JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Protein Quality, Antigenicity, and Antioxidant Activity of Soy-Based Foodstuffs

MIRYAM AMIGO-BENAVENT, JOSE MANUEL SILVÁN, FRANCISCO JAVIER MORENO, MAR VILLAMIEL, AND M. DOLORES DEL CASTILLO*

Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

Commercial soy-based foodstuffs, including beverages ($n = 15$), cow's milk supplemented with soy isoflavones ($n = 1$), snacks ($n = 1$), and biscuits ($n = 2$), were analyzed to find any link between alterations in protein quality, safety (antigenicity), functionality (antioxidant activity), and food processing. Protein content was analyzed by the Kjeldhal method and available lysine by OPA assay. Chromatographic (RP-HPLC) and electrophoretic (SDS-PAGE) protein profiles were obtained to monitor modifications in the structure of soy allergens. The antigenicity was estimated by immunoblotting against soy total antibodies. Total phenol content was measured by Folin−Ciocalteu, while peroxyl radical scavenging activity of the sample was determined by $ORAC_{FL}$ assay. Protein content did not differ of those declared by the producers. Lysine availability was higher in liquid soy beverages compared to that in other soy foodstuffs studied here. 7S and 11S soy allergens were detected by RP-HPLC and SDS-PAGE, respectively. Both data indicated changes in soy protein patterns due to processing of instant powdered soymilk, soy snacks, and biscuits. Immunoblotting assay showed modifications in the antigenic response of these foodstuffs based on soy, suggesting that their processing had altered the structure of soy allergens. RP-HPLC, SDS-PAGE, and immunoblotting resulted in adequate analytical approaches for detecting changes in protein structure due to processing and adulteration. Protein quality, antigenicity, and antioxidant activity of soy products can be affected as a function of the intensity of the thermal processing.

KEYWORDS: Antigenicity; antioxidant activity; processing; protein quality; soy

INTRODUCTION

Foodstuffs based on soy are increasingly being consumed worldwide. Soymilk-based beverages were the fastest-growing category in 2004, with a growth of 31% ordered in restaurants and heart-helping foods in supermarkets and a value of 244 million euros (*1*). Soy is a good source of high-quality proteins because it is rich in lysine and other essential amino acids (*2*) and a good alternative for vegetarians, lactose-intolerant people, and consumers allergic to cow's milk (*3*).

Many studies have indicated that soy has possible beneficial effects on human health; the most relevant physiological positive effects are reduction in cholesterol and triglycerides (*4, 5*), antioxidant activity (*2*), and capacity to prevent or block certain mammalian tumors and protect against obesity (*6*). Soy has also possible adverse effects such as poor protein digestibility and potential allergenicity (*2, 7, 8*).

Generally, soy-based foodstuffs manufacturing involves thermal processes, which provide (i) longer shelf life by reduction or elimination of microorganisms, (ii) characteristic flavor, and (iii) improvement of nutritional value by destroying antinutritional factors (Kunitz and Bowman−Birk trypsin inhibitors) and increasing protein digestibility (*9*).

It is known that allergy to soy resides in its protein fractions; 7S and 11S globulins are the most important (*10*). Proteolysis, cross-linking, amino acid racemization, oxidation, and other reactions such as Maillard reaction may cause protein deterioration during thermal processing of soy products. Food processing including both thermal treatment and proteolysis may also have an impact on the potential allergenicity of proteins because any process that modifies the structure of an allergen might be expected to affect its ability to be recognized by antibodies (*11, 12*). Determination of the processing, which might modify the allergenicity of a food ingredient, may be useful for risk analysis and risk management (*11*). Indivertibly eating foods that contain soybean proteins can occur because of undeclared allergens due to omissions and errors on labels, cross contamination of manufacturing equipment, and error made by suppliers of the ingredients. Detection of soy allergens in food products can be assayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting (*11, 13*). The antioxidant activity of soy has been also related to its content in proteins (*14*) and/or polyphenols (*15*). Both may be transformed during thermal processing, affecting the overall antioxidant activity of soy foodstuffs. The aim of the present

* Corresponding author. Telephone: +34 91 562 29 00. Fax: +34 91 564 48 53. E-mail: delcastillo@ifi.csic.es.

10.1021/jf800697n CCC: $40.75
Published on Web 07/12/2008

Table 1. Protein and Available Lysine Content of Soy-Based Foodstuffs[a]

sample code	protein content (%) (N × 6.25)	available Lys (g/100 g of protein) (w/w)	description reported on the label
A	14.56 ± 0.45^{p1}	3.52 ± 0.01^{p1}	instant powdered soymilk (soy protein isolate, maize maltodextrins, vegetable oil, calcium phosphate, minerals, lecithin, vitamins and flavor)
B	3.98 ± 0.03^{l2}	2.92 ± 0.06^{l2}	UHT liquid soymilk (mineral water, dehulled soybeans, sea salt)
C	3.72 ± 0.01^{l3}	6.67 ± 0.06^{l3}	UHT liquid soymilk (soybeans, chicory syrup, tricalcium phosphate, sea salt, and flavor)
D	3.14 ± 0.06^{l4}	6.48 ± 0.10^{l4}	UHT liquid soymilk (soybeans, fructose, tricalcium phosphate, stabilizers, flavor, and vitamins A and D)
E	3.67 ± 0.28^{l5}	8.29 ± 0.24^{l5}	UHT liquid soymilk (dehulled soybeans, raw cane sugar, dextrose, calcium bicarbonate, salt, and vitamin B_2)
F	3.58 ± 0.07^{l6}	7.72 ± 0.20^{l6}	UHT ecologic liquid soymilk (dehulled soybeans, chicory syrup, and salt)
G	2.70 ± 0.14^{l7}	5.18 ± 0.12^{l7}	UHT liquid soymilk fortified with vitamins A and D and Ca (soybeans, saccharose, calcium and phosphor, flavor, vitamins A and D)
H	3.73 ± 0.01^{l8}	6.45 ± 0.04^{l8}	UHT liquid soymilk (soybeans, soy protein isolate, vitamins C, D, A, and E, selenium, zinc, stabilizers, chicory syrup, vegetable fibers, flavor, and bicalcium phosphate)
I	4.01 ± 0.06^{l9}	6.92 ± 0.39^{l9}	UHT liquid soymilk (soybeans, sugar, fiber, salt, and calcium)
J	3.33 ± 0.18^{l10}	6.62 ± 0.19^{l10}	UHT liquid soymilk added with cereals (dehulled soybeans, fructose and cereals (barley, malt, rye, chicory))
K	3.55 ± 0.01^{l11}	6.94 ± 0.08^{l11}	UHT liquid soymilk added with cereals (dehulled soybeans, glucose syrup, fructose, soluble cereal extract)
L	2.84 ± 0.01^{l12}	5.98 ± 0.18^{l12}	UHT vanilla-flavored soy drink (soybeans, sugar, fructose, tricalcium phosphate, stabilizers, and flavor)
M	3.26 ± 0.01^{l13}	5.76 ± 0.18^{l13}	UHT vanilla-flavored soy drink (dehulled soybeans, raw cane sugar, dextrose, calcium bicarbonate, flavor, salt, and vitamin B_2)
N	3.16 ± 0.02^{l14}	6.44 ± 0.03^{l14}	UHT strawberry-flavored soy drink (dehulled soybeans, raw cane sugar, seaweeds, natural flavoring, red beet powder, sea salt)
P	3.63 ± 0.02^{c15}	9.76 ± 0.01^{c15}	UHT cow's milk fortified with soy isoflavones (milk, milk proteins, minerals, vitamins, and soy isoflavones)
Q	38.22 ± 0.01^{p16}	3.94 ± 0.01^{p16}	powdered soy shake (dehulled soybeans)
R	15.91 ± 0.04	1.54 ± 0.01	biscuits with soy and kiwi (wheat flour, vegetal oil, soy protein, dehydrated kiwi, glucose syrup, soy lecithin, vitamins, and minerals)
S	8.11 ± 0.10	7.40 ± 0.11	biscuits covered with soy and chocolate with milk (chocolate with milk, rice and maize flour, soy protein isolate, vitamins, and minerals)
T	33.01 ± 0.25	2.78 ± 0.01	soy snack (defatted soy flour, starch, salt, and soy lecithin)

