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Influence of temperature, pH and ionic strength on the production of isoflavone-rich soy protein isolates

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Abstract

Soy protein isolates (SPI) may present different isoflavone profiles and contents, depending on processing conditions. In the present work, seven different SPI, resulting from changes in the processing steps, were obtained. The best parameters for obtaining isoflavone-richer SPI were: extraction at pH 9 and temperature of 55 °C, acid precipitation performed at pH 4.5, acid-washing of the precipitate and mild centrifugation conditions for the separation of acid-precipitated proteins. Isoflavones were soluble in aqueous solution in the pH range 2–10 (73–93% of the amount solubilized in 80% methanol). The profile of isoflavones was dependent on the temperature used for the aqueous extraction. Temperatures below 50 °C caused hydrolysis of β-glucosides with increase of aglucones, by endogenous β-glucosidase activity.

Keywords: Isoflavones; Soy protein isolate; Processing

1. Introduction

Soy protein isolates (SPI) are used as ingredients in several food products; beside the nutritional and functional properties, there are some possible beneficial effects associated with their consumption. Crouse et al. (1999) demonstrated that SPI had a cholesterol-lowering effect in humans, proportional to the isoflavone content.

Isoflavones represent the most common group of phytoestrogens and are found, at particularly high levels, in soybeans. They have been associated with beneficial effects in humans, such as prevention of cancer, cardiovascular diseases, osteoporosis, and menopausal symptoms (Adlercreutz & Mazur, 1997).

Soybean contains three types of isoflavones, in four chemical forms: the aglucones daidzein, genistein, and glycitein; the β-glucosides daidzin, genistin, and glycitin, the acetyl-β-glucosides, 6″-*O*-acetyl-β-daidzin, 6″-*O*-acetyl-β-genistin, 6″-*O*-acetyl-β-glycitin and the malonyl-β-glucosides, 6″-*O*-maloyl-β-daidzin, 6″-*O*-malonyl-β-glycitin and 6″-*O*-malonyl-β-glycitin (Kudou et al., 1991). Isoflavone content depends on the variety and environmental conditions (Genovese, Hassimotto, & Lajolo, 2005; Wang, Sherrard, Pagadala, Wixon, & Scott, 2000; Wang & Murphy, 1994a).

The differences in the chemical structure of isoflavones may influence the biological activity, bioavailability and, as a result, the physiological effects of these constituents (Setchell & Cassidy, 1999). Daidzein was found to have higher bioavailability than genistein in adult women (Xu, Wang, Murphy, Cook, & Hendrich, 1994). In relation to the isoflavone form, aglucones or glucosides, there is no general consensus about the effect on bioavailability. According to Izumi et al. (2000), aglucone isoflavones were absorbed faster and in higher amounts than their glucosides in the human, but exactly

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the contrary was reported by Setchell et al. (2001). On the other hand, no differences were found by Xu, Wang, Murphy, and Hendrich (2000) or Zubik and Meydani (2003).

The conditions of soy processing may cause changes in the contents and profiles of isoflavones. Important losses are observed during soaking, heating, filtration, and alkaline extraction, used in soy products production (Jackson et al., 2002; Rickert, Meyer, Hu, & Murphy, 2004a; Wang & Murphy, 1994b). Storage was also found to alter isoflavone profiles of soy products (Pinto, Lajolo, & Genovese, 2005).

Most isoflavones in soy and soy protein products, such as defatted flour, isolate, concentrate and textured protein are found in the esterified forms (97–98%). The distribution among these forms, however, varies from product to product (Wang & Murphy, 1994b). Malonyl-β-glucosides predominate in raw unprocessed soybeans and can be degraded into acetyl-β-glucosides by dry heat treatments, such as toasting and extrusion. Most commonly, the unstable malonyl-β-glucosides are directly transformed into β-glucosides and aglucones by thermal and, enzymatic action, respectively (Coward, Smith, Kirk, & Barnes, 1998). The biological significance of the presence of these different forms in soybeans is not yet clear.

The objective of this work was to determine how soy protein isolate processing steps influence isoflavone retention and profile in the final product, aiming at the production of soy protein isolates richer in isoflavones. This could allow the development of functional food product with a higher content of these biologically active substances.

2. Materials and methods

2.1. Materials

The defatted soy flour (DSF) was supplied by Bunge Alimentos S.A. (Esteio, RS, Brazil), in 2003, and stored at −18 °C until used. All chemicals and solvent were reagent or HPLC grade.

2.2. Methods

2.2.1. Protein extraction

DSF was extracted with a mechanical stirrer in aqueous solution, and the parameters assessed were: pH (2–10), water-to-flour ratio (10:1, 20:1 and 30:1), ionic strength (0.01–1.0 M NaCl), time (1 and 3 h), and temperature (4–50 °C) extraction. The slurries obtained were centrifuged at 12,000*g* for 20 min in Sorvall Superspeed Fixed-Angle Rotors centrifuge (Newtown, CT, USA). The extracts obtained were sampled for protein content and isoflavone determination as follow. Extraction efficiency was calculated in relation to the total protein and isoflavone contents.

2.2.2. Protien content

The protein content ($N \times 6.25$) was determined in triplicate by the micro Kjeldahl method 960.52 AOAC (Association of Official Analytical Chemists, 1995).

2.2.3. Solid-phase extraction (SPE)

Aliquots (10 ml) of the different extracts obtained from DSF (see above) were passed through polyamide CC 6 (Macherey-Nagel GmbH and Co, Germany) columns (1 g/6 ml), previously conditioned with 20 ml of methanol and 60 ml of water. The columns were washed with water (20 ml) and retained isoflavones were eluted with 50 ml of 99.5:0.5 methanol/ammonia (Genovese & Lajolo, 2001; Price, Prosser, Richetin, & Rhodes, 1999). 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 rotatory evaporator (Rotavapor® 120, Büchi, Flawil, Sweden) at ⩽40 °C to a volume of 0.2–0.4 ml, and then the volume was adjusted to 2 ml with HPLC grade methanol. Aliquots of the sample were filtered through a 0.22 μm PTFE (polytetrafluoroethylene) filiter unit (Millipore Ltd., Bedford, MA) and analysed by HPLC.

