Journal of Agriculture and Food Research 3 (2021) 100113

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.journals.elsevier.com/journal-of-agriculture-and-food-research/

Determination of total isoflavones and rutin in seeds, roots, and leaves of Brazilian soybean cultivars by using voltammetric methods

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ARTICLE INFO

Keywords:
Differential pulse voltammetry
Isoflavones
Rutin
Soybean
Brazilian cultivars

ABSTRACT

Isoflavones and rutin are important bioactive compounds found in soybean plants. These compounds are widely known to have beneficial effects on human health and to contribute on soybean plant defense against herbivores. To support studies related to conventional or genetic improvement of soybean plants through the selection of plants with high levels of bioactive compounds, two highly sensitive and promising methodologies based on voltammetry have been optimized and validated to determine isoflavones and rutin levels in 24 Brazilian soybean cultivars and in its different organs (leaf, seed and root). After statistical analysis, the contrasts of total isoflavones concentration in soybean seeds indicated the formation of three groups: one group represented by the cultivar BRS 133 with 902.6 $\mu g\, g^{-1}$ and two groups with mean concentrations ranging from 313.7 to 529.0 $\mu g\, g^{-1}$ and 127.5–233.5 $\mu g\, g^{-1}$, respectively. In soybean roots, the contrasts of total isoflavones concentration indicated the formation of two groups ranging from 679.1 to 1470.6 $\mu g\, g^{-1}$ and 137.1–447.9 $\mu g\, g^{-1}$, respectively. For soybean leaves, only three cultivars presented rutin and the highest concentration was found to be 1438.8 $\mu g\, g^{-1}$. After applying the Scott-Knott test ($p < 0.05$), there was statistical evidence that BRS 133 variety differs from other varieties in terms of isoflavones concentration in seeds. Such evidence pointed out BRS 133 as a promising cultivar to ongoing projects aiming genetic manipulation of soybean plants for uses in soy food products.

1. Introduction

Soybean is an important component of the Brazilian agribusiness representing 13% of total export complex [1]. Soybean is mainly used for animal feeding, but soybean protein is also largely consumed as an ingredient of different human food products [2]. Because soybean presents isoflavones, which are related to human health, soybean is used on processing of functional foods. Several studies reported effects of isoflavones on reducing risks of chronical diseases. Human intestinal microbiota can produce the non-steroidal estrogen S-equol as metabolite, which is determinant to bioavailability of isoflavones, leading to reduced cardiovascular risk, prostate and breast cancer, and relieving menopausal symptoms, such as hot flashes and osteoporosis [3].

In soybean seeds, isoflavones chemical forms are aglycones (daidzein, genistein, and glycitein), glycon (daidzin, genistin and glycitin) forms and glucoside conjugates: the β-glucosides 6″- O-acetyl-β-glucosides (6OAcGlc) and the 6″- O-malonyl- β-glucosides (6OMalGlc) [4,5]. In general, mature soybean seeds are the most largely employed raw material to produce human or livestock processed foods. Depending on processing methods, there are changes in the form and formation of isoflavones. Aglycone forms are more common in fermented soybean foods, while the glucosides are presented in non-fermented soybean foods [6,7]. Acetyl forms are presented in toasted soy flour, while malonyl forms are decarboxylatedunder high thermal treatments [8]. Soybean molasses irradiated with gamma rays showed less amount of isoflavones than irradiated soy flour. The high water content in molasses

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https://doi.org/10.1016/j.jafr.2021.100113
Received 7 May 2020; Received in revised form 29 December 2020; Accepted 17 January 2021

reduce isoflavones concentration [9].

Isoflavones are highly affected by genetics and environmental conditions such as temperature during the seed filling [10–14]. Soybean genotypes evaluated at different locations in Brazil showed this variability [15,16]. Locations with reduced average temperature during the seed filling give rise to a higher content of isoflavones. Daydé et al. [17] also reported that low temperatures associated with late watering (rain or irrigation) increased the isoflavone content. However, long-term drought conditions most at later development stages reduced isoflavone content [18]. Britz et al. [19] reported that high temperatures and drought are most likely to affect isoflavone concentrations in seeds of early maturity soybean lines than in late maturity ones.

In general, Brazilian soybean present about 1.5 mg isoflavones per gram of seeds, American soybean contain about 2.0 mg per gram, while soybean from Japan and Canada present a higher content (4.5 and 5.5 mg per gram, respectively) [12]. Although the mean value of Brazilian soybean is relatively low [20], isoflavone content is very variable among genotypes and sowing locations [16]. Lee et al. [21] analyzed isoflavone content in different parts of the soybean sprout, with roots presenting the highest total isoflavone concentrations followed by cotyledons and hypocotyls. Daidzin concentration in sprouts was four times greater than in seeds. Apparently, accumulation of isoflavones, as daidzein and genistein in roots is related to induction of nodulation in soybeans [22].

Higher individual or total isoflavones content in soybean lines may protect soybean against biological stresses [23]. Daidzein is the precursor of prenylated 6a-hydroxy-pterocarpans (glyceollins), which is an antimicrobial phytoalexin important for resistance of cultivars to Phytophthora megasperma [24]. Carpentieri-Pipolo et al. [25] also reported high concentration of daidzein in soybean roots, after inoculation for ten days with the nematode Meloydoyne incognita race 3. The insect resistance genotype PI 227 687 also presented high concentrations of daidzin and genistin in soybean seeds [26,27]. Resistance of soybean PI 227687 to defoliating insects comes mainly from rutin, a flavonol glycoside that was identified in leaves [27,28]. Soybean leaves also synthesize and accumulate a subset of flavonoids following herbivore attack [29].

