International Journal of Food Sciences and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713425816

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To link to this article: DOI: 10.1080/09637480601149350
URL: http://dx.doi.org/10.1080/09637480601149350

International Journal of Food Sciences and Nutrition,
March 2007; 58(2): 116–124

informa
healthcare

Analysis of isoflavonoids from leguminous plant extracts by RPHPLC/DAD and electrospray ionization mass spectrometry

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Abstract
Traditionally, food is associated with energetic and nutritious characteristics such as sources of carbohydrates, proteins and lipids. Recently, however, foods with bioactive substances such as isoflavones have received great attention. The objective of this study was therefore to evaluate the presence of the isoflavones daidzein, glycitein, genistein and their conjugated forms in grains and leaves of several leguminous plants utilized largely in Brazilian cuisine. Grains used in Peruvian cuisine were also analyzed. After extracting phenolic compounds with methanol (80%), isoflavones as detected by reversed-phase high-performance liquid chromatography/diode-array detector were only found in chickpeas and soybean. Chickpea extracts showed only the isoflavone genistein at 31 μg/g defatted flour. Detection of these isoflavones was confirmed by electrospray ionization mass and tandem mass spectrometric experiments. For soybean, a distinct distribution of isoflavones was found in hypocotyls and cotyledon. The highest concentration of isoflavones found was approximately 5.9 mg/g for hypocotyls, whereas the total concentration of isoflavone was around 0.4 mg/g for the cotyledons (dry matter). These results indicate that isoflavone concentrations vary within the different tissues of the leguminous species tested.

Keywords: *Isoflavones, Fabaceae, alfalfa, leaves, grains, electrospray ionization mass spectrometry*

Introduction

Besides nutrition, foods from plants contain different chemical compounds, which present different biological activities, such as antioxidant, anti-inflammatory, antiviral, or protection against ultraviolet-B radiation (Harborne and Williams 2000; Lima et al. 2004). The isoflavonoids, for example, are a group of chemical compounds with phenolic structures that have been associated with certain biological properties in leguminous plants, such as soybeans, chickpeas and peas (Lima et al. 2004; Simmonds and Stevenson 2001). The isoflavonoids (Figure 1) are a subclass of the extensive group of flavonoids characterized by a polyphenolic structure, with two

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ISSN 0963-7486 print/ISSN 1465-3478 online
DOI: 10.1080/09637480601149350

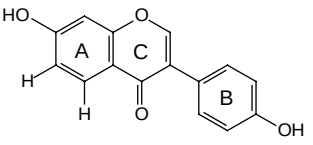

Figure 1. Chemical structure of soybean isoflavone.

phenolic rings (A and B) linked to a heterocyclic ring (C), with more than 360 aglycone forms (Dewick 1993; Heller and Forkmann 1993; Griffith and Collison 2001). Biochemical studies in plants, mainly with soybean (*Glycine max* L.), have found an enzyme capable of transform flavanones (naringenin and liquiritigenin) to isoflavones (genistein and daidzein, respectively) (Hagmann and Grisebach 1984).

The isoflavones are distributed within a small group of plants, being restricted mainly to Papilionoideae, Caesalpinioideae and Mimosoideae, which are all in the family Leguminosae (Dewick 1993). Isoflavonoids are frequently involved in the plant/bacterial signaling that results in nodule formation of leguminous roots (Hartwig et al. 1990). During seed germination, alterations in the composition of biochemical constituents, antinutritional factors, as well as biologically active compounds, such as isoflavonoids, have been observed (Bau et al. 2000).

The isoflavone content in soybeans has been widely studied, with daidzein, genistein and its glucoside forms (daidzin and genistin) comprising most of the isoflavones found in soybeans and in soy products (Ho et al. 2002; Tsukamoto et al. 2001). However, other isoflavonoids—formononetin (detected by mass spectrometry as the ion of m/z 268) (Merken and Beecher 2000), biochanin A (m/z 284) (Merken and Beecher 2000), puerarin (m/z 417) (Hirakura et al. 1997), wighteone (Katagiri et al. 2000) or luteone (Katagiri et al. 2000)—have been identified from several parts of the plant, such as roots, stems, leaves and seeds. To understand the isoflavone composition of plants used in Brazilian food, the present study was performed to verify the presence of different isoflavone forms and to compare the composition of these compounds in different plant tissues.

