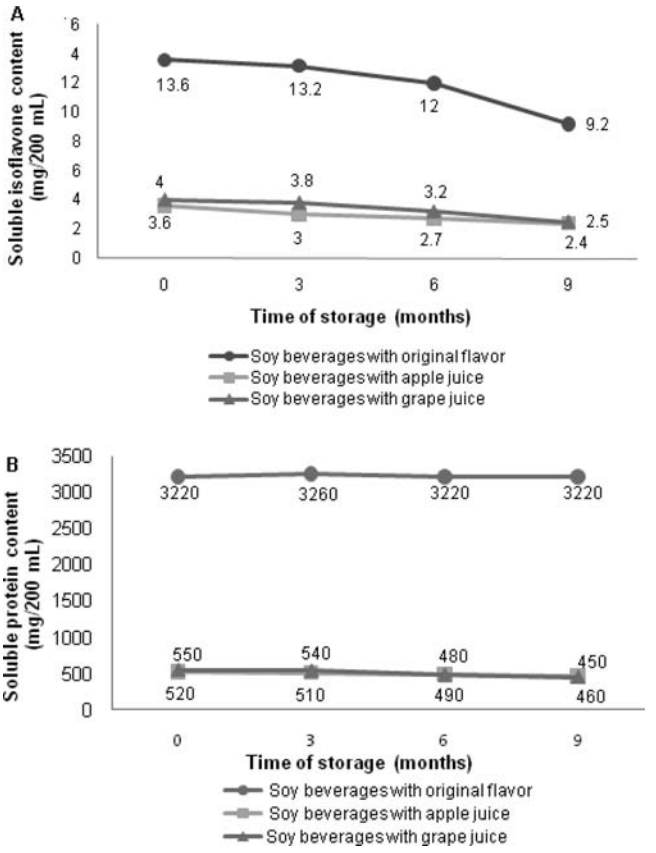

Figure 5. Soluble isoflavone (**A**) and protein (**B**) contents of soy beverages during the shelf-life period (9 months at room temperature).

The isoflavone and protein contents of three different soy milk-based beverages (apple juice, grape juice, and original flavor) were determined every 3 months during the shelf-life period (9 months at room temperature), in the soluble fraction (separated from the sediment after centrifugation). As can be seen in **Figure 5**, there was a reduction in soluble isoflavone contents of 36.5, 31.6, and 32.3%, respectively, for the apple, grape, and original beverages, over the 9 months of storage. Soluble protein contents, on the other hand, were reduced only in apple (11.5%) and grape (18.2%) beverages,

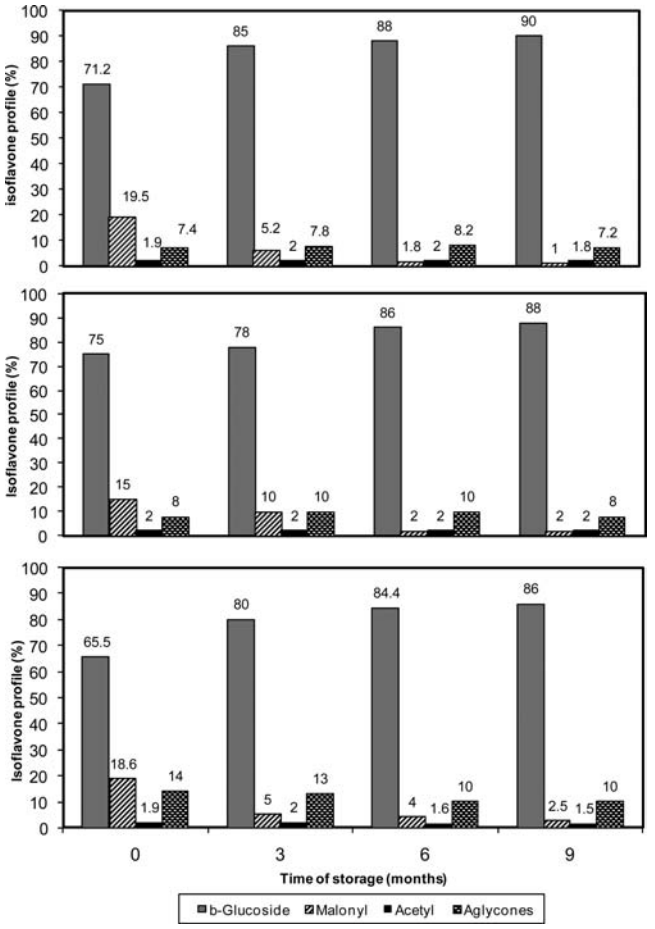

Figure 6. Soluble isoflavone profile of apple (**A**), grape (**B**), and original (**C**) soy beverages during the shelf-life period (9 months at room temperature).

