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Original Article

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

Francini K.J. Yatsu, Letícia S. Koester, Valquiria L. Bassani *

Laboratório de Desenvolvimento Galênico, Programa de Pós-graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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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

* Corresponding author.
E-mail: valquiria.bassani@ufrgs.br (V.L. Bassani).

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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.

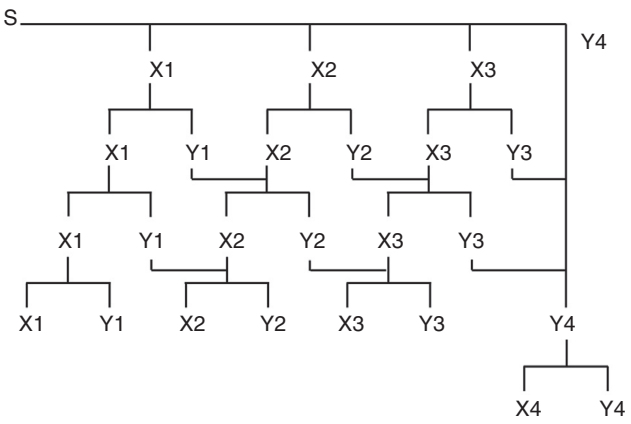

Fig. 1. Fractional crystallization scheme.

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

of gold. The particle size analysis was determined using a laser diffractometer CILAS 1180 (Madison, USA). Approximately 200 mg of samples were dispersed in silicon 250 cps and suspended with an ultrasonic system to prevent sedimentation of the particles. The information acquired was analyzed by a computational program and values of mean diameters were obtained.

Fourier transformed-infrared (FTIR) spectroscopy

Infrared spectra covering the range of 4000–400 cm^{-1} were obtained with a Spectrum BX FTIR spectrometer with *MIRacle ATR* accessory (Perkin Elmer, MA, USA). The spectra were an average of 36 scans at a resolution of 4 cm^{-1}.

Thermal analysis

Differential scanning calorimetry (DSC) curve was performed in a DSC-60 calorimeter (Shimadzu Co., Kyoto, Japan) using the following conditions: dynamic nitrogen atmosphere (50 ml/min) and heating rate of 10 °C/min. Samples were accurately weighted and submitted to further heat scanning from 25 to 500 °C in a sealed aluminum pan. An empty sealed aluminum pan was used as reference. Data analysis was carried out using TA Analysis Software.

X-ray diffraction

The X-ray diffraction (XRD) experiments were carried out on a Siemens D5000 diffractometer (Siemens, Berlin, Germany) equipped with a curved graphite crystal using Cu Kα radiation (λ = 1.5406 Å). The diffraction data were collected at room temperature in Bragg–Brentano θ–2θ geometry. The equipment was operated at 40 kV and 20 mA with a scan range between 8 and 40. The diffractograms were obtained with a constant step, step size/time of 0.02–2θ/s.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra obtained for samples were recorded in a Bruker DRX400 MHz, 24 °C, using DMSO-*d6* as solvent. Chemical shifts were reported in parts per million, using tetramethylsilane (0 ppm) as internal standard. One-dimensional ^{1}H NMR and ^{13}C NMR were obtained under standard conditions.

Solubility determination

The aqueous solubility of the isoflavone enriched-fraction and standards were determined by adding an excess of sample into 2 ml of distilled water in a glass vial. The glass vial was capped and attached to a wrist shaker in a water bath at 24 ± 2 °C for 2 days with agitation. Afterwards, the samples were centrifuged 30 min at 5400 × *g*, filtered through a 0.45 μm PTFE membrane and analyzed by LC. All the experiments were performed in triplicate.

Forced degradation studies

The isoflavone enriched-fraction was solubilized in methanol, an aliquot was transferred to a volumetric flask and the volume was completed with one of the three stressing agent solution (1 M HCl, 1 M NaOH or H_2O_2 30%). The resultant samples were stored at 5, 24 and 60 °C or in a photostability chamber (UVC 254 nm) at 24 °C for 7 days. The isoflavones content was measured in triplicate using the above described LC method. The concentrations of the remaining isoflavones determined at the different time intervals of degradations studies were plotted in three different graphs, as follows: (a) concentrations values against time (zero-order kinetics), (b) Ln of concentration versus time (first-order kinetics) and (c) reciprocal of concentration versus time (second-order kinetics). The determination coefficients (*r*) were obtained and the best fit observed. Each experiment was done in triplicate (analyzed by LC method) and average values were taken for the analysis.

Statistical analysis

The results were analyzed either by the student's test or by the analysis of variance (ANOVA) followed by Tukey's test for significance at $p < 0.05$.

Results and discussion

Isoflavones extraction and purification

The absorption of isoflavones along the gastrointestinal tract occurs when they are in the aglycone form. In soybean and most of soybean base products they are present as glycosides and the β-glucosidases present in the gastrointestinal tract microflore provide its hydrolysis to aglycones (Setchell et al., 2002a). However, the rate of hydrolysis varies within individuals (Setchell et al., 2002b). Thus, in view to obtain products with more reproducible absorption, the conversion of the isoflavone-glycosides to their aglycone form reveals to be a promising strategy. Moreover, a standardized fraction containing high concentration of *G. max* isoflavone-aglycones is a novel and potential raw material to develop isoflavone-based products, phytopharmaceuticals or nutraceuticals.

