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Changes of isoflavone in soybean cotyledons soaked in different volumes of water

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ABSTRACT

Soybean cotyledons, recently dehulled, were soaked at 50 °C for 12 h as a pre treatment to obtain defatted soy flour enriched in aglycones. Grains of cultivar BRS 213 from the crop years 2004 and 2005 were used and initially had 1.4 and 1.2 mg g^{-1} of total isoflavones, respectively. The molar mass of malonyl and β-glycosides decreased after soaking (33% and 56.5%, in grains from the crop years 2004 and 2005, respectively), while the aglycones daidzein and genistein, that were previously undetectable, increased to 0.5 and 0.8 μmol g^{-1} in grains from the crop years 2004 and 2005, respectively. Cotyledons treated with the reduced volume of water had a reduction of 4% of the total isoflavone molar mass while, for the cotyledons treated with higher volume of water, there was a reduction of 14%, due to the leaching of isoflavones to the soaking water.

1. Introduction

In Brazil, the search for soybean-derived food has increased in consequence of the health benefits attributed to the intake of this legume. Among the functional substances of soybean, the isoflavones are highlighted: a group of twelve compounds that consist of the aglycones daidzein, glycitein and genistein and their β-glucoside conjugates, malonyl and acetyl glucosides (Tsukamoto et al., 1995). According to Setchell (1998), the aglycones constitute a group of substances responsible for several biological activities, acting as anti-cancer compounds, decreasing cholesterol level and reducing loss of bone mass.

The bioavailability of isoflavones is influenced by the manner in which they are consumed. According to Izumi et al. (2000), aglycones are absorbed more rapidly, with better utilisation, than are the malonyl and acetyl conjugates. When consumed, the β-glucosides can be hydrolysed to aglycones by enzymatic action with β-glucosidase activity associated with the intestinal microflora. Nevertheless, according to Setchell (1998), the availability of the enzyme in the intestine is limited and the metabolism of isoflavones can vary among populations and as a function of the diet, medicine intake and time of food residence in the intestine. Due to these factors, the consumption of food with higher levels of aglycone is desirable.

The aglycones are present in the soybean grains in small quantities, varying from 1% to 3% of the total isoflavones (Góes-Favoni, 2002). According to Wang and Murphy (1996) the concentration of total isoflavones in non-fermented food is twice to three times higher than that in fermented food, with the conjugated forms as the main compounds. According to Murphy, Barua, and Hauck (2002), a fermented food, such as tempeh, shows a higher content of aglycones (approximately 50% of the isoflavone molar mass), due to the action of soy endogenous β-glucosidases and the associated β-glucosidase of the fermenting microbes, which promote the hydrolysis of the β-glucoside conjugates, converting them to aglycones.

The concentration of isoflavones in soy-derived food is determined by the cultivar used for processing, dilution with other ingredients and mainly by the processing conditions, especially the temperature to which the material is exposed (Barnes, Kirk, & Coward, 1994; Chien, Hsieh, Kao, & Chen, 2005; Góes-Favoni, Beléia, Carrão-Panizzi, & Mandarino, 2004; Rostagno, Palma, & Barroso, 2005). According to Wang and Murphy (1996), certain methods of processing, such as soaking, fermentation, coagulation and protein precipitation, significantly alter the distribution of the total isoflavone components and provoke loss of isoflavone components to water, serum or residue, although they are not altered in composition. Nevertheless, Chien et al. (2005) observed significant loss of aglycones due to degradation when the isoflavones were exposed to heating at temperatures of 150 and 200 °C, with malonyl conjugates as the compound most susceptible to the action of heat.

Considering the importance of aglycones to health and taking into account that they can be formed by the hydrolysis of their conjugates (Matsuura, Obata, & Fukushima, 1989), some studies have described that hydrothermal treatments were an effective way to increase aglycone levels by the action of β-glucosidase (β-D-glycoside glycohydrolase, EC 3.2.1.21), endogenous in soy

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(Carrão-Panizzi, Góes-Favoni, & Kikuchi, 2004; Góes-Favoni, 2002; Matsuura et al., 1989). During the hydrothermal treatment of soybean grains for different periods (1, 4, 8, 12, 16, 20 and 24 h), Góes-Favoni (2002) observed that the aglycone contents increased from approximately 3% of the total in the grains *in natura* to an average of 57% of the total isoflavones after 24 h of soaking at 50 °C.

Soaking is a common practice in the processing of several soy-based foods, such as tempeh, tofu and soymilk. But, despite the increase in aglycone content observed in these foods, considerable amounts of isoflavones (12 to 57%) are lost in the water during soaking (Kao, Lu, Hsieh, & Chen, 2004; Wang & Murphy, 1996). The leaching of isoflavones to the soaking water is time- and temperature-dependent and increases with the temperature and time of exposure of the soybean grains (Kao et al., 2004). Since, in the majority of processing methods, the soaking water is discarded, procedures that take into account the formation and retention of aglycone in the food, as well as the minimum generation of industrial residues, must be studied.

The objective of this work was to evaluate the transformations of isoflavones that occur during hydrothermal treatment at 50 °C for 12 h in different volumes of water, during the processing of soybean grains, to obtain defatted flour rich in aglycones.

