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Analytica Chimica Acta 522 (2004) 169–177
ANALYTICA CHIMICA ACTA
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Pressurized liquid extraction of isoflavones from soybeans

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Received 17 March 2004; received in revised form 28 May 2004; accepted 28 May 2004
Available online 5 August 2004

Abstract

Isoflavone derivatives from freeze-dried soybeans were extracted by pressurized liquid extraction (PLE) and determined by reverse-phase high performance liquid chromatography (HPLC) with both photo diode array and mass spectrometry (MS) detection. Both real and spiked samples were used in the development of the method.

Several extraction solvents (methanol (MeOH) and ethanol (EtOH), 30–80% in water and water), temperatures (60–200 °C), pressures (100–200 atm), as well as the sample size (0.5–0.05 g) and cycle length (5–10 min) were studied for the optimization of the extraction protocol. The optimized extraction conditions for quantitative recoveries were: 0.1 g of sample, 100 °C, three (7 min) static extraction cycles and ethanol 70% as extracting solvent. The stability of the isoflavones during the PLE was also determined. Under PLE conditions, degradation of malonyl glucoside forms of the isoflavones takes place using temperatures higher than 100 °C whereas degradation of glucosides takes place above 150 °C. Using the optimized protocol, isoflavones can be extracted from freeze-dried soybeans without degradation.

Keywords: Pressurized liquid extraction; Isoflavones; Soybeans; Stability studies

1. Introduction

Soybeans are one of most produced and commercialized commodities worldwide. They are known to contain high amounts of protein and oil but also have several phytochemicals. Consumption of soybeans have been associated with a decreased risk of osteoporosis, cardiovascular disease and cancer, including breast, prostate and colon cancers [1,2]. Indication that soybeans are associated with such wide range of diseases has led to several studies focusing in functional compounds present in the leguminosae.

Of all the phytochemicals present in soybeans, isoflavones are present in significant high amounts. Soybeans contain 1.2–4.2 mg of total isoflavones/g of sample, with large variation due to variety, crop year and growth location [3]. The 12 main isoflavones found in soybeans are daidzein (De), glycitein (Gle) and genistein (Ge) and their respective malonyl, acetyl and glucosyl forms [4] (Fig. 1). In soybeans, the three families of genistein, daidzein and glycitein are found in an approximately ratio of 6:3:1, respectively [5].

It has been suggested that a substantial portion of the health benefits of soy-containing foods is due to its content of isoflavones, increasing the interest in these compounds. This interest has led to the development of several extraction and analytical methods for isoflavones from soy and soy-based foods during the last years.

Extraction using soxhlet [6,7], mix-stirring [8,9] or overnight soaking [10] with either organic solvents (ethanol (EtOH), methanol (MeOH) or acetonitrile) or aqueous mixtures are the most used methods.

These methods for the extraction of soybeans isoflavones have important drawbacks, such as degradation, high solvent consumption, requiring additional clean-up and concentration steps before chromatographic analysis.

Extraction of isoflavones derivatives without degradation is very difficult since some of them are very sensitive to hydrolysis. Murphy et al. [11], for example, reported a conversion rate of 0.2–0.3 mol% per hour of malonyl isoflavones to their respective glucosides at room temperature. This is one of the reasons which make difficult the commercial availability of analytical standards of the malonyl forms.

New techniques for the extraction of isoflavones from soybeans have been recently reported, including supercritical

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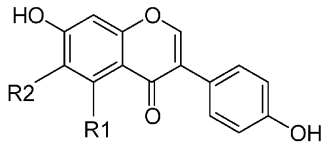
doi:10.1016/j.aca.2004.05.078

Aglycones:

Compounds	Symbol	R_1	R_2
Daidzein	De	H	H
Glycitein	Gle	H	OCH_3
Genistein	Ge	OH	H



Glucosides:

Compounds		R_1	R_2	R_3
Daidzin	Di	H	H	H
Glycitin	Gly	H	OCH_3	H
Genistin	Gi	OH	H	H
Acetyldaidzin	AcDi	H	H	$COCH_3$
Acetylglycitin	AcGly	H	OCH_3	$COCH_3$
Acetylgenistin	AcGi	OH	H	$COCH_3$
Malonyldaidzin	MDi	H	H	$COCH_2COOH$
Malonylglycitin	MGly	H	OCH_3	$COCH_2COOH$
Malonylgenistin	MGi	OH	H	$COCH_2COOH$

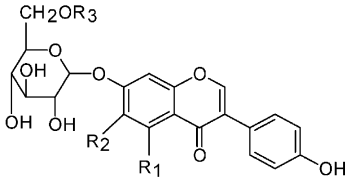


Fig. 1. Chemical structures of soybean isoflavones.

fluid extraction [12,13] and ultrasound-assisted extraction [13,14].

Pressurized liquid extraction (PLE) is a sample preparation technique where temperature and pressure are used to accelerate extraction of compounds from solid and semi-solid samples. Pressure is used to increase the contact between the extracting fluid and sample. Temperature is used to break the analyte-matrix bonds. Since PLE is conducted at elevated pressures it allows liquid extraction at temperatures above the boiling points of the solvent at atmospheric pressure, thereby improving analyte solubility and its desorption from the matrix. Moreover, temperature can dramatically modify the relative permitivity of the extracting fluid, increasing selectivity [15].

