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Supercritical fluid extraction of isoflavones from soybean flour

Maurício A. Rostagno*, Júlio M.A. Araújo, Delcio Sandi

Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, 36571-000, Viçosa, MG, Brazil

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Abstract

This study evaluated the use of supercritical carbon dioxide (SC-CO_2) for the extraction of soybeans isoflavones (genistin, genistein and daidzein), where different temperatures, pressures and modifier percentages were tested and compared with conventional extraction methods (soxhlet and ultra-sonification) conducted in parallel. The extraction conditions were 40–70 °C, 200–360 bar, adding 0, 5 and 10 mol% of methanol 70% in water (v/v^{-1}) as modifier. The HPLC analysis of the obtained extracts revealed that extraction of genistin and genistein at 70 °C/200 bar using a mixture of carbon dioxide (55.2 g) and modifier (10 mol%), resulted in lower values than conventional methods. The analytical results additionally showed a predominant effect of temperature in the amount of genistin and genistein extracted by SC-CO_2. Extraction of daidzein at 50 °C/360 bar resulted in higher values than conventional methods and a predominant effect of the pressure was observed. A strong interaction between temperature and pressure was observed in the extraction of the tested isoflavones. The maximum amount of total isoflavonoids extracted by each method were: ultra-sonification (311.55 $\mu g \cdot g^{-1}$), soxhlet (212.86 $\mu g \cdot g^{-1}$) and SC-CO_2 (86.28 $\mu g \cdot g^{-1}$).

Keywords: Supercritical fluid extraction; Isoflavones; Soybean

1. Introduction

Soybeans contain a large number of compounds of biological interest already isolated and identified. These compounds include isoflavones (Fig. 1), phytosterols, protease inhibitors, inositol hexaphosphate and saponins (Mazur, Duke, Wahala, Rasku, & Adlercreutz, 1998).

Isoflavones are heterociclic phenols with close similarity in structure to estrogens and a diphenolic character similar that of lignans displaying both estrogenic and antiestrogenic activity, influencing sex hormone metabolism and their biological activity (Adlercreutz, 1995; Setchell & Adlercreutz, 1988). They also may be important antioxidants (Arora, Valcic, Cornejo, Nair, & Timmermann, 2000; Toda & Shirataki, 2001). The isoflavones have been implicated in a wide range of health conditions ranging from menopause (Chiechi, 1999; Umland, Cauffield, Kirk, & Thomason, 2000), cardiovascular disease (Lissin & Cooke, 2000; Tikkanen & Adlercreutz, 2000), osteoporosis (Anderson & Garner, 1997; Sugimoto & Yamaguchi, 2000) and cancer (Adlercreutz, 1995; Lamartiniere, 2000; Mazur et al., 1998; McCarty, 2001; Messina, 1999; Tillem, 2000). Besides that, they are, in part, responsible for the bitterness and astringent taste of soybeans products witch have been to the present the most important limiting factor in the utilization of soy as a food ingredient (Araújo, Carlos, & Sedyama, 1997; Carrao-Panizzi, Pinobeléia, Ferreira, Oliveira, & Kitamura, 1999).

Common methods for the extraction of the isoflavones from soybeans and soy products include organic solvent extraction with aqueous methanol (Barnes, Kirk, & Coward, 1994; Liggins, Bluck, Coward, & Binham, 1998; Liggins, Grimwood, & Bingham, 2000), ethanol (Franke, Custer, Cerna, & Narala, 1994; Hutabarat, Mulholland, & Greenfield, 1998) or acetonitrile (Barnes et al., 1994; Liggins et al.,1998; Wang & Murphy, 1996) using simple mixing, soxhlet or ultrasonification techniques for a few to several hours. These procedures have important drawbacks such as long

* Corresponding author.
E-mail address: mrostagno@hotmail.com (M.A. Rostagno).

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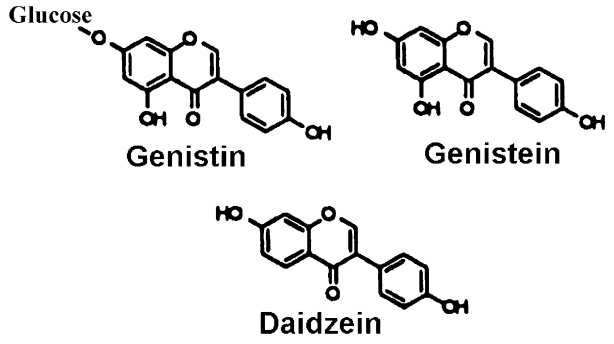

Fig. 1. Chemical structure of analyzed soybean isoflavones.

extraction times, consuming large quantities of solvents and requiring additional pre-purification and concentration steps which can result in the loss or degradation of target analytes and spendious work.