[a] Values correspond to mean values ($n = 2$) ± standard deviation. Values followed by a different superscript code denote (l*n*, ready to drink soymilks; p*n*, powdered soy drinks; and c*n*, cow milk) significant differences ($p < 0.05$) among samples. Statistical analysis was performed to look at differences in quality among drinks.

study was to evaluate protein quality, antigenic response, and antioxidant activity of commercial soy products. Additionally, the feasibility of analytical approaches such as RP-HPLC, SDS-PAGE, and immunoblotting to determine soy allergens in processed foods was also undertaken.

MATERIALS AND METHODS

Chemicals and Samples. *Chemical Reagents.* All the chemicals were of analytical grade. *O*-Phthaldialdehyde (OPA), methanol, sodium fluorescein, and ferulic acid were purchased from Fluka. Sodium tetraborate decahydrate, SDS, N^{α}-acetyl-L-lysine, 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), DL-dithiothreitol (DTT), and 6-hydroxy-2,5,7,8-tetramethylchroma-2-carboxylic acid (Trolox) were supplied by Sigma-Aldrich. Sodium dihydrogen orthophosphate dehydrate, disodium hydrogen orthophosphate anhydrous, boric acid, trifluoroacetic acid, and β-mercaptoethanol were from Merck. Soy protein isolate (SPI) was from Manuel Riesgo.

Food Samples. Beverages based on soymilk ($n = 15$), cow's milk supplemented with soy isoflavones ($n = 1$), soy snacks ($n = 1$), and soy biscuits ($n = 2$) were obtained from local markets. Selection of the foodstuffs was made taking into account their presence in the local market. **Table 1** shows information provided by the manufacturers related to food composition.

Experimental Analysis. *Total Protein Content.* The protein content was determined by means of the Kjeldahl method (*16*). The nitrogen data were converted into protein values employing a conversion factor of 6.25 and were expressed as grams per 100 g of product. Analysis was performed in duplicate.

Available Lysine. Free lysine content was estimated by OPA assay described by Goodno et al. (*17*) as modified by Ramirez-Jiménez et al. (*18*). The OPA reagent was prepared by dissolving 16.4 mg of OPA in 2.5 mL of 95% (v/v) methanol, followed by the addition of 25 mL of 0.1 M borate buffer (pH 9.5), 400 μL of 10% (v/v) β-mercaptoethanol, 5 mL of aqueous SDS solution (20% w/v) and made up to 100 mL with Milli-Q water. Samples were dissolved to obtain 0.01 mg of protein/mL. Protein amino groups were determined by reacting 0.25−50 μg of protein sample with 3 mL of OPA reagent, incubated at 25 °C for 2 min. Fluorescence readings were measured at 340-nm excitation and 455-nm emission wavelengths using a spectrofluorometer (model RF-1501, Shimadzu Corporation). Calibration curves were constructed employing final concentrations of N^{α}-acetyl-L-lysine and SPI ranging from 10 to 250 μM and 0.1 to 50 mg/mL, respectively. All measurements were performed at least in duplicate, and data were expressed as grams per 100 g of protein.

Protein Analysis by RP-HPLC-UV. Protein fraction was partially purified by isoelectric precipitation. Briefly, an aqueous solution of food samples containing 35 mg of protein was extracted at pH of 8.5−9.0 with 2 M NaOH followed by centrifugation at 12000*g* for 5 min. The extraction procedure was repeated twice, and the obtained supernatants were mixed. Proteins were precipitated at pH 4.5 with 2 M HCl and centrifuged at 12000*g* for 10 min. The precipitated proteins were diluted with 1 mL of 2% (w/v) SDS and 2% (v/v) β-mercaptoethanol. Before chromatographic analysis, further dilution of protein samples (1/100) in 2% (w/v) SDS and 2% (v/v) β-mercaptoethanol and filtration through PVDF 0.45-μm filters was carried out.

RP-HPLC analysis was performed combining chromatographic conditions previously described by others (*19–21*) with some modifications. The RP-HPLC system consisted of a Beckman binary gradient 125 pump HPLC equipped with a Metrohm-Spark Triathlon autosampler and a Beckman 166 UV detector. System Gold software was used for data acquisition. Separations were performed on a Phenomenex Jupiter 300 Å (C_4, 5-μm particle size, 250 × 4.60 mm i.d.) employing a linear binary gradient from 35 to 70% B in 45 min at room temperature. Mobile phases were: phase A, 0.025% (w/v) SDS and 0.1% (v/v) TFA in Milli-Q water; and phase B, 0.025% (w/v) SDS

and 0.085% (v/v) TFA in Milli-Q water−acetonitrile (10:90, v/v) (Laboratory-Scan Analytical Sciences). The flow rate was 1 mL/min, 100 μL was injected, and UV detection was performed at 214 nm.

When required, soy protein RP-HPLC fractions were collected into Eppendorf tubes, concentrated in a vacuum centrifuge (SpeedVac concentrator A 160, Savant Instruments), and stored at −18 °C, before analysis by SDS-PAGE.