This technique allows the required volume of extraction solvent to be reduced, the analysis time to be shortened and the handling necessary to produce more precise results to be decreased [16]. Also, PLE offers the possibility of performing the extractions under an inert atmosphere and protected from light, which represents an attractive advantage since many compounds are sensitive to these two factors [17].

Several researchers have successfully used PLE to extract chemically similar natural products from different matrices, like phenolic compounds from grape seeds and from apple [16,18], and proanthocyanidins from malt [19]. Other natural bioactive compounds have been successfully extracted by PLE [20,21]. Until the moment, the use of PLE to extract isoflavones from soybeans has not been reported. Only a method using superheated water extraction from defatted soybean flakes has been recently proposed [22], however, the long extraction time (more than 2.3 h) and the high amount of sample (180 g) proposed are not suitable for analytical purposes. Moreover, no stability studies were performed.

This way, in this work we evaluate the feasibility of PLE for isolating isoflavones present in soybeans, providing a reliable analytical extraction method.

2. Experimental

2.1. Sample

Soybeans were ground in a coffee grinder, freeze-dried and stored at −20 °C until the analysis.

2.2. Chemicals and solvents

Ethanol (Panreac, Barcelona, Spain) and methanol (Merck, Darmstadt, Germany) used were HPLC grade. Water was supplied by a Milli-Q water purifier system from Milipore (Bedford, MA, USA). Isoflavones genistin, genistein and daidzein were obtained from Sigma Chemical Co. (St. Luis, MO, USA). Isoflavones daidzin (Di), glycitin (Gly), malonyl daidzin (MDi), malonyl glycitin (MGly) and malonyl genistin (MGi) were obtained from LC Labs (Woburn, MA, USA).

2.3. Extraction of soy isoflavones

Extractions were performed on a Dionex ASE 200 extractor (Dionex Corp., Sunnyvale, CA, USA). The freeze-dried sample was mixed with sea sand (Panreac, Barcelona, Spain)

and placed in an 11-mL stainless steel extraction cell. A cellulose filter (Dionex Corp.) was placed at the bottom of the extraction cell.

The extraction cell was filled with the extraction solvent (water, EtOH or MeOH (30–80% in water)), pressurized (100–200 atm), and then heated (60–200 °C). The cell is heated for a fixed time to ensure that the sample reaches thermal equilibrium. The sample was then extracted by three static extraction cycles of 5, 7 or 10 min with the solvent at the experimental temperature and pressure. After the extraction, the cell was rinsed with fresh solvent (100% of the extraction cell volume) and purged with a flow of nitrogen for 300 s. The extract was collected into a 60 mL amber glass vial. The process consumes approximately 22-mL of the solvent. The sample volume was brought up to 25 mL, then filtered through a 0.45-μm nylon syringe filter (Millex-HN, Ireland) before chromatographic analysis. All experiments were performed in duplicate.

2.4. Stability of isoflavones

To determine the stability of the isoflavones during the PLE a series of experiments were performed using a reference extract containing all tested isoflavones. The extract contained all analyzed isoflavones in the range of 25–50 mg/L. It was obtained by ultrasound-assisted extraction of soybeans (see Ref. [14] for a complete description of the extraction process). One milliliter of the reference extract was mixed with a solid phase (sea sand) inside the extraction cell and let rest for 1 h before being submitted to the extraction conditions.

In order to ensure that the extracts inside the extraction cell were not washed out by the automatic flushing prior the extraction, the valves in the system were controlled manually, avoiding automatic flushing. All experiments were performed in duplicate.

2.5. High performance liquid chromatography (HPLC)

The extracts obtained from all experimental conditions were analyzed by HPLC in a Waters system, composed by an autosampler (717 plus), pump controller (600 S), pump (616), and a photodiode array detector (996) using a RP-18 column (LiChrosphere 100, 250 μm × 4 μm, MERCK, Germany) and a gradient of acidified water (0.1% acetic acid, solvent A) and methanol (0.1% acetic acid, solvent B) at a flow rate of 0.5 mL/min. The gradient was as follows: 0 min, 15% B; 10 min, 30% B; 20 min, 30% B; 25 min, 100%B; and 35 min 100%B. The sample volume injected was 10 μL. The UV–vis absorbance was monitored from 200 to 400 nm. UV–vis spectra were recorded and isoflavone peak areas were quantified at 254 nm. The analyzed isoflavone conjugates were daidzin, glycitin, genistin and malonyl genistin. The aglucon forms, genistein, daidzein and glycitein, were also analysed. Although we could not achieve a complete resolution of all isoflavones in a single run, the resulting separation (Fig. 2) was fairly satisfactory for the purpose of the study. Malonyl daidzin and malonyl glycitin were not quantified since under the experimental HPLC conditions their peaks were unresolved. The identification of each isoflavone was done by comparison of retention times with pure standards as well as by UV–vis and mass spectra [14]. Quantification was made using calibration curves obtained by injecting known amounts of pure compounds as external standards. Detection limits (mg/L) for Di, Gly, Gi, MGi, Ge, De and Gle, were 0.485, 0.390, 0.480, 0.430,