Several analytical techniques have been applied in the determination of isoflavones and rutin in soybeans and soybean products. These include thin layer chromatography [30], capillary electrophoresis [31], gas chromatography coupled to mass spectrometry [32], high performance and ultra-high performance liquid chromatography with various detectors [33–37] and voltammetry [38–42]. Among these, voltammetry is a powerful and versatile tool due to the advantages of high sensitivity, simplicity, good stability, inexpensive instrumentation and reduced interference from non-electroactive substances. Isoflavones were determined in soybeans and soy-based foods by linear sweep voltammetric and amperometric methods using mercury and glassy carbon electrodes [38,39]. Square-wave voltammetry was successfully used to determine genistein in soy flour and soy based supplements [40]. A voltammetric method based on the accumulation of a Cu(II)–rutin complex at a hanging mercury drop electrode was used for the determination of rutin in soybean cultivars [41]. In a previous study, we reported the application of differential pulse voltammetric methods based on carbon electrodes for the determination of rutin and total isoflavones in different growth stages of a Brazilian soybean cultivar (BRS 217 Flora) [42].

The main objective of this work is to present highly sensitive validated voltammetric methods to determine rutin and total isoflavones content in seeds, leaves and roots of twenty-four Brazilian soybean cultivars. The isoflavones and rutin determination in different organs of soybean plants can show the different distribution of these phenolic compounds among studied cultivars. Besides this, the new data give support to the genetic breeding program of Brazilian Agriculture Research Company (EMBRAPA) by indicating the differences among soybean cultivars, contributing to the development of new soybean cultivars with special characteristics for different uses, including human food.

2. Experimental

2.1. Plant material and growth conditions

Soybean plants of twenty-four different cultivars (BRS 133, BRS 184, BRS 213, BRS 216, BRS 232, BRS 243, BRS 245, BRS 246, BRS 255, BRS 257, BRS 258, BRS 259, BRS 260, BRS 261, BRS 262, BRS 268, BRS 282, BRS 283, BRS 284, BRS 294, BRS 295, BRS 316, BRS 317, Embrapa 48) were grown on experimental fields at Embrapa Soja, Londrina, Brazil. Seeds, leaves and roots were collected in the stage of full maturity, R6 to R8. The cultivars were planted in duplicate on October 26th/2009. The materials were planted in no-till system with NPK fertilization of 300 kg per hectare. Appropriate chemicals were applied for weed, diseases and pests control. Seeds, leaves and roots were frozen at −20 °C in plastic bags until the extract preparation for determination of isoflavones and rutin content.

2.2. Preparation of soybean seeds, leaves and roots

Flavonoids (isoflavones and rutin) were extracted from 0.1 g sample of dried and ground soybean leaves, seeds and roots by ultrasonication (20 min, room temperature) in 2.0 mL of 70% aqueous ethanol (Sigma-Aldrich, St Louis, MO). The resulting extract was decanted and the supernatant was transferred to 2.0 mL microtubes and centrifuged for 10 min at 12,100 g. Then, the new supernatant was treated by solid-phase extraction (SPE). After cartridges conditioning with 2 mL of pure water (Merck-Millipore, Bilerica, MA) and 2 mL of methanol (Sigma-Aldrich, St Louis, MO), the soybean seed, leaf, and root extracts were passed through the cartridge (HLB, 200 mg, Perkin Elmer, Waltham, MA). Impurities were washed out with 2 mL of 10% aqueous methanol. Retained flavonoids were eluted with 2 mL of methanol and 2 mL of acetonitrile (Sigma-Aldrich, St Louis, MO). After SPE, soybean seed, leaf and root samples were collected and immediately analyzed. The ultrasonic cleaner (Cristófoli Equipamentos de Biossegurança LTDA, Campo Mourão, Brazil) was used for extraction of flavonoids (isoflavones and rutin) in soybean seeds, leaves and roots.

Analysis of Isoflavones and rutin in soybean seeds, leaves and roots.