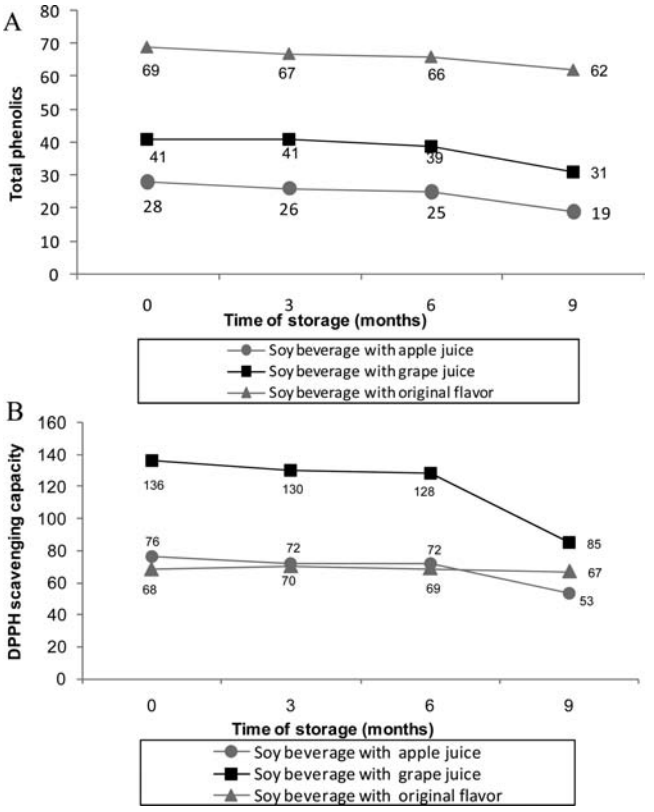

Figure 7. Total phenolics content (mg equiv of catechin/200 mL) (**A**) and DPPH radical scavenging capacity (mg equiv of Trolox/200 mL) (**B**) of apple, grape, and original soy beverages during the shelf-life period (9 months at room temperature).

maybe related to the pH (of 4.5 and 4.3, for apple and grape, respectively). In the original beverage, the pH of which was of 7.0, such a decrease was not observed, and this could be also related to microcrystalline cellulose and carboxymethylcellulose stabilizers, not present in the juice-amended beverages. As the products are subjected to ultrahigh-temperature (UHT) processing and packed in Tetra Brik Aseptic, no enzymatic activity or light effects such as oxidation are expected. In this way, other factors seem to account for isoflavone insolubilization not related to protein insolubilization. In fact, it was previously shown that part of the isoflavones extracted from soy flour is not associated with proteins (*19*). As beverages containing fruit juices have pectin as thickener, and the original uses carrageenin instead, interactions with these polysaccharides could account for these differences.

When the conjugation of soluble isoflavones was analyzed, it could be observed that it changed drastically, and a significant reduction of malonylglucosides was observed for all samples stored at room temperature while the percentage of de-esterified β-glucosides increased (**Figure 6**).

Except for the original flavor, DPPH radical scavenging capacity and total phenolics content also decreased during storage (**Figure 7**), and the decrease was more pronounced after 9 months of storage (of 30 and 37% for DPPH scavenging and of 30 and 24% for total phenolics for apple and grape soy beverages, respectively).

Previously, it was shown that the total isoflavone concentration remained unchanged with aging of the soy beverages within the range of 61−359 days of storage (*31*). To our knowledge, this is the first report showing that the soluble isoflavones and antioxidant capacity are reduced by storage.

Table 4. Effect of Soybean Storage in Silos on the Isoflavone, Protein, and Total Phenolics Content and DPPH Radical Scavenging Capacity of Soy Beverages with Apple Juice, Grape Juice, and Original Flavor[a]

	time of soybean storage		
soy beverage	0 months	3 months	6 months
apple			
isoflavone (mg/200 mL)	3.4 a	4.5 b	3.8 a
protein (g/200 mL)	1.0 a	1.3 b	1.1 a
total phenolics (mg equiv of catechin/200 mL)	28 a	30 a	30 a
DPPH (μmol equiv of Trolox/200 mL)	72 a	86 b	84 b
grape			
isoflavone (mg/200 mL)	4 b	5.6 c	3.3 a
protein (g/200 mL)	1.3 b	1.4 b	1.1 a
total phenolics (mg equiv of catechin/200 mL)	41 b	49.5 c	36 a
DPPH (μmol equiv of Trolox/200 mL)	136 b	138 b	99 a
original			
isoflavone (mg/200 mL)	14 a	15 a	14 a
protein (g/200 mL)	5.0 a	5.3 a	5.2 a
total phenolics (mg equiv of catechin/200 mL)	70 b	66 a	66 a
DPPH (μmol equiv of Trolox/200 mL)	68 a	69 a	70 a

[a] Values are expressed as means ± SD for triplicates. Means in the same row with common letters are not significantly different ($p < 0.05$).

