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The use of HPLC identification and quantification of isoflavones content in samples obtained in pharmacies

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

*[1]Programa de Pós-graduação em Tecnologia de Alimentos, Departamento de Engenharia Química, Universidade Federal do Paraná, Centro Politécnico, Setor de Tecnologia, Rua Francisco H. dos Santos, s/n, Cx. Postal 19011, 81531-980, Curitiba, Paraná, Brazil. [2]Departamento de Agronomia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. [3]Departamento de Engenharia Elétrica, Unversidade Estadual do Oeste do Paraná, Cascavel, Paraná, Brazil. [4]Departamento de Zootecnia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. [5]Departamento de Farmácia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. *Author for correspondence. Email: mantovanidaniel@hotmail.com*

ABSTRACT. Nowadays, there is a great interest in the research and the production of functional foods, such as isoflavones. Which present proven action on the prevention of health problems, such as cancer and cardiovascular diseases. Considering the importance of soybean supplements standardization, this study aimed identifying and quantifying the isoflavones in products sold at different pharmacies. Isoflavones samples were acquired in six different pharmacies specialized in the production of phytotherapic medications. The isoflavones were extracted, quantified and identified in HPLC. None of the samples, from different pharmacies, presented the isoflavones content presented on the label, i.e., 20 mg. In relation to the isoflavones profile, the highest levels found were of daidzein, with up to 66.8 $\pm$ 0.09 μg, being the lowest levels the ones of genistein. It is necessary, therefore, the adoption of methods for the standardization and characterization of the raw materials used by different pharmacies. It would assure the isoflavones content, once they are essential for the effects prescribed by the professionals in the medical area.

Key words: daidzein, genistein, functional compounds, soybean, quantity.

RESUMO. O uso do HPLC na identificação e quantificação dos teores de isoflavonas em amostras obtidas de farmácias de manipulação. O uso do HPLC na identificação e quantificação dos teores de isoflavonas em amostras obtidas de farmácias de manipulação. Atualmente há um grande interesse na pesquisa e produção de alimentos funcionais, como as isoflavonas, que apresentam comprovada ação na prevenção de males, como o câncer e as doenças cardiovasculares. Considerando a importância da padronização dos suplementos de soja, o objetivo do presente estudo foi de identificar e quantificar as isoflavonas em produtos comercializados em diferentes farmácias de manipulação. Amostras de isoflavonas foram adquiridas em seis diferentes farmácias especializadas em manipulação de fitoterápicos. As isoflavonas foram extraídas, identificadas e quantificadas em CLAE. Nenhuma das amostras, das diferentes farmácias de manipulação, apresentou os teores de isoflavonas indicado no rótulo, ou seja, 20 mg. Quanto ao perfil de isoflavonas, os maiores teores encontrados nas amostras foi de daidzeína com até 66,8 $\pm$ 0,09 μg, e os menores de genistina. Sendo assim, é necessário que sejam adotados métodos de padronização e caracterização das matérias-primas utilizadas nas diferentes farmácias de manipulação. Assegurando os teores de isoflavonas, os quais são imprescindíveis para os efeitos prescritos pelos profissionais da área médica.

Palavras-chave: daidzeína, genisteína, compostos funcionais, soja, quantidade.

Introduction

Soybean (*Glycine max* (L.) Merrill) is originally from China, belonging to the Leguminosae; its consumption, in derivate products, has been associated to the reduction of chronic diseases risk (MESSINA; BARNES, 1991).

Recently, there has been a great interest in the research and in the production of functional foods, for they present proven action on the prevention of health problems, such as cancer (MESSINA, 1999), including cancer of the endometrium and prostate as well as breast, cardiovascular diseases, osteoporosis, and menopausal symptoms (MESSINA et al., 2006). Among the functional foods, we can mention soybean derivates, rich in isoflavones, which recently have been gaining market space, due to the beneficial effects to human (WANG; MURPHY, 1994).