The voltammetric measurements were carried out on a 797 Voltammetric Analyzer (VA) Computrace (Metrohm Autolab, Utrecht, The Netherlands) with an electrochemical cell composed of a glassy carbon (GC) rotating disk ($\Phi = 2.0$ mm) as working electrode, Ag/AgCl (3 mol L^{-1} KCl) as reference electrode and a platinum wire as auxiliary electrode. The hydrogen-ion potential (pH) of the solutions was determined using a LUCA-210 pH-meter (MS Tecnopon Equipamentos Especiais LTDA, Piracicaba, Brazil). The ultrasonic cleaner (Cristófoli Equipamentos de Biossegurança LTDA, Campo Mourão, Brazil) was used for GC electrode cleaning. Analytical-grade chemicals and ultrapure water (Merck-Millipore, Bilerica, MA) were used to prepare all solutions. Genistein (>99% purity) was purchased from L. C. Laboratories (Woburn, MA) and Rutin hydrate (95% purity) was purchased from Sigma-Aldrich (St Louis, MO). All flavonoids were used without further purification. Flavonoid standard stock solutions (1.0×10^{-3} mol L^{-1}) were prepared in 5 mL of ethanol (Sigma-Aldrich, St Louis, MO), diluting with water to 10 mL and then stored in dark bottles under freezing to prevent photodegradation. Phosphate buffers, pH 6.0, 0.2 mol L^{-1}, were prepared using dibasic sodium phosphate and monobasic potassium phosphate (Sigma-Aldrich, St Louis, MO) and used as supporting electrolytes. Sodium hydroxide and hydrochloric acid (Sigma-Aldrich, St Louis, MO) were used for pH adjustment. Alumina oxide powder <10 μm and acetone (Sigma-Aldrich, St Louis, MO) were used for GC electrode cleaning. Nitric acid (Quimex, São Paulo, Brazil) was used to clean laboratory glassware. The determination of total isoflavones and rutin was done by differential pulse voltammetric technique (DPV). The measurements were performed in the potential range of 0 V (initial potential, Ei) to 1.000 V (final potential, Ef) at 50 mV pulse amplitude and 50 mV s^{-1} scan rate. GC electrode surface treatment (cleaning and activation) was

based on the following procedure: the GC electrode surface was manually polished with alumina suspension on polishing cloth for 1 min and rinsed with distilled water; the electrode was then immersed in acetone and submitted to ultrasonication for 4 min and rinsed with ultra pure water; the electrode was immersed in 0.2 mol L^{-1} phosphate buffer, pH 6.0, and submitted to continuous potential cycling from 0 to 1.8 V at 50 mV s^{-1} for 3 min. Voltammograms were recorded until steady state baseline voltammograms were obtained.

Analytical curves were obtained by using differential pulse voltammetry by six successive (10 μL) additions of 1.0×10^{-3} mol L^{-1} genistein and by seven successive (20 μL) additions of 1.0×10^{-3} mol L^{-1} rutin to the electrochemical cell containing 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6.0.

Seven parameters (linearity, linear range, limits of detection and quantitation, repeatability, intermediate precision) were determined for the validation of DPV methods in soybean using the guidelines of National Institute of Metrology, Quality and Technology [43]. The trueness of the DPV method was assessed by recovery assays in which known amounts of standards of genistein (1.5×10^{-6} mol L^{-1}; 4.5×10^{-6} mol L^{-1}; 9.0×10^{-6} mol L^{-1}) and rutin (1.0×10^{-6} mol L^{-1}; 4.0×10^{-6} mol L^{-1}; 7.0×10^{-6} mol L^{-1}) were added to the electrochemical cell containing 100 μL of soybeans extracts of seeds, leaves and roots.

Total isoflavones were determined in soybean seeds, leaves, and roots by three successive standard (10–20 μL) additions of 1.0×10^{-3} mol L^{-1} genistein. Rutin was determined in leaves by three successive (20 μL) additions of 1.0×10^{-3} mol L^{-1} rutin to the electrochemical cell containing 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6.0, and 1.0–2.0 mL of soybean sample. The concentration of rutin and isoflavones in the samples were determined by averaging the current measurements obtained in triplicate.

Reagents were manipulated using appropriate personal protective equipment, such as nitrile gloves, splash goggles and laboratory coat. Sample extraction was optimized to minimize the amounts of solvents used. Solvent waste (methanol, ethanol, acetone and acetonitrile) was classified as a hazardous waste and it was disposed into suitable containers and taken to the Central Waste Management of the Institution. With respect to other experimental procedures, DPV technique has the advantage to generate minimal amounts of waste, it is a very sensitive technique, and therefore, little quantities of sample extracts and flavonoid standards were required for analysis. However, the waste generated was classified as a hazardous waste and was placed in a suitable container to its final disposal.

2.3. Statistical analysis

Data were summarized with Microsoft Office Excel software (version 2010; Microsoft Corp, Redmond, WA, USA). Cochran's test ($\alpha = 0.05$) was used to test the homogeneity of variances of linear regression data. The means of flavonoids content of the cultivars were compared through Scott-Knott test ($p < 0.05$). Additionally, the content of flavonoids in soybean seeds was correlated with the content of flavonoids in the roots using Pearson's correlation.

3. Results and discussion

Voltammetric profiles of genistein and rutin standards and validation parameters of the voltammetric methods.

The differential pulse voltammograms obtained for standard additions of genistein and rutin in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6.0, at a glassy carbon electrode are shown in Figs. 1 and 2. Isoflavones in aglycone (genistein, glycitein, daidzein) and glycoside (daidzin, genistin and glycitin) forms exhibit similar voltammetric profiles in the range of 0.500–0.600 V due to the oxidation of 4′- hydroxyl group located in the B-ring of the isofavones [44–46] at the glassy carbon electrode, as it has already been discussed in our previous report [42]. Genistein was chosen as the standard in the determination of total isoflavones due to its higher availability and lower cost (Fig. 1). Rutin showed a distinct and intense anodic peak in the potential of 0.300 V, which corresponds to the oxidation reaction of the 3′,4′-dihydroxyl groups on the B-ring of flavonoid, as predicted by previous studies [46–48]. Thus, rutin content was separately determined (Fig. 2).

