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Development of an analytical method to quantify total isoflavones in phytotherapic capsules using high-performance liquid chromatography

Liliane C. C. Auwerter,*[*,1] Ana E. Wanczinski,[2] Roberto S. Chiandotti[2]

[1]Departamento de Química e Biologia, Universidade Tecnológica Federal do Paraná, Brazil,
[2]Departamento de Alimentos e Drogas, Instituto de Tecnologia do Paraná, Brazil.

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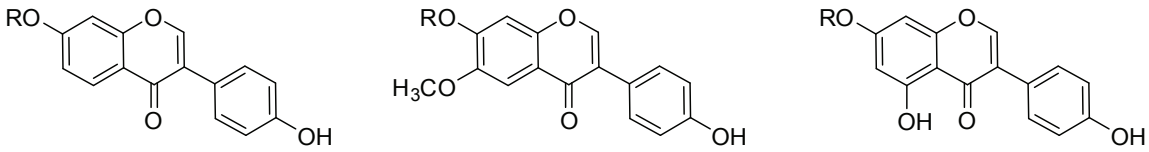
Abstract: Isoflavones can be found in grains and leaves of soybean. Currently, these are sold in pharmacies as phytotherapic capsules. Isoflavones have been recommended by doctors, especially for women, due to their ability to relieve menopause symptoms, among other benefits. However, no method exists for the official control of isoflavone content in capsules sold in the Brazilian market. This study aims to develop an appropriate analytical method to determine the total isoflavone content (daidzin, glycitin, and genistin, and their respective aglycone forms) in phytotherapic capsules purchased in pharmacies in Curitiba, Parana State, Brazil, using the technique of high-performance liquid chromatography with UV detection (UV-HPLC). The HPLC system consisted of a quaternary pump, an autosampler, and Waters reversed-phase C_{18} column (5 µm × 300 mm. Analyses were carried out at 40 °C, using a flow rate of 1.0 mL/min (acetonitrile and acetic acid 0.1%), and detection was performed at 254 nm. The method was validated as required by ANVISA and showed to be reliable for the following parameters: linearity (r^2 >0.99), selectivity (correlation between 0.99 and 1.00), precision (relative standard derivation <1.59%), accuracy (from 80% to 111.63% intraday and from 80% to 117.88% interday recovery), and robustness.

Introduction

Soybean (*Glycine max* (L.) Merr., Fabaceae) and its derivatives have been used as food products for several millennia. According to Ho et al. (2002), soy products, which are used as a major source of nutrition in China, can be divided into two categories: fermented and unfermentedproducts. The first category includes soy sauce, while the second category is represented by soy milk, soy sprouts, and soy flour. However, soy products are consumed not only as food, but also as isoflavone-containing capsules currently available in pharmacies. Currently, isoflavones have attracted considerable interest from the scientific community. Isoflavones are a subclass of flavonoids, which are described as phytoestrogens because of their estrogenic activity (Valls et al., 2009). They belong to the family of polyphenols, and, in publications and scientific reviews, have been linked to cancer prevention and relief of menopausal, such as prevention of breast cancer (Loibl et al., 2011; Magee et al., 2004; Patisaul & Jefferson, 2010), prevention of prostate cancer (Ganry, 2005), and even prevention of virus infections (Andres et al, 2009).

Many articles have been written on the identification and quantification of isoflavones or phytoestrogens (Wang et al., 2002) in soy products (such as capsules of soy germ, and nutritional supplements or other products containing soy) using high-performance liquid chromatography (HPLC) detection method (Micke et al., 2006; Griffith & Collison, 2001), nuclear magnetic resonance spectroscopy (Xiao et al., 2005), and other chromatographic. However, only a few researchers (such as César et al., 2006) have developed methods for quantifying isoflavones in capsules. If methods of different matrices are used to quantify isoflavones, it would generate heterogeneity in the quality control procedures, as is evident from the varying isoflavone contents in capsules nowadays.

Isoflavones can be found mainly in grains and leaves of soybean, in two types of structures: aglycones (or hydrolyzed, *i.e.*, daidzein (**1**), glycitein (**3**), and genistein (**5**)) and their glycosidic form (*i.e.*, daidzin (**2**), glycitin (**4**), and genistin (**6**)).



1 R=H
2 R=Glc

3 R=H
4 R=Glc

5 R=H
6 R=Glc

Because of the health benefits of isoflavones, their intake in the form of phytotherapic capsules is now recommended. However, until now, no specific methodology is used to control the concentration of this substance in soy products available in Brazil; thus, consumers do not have guarantee of quality for products they are buying.

Keeping in mind the growing consumption of the isoflavones, this study aimed at developing an appropriate analytical method to quantify the content of isoflavone capsules acquired from three different Brazilian manufacturers, using the technique of HPLC, following the established parameters of Anvisa (2003).