SDS-PAGE and Immunoblotting. For SDS-PAGE analyses, samples were diluted with Milli-Q water and aliquots of 32.5 μL were mixed with 5 μL of DTT (0.5 M) and 12.5 μL of NuPAGE LDS sample buffer (4×) (Invitrogen) and heated at 100 °C for 10 min. Samples were loaded (20 μL equivalent to 20 μg of protein) on a 4−12% linear gradient polyacrylamide NuPAGE Novex Bis-Tris precast gel. A continuous buffer system (NuPAGE MES SDS running buffer, Invitrogen) was used. Gels were run for 50 min at initial current of 120 mA/gel and at constant voltage of 200 V and stained using Colloidal Blue Staining Kit (Invitrogen). Marker proteins were: insulin A chain (M_r 2500), insulin B chain (M_r 3500), aprotinin (M_r 6000), lysozyme (M_r 14 400), trypsin inhibitor (M_r 21 500), carbonic anhydrase (M_r 31 000), lactate dehydrogenase (M_r 36 500), glutamic dehydrogenase (M_r 55 400), bovine serum albumin (M_r 66 300), and phosphorylase B (M_r 97 400).

After electrophoresis, proteins were electroblotted onto a 0.45-μm Invitrolon PVDF membrane using the XCell II blot module (Invitrogen), at 30 V for 2 h, following the manufacturer's instructions. Blots were blocked with TBST [0.05% (v/v) Tween-20 in Tris-buffered saline] containing 10% (w/v) milk powder for 1 h at room temperature. After being washed three times with TBST, blots were incubated with a polyclonal rabbit *anti*-soy sera diluted 1:1000 (v/v) in TBST overnight at 4 °C, washed, and incubated with a horseradish peroxidase-labeled goat *anti*-rabbit IgG diluted 1:1000 (v/v) in TBST containing 10% (w/v) milk powder for 1 h at room temperature. Antibodies were from Sigma. Blots were again washed five times with TBST and stained with a liquid substrate system for membranes of 3,3′,5,5′-tetramethylbenzidine (Sigma).

Antioxidant Properties. Total phenol content (natural antioxidants) was estimated by analysis of the content of OH or potentially oxidazable groups by means of the Folin−Ciocalteu method in a 96-well microplate as described by Schmidt et al. (*22*). Food samples containing 3.5 g/100 g of products were dissolved in DMSO (dilution ranging from 1:5 to 1:8 v:v) and centrifuged twice at 12000*g* for 10 min. Ten microliters of these supernatants was added with 150 μL of aqueous Folin−Ciocalteu reagent (1 mL of reagent/14 mL of Milli-Q water) and incubated at room temperature for 3 min. After the incubation, 50 μL of sodium bicarbonate solution (2 mL of saturated sodium bicarbonate to 3 mL of water) was added to each well and incubated at room temperature for 2 h. Then, the plate was read at 725 nm using a PowerWave XS microplate spectrophotometer (Bio-Tek). Measurements were carried out in triplicate. Data were expressed as milligrams of ferulic acid equivalent (FAE)/100 g of product. A calibration curve of ferulic acid (0.1−0.6 mg/mL) was constructed and employed for phenol quantification in the samples.

The overall peroxyl radical scavenging activity of peroxyl radicals of soy products was calculated by $ORAC_{FL}$ assay as described by del Castillo et al. (*23*), employing a calibration curve of ferulic acid ranged from 0.1 to 0.6 mg/mL. Biscuits and soy snacks (samples R, S, and T) were diluted in phosphate buffer (75 mM, pH 7.5) to a final protein concentration of 3.5 mg/mL and twice centrifuged at 12000*g* for 10 min, and then the clear supernatant was collected and employed for analysis. Fluorescein stock solution (100 μM) in phosphate buffer (75 mM, pH 7.5) was prepared and stored at 4 °C in the dark. Trolox stock solutions in phosphate buffer (75 mM, pH 7.5) were aliquoted (2 mL) and stored at −20 °C until analysis. Further dilutions (12.5, 25, 40, 50, and 100 μM) were performed to construct a calibration curve.

A fresh working solution of fluorescein (48 nM, 2.225 mL) was premixed with sample, standard or phosphate buffer (375 μL), and incubated for 30 s. The fluorescence (λ_{exc} = 493 nm and λ_{em} = 515 nm) was measured in a spectrofluorophotometer (model RF-1501, Shimadzu Corporation). This reading was the fluorescence at time zero (f_0). The assay was initiated by adding AAPH (143 mM, 375 μL). Mixtures were kept in a water bath at 37 °C for 40 min. Fluorescence readings were taken every 5 min after AAPH addition. The fluorescence decay curve was plotted and the area under the curve calculated. Blanks were run by replacing sample with phosphate buffer. Sample fluorescence values were corrected for the blank value. Trolox (20 μM) was analyzed with every batch of sample as a quality control measure. Determinations were carried out at least in duplicate. Data were expressed as micromole Trolox equivalents (TE)/g of product.

Statistical Analysis. Microsoft Excel 2000 program was employed for statistical analysis of the data with the level of significance set at 95%. One-way analysis of variance was used to look for differences between means of more than two groups of samples for the protein content, available lysine, and antioxidant activity. Correlation between Folin−Ciocalteu and ORAC data was also determined employing this program.

RESULTS

Protein Quality. The protein content of soy foodstuffs is shown in **Table 1**. Protein values detected for liquid soy beverages (samples B−N) ranged from 2.70 to 4.01 g/100 g of product ($p < 0.05$). Ready to drink powdered samples prepared as recommended by the manufacturer, 13.76 g of sample A and 5.86 g of sample Q in 200 mL of water, corresponded to protein values of 0.98 and 1.12 g/100 g of product for A and Q, respectively.

Available lysine values ranging from 1.54 to 9.76 g/100 g of protein were obtained for soy products (**Table 1**). Instant powdered soy milk (sample A), liquid soymilk beverage (sample B), powdered soy shake (sample Q), biscuits containing soy and kiwi (sample R), and soy snack (sample T) showed low levels of availability of this essential amino acid. As expected, the highest value of available lysine corresponded to the cow's milk supplemented with soy isoflavones (sample P). Available lysine values of ready to drink powdered samples were 0.24 g/100 g of protein (A) and 0.12 g/100 g of protein (Q).

