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Original Article

Isoflavone-aglycone fraction from *Glycine max*: a promising raw material for isoflavone-based pharmaceutical or nutraceutical products

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ABSTRACT

The present work was designed to obtain a fraction containing high concentration of isoflavone-aglycones from *Glycine max* (L.) Merr., Fabaceae, named isoflavone enriched-fraction, from a dry extract containing isoflavones-glycosides. A simple and low cost method was proposed: extraction of isoflavone glycosides from the *G. max* dry extract with a proper solvent, hydrolysis of the glycosides, recovery of the aglycones, and purification of the fraction containing high isoflavone-aglycones concentration. All the extraction and purification parameters were optimized based on the isoflavones yields, which were analyzed by liquid chromatography and expressed as total isoflavone aglycones. The optimization of the process conditions was accomplished using the classical one-variable-at-a-time method. The identity and purity of the isoflavones contained in this enriched fraction were determined by LC/UV/ESI/MS analysis, Fourier transformed-infrared spectroscopy, and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The physicochemical properties of the isoflavone enriched-fraction were evaluated by scanning electron microscopy, X-ray diffraction and differential scanning calorimetry. The moisture content, particle size, equilibrium solubility and thermal and photostability were also determined. The high isoflavone-aglycone content (daidzein, 489.35 mg/g; glycitein, 251.02 mg/g and genistein, 158.96 mg/g) as well as the high purity obtained (90% of total isoflavones) make this fraction a promising novel raw material for the production of isoflavone-aglycones based pharmaceuticals or functional foods.

Introduction

The isoflavones belong to the class of flavonoides and are especially present in the species of the Fabaceae as *Glycine max* (L.) Merr. (soy) (Kuiper et al., 1998; Yamaguchi, 2002; Howes et al., 2006). Since 1950, isoflavones have been extensively studied due to their estrogenic effect (Gardner et al., 2001; Adlercreutz, 2002) being related to most of their beneficial health effects, like the relieve of symptoms associated to the menopause (Murphy et al., 2002), prevention of bone mass loss due to ovarian hormone deficiency (Rostagno et al., 2007), reduction of the levels of postprandial glucose, triacylglycerides, cholesterol and weight in patients with type 2 diabetes (Li-Hsun et al., 2004; Rostagno et al., 2004).

Thus, the development of isoflavone-based pharmaceutical products, functional foods or dietary supplements has demonstrated its interest to promote health benefits. Despite most available products contain isoflavones in the glycoside form, the isoflavones absorption only occurs in the aglycone form, which is provided by the β-glucosidases. Considering that this step varies within individuals (Rostagno et al., 2009), the conversion of the isoflavone-glycoside into their aglycone form reveals to be an interesting way to overcome this limitation.

Different extraction methods have been employed for isoflavone extraction from soybeans, such as simple stirring (Murphy et al., 2002), sonication (Rostagno et al., 2007b), soxhlet (Li-Hsun et al., 2004) pressurized liquid extraction (Rostagno et al., 2004), supercritical fluid extraction (Kao et al., 2008) and microwave-assisted extraction (Rostagno et al., 2007a). Nevertheless, some of the methods are expensive and/or energy and time consuming, or even, require large amounts of solvent and are ineffective for isoflavone extraction (Terigar et al., 2010).

In the same way, different chromatographic methods are used to fractionate or isolate pure products. Generally, conventional methods such as low pressure chromatography and preparative reversed-phase liquid chromatography are used, but they are time and solvent consuming, require multiple chromatographic

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steps and present the risk of irreversible adsorption on the solid-phase materials (Choi and BeKim, 2005). Separation methods of isoflavones by high-speed counter-current chromatography have been also described (Yang et al., 2001). The main limitation of this technique is to find a solvent system able to provide partition coefficients different enough for all the isoflavones (Valls et al., 2009). Moreover, it usually requires lengthy steps using solvents which are inappropriate to large-scale production.

In this context, the present work was designed to produce a standardized fraction containing high concentration of *G. max* isoflavone-aglycones that could be used as a new raw material to develop isoflavone-based products, phytopharmaceuticals or nutraceuticals. The physicochemical properties and preliminary stability tests of the isoflavone enriched fraction were also evaluated.

Materials and methods

Chemicals

Liquid chromatography (LC)-grade acetonitrile (Tedia, Fairfield, OH, USA), trifluoroacetic acid (Merck, Hohonbrunn, Germany), and purified water (Milli-Q^{TM} system, Millipore, Bedford, MA, USA) were used for mobile phase preparation. Daidzein (98%, HPLC purity), glycitein (97%, HPLC purity), and genistein (98%, HPLC purity) were obtained from Sigma–Aldrich (Steinheim, Germany) and were used as external standards. Ethanol 96% (v/v) (Rebeschini, Porto Alegre, Brazil) and concentrated hydrochloric acid (Fmaia, São Paulo, Brazil) was used to extract and hydrolyze the isoflavone glycosidic conjugates, respectively. *Glycine max* (L.) Merr., Fabaceae, dry extract (GMDE) was purchased from JieJing Biology Technology (Xiamen, China).