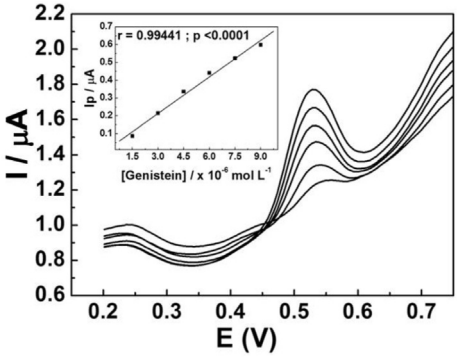

Fig. 1. Differential pulse voltammograms obtained for standard additions of 10 μL of genistein (1.0×10^{-3} mol L^{-1}) in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6.0. Initial potential (E_i) = 0.200 V, final potential (E_f) = 0.800 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 mol L^{-1}), auxiliary electrode: platinum wire.

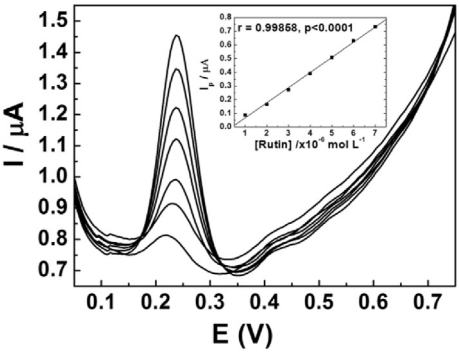

Fig. 2. Differential pulse voltammograms obtained for standard additions of 20 μL of rutin (1.0×10^{-5} mol L^{-1}) in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6.0. Initial potential (E_i) = 0.000 V, final potential (E_f) = 0.800 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 mol L^{-1}), auxiliary electrode: platinum wire.

Two highly sensitive voltammetric methods based on a glassy carbon electrode for determination of rutin and total isoflavones (genistein, glycitein, daidzein, daidzin, genistin and glycitin) were validated under the optimized experimental conditions. A linear response of the oxidation peak current as a function of concentration was observed for genistein and rutin over the concentration range studied (Figs. 1 and 2). The overall performance of the voltammetric methods is summarized in Table 1. Correlation coefficients (r) were higher than 0.99 for both determinations, which attest to the linearity of the proposed methods. Homocedastic results from Cochran's test (C calculated < C critical - 95% confidence level, Table 1) are another evidence of the linearity of the methods. The detection limits were found to be 6.0×10^{-7} mol L^{-1} for genistein and 5.0×10^{-7} mol L^{-1} for rutin (Table 1). The calculated repeatability and intermediate precision values were considered satisfactory (Table 1). The repeatability index was smaller than the intermediate index for genistein and rutin, which indicates that the variation for measurements performed in different days was larger than the variation for independent experiments conducted within a day, as expected. The results obtained for recovery tests (Table 1) were higher than 85%

Table 1
Validation parameters for determination of genistein and rutin by differential pulse voltammetry using glassy carbon electrode.

Parameters	Genistein	Rutin
Linear regression equation	$I\ (A) = -8.1 \times 10^{-9} + 0.068$ [genistein]/mol L^{-1}	$I\ (A) = -4.4 \times 10^{-8} + 0.110$ [rutin]/mol L^{-1}
Linearity range/ Working range (mol L^{-1})	$1.5 \times 10^{-6} - 9.0 \times 10^{-6}$	$1.0 \times 10^{-6} - 7.0 \times 10^{-6}$
Regression coefficient	$r = 0.9944$ ($p < 0.0001$) C_{calc} (0.1205) < C_{tab}	$r = 0.9986$ ($p < 0.0001$) C_{calc} (0.2235) < C_{tab}
Cochran's test	(0.6161) ($\alpha = 95\%$; N = 6; n = 3)	(0.5612) ($\alpha = 95\%$; N = 7; n = 3)
Limit of Detection (mol L^{-1})	6.0×10^{-7}	5.0×10^{-7}
Limit of Quantification (mol L^{-1})	1.5×10^{-6}	1.0×10^{-6}
Repeatability (%) ($N^a = 3$; $n^b = 7$)	7–15	7–10
Intermediate Precision (%) ($N^a = 3$; $n^b = 3$)	16–28	10–21
Recovery (%)	92–111	85–107

N^a number of concentrations levels.
n^b number of replicates per concentration.

for rutin concentration below 6.0×10^{-6} mol L^{-1} and higher than 92% for genistein concentration upper to 1.5×10^{-6} mol L^{-1}, suggesting that the accuracies of the proposed methods were acceptable along the working ranges.

Voltammetric profiles of soybean samples and concentration of total isoflavones and rutin in soybean seeds, roots and leaves of different cultivars.