2.2.4. Isoflavone extraction

Powdered samples (1 g) of the DSF and freeze-dried soy protein isolate (SPI) were extracted with 80% aqueous methanol (20:1 v/w) under agitation for 2 h at 4 °C, according to Genovese and Lajolo (2001, 2002). The homogenate was filtered through filter paper Whatman No. 06 and concentrated until methanol elimination was completed on a rotatory evaporator at ⩽40 °C. The volume of the extracts was adjusted to 5 ml with HPLC grade methanol, and aliquots were filtered through a 0.22 μm PTFE filter unit and analysed by HPLC.

2.2.5. HPLC quantitation of isoflavones

Isoflavone quantitation was performed according to Song, Barua, Buseman, and Murphy (1998) with a C18 NovaPak (30 cm × 4.6 mm i.d.) column (Waters, Milford, MA) and a Hewlett–Packard 1100 system with autosampler and quaternary pump coupled to a diode array detector (Palo Alto, CA), based on external calibration. Standards of daidzein and genistein were from Sigma Chemicals Co. (St. Louis, MO), daidzin and genistein were from Apin Chemicals Ltd. (Abingdon, UK), and glycitin and glycitein were from Nacalai-Tesque Inc. (Kyoto, Japan). Concentrations of malonyl and acetyl-β-glucosides were calculated using standard curves for the respective β-glucosides, adjusting for differences in molecular weight. Total isoflavone contents were expressed as μmol/g of sample.

2.2.6. Solubility of the isoflavones

Solubility of the isoflavones extracted from the DSF (20:1 water-to-flour ratio, at 4 °C for 1 h) was determined after partial purification of the extract through SPE. Ten ml of the aqueous extract were passed through a 1 g polyamide column previously conditioned with 20 m1, of methanol and 60 ml of water. The columns were washed with water (20 ml) and retained isoflavones were eluted with 50 ml 99.5:0.5 methanol/ammonia (Genovese & Lajolo, 2001, 2002; Price et al., 1999). The effluents were evaporated on a rotatory evaporator to a volume of 2 ml, and then the volume was adjusted to 5 ml with water. The samples, with the pH adjusted to values in the pH range 2–10, were allowed to stand for 30 min, and then filtered through a 0.22 μm PTFE (polytetrafluoroethylene) filter unit (Millipore Ltd., Bedford, MA). The volume was then completed to 10 ml with HPLC-grade methanol and the samples were analysed by HPLC.

2.2.7. Endogenous β-glucosidase

β-Glucosidase activity was determined according to Matsuura, Sasaki, and Murao (1995), using, as a synthetic substrate, *p*-nitrophenyl-β-D-glucoside (*p*-NPG), Sigma Chemicals Co. (St. Louis, EUA). DSF (2 g) was incubated in 40 ml of 0.5 mM *p*-NPG in 0.1 M phosphate–citrate buffer, pH 5, under agitation, at two different temperatures: at 45 °C, the optimal temperature for the enzyme, and at 60 °C, the temperature of inactivation of the enzyme. Aliquots of 1 ml of the suspension were collected at time intervals of 0, l, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min and transferred to vials containing 1 ml of 0.625 M sodium carbonate solution and 0.25 mL of 0.1 M phosphate–citrate buffer, pH 5. The resulting yellow colour, was immediately measured at 420 nm with a spectrophotometer, model Ultrospec 2000 UV/Vis (Amersham Biosciences, Cambridge, UK). The hydrolyzed *p*-nitrophenol was determined by referring to a calibration curve prepared with 10–100 μl of *p*-nitrophenol.

In parallel, DSF was incubated for 3 h, at a 20:1 water-to-flour ratio, in the presence or absence of the substrate *p*-NPG. The slurry was centrifuged at 12,000*g* for 20 min (25 °C), and the supernatant was sampled for isoflavone determination.

2.2.8. Soy protein isolate (SPI) production

The DSF (50 g) was extracted at a 20:1 water-to flour ratio with a magnetic stirrer for 1 h, and the temperature and pH of extraction were varied according to Table 1. The slurry was centrifuged at 16000*g*, for 20 min (25 °C) and the supernatant (total extract) was sampled for protein content and isoflavone determination. The clarified extract was adjusted to pH 4.5 with HCl 6 N. The precipitate was allowed to stand for 30 min at room temperature and was centrifuged in the conditions specified in Table 1. The precipitate was washed with acidified water (pH 4.5) for 5 min under agitation, and centrifuged in the same condition as above. The supernatant obtained in pH 4.5 (whey) and the washing water were sampled for protein content and isoflavone determination. The precipitate was freeze-dried and analysed for protein content and isoflavone. The extraction and analysis were carried out in triplicate. Seven soy protein isolates (SPI) were prepared (I–VII), and the parameters modified for each of show in Table 1.

2.2.9. Statistical analysis

All analyses were run in triplicate. Isoflavone and protein concentrations were expressed as means ± standard derivation (SD). Statistical analysis was done by using the Statistic software package version 5.0 (StatSoft, Inc., Tulsa, OK). Differences between means were first analysed by ANOVA test and then least significant difference (LSD) test ($p < 0.05$).

3. Results and discussion

3.1. General

Three water-to-flour ratios (10:1, 20:1, and 30:1) were tested for protein yield during water extraction (pH 7 and 4 °C) of proteins from DSF. There were no differences among the three ratios tested and the protein yield was around 65% of the total content of the DSF.