Liquid chromatography analysis

Chromatographic conditions

The LC analysis was performed as described by Yatsu et al. (2014) on a Shimadzu Prominence device coupled to diode array detection (DAD) instrument and an autosampler (Kyoto, Japan). The stationary phase was a Phenomenex RP-18 column (Synergi Fusion 150 × 4.6 mm i.d.; particle size, 4 μm) guarded by a Waters RP-18 precolumn (20 × 3.9 mm i.d.; particle size, 10 mm) (Milford, MA, USA). The mobile phase consisted of (A) trifluoroacetic acid 0.1% (v/v) and (B) acetonitrile:trifluoroacetic acid (100:0.01, v/v). The gradient elution was 20–25% B (0–10 min), 25–30% B (10–15 min), and 30–35% B (15–23 min). The column was washed with acetonitrile for 3 min and re-equilibrated with 20% B for 4 min before the next analysis. The flow rate was 1 ml/min and the injection volume was 10 μl. The detection wavelength was 260 nm and the analysis was carried out at 40 °C.

LC–ESI-MS system was an Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA) was coupled to triple quadrupole API 5000 mass spectrometer (Applied Biosystems/Sciex, Foster City, CA, USA) using electrospray ionization interface in negative ionization mode (ESI). Data acquisition and data processing was performed by Analyst 1.4.2 software (Applied Biosystems/Sciex). The isoflavone enriched-fraction was identified based on the aglycones UV spectra between 200 and 400 nm, their ESI mass spectra, and their retention times, with respect to the reference materials.

Determination of aglycones in the Glycine max *dry extract*

It was performed according to Delmonte et al. (2006). Briefly, about 100 mg *G. max* dry extract was accurately weighed in a 50 ml Erlenmeyer and 10 ml of acetonitrile was added to suspend the sample. Deionized water (6 ml) was added and shaken for 2 h at 60 °C. After cooling down to room temperature, the volume was adjusted to 20 ml with deionized water. The resulting solution was centrifuged for 10 min at 5400 × *g*. The clear solution was diluted with acetonitrile 50% (v/v) and filtered through a 0.45 μm PTFE membrane for direct analysis or hydrolyzed (4 ml) with 0.5 ml of concentrated HCL for 2 h at 80 °C. The hydrolyzed solution was diluted with acetonitrile 50% (v/v) and filtered through a 0.45 μm PTFE membrane for direct analysis.

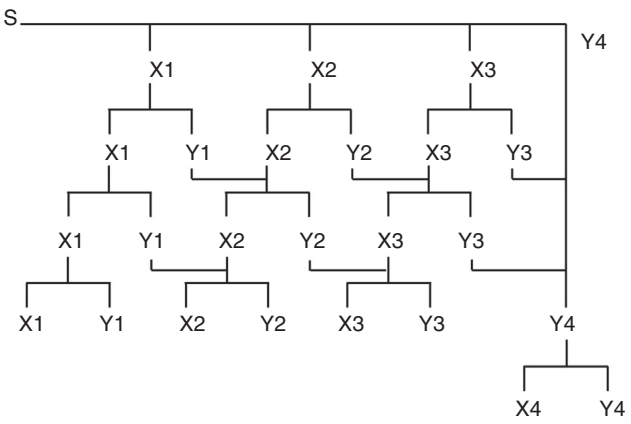

Fig. 1. Fractional crystallization scheme.

Isoflavones extraction and purification

Extraction of isoflavones from *G. max* dry extract was performed with ethanol at the ratio of dry extract to ethanol of 1:12 to 1:20 (g/ml). The resulting mixture was heated at 25–60 °C for 2–12 h under constant agitation. The extractive solution was separated from the insoluble material by filtration and the glycosides were hydrolyzed using different amount of 37% hydrochloric acid at 80 °C for 2–12 h under constant agitation. The resulting solution was mixed with distilled water at the volume ratio of 1:1 to 1:6 (ml/ml), stayed for 12 h at 10 °C. The supernatant was separated by decantation. The solid was dissolved in ethanol yielding an initial solution S. Three successive crystallizations were performed, being X1, X2 and X3 the precipitates obtained and Y4 the mother solution. Each of these precipitates (Xi) was dissolved in 96% ethanol and then recrystallized, thus obtaining a precipitate Xi and a solution Yi from each Xi. Each solution Yi was separated from the solid Xi, by filtration, and used to dissolve the solid Xi + 1. Y3 was mixed with the mother liquor Y4. The process was repeated 3 times and those solutes present in Y4 were also recovered by crystallization, which gave the solid X4 (Fig. 1). All the experiments were performed in triplicate.

Isoflavone enriched-fraction physicochemical characterization

Yield and moisture content

The yield of the optimized process of extraction and purification was calculated as the difference between the theoretical amount of isoflavone aglycones present in the *G. max* dry extract and the amount of the dry isoflavone enriched-fraction obtained at the end of the process, taking into account the residual moisture content determined by the titrimetric method (USP, 2009).

Scanning electron microscopy and particle size

The photomicrographs of the samples were taken using a Jeol JSM 6060 microscope (Tokyo, Japan) at a voltage of 10 kV. The samples were previously mounted on aluminum stubs using double-sided adhesive tape and vacuum-coated with a thin layer