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Supercritical fluid extraction of daidzein and genistein isoflavones from soybean hypocotyl after hydrolysis with endogenous β-glucosidases

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Abstract

An optimal condition of supercritical fluid extraction (SFE) for isoflavone aglycones (daidzein and genistein) in soybean hypocotyls previously subjected to thermohydration at pH 5.0 and a temperature of 50 °C for 6, 12 and 18 h was developed. Different temperatures, pressures and cosolvents (methanol, ethanol, and acetonitrile) was tested and compared with solid–liquid extraction using aqueous methanol solution (80% v v^{-1}) conducted in parallel for comparison. The extraction conditions were 50–70 °C, 176–380 bar, adding 0, 5, 10 mol% of cosolvents 80% in water as a modifier. The results from SC–CO_2 showed that the cosolvent and pressure have significant effects in the extraction efficiency. It was found that the extraction conditions promoting the highest extraction of daidzein and genistein were at the temperature of 60 °C, pressure of 380 bar and both static and dynamic extraction of 15 min with the addition of 10% acetonitrile (80% v v^{-1}). The maximum amounts of daidzein and genistein extracted by each method were solid–liquid extraction (70.07 mg 100 g^{-1}) and carbon dioxide–acetonitrile (17.97 mg 100 g^{-1}). The yield of daidzein and genistein achieved by a 30 min SC–CO_2 extraction on soybean hypocotyls after 12 h soaking time was markedly improved by the addition of a modifier (acetonitrile) to the CO_2 fluid. HPLC analysis of the obtained extracts revealed that extraction of isoflavone aglycones by SC–CO_2 was 4.78 and 13.19 mg 100 g^{-1} for daidzein and genistein, respectively. The contents of daidzein and genistein obtained in the solid–liquid extraction were superior to 86% and 63%, respectively, compared to supercritical extraction.

Keywords: Extraction methods; Daidzein; Genistein; HPLC

1. Introduction

Daidzein and genistein, the major isoflavones in soybeans, are associated with a broad variety of beneficial properties on human health and are found in high concentrations in soybean hypocotyl (10 times the concentration in soybeans) (Liu, 1999; Zhu, Hettiarchchy, Horax, & Chen, 2005). After consumption, primary isoflavonoids are metabolized in the gut and transformed into active aglycones, some of which are absorbed as free isoflavones (Arjmandi & Smith, 2002; Fritz, Seppanen, Kurzer, & Csallany, 2003; Morel, Stermitz, Tegos, & Lewis, 2003; Wei, Zhang, Wang, & Lebwohl, 2002; Wuttke, Jary, Becker, Schultens, & Christoffel, 2003 & Yamaguchi, 2002).

From the structure of the isoflavone glucosides and their aglycones we presume that the isoflavone glucoside is hydrolyzed by the β-glucosidase. Matsuura, Obata, and Fukushima (1989), Matsuura and Obata (1993), Pandjaitan, Hettiarachchy, and Ju (2000) reported that β-glucosidase was related to the production of daidzein and genistein during the soaking of soybeans.

The traditional method for the extraction of plant materials include steam distillation and organic solvent extraction using percolation, maceration or Soxhlet techniques (Liggins, Blunk, Runswick, Atkinson, & Coward,

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2000; Simonne et al., 2000; Tura & Robards, 2002). These procedures however they are have distinct drawbacks such as time-consuming and labour-intensive operations, handling of large volumes of hazardous solvents and extended concentration steps which can result in the loss or degradation of target analytes. Moreover, there is an increasing interest for alternative extraction technologies consuming less organic solvents, because of the rising solvent acquisition and disposal cost and regulatory restriction.

