Chromatographic Quantification of Isoflavone Content from Soy Derivates Using HPLC Technique

Daniel Mantovani[1,*], Lúcio Cardozo Filho[2], Leonilda Correia Santos[3], Vera Lúcia Ferreira de Souza[4], and **Cecília Sumie Watanabe[5]**

[1]Department of Agronomy, State University of Maringa; [2]Department of Chemical Engineering, State University of Maringa; [3]Department of Chemical Engineering, State University of Parana West; [4]Department Animal Science, State University of Maringa; [5]Department Pharmacy State, University of Maringa, Av. Colombo 5790, CEP 87020-900 Maringa, PR, Brazil and State University of Parana Av. Tarquínio Joslin dos Santos, 1300, CEP 85870-650 Foz do Iguaçu, PR, Brazil

Abstract

***Glycine max* soybean and its derivates stand out as functional food due to its content being free of aglycon isoflavones and their respective β-glucosides. This study is aimed at classifying and quantifying the isoflavones content in defatted soy flour (DSF) derivates and textured soy protein (TSP). From DSF and TSP samples found in the market, the soy protein isolates (SPI) and the soluble soy protein concentrate (SSPC) were obtained. The isoflavones were extracted from SPI and from SSPC, classified, and quantified through HPLC. The isoflavones daidzein and genistein and their glucosides daidzin, genistin, were found. For the SPI obtained from the TSP, values of β-glucosides came out with an average of 32.08 mg/100 g. However, for the SPI obtained from the DSF, values averaged 13.32 ± 0.172 mg/100 g. The highest content levels were observed in the derivates obtained from TSP. SPI was found with the lowest levels of daidzin in SSPC obtained from the DSF. Genistein was only observed in the supernatant, a derivate of SSPC obtained from TSP. During the derivatization process, losses of up to 35.7% of isoflavones in the precipitate occurred. Both SPI and SSPC, obtained from DSF and from TSP, presented great concentration of β-glucosides compounds.**

Introduction

Soy protein isolates (SPI) and soluble soy protein concentrate (SSPC) are widely used in the food industry, mostly in application to meat, bakery products, drinks, and cereals among others. The processes for obtaining them were established mainly to obtain extraction yield and the maintenance of technological functional properties such as water retention, gel segregation, oil absorption, and emulsification (1). Once soybean isoflavones biological potential is high, it is interesting to know how the processing of these ingredients affects the isoflavones quantity and profile and offer alternatives to minimize losses.

SPI is, by definition, a product obtained from clean, peeled, soybean grains, which are also unfatted by the removal of non-protein components that must contain at least 90% protein (2). The proteins are extracted from soybean flocks or flour unfatted with alkaline solution and clarified extract through centrifugation for the precipitate removal. After this, the globulins are precipitated in their isoeletric point pH 4.5 through the addition of an acid of alimentary level. The suspension is centrifuged and washed, and it might or not be neutralized before spray-dryer drying process (3).

Nowadays, there is widespread consumption of soybean in Brazil, either in grain form or in traditional products such as tofu, soy milk, miso, and natto. Their acceptability is limited mainly due to their characteristic taste. SPI and SSPC present a milder taste in relation to soybean grains because compounds responsible for the astringent taste are removed or inactivated during the processing (3).

Studies on soybean and its derivates have generated interest on the research and the production of functional foods due to the fact that diverse studies have proved the relation between diet and the prevention of health problems such as cancer and cardiovascular diseases (4).

Aglycon isoflavones found in soybean and its derivates are: daidzein (7,4'-dihidroxiisoflavone), glycitein (7,4'-dihidroxi-6-metoxiisoflavone), and genistein (5,7,4'-trihidroxiisoflavone). Daidzein and genistein are frequently used as medicines for fighting prostate cancer (5).

The isoflavone daidzein with genistein is considered to be the most abundant phytoestrogen in soybean and its derivates. On the other hand, glycitein is the least abundant isoflavone and also the least studied. Glycitein chemical structure is similar to the structure of genistein and daidzein, thus it can be supposed that it presents physiological activities similar to human metabolism (6).

