

QUALIDADE MICROBIOLÓGICA DAS CÁPSULAS DE ISOFLAVONAS COMERCIAIS E QUANTIFICAÇÃO DE SEUS TEORES

MICROBIOLOGICAL QUALITY OF COMMERCIAL CAPSULES OF ISOFLAVONES AND QUANTIFICATION OF THEIR CONTENT

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Resumo

A pesquisa despertou um grande interesse na produção de alimentos funcionais, como as isoflavonas, com comprovada ação na prevenção de problemas de saúde, como o câncer e doenças cardiovasculares. Considerando a importância da normalização de suplementos de soja, o estudo objetivou identificar e quantificar as isoflavonas nos produtos vendidos em diferentes farmácias. Amostras de isoflavonas foram adquiridas em seis diferentes farmácias especializadas na produção de medicamentos fitoterápicos. As isoflavonas foram extraídas, quantificadas e identificadas em HPLC. Nenhuma das amostras nas diferentes farmácias apresentou o conteúdo de isoflavonas descrito no rótulo, ou seja, 20 mg. Em relação ao perfil de isoflavonas, os mais altos níveis encontrados foram de daidzeína, com até 66,8 µg e menores valores de genisteína. Portanto, é necessário a adoção de métodos de padronização e caracterização das matérias-primas e certificar que o conteúdo de isoflavonas, uma vez que são essenciais para os efeitos prescritos por profissionais da área médica.

Palavras-chave: *quantidade; supervisão; compostos funcionais; micro-organismos.*

1 INTRODUCTION

Soybean (*Glycine Max* (L.) Merrill) is originally from China, belonging to the Leguminosae group. The Isoflavones phytoestrogens (diphenol compounds), present in this group are similar to the human estrogen (estradiol) but much less potent. Because of this similarity, isoflavones have been suggested to have preventive effects for many kinds of hormone-dependent diseases (CHOI and RHEE, 2006). However, one must be very cautious in using isoflavones products, because an increasing number of studies are showing little or no effect of isoflavones on many such diseases (PARK; YU-MI; KWON, 2010).

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

In Brazil evaluated soybean participation in human diet through grain consumption and foods made of it in soybean derivatives production, from which isoflavones are extracted and commercialized in powder, pills and similar forms designated as nutritional supplement (GENOVESE and LAJOLO, 2001; MANTOVANI et al. 2011).

Further microbiological contaminants research which may complement the recommendations reported by World Health Organization (WHO) is also possible (WHO, 1998). Researches carried out by Kneifel, Czech, Kopp (2002) investigated microbiological quality of vegetal drugs and derivatives in other countries, which demonstrate microbial contaminations in disagreement with internationally established rules for medicaments (MESSINA and BARNES, 1991; MARTINS et al. 2001). In Brazil herbal commercialized in pharmacies showed similar results (MACIEL et al. 2002). Depending on prescriptions, you can obtain capsules containing various amounts of isoflavones on compounding pharmacies Mantovani et al. (2009) and Mantovani (2013b) quantified the isoflavones levels presents in defatted soy flour (DSF) derivative and textured soy protein (TSP) resulting in values below the expected levels of isoflavones.

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 non steroidal compounds of different structures, falling into three main classes: isoflavones, coumestans, and lignin's. These molecules have a common diphenolic group which gives them stability and which has been shown to bind to estrogen receptors (CHOI and RHEE, 2006). Phytoestrogens have similarity in structure to the human female hormone 17- 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; PEREZ; PARTRIDGE, 2007; SYED et al. 2007).

Among the main fungi genera found in Brazil are *Cladosporium*, *Fusarium*, *Aspergillus*, *Penicillium* and *Rhizopus*. The presence of these fungi in herbal medicine might be harmful to human health due to the appearance of illnesses when (KNEIFEL, CZECH, KOPP, 2002).

Currently, depending on medical prescriptions, capsules with different contents of isoflavones can be obtained in manipulation pharmacies. Therefore raw materials isoflavones are

fractionated by the suppliers to provide orders with specific quantities, which might increase the microbial contaminant load in this process (MARTINS et al. 2001).

Considering the significant increase in commercialization and consumption of isoflavones capsules in manipulation pharmacies, the present study was aimed at evaluating the microbiological quality of such products, and their adequacy according to the Brazilian legislation for products from herbal medicines manipulated in Brazil, using the high-performance liquid chromatography (HPLC) identification and quantification of isoflavones content in samples obtained in pharmacies.