Table 1
Parameters modified during soy protein isolates production

	Extraction conditions		Precipitation pH	Centrifugation conditions
	pH	Temperature (°C)		
IPS I	7	26	4.5	16,000*g*/20 min
IPS II	7	24	4.0	16,000*g*/20 min
IPS III	7	22	4.5	16,000*g*/20 min
IPS IV	7	26	4.5	162*g*/2 min
IPS V	7	24	4.5	162*g*/1 min
IPS VI	9	24	4.5	162*g*/2 min
IPS VII	9	55	4.5	162*g*/2 min

Further extractions were then performed using a 20:1 water-to-flour ratio.

3.2. Effect of time and temperature on extraction efficiency of isoflavones and proteins in water

Times of extraction of 1 and 3 h were tested and it was observed that regardless the temperature used (4, 25 or 50 °C), the increase of time did not result in a higher yield of proteins (Fig. 1). However, the increase of temperature provided an increase in the efficiency of the extraction of proteins, of 64–88% of the total content of the DSF, at 4 and 50 °C, respectively.

Next, using the time of 1 h of extraction, the effect of the temperature on the extraction of isoflavones in aqueous medium was determined. Contrary to what was observed for proteins, there was no significant difference among the three temperatures, with a mean yield of about 76% of the total amount of isoflavones of DSF extracted.

Since the temperature of extraction did not interfere with the yield of isoflavones, the time and temperature of extraction used for further tests were 1 h and 4 °C, respectively, because room temperature may vary significantly and the temperature of 50 °C led to a significant change in the profile of isoflavones extracted, as shown later.

3.3. Effects of ionic strength on extraction efficiency of isoflavones and proteins

Solutions of concentrations of NaCl at concentrations of 0.01, 0.1, 0.2, 0.5 and 1.0 M were used for the aqueous extraction of the DSF. Fig. 2 shows that ionic strengths of 0.1 and 0.2 M caused a decrease in the extraction of proteins and isoflavones, and increase or decrease of the ionic strength beyond this range resulted in an increase of extraction of both proteins and isoflavones, reaching a maximum at about 0.5 M. However, at this ionic strength, the yield of isoflavones was lower than in water, and the efficiency of acid precipitation was very reduced (Hermansson, 1978).

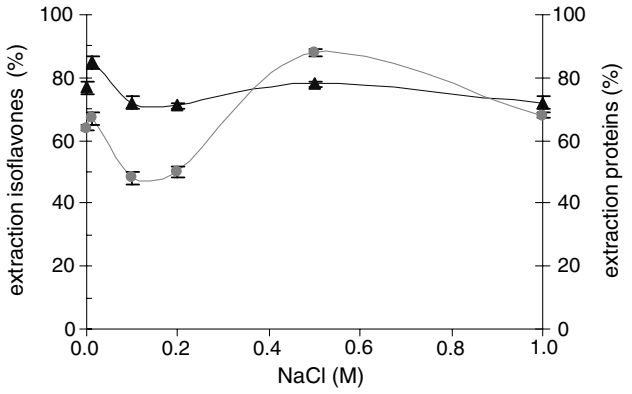

Fig. 2. Effect of ionic strength on the extraction of proteins (●) and isoflavones (▲) from the defatted soy flour. Extraction efficiency was calculated in relation to total protein content ($N \times 6.25$) and total isoflavone content extracted by means of 80% methanol.

3.4. Effects of pH on extraction efficiency of isoflavones and proteins

The range of pH tested was 2–10, since values beyond this range may cause hydrolytic reactions in proteins. Fig. 3 shows that the curve of isoflavone extraction followed that for proteins. In the region close to the isoelectric pH of soy proteins (p$I \sim 4.5$) there was a drastic decrease in the efficiency of protein extraction and the curve of isoflavones followed this, drop. Values over or under the pI resulted in higher efficiency of protein and isoflavone extraction. However, although val-

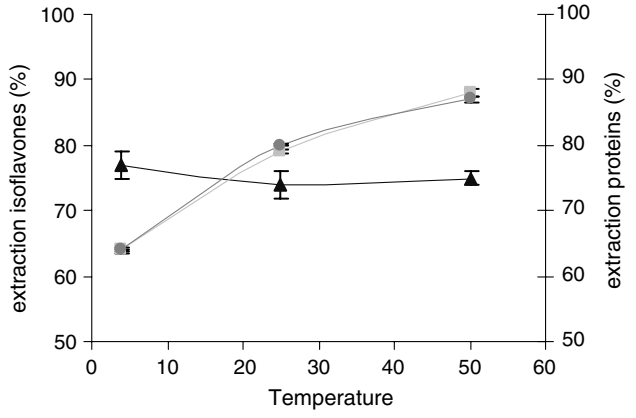

Fig. 1. Effect of time and temperature on the extraction of proteins from the defatted soy flour. Extraction efficiency was calculated in relation to total protein content ($N \times 6.25$) and total isoflavone content extracted by means of 80% methanol. Protein (%) extracted after 1-h extraction (■) and 3-h extraction (●); isoflavone extracted after 1-h extraction (▲).