Considering the importance of studies on products used as phytoestrogen present in soy derivates, the aim of this work was

*Author to whom correspondence should be addressed: email mantovanidaniel@hotmail.com.

to obtain SPI and SSPC from textured soy protein and defatted soy flour and, after obtaining the derivates, identify and quantify isoflavone compounds present in them, especially the free aglycons and their respective β-glucosides.

Materials and Methods

Raw material used for obtaining soybean protein concentrate and isolate

The samples of texturized soy protein (TSP) and defatted soy flour (DSF) were acquired in supermarkets. They were packed in sealed plastic bags; its shelf life is one year. All the samples were stored at 4°C until extraction of SPI and SSPC.

Obtaining SPI

For obtaining SPI, a method used by Wang et al. (7) was modified for adaptations of optimizations and protein yield (2,8). Twenty-five grams of TSP and DSF were weighed, suspended in 500 mL of distilled water, and the pH was adjusted to 9.0 with the addition of 4 N NaOH. The extraction time was 45 min at 55°C on a shaking table (Model 185, Technal, Wakefield, UK). After this, the suspension was centrifuged at 13,000 RPM for 20 min under room temperature. The supernatant had its pH readjusted to 4.5 with the addition of 6 N HCl and homogenized for 1 h at room temperature. After this, the suspension was centrifuged again for 20 min at 13,000 RPM at 5°C. The precipitate was separated from the supernatant; both were lyophilized in a lyophilizer (Labconco, Kansas City, MO) and placed in an eppendorf-type micro tube before being stored at 4°C until the analysis.

Obtaining SSPC

For obtaining SSPC, a method described by Berk (2) was used. Twenty-five grams of TSP and DSF were weighed, suspended in 500 mL of distilled water, and the pH was adjusted to 4.5 with the addition of 6 N HCl. After 1 h of shaking in water bath (Model TE 0541/1, Technal) at room temperature, the mixture was centrifuged for 20 min at 13,000 RPM at 5°C. The precipitate was separated from the supernatant; both were lyophilized in a lyophilizer (Labconco) and placed in an eppendorf-type microtube before being stored at 4ºC until the analysis.

Reagents and standards

The standards of the compounds daidzin, genistin, daidzein, and genistein isoflavones were acquired from Chemical Co. (St. Louis, MO). The solvents used, chromatographic-grade methanol and acetic acid, were acquired from J.T. Baker (Phillipsburg, NJ). The water was purified at Milli-Q Millipore system (Bedford, MA). Before the injection, all of them were filtered in membranes of 0.45-µm nylon (Alltech, Deerfield, IL).

Preparation for chromographical analysis

The extraction of isoflavone compounds, aglycons and their β-glucosides were carried out according to the methodology described by Grün et al (11). Derivates formed from SPI and SSPC were sampled 1.00 g from the precipitate and 0.50 g from the supernatant, diluted in 15 mL of 80% methanol under constant shaking for 30 min at room temperature. The mixture was centrifuged for 15 min at 5,000 RPM before the supernatant was separated from the precipitate and filtered with paper filter Whatman no. 1. The precipitate was diluted again in 10 mL of 80% methanol, shaken for 30 min at room temperature, and centrifuged for 5 min at 5,000 RPM. The supernatant was separated from the precipitate and filtered in filter paper Whatman no. 1. The supernatant for both extractions was concentrated in a rotatory evaporator (Rotavapor Fisatom) at bath temperatures of 40°C.

Extracts purification

The concentrated extract, free of 80% methanol, was purified through a column for solid phase extraction C_{18} (Bondesil Sorbents, Varian) with 6 mL of volume, adsorbent mass of 0.8 g, and retention capacity of about 0.3 g of apolar substances. In each extraction, the volume correspondent to 0.20 g of isoflavones was applied. The column was washed with 4 mL of cold water, and the isoflavones were eluted in 6 mL of 80% methanol. The purified extract was concentrated in a rotatory evaporator (Rotavapor Fisatom) in a bath at 40°C.

Isoflavones identification and quantification

Samples of purified extract had their volume adjusted with solvent 80% methanol, and were filtered in polyethylene filters with PTFE membrane (Millipore) 0.45-µm pore before the injection.