2 MATERIALS AND METHODS

Samples acquisition

Isoflavones samples were acquired in six different pharmacies, (A, B, C, D, E and F) of Brazil. The pharmacies are specialized in herbal medicines. The compounds prepared in the pharmacies were acquired in capsules. 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 compounds used in production.

Microbiology Analyses

The methodology used for the microbiological analysis was carried out as described by VANDERZANT et al. (1992) following the Compendium of Methods for the Microbiological Examination of Foods. Analyses to detect coliforms were carried out at 45°C. Analyses to detect Coliforms at 45°C, most probable number (NMP/g⁻¹), *Staphylococcus* sp, *Bacillus* sp, fungi and yeasts, colony formation unity (UFC/g⁻¹), and the presence or absence of *Salmonella* sp in 25g were carried out. For the fungus and yeast analyses, the quantification method described by US methodological Pharmacopeia was used (PHARMACOPEIA, 2005).

Isoflavones Extraction

Isoflavones extraction was carried out according to the methodology mentioned by (GRÜN et al. 2001; MANTOVANI et al. 2013a). Aliquots of 1.0 g were removed and homogenized in 15 mL of methanol 80%, under constant agitation for 30 minutes. After that, the centrifugation at 5.000 rpm for 15 minutes was carried out. The supernatant was filtered with filter paper (Whatman n° 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 rpm, and the supernatant was filtered with filter paper (Whatman n° 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

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, desaerator, automatic injector, detector (UV-Visible), and the software program Brown version 1.5[©] JMBS. The chromatographic conditions described by (SONG et al. 1998) were used C₁₈ 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 glycosides 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 weight 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 duplicate. 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.

4 RESULTS AND DISCUSSION

Among the several parameters which determine food quality, the most important are undoubtedly those which define its microbiological characteristics (ABOU-ARAB et al. 1999).

Therefore, the 357 Resolution in April 20th 2001 technical regulation of the Practice of Pharmacy (BRASIL, 2001a). The results for microbiological analyses of bacteria in the isoflavones capsules samples manipulated by pharmacies according to the 12th RDC establishes maximum of 10 UFC/g for Coliforms at 45°C and 5×10^2 for *Staphylococcus* Coagulase positive. According to results obtained and presented in Table 1, all analyzed samples are in agreement with legislation.

Table 1- Microbial analysis of bacteria present in isoflavones capsules manipulated by pharmacies.

<i>Manipulation pharmacies</i>	A	B	C	D	E	F
<i>Coliforms</i> at 45 °C NMP/mL ⁻¹						
<i>Staphylococcus coagulase</i> (+) UFC/g ⁻¹						
<i>Staphylococcus sp</i> UFC/g ⁻¹		1.2×10^3				
<i>Bacillus sp</i> UFC/g ⁻¹		9.8×10^2	3.1×10^2			6.9×10
<i>Salmonella sp</i> 25 g ⁻¹	abs.	abs.	abs.	abs.	abs.	abs.

For microorganisms *Staphylococcus sp* and *Bacillus sp* the 12th RDC resolution of January, 2001 does not cite values of contamination (BRASIL, 2001b). The presence of *Staphylococcus sp* indicates food contamination, which might be caused by lack of hygiene during manipulation (BUGNO, 2006). All isoflavones capsules analyzed presented absence of *Salmonella sp*. This way they are agreement with current legislation, which requires the absence of *Salmonella sp* in 25 grams of the product (BUGNO, 2006).

From the forty isoflavones capsules analyzed by 25 only four (10%) were considered suitable for consumption whereas thirty-six (90%) were declared unfit for human consumption, from which thirty-two (80%) presented more than 10^3 UFC/g⁻¹, besides identification and isolation of the following bacteria: 15% *Escherichia coli*, 2.5% *Staphylococcus aureus*, 2% *Pseudomonas aeruginosa*, *Salmonella sp* and *Shigella sp* 27.5% among samples.

The 12th RDC resolution of January, 2001 Brasil, (2001b) do not predict maximum and minimum values for fungus and yeast in foods and similar products. In the case of herbal medicines, the maximum limit allowed by the World Health Organization for filamentous fungi is 5×10^2 UFC/g⁻¹ (VANDERZANT et al. 1992). Table 2 presents microbiological values found for fungus and yeasts, which makes the problems related to packaging during storage evident once all samples analyzed showed the presence of one or more types of fungi and yeasts such as *Rhodotorula sp*, *Mucor*, *Candida sp*, *Aspergillus niger* and *Cladosporium sp*.