Materials and Methods

Samples, standards, and reagents

Three samples (named as Samples A, B, and C) from different Brazilian manufacturers were considered for the study. These samples were purchased from pharmacies at Curitiba, Parana State, Brazil. The manufacturers declared that each capsule of Samples A, B, and C contains 60, 170, and 60 mg of total isoflavones, respectively. The standards were donated by the Laboratory of Fine Chemistry at TECPAR, Curitiba, which refined these substances. Reagents used in this study included ethanol, methanol, acetonitrile, and acetic acid (HPLC grade) purchased from J.T. Bake; dimethyl sulfoxide (DMSO) purchased from Mallinckrodt; and distilled water purified in a Millipore system.

Instrument and HPLC conditions

Initial chromatographic conditions were based on those described in related publications (Adlercreutz, 2003; Griffith & Collison, 2001; Wu et al., 2004; Klejdus et al., 2005), which described methods of quantification of soy isoflavones by liquid chromatography. Following the procedures of these studies, trials were conducted to develop the mobile phase. The gradients of acetonitrile (gradient A) and 0.1% acetic acid (gradient B) were modified and optimized; the time period of analysis extended until the separation of peaks was satisfactory. After optimization, the mobile phase was gradient elution of acetonitrile/water (0-6 min 5.00% A and 95.00% B, 6-15 min 20.00% A and 80.00% B, and 15-20 min 25.00% A and 75.00% B). A chromatogram of this condition can be seen in Figure 1. The HPLC equipment used was a Hewlett Packard HP-1100 system, consisting of a quaternary pump, an autosampler, and a Waters reversed-phase C_{18} column (5 µm × 300 mm). Analyses were carried out at 40 °C, using a flow rate of 1.0 mL/min, and the detection was performed at 254 nm.

Preparation of standards and samples

Preparation of standards

Each standard was dissolved individually in DMSO (10% v/v) and methanol (90% v/v), then sonicated for 15 min for a better dissolution of the substance, and stored at 4 °C. Concentration of the stock solutions was around 100 µg/mL, which was diluted six times, with a solution of acetonitrile and water (1:1 v/v), to reach a concentration of 1 µg/mL.

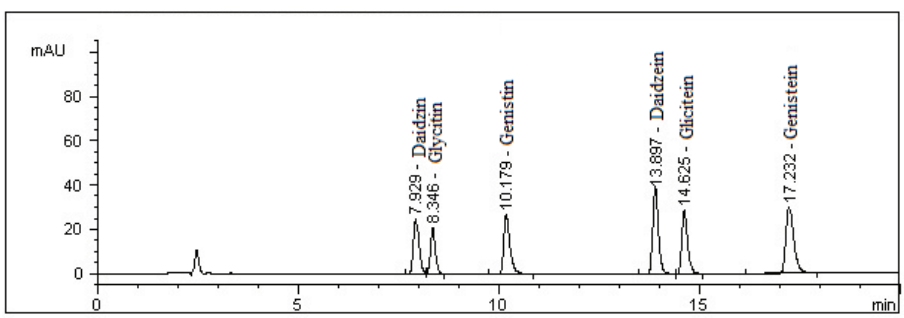


Figure 1. Chromatogram of the six isoflavones with the chosen conditions.

Preparation of samples

Ten capsules of Samples A, B, and C were mixed. From this mixture, the amount equivalent to one capsule was measured and added to a 100 mL volumetric flask containing 10 mL DMSO, and the final volume was made up with a solution of acetonitrile and water (1:1 v/v). Then, the samples were injected in the HPLC equipment and from the chromatogram the areas correspondent to each concentration were obtained.

Parameters of validation

The parameters used in this work followed a Brazilian regulation (Anvisa, 2003) that guides researches on validation methods. These parameters, which include selectivity, linearity, precision, accuracy, and robustness, should be evaluated in order to guarantee the reliability of the method.

Selectivity

Each sample was analyzed and compared with the six isoflavone standards in an absorption test of UV light using different wavelengths (230, 240, 250, 260, 270, 280, 290, and 300 nm) to ensure that the isoflavone peaks were free from any interference.

Linearity

Aliquots of a minimum of five concentrations (in a linear range) were prepared from the stock solutions: daidzin (1.62-104.02 µg/mL), glycitin (0.71-89.88 µg/mL), genistin (0.94-119.92 µg/mL), daidzein (0.81-103.68 µg/mL), glycitein (1.52-97.46 µg/mL), and genistein (0.79-100.76 µg/mL). The data obtained for each isoflavone sample were submitted to regression analysis and the correlation coefficients were calculated using Microsoft Office Excel®.

Precision

Intra- and interday precision data were analyzed using three samples of each manufacturer and the relative standard derivation (RSD) was calculated; the interday precision was evaluated two days after the injection of sample for intraday precision.