Supercritical fluid extraction (SFE) offers several advantages over conventional solvent extraction methods. SFE can penetrate into the pores of solid materials more effectively than techniques based upon liquid solvents, so it enables a much faster mass transfer, resulting in faster extractions. For instance, the extraction time can be reduced from hours or days for a liquid–solid extraction to a few minutes for SFE, with comparable or better recoveries. Also, in SFE, fresh fluid is continuously pumped through the samples, so it can provide quantitative or complete extraction, and the solvation power of the fluid can be manipulated by changing pressure and/or temperature, facilitating a remarkable high selectivity (Lang & Wai, 2001; Smith, 1999; Tura & Robards, 2002). The most important advantage of utilizing SC–CO_2 is the easy separation of the solvent from the extracted material and it operates at an ambient temperature that does not affect the heat sensitive compounds. Further, SC–CO_2 provides lower mass transfer resistance than do those in conventional separation process. These advantages have attracted an increasing interest from researchers, especially from the food, pharmacy and environmental-engineering industries.

However, due to the limited solubility of polar organic compounds in SC–CO_2 or to their interaction with the matrix, quantitative extraction of these compounds with pure SC–CO_2 is not possible. The addition of a polar modifier (e.g. methanol) to SC–CO_2 is the simplest and the most effective way to obtain the desired polarity of CO_2-based fluids. Modifiers can also overcome interactions between the analyte and the matrix, increasing the extraction efficiency of polar organic compounds (Lang & Wai, 2001).

Several researchers successfully applied SFE to extract similar compounds from different matrices, like phenolic compounds from olive leaves (Le Floch, Tena, Rios, & Valcarel, 1998), flavonoids from *Scutellaria radix* (Lin, Tsai, & Wen, 1999), from *Ginkgo biloba* (Liu, Zhao, Wang, & Yang, 1999) and from Chamomile flowers (Scalia, Guiffreda, & Pallado, 1999), polyphenols from grape seeds (Palma, Taylor, Zoecklein, & Douglas, 2000), isoflavones from soybean flour (Rostagno, Araújo, & Sandi, 2002) and isoflavone from soybean products (Chandra & Nair, 1996).

In the present study, SFE was used to extract isoflavone aglycones (daidzein and genistein) from soybean hypocotyls after enzymatic hydrolysis of the glucosidic isoflavones by endogenous β-glucosidases, and the extracts were analyzed by HPLC. Extraction conditions were adjusted in order to obtain the highest yield of daidzein and genistein, and the influence of the extraction conditions of the method was examined. The results obtained was compared with the results obtained using solid–liquid extraction.

2. Materials and method

2.1. Materials

SFE grade carbon dioxide (99.99% pure) supplied in a cylinder with a dip tube was purchased from White Martins (Brazil); genistin, genistein, daidzin, daidzein, flavone and *p*-nitrophenyl-β-D-glucoside (*p*-NPG) were obtained from Sigma Chemical Co. (USA). Acetronitrile, methanol, dimethyl sulfoxide (DMSO), trifluoroacetic (TFA) (Vetec Química-Brazil) were of analytical grade (99%). The solvents used were filtered through a 0.45 μm nylon membrane filter prior to utilization.

2.2. Sample preparation

The hypocotyls of the cultivar UFVS-2008 cultivated between November of 2001 and March of 2003 were used in this study. The soybean seeds were first soaked in tap water acidified to pH 5.0 with 0.1 N HCl (250 g seeds 750 mL^{-1}) at 50 °C for 6, 12 and 18 h in order to promote the enzymatic hydrolysis of the glycosidic isoflavones by endogenous β-glucosidases, drained and rinsed in cool water (Matsuura & Obata, 1993). The hypocotyls were removed manually, lyophilized and ground in a bench coffee grinder and sieved to obtain particles with diameters ranging from 0.297 to 0.35 mm and stored in vials kept in nitrogen at −20 °C. For a blank experiment, soybean seeds were heated in an oven at 90 °C for 6 h to inactivate the enzyme, in accordance with the method described earlier (Chiang, Shieh, & Chu, 2001).

