Isoflavone quantitation in soymilk: Genistein content and its biological effect

Natália Freddo\textsuperscript{a}, Jessica Nardi\textsuperscript{b}, Charise Dallazem Bertol\textsuperscript{a,b}, Eliane Dallegre\textsuperscript{c}, Mirna B. Leal\textsuperscript{d}, Fabiano Barreto\textsuperscript{e}, Izadora Borgmann Frizzo\textsuperscript{a} and Luciana Grazziotin Rossato-Grando\textsuperscript{a,f}

\textsuperscript{a}Institute of Biological Sciences, University of Passo Fundo, Passo Fundo, Brazil; \textsuperscript{b}Post-Graduation Program in Human Aging, University of Passo Fundo, Passo Fundo, Brazil; \textsuperscript{c}Department of Pharmacoscience, Federal University of Health Sciences of Porto Alegre, Porto Alegre, Brazil; \textsuperscript{d}Department of Pharmacology, Institute of Basic Health Science, Federal University of Rio Grande do Sul, Porto Alegre, Brazil; \textsuperscript{e}Department of Pesticide Residues and Veterinary Medicine Analysis, Agriculture and Livestock Laboratory (LANAGRO), Porto Alegre, Rio Grande do Sul, Brazil; \textsuperscript{f}Post-Graduation Program in Bioexperimentation, University of Passo Fundo, Passo Fundo, Brazil

**ABSTRACT**

Soy derivatives contain isoflavones with structural similarity to estrogen hormones known as phytoestrogens. The levels of isoflavones in these foods are quite variable and often unknown. The objective of this work was to develop and validate a methodology for the quantification of isoflavone genistein in soymilk by liquid chromatography coupled with an ultraviolet detector. After quantifying the levels of genistein in soymilk, the behavioral effects of daily administration of soymilk supplemented or not with glyphosate (50 or 100 mg/kg) throughout the pre-pubertal period in male rats were evaluated. The method developed is selective, sensitive, specific, and precise for the quantification of genistein with a total execution time of 20 min. Behavioral test highlights changes in anxiety pattern after chronic prepubertal exposure to soymilk containing 26.46 µg/mL genistein.

**Introduction**

The consumption of soy products has increased significantly in recent years, considering the increase in the number of vegetarians, vegans, and lactose-intolerant individuals who seek soy derivatives as a healthy protein alternative (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). These products contain isoflavones that have estrogenic activity and for this reason are known as phytoestrogens, being genistein the prototype of the group. There is variability in the levels of isoflavones found in soy since their quantity varies according to the morphological part of the plant, climate, planting location, temperature, lightness, and availability of soil water (Budryn, Czarnecka, Carascos, & Sánchez, 2017).

The regular use of soy derivatives has a number of benefits, such as the reduction of serum cholesterol, relief of menopausal symptoms, reduction of cardiovascular diseases, protective effect of carcinogenesis, and antioxidant function (Monteiro, Queirós, Lopes, & Pedro, 2018). On the other hand, phytoestrogens are also recognized as anxiolytic substances and might negatively interfere with endocrine system (Nardi et al., 2017), corroborating the importance of knowing the concentrations of these compounds that people are exposed through the diet.

Thus, this work aims to develop and validate a method for quantification of genistein in soymilk through liquid chromatography coupled with ultraviolet photodiode array detector (LC-PDA). It was measured genistein content in commercial soymilk and evaluated the behavioral effect of a subchronic exposure to soymilk daily administered to male rats in the prepubertal period. Glyphosate-based herbicides are the most used agrochemical in soy crops and soybeans frequently contain elevated levels of glyphosate residues, sometimes above the allowed limits (Bohm, Genoves, Pigosso, Trichez, & Rombaldi, 2008). Soymilk supplemented with glyphosate was also tested.
Materials and methods

Chemicals

Acetic acid and acetonitrile (HPLC grade) were purchased from Merck (São Paulo, Brazil). The genistein standard was purchased from Sigma–Aldrich (São Paulo, Brazil). Roundup Original®, produced by Monsanto, was used in the experiment. Soymilk (5.2 g protein per serving) was used as feed control and in method validation.

Method validation

The analytical method was validated in accordance with both Brazilian Sanitary Surveillance Agency (ANVISA) and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. Linearity, limits of detection (LD) and quantification (LQ), precision, accuracy, specificity, robustness, and stability were evaluated.

HPLC-UV analysis

The analyses were performed on HPLC Flexar LC Perkin Elmer equipped with Flexar LC binary pump, Flexar PDA detector, and autosampler. Peak area data is integrated into Software Chromera Workstation. A Brownlee C8 reverse phase column (250 mm × 4.6 mm, 5 μm) was used. For the mobile phase in the equipment, 5 μL injections with a constant flow rate of 0.35 mL were performed along a gradient of acetic acid 0.5% and acetonitrile 100% (70:30). The analysis wavelength was 254 nm, the genistein retention time was 7.6 min, and the total analysis time was 20 min due to the matrix interferers (soymilk).