The differential pulse voltammograms obtained for BRS 133 soybean seed sample in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6, with different genistein concentrations is presented in Fig. 3. A well-defined peak at 0.549 V was observed with the addition of 1 mL of soybean seed extract, which can be assigned to the oxidation of total isoflavones. The studied soybean seed samples from the other cultivars presented similar voltammograms. In Fig. 4, the differential pulse voltammograms obtained for BRS 294 soybean leaf sample in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6, with different concentrations of rutin are presented. A well-defined peak at 0.282 V was also observed with the addition of 2 mL of soybean leaf extract, which can be assigned to the

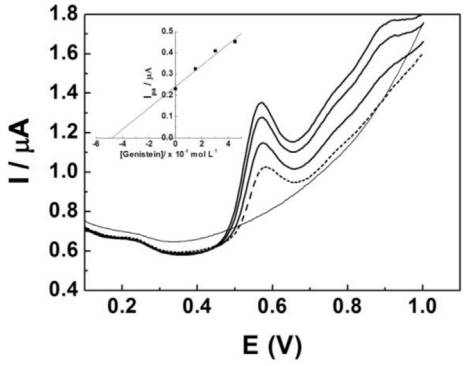

Fig. 3. Differential pulse voltammograms obtained from seed sample of the soybean cultivar BRS 133 (dashed line), in 10 mL of 0.2 mol L^{-1} phosphate buffer (thinner solid line), pH 6.0, with three standard additions of 15 µL of 1.0×10^{-3} mol L^{-1} of genistein (thicker solid lines). Initial potential (E_i) = 0.000 V, final potential (E_f) = 1.000 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 mol L^{-1}), auxiliary electrode: platinum wire. Insert: Standard addition curve of genistein over the sample used to determine the isoflavones content in the sample.

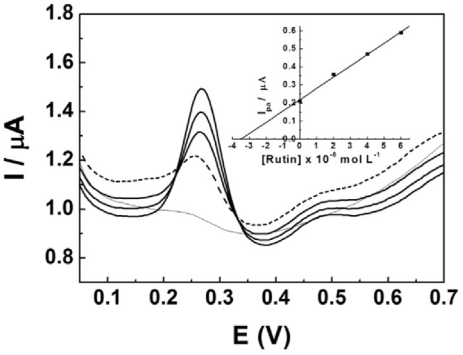

Fig. 4. Differential pulse voltammograms obtained from leaf of the soybean cultivar BRS 294 (dashed line), in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6.0 (thinner solid line), with three standard additions of 20 µL of 1.0×10^{-3} mol L^{-1} of rutin (thicker solid line). Initial potential (E_i) = 0.000 V, final potential (E_f) = 0.700 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 mol L^{-1}), auxiliary electrode: platinum wire. Insert: Standard addition curve of rutin over the sample used to determine the rutin content in the sample.

oxidation of rutin [46]. The other studied soybean leaf samples from cultivars BRS 268 and BRS 295 presented similar voltammograms. The differential pulse voltammograms obtained for BRS 268 soybean root sample in 10 mL of 0.2 mol L^{-1} phosphate buffer, pH 6, with different genistein concentrations, are presented in Fig. 5. A well-defined peak at 0.559 V was observed with the addition of 500 µL of soybean root extract, which can be assigned to the oxidation of total isoflavones. The other studied soybean root samples presented similar voltammograms.

The voltammetric method based on differential pulse voltammetry and glassy carbon electrode showed to be a powerful and versatile tool for the total isoflavones (daidzein, genistein, glycitein, daidzin, genistin, glycitin) and rutin determination in twenty-four Brazilian soybean cultivars. This study was original for analyzing the flavonoids in different parts of soybean plants, as seeds, roots and leaves of many cultivars (Table 2). Isoflavones content was found in specific parts of the soybean plants, i.e., they were detected exclusively in seeds and roots. The range of mean isoflavone concentrations in the roots (271.2–1471 µg g^{-1}) was higher than in soybean seeds (127.5–902.6 µg g^{-1}). Rutin was detected only in leaves of a small number of plants from cultivars BRS 294, BRS 295 and BRS 268.

Significance differences among cultivars were observed for mean

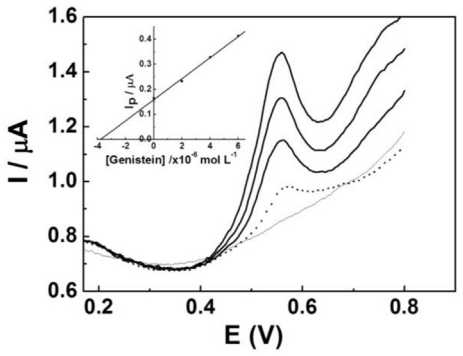

Fig. 5. Differential pulse voltammograms obtained from root sample of the soybean cultivar BRS 268 (dashed line), in 10 mL of 0.2 mol L^{-1} phosphate buffer (thinner solid line), pH 6.0, with three standard additions of 20 µL of 1.0×10^{-3} mol L^{-1} of genistein (thicker solid line). Initial potential (E_i) = 0.000 V, final potential (E_f) = 0.800 V, pulse amplitude = 50 mV, scan rate = 50 mV s^{-1}, working electrode: glassy carbon, reference electrode: Ag/AgCl (KCl 3 mol L^{-1}), auxiliary electrode: platinum wire. Insert: Standard addition curve of genistein over the sample used to determine the isoflavones content in the sample.

Table 2
Total isoflavone and rutin concentrations (μg g^{-1}) in seeds, roots and leaves of different Brazilian soybean cultivars.