2. Materials and methods

2.1. Materials

Soybean grains, cultivar BRS 213, from crop years 2004 and 2005, harvested in the region of Ponta Grossa, Paraná, Brazil (South Latitude 25°05′, altitude 975 m), were provided by Empresa Brasileira de Pesquisa Agropecuária – Centro Nacional de Pesquisa da Soja (Embrapa Soja). The reagents used in the analyses were of analytical grade or for liquid chromatography. The standards of daidzin, genistin and glycitin were obtained from Sigma Chemicals Co. (St. Louis, EUA).

2.2. Hydrothermal treatment

Soybean grains were submitted to dehulling, with removal of tegument and hypocotyl, leaving only the soybean cotyledons. The cotyledons were soaked in deionised water with two different volumes: grains from crop year 2004 were soaked in the proportion of 1:1.2 (*p*:*p*, soybean:water, just enough to cover the material) and soybean grains from crop year 2005 were soaked in the proportion of 1:3 (*p*:*p*, soybean:water). The cotyledons were maintained at 50 °C for 12 h, followed by draining off and drying in a ventilated oven at 45 °C for 24 h. The analysis of isoflavones in the soaking water used in the treatment of the cotyledons, from crop year 2004, could not be investigated since they were almost completely absorbed. A sample of the soaking water used in the treatment of the cotyledons from crop year 2005 was concentrated in a vacuum centrifuge (Eppendorf Vacufuge Mod. 5417R) at 30 °C to 500 µl, for quantification of the isoflavones (Góes-Favoni, 2002). After drying, the cotyledons were ground and defatted and the flours obtained from the whole soybean grains, and the treated and non-treated cotyledons, had their isoflavone contents analysed and the β-glucosidase activity determined. Milling of the material was with a Perten Laboratory Mill 3100 and defatting was with *n*-hexane at room temperature (24 °C).

2.3. Determination of isoflavones

The extraction was done with 100 mg of the sample added to 4.0 ml of 70% ethanol with 0.1% acetic acid for 1 h at room temperature, according to the methodology described by Carrão-Panizzi, Góes-Favoni, and Kikuchi (2002). The separation and quantification of the isoflavones was done in a High Pressure Liquid Chromatograph (Waters, Milford, USA) equipped with a reverse-phase column (YMC Pack ODS-AM, 5 µm, 250 mm × 4.6 mm ID) and diode array detector (Waters 996) adjusted to 260 nm wavelength, following procedures proposed by Berhow (2002). The mobile phase was a methanol binary linear gradient containing 0.025% of trifluoroacetic acid (TFA) (solvent A) and ultra-pure deionised distilled water containing 0.025% TFA (solvent B) in a flow of 1 ml/min. The initial condition of the gradient was 20% for solvent A, which at 40 min reached the concentration of 100% to immediately return to 20% at 41 min and it remained in this condition for up to 60 min. The software for the control of the equipment and data acquisition was Millennium 32 (version 3.05.01). The isoflavones were identified and quantified by comparing the standard curves genistin, genistein, daidzin, daidzein, glycitin and glycitein (Fig. 1). Concentrations of the malonyl glucosides were calculated from the standard curves of their corresponding β-glucosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and their β-glucosides (Coward, Smith, Kirk, & Barnes, 1998). The results were expressed on a dry basis (mg g^{-1}). The total of isoflavones was expressed as the sum of the compounds after normalisation of the differences of the glycosylated form molecular

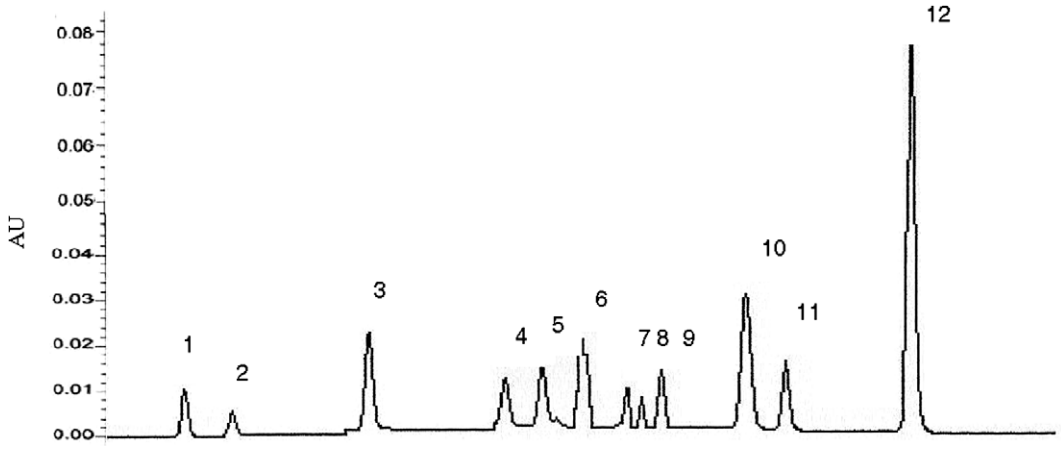

Fig. 1. Mixture of isoflavones standard analysed by high pressure liquid chromatography and diode array detector adjusted to 260 nm wavelength. 1 = daidzin; 2 = glycitin; 3 = genistin; 4 = malonyl daidzin; 5 = malonyl glycitin; 6 = malonyl genistin; 7 = acetyl daidzin; 8 = acetyl glycitin; 9 = acetyl genistin; 10 = daidzein; 11 = glycitein; 12 = genistein.

weights, using the following molecular masses: daidzin = 416.38, malonyl daidzin = 502.43, daidzein = 254.24, genistin = 432.38, malonyl genistin = 518.43, genistein = 270.23, glycitin = 446.41, malonyl glycitin = 532.46, glycitein = 284.27.

