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Journal of Chromatography A, 1012 (2003) 119–128
JOURNAL OF CHROMATOGRAPHY A
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Ultrasound-assisted extraction of soy isoflavones ☆

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Received 7 March 2003; received in revised form 24 June 2003; accepted 2 July 2003

Abstract

Efficiency in extracting four isoflavone derivatives (daidzin, glycitin, genistin and malonyl genistin) from freeze–dried ground soybeans was compared for mix-stirring extraction and ultrasound-assisted extraction, using different solvents and extraction temperatures with both. The efficiency of the extraction of soy isoflavones was improved by ultrasound but was dependent on the solvent employed. Optimization of the ratios of sample quantity to solvent volume and length of extraction time was also performed. Isoflavones can be quantitatively extracted from soybeans with 50% ethanol at 60 °C using ultrasound-assisted extraction in 20 min.
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Keywords: Ultrasound-assisted extraction; Extraction methods; Soybean; Isoflavones

1. Introduction

Isoflavones are a flavanoid subgroup found in several plants. In soybeans, they are present in 12 main forms: genistin, glycitin, daidzin and their respective acetyl, malonyl and aglucon forms [1].

There is an increasing interest in these compounds due to their biological effects, including estrogenic and fungitoxic activities. Isoflavones have been associated with a decreased risk of breast, prostate and colon cancers and are being studied for the prevention of menopausal symptoms and osteoporosis (see Ref. [2] for a review). They may be also important antioxidant agents, since they have hydroxyl groups in rings A and/or B, and are thus capable of donating hydrogen to free radicals. The antioxidant activity of isoflavonoids may be related to the number and position of hydroxyl groups [3,4].

Typically, isoflavones have been extracted using aqueous methanol (MeOH) [5], ethanol (EtOH) [6] or acetonitrile (MeCN) [7,8]. Murphy [9] tested EtOH, MeOH, acetone (Ace) and MeCN, with and without addition of hydrochloric acid, as extracting solvents by mix-stirring for 2 h at room temperature.

Griffith and Collison [10] compared 80% MeOH and 60% MeCN with and without acid to extract isoflavones from soy protein, soy foods and nutritional supplements. They observed that 60% MeCN was more efficient as the extracting solvent than 80% MeOH. More recently, Murphy et al. [11] have evaluated MeCN, Ace, MeOH and EtOH, all with the addition of 53% water, to extract the 12 main isoflavones from several soy foods, using a mix-

☆Presented at the 2nd Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November 2002.

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doi:10.1016/S0021-9673(03)01184-1

stirring technique. According to these authors, the four different solvents present different capabilities in extracting the different isoflavone forms. MeCN (47%), in general, was the best extraction solvent for the soy foods tested. In soy flour, MeOH (47%) yielded higher aglucon levels than MeCN (47%), and Ace (47%), was almost as good as MeCN (47%), but slightly underestimated the total individual isoflavone concentration. EtOH (47%), in soy flour, produced similar results to MeCN (47%), Ace (47%) and MeOH (47%) in extracting the glucosides but lower recoveries were obtained for the malonyl glucosides.

Seeking more environmentally friendly methods and increased productivity, newer extraction techniques have been developed, including supercritical fluid extraction [12,13] and ultrasound-assisted extraction (UAE) [13,14]. Among these, UAE is the cheapest technique and has the lowest instrumental requirements.

The enhancement of extraction efficiency of organic compounds by ultrasound is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave. Cavitation bubbles are produced and compressed during the application of ultrasound. The increase in the pressure and temperature caused by the compression leads to the collapse of the bubble. With the collapse of bubble, a resultant ''shock wave'' passes through the solvent enhancing the mixing [15].

Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phase. This, coupled with the enhanced mass transfer and significant disruption of cells, via cavitation bubble collapse, increases the release of intracellular product into the bulk medium. The use of higher temperatures in UAE can increase the efficiency of the extraction process due to the increase in the number of cavitation bubbles formed [15–17].

Although new techniques have been tried for the extraction of isoflavones, these studies were very limited in scope and a full evaluation of UAE is still needed to establish a general protocol. The objective of this work is to determine the best extraction conditions in a fast and reliable ultrasound-assisted extraction protocol.

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

The MeCN (Scharlau, Barcelona, Spain), EtOH (Panreac, Barcelona, Spain) and MeOH (Merck, Darmstadt, Germany) used were HPLC grade. Water was supplied by a Milli-Q water purifier system from Milipore (Bedford, MA, USA). The isoflavones standards and 2,5-dihydroxybenzaldehyde were obtained from Sigma (St. Louis, MO, USA).

2.3. Extraction of soy isoflavones

Extractions were carried out in a high-intensity ultrasound probe system of 200 W and 24 kHz (Model UP 200S, Dr. Hielscher, Germany) equipped with a 2 mm microtip. Its ultrasonic vibrations amplitude controller was set to 100% of nominal power. An ultrasonic bath of 360 W (J.P. Selecta, Barcelona, Spain) was also tested as an alternative to the ultrasonic probe. The extractions were performed at constant temperature by means of a temperature controller coupled to the ultrasonic bath. The conventional extraction method by mix-stirring was used as a reference for comparison with the UAE method. All experiments were performed in duplicate.