In the past few years, several new extraction procedures have been investigated as replacements for the traditional procedures. Supercritical fluid extraction (SFE) offers several advantages over conventional extraction methods such as increased selectivity, expeditiousness, automaticity, environmental safety, dramatically decreased use of organic solvents, higher speed and better reproducibility (Araújo, 1999; Knipe, Miles, Rowland, & Randall, 1993; Lang & Wai, 2001).

Carbon dioxide, the most commonly used supercritical fluid, as solvent, is chemically inert, non-toxic, non-flammable and is an accepted food grade solvent. These advantages have attracted increasing interest from researchers, especially from 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 a desired polarity of CO_2 based fluids. Modifiers can also overcome interactions between analyte and matrix, increasing the extraction efficiency of polar organic compounds (Björklund, Järemo, Mathiasson, Jönsson, & Karlsson, 1998; Lang & Wai, 2001).

Recently, several researchers successfully applied SFE to extract similar compounds from different matrices, like phenolic compounds from olive leaves (Le Floch et al., 1998), flavanoids from *Scutellariae radix* (Lin, Tsai, & Wen, 1999), from *Ginkgo biloba* (Liu, Zhao, Wang, & Yang, 1999), and from Chamomile flowers (Scalia, Guiffreda, & Pallado, 1999), flavanones and xanthones from the root bark of the osage orange tree (Costa, Margolis, Benner, & Horton, 1999) and polyphenols from grape seeds (Palma & Taylor, 1999). The main objective was to study the feasibility of the use of supercritical carbon dioxide for extraction of isoflavones from soybean flour.

2. Materials and methods

2.1. Soybean flour sample

Soybeans from lines without lipoxigenases of the IAC-12 variety cultivated in green houses between November of 1999 and March of 2000 were used in this study. The soybeans were first grinded in a disc-grinding mill (Brasil, model 224, São Paulo, SP-Brazil) for partial dehulling. The resulting material was grinded in a knife grinding mill (Brabender, model WI, Duisburg, Germany) and defatted in a soxhlet extractor for 9 h at 70 °C, using hexane as solvent (10 $ml \cdot g^{-1}$). The remaining material was dried at 30 °C and stored at −20 °C until used as sample for the experimental extractions.

2.2. Chemicals and solvents

SFE grade carbon dioxide (99.998% pure) supplied in cylinders with a dip tube was purchased from White Martins (Juiz de Fora, MG, Brazil), flavone, genistin, genistein and daidzein were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (Vetec Química Ltd, Duque de Caxias, RJ, Brazil) and methanol (Grupo Química, Rio de Janeiro, RJ, Brazil) used were HPLC grade. Dimetil sulfoxide (DMSO) and trifluoroacetic acid (TFA; Vetec Química Ltd) were analytical grade (99%). The solvents used were filtered through a 0,45 μm nylon membrane filter (Schleicher & Schnell, Keene, NH, USA) prior utilization.

2.3. Extraction methods

The soybean isoflavones were extracted using three different methods: soxhlet extraction, ultra-sonification extraction and supercritical fluid extraction.

2.3.1. Soxhlet method

Twenty grams of sample were weighted in filter paper and placed in a 500 ml soxhlet glass thimble. The extraction was carried out using methanol 80% in water (v/v^{-1}) as solvent (10 $ml \cdot g^{-1}$ of sample) at the solvent boiling point (70–80 °C) for 9 h (4 cycles/h^{-1}; $n = 5$; Nguyenele, Wang, & Cheung, 1995). The crude extract was concentrated by reduced pressure evaporation (30 °C; Fisatom, model 82) and centrifuged at $800 \times \boldsymbol{g}$ for 20 min (Beckman, Fullerton, CA, USA). Two milliliters of the supernatant was filtered in a PTFE filter (20 μm; Milipore,Bedford, MA, USA) and, after the addition of the internal standard (Section 2.4), analyzed by HPLC.