2.3. Assay of enzyme activity

β-Glucosidase activity was monitored with a synthetic substrate, *p*-nitrophenyl-β-D-glucoside (*p*-NPG), according to the procedure of Matsuura and Obata, 1993. The lyophilized soybean hypocotyls (100 mg) were mixed with 0.1 M sodium phosphate buffer (1.5 ml), with a pH of 5.0 for 30 min. The slurry was centrifuged at 8000*g* for 5 min, and the supernatant fraction was utilized as the source of the crude enzyme. The estimation of the hydrolyzing activity of the synthetic substrate, the *p*-NPG, was as follows: a 500 μl of 1 mM *p*-NPG in 0.1 M phosphate–citrate buffer, pH 5.0 and 300 μl water was incubated at 45 °C for 40 min after the addition of 200 μl of the enzymatic extract. The reaction was stopped by the addition of 2 ml of 0.25 M sodium carbonate. The

resulting yellow color was immediately measured at 420 nm with a spectrophotometer model 634-S UV/VIS (Varian, USA). The hydrolyzed *p*-nitrophenol was determined by referring to a calibration curve prepared, concurrently in the same manner, with 0.02–0.16 μM of *p*-NPG. A unit of enzyme activity was defined as the amount of enzyme which would liberate 1 μM of *p*-nitrophenol min^{-1}.

2.4. Extraction methods

2.4.1. Conventional solid–liquid extraction

Pulverized soybean hypocotyl (500 mg) was weighted and placed in an Erlenmeyer. The extraction was carried out using 10 ml methanol 80% in water (v v^{-1}) as the solvent for 2 h with magnetic stirrer (Simonne et al., 2000). The crude extract was centrifuged at 800*g* for 20 min (Beckman, USA) and filtered in a cellulose filter (0.45 μm; Milipore, USA).

2.4.2. Supercritical fluid extraction method

A Hewlett–Packard 7680A extraction module with a 7-ml thick-walled stainless steel thimble was used to carry out supercritical fluid extraction. The extractor employed a variable restrictor to allow an instant depressurization of the supercritical fluid and the decoupling of flow and pressure in order to control the pressure independently of the supercritical fluid flow rate. An amount of 80 mg of powdered sample (mesh 40) was weighed in a filter paper and packed into a 7.0 ml thimble. The volume of the thimble was reduced to 5.46 ml with a glass stick to increase the "thimble-volume-swept". The SFE extract was collected on a solid-phase trap held at an ambient temperature. The trap consisted of octadecylsilane (ODS), which was flushed by 80% methanol at a flow rate of 0.5 ml min^{-1} and collected in a 1-ml vial, that was used directly for chromatographic analysis. Six extractions (Table 2) were carried out at a constant static time of 15 min, temperatures of 50, 60, and 70 °C, pressures of 176, 218, 261, 370, and 380 bar, and dynamic time of 15 min. Cosolvents (methanol, ethanol and acetonitrile) depending of the experiment were added directly into the sample inside the thimble at concentrations of 0, 5, and 10 mol% of the CO_2 mass passed through the system during the dynamic extraction (20.7 g; net CO_2 mass = 0.92 × extraction time × CO_2 flow rate; were 0.92 g ml^{-1} is the density within the cooled pump head). The CO_2 flow rate was held constant at 1.5 ml min^{-1}. This extraction process was repeated three times (3 × 30 = 90 min) for the same sample, to a total CO_2 mass of 62.1 g and a total modifier volume of 0, 0.8 and 1.2 ml (0%, 5% and 10% mol%, respectively).

2.5. High performance liquid chromatography (HPLC)

A Hewlette–Packard 1050 series modular LC system equipped with a Reodyne 7125 injector linked to a 50 μl loop and variable-wavelength UV detector was used for the analysis by liquid chromatography. The HPLC method developed by Coward, Barnes, and Barnes (1993) was used, adjusting the chromatographic conditions to achieve good isoflavone separation. Separations were performed in a Hypersil-ODS C_{18} column (20 cm × 4.6 mm i.d. × 5 μm; Hewlett & Packard, USA) fitted with guard a column. The mobile phase consisted of solvent A, 0.1% TFA in water, and solvent B, acetonitrile. The initial solvent condition was 100% solvent A. A gradient was set to increase solvent B from 0 to 54.6% within 20.6 min, holding these conditions for 10 min and then returned to the original condition in 5 min. The chromatographic analysis was performed at an ambient temperature, using a flow rate of 1.0 ml $min^{-}1$ and detection wavelength set to 262 nm. Injection of 50 μl was effected with a Hewlett–Packard 100 μl syringe.