Cultivars	Seed		Root		Leaf	
	Total isoflavones (μg g^{-1}) n[a] = 2	Rutin (μg g^{-1})	Total isoflavones (μg g^{-1}) n = 2	Rutin (μg g^{-1})	Total isoflavones (μg g^{-1}) n = 2	Rutin (μg g^{-1}) n = 1
BRS 295	127.5 ± 27.2	nd[b]	271.2 ± 153.4	Nd	nd[c]	1443[e]
BRS 232	150.9 ± 4.5	nd	447.9 ± 201.5	Nd	nd	Nd
BRS 268	151.0 ± 69.7	nd	1471 ± 551	Nd	nd	1439[e]
BRS 245	152.5 ± 2.0	nd	302.6 ± 1.6	Nd	nd	Nd
BRS 216	154.5 ± 23.4	nd	na[d]	Nd	nd	Nd
BRS 316	155.3 ± 24.4	nd	na	Nd	nd	Nd
Embrapa 48	168.4 ± 57.6	nd	374.0 ± 184.5	Nd	nd	Nd
BRS 284	169.4 ± 45.4	nd	310.5 ± 64.6	Nd	nd	nd
BRS 184	172.6 ± 47.7	nd	416.2 ± 128.1	nd	nd	nd
BRS 259	173.2 ± 14.0	nd	na	nd	nd	nd
BRS 243	173.4 ± 22.5	nd	137.1 ± 83.8	nd	nd	nd
BRS 255	173.9 ± 54.0	nd	426.0 ± 156.6	nd	nd	nd
BRS 258	176.0 ± 23.7	nd	na	nd	nd	nd
BRS 213	186.0 ± 29.9	nd	808.0 ± 316.5	nd	nd	nd
BRS 257	192.3 ± 3.9	nd	na	nd	nd	nd
BRS 262	233.5 ± 28.4	nd	679.1 ± 493.1	nd	nd	nd
BRS 282	313.7[e]	nd	na	nd	nd	nd
BRS 294	336.2 ± 26.5	nd	346.6 ± 102.2	nd	nd	859.6[e]
BRS 261	421.5 ± 3.9	nd	1091 ± 378	nd	nd	nd
BRS 317	426.7 ± 37.1	nd	1283 ± 462	nd	nd	nd
BRS 260	440.4 ± 80.7	nd	na	nd	nd	nd
BRS 283	452.7 ± 43.4	nd	1028 ± 258	nd	nd	nd
BRS 246	529.0 ± 88.0	nd	352.2 ± 171.5	nd	nd	nd
BRS 133	902.6 ± 2.5	nd	na	nd	nd	nd

[a] number of analyzed plants.
[b] not detected, rutin concentrations lower than LoQ (24.4 μg g^{-1}).
[c] not detected, total isoflavone concentrations lower than LoQ (16.2 μg g^{-1}).
[d] not analyzed.
[e] Detected only in one plant.

Soybean seed analysis

concentrations of total isoflavones in soybean seeds (Scott-Knott test, df = 23, p-value < 0.05, Fig. 6). At 5% level of significance, the contrasts of the cultivars concentrations indicated the formation of three groups.

The first group consists of only one individual with the highest mean concentration of total isoflavones, corresponding to BRS 133 cultivar (902.6 μg g^{-1}), represented by the letter "a". The second largest mean concentration of total isoflavones (313.7–529.0 μg g^{-1}) is observed among the genotypes who received the letter "b": BRS 246, BRS 283, BRS 260, BRS 317, BRS 261, BRS 294 and BRS 282. The third highest mean concentration of isoflavones (127.5–233.5 μg g^{-1}) comprises cultivars indicated with letter "c", including BRS 262, BRS 257, BRS 213, BRS 258, BRS 255, BRS 243, BRS 259, BRS 184, BRS 284, Embrapa 48, BRS 316, BRS 216, BRS 245, BRS 268, BRS 232 and BRS 295.

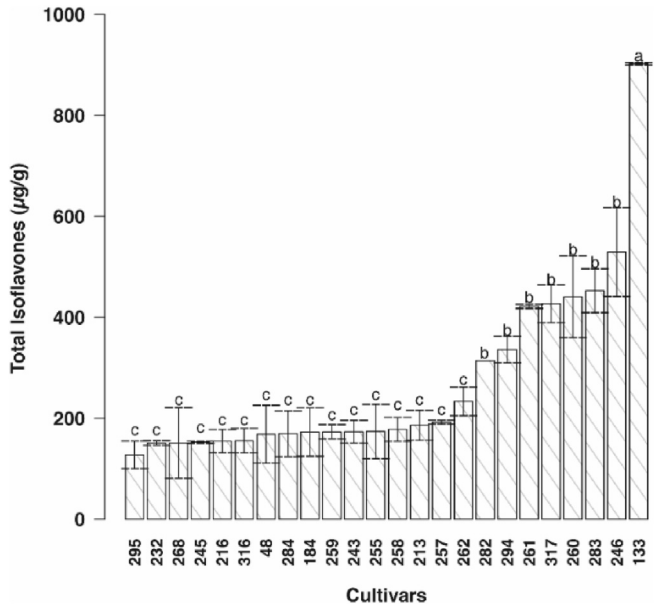

Fig. 6. Total isoflavones concentrations (μg g^{-1}) in seeds of different soybean cultivars.