2.3.2. Ultra sonification method

Extraction of 1-g sample was carried out at room temperature in a ultrasonic bath (22 kHz; Branson Cleaning Equipments, model B220, Shelton, Conn.,

USA) with a mixture of methanol and water (80:20 v/v^{-1}) as solvent (20 $ml \cdot g^{-1}$ of sample) for 1 h and repeated three times (total volume of 60 ml; $n = 5$; Lin et al., 1999). The crude extract was concentrated by reduced pressure evaporation (30 °C) and centrifuged at $800 \times \boldsymbol{g}$ for 20 min. Two milliliters of the supernatant was filtered in a PTFE filter (20 μm) and, after the addition of the internal standard (Section 2.4), analyzed by HPLC.

2.3.3. Supercritical fluid extraction method

The SFE experiments were performed on a Hewlett-Packard model 7680A SFE module. One gram of the sample was weighted in a filter paper and packed into the 7.0-ml thimble. The volume of the thimble was reduced to 5.46 ml with a glass stick to increase the "thimble-volumes-swept". A mixture of methanol 70% in water (v/v^{-1}) used as modifier, was added directly into the sample inside the thimble in concentrations of 0, 5 or 10 molar% (0, 0.8 or 1.6 ml, respectively) of the CO_2 mass passed through the system during the dynamic extraction (18.4 g; net CO_2 mass = 0.92 × extraction time × CO_2 flow rate; where 0.92 g/ml^{-1} is the density within the cooled pump head; Knipe et al., 1993). The thimble was closed with hand screw caps and the system pressurized with pressures ranging from 200 to 360 bar at temperatures from 40 to 70 °C depending of the experiment. Extractions were carried out using a static (10 min) followed by a dynamic period (20 min) to a total extraction time of 30 min. The CO_2 flow rate was 1.0 ml/min^{-1}. After the extraction, the ODS trap was automatically rinsed three times with 1.5 ml of methanol at a flow rate of 0.5 ml/min^{-1} and collected in three separate vials that, after the addition of the internal standard (Section 2.4), were used directly for chromatographic analysis. This extraction process was repeated three times (3 × 30 = 90 min) for the same sample, to a total CO_2 mass of 55.2 g and to a total modifier volume of 0, 2.4 or 4.8 ml (0, 5 and 10 molar%, respectively; $n = 2$).

2.4. High performance liquid chromatography (HPLC)

A Hewlett-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, Setchell, and Barnes (1994) was used, adjusting the chromatographic conditions to achieve good isoflavone separation. Separations were performed in a Hypersil-Mos C_8 column (20 cm × 4.6 mm i.d. × 5 μm; Hewlett & Packard, Wilmington, DE, USA) fitted with a guard column. The mobile phase was composed of TFA 0.1% in water (A) and acetonitrile (B). The elution was performed with a linear gradient (2.25 $ml \cdot min^{-1}$) from 100 to 54.6% (v/v^{-1}) of eluent A in B in 20.6 min, holding these conditions for 10 min and returning to 100% in 5 min. The chromatographic analysis was performed at ambient temperature, using a flow rate of 1.0 ml/min^{-1} and detection wavelength set to 262 nm. Injections of 50 μl were effected with a Hewlett-Packard 100-μl syringe.

Stock solutions of genistin, genistein, daidzein and flavone were first prepared in DMSO, then these solutions were diluted in the mobile phase (54.6% of eluent A in B) to a final concentration between 10 and 90 μM and, after the addition of the internal standard, ana-