The concentration of isoflavone in the sample was calculated from standard curves calibrated using the four isoflavone standards. The calibration curves (correlation coefficients) for daidzin, genistin, daidzein and genistein were $y = 0.05089x + 0.06683$ ($r = 0.9906$), $y = 0.07375x - 0.04091$ ($r = 0.9987$), $y = 0.09058x + 0.04099$ ($r = 0.9994$) and $y = 0.0379x + 0.0161$ ($r = 0.996$), respectively.

The stock solutions of genistin, genistein, daidzein and daidzin were first prepared in DMSO. These solutions were diluted in the mobile phase (54.6% of eluent A in B) to a final concentration between 0.2 and 20 μg ml^{-1} and, after the addition of the internal standard, analyzed by HPLC. Flavone was used as the internal standard with a final concentration of 2 μg ml^{-1} in the standards solutions and extracts. The identification of the separated compounds in the soybean hypocotyl, was assigned by a comparison of retention times and co-chromatography with authentic standards. Quantification was carried out by integration of the peak areas using the internal standard method. Response linearity was observed for a concentration range of 0.2–12 μg ml^{-1} with a 1% confidence level. Each extract was analyzed until reaching a reproducibility higher than 95%. The chromatograms of the standards and extracts obtained by solid–liquid extraction and SFE are presented in Fig. 1.

2.6. Statistical analysis

All values are reported as means of triplicate determinations. Data were submitted to analysis of variance in a completely randomized design, with three soaking time levels in three replicates, plus a control treatment. The soaking time effect on endogenous β-glucosidase activity on daidzein and genistein levels were analyzed by linear regression models. Honestly Significance Difference (HSD) Tukey's procedure, at a 5% significance level, was used to compare soybean hypocotyls isoflavone aglycones means from different extraction methods.