Identification, Thermal Stability, and Antigenicity of Soy Proteins. 7S and 11S soy proteins were identified combining HPLC and SDS-PAGE data. **Figure 1** shows RP-HPLC profiles of native SPI, heated SPI, and some foods subjected to different manufacturing processes. Ten chromatographic peaks were resolved from the native SPI sample under the assayed conditions (**Figure 1A**, solid line). Most of them were unstable after the thermal treatment at 95 °C for 1 h (**Figure 1A**, dash line). Differences in the chromatographic profiles of the soy foodstuffs were detected. The majority of the peaks found in the native SPI sample remained in liquid soy milk, while a hump containing low amounts and no separated soy proteins was observed in instant powdered soymilk (**Figure 1B**). Similar chromatographic profiles were obtained for biscuits containing soy and kiwi, soy snacks instant powdered soymilk (**Figure 1C**), and SPI heated at 95 °C for 1 h (**Figure 1A**). No soy proteins were detected in cow's milk supplemented with soy isoflavones. A characteristic profile of milk proteins was obtained for this sample as can be observed in **Figure 1D**.

Figure 2 shows SDS-PAGE migration patterns corresponding to protein peaks from native SPI separated and manually collected by RP-HPLC (**Figure 1A**, solid line). Seven fractions containing proteins were collected, concentrated in a vacuum centrifuge, and subjected to electrophoresis analysis. According to the estimated molecular masses of the protein bands shown in **Figure 2**, the protein peaks labeled in **Figure 1A** were assigned as 7S and 11S soy proteins.

Soy proteins were clearly detected by SDS-PAGE in all liquid samples declared by producers as soy foodstuffs (**Figure 3A**). Electrophoretic bands corresponding to α′ ($M_r \approx 64\,200$), α ($M_r \approx 61\,770$), and β ($M_r \approx 49\,580$) subunits of the 7S fraction and acidic ($M_r \approx 36\,500$) and basic ($M_r \approx 20\,500$) subunits of

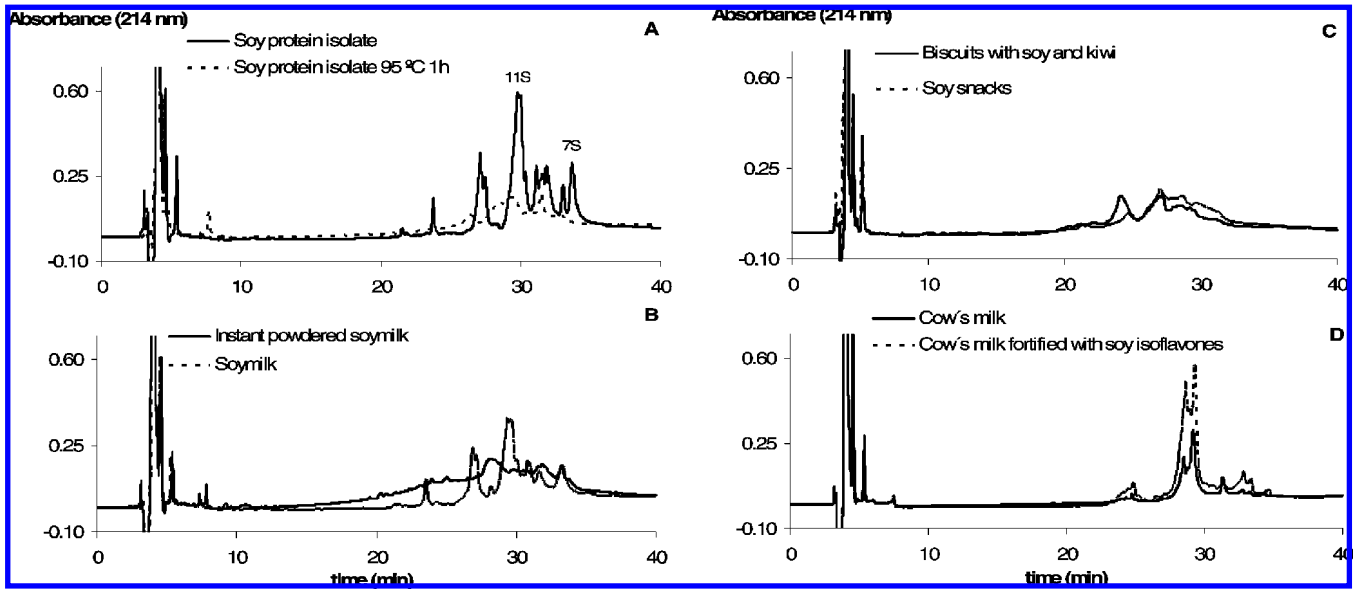

Figure 1. RP-HPLC analysis of soy protein isolate (**A**), instant powdered soymilk and liquid soymilk (**B**), snacks and biscuits with soy and kiwi (**C**), and cow's milk and cow's milk supplemented with soy isoflavones (**D**).

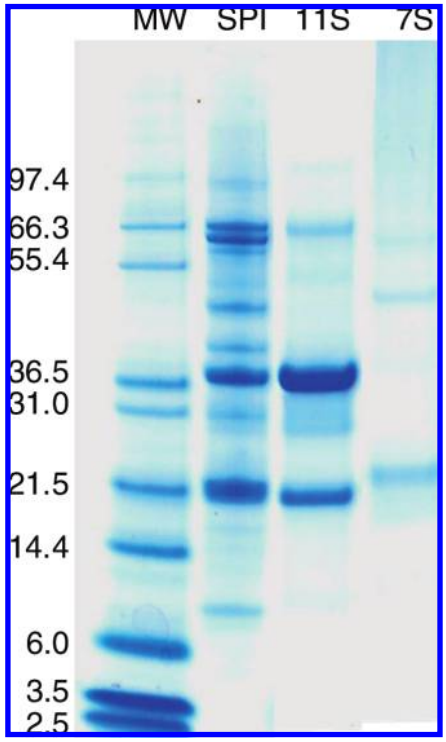

Figure 2. SDS-PAGE 4−12% gradient gel stained with Coomassie blue of the fractions obtained from soy protein isolate by RP-HPLC. 1, molecular weight marker (kDa); 2, soy protein isolate; 3, 11S globulin; and 4, 7 S globulin.

the 11S fraction of soy were identified in these samples. However, fainted and undefined bands were detected for the instant powdered (sample A, lane 3) and soy snack (sample T, lane 12) samples. A different protein pattern was found for cow's milk but was similar to soy biscuits covered with chocolate and milk (lanes 10 and 13, respectively). No soy proteins were detected in cow's milk supplemented with soy isoflavones (sample P, lane 10).

SDS-PAGE and immunoblotting data agreed (**Figure 3A**,**B**). No soy proteins bands were detected in instant powdered soymilk (**Figure 3B**, lane 3), soy snacks (**Figure 3B**, lane 12), and soy biscuits chocolate covered with chocolate with milk (**Figure 3B**, lane 13).