2.4. Activity of β-glucosidase

One hundred mg sample of soy flour, and 1.5 ml of 0.05 M citrate buffer (pH 4.5) containing 0.1 M NaCl was maintained for 1 h at room temperature. The samples were centrifuged and the supernatant was kept for enzyme activity analysis. Two millilitres of the substrate 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma Chemical Co., St. Louis, EUA) in phosphate–citrate buffer (0.1 M pH 5.0) were transferred to a test tube and kept in a water bath at 30 °C for 10 min; then 0.5 ml of the supernatant was added and the tube left in the water bath at 30 °C for 30 min. The reaction was stopped with 2.5 ml of 0.05 M sodium carbonate and the contents were immediately measured in a spectrophotometer (Cecil Instruments, England, Mod. 3000) at 420 nm. To determine the β-glucosidase activity in the soaking water 0.5 ml of this water was used as the enzyme solution. The blank solution was composed of 2.5 ml of 0.05 M sodium carbonate solution, 2.0 ml of substrate solution and 0.5 ml of 0.05 M citrate buffer (pH 4.5) containing 0.1 M NaCl. The *para*-nitrophenol (*p*-NP) released by the action of the enzyme was determined by referring to a calibration curve prepared from the *p*-NP (Sigma–Aldrich Co., St Louis, USA) in concentrations that varied from 5 to 300 μM, according to Matsuura and Obata (1993). One activity unit (UA) was defined as the quantity of enzyme necessary to release 1 μmol of *p*-NP per minute under the experimental conditions. Results were expressed as β-glucosidase activity level (UA g^{-1} of sample on dry weight or UA ml^{-1} of the soaking water).

2.5. Statistical analysis

The data were analysed by analysis of variance (ANOVA) and the treatments were compared by the Tukey test with SAS (1999) and SANEST (Zonta, Machado, & Júnior, 1982). The use of ANOVA presupposed that the residues resulting from the adopted model were random and normally distributed, and that the variances of the treatments were homogeneous and that the effects considered in the model, influencing the measured variables, were additive. The graphs of predicted variables versus residues, and the tests of Shapiro and Wilk (1965), Burr and Foster (1972), and Tukey (1949) were used, respectively, to verify these presuppositions.

3. Results and discussion

In the whole soybean, the content of total isoflavones was 1.4 and 1.2 mg g^{-1}, in the crop years 2004 and 2005, respectively (Table 1). The difference in concentrations observed between the crop years probably occurred due to differences in environmental temperature during the growing season: in the crop year 2004 the average temperature registered in the region of Ponta Grossa was 20.4 °C while, in the crop year 2005, it was 21.7 °C. According to Tsukamoto et al. (1995), the total isoflavone concentration in soybean is genetically determined and influenced by environmental conditions, with the temperature as the main factor. Carrão-Panizzi, Kitamura, Beléia, and Oliveira (1998) analysed soybean sowed in regions with different average temperatures. At 20 °C average temperature for the growing season, the isoflavone concentrations were 1.5 mg g^{-1} (IAS 5) and 0.6 mg g^{-1} (BR 36) and, in regions with an average temperature of 23 °C, the concentrations were lower: 1.2 and 0.5 mg g^{-1}, respectively. Eldridge and Kwolek (1983) detected differences in the total isoflavone content, varying from 2.4 to 3.6 mg g^{-1} when the Clark cultivar was sowed in Urbana, IL, USA, in the years 1975, 1976, 1978 and 1979, demonstrating the influence of the climatic and environmental conditions on the isoflavones.

Table 1
Total isoflavones content (mg g^{-1}) in soy flour defatted rich in aglycones processing.[a,b]

	Total Isoflavones (mg g^{-1})	
	2004 (1:1,2, soybean:water)	2005 (1:3, soybean:water)
Whole soybean grain	1.4 aA ± 0.011	1.2 aB ± 0.004
Cotyledons	1.2 bA ± 0.009	1.0 bB ± 0.013
Ground cotyledons	1.2 bA ± 0.009	1.0 bB ± 0.013
Defatted ground cotyledons	1.2 bA ± 0.007	1.0 bB ± 0.004
Treated cotyledons	0.97 dA ± 0.009	0.68 cB ± 0.006
Defatted treated cotyledons	0.98 cA ± 0.006	0.68 cB ± 0.002

[a] Averages (±standard deviation) of three repetitions on dry basis.
[b] Averages followed by the same small letters in the columns and capital letters in the lines do not differ among them (Tukey $p \leqslant 0.05$).