Materials and methods

Chemicals

Glacial acetic acid and methanol (HPLC grade; Merck, Darmstad, Germany) were filtered through 0.22-μm PTFE filters (Millipore, Sao Paulo, Brazil). Daidzein (Extrasynthese, 99%), glycitein (Fujico, 95%), genistein (Extrasynthese, 99%, Genay, France), daidzin (Extrasynthese, 99%), glycitin (Fujico, Tokyo, Japan 95%), and genistin (Extrasynthese, 97%), 6″-*O*-malonyldaidzin (Fujico, 90%), 6″-*O*-malonylglycitin (Fujico, 90%), and 6″-*O*-malonylgenistin (Fujico, 90%) were used as standards.

Plant samples

Chickpea (*Cicer arientinum*), pigeonpeas (*Cajanus cajan*), snapbean (*Phaseolus vulgaris*), favabean (*Vicia faba*), giant Peruvian lima (*Phaseolus limensis*), English peas (*Pisum sativum*) and soybean (*Glycine max* L.) grains, from the Leguminosae family, were analyzed in this work. Two common Peruvian grains, kiwicha

(*Amaranthus caudatus*) and kañiwa (*Chenopodium pallidicaule*) were collected in Peru, together with pallar (*Phaseolus lunatus*), and favabean. Fresh leaves, and stems of alfalfa (*Medicago sativa*), which were collected in the Fazenda Rio das Pedras (Campinas, Brazil), were also analyzed in this work.

Sample preparation

The isoflavones were extracted from dry grains, leaves, and stems according to Fukutake et al. (1996). An amount of each sample was macerated and passed through sieves of 100–200 mesh (Tyler series). The flour obtained was defatted with hexane (Synth, P.A., Sao Paulo, Brazil) for 30 min at 26°C, maintaining the proportion 1:20 (w/v). The suspension obtained was centrifuged and the solids (10 g) were separated from the supernatant and dried under a hood at 26°C. Samples (1 g) from each flour type were submitted to alcoholic extraction with 20 ml of 80% methanol at 26°C for 1 h. After extraction, the mixture was centrifuged at 5000 $\times g$ for 10 min to separate the supernatant, and they were used for analyses.

Chromatographic analysis

Reversed-phase high-performance liquid chromatography (RPHPLC) analysis was carried out with the Shimadzu SPD-M10Avp using a YMC Pack ODS-A column (Kyoto, Japan). Elution was carried out at a flow rate of 1 ml/min using a solvent gradient consisting of a linear increase from 20% to 80% solvent A (methanol, PA; Synth) in solvent B (water) (Park et al. 2001a, 2002). Eluted isoflavones were detected by their absorbance at 254 nm. Quantitative data for daidzein, glycitein, genistein, and their malonyl and glucoside forms were obtained by comparison with authentic standards. The isoflavone forms were identified by electrospray ionization mass spectrometry (ESI-MS).

ESI-MS analysis

ESI-MS experiments were performed on a Q-Tof (Micromass, Manchester, UK) orthogonal acceleration quadrupole – time-of-flight mass spectrometer equipped with an ESI ionization source with a Z-spray configuration and main operation conditions as described elsewhere (Aguiar 2004; Nitschke et al. 2004). The following typical operating conditions were used: 3.3 kV capillary voltage, 35 V cone voltage, and 100°C dessolvation gas temperature. Tandem ESI-MS/mass spectrometric (MS) spectra were collected after 15 eV collision-induced dissociation of mass-selected ions with argon. Mass selection was performed by Q1 using a unitary m/z window, and collisions were performed in the r.f.-only quadrupole collision cell, followed by mass analysis of product ions by the high-resolution orthogonal-reflectron time-of-flight analyzer.