RP-HPLC, SDS-PAGE, and immunoblotting analyses indicated that protein structure and antigenic response of instant powdered soymilk, soy snack, and soy biscuits were modified by processing.

Antioxidant Activity. Total phenol contents of soy-based foodstuffs ranged from 40.45 to 1708.01 mg of FAE/100 g of product (**Figure 4A**), which were significantly different ($p < 0.05$). Values corresponding to ready to drink powdered samples were 1586.73 (sample A) and 1708.01 mg of FAE/100 g of product (sample Q), respectively. Similar total phenol contents were obtained for cow's milk and liquid soy beverages.

$ORAC_{FL}$ data can be observed in **Figure 4B**. Foodstuffs studied in the present article protected fluorescein against peroxyl alkyl radical oxidation. $ORAC_{FL}$ values ranged from 2.09 to 75.08 μmol TE/g of product. Peroxyl scavenging radical behaviors of liquid and powder foods were significantly different ($p < 0.05$).

A correlation of $r = 0.77$ between total phenolic compounds and $ORAC_{FL}$ for the soymilk liquid beverages, ready to drink powdered and cow's milk samples was obtained, whereas a lower correlation ($r = 0.35$) was found between these two parameters in solid samples.

DISCUSSION

Protein Quality. Protein values reported here are lower than those described by van Buren et al. (*24*), who determined values in soluble nitrogen, and similar to those published by others for soymilks (*25, 26*).

Data on lysine content agreed with those previously published (*24, 25, 27*) for soymilks. The intensity of the thermal processes employed to manufacture each food affected the availability of this essential amino acid. Maillard and cross-linking reactions constitute favored pathways for lysine blockage during thermal processing of soy foods (*28*). Furosine, an early chemical indicator of the Maillard reaction, was reported in soy beverages thermally treated (*29, 30*). Instant food manufacturing requires high temperatures under which this reaction is also favored (*31*). UHT and sterilization processes employed for both liquid and powdered foods also involve severe thermal treat-

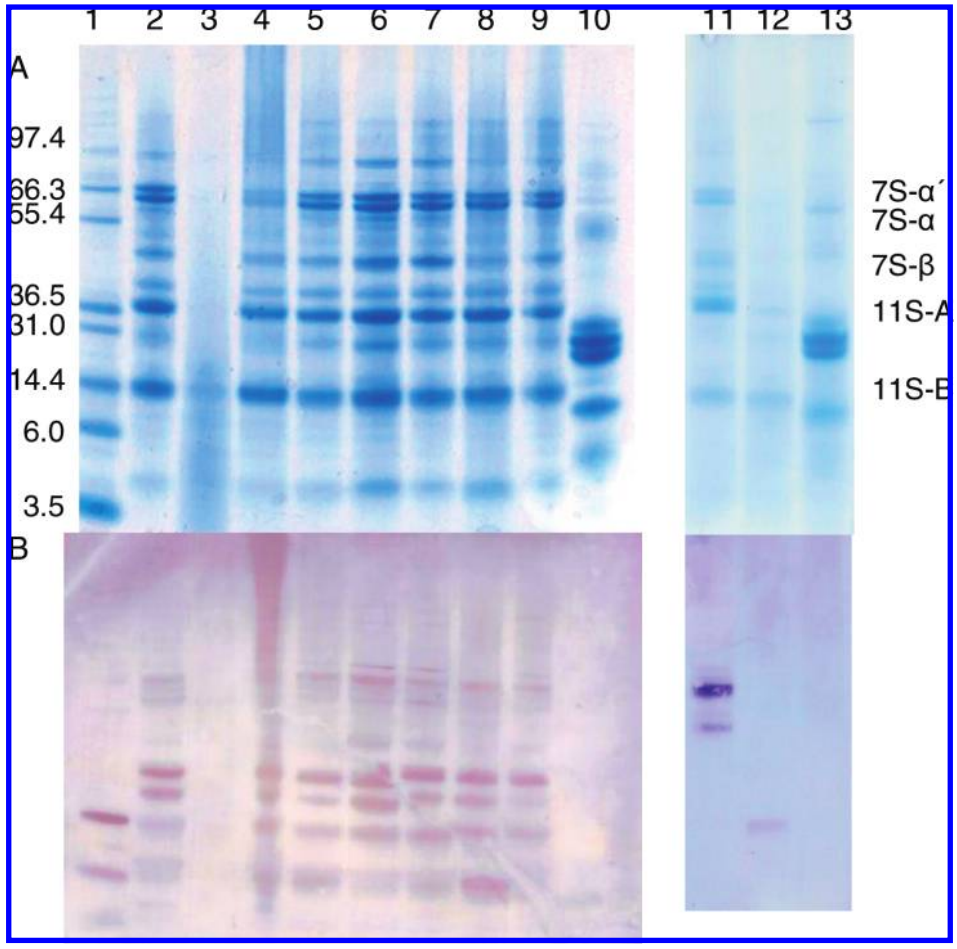

Figure 3. SDS-PAGE stained with Coomassie blue (**A**) and immunoblotting (**B**) of soy-based foodstuffs. 1, molecular weight marker (kDa); 2, isolate soy protein; 3, instant powdered soymilk; 4, powdered soy shake; 5 and 6, soymilk; 7, soymilk flavored with vanilla; 8, soymilk flavored with strawberry; 9, soymilk added with cereals; 10, cow's milk supplemented with soy isoflavones; 11, biscuits with soy and kiwi; 12, soy snack; and 13, biscuits covered with soy and chocolate with milk. α′, α, and β are subunits of the 7S protein fraction. A (acid) and B (basic) are subunits of the 11S protein fraction of the soy.

ments. Taking into account the lysine content of soy products, it can be concluded that generally the protein quality of soy beverages is comparable to that of cow's milk (7.43 g/100 g of protein) and egg whites (6.49 g/100 g of protein) (*25*). Moreover, data on lysine demonstrated that solely protein content is not a good parameter for estimating food nutritional quality. Lysine content similar to that found for powdered soy foods (sample A) was measured in a soy beverage possessing average protein content (**Table 1**, sample B).

Structural Changes and Antigenicity of Soy Proteins. Changes in protein structure and antigenicity were found, and these modifications could be related to the intensity of processing conditions (*13, 32*).