After the removal of the soybean tegument, the content of isoflavones decreased to 1.2 and 1.0 mg g^{-1} for the crop years 2004 and 2005, with reductions of 14% and 17%, respectively, due to the elimination of the hypocotyl (Table 1). According to Tsukamoto et al. (1995), isoflavones are not present in the seed teguments and 10–20% of the total isoflavones from the soy grain is concentrated in the hypocotyl.

Parts of the cotyledons from crop years 2004 and 2005, obtained after the dehulling, were submitted to grinding and defatting with *n*-hexane at room temperature (24 °C). The isoflavones content was not modified, remaining at 1.2 and 1.0 mg g^{-1} in the cotyledons of the crop years 2004 and 2005, respectively (Table 1). According to Coward et al. (1998), milling and oil extraction at room temperature do not alter the quantitative or qualitative profiles of the isoflavones.

After the hydrothermal treatment, the cotyledons were dried, ground and defatted, when the content of total isoflavones reached values of 0.98 and 0.68 mg g^{-1} in the samples from the crop years 2004 and 2005, respectively (Table 1). This decrease of 18% and 32% in the content of total isoflavones probably occurred due to the loss of isoflavones to the soaking water that was higher for the cotyledons treated with the higher water volume. The chromatograms of the whole soybean, and defatted treated cotyledon, and water from the treatment of cotyledons are shown in Fig. 2.

The analysis of the water from the treatment of cotyledons of the 2005 crop showed 0.12 mg g^{-1} of total isoflavones (Table 3), corresponding to the loss of 12% isoflavones originally present in the cotyledons. Grün et al. (2001), evaluating the influence of processing on the isoflavones, observed a decrease from 15% to 28% when the tofu was submitted to cooking in water with different temperatures (80, 90 and 100 °C) and for different times (10, 20, 30 and 40 min) and concluded that the decrease was due to the isoflavones leaching to the water and that the losses increased with increases in time and temperature of cooking. According to Wang and Murphy (1996), elevation of the temperature during soaking provokes an increase in the speed of leaching of the isoflavones, especially if the grains are dehulled.

The malonyl and β-glucoside forms corresponded to 96% and 91% of the molar mass of isoflavones in the soybean grains from the crop years 2004 and 2005, with the conjugated malonyl being the main compound detected: 1.8 and 1.6 μmol g^{-1}, corresponding to 62% and 61%, respectively (Tables 2 and 3). Murphy et al. (2002), when testing different solvents for the extraction of isoflavones in