Isoflavone detection

Isoflavones were detected through ultraviolet–visible (200–600 nm) spectroscopy using a Beckman Coulter DU-70 spectrophotometer (Sao Paulo, Brazil). Fourier transform infrared analyses were carried out with a Perkin Elmer FTIR Spectrum

One (650 − 4,000 cm^{-1}; Perkin-Elmer, Sao Paulo, Brazil) using the stretching mode (Aguiar 2004).

Statistical analysis

Data were subject to randomized block design and were evaluated by analysis of variance and the Tukey test ($P < 0.05$) to determine the significance of differences between the mean values using the Statistica for Windows Release 5.0 (1995) computer program (Statsoft Inc., Tulsa, OK, USA). All values were the mean of three repetitions, and are presented as the mean ± standard deviation.

Results and discussion

The importance of identifying the isoflavones produced by each plant species used in human diet can be illustrated by the isoflavone formononetin, which has been shown to have negative effects for sheep fertility (Vicent and Fitzpatrick 2000; Stroheker et al. 2003). This became particularly important after the recent finding showing that isoflavones are associated with various traits such as anti-cancer, anti-inflammatory and hormonal replacement (Atkinson et al. 2004).

The analysis of the chemical composition of the methanolic extracts of the leguminous plants studied herein show that only chickpea and soybean have isoflavones at a concentration high enough to be detected by liquid chromatography (RPHPLC/diode-array detector (DAD)) (Table I).

Chickpea extracts show a concentration of 31 ± 0.95 μg genistein per gram of defatted dried flour, whereas no isoflavones were detected in the alcoholic extracts of the samples from other species. Detection and quantitation of genistein in chickpea by RPHPLC/DAD, as well as the other isoflavones (data not shown) was confirmed by ESI-MS and tandem MS experiments (Aguiar 2004). For instance, the ESI tandem mass spectra (ESI-MS/MS) in Figure 2 shows characteristic fragment ions of m/z 91,

Table I. Isoflavone contents in eatable grains and leaves determined by RPHPLC (μg/g).

Common name	Scientific name	Material analyzed	Isoflavones[a]
English peas	*Pisum sativum*	Grains	Not detected
Favabean	*Vicia faba*	Grains	Not detected
Snapbean	*Phaseolus vulgaris*	Grains	Not detected
Pallar[b]	*Phaseolus lunatus*	Grains	Not detected
Pigeonpeas	*Cajanus cajan*	Grains	Not detected
Chickpeas	*Cicer arientinum*	Grains	31.0
Kiwicha[b]	*Amaranthus caudatus*	Grains	Not detected
Kañiwa[b]	*Chenopodium pallidicaule*	Grains	Not detected
Giant Peruvian lima[b]	*Phaseolus limensis*	Grains	Not detected
Alfalfa	*Medicago sativa*	Grains	Not detected
		Leaves	Not detected
		Stem	Not detected
Soybean	*Glycine max*	Hypocotyl	5,857
		Cotyledon	385
		Coat	Not detected
		Leaves	Not detected

[a]Quantity of isoflavones is μg/g grains or leaves of the various eatable plant used in Brazilian and Peruvian cuisine. [b]Peruvian plants.

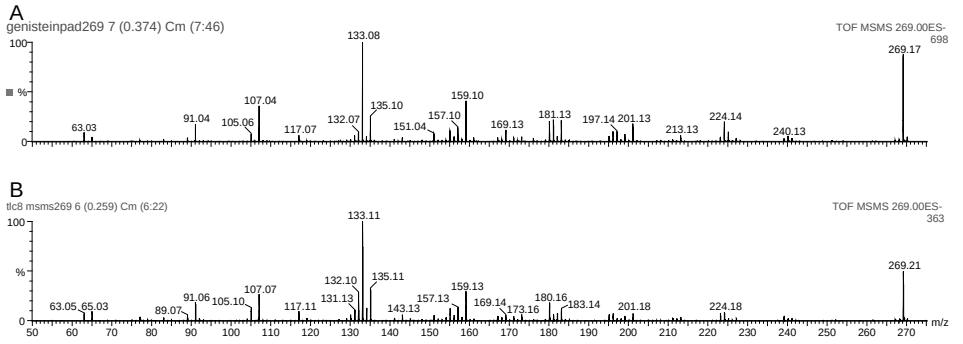

Figure 2. ESI-MS/MS spectra in the negative ion mode for the de-protonated molecule of genistein (m/z 269) from (a) a solution of a genistein standard and (b) the methanolic extract of chickpea.