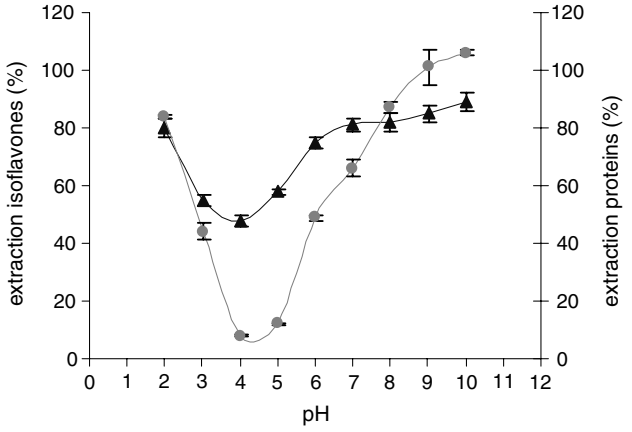

Fig. 3. Effect of pH on the extraction of proteins (●) and isoflavones (▲) from the defatted soy flour. Extraction efficiency was calculated in relation to total protein content ($N \times 6.25$) and total isoflavone content extracted by means of 80% methanol.

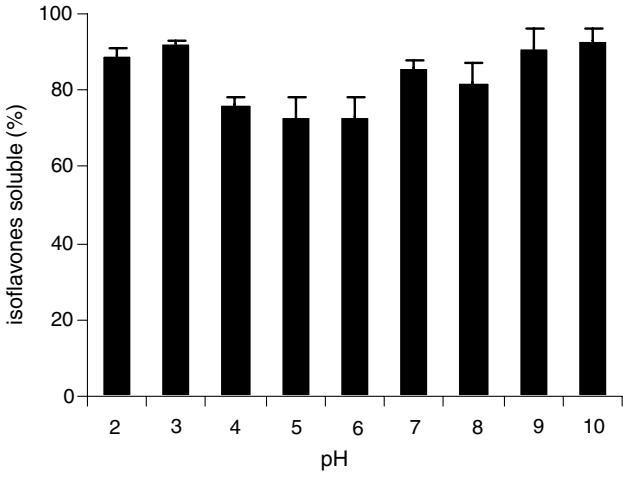

Fig. 4. Solubility, at different pH values, of isoflavanes extracted from the defatted soy flour.

ues of pH over 7 caused a significant increase in the yield of protein, this was not followed proportionally by isoflavones,whose yields, were 81% at pH 7 and 89% at pH l0, while, for proteins, they were 66% at pH 7 and 106% at pH 10.

3.5. Solubility of the isoflavones

The isoflavones extracted from the DSF and isolated through SPE showed a high solubility in aqueous solution, in the pH range 2–10 (73–93% of the amount solubilized in methanol). A lower solubility of isoflavones was observed at pH between 4 and 6 (Fig. 4). These results are similar to those for the effect of pH on isoflavone extraction from the DSF (Fig. 3), and indicate that the lower yield of isoflavones at the isoelectric pH of proteins is a result of both an inherent lower solubility and a decreased extraction due to their associations with solubilized proteins.

In relation to the isoflavone profile (Table 2), at pH $\geqslant$ 8, the de-malonylation reaction seemed to be favoured. Malonyl-β-glucosides dropped from around 50% (pH 8) to 36% (pH 10), while β-glucosides increased proportionally from 43% (pH 8) to 57% (pH 10) of the total isoforms. The acetyl-β-glucosides and aglucones showed little variation.

3.6. Effect of extraction conditions on the profile of isoflavones

Tables 3 and 5 show the variation of the distribution of isoflavones (concentration of malonyl-β-glucosides, acetyl-β-glucosides, β-glucosides and aglucones and the concentration of total daidzein, genistein and glycitein) according to the different extractions.

In the extraction performed at room temperature, there was a slight increase of aglucones, with a proportional decrease of β-glucosides, compared to the extraction at 4 °C. However, at 50 °C, the aglucones corresponded to 20% of the total amount extracted, and there was a large decrease of β-glucosides (Table 3). The increase of the temperature did not alter the percentage of either acetyl-β-glucosides or malonyl-β-glucosides.

Wang, Ma, Pagadala, Sherrard, and Krishnan (1998) reported that the DSF had 99% of the isoflavones as glucosides. In the present work, the value found was of 97% of glucosides extracted at 4 °C and 93% at 25 °C. However, the amount of glucosides extracted decreased to 80% at 50 °C, indicating that aglucones were being

Table 3
Profile of isoflavones extracted from the defatted soy flour at different temperatures (μmol/g)

	4 °C	25 °C	50 °C
β-Glucosides	2.15^{a}	1.86^{b}	1.04^{c}
M glucosides	3.18^{d}	3.09^{d}	3.22^{d}
Ac glucosides	0.09^{e}	0.09^{e}	0.11^{e}
Aglucones	0.19^{f}	0.35^{g}	1.09^{h}
Total daidzein	2.27^{a}	2.14^{b}	1.96^{b}
Total glycitein	2.19^{c}	2.12^{c}	2.23^{c}
Total genistein	2.87^{d}	2.79^{d}	3.04^{e}

Results are expressed as means ($n = 3$). Means in the same row with common letters are not significantly different ($p < 0.05$). M and Ac are used for the radical malonyl and acetyl, respectively.

Table 2
Effect of pH on the profile of water-soluble isoflavones (μmol/g)

	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
β-Glucosides	2.14^{a}	2.30^{b}	1.90^{c}	1.92^{c}	1.83^{c}	2.33^{b}	2.84^{d}	2.93^{d}	3.34^{e}
M glucosides	2.91^{f}	2.94^{f}	2.42^{g}	2.28^{h}	2.28^{h}	2.63^{i}	2.42^{g}	2.44^{g}	2.15^{j}
Ac glucosides	0.07^{k}	0.08^{k}	0.07^{k}	0.07^{k}	0.06^{k}	0.06^{k}	0.06^{k}	0.02^{l}	n.d.
Aglucones	0.49^{m}	0.46^{m}	$0.39^{m,n}$	0.36^{n}	0.35^{n}	0.36^{n}	0.36^{n}	0.36^{n}	$0.40^{m,n}$
Total daidzein	2.24^{a}	2.32^{a}	1.91^{b}	1.93^{b}	1.80^{b}	2.19^{a}	2.14^{a}	2.44^{a}	2.38^{a}
Total glycitein	1.91^{c}	1.91^{c}	1.58^{d}	1.48^{d}	1.54^{d}	1.75^{e}	$1.60^{d,f}$	1.64^{f}	1.52^{d}
Total genistein	3.04^{g}	3.15^{g}	2.60^{h}	2.45^{i}	2.45^{i}	2.89^{i}	2.74^{j}	2.97^{g}	3.17^{g}

Results are expressed as means ($n = 3$). n.d., not detected. Means in the same row with common letters are not significantly different ($p < 0.05$). M and Ac are used for the radical malonyl and acetyl, respectively.