Isoflavones identification and quantification were carried out

Table I. Isoflavones Levels Found in SPI Obtained From the TSP and DSF

Isoflavones	Daidzin	Genistin	Daidzein	Genistein	Total isoflavones*
SPI from the TSP	7.54 ± 0.096	24.54 ± 0.105	nd†	nd	32.08 ± 0.045
SPI from the DSF	3.34 ± 0.050	9.06 ± 0.127	0.92 ± 0.020	nd	13.32 ± 0.172

* Total isoflavones are in mg/100g;
† nd = not detected.

Table II. Isoflavones Levels Found in the Supernatant and the Insoluble Residue for the Process of Obtaining SSPC from TSP and DSF*

Isoflavones	Daidzin	Genistin	Daidzein	Genistein	Total isoflavones
TSP Supernatant	19.84 ± 0.032b	31.56 ± 0.073b	nd†	nd	51.40 ± 0.025b
DSF Supernatant	26.5 ± 0.022a	47.89 ± 0.045a	nd	1.09 ± 0.032	75.48 ± 0.067a
TSP Insoluble residue	4.19 ± 0.074d	7.60 ± 0.065c	nd	nd	11.79 ± 0.122d
DSF Insoluble residue	3.14 ± 0.053e	7.79 ± 0.034c	nd	nd	10.93 ± 0.085e
SSPC from the TSP	11.03 ± 0.125c	7.14 ± 0.060d	nd	10.14 ± 0.075	28.31 ± 0.126c
SSPC from the DSF	1.38 ± 0.026f	5.15 ± 0.052e	nd	nd	6.53 ± 0.018f

* Different letters in the same column indicate significant difference at the level of 5%;
† Total isoflavones are in mg/100 g;
‡ nd = non detected.

through high-performance liquid chromatography (Gilson 321) with a secondary pump, deaerator, automatic injector, detector (UV–Vis), and the software program Boriwn version 1.5 JMBS. The chromatographic conditions described by Song et al. (10) were used. C_{18} coated column Lichrospher of Merck (250 × 4.6 mm, 5 µm, Whitehouse Station, NJ) was used at 30°C. Mobile phase was constituted of acetic acid–methanol (19:1, v/v), initial flow at 1 mL/min, UV–Vis detection at 254 nm, and injection volume of 20 µL.

The concentrations of daidzin, genistin, daidzein, and genistein were calculated upon authentic (Sigma Co.) standard curves. Its concentrations and its conjugated were calculated by standard curve of daidzin (13,15).

Protein determination and humidity

The content levels of each part of the processing and on the final products were determined by the semi-micromethods of Kjeldahl – AOAC 960.52 (9). The humidity percentage was realized only in raw materials TSP and DSF and determined by the method AOAC 925.10 (9).

Statistical analysis

The statistical analysis was carried out through the software SAS version 9.1.3. Significant differences were at 5% level. The results were evaluated with standard deviation mean $n = 3$ in the application of Tukey's test.

Results and Discussion

Table I presents isoflavones values present at the process of producing and obtaining of SPI extracted from TSP and DSF. The compounds obtained were daidzin, genistin, daidzein, and genistein. The higher isoflavones content levels observed in derivates extracted from SPI obtained from the TSP averaged 32.08 ± 0.045 mg/100 g. SSPC obtained from the DSF averaged 13.32 ± 0.172 mg/100 g. The higher concentration of isoflavone compound was genistin obtained for derivate SPI probably due to high solubility of β-glucosides compounds when compared to aglycons compounds. Table II shows isoflavones levels found in the supernatant and the insoluble residue during the process for obtaining SSPC from TSP and DSF. Although the supernatants presented the high levels of compounds daidzin and genistin, these compounds were retained in the precipitate carrying to losses during the obtention process of SPI values as demonstrated in the Table I.