Isoflavones structural and chemical variation is high, and only soybeans contain three kinds of isoflavones with 4 isomeric forms, in total, 12 different kinds of this compound. The forms that appear to be the most active ones in human body are: daidzein, genistein and glycitein (DEWICK, 1994). The genistein is one of the two most important isoflavones in soya; it has been calling much attention, not only for its potential anti-estrogenic effect, but also because it inhibits various enzymes involved in carcinogenisis processes (MACKEY; EDEN, 1998). The genistein concentration in most part of soy products varies from 1 to 2 mg g^{-1} (BARNES et al., 1995). Values of concentrations of isoflavones in soya beans are affected by genetic and environmental factors: type of cultivate, planting location, climate, year of harvest, soil type and the interactions between these factors (SETCHELL, 1998).

Phytoestrogens are a broad group of nonsteroidal compounds of different structures, falling into three main classes: isoflavones, coumestans, and lignans. These molecules have a common diphenolic group which gives them stability and which has been shown to bind to estrogen receptors (TURNER et al., 2007). Phytoestrogens have similarity in structure to the human female hormone17-β-estradiol, bind to both alpha and beta estrogen receptors, mimic the action of estrogens on target organs, and exert many health benefits against hormone-dependent diseases (CASHMAN, 2007; DUFFY et al., 2007; SYED et al., 2007). The aglycones forms daidzein and genistein are also very frequently used as medicines against prostate cancer, when associated with glucosilated forms daidzin and genistin (LI et al., 2006).

The daidzein isoflavone, together with genistein, is considered to be the most abundant phytoestrogen in soybean and its derivates. On the other hand, glycitein is the least abundant and also the least studied isoflavone. The chemical structure of glycitein is similar to the one of genistein and daidzein, thus it can be supposed that it presents physiological activities similar to human metabolism (SONG et al., 1999).

The aim of this study was identifying and quantifying isoflavones compounds in products sold in different pharmacies.

Material and methods

Samples obtaining

Isoflavones samples were acquired in six different pharmacies three in Maringá city (A, B and C) in northwest of Paraná and three in Foz do Iguaçu (D, E and F) west of Paraná, Brazil. The pharmacies specialized in phytotherapic products. The compounds prepared in the pharmacies were acquired in capsule form. According to their label, each of them should contain 20 mg of isoflavones. The shelf life, indicated in their labels, was six months for all the samples. It was not possible to obtain information about the suppliers of the isoflavones compounds used in their production. The samples were stocked at the temperature of 4°C, until the analysis was started.

Isoflavones Extraction

Isoflavones extraction was carried out according to the methodology mentioned by Grün et al. (2001). Aliquots of 1.00 g were removed and homogenized in 15 mL of methanol 80%, under constant agitation for 30 minutes. After that, the centrifugation at 5,000 g for 15 minutes was carried out. The supernatant was filtered with filter paper (Whatman nº 1), and transferred to a volumetric flask. The precipitate in the centrifuge tube received then 10 mL of methanol 80%, was homogenized for 30 minutes, centrifuged for 5 minutes at 5,000 g, and the supernatant was filtered with filter paper (Whatman no. 1). The supernatants were gathered in a volumetric flask and concentrated in a rotaevaporator (Rotavapor® Fisatom), with 40°C bath temperature. For the chromatographic analysis, the concentrated samples had their volume adjusted with methanol 80%, and were filtered in membrane 0.20 μm (Alltech, Deerfield, IL), before the injection.

Reactants and standards

The isoflavones standards daidzin, genistin, daidzein and genistein were acquired from Sigma Chemical Co (St. Louis, EUA). Methanol and acetic acid chromatographic level were acquired from J. Backer, and water was purified in the system Milli-Q Millipore (Bedford, MA, USA), and filtered in membrane 0.45 μm (Alltech, Deerfield, IL).

Isoflavones identification and quantification through HPLC

Samples of the purified extract had their bulk adjusted with methanol 80%, and filtered in polyethylene filters with PTFE membrane (Millipore Ltd. Bedford, E.U.A.) of 0.45 μm pore, before the injection.