In this work, a standardized *G. max* dry extract containing 22% of isoflavone glycosides was used as raw material. The process could be generally resumed in the following steps: 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 LC and expressed as total isoflavone aglycones. The optimization of the process conditions was accomplished using the classical one-variable-at-a-time method.

The solid–liquid extraction method by mechanical stirring was chosen to extract the isoflavone glycosides because it is a simple, versatile and low cost technique. Since isoflavone heterosides are polyphenols with several hydroxyl groups linked to sugar moiety, they are more soluble in polar solvents. The study performed by Murphy (1981) compares the use of different organic solvents (methanol, ethanol, acetonitrile, ethyl acetate, and chloroform) combined to different water and acid proportions for the extraction of isoflavones from many soy products (Rostagno et al., 2009). Among the solvent used, ethanol was the best choice to extract these compounds, due to its high efficiency, low cost, low toxicity, environmental compatibility, ease recovery and recycling. Thus, in the present study, the extraction efficiency of ethanol containing different proportions of water (6:4; 8:2; 9.6:0.4) was comparatively evaluated after 8 h of mechanical stirring at room temperature; the ratio of *G. max* dry extract to solvent was 1:50 (g/ml) in view to avoid the solvent saturation (Fig. 2).

Despite no significant difference ($p < 0.05$) in the extraction efficiency between the different proportions of water added to the ethanol has been observed, 96% (v/v) ethanol was selected as the solvent for extraction because higher water concentration also co-extracted many other water-soluble compounds present in the *G. max* dry extract (data not shown).

In order to establish the volume of solvent necessary to achieve the highest yield of isoflavones, different proportions of dry extract:solvent were evaluated after 8 h of mechanical stirring at room temperature (Fig. 3). There was no significant difference between the ratios 1:20 and 1:50 of sample: 96% (v/v) ethanol proportions ($p > 0.05$), and therefore the ratio of 1:20 (g/ml) was selected. Moreover, the relation between time and temperature of extraction was evaluated (Fig. 4). The yield of total isoflavones

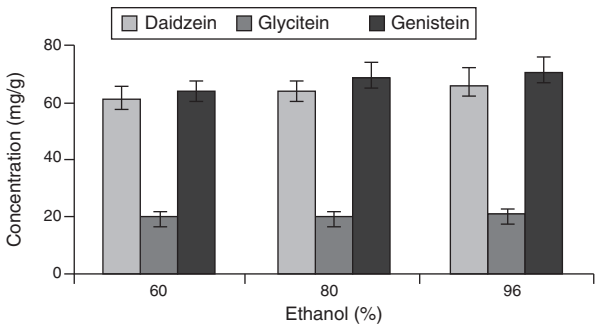

Fig. 2. Solvent extraction efficiency at sample:solvent ratio of 1:50 (g/ml) after 8 h of mechanical stirring at room temperature.

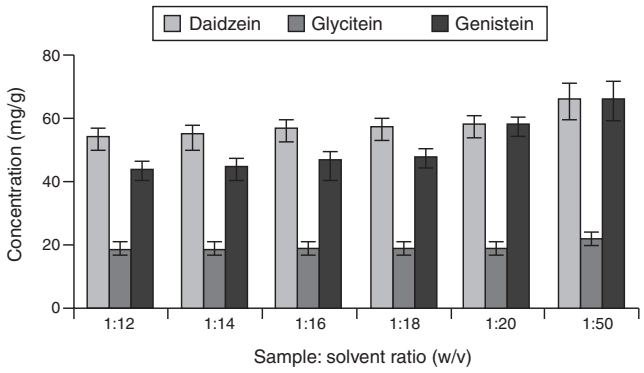

Fig. 3. Yield of total isoflavones at different sample:solvent proportions after 8 h of mechanical stirring at room temperature.

increased with increasing temperature ($p < 0.05$) reaching the highest concentration at 60 °C, but did not increase over time.

In summary, the optimum extraction conditions were found to be 60 °C for 2 h at the dry extract:solvent ratio of 1:20 (g/ml) using ethanol 96% (v/v) as solvent. In these conditions, it was possible to extract practically 100% of the isoflavones aglycones from the *G. max* dry extract, about 144.04 ± 4.25 mg of total isoflavones from 1 g of *G. max* dry extract.

In order to convert isoflavone glycosides to biologically active isoflavone-aglycones, the β-glycosidic linkage must be broken. In the last few years, several studies have reported the transformation of isoflavone glycosides to aglycones by acid and enzymatic hydrolysis (Utkina et al., 2004; Chun et al., 2008). Considering the cost of acquisition or production of β-glucosidases from microorganisms and the complexity of the hydrolytic enzymatic processes, we have chosen acid hydrolysis. The acid hydrolysis efficiency (acid concentration and hydrolysis time) was assessed at 80 °C with hydrochloric acid and it was also evaluated by LC (Fig. 5).