The values for the aglycons compounds, especially genistein was observed in the supernatant SSPC obtained from the TSP (Table II). On the other hand, daidzin and genistin were found in all samples analyzed. This is in accordance with the study realized by Coward et al. (13), where β-glucosides compounds showed higher solubility compared to the aglycons compounds. Studies led by Wang and Murphy (8) presented isoflavones compounds contents in SPI and SSPC and found similar averages for the different isoflavones compounds. Further research indicated a rise on isoflavones content, after the production of soybean derivates (2,3). Figure 1 presents the chromatographic profile of SPI isoflavones obtained from DSF.

Considering DSF as raw material, approximately 35% of daidzin and 33% of genistin remained in insoluble residue. On the other hand, when the raw material used was TSP, the losses of insoluble residues were lower, with approximately 18% of genistin. In the supernatant, we found only genistein, indicating that the process using acid pH 4.5 was efficient for the segregation of aglycons isoflavones compounds during the process for obtaining SPI (Table I) and SSPC (Table II).

However, SPI and SSPC production obtained from raw materials DSF and TSP presented great variations for SPI with an average 61.34%. For SSPC the average variations of 76.93% were

Table III. Mass Global Balance for Isoflavones Levels Found SPI and SSPC Obtained From DSF and TSP*

Isoflavones	Humidity (%)	Total mass (g)	Protein (%)	Total isoflavones (mg/100 g)
DSF	7.87 + 1.004	25.006 + 0.010a	51.18 + 0.014e	21.82 + 0.017e
SPI from DSF		8.021 ± 0.034c	95.82 ± 0.246a	13.32 ± 0.172g
SSPC from DSF		6.851 ± 0.043e	66.36 ± 0.086c	6.53 ± 0.018j
DSF supernatant		7.050 ± 0.037d	16.31 ± 0.055h	75.48 ± 0.067a
DSF IR†		3.078 ± 0.024i	34.91 ± 0.097f	10.93 ± 0.085i
TSP	2.83 ± 1.089	25.008 ± 0.105a	51.54 ± 0.208e	14.23 ± 0.055 f
SPI from TSP		9.437 ± 0.040b	64.98 ± 0.118d	32.08 ± 0.045c
SSPC from TSP		6.304 ± 0.072f	82.16 ± 0.146b	28.31 ± 0.126d
TSP supernatant		5.875 ± 0.037g	12.57 ± 0.114i	51.40 ± 0.025b
TSP insoluble residue		3.392 ± 0.030h	28.48 ± 0.079g	11.79 ± 0.122h

* Different letters in the same column indicate significant difference at the level of 5%.
† IR = insoluble residue.

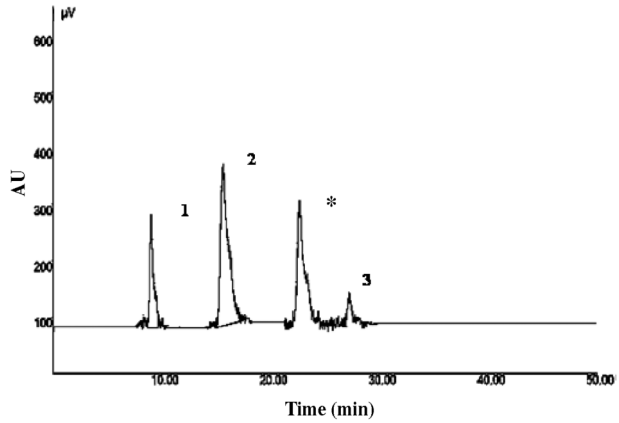

Figure 1. Chromatographic profile of the SPI isoflavones obtained from DSF. Chromatographic conditions: C_{18} coated column Lichrospher of Merck (250 × 4.6 mm, 5 µm) at 30°C. Mobile phase consisted of acetic acid–methanol (19:1, v/v), initial flow 1 mL/min, temperature: 30ºC, UV–vis detection at 254 nm; and injection volume of 20 µL. Peaks: daidzin, 1; genistin, 2; daidzein, 3; and Unknown, *.

still higher than the one verified in SPI. Therefore, this process produced higher concentration of compounds β-glucosides, daidzin, and genistin.

