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Rutin and total isoflavone determination in soybean at different growth stages by using voltammetric methods ☆

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ABSTRACT

Two highly sensitive differential pulse voltammetric (DPV) methods based on the oxidation of genistein and rutin in a glassy carbon electrode is presented. Under optimized conditions (0.2 M phosphate buffer, pH 6.0, 50 mV pulse amplitude, 50 mV s^{-1} scan rate), the oxidation peak currents (I_p) of genistein and rutin are linear ($I_{pa}(A) = 5.0 \times 10^{-8} + 0.056$ [genistein] $r = 0.9969$; $I_{pa}(A) = -3.0 \times 10^{-8} + 0.112$ [rutin] $r = 0.9974$) to genistein and rutin concentrations in the range of 1.0×10^{-6}–6.0×10^{-6} M and 1.0×10^{-6}–1.2×10^{-5} M, respectively. The detection limits obtained for genistein and rutin were 6.1×10^{-7} M and 3.8×10^{-7} M, respectively. The quantitation limit for both genistein and rutin was 1.0×10^{-6} M. The repeatability of DPV methods was acceptable (relative standard deviation (RSD) < 10%). The presence of matrix effect on the genistein determination and the absence of matrix effect on the rutin determination were attested by t-test (95% confidence level). The DPV methods were applied to the determination of rutin and total isoflavones in a Brazilian soybean cultivar (BRS 216 Flora) in ten different growth stages, with recoveries of 73–109%. Flora exhibited concentration levels of rutin and total isoflavones in leaves, seeds and pods ranging from 0.44 to 1.7 mg g^{-1} and 72 to 128 μg g^{-1}, respectively.

1. Introduction

Soybean [*Glycine max* (L.) Merrill, family Leguminosae, subfamily Papilionoidae] originated in Eastern Asia and is one of the most frequently cultivated crops worldwide. World production of soybeans has increased by a factor of eight in the last half century to reach its present level of over 100 million metric tons per year. The leading producers are the USA (45%), Brazil (20%) and China (12%) [1]. Soybean was introduced in Brazil at the end of XIX century by USA and today is grown in most states, with Mato Grosso providing the largest portion of Brazilian production (81 million tonnes during the 2012/2013 harvest) [2]. Soybean is rich in oil (18%) and protein (40%) and is used for both human and animal consumption as well as for industrial purposes, such as biofuels. The use of soybean as an important food resource has increased because of its nutritional properties and the functional characteristics of flavonoids.

Flavonoids are a large family of over 4000 ubiquitous secondary plant metabolites, comprising six subclasses, anthocyanins (e.g. apigenidin), flavones (e.g. rutin), flavanol (e.g. catechin), flavonols (e.g. quercetin), isoflavones (e.g. genistein) and flavanones (e.g. naringenin) [3]. The interest in flavonoids can be explained by their biological and physiological roles in plants (e.g. defense and signaling actions in reproduction, pathogenesis and symbiosis) [4,5] and in animals and human (e.g. estrogenic and antioxidant actions) [6–8]. Given the variety of functional and structural of flavonoids, separation, identification and trace-level determination of these metabolites is challenging and essential. A wide range of analytical techniques have been used in the determination of flavonoids such as high performance liquid chromatography and electrochemical techniques [9–14]. Besides the analytical determination, electrochemical characterization of flavonoids is suitable to evaluate the mechanisms involved in their antioxidant activity [15–19]. Generally, in electrochemical studies of flavonoids, such as isoflavones and rutin, a highly variety of working electrode materials are used; however, the most involved in these studies are carbon electrodes, mainly glassy carbon electrodes [20–26].

Rutin (3′,4′,5,7-tetrahydroxyflavone-3-O-β-D-rutinoside) is a kind of flavonoid glycoside, called vitamin P, that has been identified in the leaves of soybean [27–29]. Rutin has many physiological (anti-inflammatory, anti-tumor and anti-bacterial) and pharmacological (anti-allergic, vasoactive, anti-viral and anti-protozoal) activities [30,31] and is considered a model in studies of soybean anti-herbivore defense [32–34]. The determination of rutin in soybean has been performed using high performance liquid chromatography [27,28] and voltammetry [29] in order to identify soybean genotypes

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that are resistant to pests. The concentration of rutin in soybean in these studies varied from 0.0554 to 0.972 mg g^{-1}.