Soy protein molecular masses estimated by SDS-PAGE (**Figure 3A**) agreed with those previously described (*33*). Slight modifications in electromigration might be attributable to glycation by Maillard reaction (*34*). In addition, SDS-PAGE results seem to indicate that the primary structure of 7S and 11S globulins survived soy liquid beverage manufacturing conditions. Thus, 7S and 11S globulins, which have been shown to be thermostable up to 75 and 95 °C, respectively (*35, 36*), were detected in processed soy liquid beverages. A different behavior was observed for powdered soy beverage, biscuits with soy and kiwi, soy snacks, and biscuits covered with soy and chocolate with milk. Fainted and nonsoy protein bands were observed in these samples, suggesting the employment of more drastic thermal processing conditions that caused changes in the structure of soy proteins. Treatments at 180 °C for 1 to 2 min favor changes in protein structure by Maillard and cross-linking reactions (*37, 38*), which are reflected in the SDS-PAGE profile. The occurrence of the Maillard reaction was previously checked by furosine analysis in soy beverages (*30*). SDS-PAGE might be more adequate to look at changes in the primary structure of the protein than the analysis of a unique product of the Maillard reaction. Results also suggested no adulteration of analyzed cow's milk with soy proteins (sample P), demonstrating that this technique might be feasible for detecting this type of adulteration. The protein SDS-PAGE profiles found for cow's milk and cow milk supplemented with soy isoflavones matched and agreed with those previously described (*39, 40*). Only milk proteins were observed in soy biscuits covered with chocolate with milk (**Figure 3A**, lane 13). Data suggested that milk proteins were in higher concentration or were more thermostable than soy proteins. Producers declared both soy and milk proteins as components of these biscuits (**Table 1**).

In regard to immunoblotting data, milk proteins were not detected, confirming that no cross reactions took place. Processing masked the antigenic response of soy allergens in instant soy beverage, biscuits with soy and kiwi, soy snacks, and soy-chocolate biscuits. Further studies employing sera of soy

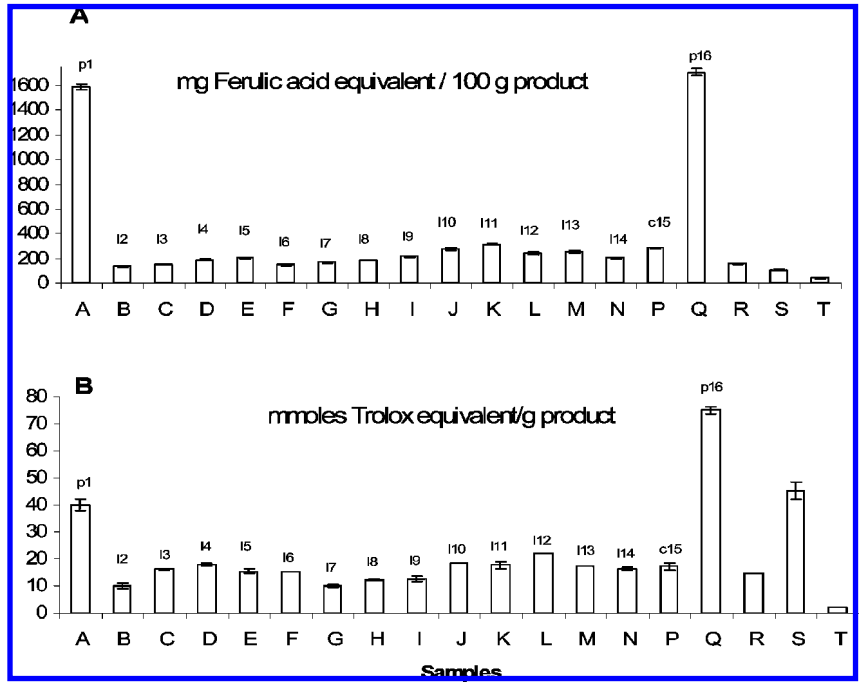

Figure 4. Total phenolic compounds (**A**) and $ORAC_{FL}$ values (**B**) of soy-based foodstuffs. Data are expressed in milligram equivalents of ferulic acid/100 g of product (**A**) and micromolar equivalents of Trolox per gram of product (**B**). Values are the means of triplicate analyses; error bars denote the relative standard deviation. Sample codes are explained in **Table 1**. Bars with different codes (l*n*, ready to drink soymilks; p*n*, powdered soy drinks; and c*n*, cow milk) are significantly different ($p < 0.05$). Statistical analysis was performed to look at differences in quality among drinks.

allergenic patients are needed to check if the structural changes of soy allergens caused by thermal processing also involve changes in their allergenic response. Food processing may decrease the intrinsic allergenicity of protein. Although data published thus far related to that subject are not conclusive, alteration of protein conformation by heating has been associated to loss of conformational epitopes and consequently reduction or abolition of allergenic potential of proteins (*11*).

Antioxidant Activity. Data demonstrated that food composition (**Table 1**) and processing can have significant effects ($p < 0.05$) on total phenolic compounds and $ORAC_{FL}$ values.

Soybeans contain many phenolic compounds such as chlorogenic acid, caffeic acid, ferulic acid, and *p*-coumaric acid possessing antioxidant effect beneficial to human health (*41*). These compounds constitute from 28 to 72% of the total phenol level in soybean seeds. Isoflavones were also found to possess important biological functions, including antioxidant and anticarcinogenic properties. Genistein and daidzein were described as the major isoflavones in soybeans, with the former possessing the strongest antioxidant properties (*42*).

According to **Figure 4**, most soy foodstuffs studied in the present article had greater scavenger potential of peroxyl radicals than other vegetable foods such as carrots, peas, fruit juices, among others (*43, 44*). Values presented here are lower than those reported by Monje et al. (*45*), who determined antioxidant capacity of cotyledons and germs of soybean from two geographical locations and two cultivars. Both phenolics and isoflavones were previously described as good scavengers of peroxyl radicals measured by ORAC using fluorescein and phycoerythrin as fluorescence probes. Phenolic compounds were described to be stronger peroxyl radical scavengers than Trolox and ascorbic acid (*23, 46, 47*). Genistein, daidzein, and their metabolites exhibited peroxyl radical scavenging activity comparable or higher than that of quercetin, a very well known dietary flavonoid. $ORAC_{PE}$ of 4 and 5 mmol Trolox equivalents were reported for daidzein and genistein, respectively. Soy naturally occurring isoflavones were more effective than Trolox and ascorbic acid determined with the $ORAC_{PE}$ assay (*48*). A correlation between soy isoflavone content and $ORAC_{FL}$ values was reported (*45*). However, our data seem to indicate that other food constituents in addition to total phenols may be contributing to the peroxyl radical scavenging activity of soy foods studied here. The chemical form of soy isoflavones in foods is affected during processing (*49*), which might be relevant to their antioxidant character. Additionally, chemical events such as Maillard reaction and caramelization simultaneously occurring during food processing might give rise to neoantioxidants. Therefore, it may be suggested that both remaining natural antioxidants and those formed during processing could contribute to the overall antioxidant capacity of the final product.