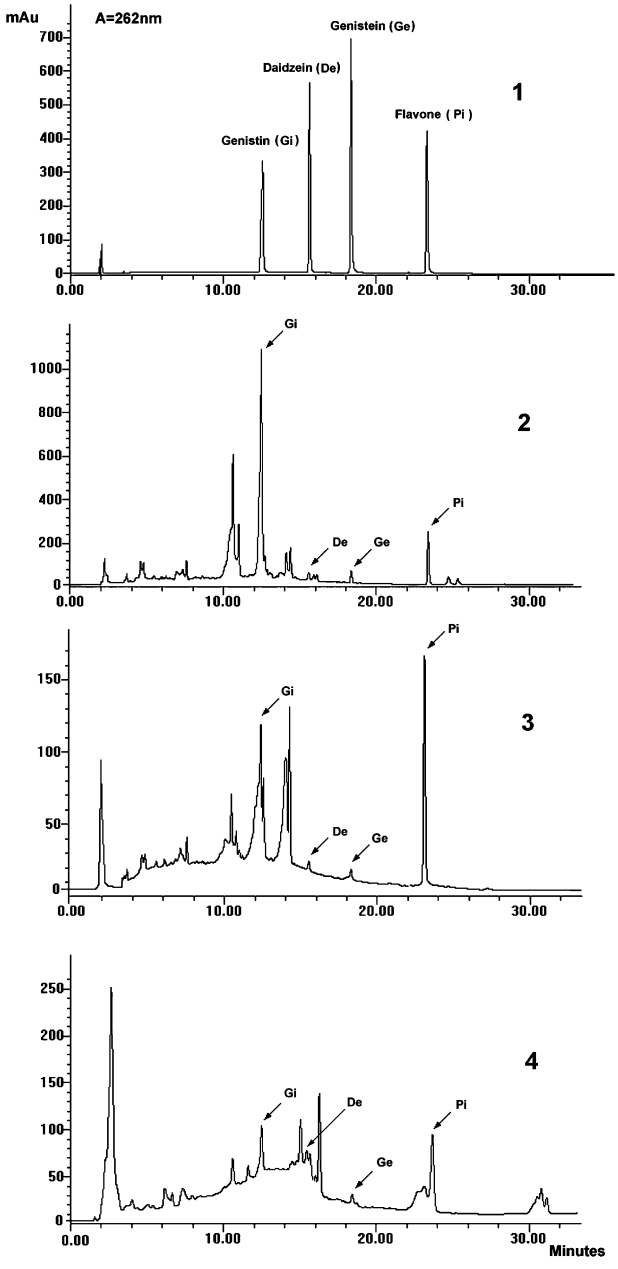

Fig. 2. Chromatograms of isoflavones standards (1) and extracts obtained by soxhlet (2), ultra-sonification (3) and supercritical fluid extraction (4) methods.

lyzed by HPLC. Flavone was used as internal standard with a final concentration of 90 μM in the standard solutions and extracts. The identity of the separated compounds in the soybean extracts was assigned by 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. The regression equation was calculated in terms of the ratio between of area of genistin peak and the area of the internal standard peak and related with the concentration of each isoflavone standard calibrators. Response linearity was observed for a concentration range of 10–50 μM with a 1% confidence level. The calibration curves (correlation coefficient) for genistin, genistein and daidzein were $Y = 0.0399x + 0.0463$ ($r^2 = 0.997$), $Y = 0.0379x + 0.0161$ ($r^2 = 0.996$) and $Y = 0.0273x + 0.0014$ ($r^2 = 0.996$), respectively. Each soybean extract was analyzed until reaching reproducibility higher than 95%. The results were then converted to μg of isoflavone/g of sample (μg/g^{-1}). The chromatograms of the standards and extracts obtained by soxhlet, sonification and SFE methods are presented in Fig. 2.

3. Results and discussion

Although several researchers have reported methods for the extraction of isoflavanoids from soybeans, (Barnes et al., 1994; Hutabarat et al., 1998; Liggins, Bluck, Runswick, Atkinson, Coward, & Bingham, 2000; Liggins et al., 1998) the SFE of these components has not been investigated to the moment.

The initial development of the conditions for analytical SFE of isoflavones from soybeans was performed in pressure range of 200–400 bar and temperatures between 40 and 70 °C, using 18.4 g of CO_2 in the dynamic extraction with 5 and 10 molar% (0.8 and 1.6 ml) of modifier (methanol: water 70:30 v/v^{-1}) and samples of 0.1, 0.5 and 1.0 g. Increasing the pressure and temperature as well the modifier concentration and sample mass did not achieve any significant improvement in the extraction efficiency of the isoflavones which were detected only in trace levels. Higher percentages of the modifier were not employed because it would compromise, in part, the aim of minimizing the volume of hazardous solvents used.

Increasing the SC-CO_2 mass used in the dynamic extraction from 18.4 to 55.2 g significantly improved the isoflavones recovery (1.0 g of sample) with the modifier at 10 molar%. This was expected since the extraction efficiency is strongly dependent on mass transfer rates. The values for each isoflavone extracted using 55.2g of SC-CO_2 are listed in Table 1 and the amount extracted at constant density (0.84 g·ml^{-1}) is shown in Fig. 3. A strong interaction between temperature and pressure was observed. Extraction at 50 °C and 360 bar (53.64 μg·g^{-1}) or 70 °C and 200 bar (51.94 μg·g^{-1}) recovered the highest amount of genistin. For genistein, the best extraction condition was at 70 °C and 200 bar (2.46 μg·g^{-1}). The predominant effect of the temperature in the amount extracted of these two isoflavones (genistin and genistein) can also be noted. This is probably due to an increase in the extract steam pressure in detriment of the fluid's density and a higher kinetics of desorption of the compounds from the sample matrix. As the tem-