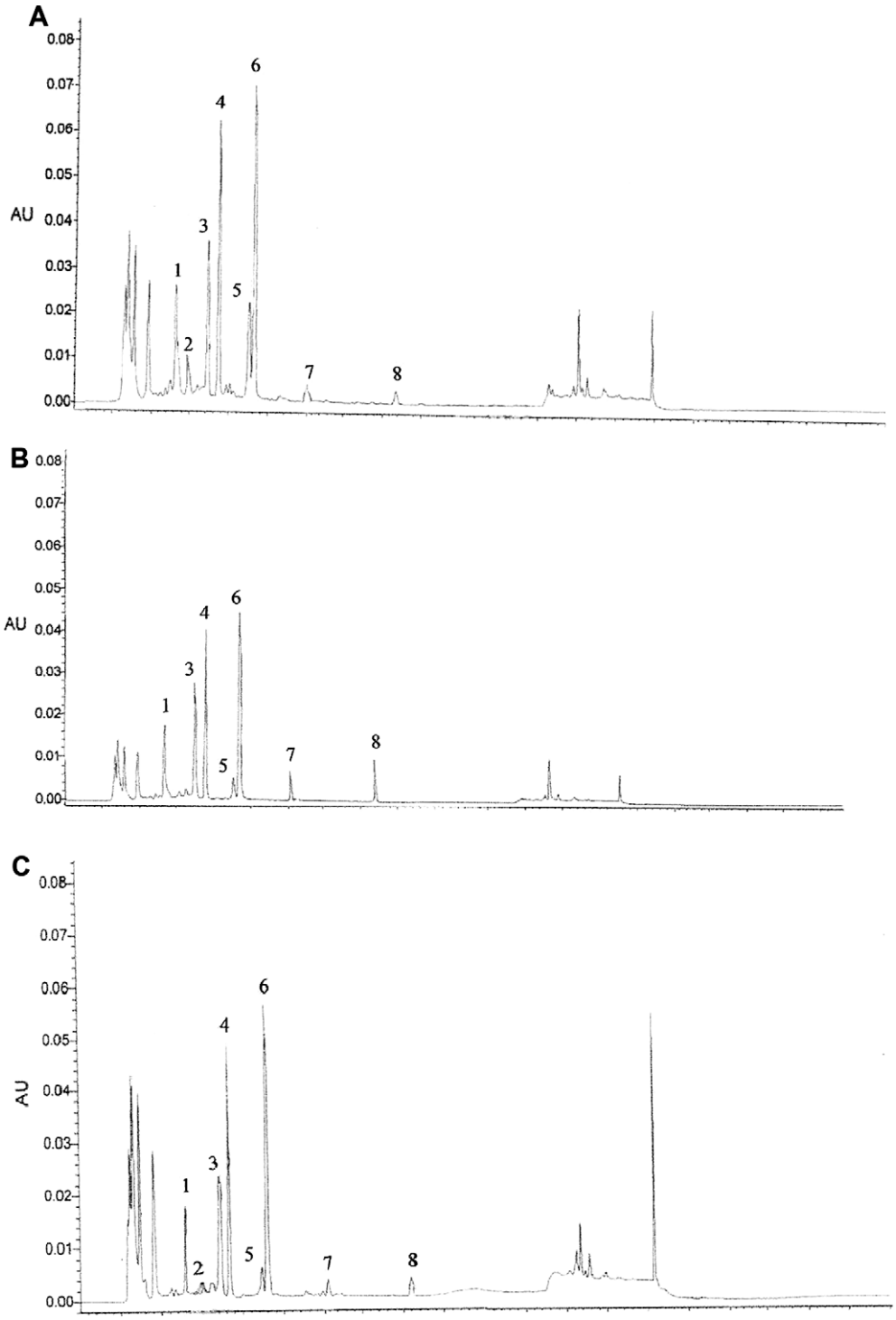

Fig. 2. Chromatograms of isoflavone in the whole soybean: A (2004); C (2005), and isoflavones in the defatted treated cotyledon: B (2004); D (2005), and isoflavones in the water from the treatment of cotyledons: E (2005). 1 = daidzin; 2 = glycitin; 3 = genistin; 4 = malonyl daidzin; 5 = malonyl glycitin; 6 = malonyl genistin; 7 = daidzein; 8 = genistein.

soy-derived food observed that, in soy flour, the malonyl conjugates and β-glucosides corresponded to 90% of the isoflavone molar mass, when extracted with ethanol.

In the cotyledons soaked at 50 °C for 12 h, the concentration of the malonyl conjugate decreased to 1.1 μmol g^{-1} (2004), treated with a reduced volume of water, and to 0.7 μmol g^{-1} (2005), treated with a higher volume of water (Tables 2 and 3). The reduction of the malonyl conjugates occurred, in part, due to their transformation into β-glucosides, since, according to Barnes et al. (1994), the malonyl compounds are thermally unstable and their ester bonds can be cleaved to form β-glucosides. These authors conducted experiments for the extraction of isoflavones in different soy-derived foods and observed that the conversion of malonyl conjugates into β-glucosides was time- and temperature-dependent. When the extraction of isoflavones of aqueous soy extract was done at 60 °C for 4 h, the content of malonyl genistin was 9%

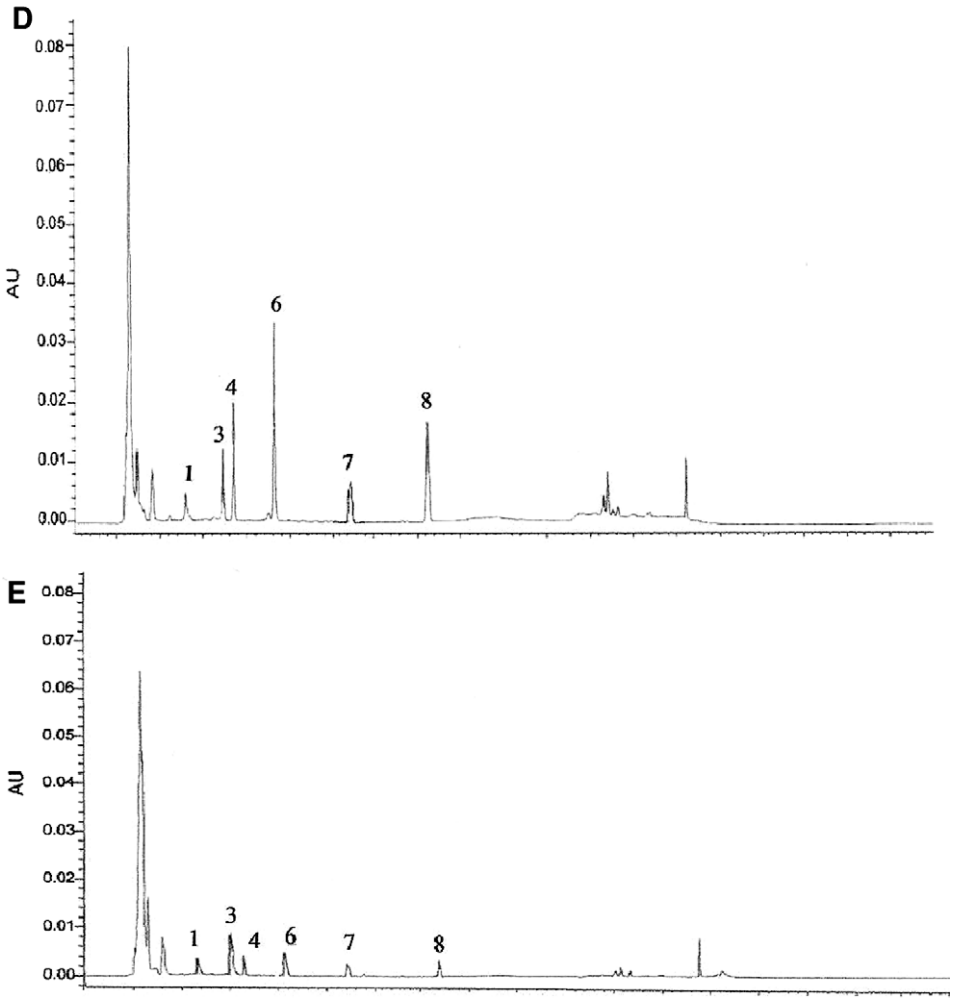

Fig. 2 (*continued*)