Table 4
Profile of the isoflavones extracted from the defatted soy flour in 0.1 M phosphate–citrate buffer (pH 5) at 45 and 60 °C, in the presence or absence of *p*-NPG (μmol/g)

	45 °C	45 °C/ρ-NPG	60 °C	60 °C/ρ-NPG
β-Glucosides	1.18[a]	1.05[b]	3.10[c]	3.48[d]
M glucosides	2.58[e]	2.59[e]	2.67[f]	2.43[g]
Ac glucosides	0.06[h]	0.06[h]	0.06[h]	0.06[h]
Aglucones	1.51[i]	1.556[i]	0.72[j]	0.43[k]
Total daidzein	2.00[a]	2.19[b]	2.50[c]	2.63[d]
Total glycitein	1.76[e]	1.66[e]	1.97[f]	1.77[e]
Total genistein	3.05[g]	2.81[h]	3.70[i]	3.38[j]

Results are expressed as mean ($n = 3$). Means in the same row with common letters different ($p < 0.05$). M and Ac are used for the radical malonyl and acetyl, respectively.

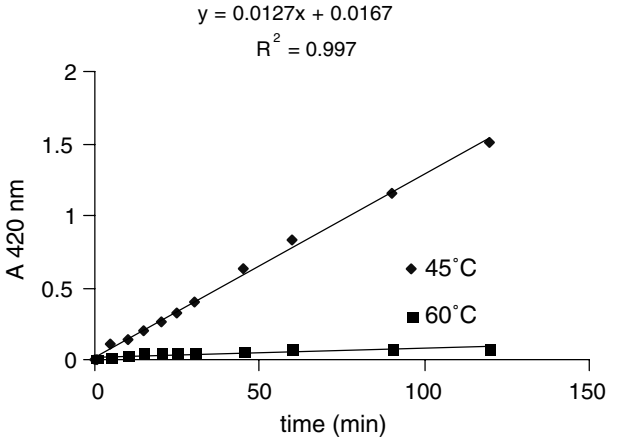

Fig. 5. Effect of temperature on β-glucosidase activity (production of *p*-nitrophenol) during protein extraction from the defatted say flour.

formed, probably as a result of enzymathic hydrolysis (Table 3.)

In order to find whether there was β-glucosidase activity, the aqueous extraction of DSF, was performed in the presence of the synthetic substrate, *p*-nitrophenyl-β-D-glucoside (*p*-NPG),and the *p*-nitrophenol formed was measured. The extraction was performed at two temperatures: the optimum (45 °C), and the temperature of inactivation of the enzyme (60 °C). As can be seen in Fig. 5, during the extraction at 45 °C there was a significant activity of β-glucosidases, with a formation of *p*-nitrophenol proportional to the time of extraction. On the other hand, no activity was observed at 60 °C. These results are in accordance with the report of Matsuura and Obata (1993) and Matsuura et al. (1995), showing β-glycosidase activity in the temperature range 10–50 °C (optimum 45 °C) and pH range 3.5–7 (optimum 4.5–5.5).

Table 4 shows the profile of isoflavones in the extracts obtained at 45 and 60 °C, in the presence or absence of *p*-NPG. During extraction at 45 °C, the β-glucosidases acted by hydrolyzing β-glucosides (~20% of total) with an increase of aglucones (~29% of total). At 60 °C, on the other hand, the β-glucosides represented ~50% of the total and aglucones, only ~9%. The malonyl-β-glucosides decreased at 60 °C compared to 45 °C, probably due to their thermal unstability (Barnes, Kirk, & Coward, 1994; Coward, Barnes, Setchell, & Barnes, 1993; Kudou et al., 1991). Rickert et al. (2004a) also reported a decrease of the malonyl-β-glucosides during extraction at 60 °C compared to 25 °C, for the insoluble residue, isolate and whey fractions. The percentage of malonyl-β-glucosides in the extract obtained at 45 °C (Table 4) was similar to those observed at 4, 25 and 50 °C (Table 3), indicating that β-glucosidases did not hydrolyse sugar esterified to malonyl or acetyl groups.

The profile of isoflavones depends on the temperature used during soy product processing, and heat would cause deesterification of malonyl-β-glucosides and acetyl-β-glucosides to the respective β-glucosides, as during tofu and soymilk production (Coward et al., 1998; Grün et al., 2001). Coward et al. (1998) and Barnes et al. (1994) reported that, during the methanolic extraction at 80 °C, the complete conversion of malonyl-β-glucosides and acetyl-β-glucosides occurred. Similarly, in soy milk held at 80 °C for 3 h, malonyl-β-glucosides converted to the β-glucoside form (Murphy, Barua, & Hauck, 2002). Dry heat, on the other hand, was shown to cause a loss of carbon dioxide (decarboxylation) leading to the formation of substantial amounts of the acetyl-β-glucosides from malonyl-β-glucosides, as in toasting of soy flour or extrusion, used to produce tex-

Table 5
Profile of isoflavones extracted from the defatted soy flour at different pH(μmol/g)