ORAC values found in biscuits can be explained on the basis of their composition. Chocolate, cereals, strawberry, and kiwi fruit were previously reported as scavengers of peroxyl radicals determined by $ORAC_{FL}$ assay (*44*). Chocolate is very effective in protecting fluorescein against peroxyl radical action. In addition, most of the foods were supplemented with minerals and vitamins which can also act as antioxidants. Our findings seem to indicate that manufacturing of instant soy milk and soy shake samples may damage antioxidants and consequently decrease the overall antioxidant activity of the ready to drink beverages. More studies should be carried out to elucidate either the individual role or the synergic effect of soy food ingredients contributing to their overall antioxidant activity.

In conclusion, the protein quality, antigenicity, and antioxidant activity of soy products can be affected as a function of the intensity of the thermal processing. Data presented here suggested that solely protein values are not a good indicator of protein quality of soy product. Denaturation and loss of available lysine by means of the Maillard reaction seem to be the main changes affecting the protein fraction. A relationship between

structural modifications of protein estimated by HPLC, SDS-PAGE, and immunoblotting data was observed. Therefore, chromatographic, electrophoretic, and immunological assays may be employed for the simultaneous identification of soy products, assessment of protein quality, detection of adulterations, and search for soy allergens. Soy foods scavenged peroxyl radicals and total phenols seemed to be the major contributors to this activity in soy liquid beverages.

ABBREVIATIONS USED

DMSO, dimethylsulfoxide; FAE, ferulic acid equivalent; OPA, *O*-phthaldialdehyde; $ORAC_{FL}$, oxygen radical absorbance capacity using fluorescein as fluorescent probe; $ORAC_{PE}$, oxygen radical absorbance capacity using B-phycoerythrin as fluorescent probe; SPI, soy protein isolate; TE, Trolox equivalent; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

We thank Noelia Herrero Escudero for kind support.

LITERATURE CITED

(1) Sloan, A. E. Top 10 global food trends. *Food Technol.* **2005**, *59*, 20–32.
(2) Friedman, M.; Brandon, D. L. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **2001**, *49*, 1069–1086.
(3) Golbitz, P.; Jordan, J. Soyfoods: Market and Products. In *Soy Applications in Food*; Riaz, M. N., Ed.; Taylor & Francis: Boca Raton, FL, 2006.
(4) Takamatsu, K.; Tachibana, N.; Matsumoto, I.; Abe, K. Soy protein functionality and nutrigenomic analysis. *Biofactors* **2004**, *21*, 49–53.
(5) Torres, N.; Torre-Villalvazo, I.; Tovar, R. Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders. *J. Nutr. Biochem.* **2006**, *17*, 365–373.
(6) Duranti, M. Grain legume proteins and nutraceutical properties. *Fitoterapia* **2006**, *77*, 67–82.
(7) Lessof, M. H. *Food Allergy and Other Adverse Reactions to Food*; ILSI Press: Washington, DC, 1994.
(8) U.S. Food and Drug Administration. Food allergies. What you need to know. Foodfacts; February 2007; http://www.cfsan-.fda.gov/~dms/ffalrgn.html.
(9) Kwok, K. C.; Liang, H. H.; Niranjan, K. Optimizing conditions for thermal processes of soy milk. *J. Agric. Food Chem.* **2002**, *50*, 4834–4838.
(10) Ogawa, T. Soy Allergy. In *Soy in Health and Disease Prevention*; Sugano, M., Ed.; Taylor & Francis: Boca Raton, FL, 2006.
(11) Thomas, K.; Herouet-Guicheney, C.; Ladics, G.; Bannon, G.; Cockburn, A.; Crevel, R.; Fitzpatrick, J.; Mills, C.; Privalle, L.; Vieths, S. Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. *Food Chem. Toxicol.* **2007**, *45*, 1116–1122.
(12) Frias, J.; Song, Y. S.; Martı´nez-Villaluenga, C.; Gonzále de Mejia, E.; Vidal-Valverde, C. Immunoreactivity and amino acid content of fermented soybean products. *J. Agric. Food Chem.* **2008**, *56*, 99–105.
(13) Franck, P.; Moneret Vautrin, D. A.; Dousset, B.; Kanny, G.; Nabet, P.; Guénard-Bilbaut, L.; Parisot, L. The allergenicity of soybean-based products is modified by food technologies. *Int. Arch. Allergy Immunol.* **2002**, *128*, 212–219.
(14) Manzoni, C.; Lovati, M. R.; Gianazza, E.; Morita, Y.; Sirtori, C. R. Soybean protein products as regulators of liver low-density lipoprotein receptors. II. α-α′ Rich commercial soy concentrate and α′ deficient mutant differently affect low-density lipoprotein receptor activation. *J. Agric. Food Chem.* **1998**, *46*, 2481–2484.
(15) Saura-Calixto, F.; Goñi, I. Fibra dietéticay antioxidantes en la dieta española y en los alimentos funcionales. In *Alimentos Funcionales*; Juárez, M., Olano, A., Morais, F., Eds.; FECYT: Madrid, Spain, 2005; pp 267−214.
(16) AOAC Official Method 979.09. Protein in grains In Official Methods of Analysis; Association of Analytical Communities: Washington, DC, 2002.
(17) Goodno, C. C.; Swaisgood, H. E.; Catignani, G. L. A fluorimetric assay for available lysine in proteins. *Anal. Biochem.* **1981**, *115*, 203–211.
(18) Ramirez-Jimenez, A.; Guerra-Hernandez, E. J.; Garcia-Villanova, B. Evaluation of amino group losses for monitoring storage of infant cereals containing milk. *Food Control* **2004**, *15*, 351–354.
(19) Buehler, R. E.; Vantoai, T. T.; McIntosh, G. H. The optimisation of elution and extraction conditions for the separation of soybean seed proteins using RP-HPLC. *Seed Sci. Technol.* **1989**, *17*, 193–204.
(20) Peterson, R. E.; Wolf, W. J. Enhancement of high-performance liquid chromatography of soybean proteins by addition of sodium dodecyl sulfate. *Cereal Chem.* **1992**, *69*, 101–102.
(21) Oomah, B. D.; Voldeng, H.; Fregeau-Reid, J. A. Characterization of soybean proteins by HPLC. *Plant Foods Hum. Nutr.* **1994**, *45*, 251–263.
(22) Schmidt, B. M.; Erdman, J.; Lila, M. A. Effects of food processing on blueberry antiproliferation and antioxidant activity. *J. Food Sci.* **2005**, *70*, S389–S394.
(23) del Castillo, M. D.; Gordon, M. H.; Ames, J. M. Peroxyl radical-scavenging activity of coffee brews. *Eur. Food Res. Technol.* **2005**, *221*, 471–477.
(24) van Buren, J. P.; Hackler, L. R.; Elrawi, I.; Steinkraus, K. H.; Hand, D. B. Heat effects on soymilk - Indices of protein quality in dried soymilks. *J. Agric. Food Chem.* **1964**, *12*, 524–528.
(25) Souci, S. W.; Fachmann, W.; Kraut, H. *Food Composition and Nutrition Tables*; Medpharm Scientific Publishers: London, 2000.
(26) Ortega Anta, R. M.; López Sobaler, A. M.; Requejo Marcos, A. M.; Andrés Carvajales, P. *La Composición de los Alimentos. Herramienta Básica para la Valoración Nutricional*, 1st ed.; Editorial Complutense: Madrid, Spain, 2004.
(27) Kwok, K. C.; Shiu, Y. W.; Yeung, C. H.; Niranjan, K. Effect of thermal processing on available lysine, thiamine and riboflavin content in soymilk. *J. Sci. Food Agric.* **1998**, *77*, 473–478.
(28) Meade, S. J.; Reid, E. A.; Gerrard, J. A. The impact of processing on the nutritional quality of food proteins. *J. AOAC Int.* **2005**, *88*, 904–922.
(29) Marconi, E.; Acquistucci, R.; Panfili, G. *Furosine Determination in Commercial Samples of Soymilk, COST Action 98, Effects of Antinutrients on the Nutritional Value of Legume Diets*; Proceedings of the 6th Scientific Workshop in Rome, Italy, 1999; pp 39−45.
(30) Amigo-Benavent, M.; Villamiel, M.; del Castillo, M. D. Chromatographic and electrophoretic approaches for the analysis of protein quality of soy beverages. *J. Sep. Sci.* **2007**, *30*, 502–507.
(31) Villamiel, M.; del Castillo, M. D.; Corzo, N. Browning Reactions. In *Food Biochemistry and Food Processing*; Hui, Y. H., Nip, W.-K., Mollet, L., Paliyath, G., Simpson, B. K., Eds.; Blackwell Publishing: Oxford, 2006; pp 71−100.
(32) Saz, J. M.; Marina, M. L. High performance liquid chromatography and capillary electrophoresis in the analysis of soybean proteins and peptides in foodstuffs. *J. Sep. Sci.* **2007**, *30*, 431–451.
(33) Garcia, M. C.; Torre, M.; Marina, M. L.; Laborda, F. Composition and characterization of soyabean and related products. *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 361–391.
(34) van de Lagemaat, J.; Silván, J. M.; Moreno, F. J.; Olano, A.; del Castillo, M. D. In vitro glycation and antigenicity of soy proteins. *Food Res. Int.* **2007**, *40*, 153–160.
(35) Mills, E. N. C.; Huang, L.; Noel, T. R.; Gunning, A. P.; Morris, V. J. Formation of thermally induced aggregates of the soya globulin β-conglycinin. *Biochim. Biophys. Acta* **2001**, *1547*, 339–350.
(36) Mills, E. N. C.; Marigheto, N. A.; Wellner, N.; Fairhurst, S. A.; Jenkins, J. A.; Mann, R.; Belton, P. S. Thermally induced structural changes in glycinin, the 11S globulin of soya bean (*Glycine max*)−An in situ spectroscopic study. *Biochim. Biophys. Acta* **2003**, *1648*, 105–114.