Table 1
Mean values of the amount of isoflavones extracted with 55.2 g of SC-CO_2 with 10 molar% of modifier (methanol 70% in water, v/v^{-1}; $n = 2$)

Temperature (°C)	Pressure (bar)	Density (g/ml)	Amount (mean ± S.D.; μg.g^{-1})			
			Genistin	Genistein	Dadzein	Total
40	200	0.84	16.61 ± 2.89	1.64 ± 0.04	6.56 ± 1.36	24.81
	250	0.88	14.00 ± 1.43	0.98 ± 0.04	6.54 ± 0.93	21.52
	300	0.91	11.76 ± 1.64	1.38 ± 0.08	11.85 ± 0.13	24.99
	360	0.94	14.31 ± 0.34	0.83 ± 0.16	13.62 ± 3.09	28.76
50	200	0.78	38.01 ± 0.01	1.16 ± 0.01	12.28 ± 0.03	51.45
	250	0.84	42.98 ± 0.06	1.35 ± 0.01	15.14 ± 0.06	59.47
	300	0.87	23.21 ± 0.86	1.44 ± 0.01	10.08 ± 0.71	34.73
	360	0.90	53.64 ± 0.06	1.71 ± 0.01	30.93 ± 0.04	86.28
60	200	0.72	8.78 ± 1.64	0.79 ± 0.02	3.58 ± 0.14	13.15
	250	0.79	27.07 ± 0.32	2.28 ± 0.06	11.35 ± 2.11	40.70
	300	0.84	1.24 ± 0.26	0.00 ± 0.00	0.74 ± 0.02	1.98
	360	0.88	17.18 ± 4.38	0.54 ± 0.14	6.00 ± 1.90	23.72
70	200	0.66	51.94 ± 5.35	2.46 ± 0.18	16.63 ± 4.36	71.03
	250	0.74	8.51 ± 1.10	1.03 ± 0.06	2.11 ± 0.93	11.65
	300	0.79	24.54 ± 0.83	1.54 ± 0.01	7.95 ± 0.13	34.03
	360	0.84	27.68 ± 2.97	1.22 ± 0.04	10.52 ± 1.20	39.42

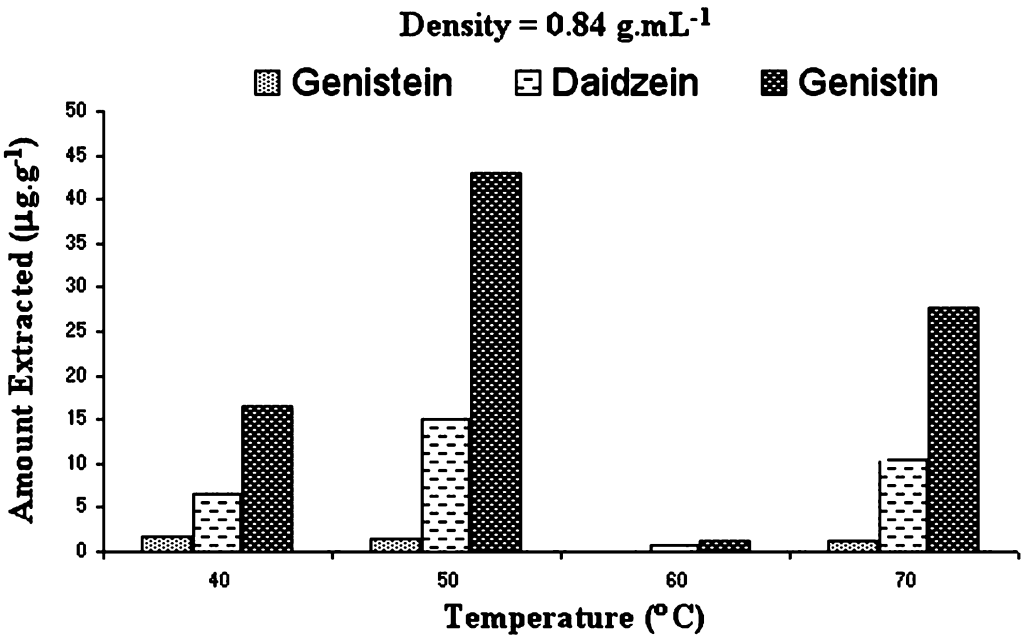

Fig. 3. Effect of the temperature in the recovery of each isoflavone ($\mu g \cdot g^{-1}$) using 55.2 g of SC-CO_2 at constant density ($d = 0.84\ g \cdot ml^{-1}$).

perature increases, desorption is faster and more solute is available for extraction.