	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
β-Glucosides	2.55[a]	2.60[a,d]	1.73[b]	1.85[c]	2.66[d]	2.62[d]	2.71[d]	3.15[e]	3.45[f]
M-glucosides	2.83[g]	2.81[g]	1.52[h]	2.12[i]	2.85[g]	2.88[g]	2.33[j]	2.52[k]	2.53[k]
Ac-glucosides	0.08[l]	0.06[l,m]	0.04[l,m]	0.04[l,m]	0.03[m]	0.02[m]	0.03[m]	0.03[m]	0.03[m]
Aglucones	0.36[n,p]	0.33[n]	0.22[o]	0.20[o]	0.31[n]	0.28[n,o]	0.42[p]	0.48[p]	0.48[p]
Total daidzein	2.35[a]	0.92[b]	1.53[c]	1.77[d]	2.41[a]	2.32[a]	2.34[a]	2.40[a]	2.46[a]
Total glycitein	1.93[e]	1.19[f]	1.07[g]	1.48[h]	1.93[e]	1.97[e]	1.54[h]	1.65[i]	1.99[e]
Total genistein	3.02[j]	3.17[k]	1.56[l]	1.99[m]	2.95[j]	3.13[k]	2.82[n]	3.45[o]	3.64[p]

Results are expressed as means ($n = 3$). Means in the same row with common letters are not significantly different ($p < 0.05$). M and Ac are for the radical malonyl and acetyl, respectively.

Table 6
Isoflavone and protein recovery after isoeletric precipitation at pH 4, 4.5 and 5 of aqueous extracts (pH 7) from the defatted soy flour

	pH 4	pH 4.5	pH 5
Isoflavones (%)			
Acid whey	62 ± 3^{a}	67 ± 2^{a}	72 ± 2^{b}
Precipitate	38 ± 3^{c}	33 ± 2^{c}	28 ± 2^{d}
Proteins (%)			
Acid whey	15.5 ± 0.3^{g}	12.1 ± 0.2^{h}	19.0 ± 0.2^{i}
Precipitate	84.5 ± 0.3^{j}	87.9 ± 0.2^{k}	81.0 ± 0.2^{l}

Results are expressed as means ± SD ($n = 3$). Means in the same row with common letters are not significantly different ($p < 0.05$).

tured vegetable protein (Coward et al., 1993; Mahungu et al., 1999). Also, during soaking at temperatures below 50 °C (the first step in soymilk processing) endogenous β-glucosidases would hydrolyze β-glucosides forming aglucones. (Matsuura, Obata, & Fukushima, 1989; Matsuura & Obata, 1993).

The ionic strength did not influence the profile of isoflavones extracted in aqueous medium at NaCl concentrations of 0.01, 0.1, 0.2, 0.5 and, 1.0 M. On the contrary, pH showed a strong effect on the isoflavone profile (Table 5). At pH $\geqslant$ 8 and $\leqslant$4, the deesterification reaction seemed to be favoured. Malonyl-β-glucosides dropped from around 50–43% while β-glucosides increased proportionally from 44% to 53%. The acetyl-β-glucosides and aglucones showed little variation. As expected, genistein was the isoflavone that appeared in the highest proportion, and the percentage of glycitein was higher, between pH 3 and 5.

3.7. Precipitation of soy proteins

Commercial SPI are usually prepared through the extraction of the DSF in alkaline medium, utilizing a pH of 8–10, and precipitation of proteins at pH 4.5. To find whether the pH of protein precipitation could have some influence on the isoflavone content of acid-precipitated protein, the pH values 4, 4.5 and 5 were tested.

Table 6 shows the yield of precipitation of isoflavones and proteins at the three pHs. The precipitate obtained at pH 4 presented a higher percentage of isoflavones than that at pH 5, but it was not significantly different from the yield obtained at pH 4.5. However, at pH 4.5, the yield of protein was significantly higher. In this way, the isoflavone-richer acid precipitated protein obtained at pH 4 was at the expense of protein yield, which is not interesting from the industrial point of view. This shows the importance of proper pH control.

3.8. Production of a soy protein isolate (SPI) with increased content of isoflavones

From the results obtained, seven different isolates were prepared, changing a parameter in the processing step (Table 1) for each one.

The water, protein, and isoflavone contents of freeze-dried SPI are shown in Table 7. The products obtained corresponded to the definition of SPI, presenting over 90% protein ($N \times 6.25$).

The yield in soy protein isolate processing varied from 27% to 35% (w/w), higher than the value of 17.8% obtained by Wang and Murphy (1996) and compatible with the 28.9% report by Wang et al. (1998). These differences probably result from the different conditions of extraction utilized, such as pH, temperature, precipitation condition and centrifugation, as verified by Rickert et al. (2004a).

The isolates I, II and III were obtained under more rigorous conditions of centrifugation. As the isolates obtained industrially, before being submitted to drying, present a pastier consistency (~18% solids) than these (~50% solids), for the isolates IV, V, VI and VII much milder conditions of centrifugation were utilized. The aim was to check whether the higher presence of acid whey in the precipitate would result in a higher concentration of isoflavones. As shown in Table 7, the utilization of a milder centrifugation resulted in an increase of 52–80% in the content of isoflavones for the isolates IV, V, VI and VII, as a consequence of entrapped whey in isolate.

The isolate I presented a low isoflavone content corresponding to only 10% of the total extracted at pH 7, remaining the highest proportion of the acid whey (~75%), similar to the results of Wang and Murphy (1996). The same was observed for the isolates II and III (9%), and the pH of precipitation did not influence the isoflavone content of the SPI (Table 8).