Isoflavones compounds identification and quantification were carried out through high performance liquid chromatography (Gilson 321), with a secondary pump, deaerator, automatic injector, detector (UV-Visible), and the software program Boriwn version 1.5 ©JMBS. The chromatographic conditions described by Song et al. (1998) were used C_{18} coated column Lichrospher, of Merck (250 x 4,6 mm, 5 μm) was used, at 30°C; mobile phase was constituted of acetic acid and methanol (19:1, v v^{-1}) with 1 mL $min.^{-1}$ initial flow; detection at UV-Visible 254 nm; and injection volume of 20 μL. The calibration curve was prepared using authentic standards in concentration of 0.25 to 0.1 mg mL^{-1} diluted in mobile phase. Peaks of soy isoflavone glucosides and aglycones were identified by matching retention times (dadzin, genistin, daidzein and genistein). Samples were injected in duplicate. The results were expressed in (100 μg g^{-1}) after normalization of differences in molecular wight glycosylated forms of the multiplying it made the mass of each derivative by the ratio between the molecular weight of the aglycones and the molecular weight glycosylated form.

Data statistical analysis

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

Results and discussion

Table 1 presents the content of isoflavones, daidzin, genistin, daidzein and genistein compounds, found in samples from six different pharmacies.

The samples of pharmacy C presented the highest total content of isoflavones, with practically 60% of daidzein.

The lowest total content of isoflavones was observed in samples from pharmacy E, almost 12% of the total content found in pharmacy C. It showed the great disparity among the products found in pharmacies.

None of the samples analyzed presented the isoflavone content indicated on the label, i.e. 20 mg. Studies carried out by Nurmi et al. (2002), with fifteen soybean-based supplements sold in Finland showed that, out of the eleven ones that specified the isoflavones content, only one had the content presented on the label. The ten remaining products presented isoflavones content from 23 to 69% lower than the content indicated in their labels. Setchell et al. (2001), studying samples of supplements containing isoflavones, observed that, among 30 markets that declared the isoflavones content, 24 of them declared lower content than indicated.

In relation to the isoflavones found in the pharmacies samples (Table 1), it can be observed a higher concentration of daidzein, with average up to 66.8 ± 0.09 μg. On the other hand, genistin was the isoflavone found to be with the lowest concentration, not being detected in the samples of pharmacy E. Genistein was not found in the samples of pharmacies D and E. This great difference observed in the isoflavones profile is mainly due to the lack of standardization of the raw materials used in the production of these compounds.

The raw materials most frequently used as isoflavones sources are soybean germen and concentrated soybean extract, which present great differences in their composition: in the hypocotyls of the plant, daidzin and glycitein are found, while in the cotyledon, there is 20 times as much genistin (ELDRIDGE; KWOLEK, 1983).

Considering the fact that none of the samples studied presented the isoflavones content presented on the label, it is necessary the adoption of methods for the standardization and characterization of the raw materials used by different pharmacies. It would assure the isoflavones content, which is extremely necessary for the effects prescribed by professionals in the medical area. On the other hand, in Brazil, ANVISA itself still does not have methodological standardization for the analysis and for the way of expressing isoflavones content so that these products can be inspected.

Table 1. Isoflavones level (100 μg g^{-1}) found in sample obtained from six different pharmacies.

Pharmacies	Daidzin	Genistin	Daidzein	Genistein	Isoflavones level (100 μg g^{-1})
A	8.51 ± 0.11c	7.12 ± 0.11a	3.41 ± 0.10f	16.21 ± 0.55b	35.20 ± 0.21d
B	7.06 ± 0.51c	5.22 ± 0.12c	28.30 ± 0.34d	18.02 ± 0.14a	58.50 ± 0.11c
C	18.20 ± 0.15b	6.03 ± 0.05b	61.31 ± 0.10b	18.01 ± 0.56a	103.50 ± 0.04a
D	20.03 ± 0.73a	7.61 ± 0.06a	66.82 ± 0.09a	nd	94.40 ± 0.02b
E	2.41 ± 0.03d	nd	10.23 ± 0.17e	nd	12.60 ± 0.09e
F	7.73 ± 0.06c	6.30 ± 0.05b	34.01 ± 0.39c	9.91 ± 0.17c	57.90 ± 0.15c

nd = non detected. *Different letters in the same column indicate significant difference at 5% level.

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