According to Zhang et al. (2007), hydrolysis is not efficient at low temperatures (<75 °C) but temperatures higher than 80 °C did not increase hydrolysis efficiency besides being more energy consuming. Regarding the choice of the acid, hydrochloric acid is one of the least hazardous strong acids to handle and their solutions are quite stable upon storage, maintaining their concentrations over time. These attributes, plus the fact that it is available as high quality reagent, make hydrochloric acid an excellent acidifying reagent. Moreover, the hydrolysis was optimized using hydrochloric acid concentration in the range of 0.1–0.5 M at 80 °C. The yields of aglycones were found to be correlated with the acid concentration and hydrolysis time, where the last parameter was more significant. The optimum hydrolysis condition was found to be 80 °C for 12 h at the acid concentration of 0.5 M to achieve the best yield of isoflavones, about 130.33 ± 1.08 mg of total isoflavones/g of *G. max* dry extract, which corresponds to 90% of the total isoflavone aglycones present

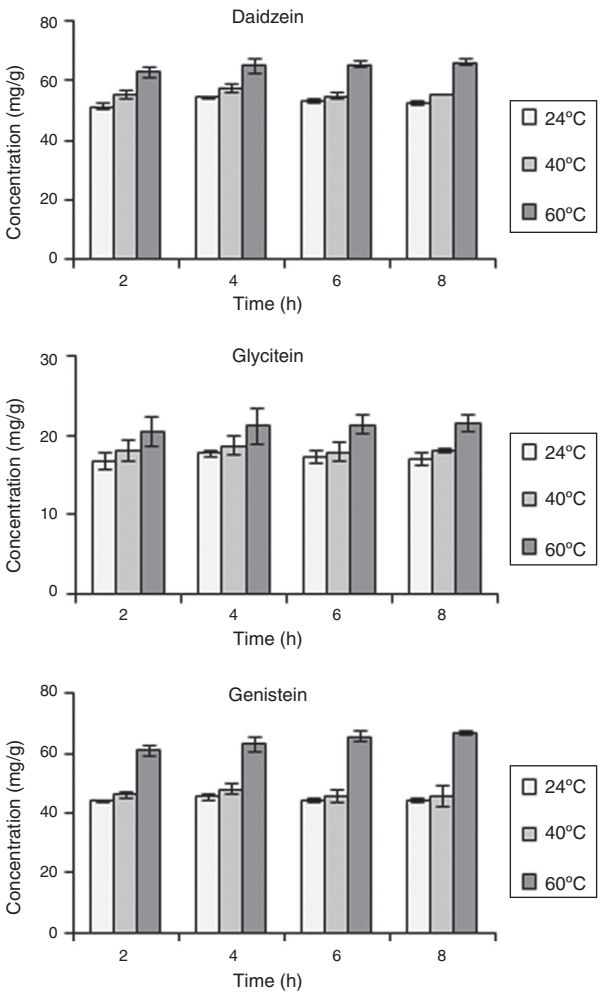

Fig. 4. Yield of total isoflavones as function of time and temperature at sample:solvent ratio of 1:20 (g/ml).

in the extractive solution. Daidzein, glycitein and genistein correspond, respectively to 85%, 100%, and 92% of total isoflavones. In the LC chromatogram it was observed that glycitin and genistin peaks disappeared while daidzin peak was significantly reduced, but it remain present in low amount, demonstrating to be more resistant to the acid hydrolysis.

Since the isoflavone aglycones are practically insoluble in water, its addition as antisolvent is a promising alternative for recovering them by crystallization from the hydrolyzed extract. Thus, the aglycone crystallization was performed by adding a known amount of water to the hydrolyzed extract. The mixture was filtered through a 0.2 μm membrane to separate the solids from the mother liquor. The supernatant was dissolved in methanol and analyzed by LC. The recovery was calculated as the difference between the amount of isoflavone aglycones present in the hydrolyzed extract and the amount of aglycones obtained from the crystallization process (Fig. 6). The results demonstrated that the best ratio of water added to the hydrolyzed extract for crystallization was 1:5 (v/v). Higher ratios did not improve the yields. Furthermore, higher ratios lead to high loading on the crystallization and filtration step, which would result in difficulties in manufacturing. At this ratio, the recovery of the total isoflavone aglycones was 61% (51.5% for daidzein and glycitein and 74% for genistein). Similar results were obtained by Harjo and coworkers (Harjo et al., 2007), who observed recovery of 35% for glycitein, 45% for daidzein and 90% for genistein employing the same technique to extract and purify isoflavones from a soybean

Table 1
The content (mg/g) of isoflavone-aglycones in the products obtained in the extraction and purification optimized processes.