Table 2
Isoflavones content in grains from cultivar BRS 213 crop year 2004, during processing of defatted soy flour rich in aglycones, by soaking with a reduced volume of water (1:1,2, *p*:*p*, soybean:water).[a,b]

	β-Glycosides (mg g^{-1})			Malonyl (mg g^{-1})			Aglycones (mg g^{-1})			Total[c] (μmol g^{-1})
	Din	Gly	Gin	Din	Gly	Gin	Dein	Glein	Gein	
Whole soybean grain	0.15 b ± 0.010	0.08 a ± 0.004	0.18 b ± 0.002	0.39 b ± 0.002	0.13 a ± 0.004	0.42 b ± 0.004	0.01 c ± 0.00	ND	0.01 b ± 0.00	2.9
Cotyledons	0.18 a ± 0.003	ND[d]	0.20 a ± 0.004	0.40 a ± 0.008	ND	0.42 b ± 0.002	ND	ND	ND	2.4
Ground cotyledons	0.17 a ± 0.003	ND	0.20 a ± 0.002	0.39 b ± 0.003	ND	0.44 a ± 0.002	ND	ND	ND	2.4
Defatted ground cotyledons	0.17 a ± 0.003	ND	0.20 a ± 0.002	0.39 b ± 0.003	ND	0.44 a ± 0.002	0.01 c ± 0.002	ND	ND	2.5
Treated cotyledons	0.11 d ± 0.001	ND	0.18 b ± 0.002	0.25 c ± 0.005	ND	0.30 c ± 0.002	0.05 b ± 0.00	ND	0.08 a ± 0.001	2.3
Defatted treated cotyledons	0.12 c ± 0.003	ND	0.17 c ± 0.002	0.26 c ± 0.002	ND	0.29 d ± 0.003	0.06 a ± 0.00	ND	0.08 a ± 0.00	2.3

Din = daidzin; Gly = glycitin; Gin = genistin; Dein = daidzein; Glein = glycitein; Gein = genistein.
[a] Averages (±standard deviation) of three repetitions on dry basis.
[b] Averages followed by the same small letters in the columns do not differ among them (Tukey $p \leqslant 0.05$).
[c] Total express the sum of the conjugates after the difference of molecular weight of the glycosylated forms is normalised.
[d] ND = non detected.

lower than when they were extracted for 24 h at 22 °C. Toda, Sakamoto, Takayanagi, and Yokotsuka (2001) maintained whole soybean grains soaking for 20 h at 15 °C and did not observe significant alterations of the malonyl conjugates, attributing this fact to the low temperature used.

According to Chien et al. (2005), the heating treatment used during the processing of the soybean grains has a significant influence on the compounds' conversions. When the standard malonyl genistin (50 μg ml^{-1}) was submitted to dry heating (150 °C) for 15 min, acetyl genistin and genistin were formed, increasing to

Table 3
Isoflavones content in grains from cultivar BRS 213 crop year 2005, during processing of defatted soy flour rich in aglycones, by soaking with a higher volume of water (1:3, *p*:*p*, soybean:water).[a,b]

	β-Glycosides (mg g^{-1})			Malonil (mg g^{-1})			Aglycones (mg g^{-1})			Total[c] (μmol g^{-1})
	Din	Gly	Gin	Din	Gly	Gin	Dein	Glein	Gein	
Whole soybean grain	0.13 a ± 0.008	0.05 a ± 0.001	0.16 a ± 0.009	0.34 a ± 0.003	0.08 a ± 0.007	0.40 a ± 0.005	0.03 c ± 0.002	ND	0.03 b ± 0.002	2.6
Cotyledons	0.13 a ± 0.002	ND[d]	0.15 b ± 0.003	0.34 a ± 0.006	ND	0.37 b ± 0.002	0.01 d ± 0.001	ND	0.01 c ± 0.001	2.1
Ground cotyledons	0.13 a ± 0.002	ND	0.15 b ± 0.003	0.34 a ± 0.006	ND	0.37 b ± 0.003	0.01 d ± 0.001	ND	0.01 c ± 0.001	2.1
Defatted ground cotyledons	0.11 b ± 0.001	ND	0.17 a ± 0.006	0.34 a ± 0.003	ND	0.37 b ± 0.003	0.01 d ± 0.001	ND	0.01 c ± 0.002	2.1
Treated cotyledons	0.03 c ± 0.003	ND	0.09 c ± 0.001	0.15 b ± 0.002	ND	0.21 c ± 0.002	0.07 b ± 0.001	ND	0.13 a ± 0.004	1.7
Defatted treated cotyledons	0.03 c ± 0.001	ND	0.09 c ± 0.00	0.14 b ± 0.002	ND	0.21 c ± 0.001	0.08 a ± 0.001	ND	0.13 a ± 0.00	1.8
Soaking water	0.01	ND	0.04	0.01	ND	0.03	0.01	ND	0.02	0.3