Isoflavones are a group of phenolic compounds that has been found in soybean seeds as aglycones (daidzein, genistein and glycitein), β-glucosides (genistin, daidzin and glycitin), β-glucosides conjugated with malonyl (6″-O-malonyldaidzin, 6″-O-malonylgenistin and 6″-O-malonylglycitin) and acetyl groups (6″-O-acetyl-daidzin, 6″-O-acetyl-genistin and 6″-O-acetyl-glycitin) [35,36]. The interest in soybean isoflavones is increasing due to their biological properties, such as reducing the risk of cardiovascular, atherosclerotic, hemolytic and carcinogenic diseases and improvement of bone health, osteoporosis, menopausal symptoms and blood cholesterol levels [37–39]. The isoflavone content in soybean seeds, which varies from 126.1 to 409.2 mg/100 g of seeds), depends on genetic factors, sowing conditions, geographic location, temperature during cultivation and growth stage [36,40–43]. Genistein is one of the best known isoflavones and its content in soybean has been determined by high performance liquid chromatography and voltammmetry [41,44–46] and is in the range of 12–450 μg g^{-1}. The determination of total isoflavones in soybean cultivars from different countries (Korea, Italia, Brazil, USA) has been done by high performance liquid chromatography with UV and photo diode array detectors [36,42,44,45,47–49] in order to evaluate the influence of genetics, grown location and conditions, planting dates, seed weight and maturity groups on isoflavone contents. The concentration of total isoflavones in soybean in these studies varied from 1.8 to 766 mg/100 g.

Rutin and isoflavones have been associated with soybean resistance to pests. Some Brazilian soybean cultivars, with high concentrations of rutin and genistin, showed accentuated resistance to *Anticarsia gemmatalis* [32]. The adverse effects of a soybean genotype (PI 227687) on the physiology of *Trchoplusia ni* were assigned to the action of rutin as an antibiotic [33]. PI 227687, which possesses the highest concentration of genistin and daidzin, was considered the most promising genotype for use in soybean breeding programs as sources of resistance to stink bug [50]. These flavonoids are not evenly distributed within soybean tissues and organs. The distribution of these chemical defense compounds within soybean is a function of tissue or organ value in terms of fitness as it is predicted by the optimal defense theory (ODT) [51].

This work describes two highly sensitive DPV methods based on a glassy carbon electrode for the determination of rutin and total isoflavones in a Brazilian soybean cultivar (BRS 216 Flora) in different growth stages (VC, V1, V2, V3, V5, R4, R5, R6, R7 and R8). Thus, different parameters were evaluated for their quantification and for the validation of DPV methods in soybean. Compared to HPLC, the proposed methods present good selectivity, high sensitivity, rapid responses, low cost instrumentation and reduced sample size for the determination of rutin and total isoflavones. Moreover, the study presents new data about rutin and total isoflavone contents in seeds, leaves and pods of a Brazilian soybean cultivar in ten growth stages, which give support to the optimal defense theory. The data have also many applications in integrated pest management and breeding programs, contributing to the sustainability of soybean-based agricultural systems.

2. Experimental

2.1. Apparatus

DPV measurements were carried out on a 797 Voltammetric Analyzer (VA) Computrace (Metrohm, Switzerland) with an electrochemical cell composed of a glassy carbon (GC) rotating disk ($\Phi = 2.0$ mm) as working electrode, Ag/AgCl (3 M KCl) electrode as reference electrode and a platinum wire as auxiliary electrode. DPV measurements were performed in the potential range of 0.000 V (initial potential, E_i) to 1.000 V (final potential, E_f) at the following settings: 50 mV pulse amplitude and $\nu = 50$ mV s^{-1} scan rate. The hydrogen-ion potential (pH) of the solutions was determined using a LUCA-210 pH-meter (MS Tecnopon Equipamentos Especiais LTDA, BRAZIL). The ultrasonic cleaner (Cristófoli Equipamentos de Biossegurança LTDA, BRAZIL) was used for GC electrode cleaning and flavonoids (isoflavones and rutin) extraction in soybean seeds and leaves.