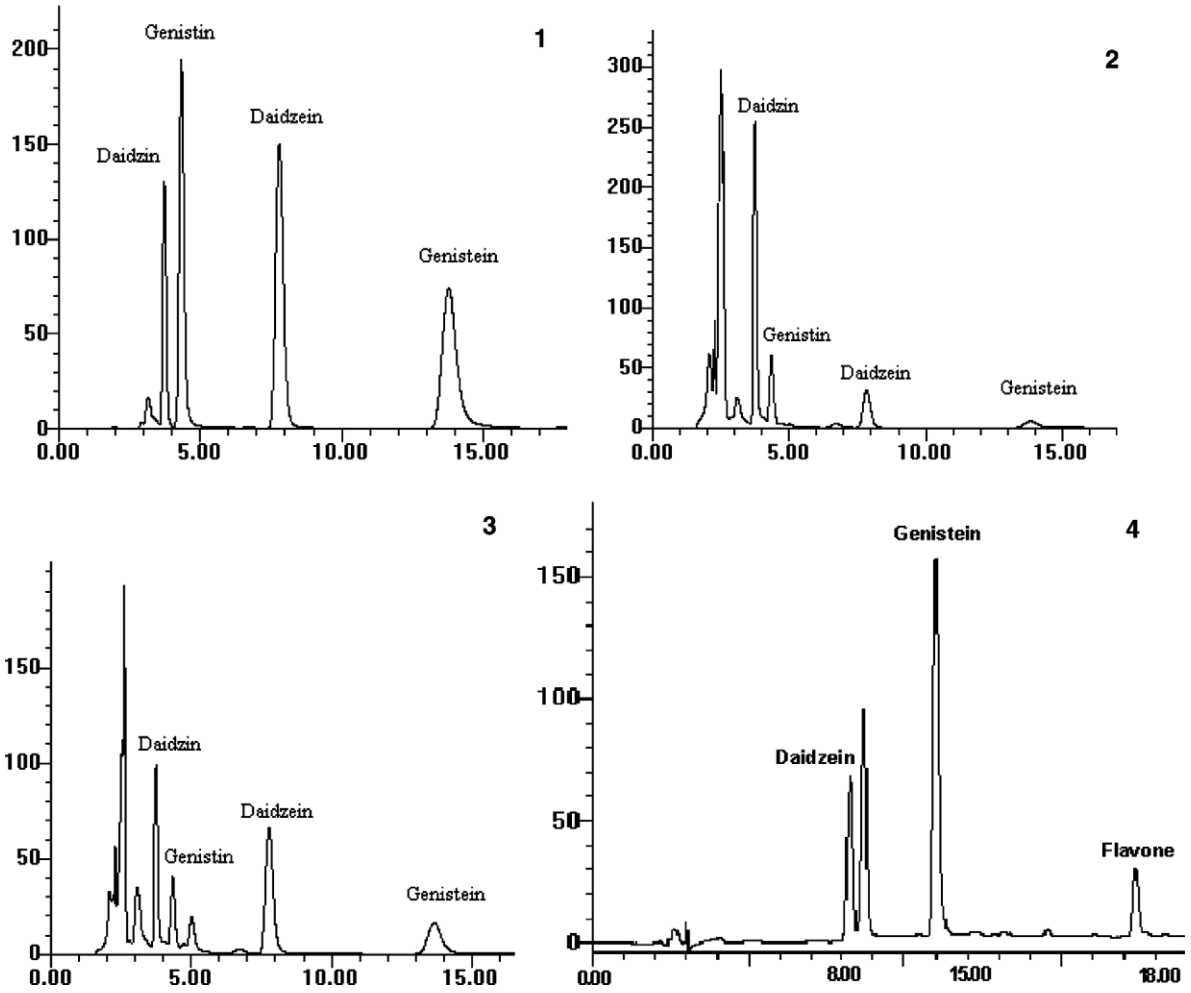

Fig. 1. HPLC Chromatograms of isoflavone standards (1) and various extracts: solid–liquid extraction of the control (2), after 6 h thermohydration (3) and SFE-acetonitrile (4).

3. Results and discussion

3.1. Hydrolysis of isoflavone in soybean hypocotyl by endogenous β-glucosidases

The effect of soaking time on enzyme activity was investigated by using *p*-NPG at 50 °C and pH 5.0, incubated for 18 h (Fig. 2). The quantities of daidzein and genistein increased with soaking time up to 12 h. Thus, isoflavone glucosides hydrolysis was time dependent. At 12 h incubation time, the percent hydrolysis was 60% for daidzin and 56% for genistin. Matsuura and Obata (1993) *found 50% hydrolysis for daidzin and 51% for genistin.* In this experiment, daidzin was more easily hydrolyzed than genistin by β-glucosidases. Zhu et al. (2005) observed that the maximum amounts of isoflavone content were achieved when the hypocotyl length of seeds was 0.5–2.5 mm, and a decrease was observed after this stage. The higher values (>90%) for hydrolysis from glycosides to aglycones were obtained by using β-glucosidase in soymeal (Xie, Hattiarachchy, Cai, Tsurahami, & Koikeda, 2003). The work conducted by Chiang et al. (2001) using HCL to hydrolyze glucosides to aglucones in soybean hypocotyls achieved an recovery higher than 90%.