In our studies, since the cultivars were grown in the same area with the same environmental conditions, it was verified that genetic factor plays the most important role for isoflavone accumulation in seeds. This statement was confirmed by other authors such as Carrão-Panizzi [20], Hoeck et al. [49], Ribeiro et al. [50], Xu & Chang [51], Zhang et al. [52], and Rigo et al. [53], that reported significant differences in isoflavones content for soybean cultivars due to genetic variability.

The total isoflavones concentration in soybean seeds observed in this work were lower than those reported by Xu and Chang [51] and Hoeck et al. [54] in North America, which range of concentrations are 1180–2860 μg g^{-1} and 1212–2547 μg g^{-1}, respectively. In Korea it was observed concentrations ranging from 1519 to 7657 μg g^{-1} [55], in China from 551 to 7584 μg g^{-1} [52], and in Thailand from 340 to 1990 μg g^{-1} [56]. In those studies, the analysis of total isoflavones in soybean seeds was performed in cultivars that were grown under different environmental conditions (location, sowing date, temperature and soil nutrition), which were significant for the variability of total isoflavone content. In addition, most of these studies reported concentrations of total isoflavones including other forms of isoflavones such as β-glycosides conjugated as acetyl and malonyl.

In comparison with other Brazilian cultivars, sowed at the same region of the soybean genotypes described in this work (Londrina, Southern Brazil), but in different soybean season and with different environmental conditions, the total isoflavone content, including aglycone forms (genistein, glycitein, daidzein) and glycosidic forms (daidzin, genistin and glycitin) presented comparable values. Carrão-Panizzi et al. [20] analyzed 100 Brazilian soybean genotypes sowed during 1993/1994 and they reported total isoflavones concentration in the

range of 167.8–1729 μg g^{-1}. Considering only the glycosidic isoflavone forms found in those cultivars (daidzin and genistin), the concentration range was found to be 40.5–361.3 μg g^{-1}. Ribeiro et al. [50] determined isoflavones content in eighteen Brazilian soybean cultivars from different maturity groups during 2002/2003. The total isoflavones concentration found ranged from 618.3 to 1743 μg g^{-1}, and when considering only the content of β-glycosides and aglycones (genistin, daidzin, glicitin, genistein and daidzein) the concentrations ranged from 214.2 to 565.1 μg g^{-1} Ávila et al. [57] analyzed the total isoflavones content in six cultivars, sowed at Maringa, Southern Brazil, in 2004/2005, including Embrapa 48, BRS 133 and BRS 184. The total concentration of isoflavones ranged from 883 to 1852 μg g^{-1}, with the highest concentration for BRS 133. Considering only the content of β-glycoside derivatives (daidzin, glicitin and genistin), the concentrations that were found by them ranged from 192.6 to 485.1 μg g^{-1}. Brazilian soybean cultivars exhibit variable isoflavone content, which are independent of their maturity group, but highly dependent on the genetic factor and environmental conditions.

Thus, the analysis of isoflavones content in soybean seeds of the studied cultivars pointed out that BRS 133 and the cultivars that received the letter "b" as the most promising materials to ongoing projects aiming genetic manipulation of soybean ~~plants with resistance to pests or~~ for uses in soy food products.

Soybean root analysis

The mean concentration of total isoflavones in soybean roots is different among cultivars (Scott-Knott test, df = 15, p-value < 0.05, Fig. 7). At 5% level of significance, the contrasts of the cultivars concentrations indicated the formation of two groups. The first group with the highest mean concentration of total isoflavones (679.1–1470.6 μg g^{-1}) consists of the cultivars which received the letter "a": BRS 268, BRS 317, BRS 261, BRS 283, BRS 213 and BRS 262. The second highest mean concentration of total isoflavones (137.1–447.9 μg g^{-1}) comprises cultivars assigned with letter "b", including: BRS 232, BRS 255, BRS 184, Embrapa 48, BRS 246, BRS 294, BRS 284, BRS 245, BRS 295 and BRS 243. The determination of isoflavones in soybean roots is not so usual and there are few articles reporting flavonoids in soybean roots in the literature. Among the isoflavone compounds that were identified in roots, genistein and daidzein are the most reported ones [58,59]. In this work, the total isoflavones content in roots was higher than in seeds for most of the studied cultivars and this article was the first to report these results for many different cultivars. These results can be used on future studies concerning the distribution of bioactive compounds and its content throughout the soybean plant and to select soybean plants that are more resistant to pests. Moreover, the information about the total isoflavones content in soybean roots of these cultivars may be useful for studies on root–rhizosphere interactions [60–62].

Among the studied genotypes, only BRS 268, BRS 295 and BRS 294 showed detectable amounts of the flavonoid rutin in leaves, and the concentrations were found to be 1438.8 μg g^{-1}, 1443.1 μg g^{-1} and 859.6 μg g^{-1}, respectively. The absence of rutin in most of analyzed soybean leaves can be explained by the optimal defense theory involving the distribution of chemical anti-herbivore defenses in plants [63,64]. This theory predicts that defense chemicals such as flavonoids are allocated within the plant as a function of tissue value, which also depends upon the developmental stage. In soybean plants from R6 to R8 stages, the seeds and roots have higher concentration of flavonoids when compared to the leaves because they are more valuable tissues to the plant and they are more susceptible to pest attack than the leaves. Moreover, in this stage of development, the leaves are older and its bioactive substances that assist in plant defense are present in lower concentrations. Soybean leaves at V6 stage are more valuable and more susceptible to the defoliator insects than older leaves and for these reason they have higher content of bioactive substances, as flavonoids [28,65].