Din = daidzin; Gly = glycitin; Gin = genistin; Dein = daidzein; Glein = glycitein; Gein = genistein.
[a] Averages (±standard deviation) of three repetitions on dry basis in the flours and average (±standard deviation) of two repetitions in the soaking water.
[b] Averages followed by the same small letters in the columns do not differ among them (Tukey $p \leqslant 0.05$).
[c] Total expressed as the sum of the conjugates after the difference of molecular weight of the glycosylated forms is normalised.
[d] ND = non detected.

12.6 and 7.6 μg ml^{-1}, respectively. Nevertheless, under wet heating at 150 °C, more genistin (15.0 μg ml^{-1}) was formed than acetyl genistin (7.8 μg ml^{-1}). Rostagno et al. (2005), studied the stability of the isoflavones at 25 and 40 °C, and observed that the conversion rate of malonyl genistin, malonyl daidzin and malonyl glycitin into their β-glucosides was twice higher when stored for seven days at 40 °C (0.236, 0.038 and 0.021 μmol h^{-1}, respectively) than at 25 °C (0.117, 0.018 and 0.010 μmol h^{-1}, respectively). There were corresponding increases of genistin, daidzin and glycitin, while the content of total isoflavones remained unaltered, proving that the decrease in the malonyl conjugates content, under the evaluated conditions, occurred exclusively due to their conversion to β-glucosides.

Góes-Favoni et al. (2004) analysed different commercially available soy-based foods and observed that soy flours obtained from the same cultivar and from the same environmental conditions during the development of the grains, but submitted to thermal treatment during processing, had lower content of malonyl conjugates (0.8 mg g^{-1}) than had those of flour without thermal treatment (1.5 mg g^{-1}), while the β-glucoside contents and aglycone increased.

Considering the thermal instability of the malonyl conjugates, the content of β-glucosides should increase during the hydrothermal treatment. Nevertheless, a decrease in the amount of these compounds was observed from 0.9 to 0.7 μmol g^{-1} (2004), upon treatment with a reduced volume of water, and from 0.7 to 0.3 μmol g^{-1} (2005), upon treatment with a higher water volume (Tables 2 and 3). This reduction of β-glucosides occurred, in part, due to its conversion into aglycones in a reaction catalysed by β-glucosidase endogenous in the soy. Another fact that contributed to the decrease of these compounds after soaking was their leaching to the water, comprising 5% of total isoflavones originally contained in the soy cotyledons from crop year 2005 (Table 3). According to Matsuura et al. (1989), soy endogenous β-glucosidases, under ideal conditions of temperature and pH (50 °C, pH 5.5–6.0) are capable of hydrolysing the glycosidic bond, liberating glucose and aglycones. Kao et al. (2004), analysing the effect of soaking conditions of whole soybean, observed that β-glucosides decreased with increase in soaking time (0, 4, 8 and 12 h) and temperature of soaking (25, 35 and 45 °C), decreasing from 1.2 to 0.1 mg g^{-1} after 12 h at 45 °C, while the content of aglycone increased from 0.3 to 1.6 mg g^{-1}.

The aglycones, that initially were 0.02 and 0.06 mg g^{-1} in the whole soybean grains and corresponded to 2.4% and 8.5% of the molar mass of total isoflavones in the grains from crop years 2004 and 2005, respectively, were reduced after the dehulling. Aglycones disappeared from the cotyledons (2004) and were reduced to 3% of the total isoflavone molar mass in the cotyledons from the crop year 2005 (Tables 2 and 3).

Aglycones that were not detectable (2004) increased to 0.5 μmol g^{-1} and increased from 0.1 to 0.8 μmol g^{-1} (2005) after the hydrothermal treatment. This increase of aglycones is probably due to β-glucosidase since, even with the evidences that the malonyl conjugates can be converted directly to aglycone (Chien et al., 2005), this occurs at elevated temperatures (200 °C). Toda et al. (2001), observed an increase of aglycones from 4.0% to 13.1% of the total isoflavones in soybean (cultivar Tamahomare) soaked for 20 h at 15 °C. These authors also observed a decrease of β-glucosides from 34.2% to 27.5% of the total isoflavones and, although soy β-glucosidases show maximum activity at 50 °C (Matsuura et al., 1989), the alterations were attributed to enzymatic hydrolysis. Murphy et al. (2002), analysing different soy-based foods observed that, in tofu (product obtained from the coagulation of aqueous soy extract), the aglycones corresponded to 37% of the total molar mass of isoflavones, while in soy flours the aglycones corresponded to only 1.6%. The higher content of aglycones in tofu is attributed to the action of soy endogenous β-glucosidases activated during the soaking of the grains to make soy extract (Kao et al., 2004). Nevertheless, the soaking and coagulation steps during the processing of the tofu provoked loss of isoflavones to the water and serum, which made the content of total isoflavones lower in tofu than in soy flours (Wang & Murphy, 1996). Nowadays the ingestion of 25 g of soy proteins per day associated with 30–50 mg of isoflavones, which are capable of decreasing the serum cholesterol (Setchell, 1998), is recommended. Considering that treated and defatted soy flours had, on average, 46% of proteins (data not shown), the daily consumption of 54 g of these flours would contribute 25 g of proteins and 53 mg of total isoflavones (2004) and 37 mg if from year 2005.