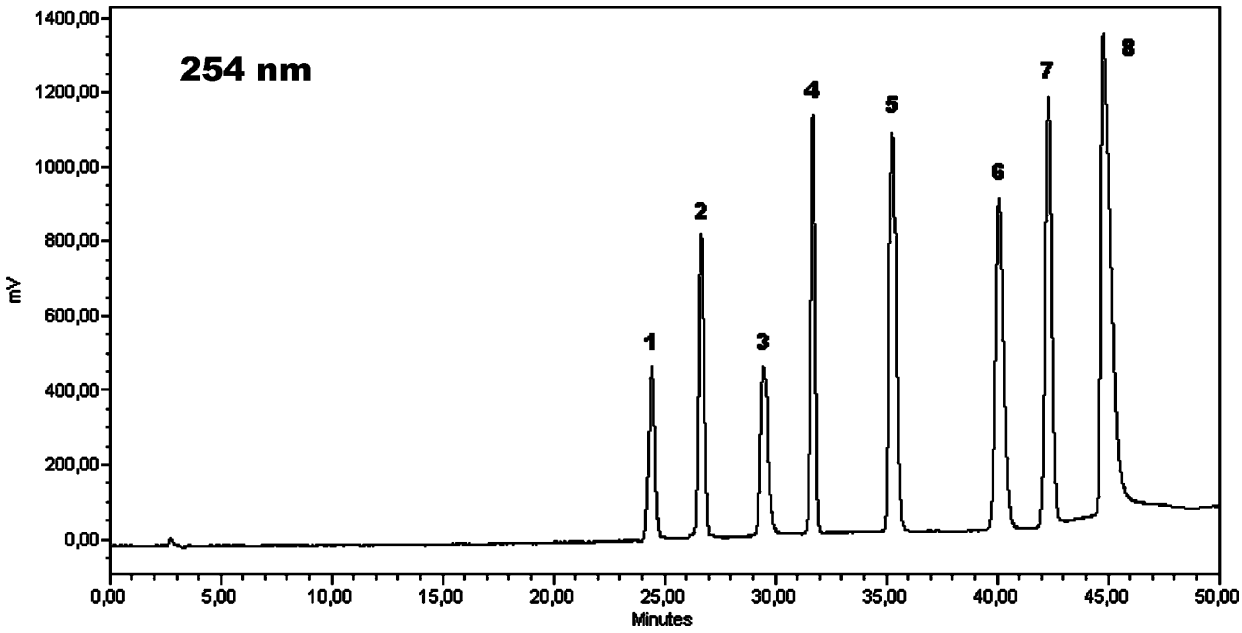


Fig. 2. Chromatogram of standard isoflavones (254 nm): (1) daidzin; (2) glycitin; (3) genistin; (4) malonyl daidzin and malonyl glycitin; (5) malonyl genistin; (6) daidzein; (7) glycitein; and (8) genistein.

0.353, 0.488 and 0.453, respectively. Quantification limits (mg/L) for Di, Gly, Gi, MGi, Ge, De and Gle, were 1.617, 1.301, 1.600, 1.434, 1.177, 1.627 and 1.510, respectively. Detection and quantification limits were calculated using the ALAMIN software [23].

The HPLC–MS analyses of the extracts were performed in a Finnigan LCQTM coupled LC–MS system, of Finnigan MAT (Thermo Electron Co., San Jose, USA). This equipment is fitted with a Spectra SYSTEM 2000 model gradient pump (Thermo Separation Products, Fremont, USA) and a mass detector (model LCQ) that consists of an electrospray interface and an ion trap mass analyzer. The chromatographic column and elution conditions used were same as in HPLC–PDA analyses. The software for the control of the equipment, and the acquisition and treatment of data is Xcalibur, version 1.2. The sample injection volume was 100 μL. The interface conditions were: positive ionization mode; temperature of the capillary: 220 °C; spray voltage: 4.6 kV; capillary voltage: −5 V; sheath gas flow: 80 (arbitrary units); and auxiliary gas flow: 10 (arbitrary units). ESI–MS spectra were acquired in the *m/z* range of 200–600.

3. Results and discussion

3.1. Solvent selection

The initial experiments used 0.5 g of freeze-dried soybeans extracted by water, methanol or ethanol (30–80% in water) at 60 °C and 100 atm of pressure using three static extraction cycles of 5 min to determine the best extraction conditions to be further studied. Fig. 3 shows the extraction efficiency for isoflavones as different solvents were employed. R.S.D.s for the extractions were always lower than 5%.

Using EtOH/water mixtures, extraction efficiency increased when increasing the water percentage in the extraction solvent from 0% (EtOH: 394.73 μg of total isoflavones/g) to 30% (EtOH 70%: 917.48 μg of total isoflavones/g). However, higher amounts of water in the extraction solvent resulted in lower extraction efficiency. Hundred percent water was the solvent that extracted the lowest amount of isoflavones (314.59 μg of total isoflavones/g) within the solvents assayed. Using MeOH/water mixtures, similar results were obtained and the highest extraction efficiency was achieved with a water percentage of 40% (MeOH 60% − 870.62 μg of total isoflavones/g).