107, 133, 159, 224 and 269 for both the sample and a genistein standard, thus confirming the detection of this isoflavone.

The ultraviolet spectra (200 – 600 nm) for the leguminous plants studied, with the exception of chickpea, alfalfa and soybean, fail to show the maximum absorption at 250–270 nm that is characteristic for isoflavones. The ultraviolet data corroborate with the RPHPLC/DAD analyses, for which no compounds were detected with absorptions at 254 nm.

A large number of compounds were found in the alfalfa leaves samples, but none of them correlate to the isoflavones standards used in this study. Isoflavones such as maackiain (3-hydroxy-8,9-methylenodyoxypterocarpan) have been extracted from legumines such as *Trifolium* sp. and *P. sativum* (Sun et al. 1991). Simmonds and Stevenson (2001) reported the presence of maackiain in samples of *Cicer bijugum* and *Cicer arietinum* at 200–300 μg/g dry leaves. Another flavonoid known as quercetin, which is associated with plant viral resistance, has been extracted from *Chenopodium quinoa* (Malhotra et al. 1996). Therefore, the compounds that were not identified in alfalfa samples (stem and leaves) are probably other forms of isoflavones besides those usually found in soybean (Table I and Figure 3).

Infrared spectra from the methanolic leaves and stems extracts of alfalfa show mainly the presence of compounds with hydroxyl (3,509 cm^{-1}) and carbonyl groups (1,646 cm^{-1}), such as those expected for a mixture of isoflavones standards (daidzin, genistin, daidzein and genistein). Absorption peaks at values different from those found in the methanolic extraction of chickpea (2,020–2,714 cm^{-1}) also correlate with a mixture of the mentioned standards (2,118–2,740 cm^{-1}) and are attributed to the –C=C=C– and COH groups, respectively. These results indicate the presence of flavonoids in the extracts of leaves and stems of alfalfa.

Soybeans that have traditionally been utilized in the human diets are known to be relatively rich in isoflavones. We therefore mapped he distribution of isoflavones in different parts of soybean grains and plant (Table II). This mapping is important particularly for industries that produce soybean-based products since it can guide grinding and processing to avoid excessive losses of isoflavones.

According to Tsukamoto et al. (1995), the cotyledon has around 90% of the total mass of soybean, whereas the hypocotyls have 2–3% of the total and the tegument represents about 6% of the bean mass. Several studies reported that the content of isoflavones in soybean hypocotyls is two-fold to 10-fold greater than that from the