Sample treatment

Soy milk samples were centrifuged for 10 min at 600 g. In order to purify and concentrate the isoflavones, the sample was passed through a solid-phase extraction column (SPE) (Strata-X 100 mg/1 mL, Phenomenex). The samples were concentrated under nitrogen flow in the concentration (SPE) (Strata-X 100 mg/1 mL, Phenomenex). The samples were passed through a solid-phase extraction column (SPE) (Strata-X 100 mg/1 mL, Phenomenex). The samples were concentrated under nitrogen flow in the concentrato (Tecnal – TE/0195) for 30 min and subjected to ultrasound (Unique model USC 2850) for 5 min. The samples were then resuspended in 2 mL methanol (MeOH) and injected into the HPLC.

Linearity

The 1 mg/mL isoflavone stock solution was obtained by means of 10 mg of genistein standard solubilized in 10 mL of MeOH. The linearity, as indicated by the regression curves and the square correlation coefficients (r²), was performed in 3 days using five different concentration levels in a range of 10–100 μg/mL of genistein in soy milk. The results were analyzed through ANOVA (Portal Action software, Estatcamp and DigUp, São Carlos – SP, Brazil) and the minimum accepted r² was 0.99. A normality evaluated by Anderson–Darling, Kolmogorov–Smirnov, Shapiro–Wilk and Ryan–Joiner. The autocorrelation in the residues was evaluated by the Durbin–Watson test and the homoscedasticity by the Cochran test.

Limits of detection and quantification

The LD and LQ were determined from the data of the calibration curves (BRASIL, 2003; ICH, 2005). LD was estimated after five injections based on a signal-to-noise ratio of 10. A signal-to-noise ratio of 3 was considered acceptable for estimating LD (ICH, 2005).

Precision and accuracy

Precision and accuracy were calculated by analyzing three replicates of three different concentrations (20, 60, and 90 μg/mL) of genistein. Accuracy was assessed by the calculation of the mean, standard deviation, and coefficient of variation (CV%) of the values. The precision was calculated by the percentage value between the calculated value and the nominal value (ICH, 2005).

Specificity and recovery

To evaluate the recovery, three different concentrations (20, 60, and 90 μg/mL) of isoflavones were made from a stock solution (1 mg/mL). Samples were injected after SPE and without passing the extraction process in SPE, and the signal generated by the LC-PDA was compared.

Proof of applicability

The applicability of the method was proved by the analysis of isoflavones in three different brands of commercially available soymilk. Samples were obtained commercially and were processed in the same way as the standards.

Behavioral test

Experimental model

The experiment was conducted in 23-day-old male Wistar rats, divided into four groups (seven animals per group). The treatment was administered orally (through gavage), daily, for 35 days. The control group received a saline solution, the soymilk control group received only soymilk (containing 26.46 μg/mL genistein), the glyphosate 50 group received soymilk plus 50 mg/kg glyphosate, and the glyphosate 100 group received soymilk plus 100 mg/kg glyphosate. The doses were chosen according to the preliminary studies (Dallegrove et al., 2007; Nardi et al., 2017). The animals were housed in individual cages, allowing individualized evaluation of food and water intake. Individual body weight gain and toxicity signs (piloerection, diarrhea, mucosal pallor, cyanosis, and respiratory function) were daily evaluated. After 35 days of treatment, when the rats were 58 days old, a behavioral test was performed. The experimentation, transportation, and care of the animals were performed in compliance with the relevant laws and institutional guidelines. This project was approved by the Ethics Committee on Animal Use of University of Passo Fundo (protocol number 008/2014).

Elevated plus maze (EPM) test

The apparatus consists of two closed arms (50 × 10 × 40 cm) with walls made of acrylic and two open arms (10 × 50 cm). The arms are arranged in a cross shape, that lies on an elevated base, 50 cm above the floor. Animals were evaluated and filmed during 5 min, in a darkroom, illuminated by a red light. Each animal was individually placed on the central space of the maze, facing the open arm. A longer stay in the open arms of the apparatus is compatible with an anxiogenic behavior. The observed parameters are shown in Table 3. Increased grooming, rearing, and dung are compatible with anxiogenic effect. After each test, the apparatus was cleaned with ethanol 5% (Hei et al., 2012).
Statistical analysis
Data distribution was considered normal by Shapiro–Wilk test; thus, statistical comparison was performed by one-way ANOVA, followed by Tukey post hoc test. Significance was accepted at p values <0.05 vs control.

Glyphosate analysis in soymilk by liquid chromatography coupled with mass spectrometry (LC-MS)
Glyphosate content in soymilk samples was analyzed before glyphosate spike. Soymilk sample was extracted with 10 mL methanol and 1% formic acid and followed by centrifugation at 3,500 xg for 10 min. An aliquot of 500 µL was diluted with 500 µL of acetonitrile and analyzed by LC-MS.