In the cotyledons of the crop year 2004, the isoflavones total molar mass decreased by 4% after soaking with a reduced volume of water and varied from 2.4 to 2.3 μmol g^{-1}, since almost all of the water added for the soaking formed a continuous phase with the cotyledons and the isoflavones remained in the tissue after drying. In the cotyledons from the crop year 2005 the decrease after soaking with a higher volume of water was 14%, from 2.1 to 1.8 μmol g^{-1}. The soaking water (2005) contained 14% of the total

Table 4
β-Glycosidase activity levels in defatted cotyledons, defatted soaked cotyledons and in the soaking water from the treatment of cultivar BRS 213 with a reduced volume of water (crop year 2004) and with a greater volume of water (crop year 2005).[a,b]

	β-Glycosidase activity	
	2004 (1:1,2, soybean:water)	2005 (1:3, soybean:water)
Defatted Cotyledons (UA g^{-1})	78.3 aA ± 2.4	76.1 aB ± 3.0
Defatted Treated Cotyledons (UA g^{-1})	24.7 bA ± 1.3	14.3 bB ± 2.3
Soaking water (UA ml^{-1})	7.0 cB ± 0.13	10.6 cA ± 0.24

[a] Averages (±standard deviation) of three repetitions.
[b] Averages followed by the same small letters in the columns and capital letters in the lines do not differ among them (Tukey $p \leqslant 0.05$).

molar mass (0.3 μmol g^{-1}) of isoflavones originally in the soy cotyledons, with 42% β-glucosides, 33% malonyl conjugates and 25% aglycones (Table 3). Góes-Favoni (2002), when analysing the soaking water in which whole soybean grains were maintained for 24 h at 50 °C, observed that around 45% of the total isoflavones originally in the soy grains migrated to the water. During the processing of tempeh, Wang and Murphy (1996) observed losses of 12% and 49% of isoflavones to the water during soaking and cooking, respectively, and observed insignificant quantities of isoflavones in the okara (insoluble residue), suggesting that isoflavones can be associated with soluble components (probably soy soluble proteins).

Since more conjugates migrated from the cotyledons to the soaking water when treated with a higher volume of water, a smaller quantity of these compounds remained in the cotyledons to be substrate of the β-glucosidase, thus justifying the higher increase in aglycones (2004) treated with a reduced volume of water (Tables 2 and 3). Besides, part of the formed aglycones also migrated to the soaking water (Table 3).

Another fact that contributed to the lower formation of aglycones in the cotyledons of the crop year 2005, was the migration of the β-glucosidase to the soaking water. The activities of the β-glucosidases were 78.3 and 76.1 UA g^{-1} in the defatted cotyledon flours from the crop years 2004 and 2005, respectively. After soaking for 12 h at 50 °C and defatting, the activities of the enzyme were 24.7 UA g^{-1} (2004), treated with a reduced volume of water, and 14.3 UA g^{-1} (2005) treated with a higher volume of water, decreasing 68% and 81%, respectively. The soaking water had 7.0 and 10.6 UA ml^{-1} from crop years 2004 and 2005, respectively (Table 4). Góes-Favoni, Beléia, and Carrão-Panizzi (2004) observed a decrease of 80% on the average, in the activity of β-glucosidase after soybean grains were treated (IAS 5) at 50 °C for 12 h, while Toda et al. (2001) observed that the activity of β-glucosidase in the water of hydration increased with the time of soaking at 15 °C for 5, 10, 15 and 20 h, evidencing the solubility of the enzyme.

4. Conclusion

Grinding and defatting of soybean grains, when done at room temperature, did not alter the profile or concentrations of isoflavones. Nevertheless, dehulling, due to the elimination of the hypocotyl, provoked a significant reduction in the content of isoflavones. The hydrothermal treatment at 50 °C for 12 h resulted in a reduction in the content of malonyl conjugates due to their conversion to β-glucosides and to leaching to the soaking water. β-Glucosides that also migrated to the water markedly decreased because of the hydrolysis by the soy endogenous β-glucosidases in the cotyledons, originating the aglycones. The hydrothermal treatment, done with a reduced volume of water, provided higher amounts of aglycones as a result of the lower migration of isoflavones and β-glucosidase to the soaking water. The lower amount of water makes the industrial processing feasible since lower amounts of residues will be generated. Considering the recommended isoflavones intake (30–50 mg/day), the hydrothermal treatment at 50 °C for 12 h is efficient in the formation of aglycones and, when done with a reduced volume of water, makes this feasible for the development of soy flour enriched in aglycones, with minimum generation of industrial residues.

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