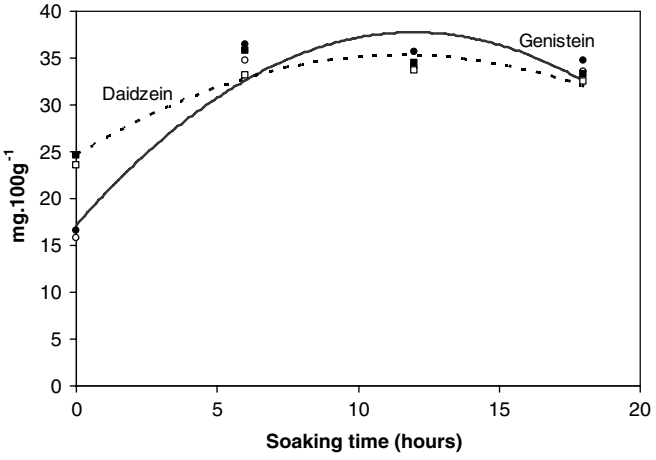

Fig. 2. Soaking time effect on endogenous β-glucosidase activity on daidzein and genistein. Daidzein = 24.62 + 1.8566(time) − 0.0804(time)^2 R^2 = 0.8312 Genistein = 17.17 + 3.4429(time) − 0.1439(time)^2 R^2 = 0.8786.

Table 1
Change in the content (mg 100 g^{-1}) of soybean hypocotyls isoflavone extracted using conventional method after thermohydration and ANOVA table for the soaking time experiment

Isoflavone[b]	Thermohydration (h)[a]			
	Control[c]	6	12	18
Daidzin	919.54	551.72	597.72	643.68
Genistin	170.92	95.72	99.17	102.55
Daidzein (D)	24.10 ± 0.78	34.46 ± 1.81	33.74 ± 1.10	32.52 ± 1.06
Genistein (G)	16.19 ± 0.53	35.61 ± 1.16	34.80 ± 1.14	34.49 ± 1.81
D + G	40.29	70.07	68.54	66.01
Total	1130.75	717.51	765.43	812.24
Source of variance	Sum of square	Degree of freedom	Mean square	*F* probability (%)[d]
Daidzein				
Time	138.55	3	46.18	0.341
Quadratic model	127.32	2	63.66	
Error	6.22	4	1.55	
Genistein				
Time	514.99	3	171.66	0.026
Quadratic model	475.9875	2	237.99	
Error	6.20	4	1.55	

[a] Temperature of incubation was maintained at 50°C/pH 5.0.
[b] Average of three replicates.
[c] Heat soybean hypocotyls (90 °C).
[d] Critical value of $F_{(2,4)}$ is 6.94 with 95% confidence level.

Analysis of variance showed a significant ($p < 0.05$) effect of soaking time on both daidzein and genistein concentrations (Table 1). A quadratic model fitted well on daidzein concentration with soaking time, with no significant lack of fit and 91.9% explanation as compared to soaking time sum of square (Table 1). As for genistein concentration, there was a significant lack of fit for the same quadratic model, however, there is also a 91.9% explanation by this model, as compared to soaking time sum of square (Table 1). The results in Fig. 2 show that after a 12 h soaking time there is no increase in daidzein or genistein concentrations.

3.2. Supercritical extraction of isoflavones after hydrolysis with β-glucosidases

Solid–liquid extraction has traditionally been used to extract isoflavones. In this study, we compare the efficiency of this method with supercritical fluid extraction. Since various parameters can potentially affect the extraction process, optimization of the experimental conditions is a critical step in the development of the SFE method. In fact, the fluid pressures and temperatures, the percentage of cosolvent and the extraction times are generally considered to be the most important factors.

The initial development of the conditions for analytical SFE of isoflavones from soybean hypocotyls was performed in the pressure range of 176–380 bar and temperatures between 50 and 70 °C, using 13.8 g of CO_2 in the dynamic extraction with 5 and 10 mol% (0.4 and 0.8 ml) of cosolvent (methanol 80%) and sample of 80 mg. Because the yield extracted by pure supercritical carbon dioxide was not satisfactory, and changes in pressure and temperature negligibly improved the yield, it was thought that increasing the polarity of the extraction solvent might overcome the low yield. At least 17 cosolvents have been studied in SFEs of natural products. Among these cosolvents, methanol is the most commonly used because it is effective polar modifier and is miscible up to 20% with CO_2. Methanol 80% v v^{-1} was initially chosen as the cosolvent in this study (Palma et al., 2000; Rostagno et al., 2002). The amounts of crude extract by SFE under various conditions are listed in Table 3. However, as our preliminary experiments showed, methanol was not a good cosolvent in this case. Table 2