During the global mass balance study, presented in Table III, for the production of SPI and its derivates, it was observed that the product obtained corresponds to the definition of SPI, for it presents more than 90% protein. As for the values obtained for protein isolates derivates, they present low content of isoflavones due to the losses occurred during the extraction because alkaline pH 4.5 may modify the protein molecules and alter linkages with isoflavones (12). This fact was observed in this study, mainly for the soybean protein isolate derivate from DSF. Other reported studies (13) analyzed different brands of hydro-soluble extracts and conclude that extracts obtained from soybean protein isolates present a quantity of isoflavones four times lower than the ones prepared from soybean grains.

Conclusions

The production of SPI and SSPC obtained from raw materials DSF and TSP presented great variations for SPI with average 61.34%, probably because of the endogenous soy β-glucosides. For SSPC the average variations of 76.93% were still higher than the one verified in SPI, indicating possible hydrolysis of the parent isoflavones glucosides. Therefore, this production process generated concentrations of β-glucosidic compounds, genistin, and daidzin, which act together with aglycons, daidzein, and genistein to fight against many kinds of deseases.

References

1. N. Hettiarachchy and U. Kalapathy. Soybean protein products. In: *Soybeans: Chemistry, Technology and Utilization*, 2nd ed. Gaitherbursg, Aspen Publishers, Ch. 8. 1999, pp. 379–411.
2. Z. Berk. *Protein isolate. In: Technology of Edible Flours and Protein Products From Soybean*, 3th ed. Roma, FAO (United Nations). Ch. 6. 1979, pp. 112–124. FAO Agricultural Service Bulletin 11.
3. J.S. Circle and K.A. Smith. "Processing flours, concentrates and isolates". A.K. Smith and S.J. Circle (Eds). In *Soybeans: Chemistry and Technology* (Vol. 1 – Proteins). AVI Publishing Company Inc., Ch. 9. 1972, pp. 294–338.
4. J.M. Messina. "Soyfoods: their role in disease prevention and treatment". K. Liu (Eds). In *Soybeans: Chemistry, Technology and Utilization*. 2nd ed. Gaitherburg, Aspen Publishers., Ch. 10. 1999, pp. 443–477.
5. W. Li, M. Zhang, J. Zhang, H.X. Li Zhang, and Q.C.O. Sun. Interactions of daidzin with intramolecular G-quadruplex. *FEBS Lett* **580:** 4905–4910 (2006).
6. T.T. Song, S. Hendrich, and A.P. Murphy. Estrogenic activity of glycitein, a soy isoflavone. *J. Agric. Food Chem.* **130:** 1607–1610. (1999).
7. C. Wang, M.S. Pagadala, S.M. Sherrad, and G. P. Krishnan. Changes of isoflavones during processing of soy protein isolates. *J. Am. Oil Chem. Soc.* **75:** 337–341 (1998).
8. H. Wang and A.P. Murphy. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem.* **44:** 2377–2383 (1996).
9. D.C. Washington W. Horwits. AOAC–Association of Official Analytical Chemists Official Methods of Analysis, 16nd ed. USA (1997).
10. T. Song, K. Barua, G. Buseman, and P.A. Murphy. Soy isoflavones analysis: quality control and a new internal standard. *Am. J. Clin. Nutr.* **68:** 1474S–1479S (1998).
11. U.I. Grün, K.A.C. Li, B. Lin, J. Zhang, and F.N. Lakdas. Changes in the profile of Genistein, Daidzein, and their Conjugates during Thermal Processing of Tofu. *J. Agric. Food Chem.* **49:** 2839–2843 (2001).
12. J.H. Wang and A.P. Murphy. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **42:** 1666–1673 (1994).
13. L. Coward, M. Smith, M. Kirk, and S. Barnes. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **68:** 1486S–1491S (1998).
14. A.C. Eldridge. Determination of isoflavones in soybean flours, protein concentrates, and isolates. *J. Agric. Food Chem.* **30(2):** 353–355 (1982).
15. S. Barnes, L. Kirk, and J. Coward. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-Mass spectrometry. *J. Agric. Food Chem.* **42:** 2466–2474 (1994).

Manuscript received November 15, 2008;
Revision received March 19, 2009.