As can be seen, the necessity of the addition of certain amount of water (30–40%) to the extracting solvent in order to improve the extraction of isoflavones is evident. Since polyphenols with several hydroxyl groups, such as glucosides, are hydrophilic and generally present higher solubilities in hydroalcoholic mixtures than in a pure alcoholic solvent [16,24]; it was expected that use of pure solvents resulted in a low isoflavone extraction efficiency, although in some cases (i.e. Di and Gi), using mixtures (EtOH 30%) extracted lower amounts than pure solvents

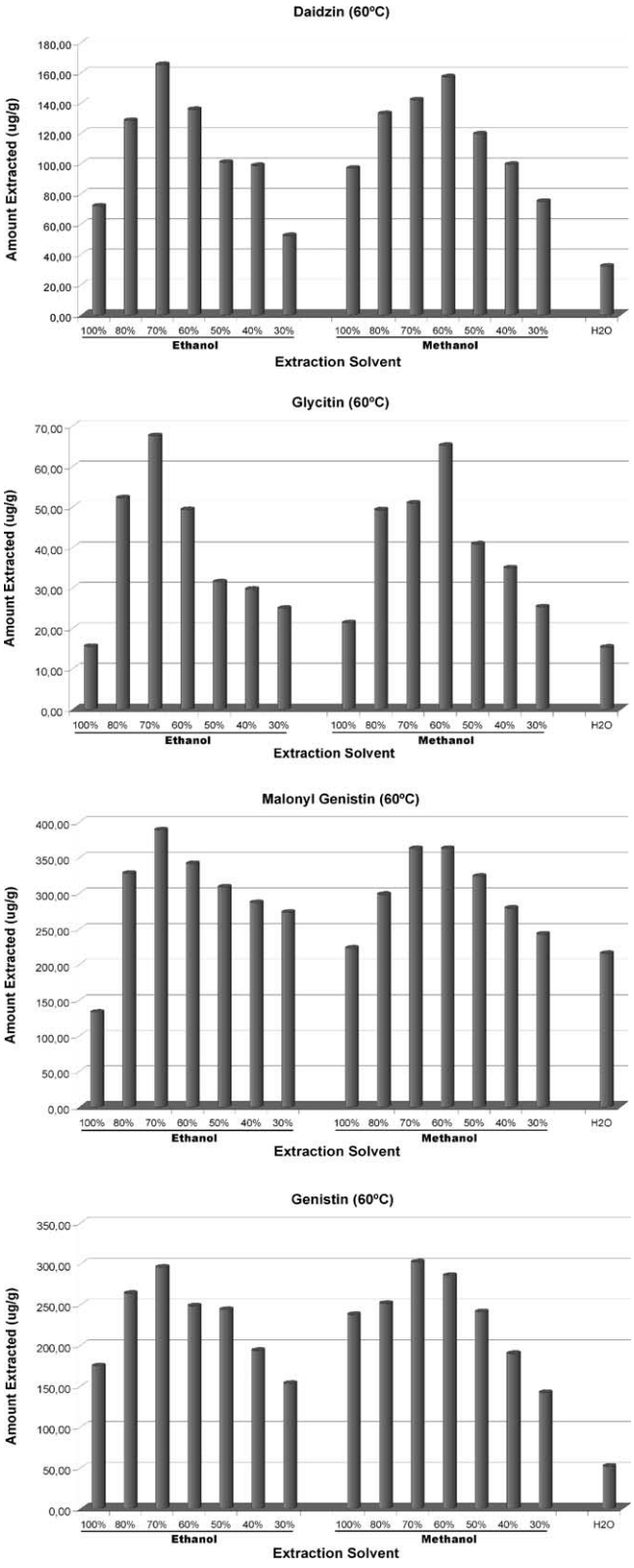


Fig. 3. Extraction efficiency for tested isoflavones as different solvents were employed (60 °C, 100 atm, 0.5 g of sample, and three static extraction cycles of 5 min each).

Table 1
Amount of isoflavones extracted from the soy sample (μg/g) as different temperatures were employed

Temperature (°C)	Di	De	Gly	Gle	MGi	Gi	Ge
60	165.41 ± 3.2	n.d.	66.33 ± 2.0	n.d.	386.48 ± 4.2	294.94 ± 5.7	10.23 ± 0.5
80	176.19 ± 3.2	n.d.	72.09 ± 2.2	n.d.	396.74 ± 5.3	337.78 ± 7.1	10.31 ± 0.5
100	202.34 ± 3.4	n.d.	91.83 ± 3.0	n.d.	445.70 ± 11.8	383.34 ± 10.7	10.68 ± 0.6
150	290.49 ± 4.4	n.d.	128.63 ± 3.5	n.d.	203.35 ± 3.8	616.08 ± 14.7	10.59 ± 0.4
200	117.82 ± 2.7	108.66 ± 4.0	53.64 ± 1.6	90.12 ± 3.9	n.d.	382.72 ± 11.6	153.74 ± 4.9

n.d.: non detected; 60–200 °C, 100 atm, 0.5 g of sample and three static extraction cycles of 5 min.

(EtOH–MeOH). Therefore, EtOH 70% was chosen to be used as extraction solvent not only for its superior efficiency but also for its lower cost, toxicity and superior environmental compatibility than MeOH.

3.2. Temperature

Since the increase of the temperature can improve the extraction of natural compounds, a series of experiments using EtOH 70% at different temperatures (60–200 °C) was performed to determine the best extraction temperature. The results for the extractions at different temperatures are shown in Table 1. Approximately 0.5 g of freeze-dried soybeans were used for each experiment.

One important point, when increasing the temperature of an extraction method is the stability of the aimed compounds. Since the most unstable isoflavone tested was MGi, careful observation of the extraction efficiency for this compound may indicate the highest possible extraction temperature in which it is possible to work without causing degradation of isoflavones.

In the experiments, the extraction efficiency of MGi increased up to 100 °C and above this temperature it started to decrease. The decrease in the extraction efficiency was possibly due to degradation of this compound at temperatures higher than 100 °C.