2.2. Chemicals and samples

Analytical-reagent grade chemicals and ultrapure water (Milli-pore, United States) were used to prepare all solutions. Genistein (>99% purity), daidzein (>99% purity), glycitin (99% purity), glycitein (>99% purity), daidzin (>99% purity), and genistin (>99.5% purity) were purchased from L. C. Laboratories (Canada) and Rutin hydrate (95% purity) was purchased from Sigma-Aldrich (Brazil). All flavonoids were used without further purification. Flavonoid Standard Stock solutions (1.0×10^{-3} M) were prepared in 5 mL of ethanol (Sigma-Aldrich, United States), diluting with water to 10 mL and then stored in dark bottles under freezing to prevent photodegradation. Phosphate buffers in the pH range of 3.0–9.0 were prepared using dibasic sodium phosphate, monobasic potassium phosphate and phosphoric acid (Sigma-Aldrich, United States, Brazil) and used as supporting electrolytes. Britton Robinson buffers in the pH range of 5.0–9.0 were prepared using phosphoric, acetic and boric acids purchased from Sigma-Aldrich (Brazil). Sodium hydroxide and hydrochloric acid (Sigma-Aldrich, Germany) were used for pH adjustment. Ethanol (Sigma-Aldrich, Germany) was used for flavonoids (isoflavones and rutin) extraction in soybean seeds and leaves. Alumina oxide powder <10 μm (Sigma-Aldrich, United States) and acetone (Sigma-Aldrich, Netherlands) were used for GC electrode cleaning. Nitric acid (Quimex, Brazil) was used to prepare laboratory glassware. Soybean seeds and leaves of Flora cultivar (Embrapa Cerrados, Brazil), produced under greenhouse conditions (temperature (T) = 20–30 °C; relative humidity = 50–85%), were harvested at VC, V1, V2, V3, V5, R4, R5, R6, R7 and R8 growth stages.

2.3. Procedures

The laboratory glassware was kept in a 20% (by volume) nitric acid solution overnight. Afterwards, it was kept in an ultra pure water bath overnight, rinsed thoroughly with ultra pure water and air-dried.

GC electrode cleaning was based on the following procedure: the GC electrode surface was polished manually with alumina suspension on polishing cloth for 1 min and rinsed with distilled water; The electrode was immersed in acetone and submitted to ultrasonication for 4 min and rinsed with ultra pure water; the electrode was immersed in phosphate buffer pH 6.0 and submitted to continuous potential cycling from 0.000 to 1.800 V at 50 mV s^{-1} for 3 min. Voltammograms were recorded until steady state baseline voltammograms were obtained.

Experiments were performed with six isoflavones (genistein, daidzein, glycitin, glycitein, daidzin, genistin) and rutin at room temperature in three replicates.

The voltammetric profile of six isoflavones (genistein, daidzein, glycitin, glycitein, daidzin, genistin) and rutin was studied by differential pulse voltammetry through additions of 100 μL of each isoflavone and rutin at 1.0×10^{-3} M to the electrochemical cell containing 10 mL of phosphate buffer pH 6.0. In another experiment, the voltammetric profile of a mixture of five isoflavones (genistin, daidzin, glycitin, genistein and daidzein) at 4.0×10^{-3} M was studied by differential pulse voltammetry through additions of 200 μL of 1.0×10^{-3} M genistin, 120 μL of 1.0×10^{-3} M daidzin, 60 μL of 1.0×10^{-3} M glycitin, 12 μL of 1.0×10^{-3} M daidzein and 2 μL of 4.0×10^{-3} M genistein to the electrochemical cell containing 10 mL of phosphate buffer pH 6.0. In order to evaluate the use of genistein as the quantification standard of total isoflavone determination in the hypothetical mixture, three additions of 20 μL of 1.0×10^{-3} M genistein were done and the recovery results of total isoflavones and relative error were calculated.

In order to optimize the experimental conditions and gain the highest sensitivity for the DPV methods, the influence of the pH, scan