Table 2
Mean values of the amount of isoflavones extracted from soybean hypocotyls using SC–CO_2 with 10 mol% of cosolvent (methanol 80% in water, v v^{-1})

Run number	*T* (°C)	*P* (bar)	Amount (mean ± S.D. mg 100 g^{-1})				
			Daidzin	Genistin	Daidzein	Genistein	Total
1	50	176	nd	nd	0.43 ± 0.04	1.30 ± 0.05	1.73
2		370	nd	nd	0.81 ± 0.01	2.51 ± 0.04	3.32
3	60	218	nd	nd	1.09 ± 0.04	2.78 ± 0.04	3.87
4		380	nd	nd	1.72 ± 0.01	5.02 ± 0.02	6.74
5	70	261	nd	nd	0.83 ± 0.01	1.98 ± 0.01	2.81
6		370	nd	nd	1.21 ± 0.01	3.68 ± 0.03	4.89

Table 3
Mean value of the amount of soybean hypocotyls isoflavones extracted (mg 100 g^{-1}) for each extraction method after 12 h thermohydration[e]

Isoflavone (mg 100 g^{-1})	Extraction method			
	Solid–liquid[a]	SFE[b]	SFE[c]	SFE[d]
Daidzin	551.72	nd	nd	nd
Genistin	95.72	nd	nd	nd
Daidzein (D)	34.46[a] ± 1.81	1.72[d] ± 0.01	1.98[c] ± 0.01	4.78[b] ± 0.03
Genistein (G)	35.61[a] ± 2.91	5.02[d] ± 0.02	5.92[c] ± 0.03	13.19[b] ± 0.37
D + G	70.07	6.74	7.90	17.97
Total	717.51	6.74	7.90	17.97

[a] Methanol 80% v v^{-1}; $n = 3$.
[b] 60 °C/380 bar; methanol 80% v v^{-1}; $n = 3$.
[c] 60 °C/380 bar; ethanol 80% v v^{-1}; $n = 3$.
[d] 60 °C/380 bar; acetonitrile 80% v v^{-1}; $n = 3$.
[e] Values in a line with different letters are significantly different ($p < 0.05$), by HSD Tukey's test.

shows that at a certain pressure, with 80% methanol as cosolvent, when the temperature was raised from 50 to 60 °C, the total isoflavone aglycone extraction yield increased. The increment rates were 55.87%, 51.15% at 370 bar and 380 bar for daidzein, genistein, respectively. When the temperature was increased from 60 to 70 °C, the extraction yield decreased, and these results suggested that 60 °C was the optimal temperature for extraction.

At 50 °C, pressure changes from 176 bar to 370 bar had a significant influence on the extraction yield, and at 60 °C as the pressure changes from 218 bar to 380 bar the yield also increased; as the pressure changed from 261 bar to 370 bar at 70 °C, the yield decreased. As a result, 380 bar was the critical pressure. At 60 °C, with the cosolvent of 10% v v^{-1}, when the pressure increased from 218 bar to 380 bar, the yield increased. The increased amount was 57.79% and 80.57% for daidzein and genistein, respectively.

Increasing the SC–CO_2 mass used in the dynamic extraction from 13.8 to 20.7 g significantly improved the isoflavones recovery with the modifier at 10 mol%. This was expected since the extraction efficiency is strongly dependent on the mass transfer rates. The values of each isoflavones extracted using 20.7 g of SC–CO_2 are listed in Table 2. For total isoflavone aglycones, the best extraction condition was at 60 °C and 380 bar (6.74 mg 100 g^{-1}) with cosolvent (methanol 80%) at 10 mol%, while no isoflavone was extracted without a cosolvent. The predominant effect of the pressure in the amount extracted of these two isoflavones (genistein and daidzein) was observed. This is probably due to a decrease in the extract steam pressure and increase in the density of fluid and a higher kinetics of desorption of the compounds from the sample matrix. As the pressure increases, desorption is faster and more solute is available for extraction.