Accuracy

Three concentration levels of each isoflavone standard were added to the three samples (in triplicate). The error was calculated using the recovery percentage, and an interday injection was obtained at two consecutive days to ensure the accuracy reliability.

Robustness

Three modified analytical parameters of the established method were as follows: pH of the mobile phase, use of sonication, and dilution of samples. The pH was modified by increasing the concentration of acetic acid from 0.1% to 0.5% on the mobile phase. In the second step, the use of sonication was discontinued, and finally the acetonitrile/water dilution solvent media was replaced with an ethanol/water mixture. The Student's *t*-test was applied for the obtained data (95% confidence interval) to ensure that the modifications do not interfere with the results statistically.

Quantification of the pharmaceutical isoflavones capsules

Once the regression analysis was done, it was possible to obtain the linear equations of each standard. Therefore, data from sample analyses were used to solve the linear equations to obtain the concentrations of total isoflavones.

Results and Discussion

Selectivity

All the samples showed the same profile of standards and correlations between 0.99 and 1.00, ensuring that the peaks of interest are free from any interference.

Table 1. Linearity parameters of isoflavones standards.

Isoflavones	Linear equations $y = ax + b$	Correlation coefficients (R^2)	Linear range (µg/mL)	Angular coefficient	Standard of significance (%)
daidzin	y = 43.60x – 11.10	0.99870	1.62–104.02	43.60	<5.00
glycitin	y = 37.94x + 3.72	0.99998	0.71–89.88	37.94	<5.00
genistin	y = 48.82x + 47.96	0.99880	0.94–119.92	48.82	<5.00
daidzein	y = 65.05x + 42.72	0.99870	0.81–103.68	65.05	<5.00
glycitein	y = 52.83x – 5.00	0.99996	1.52–97.46	52.83	<5.00
genistein	y = 76.46x – 7.62	0.99992	0.79–100.76	76.46	<5.00

Linearity

Linearity parameters given in Table 1 show a linear relationship among data.

Precision

Average values of RSD for intraday precision of the six isoflavones analyzed were no higher than 2.69%. The interday precision results showed no RSD higher than 1.59%.

Accuracy

The method applied for the samples showed that the recovery was 80-111.63% for the six isoflavones and 80-117.88% for the interday test.

Robustness

The Student's *t*-test showed that the obtained data were not considered to be statistically significant for the three changes made.

Quantification of the pharmaceutical isoflavone capsules

Concentrations of the six isoflavones in each sample (Table 2) were calculated using the average area of the chromatograms in linear equations and compared with the concentrations stated on the manufacturer's labels (see Figure 2).

Table 2. Concentrations of the six isoflavones on the samples compared with the concentrations stated on the labels (mg per capsule).

Isoflavones	Concentration (mg)		
	Sample A	Sample B	Sample C
daidzin	02.813	00.000	20.587
glycitin	01.018	00.000	01.767
genistin	01.212	00.000	36.240
daidzein	24.654	00.000	03.012
glycitein	01.072	00.026	00.691
genistein	00.854	00.080	02.433
Total (mg per capsule)	31.623	00.106	64.731
Concentration stated on the labels (mg per capsule)	60.000	270.000	60.000

As can be seen from Figure 2, only Sample C had concentration of isoflavones close to the value stated on its label. Sample A had about half the stated value, and Sample B had very low concentrations of isoflavones, most of which could not be detected. This could be explained by a large variation in isoflavone content as soybeans grow at different locations; however, the result of Sample B puts the reliability of the manufacturer in doubt.

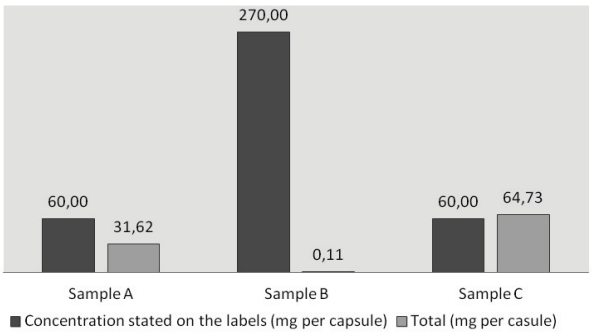


Figure 2. Concentration of total isoflavones in Samples A, B, and C (mg per capsule).

Conclusions

This study describes an effective and appropriate method for quantifying the six most common isoflavones present in commercial capsules available in Brazil, using HPLC technique. In addition, it was observed that the quality of this type of medicine must be monitored to provide consumer with a guarantee that the product has the same concentration of isoflavone as described on the label.

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***Correspondence**

Liliane C. C. Auwerter
Departamento de Química e Biologia, Universidade Tecnológica Federal do Paraná
Rua Professor Assis Gonçalves, 1152, Água Verde, 80 620-250 Curitiba-PR, Brazil
anaelisa@tecpar.br
Tel. +55 41 9941 8334