Table 2- Microbiological analysis of fungi and yeasts detected in isoflavone capsules.

Samples	Manipulation pharmacies	
	Counting in (UFC/g ⁻¹)	microorganisms
A	5.3x10	<i>Rhodotorula</i> sp.; <i>Mucor</i> sp.; Yeasts
B	5.4x10 ²	Yeasts
C	1.0x10 ²	<i>Rhodotorula</i> sp.; Yeasts
D	2.8x10 ²	<i>Mucor</i> sp.; <i>Cladosporium</i> sp
E	2.1x10 ²	<i>Candida</i> sp.; Yeasts
F	6.6x10 ²	<i>Aspergillus niger</i> ; <i>Mucor</i> sp.; <i>Cladosporium</i> sp

Studies conducted by Bugno, (2006) in forty capsules samples described that 25% showed contamination by fungus and yeasts with values superior to 10² UFC/g⁻¹. They identified the presence of *Aspergillus* spp, *Rhizomucor* sp, *Acremonium* sp, *Cândida* sp, *Fusarium* spp, *Penicillium* spp and *Rhizopus* sp in approximately 5% of the samples.

Molds and yeasts are more resistant to low water activity and acid pH than bacteria. Hence, they deteriorate foods from vegetables, which might even cause food spoiling depending on the development stage. Among deteriorating microorganisms are *Mucor* ssp, *Rhizopus* ssp, and *Aspergillus niger* (MARTINS, 2001). Samples of isoflavone capsules presented acceptable contamination rates by bacteria (Brasil, 2001b). However, the incidence of fungus and yeasts was expressive, which demonstrates the necessity for a better microbiological control of raw material in order to guarantee safety, efficiency and quality of manipulated products (CALIXTO, (2000).

According to Mantovani et al. (2011), relating the levels of isoflavones present obtained in manipulation pharmacies. The Table 3 presents the content of isoflavones, daidzin, genistin, daidzein and genisteína compounds, found in samples from six different pharmacies.

Table 3- 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	8.5 ± 0.1c	7.1 ± 0.1a	3.4 ± 0.1f	16.2 ± 0.5b	35.2 ± 0.2d
B	7.0 ± 0.5c	5.2 ± 0.1c	28.3 ± 0.3d	18.0 ± 0.1a	58.5 ± 0.1c
C	18.2 ± 0.1b	6.0 ± 0.0b	61.3 ± 0.1b	18.0 ± 0.5a	103.5 ± 0.0a
D	20.0 ± 0.7a	7.6 ± 0.0a	66.8 ± 0.0a	0.00	94.4 ± 0.0b
E	2.4 ± 0.0d	0.00	10.2 ± 0.1e	0.00	12.6 ± 0.0e
F	7.7 ± 0.0c	6.3 ± 0.0b	34.0 ± 0.3c	9.9 ± 0.1c	57.9 ± 0.1c

*Different letters in the same column indicate significant difference at 5% level.

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. According Song et al. (1998); Nurmi et al. (2002) is recommended express the forms that are absorbed the aglycones, express the isoflavones content of commercial products is to normalize the results to the mass of aglycones and/or present the molar quantities of isoflavones.

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 µg. On the other hand, genistein 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 genistina (ELDRIDGE and 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, National Health Surveillance Agency (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.

4 CONCLUSIONS

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. On the other hand, in Brazil, ANVISA itself still does not have methodological standardization for analyses isoflavones and

content expression. However microbiological analysis of isoflavones capsules presented acceptable contamination rates by bacteria. However, the incidence of fungus and yeasts was expressive, which demonstrates the necessity for a better microbiological control of raw material in order to guarantee safety, efficiency and quality of manipulated products.

Abstract

The survey awakened a big interest in the production of functional, such as isoflavones, with 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 phytotherapeutic 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 µg, being the lowest levels the ones of genistein. Therefore, it is necessary to adopt methods of standardization and characterization of raw ensure that the content of isoflavones, because they are essential for the purposes prescribed by medical professionals.

Key-words: quantity; supervision; functional compounds; micro-organisms.

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