Isoflavones	Concentration (mg/g) (RSD)			
	Glycine max dry extract	Extractive solution*	Hydrolyzed extract*	Crystallized product*
Daidzein	60.77 ± 4.24	62.80 ± 1.72	53.47 ± 0.38	27.53 ± 0.47
Glycitein	19.80 ± 1.38	20.46 ± 0.56	20.97 ± 0.38	10.80 ± 0.20
Genistein	63.79 ± 3.84	60.79 ± 1.97	55.90 ± 0.70	41.27 ± 0.19
Total	144.36 ± 9.46	144.05 ± 4.25	130.34 ± 1.08	79.60 ± 1.04

RSD, relative standard deviation.
* The content (mg/g) was calculated based on the amount of isoflavones in the *Glycine max* dry extract.

flour using methanol as the extraction solvent, 37% hydrochloric acid to perform the hydrolysis and the same ratio of hydrolyzed extract:water.

The recrystallized powder were analyzed by LC and presented a content of 54% of total isoflavones. An overview of the isoflavone recoveries along of the process, extraction, hydrolysis and purification is shown in Table 1. The recrystallization steps are detailed in Fig. 1 and the isoflavone aglycones content assessed at each step summarized in Table 2. The results are expressed as total isoflavone aglycones. The LC analysis of the recovered aglycones also demonstrated that most of the impurities in the hydrolyzed extract were removed by the antisolvent used in the crystallization (data not shown). Thus, the crystallization using water as antisolvent proved to be a faster and cheaper alternative method to isoflavone purification. Ethanol 96% was selected as the solvent for recrystallization because the isoflavones are more soluble in this solvent (Wu et al., 2010; Nan et al., 2014).

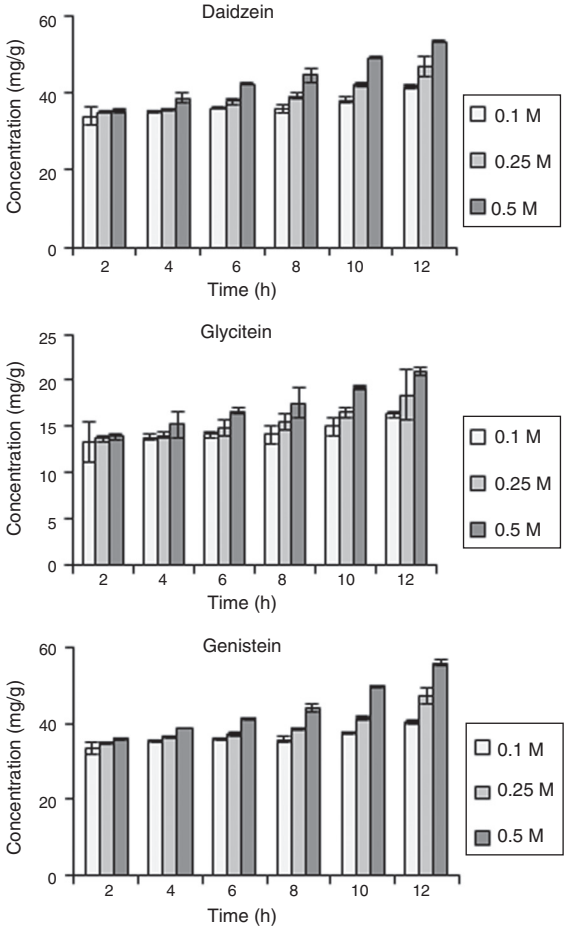

Fig. 5. Acid hydrolysis efficiency at 80 °C as function of hydrochloric acid concentration and hydrolysis time.

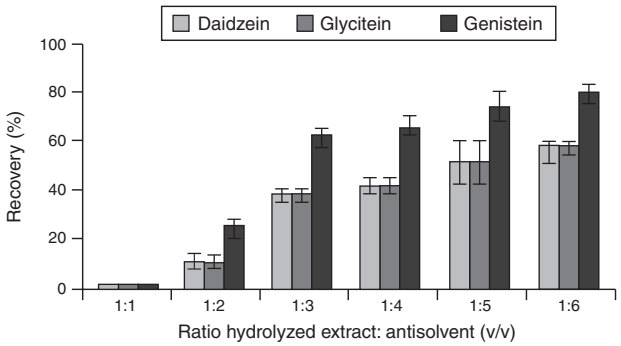

Fig. 6. Recovery of total isoflavone aglycones from the hydrolyzed extract after the antisolvent crystallization process.

Recrystallizations are usually made using portions of pure solvent and the desired degree of purity can be obtained after one or two controlled recrystallizations. However, in a mixture of components with similar solubilities or that forming solid solutions, only fractional crystallization is able to obtain the different components with an acceptable purity grade and recovery (Pombeiro and Latourrette, 1998).

Fractional crystallization is a method of purification substances based on differences in their solubility. If a mixture of two or more substances in solution is allowed to crystallize, for example by allowing the concentration of the solution to increase, the precipitate will contain more of the least soluble substance. If the solubility products are very similar, a cascade process will be needed to effectuate a complete separation. Cascade process is any process that takes place in a number of steps, usually because the single step is too inefficient to produce the desired result. Therefore, to achieve better separation the process has to be repeated a number of times, in a series, with the enriched fraction of one stage being fed to the succeeding stage for further enrichment (Pombeiro and Latourrette, 1998).