The highest amount of daidzein extracted was achieved at 50C and 360 bar (30.93 $\mu g \cdot g^{-1}$). The predominant effect of the pressure in the amount extracted of daidzein was observed and probably is linked with the increase in the fluid's density. For total isoflavones, the best extraction condition was also at 50 °C and 360 bar (86.28 $\mu g \cdot g^{-1}$) while a low extraction efficiency was observed at 60 °C and 300 bar (1.98 $\mu g \cdot g^{-1}$). Only trace levels of the isoflavones were extracted with the modifier at 5 molar%.

The mean values of the amount of soy isoflavones extracted ($\mu g \cdot g^{-1}$) for each extraction method is presented in Table 2. For genistin and genistein, the results indicated that a higher amount extracted was achieved with the ultra-sonification method than with soxhlet and SC-CO_2 (70 °C/200 bar) methods. For daidzein, SC-CO_2 (50 °C/360 bar) achieved higher extraction yield than soxhlet and sonification methods.

The SFE method used the same extraction period and amount of solvent of the ultra-sonification method (90 min, 60 ml) but less than the soxhlet method (540 min, 200 ml). The temperature used in the SFE method (50 °C) was lower than in the soxhlet (solvent boiling point) but higher than in the sonification (room temperature). SFE was the most selective of the three tested methods as fewer co-extracts were obtained in the final samples. SFE was also the most practical method for isoflavone extraction requiring fewer steps than conventional methods.

The higher amount of genistin and genistein extracted in the ultra-sonification method (methanol 70% in water, v/v^{-1}) than in the soxhlet method (methanol 80% in water, v/v^{-1}), and the higher amount of daidzein extracted in the soxhlet method than in the sonification method may be conferred, in part, to the solvents used. Genistin and genistein are more polar than daidzein and consequently are more soluble in the solvent used in the ultra-sonification method, benefiting the

Table 2
Mean values of the amount of soybean isoflavones extracted ($\mu g.g^{-1}$) for each extraction method

Isoflavones ($\mu g.g^{-1}$)	Extraction method			
	Soxhlet[a]	Ultra-sonification[b]	CO_2-SC 50 °C/360 bar[c]	CO_2-SC 70 °C/200 bar[c]
Genistin	205.2±1.82	300.3±3.93	53.64±0.06	51.94±5.35
Genistein	4.19±0.23	8.40±0.47	1.71±0.01	2.46±0.18
Daidzein	3.47±0.20	2.85±0.14	30.93±0.04	16.63±4.36
Total	212.86	311.55	86.28	71.03

[a] MeOH:H_2O (80:20 v/v^{-1}; $n = 5$).
[b] MeOH:H_2O (70:30 v/v^{-1}; $n = 5$).
[c] CO_2–MeOH:H_2O (92.6:5.2:2.2; $v/v^{-1}/v^{-1}$; $n = 2$).

amount extracted of these two isoflavones by this method. The same is true for daidzein, being more soluble in the solvent used in the soxhlet method.

In soybeans, the glucosidic form of the isoflavones is present in higher concentration than the respective aglucone form. Similar distribution was observed in the isoflavones extracted by all methods. The amount of daidzein extracted with SC-CO_2 (30.93 $\mu g \cdot g^{-1}$) was higher than the normal levels for soybean flour (1–12 $\mu g \cdot g^{-1}$). This can be due a high efficiency of the SFE method or co-elution of another compound with daidzein during the chromatographic analysis, increasing daidzein peak area and consequently superestimating it's concentration in the sample. Since the HPLC equipment was not equipped with a mass detector, we can only speculate. Enzymatic hydrolysis of daidzein precursors might as well have taken place, especially at 50 °C, which is the optimal temperature for the activity of β-glucosidases (Matsuura & Obata, 1993). The extraction with SC-CO_2 has a lot of potential in the extraction of isoflavones from fermented products (miso, tempeh), as they have high concentration of aglucone isoflavones.

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