At 150 °C, it was observed an increase in the extraction efficiency of Di, Gly and Gi. At this temperature, the increase of the extraction efficiency is due to degradation of the malonyl forms of the isoflavones leading to their glucosidic form, increasing its concentration in the sample and thus, the amount extracted.

The levels of aglucone forms (De, Gle and Ge) remained constant indicating that isoflavone glycosides (Di, Gly and Gi) are not subjected to degradation under PLE conditions below 150 °C. Aglucone levels showed a significant increase at 200 °C whereas glycoside levels showed a significant decrease; it means that isoflavone glucosides are subjected to degradation leading to their respective aglucon form at this temperature. No malonyl forms of the isoflavones were detected at 200 °C.

3.3. Stability of the isoflavones during PLE

A stability study was performed to know the influence of both the temperature and the air in the extraction results. Using a previously obtained isoflavone extract [14], a series of extractions with and without solvent inside the extraction cell were performed. When the sample is maintained in the cell without solvent, the contact with the air is allowed, so oxidation of compounds can occur. When the extraction cell is filled with solvent, N_2 is used as pressurizing gas and no contact with air is produced.

One milliliter of the reference isoflavone extract was added to the sea sand inside the extraction cell and submitted to extraction conditions after 1 h. The extractions with solvent/N_2 inside the extraction cell (SOL) were performed using three static extraction cycles of 5 min and temperatures ranging 60–200 °C. The extractions with no solvent nor N_2 inside the extractions cell (AIR) were performed using a heating time of 15 min and three static extraction cycles of 1 min using the same temperatures. Three 1-min cycles were used to wash the solution from the extraction cell since no solvent was present during the heating time. By this way, both extraction conditions maintained the sample for 15 min inside the extraction cell at high temperature. EtOH 70% was used as solvent in the experiments.

The results obtained for individual isoflavones from the natural extract are shown in Fig. 4. All values are relative to the amount of each isoflavone found in the reference extract (100%). The results indicate that without solvent/N_2 inside the extraction cell, MGi is readily degraded. Degradation of MGi takes place above 60 °C and an increase in Gi concentration is observed. Di and Gly concentrations also increased, most likely due to the decrease in MDi and MGly. No malonyl forms were observed above 150 °C.

The amount of Ge and De increased significantly, mainly above 100 °C, where Di, Gly and Gi concentration started to decrease, indicating that isoflavone glucosides are degraded leading to their respective aglucon forms. An interesting result was the decrease of aglucon (Ge and De) levels at 200 °C, suggesting that they are also subjected to degradation at this temperature.

In contrast, using PLE conditions (with solvent/N_2 inside the extraction cell), isoflavones seemed to resist better the increase in temperature without degradation. Degradation started above 100 °C for the malonyl forms and above 150 °C for the isoflavone glucosides. Above 100 °C, with the decrease of MGi, a correspondent increase in Gi was observed. Di and Gly concentrations also increased working at this temperature. De and Ge levels remained constant