The isolates IV, V and VI presented higher amounts of isoflavones (average 17% of the total extracted), after

Table 7
Content of moisture, proteins and isoflavones of soy protein isolates

	SPI I	SPI II	SPI III	SPI IV	SPI V	SPI VI	SPI VII
Moisture (g/100 g)	1.8 ± 0.2^{a}	2.0 ± 0.7^{a}	2.0 ± 0.2^{a}	2.5 ± 0.4^{a}	1.9 ± 0.1^{a}	2.1 ± 0.2^{a}	2.0 ± 0.4^{a}
Proteins (g/100 g)	93.5 ± 0.5^{b}	91.2 ± 0.4^{c}	$93 \pm 2^{b,c}$	92.5 ± 0.3^{b}	92.1 ± 1^{b}	$90 \pm 2^{b,c}$	90.5 ± 0.5^{c}
Isoflavones[a] (μmol/g)	1.93^{d}	1.92^{d}	1.85^{d}	3.00^{e}	3.23^{f}	2.95^{e}	3.45^{g}

Results are expressed as means ± SD ($n = 3$). Means in the same row with common letters are not significantly different ($p < 0.05$).
[a] The isoflavones total content is expressed as aglucones.

Table 8
Isoflavone and protein recovery (%) during the production of SPI I–VII

	Isoflavone (%)	Protein (%)
Acid whey I (pH 4.5)	70 ± 2	13.5 ± 0.1
SPI I (pH 4.5)	10 ± 1	74.6 ± 0.2
Acidified water I	6.1 ± 0.2	0.4 ± 0.1
Acid whey II (pH 4)	67 ± 2	13.6 ± 0.1
SPI II	9.0 ± 0.2	67.7 ± 0.2
Acidified water II	5.7 ± 0.5	0.3 ± 0.1
Acid whey III (pH 4.5)	74 ± 2	12.8 ± 0.2
SPI III	8.7 ± 0.3	67 ± 1
Acidified water III	5.1 ± 0.3	0.3 ± 0.2
Acid whey IV	72 ± 1	12.0 ± 0.3
SPI IV	15.6 ± 0.4	65.4 ± 0.2
Acidified water IV	7.9 ± 0.3	1.3 ± 0.1
Acid whey V	75 ± 3	10.7 ± 0.1
SPI V	19 ± 1	66.6 ± 0.6
Acidified water V	9.6 ± 0.3	1.3 ± 0.1
Acid whey VI	78 ± 2	9.9 ± 0.6
SPI VI	17 ± 1	67 ± 1
Acidified water VI	4.1 ± 0.1	2.2 ± 0.1
Acid whey VII	37 ± 4	8 ± 1
SPI VII	23 ± 1	65 ± 3
Acidified water VII	3.2 ± 0.3	2.0 ± 0.2

Results are expressed as means ± SD ($n = 3$).

adjusting the conditions of centrifugation (Table 8). However, the amount of isoflavones lost in the acid whey remained high. The isolate VI was obtained after extraction of the defatted flour at pH 9, which resulted in a higher extraction of proteins, as previously shown. However, the isolate did not present a higher content of isoflavones compared to those obtained after extraction at pH 7 (IV and V).

The isolate VII, on the other hand, was obtained after extraction of the DSF at pH 9 and 55 °C, which resulted in a higher isoflavone content, corresponding to 23% of the total extracted (Table 8). Compared to isolates I, II, and, III, there was an average increase of 80% of the isoflavone content and a decrease of 48% of the isoflavone loss in the acid whey. There was also a significant increase of the proteins extracted (48% of the total present in the defatted flour) compared to the extraction done at pH 9 at room temperature (42% of the total present in the defatted flour), confirming the data in Fig. 1.

A loss of protein, from 10% to 13% of the total extracted, was observed in the acid whey, and 0.5–1% in the water used for washing the protein precipitate. A higher amount of proteins was extracted at pH 9 than at pH 7, but it did not result in higher protein precipitation, which was around 66% of the protein extracted.

In the washing step of the protein precipitate there was a loss of 3–8% of isoflavones, much below the value of 22% reported by Wang et al. (1998). These authors, however, used water instead of acidified water for the washing step. As previously seen, isoflavones from the DSF are soluble in water and, thus, by washing the isoelectric precipitate they were removed, along with the washing water, causing a large decrease in the final content of the SPI. On the other hand, at pH 4.5 there was a decrease of the solubility of isoflavones (Fig. 4) and, as a result, the amount retained in the SPI would be higher.

Table 9 shows the profile of isoflavones during SPI production from DSF. The aqueous extracts obtained at pH 7 and 9 presented higher amounts of malonyl-β-glucosides and β-glucosides, respectively, because the alkaline pH caused the deesterification reaction (Rickert et al., 2004a).

SPI I–VI presented high amounts of aglucones (27–48%), different from isolate VII which presented only 15%. SPI I–VI were obtained at room temperature (~25 °C) and I–V at pH 7, which would allow the action of β-glucosidases, as previously discussed. The lower aglucone content of SPI VI could be explained by the pH 9 used in the extraction which, along with an extraction temperature of 55 °C, resulted in the lowest aglucone content observed for the SPI VII.

Comparing the profile of isoflavones of the DSF (Table 3) to those of SPI I to VII (Table 9), it can be noticed that the SPI presented a higher content of aglucones, similar to the report of Wang et al. (1998), which found 2% and 28% of aglucones for DSF and SPI, respectively.

The acid whey and the acidified washing water presented very similar profiles of isoflavones, as expected, having high contents of malonyl-β-glucosides and β-glucosides. However, the acid whey and the washing water resulting from the processes of production of SPI after DSF extraction at pH 9 presented higher percentages of β-glucosides, while those obtained after extraction at neutral pH presented malonyl-β-glucosides as the main conjugates (Table 9). This result can be explained by the fact that the alkaline pH used in the extraction was favourable to the deesterification reaction, increasing, in this way, the β-glucosides content.