As expected, the results demonstrated that glycitein concentration decreased at each new recrystallization step. Since this

Table 2
The content (mg/g) of isoflavone-aglycones in the products obtained in the fractional recrystallization processes.

Fractions	Isoflavones concentration (mg/g) (RSD)*			
	Daidzein	Glycitein	Genistein	Total
X1	124.82 ± 3.12	727.13 ± 5.23	65.75 ± 2.07	917.70 ± 9.18[a]
X2	489.35 ± 5.30	251.02 ± 3.33	158.96 ± 3.06	899.33 ± 9.26[a]
X3	338.70 ± 9.84	257.57 ± 3.40	205.86 ± 3.54	802.13 ± 13.23[b]
X4	522.32 ± 5.07	71.86 ± 2.03	260.36 ± 4.14	854.54 ± 8.84[c]

RSD, relative standard deviation.
* The content (mg/g) was calculated based on the amount of isoflavones in the crystals obtained in the fractional recrystallization experiment performed in triplicate; significant statistical differences were determined by Tukey's test ($p < 0.05$); differences between the concentrations were represented by different letters (a, b, c).

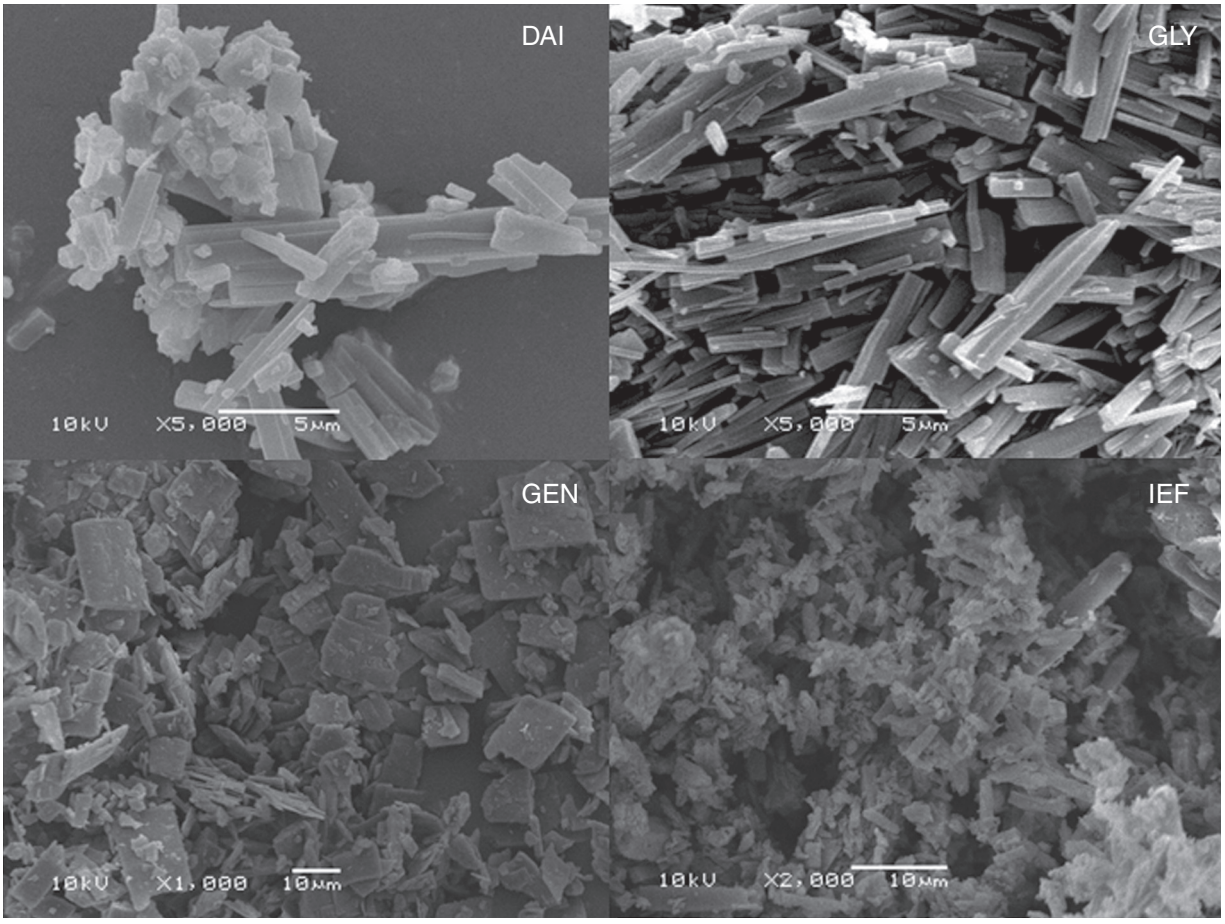

Fig. 7. Scanning electron microscopy photograph of isoflavone standards daidzein (DAI), glycitein (GLY), genistein (GEN) and enriched fraction (IEF).

isoflavone has the lowest solubility, the crystals will contain more of it in the early stages. On other hand, daidzein and genistein concentration increased at each new recrystallization step due to their higher solubility in the solvent. All fractions (X1, X2, X3 and X4) presented a high purity (80–92%). However, X2 presented the highest purity (90% of total isoflavones) with a more balanced isoflavone content, which makes this fraction a promising novel raw material for the production of isoflavone-aglycones based pharmaceutical or functional foods.