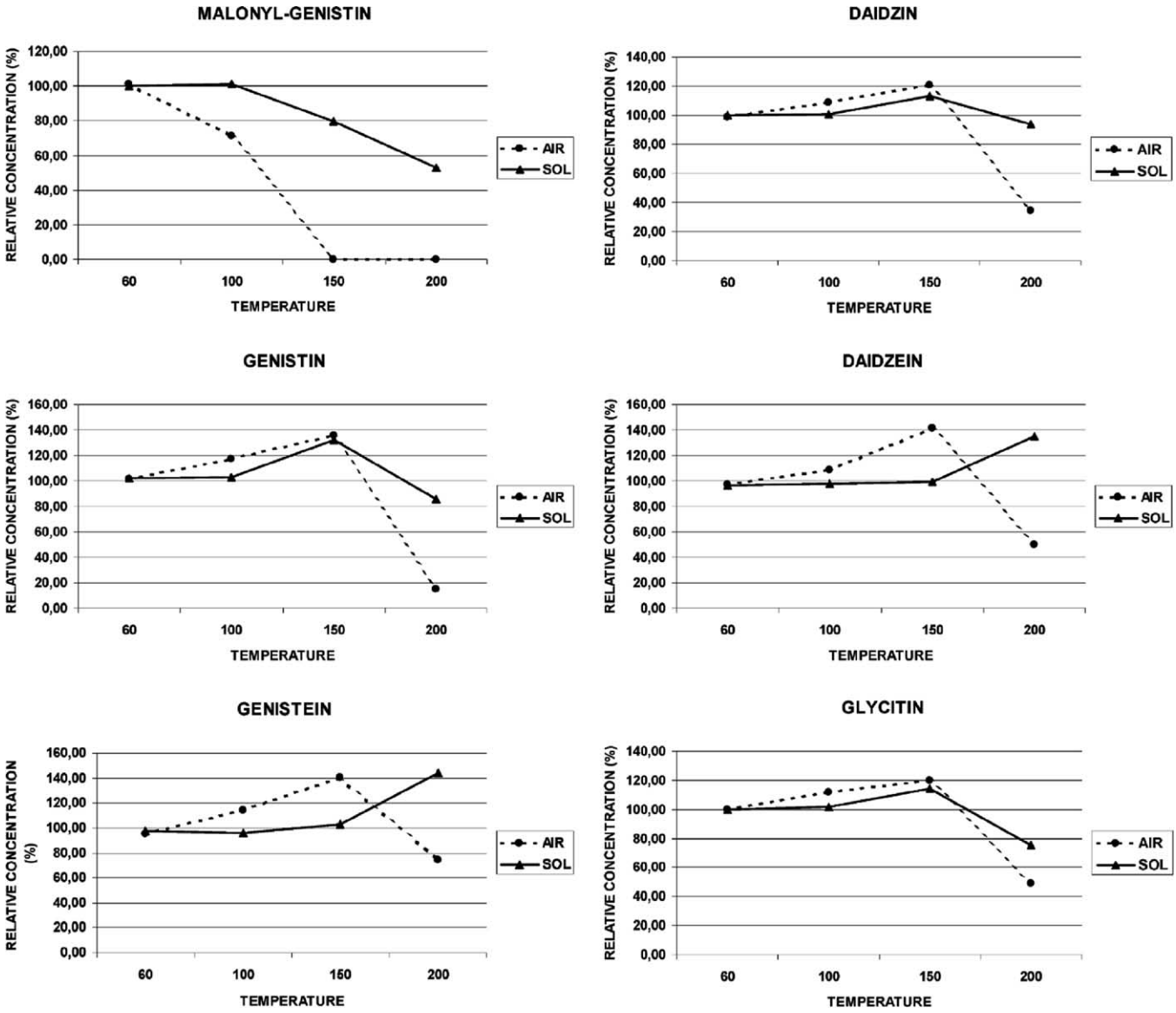


Fig. 4. Relative concentrations of individual isoflavones in the natural extract as different temperatures were employed. The extraction conditions were: (AIR) with neither solvent nor N_2 inside the extraction: 60–200 °C, 100 atm, 15 min of preheating time, 1 ml of sample (reference extract), three static extraction cycles of 1 min and EtOH 70% as extracting solvent; (SOL) with solvent/N_2 inside the extraction cell: no pre-heating time, 60–200 °C, 100 atm, 1 ml of sample (reference extract) three static extraction cycles of 5 min and EtOH 70% as extracting solvent.

below 150 °C indicating that no glucoside degradation was taking place during the extraction below this temperature.

Also De and Ge levels showed a small increase above 150 °C with its correspondent decrease of Di and Gi levels indicating conversion of these glucosides to their respective aglucon forms. Despite of longer heating time at higher temperatures (150–200 °C) malonyl glucosides were still present in the extracts obtained under PLE conditions (53.07%).

The stability study confirmed the observations made in the extractions using different temperatures, indicating that 100 °C is the maximum temperature for PLE of isoflavones since no degradation takes place and best extraction efficiency is achieved.

3.4. Pressure, sample size and static extraction time

To evaluate if pressure could influence extraction of isoflavones in a PLE method, duplicate extractions (100 °C; 100 and 200 atm; 3 × 5 min cycles) of freeze-dried soybeans (0.5 g) were performed (Table 2). Increasing the pressure from 100 to 200 atm did not influence the extraction of tested isoflavones.

The extraction time and the amount of sample must be adjusted to obtain quantitative recoveries. The amount of freeze-dried soybeans used was reduced from 0.5 to 0.25, 0.1 and 0.05 g (Table 3) using the extraction conditions optimized previously (100 °C, EtOH 70%, 100 atm and three 5-min extraction cycles).

Table 2
Amount of isoflavones extracted from the soy sample (μg/g) using different pressures

Pressure (atm)	Di	Gly	MGi	Gi
100	202.34 ± 3.4	91.83 ± 3.0	445.70 ± 11.8	383.34 ± 10.7
200	203.06 ± 4.6	84.97 ± 3.8	447.43 ± 12.7	384.30 ± 14.4

100 °C, 0.5 g of freeze-dried soybeans, three static extraction cycles of 5 min and EtOH 70% as extracting solvent.

Table 3
Amount of isoflavones extracted from the soy sample (μg/g) using different sample amounts

Sample size (g)	Di	Gly	MGi	Gi
0.50	202.34 ± 3.4	91.83 ± 3.0	445.70 ± 11.8	383.34 ± 10.7
0.25	205.38 ± 3.6	94.08 ± 2.4	448.74 ± 11.8	398.57 ± 15.6
0.10	213.46 ± 3.3	94.87 ± 3.8	462.07 ± 12.5	439.97 ± 17.0
0.05	225.44 ± 5.7	97.39 ± 4.9	475.75 ± 22.8	477.53 ± 20.6

100 °C, 100 atm, three static extraction cycles of 5 min and EtOH 70% as extracting solvent.

Table 4
Amount of isoflavones extracted from the soy sample (μg/g) of isoflavones (μg/g) using different static extraction cycle length

Static extraction cycle length (min)	Di	Gly	MGi	Gi
5	213.46 ± 3.3	94.87 ± 3.8	462.07 ± 12.5	439.97 ± 17.0
7	236.81 ± 6.2	102.38 ± 4.8	477.61 ± 14.8	510.62 ± 19.7
10	239.41 ± 6.8	101.84 ± 5.1	483.65 ±21.0	507.57 ±14.3

100 °C, 100 atm, 0.1 g of freeze-dried soybeans, three static extraction cycle and EtOH 70% as extracting solvent.