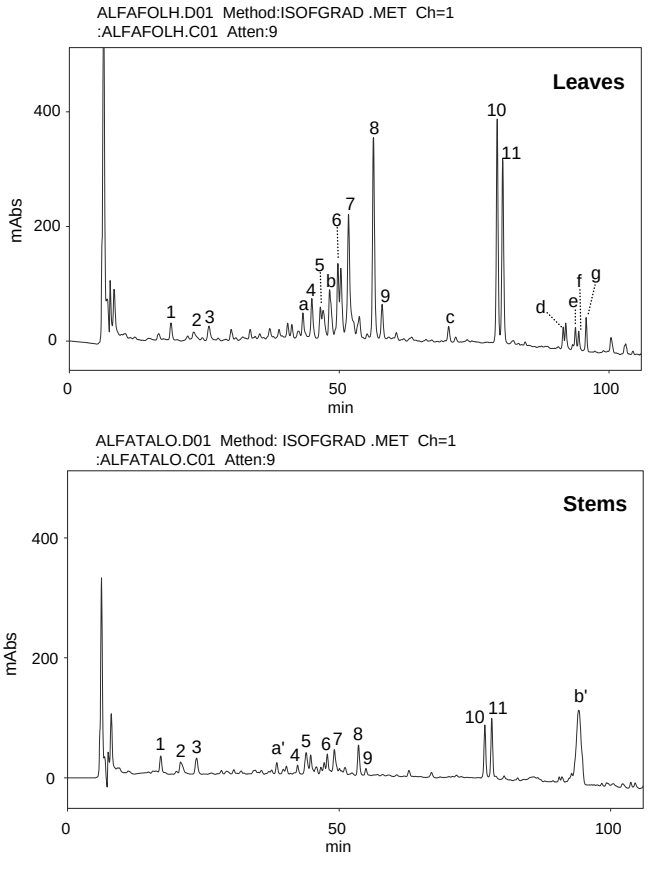

Figure 3. RPHPLC/DAD chromatograms of methanolic extracts of alfalfa. 1, λ_{max} =258 nm; 2, λ_{max} = 249 nm; 3, λ_{max} =243 nm; 4, λ_{max} =246,270 nm; 5, λ_{max} =244 nm; 6, λ_{max} =250 nm; 7, λ_{max} =250 nm; 8, λ_{max} =246,272 nm; 9, λ_{max} =242,274 nm; 10, λ_{max} =243,269 nm; 11, λ_{max} =249 nm; a, λ_{max} =270 nm; b, λ_{max} =245,270 nm; c, λ_{max} =257 nm; d, λ_{max} =271 nm; e, λ_{max} =269 nm; f, λ_{max} =270 nm; g, λ_{max} = 269 nm; a′, λ_{max} =247 nm; b′, λ_{max} =243 nm.

cotyledon. Overall, glycitein and glycitin are not detected in the cotyledon, but only in the hypocotyls (Tsukamoto et al. 2001). The present results show that the highest concentrations of isoflavones are found in the hypocotyls at around 5.9 mg/g hypocotyls, whereas total isoflavone content in the cotyledon is about 0.4 mg/g (dry matter). The total content of isoflavones in soybeans can vary depending on the cultivar analyzed (Park et al. 2001b). Tsukamoto et al. (2001) reported that the content of total isoflavones in hypocotyls is higher than 2000 mg/100 g beans, and this content corroborates with those found herein. They also reported that isoflavones found in the soybean cotyledons are mainly daidzin, malonildaidzin, malonilgenistin and genistein.

Conclusion

Isoflavones were monitored in various eatable plant species common in Brazilian and Peruvian cuisine. The presence of daidzein, glycitein, genistein and their conjugated forms has been found to be restricted to soybean, in which these important isoflavones

Table II. Isoflavone contents in the hypocotyl, cotyledon, grain coat and leaves from Brazilian soybeans (μg/g)[a].

	Glucosides			Malonates			Aglycones		
Fractions	Daidzin	Glycitin	Genistin	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
Hypocotyl	703±16	2,060±334	351±2	668±3	1,456±59	320±2	83±2	150±4	63±3
Cotyledon	52±2	7±0.2	107±2	60±2	0.0	120±2	9±2	0.0	26±2
Grain coat	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Leaves	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]BRS156 cultivar obtained from Empresa Brasileira de Pesquisa Agropecuária. Quantity of constituents as μg/g each soy fraction. Values represent the average of analyses±standard deviation, $n=3$.

are found mainly in the hypocotyls and to some extent in the cotyledon. Genistein has also been found in chickpea.

Acknowledgements

The authors thank Dr Júlio F. P. Guzmán (Universidad Peruana Unión, Lima, Peru) and Dr Mercedes C. C. Panizzi (CNPSo/EMBRAPA, PR., Brazil) for donating the grains analyzed in this work, Dr Gizelda M. Alves (DEQUI/FAENQUIL, SP., Brazil) for Fourier transform infrared analyses, and the Brazilian research foundations Fapesp and CAPES for financial assistance.

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