Isoflavone enriched-fraction characterization

With the purpose to confirm the identity and purity of the isoflavones present in the isoflavone enriched-fraction, the purified fraction was submitted to LC/UV/ESI/MS analysis, FTIR spectroscopy, and ^{1}H and ^{13}C NMR spectroscopy.

The *m*/*z* values of the analyzed isoflavones were (for negative ESI mode): daidzein (*m*/*z* 253.1, 223.1 and 195.2), glycitein (*m*/*z* 283.2, 268.1, 240.1, 211.1 and 184.1) and genistein (*m*/*z* 269.2, 225.3, 213.0, 197.2, 181.1 and 169.1). Their λ_{max} were 249 and 301 nm; 257 and 320 nm; and 259 nm, respectively. These results are all in agreement with those found in the standard materials and with the literature (Delmonte et al., 2006).

The isoflavone enriched-fraction FTIR spectrum showed a mixture of characteristic absorption bands of the three isoflavone aglycones. These results are in agreement with the isoflavone standards (Supplementary material Fig. S1). In the same way, the ^{1}H and ^{13}C NMR spectrum of the fraction demonstrated to be a mixture of chemical shifts of the three isoflavone aglycones, but was possible to identify some characteristic chemical shifts of each one (Supplementary material Fig. S2) (Jha et al., 1980; Coward et al., 1993; Park et al., 1999; Sung et al., 2004).

Supplementary Figs. S1 and S2 related to this article can be found, in the online version, at doi:10.1016/j.bjp.2015.12.004.

The isoflavone enriched-fraction showed to be a fine and pale brown powder with a mean particle size of 14.32 μm and residual moisture content of 0.52 ± 0.04%. The scanning electron microscopy (Fig. 7) revealed particles with habit and surface very similar to the native forms of daidzein and glycitein, the main components of the isoflavone enriched-fraction.

In the equilibrium solubility study (Table 3), the isoflavone aglycones presented solubility in water higher than that observed for the standards. Similar findings were reported for an aglycone mixture of soy isoflavones (consisting of 55% genistein, 43% daidzein, and 1.8% glycitein) (Huang et al., 2008). The authors verified that the solubility of genistein and daidzein in the mixture was 5.2- and 3.3-fold higher than that of the isolated compounds in pH 6 buffer. They suggested that the aglycone mixture of soy isoflavones form a eutectic solid dispersion, which indicates the existence of intermolecular interactions between the two isoflavones forming aggregates or other entities with different solubility from those of the individual molecules.

Table 3
Equilibrium solubility study.

Isoflavones	Purified fraction (μg/ml) (RSD)	Standards (μg/ml) (RSD)
Daidzein	12.54 ± 0.05	1.47 ± 0.08
Glycitein	11.32 ± 1.97	0.59 ± 0.12
Genistein	20.69 ± 1.29	5.77 ± 0.24

RSD, relative standard deviation.

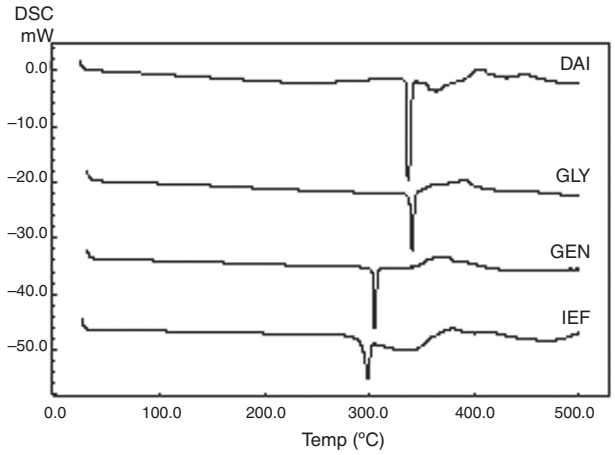

Fig. 8. The differential scanning calorimetry curve isoflavone enriched-fraction (IEF) and isoflavone standards daidzein (DAI), glycitein (GLY) and genistein (GEN).

Thus, the melting points of the isoflavone enriched-fraction and the isoflavones standards were determined by DSC (Fig. 8). Isoflavone enriched-fraction, daidzein, glycitein and genistein DSC curves showed a single sharp endothermic event at 300 °C, 337 °C, 341 °C and 305 °C, respectively.

The thermogram shows that the isoflavone enriched-fraction showed a main endothermic peak, at lower temperature than that reported for the standards, suggesting that an eutectic solid dispersion could have been formed with the aglycone molecules.

However, the difference in solubility between the isoflavones in isolated form and as a mixture can be additionally related to the different crystal structures (polymorphism) and crystal habits (morphology). XRD pattern of the isolated isoflavones used as reference revealed a well-defined crystal, whereas IEF exhibited a considerable diminution of the diffraction peaks and an absence of sharp peaks, suggesting that it is less crystalline than the standards (Fig. 9). These results point to further studies for enlightening this phenomenon.