Extraction efficiency of some isoflavones constantly increased with the reduction of sample size from 0.5 to 0.05 g. This makes necessary to increase the extraction time in order to determine when quantitative recoveries are achieved. As can be seen, the lower the sample size, the higher the R.S.D., due to higher chromatographic errors, besides of sampling errors due to the heterogeneity of the sample. Since the chromatographic peaks of 0.05 g samples were small, increasing R.S.D.s values, the optimization of the static extraction cycle length was performed using 0.1 g samples.

Then the length of the static extraction cycle was extended from three cycles of 5 min to three cycles of 7 and 10 min using the optimized extraction conditions (100 °C, EtOH 70%, 100 atm and 0.1 g of sample) (Table 4).

Extraction efficiency of all tested isoflavones increased with the extension of the static extraction cycle length from 5 to 7 min and no clear difference between 7 and 10 min was observed, indicating that quantitative extraction was obtained using three 7 min cycles. To check if all three extraction cycles are needed to guarantee the full extraction of compounds from the samples, a series of extractions were performed using one, two and three static extraction cycles of 7 min. The results for the extractions are shown in Table 5. Increasing the number of static extraction cycles of 7 min increased the amount extracted of all the tested isoflavones indicating the necessity of all three cycles.

Also, it would be very useful to reduce the number of extracting cycles since two cycles of 10 min consume less time than three cycles of 7 min. Much time is lost between static extraction cycles during de-pressurization, flushing, purging and pressurization of the system to start a new extraction cycle.

The results for the extractions using two static extraction cycles of 10 min are also shown in Table 5. Extractions carried out with two cycles of 10 min extracted lower amounts of isoflavones than extractions with three cycles of 7 min. This is probably due to the higher amount of solvent used with three extraction cycles than with two cycles.

With that, three static extraction cycles are recommendable and the total extraction time using the optimized

Table 5
Amount of isoflavones extracted from the soy sample (μg/g) of isoflavones (μg/g) using one, two and three static extraction cycles of 7 min and two static extraction cycles of 10 min

Static extraction cycles length (min)	Static extraction cycles	Di	Gly	MGi	Gi
7	1	181.29 ± 2.7	44.85 ± 2.1	348.41 ± 10.4	236.25 ± 9.7
	2	189.59 ± 3.1	77.16 ± 3.4	428.50 ± 11.9	404.66 ± 10.9
	3	237.53 ± 6.7	103.10 ± 5.5	477.87 ± 20.4	510.87 ± 13.7
10	2	203.78 ± 3.8	87.19 ± 4.1	445.41 ± 14.4	451.99 ± 14.9

100 °C, 100 atm, 0.1 g of freeze-dried soybeans and EtOH 70% as extracting solvent.

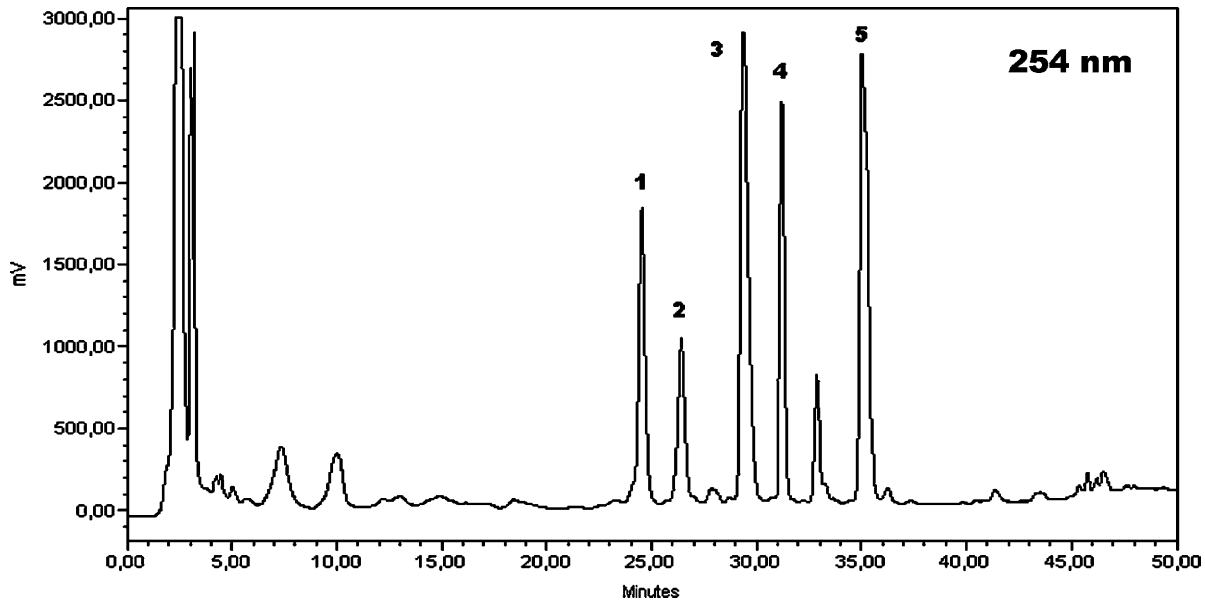


Fig. 5. Chromatogram obtained for the spiked sample: (1) daidzin; (2) glycitin; (3) genistin; (4) malonyl daidzin and malonyl glicitin; and (5) malonyl genistin.

protocol is 32 min (5 min of pre-heating, 21 min of extraction, 1 min of flushing and 5 min of purging).

In order to verify the amount of isoflavones present in the sample and to be sure that quantitative extraction are obtained using three static extraction cycles of 7 min, four extractions of the same sample were performed. The amounts of individual and total isoflavones present in the sample obtained with the optimized extraction method were comparable to those obtained with four extractions.