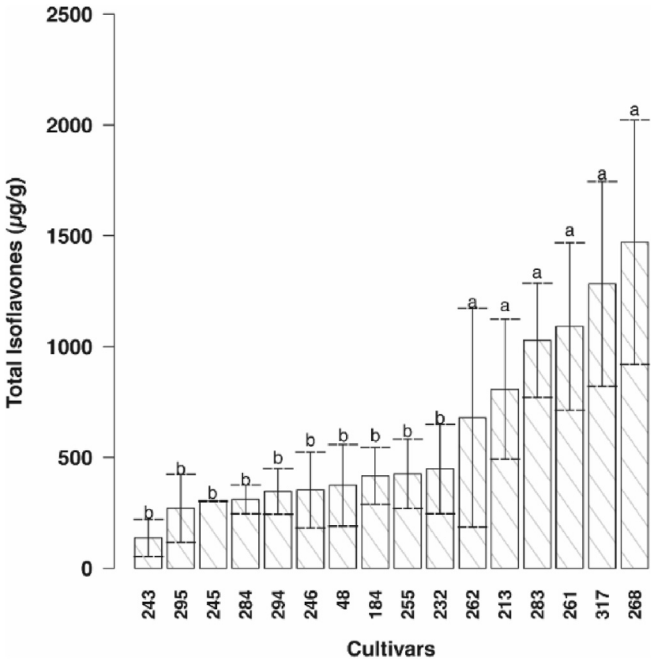

Fig. 7. Total isoflavones concentrations (μg g^{-1}) in roots of different soybean cultivars.

Soybean leaf analysis

3.1. Correlation between total isoflavone concentrations in soybean seeds and in roots

The Pearson correlation coefficient was not significant (0.22) for total isoflavone concentrations in soybean seeds and in roots (Fig. 8). Thus, this result shows that the allocation of defensive chemicals within the soybean plant has a heterogeneous distribution resulting in disproportionate isoflavones content between seeds and roots. According to the optimal defense theory, high concentrations of secondary compounds are probably allocated in the plant tissue that is more physiologically valuable and more susceptible to pest attack [63,64].

4. Conclusions

Sensitive, stable, inexpensive and simple electroanalytical methods for total isoflavones (daidzein, genistein, glycitein, daidzin, genistin and glycitin) and rutin determination, based on differential pulse voltammetry and glassy carbon electrode, showed to be a powerful and versatile tool for the analysis of soybean samples. Under optimized conditions of sample treatment and electrochemical parameters, relative low quantitation limits and good precisions and accuracies were obtained. After statistical analysis, significant differences among genotypes were observed for mean concentrations of total isoflavones in soybean seeds and roots. The analysis of isoflavone content in soybean seeds pointed the cultivar BRS 133 with the highest concentration. In comparison with other Brazilian cultivars, sowed at the same region of the soybean genotypes described in this work (Londrina, South Brazil), the total isoflavone content in seeds (aglycone and glycosidic forms) presented comparable values. The total isoflavone content in roots was higher than in seeds for almost studied genotypes, with no correlation for isoflavones concentrations between soybean seeds and roots. The significant isoflavone content obtained for soybean roots shows perspectives to the studies of root-rhizosphere interactions. The stage of development of soybean plants is an important factor that influenced the flavonoids content in leaves. At R6-R8, these compounds were almost absence in the majority of the analyzed leaf samples. The information obtained in the present work also provides elements for selection of soybean genotypes

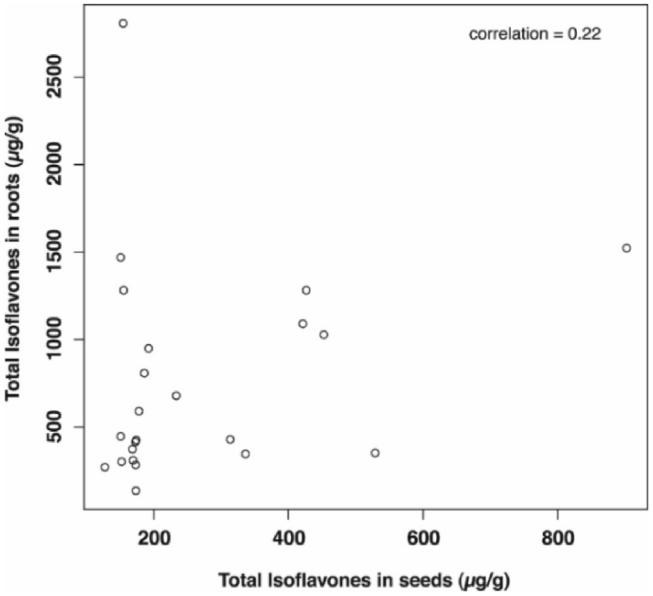

Fig. 8. Total isoflavones concentrations in soybean sedes and in roots.

with higher isoflavone content to produce soybean products with highly functional properties to human health.

Funding

This project was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA).

Declaration of competing interest

The authors declare that the submitted work was carried out in the absence of any personal, professional, or financial relationships that could potentially be construed as a conflict of interest.

Acknowledgments

The authors thank Embrapa Soja for providing soybean samples.

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