DSF and SPI presented similar percentages of acetyl-β-glucosides, corresponding to 1–2% of isoflavones, which was also reported by Wang and Murphy (1996). Rickert, Johnson, and Murphy (2004b) also observed no conversion to the acetyl-β-glucoside form during production of SPI. The production of these conjugates seems to be related to the use of dry heat, as in the case of toasting and extrusion (Wang & Murphy, 1994b).

The distribution of the total forms of isoflavones (daidzein, genistein and glycitein) during production of SPI I–VII is shown in Table 9. SPI presented the three main isoflavones in proportions similar to those found in the flour, with a higher amount of genistein, between 59% and 64% (Table 9). Acid wheys I, II, and III (resulting from more drastic centrifugation conditions) pre-

Table 9
Profile and distribution of the total forms of isoflavones (daidzein, glycitein and genistein) during SPI production from DSF (μmol/g)

	β-Glucosides	M glucosides	Ac glucosides	Aglucones	Total daidzein	Total glycitein	Total genistein
Extract pH 7/25 °C	1.97[a]	3.29[a]	0.02[a]	0.67[a]	2.44[a]	2.14[a]	3.20[a]
SPI I	0.31[b]	0.67[b]	0.02[a]	0.92[b]	0.65[b]	0.51[b]	1.21[b]
SPI II	0.36[b]	0.83[c]	0.03[a]	0.70[a]	0.64[b]	0.61[b]	1.20[b]
SPI III	0.43[b,j]	0.72[b,c]	0.03[a]	0.70[a]	0.61[b]	0.54[b]	1.16[b]
SPI IV	0.67[c]	1.20[d]	0.05[a]	1.09[c]	1.02[c]	1.91[c]	1.83[c,e]
SPI V	0.92[d]	1.36[e]	0.04[a]	0.90[b]	1.15[c]	1.01[c]	1.91[c,f]
Acid whey I	3.30[e]	6.06[f]	0.05[a]	0.92[b]	4.98[d]	3.52[d]	4.70[d]
Acid whey II	1.54[f]	2.12[g]	0.03[a]	0.32[d,e]	1.95[e]	1.24[e]	1.78[e]
Acid whey III	1.64[f]	2.36[h]	0.03[a]	0.40[d]	2.11[f]	1.40[f]	2.02[f]
Acid whey IV	1.18[g]	2.18[g]	0.03[a]	0.31[d,e]	1.65[g]	1.39[f]	1.73[e]
Acid whey V	1.43[h]	2.16[g]	0.03[a]	0.26[e]	1.74[g,j]	1.37[f]	1.80[e]
Acidified water I	0.18[i]	0.32[i]	n.d.	0.03[f]	0.18[h]	0.22[g]	0.31[g]
Acidified water II	0.14[i]	0.21[i]	n.d.	0.06[f]	0.16[h]	0.14[e]	0.22[g]
Acidified water III	0.15[i]	0.22[i]	n.d.	0.05[f]	0.16[h]	0.15[g]	0.23[g]
Acidified water IV	0.50[j]	0.93[c]	0.01[a]	0.17[g]	0.70[b]	0.60[b]	0.77[h]
Acidified water V	0.56[j]	0.82[c]	0.01[a]	0.10[g,f]	0.65[b]	0.53[b]	0.71[h]
Extract pH 9/25 °C	2.82[k]	2.30[h]	0.01[a]	0.46[d]	1.96[i]	1.85[h]	3.30[i]
SPI VI	1.01[d]	1.02[i]	0.05[a]	0.776[a]	0.94[c]	0.81[c]	1.73[e]
Acid whey VI	2.07[a]	1.94[j]	0.01[a]	0.26[e]	1.83[j]	1.35[f]	2.11[j]
Acidified water VI	0.71[c]	0.70[b]	n.d.	0.09[g,f]	0.58[b]	0.53[b]	0.79[h]
Extract pH 9/55 °C	2.92[k]	1.73[k]	n.d.	0.46[d]	1.75[g]	1.62[i]	3.00[k]
SPI VII	1.66[f]	1.23[d]	0.02[a]	0.53[d]	1.19[c]	1.03[c]	2.00[j]
Acid whey VII	1.11[d]	0.75[b]	0.01[a]	0.05[g,f]	0.88[k]	0.50[b]	0.92[l]
Acidified water VII	0.48[j]	0.36[i]	n.d.	0.04[f]	0.41[l]	0.25[g]	0.40[g]

Results are expressed as means ($n = 3$). n.d., not detected. Means in the same column with common letters are not significantly different ($p < 0.05$). M and Ac are used for the radical malonyl and acetyl, respectively.

sented lower proportions of genistein compared to the washing water, similar to the report of Wang et al. (1998). They also had the same proportions of genistein and daidzein. Acid wheys VI–VII, on the other hand, presented isoflavone compositions similar to the washing water, as a result of the milder centrifugation conditions.

Acid wheys presented a significantly higher amount of daidzein compared to the SPI and DSF, indicating that this would rather be lost in the production of isolate, as also observed by Wang et al. (1998). An explanation would be the more hydrophilic nature of daidzein compared to genistein (Coward et al., 1993).

4. Conclusion

The results of this investigation showed that the production of soy protein isolate with increased content of isoflavones depends on the utilization of mild condition of centrifugation for the separation of isoelectric precipitate (162*g* for 2 min at 25 °C), pH 9 for the extraction, pH 4.5 for the isoelectric precipitation, and utilization of acidified water for the precipitate washing.

The difference in the isoflavones profile between the defatted flour and soy protein isolates can be explained by the endogenous β-glucosidase activity in the processing of isolates, resulting in a higher percentage of aglucones. Thus, through the processing, we may obtain products with increased contents of aglucones, which are known to be faster absorbed in the intestine.

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