(37) Camire, M. E.; Camire, A.; Krumhar, K. Chemical and nutritional changes in foods during extrusion. *Crit. Rev. Food Sci. Nutr.* **1990**, *29*, 35–57.

(38) Villamiel, M. Nonenzymatic Browning of Cookies, Crackers and Breakfast Cereals. In *Food Biochemistry and Food Processing*; Hui, Y. H., Nip, W.-K., Mollet, L., Paliyath, G., Simpson, B. K., Eds.; Blackwell Publishing: Oxford, 2006; pp 555−565.

(39) Molina, E.; Amigo, L.; Ramos, M. Detection of bovine milk proteins in soymilk by western blotting. *J. Food Prot.* **1998**, *61*, 1691–1694.

(40) Restani, P.; Gaiaschi, A.; Plebani, A.; Beretta, B.; Cavagni, G.; Fiocchi, A.; Poiesi, C.; Velona, T.; Ugazio, A. G.; Galli, C. L. Cross-reactivity between milk proteins from different animal species. *Clin. Exp. Allergy* **1999**, *29*, 997–1004.

(41) Kim, S. H.; Jung, W. S.; Ahn, J. K.; Chung, I. M. Analysis of isoflavone concentration and composition in soybean [*Glycine max* (L.)] seeds between the cropping year and storage for 3 years. *Eur. Food Res. Technol.* **2005**, *220*, 207–214.

(42) Kim, E. H.; Kim, S. H.; Chung, J. I.; Chi, H. Y.; Kim, J. A.; Chung, I. M. Analysis of phenolic compounds and isofavones in soybean seeds [*Glycine max* (L.) Merill] and sprouts grown under different conditions. *Eur. Food Res. Technol.* **2006**, *222*, 201–207.

(43) Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J. Agric. Food Chem.* **2002**, *50*, 3122–3128.

(44) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026–4037.

(45) Monje, M. C.; Berger, M.; Farines, V.; Reybier, K.; Verger, A.; Dayde, J.; Theodorou, V.; Nepveu, F. Antioxidant capacity of cotyledons and germs of soybean in relation to their isoflavone content. *Food Technol. Biotechnol.* **2006**, *44*, 493–498.

(46) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619–4626.

(47) Davalos, A.; Gomez-Cordoves, C.; Bartolome, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *J. Agric. Food Chem.* **2004**, *52*, 48–54.

(48) Rüfer, C. E.; Kulling, S. E. Antioxidant activity of isoflavones and their major metabolites using different in vitro assays. *J. Agric. Food Chem.* **2006**, *54*, 2926–2931.

(49) Coward, L.; Smith, M.; Kirk, M.; Barnes, S. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S–1491S.

Received for review March 6, 2008. Revised manuscript received May 28, 2008. Accepted June 3, 2008. This work has been funded by the Ministerio de Educación y Ciencia (Ph.D. Grant BES-2005-7628 and Project AGL 2004-005031) and Comunidad Autonóma de Madrid and Consejo Superior de Investigaciones Científicas (Project 2005570M066 and ALIBIRD-CM-S0505/AGR-0153).

JF800697N