To evaluate the repeatability of the extraction procedure, a series of five replicated extractions of freeze-dried soybeans were performed in the same and different days. The results obtained for all isoflavones revealed a R.S.D. lower than 5%.

3.5. Recovery of isoflavones from a spiked sample

Recovery of isoflavones spiked to the soy sample was determined using the optimized extraction protocol. One milliliter of the reference extract was spiked directly into the soy sample inside the extraction cell. The spiked sample was let rest for 1 h before being submitted to extraction conditions. The rest time was used to allow the spiked extract interact with the soy sample. The results obtained with the extractions (duplicates) are shown in Table 6 and a chromatogram of the extract obtained in Fig. 5. Recoveries of spiked isoflavones were between 92 and 108%. The result obtained for MGi (108%) may be due to interfering compounds from the extract that may increase the analysis errors for MGi resulting in an increased peak area and thus, a higher recovery for this compound.

Table 6
Recovery of isoflavones (%) from the spiked soy sample

	Di	Gly	MGi	Gi
Recovery (%)	97.4 ± 2.8	97.4 ± 5.3	108.5 ± 4.3	92.1 ± 3.4

1 mL of reference extract, 0.1 g of freeze-dried soybeans, 100 °C, 100 atm, three static extraction cycles of 7 min and EtOH 70% as extracting solvent.

4. Conclusions

Using the optimized extraction conditions (0.1 g of sample, 100 °C, 100 atm, three static extraction cycles of 7 min, extraction solvent: ethanol 70%) quantitative extractions were achieved and no degradation was observed. The results are supported by the stability studies. Under PLE conditions, degradation of malonyl glucoside forms of the isoflavones takes place above 100 °C leading to their respective glucosidic form and degradation of glucosides above 150 °C, leading to their respective aglucone form. Using the optimized protocol, isoflavones can be reliable extracted from freeze-dried soybeans without degradation. The method has been validated with both spiked and real samples.

Acknowledgements

MAR gratefully acknowledges support of the Brazilian government for a Doctoral scholarship by the "Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES, Brasil).

References

[1] M.J. Messina, Am. J. Clin. Nutr. 70 Suppl. (1999) 439S.
[2] H. Adlercreutz, Environ. Toxicol. Pharmacol. 7 (1999) 201.

[3] H.J. Wang, P.A. Murphy, J. Agric. Food Chem. 42 (1994) 1674.
[4] P.A. Murphy, T. Song, G. Buseman, K. Barua, G.R. Beecher, D. Trainer, J. Holden, J. Agric. Food Chem. 47 (1999) 2697.
[5] H.J. Wang, P.A. Murphy, J. Agric. Food Chem. 44 (1996) 2377.
[6] T. Nguyenle, E. Wang, A.P. Cheung, J. Pharm. Biomed. Anal. 14 (1995) 221.
[7] L.S. Hutabarat, H. Greenfield, M. Mulholland, J. Chromatogr. A 886 (2000) 55.
[8] P.A. Murphy, K. Barua, C.C. Hauck, J. Chromatogr. B 777 (2002) 129.
[9] I.U. Grün, K. Adhikari, C. Li, Y. Li, B. Lin, J. Zhang, L.N. Fernando, J. Agric. Food Chem. 49 (2001) 2839.
[10] L. Liggins, L.J.C. Bluck, S. Runswick, C. Atkinson, A. Coward, S. Bingham, J. Nutr. Biochem. 11 (2000) 326.
[11] P.A. Murphy, T.T. Song, G. Buseman, K. Barua, G.R. Beecher, D. Trainer, J. Holden, J. Agric. Food Chem. 45 (1997) 4635.
[12] A. Chandra, M.G. Nair, Phytochem. Anal. 4 (1996) 259.
[13] M.A. Rostagno, J.M.A. Araujo, D. Sandi, Food Chem. 78 (2002) 111.
[14] M.A. Rostagno, M. Palma, C.G. Barroso, J. Chromatogr. A 1012 (2003) 119.
[15] C.W. Huie, Anal. Bioanal. Chem. 373 (2002) 23.
[16] R.M. Alonso-Salces, E. Korta, A. Barranco, L.A. Berrueta, B. Gallo, F. Vicente, J. Chromatogr. A 993 (2001) 37.
[17] M. Palma, Z. Piñeiro, C.G. Barroso, J. Chromatogr. A 921 (2001) 169.
[18] M. Palma, C.G. Barroso, Anal. Chim. Acta 458 (2002) 119.
[19] M. Papagiannopoulos, B. Zimmermann, A. Mellenthin, M. Krappe, G. Maio, R. Galensa, J. Chromatogr. A 958 (2002) 9.
[20] J.R. Denery, K. Dragull, C.S. Tang, Q.X. Li, Anal. Chim. Acta 501 (2004) 175.
[21] E.S. Ong, S.M. Len, Anal. Chim. Acta 482 (2003) 81.
[22] C. Li-Hsun, C. Ya-Chuan, C. Chieh-Ming, Food Chem. 84 (2004) 279.
[23] A.M.G. Campana, L.C. Rodriguez, F.A. Barrero, M.R. Ceba, J.L.S. Fernández, Trends Anal. Chem. 16 (1997) 381.
[24] K.R. Markham (Ed.), Techniques of Flavanoid Identification, Academic Press Inc., London, 1982, p. 15.