Using the optimized condition described above (60 °C and 380 bar), we tested different types of cosolvents: ethanol and acetonitrile 80%, v v^{-1}. The mean values of the amount of soybean hypocotyl isoflavones extracted (mg 100 g^{-1}) for each extraction method are presented in Table 3. For daidzein and genistein, the results indicated that higher amount extracted was achieved with the solid–liquid extraction method than the SFE. In soybeans, the glucosidic form of the isoflavones is present in a higher concentration than the respective aglucone form. A similar distribution was observed in the isoflavones extracted by all methods.

It was found that the addition of acetonitrile (10 mol%) as the cosolvent, recovered the highest amount of isoflavone aglycones (17.97 mg 100 g^{-1}), and the contents of daidzein and genistein were 4.70 and 13.19, respectively. These results were superior to found by Chandra and Nair, 1996 for soybean products using ethanol as cosolvent and Rostagno et al. (2002) for soybean flour using methanol as the cosolvent. From Table 3, different results were obtained between conventional extraction method and SFE. When using methanol, ethanol and acetonitrile as the cosolvent, the SFE method was able to extract 9.61%, 11.27% and 25.64% of isoflavones aglycone, respectively. Pressure and modifier were found to be the most important factors, where the higher levels of cosolvent and pressure significantly increase the yield of isoflavone aglycones, as illustrated in Table 3. The cosolvent was introduced by pipetting a specific amount of cosolvent and dropping it directly onto the sample in an extraction vessel. It has been reported previously (Marsili & Callahan, 1993) that this method of introduction of cosolvent in SFE, was not efficient to extract α-and β-carotene in vegetables. The methods in which the cosolvent is introduced by means of a second pump and mixer might be more efficient. These results show that SFE has the potential for the extraction of isoflavones from fermented products, as they have a high concentration of active isoflavone aglycones.

3.3. Assay by HPLC

The HPLC chromatograms of the standard solution, control, extracts from 80% methanol and SFE after thermohydration are shown in Fig. 1, as well as the retention times for the four standards and internal standard. The contents of daidzin, daidzein, genistin and genistein in various extracts of soybean hypocotyls are listed in Table 3. With 80% methanol, the yield of isoflavones aglycone

ranged from 34.46 to 35.61 mg $100\ g^{-1}$ for daidzein and genistein, respectively, and as the cosolvent in the SFE were 1.72 mg $100\ g^{-1}$ for daidzein and 5.02 mg $100\ g^{-1}$ for genistein. With ethanol as cosolvent in SFE, the yields of the above constituents were 1.98 and 5.92 mg $100\ g^{-1}$, respectively. When 10% acetonitrile was used as a cosolvent in SFE, the yields of daidzein and genistein were 4.78 and 13.19 mg $100\ g^{-1}$, respectively. The increments of daidzein and genistein content were 1.15, 2.27 times and 1.17, 1.73 times, respectively, superior to methanol and ethanol as cosolvent.

4. Conclusions

These results indicate that extraction efficiencies achieved by solid–liquid extraction are higher than those attained by SFE. However, the higher complexity of the chromatographic patterns produced by solid–liquid extraction indicates that SFE affords enhanced extraction selectivity compared to the classical techniques. The SFE extraction with the addition of 10% acetonitrile after 12 h soaking time of the soybean hypocotyls resulted in 13.87% and 37% recoveries of active aglycones daidzein and genistein, respectively, without any remaining solvent and less extraction time. *Controlled germination can be used to enhance isoflavone content in soybean and better recovery of daidzein and a genistein could be obtained by introducing of the cosolvent by means of a second pump.* The extraction with $SC–CO_2$ has a potential in the extraction of aglycones from fermented products and germinated soybean seeds, as they have a high concentration of isoflavone aglycones.

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