The effect of soybean storage in silos on the quality of soy milk-based beverages was verified by analyzing samples prepared sooner after the arrival of the 2008 harvest and after 3 and 6 months of storage of the grain. In general, no trends were detected for total phenolics, protein, and isoflavone contents of soy beverages over the 6 months of grain storage, similar for the

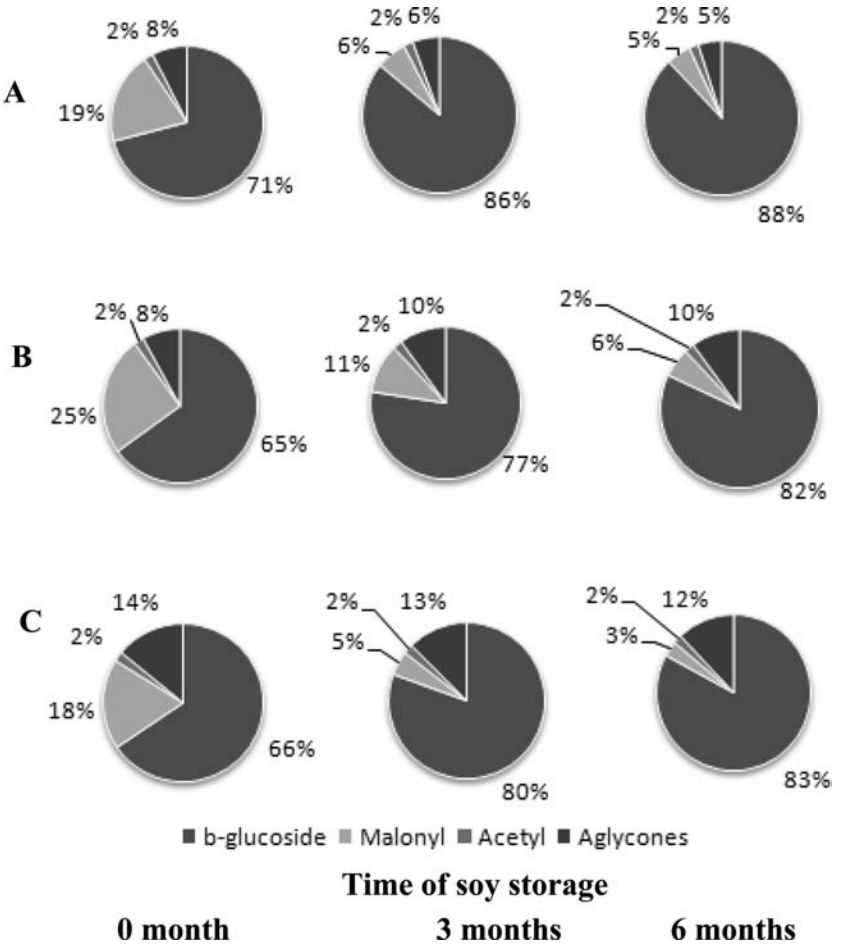

Figure 8. Effect of soybean storage in silos on the isoflavone profile of soy beverages with apple juice (**A**), grape juice (**B**), and original flavor (**C**).

DPPH scavenging capacity (**Table 4**). There was a slight increase of isoflavone and protein contents for soy beverages produced after 3 months of grain storage. However, the more significant differences were observed for isoflavone profiles: 6 months of storage favored the reduction of malonylglycosydes and increased β-glucosides in soy beverages (**Figure 8**), presumably reflecting differences in the isoflavone profile of the soybeans from which the extract was prepared. This result corroborated those reported by Hou and Chang (*32*) for soybean seeds stored at room temperature. Pinto et al. (*5*) also obtained similar results after storage of defatted soybean flour and SPI.

In conclusion, there are wide variations among different soy-based beverages in relation to isoflavone contents and antioxidant capacity, which are reduced during shelf life at least in the soluble portion. Interconversion of the individual conjugate forms of isoflavones in beverages occurs as a consequence of both product and grain storage.

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