Taking in mind that the isoflavone enriched-fraction may be used as raw material for the production of aglycone-based pharmaceutical or nutraceutical products, its stability was evaluated preliminarily in solution under acidic, basic, neutral, oxidative and photolytic stress conditions (Fig. 10). The results showed that isoflavones degraded at elevated temperature in the presence of hydrogen peroxide or in alkaline pH. These results are in agreement with Ungar et al. (2003), who reported that, in alkaline solution, the concentration of genistein and daidzein were reduced 60% and 15%, respectively.

Under acidic and neutral stress conditions, the isoflavones proved to be stable for 7 days even at 60 °C (Fig. 10A). This observation proves that the extraction and purification method developed has no negative effect on the stability of the aglycones, in other words, they do not undergo degradation during the process.

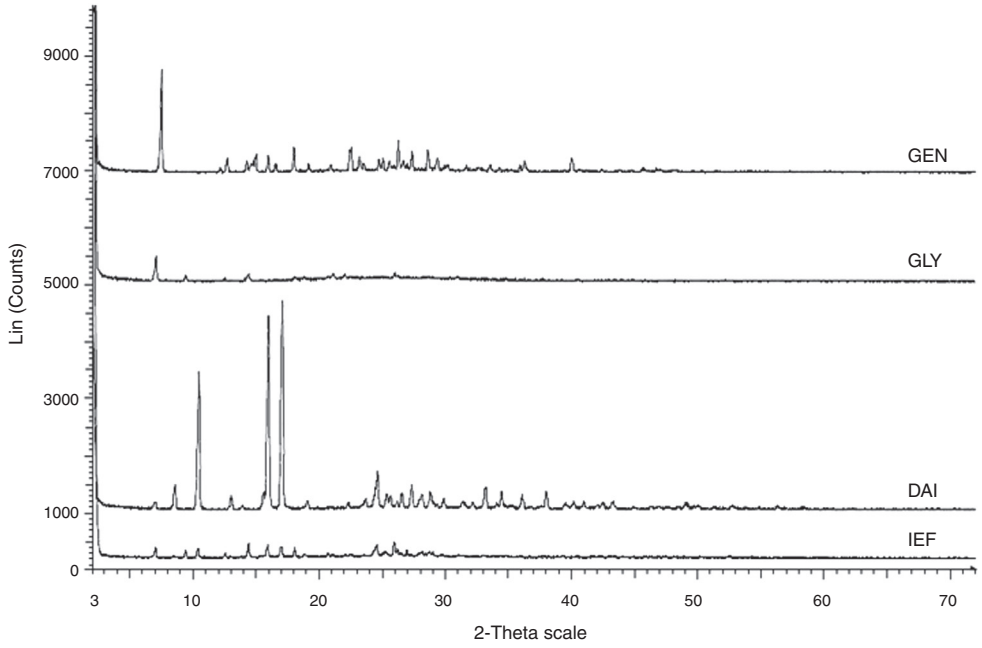

Fig. 9. X-ray diffraction pattern of isoflavone enriched-fraction (IEF) and isoflavone standards daidzein (DAI), glycitein (GLY) and genistein (GEN).

Table 4
Kinetics of degradation of isoflavones submitted to stress conditions.

Stress conditions	K (mg/day)					
	NaOH			H_2O_2		
	Daidzein	Glycitein	Genistein	Daidzein	Glycitein	Genistein
60 °C	−0.53[b,c]	−0.09[b,c]	−0.15[b,c]	−0.37[b]	−1.26[a]	−0.53[b]
UV/24 °C	−0.75[b]	−0.78[a]	−0.37[b]	−0.74[b]	−0.69[b]	−0.97[b]

K, Degradation rate constant.
[a] Zero-order kinetic.
[b] First-order kinetic.
[c] mg/h.