An Agilent 1260 LC system (Waldbronn, Germany) coupled to ABSciex 5500 QTRAP triple quadrupole mass spectrometer (Toronto, Canada) was employed. The chromatographic separation was carried out on Acclaim Trinity Q1 column (3.0 µm, 100 mm x 3 mm; Thermo Scientific, Waltham, MA, USA). The mobile phase gradient elution was 100 mM ammonium formate 1% formic acid (A) and acetonitrile (B). The flow rate was 500 µL/min and a 2-min equilibrate time was applied. The gradient started with 100% of A, kept for 3 min and decreasing to 5% in 4 min, and maintained until 7 min returning to initial condition at 8 min and kept until 10 min. The column temperature was maintained at 30°C. The injection volume was 20 µL. Mass spectrometer resolution in multiple reactions monitoring was unitary, and dwell time applied was 100 ms for all transitions. The mass spectrometer was operated in negative electrospray ionization mode, and precursor ions monitored were [M-H]. Nitrogen was used as nebulizer gas, curtain gas, heater gas, and collision gas (psi units). Collision gas (CAD) was set at medium, and nebulizer gas (GS1) and dryer gas (GS2) were set at 55 psi. The curtain gas pressure and the temperature were set at 25 psi and 650°C, respectively. Electrospray capillary voltage was set at −4.5 kV.

Results and discussion
The proposed LC-PDA method was successfully developed and validated. In this way, it is possible to quantify the isoflavone content in a complex matrix, such as soymilk, using an easy-to-perform and low-cost methodology. Behavioral tests performed in the present work highlight changes in anxiety pattern after subchronic exposure to soymilk.

Chromatographic conditions were optimized to provide a simple, fast, and reliable LC-PDA method for quantifying the genistein content present in soymilk. Samples show normal distribution, independence, and homoscedasticity (Table 1). The results of precision and accuracy show an acceptable range (Table 2), as recommended by the guidelines (BRASIL, 2003; ICH, 2005). The method presents an LQ of 8.81 µg/mL and an LD of 2.64 µg/mL. Genistein recovery is 100%. The interferents present in the soymilk sample do not interfere with isoflavone analysis using the PDA detector, as shown in Figure 1.

To prove its applicability, we applied this method in the quantification of genistein in the three different brands of commercially available soymilk. The mean genistein content is 17.58 ± 8.38 µg/mL. As expected, the highest levels of genistein are found in the products with the highest declared protein content. This methodology allows quantification of genistein content in soymilk in a simple and efficient way, while previous studies quantified isoflavones using mass spectrometry (Benlhabib, Baker, Keyler, & Singh, 2004), which makes analysis more expensive and is not available in most

**Table 1. Requirements assessed for the validation of the LC-PDA method.**

| Parameter                              | Statistical test | LC-PDA method |
|----------------------------------------|------------------|---------------|
| Regression (ANOVA)                     | p value1         | 2.607E-19     |
| Autocorrelation (Durbin–Watson)        | p value1         | 0.006803*     |
| Homoscedasticity (Cochran)             | Cochran2         | 0.515         |
| Normality test                         | Anderson–Darling | p value3      |
|                                        | p value3         | 0.3*          |
|                                        | Shapiro–Wilk     | p value3      |
|                                        | Kolmogorov–Smirnov | p value3 | 0.4*   |
|                                        | Ryan–Joiner      | p value3      | 0.4*   |

*p < 0.05.

**Table 2. Repeatability, precision, and accuracy of the method for the determination of isoflavones in soymilk.**

| Concentration | Mean ± SD | Precision (%) | CV (%) |
|---------------|-----------|---------------|--------|
| 20 µg/mL      | 19.1 ± 0.38 µg/mL | 95.4 | 1.98 |
| 60 µg/mL      | 58.1 ± 0.46 µg/mL | 96.8 | 0.8 |
| 90 µg/mL      | 90.0 ± 0.26 µg/mL | 100.0 | 0.28 |

Figure 1. Chromatogram of soymilk containing genistein (80 µg/mL) in 254 nm.

Figura 1. Cromatograma de leche de soya adicionada con genisteína (80 µg/mL) en 254 nm.
Figure 2. Time spent on open arms, on closed arms, and in the center of EPM apparatus. The results are presented as means ± standard deviation (n = 7 animals). Statistical analysis was performed by one-way ANOVA, followed by Tukey post hoc test (*p < 0.05 vs soymilk control). SM: soymilk; Gly: glyphosate.

The results evidence that rats exposed to soymilk enriched with 100 mg/kg of glyphosate on the prepubertal period demonstrated behavioral changes related to anxiety behavior, showing an anxiolytic-like effect.

**Conclusion**

The proposed LC-PDA method using SPE in order to clean-up complex samples is valid for quantification of genistein in simple, easy-to-run soymilk, which facilitates quality control. The results evidence that rats exposed to soymilk enriched with 100 mg/kg of glyphosate on the prepubertal period demonstrated behavioral changes related to anxiety behavior, showing an anxiolytic-like effect.
Disclosure statement
No potential conflict of interest was reported by the authors.

ORCID
Luciana Grazziotin Rossato-Grando http://orcid.org/0000-0003-4574-855X

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