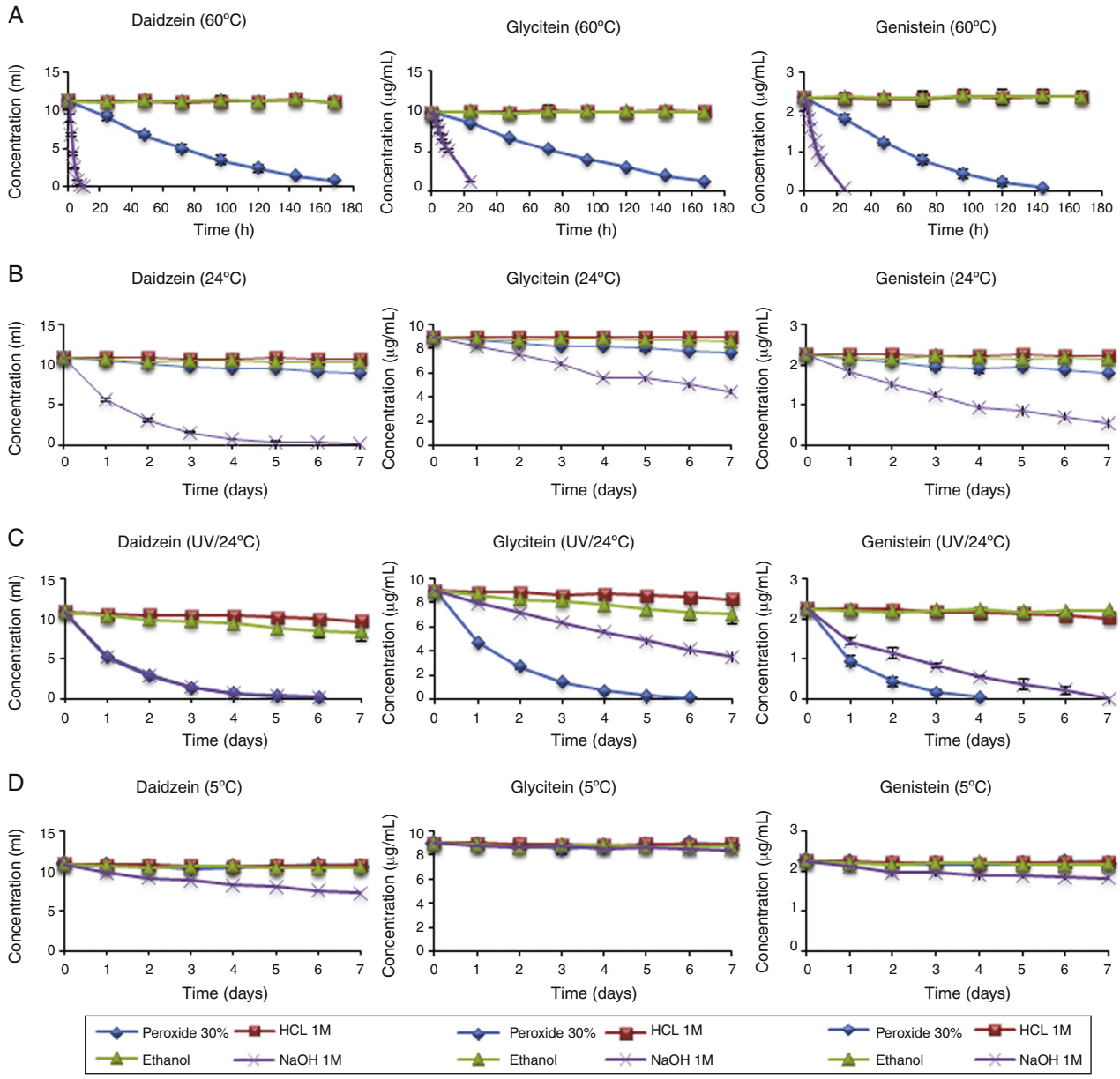

Fig. 10. Total isoflavone aglycone content under acidic, alkali, neutral and oxidative stress conditions at 60 °C (A), 24 °C (B), 24 °C/UVC radiation (C) and 5 °C (D).

All isoflavones were also unstable when exposed to UVC radiation, regardless of the stress conditions (Fig. 10C). The kinetics of degradation of isoflavones submitted to stress conditions is summarized in Table 4. Through the evaluation of the correlation coefficients, it can be demonstrated that most isoflavones showed a first-order kinetics under the stressing conditions except glycitein, that followed a zero-order kinetics when exposed to hydrogen peroxide at 60 °C and to UV radiation.

These results are partially in accordance with Rostagno et al. (2005), who demonstrated that only daidzein and genistein, in ethanol–water extracts and standard solutions, were sensitive to UV–VIS light. Therefore, during the extraction and purification process, the isoflavones should be protected from UV radiation to avoid degradation. In the present work, the main observed degradation factors were the hydrogen peroxide, alkaline pH and elevated temperature. Therefore, isoflavones must be kept at low temperatures and protected from light to avoid degradation.

Taken together, the optimized process of extraction and purification of isoflavones from *G. max* dry extract was performed with ethanol 96% (v/v) in a ratio of dry extract:ethanol of 1:20 (g/ml), at 60 °C for 2 h. The isoflavones-heterosides present in the extractive solution were successfully hydrolyzed (HCl 0.5 M, 80 °C, 12 h) and the aglycones were separated using water as antisolvent (ratio 1:5, v/v; 12 h at 10 °C). The supernatant was recrystallized in ethanol 96% (v/v) at 80 °C, filtrated and dried in oven at 40 °C for 24 h.

Conclusion

The feasibility of a high concentration isoflavone-aglycone fraction (around to 90%) was successfully demonstrated in the present work suggesting its potential as novel raw material for isoflavone-based pharmaceutical or nutraceutical products. The fraction represents an excellent perspective of improvement of absorption as well as to reduce the variability resulting from the step of enzyme hydrolysis of the glycosides along the gastrointestinal tract.

Authors' contribution

FKJY carried out the main laboratory work as part of her PhD thesis. VLB and LSK are the supervisors who provided intellectual input. All authors participated in the writing of the manuscript and approved its content.

Conflicts of interest

The authors declare no conflicts of interest.

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