Three different solvent systems, EtOH, MeOH or MeCN, with several water percentages (between 30 and 70%) and two temperatures (10 and 60 °C) were evaluated for the extraction of soy isoflavones. The initial extraction protocol used 0.5 g of freeze–dried ground soybeans in 25 ml of the extraction solvent for 10 min. This protocol was further studied to optimize the extraction method. After the extraction, 1 ml of 2,5-dihydroxybenzaldehyde was used as internal standard. The extracts were then centrifuged for 10 min and filtered though a 0.45 μm nylon syringe filter (Millex-HN, Ireland) before chromatographic analysis.

2.4. High-performance liquid chromatography (HPLC)

The HPLC–UV analyses of the extracts were performed in a Waters system consisting of an autosampler (717 plus), pump controller (600S), pump (616), and a photodiode array detector (996), using an RP-18 column (LiChrospher 100, 5 μm, Merck) and a gradient of acidified water (0.1% acetic acid) (solvent A) and acetonitrile (0.1% acetic acid) (solvent B) at a flow-rate of 0.3 ml/min. The gradient was as follows: 0 min, 15% B; 10 min, 30% B; 20 min, 30% B; 25 min, 100% B; 35 min 100% B. The UV absorbance was monitored from 200 to 400 nm. UV spectra were recorded and isoflavone peak areas were quantified at 254 nm. The sample volume injected was 10 μl. Calibration curves (correlation coefficient) for daidzein, genistin and genistein were $y=44\,751x+8805$ ($r^2=0.9980$), $y=77\,969x-31\,141$ ($r^2=0.9967$) and $y=79\,785x-55\,987$ ($r^2=0.9952$), respectively.

The detection limits (mg/g) for genistin, genistein and daidzein were, respectively, 1.3, 1.6 and 2.1. Detection limits were calculated using ALAMIN software [18]. Quantification of each isoflavone was accomplished by using the response factor of the corresponding β-glucoside group and correcting for the molecular mass difference. This method is reported to give values close to correct [11].

The HPLC–mass spectrometry (MS) analyses of the extracts were performed in a Finnigan LCQ coupled LC–MS system, of Finnigan MAT (Thermo Electron, San Jose, CA, USA). This equipment is fitted with a Spectra SYSTEM 2000 Model gradient pump (Thermo Separation Products, Fremont, CA, USA) and a mass detector (Model LCQ) that consists of an electrospray interface and an ion trap mass analyzer. The software for the control of the equipment, and the acquisition and treatment of data is Xcalibur, version 1.2. The same gradient as in LC–UV was applied. 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, focus 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.

The analyzed isoflavone glucosides were daidzin, glycitin, genistin and malonyl genistin. The identification of each isoflavone was made by comparison of retention times with pure standards, as well as by photodiode array detection (DAD) and MS spectra (Fig. 1). Chromatogram of isoflavone extracts analysis by HPLC–UV is shown in Fig. 2.

3. Results and discussion

The soy sample was extracted using the initial protocol (0.5 g, 25 ml, 10 min) and different solvent systems at 60 °C (EtOH, MeOH and MeCN, 30–70%) using both mix-stirring and UAE methods. Fig. 3 shows the yields (μg/g) of individual isoflavones and Fig. 4 the yields (μg/g) of total isoflavones obtained with different extraction methods and extracting solvents at 60 °C for 10 min.

The efficiency of the extraction of soy isoflavones was improved by ultrasound but it was dependent on the solvent employed. For almost all the cases shown, the total and individual isoflavone yields obtained with UAE were higher (0–15%) than those obtained with mix-stirring. The extractions using mix-stirring also revealed a similar behavior to UAE: when using pure solvents a low extraction efficiency was obtained and the maximum amount extracted was obtained using solvents with 40–60% of water.

EtOH (50%), MeOH (50%) and MeCN (40%) were the solvents that produced the highest yields of total isoflavones, with only small differences between them. The best extraction solvents for daidzin were 50% EtOH and 50% MeOH with no difference between them. For glycitin, the best extraction solvent was 50% EtOH. For genistin, no difference was observed between 50% EtOH, 60% MeOH and 40% MeCN, and similar results were obtained for the malonyl derivative of genistin.

These data are similar to those obtained by Murphy et al. [11] using the mix-stirring technique for 2 h; they state that 47% EtOH was as efficient as 47% MeOH and 47% MeCN for the extraction of isoflavone glucosides.

It is clearly necessary to add a certain amount of water (40–60%) to the extracting solvent in order to improve the extraction of isoflavones from soybeans. This is probably due to the relative polarity of these compounds, and to the increased propagation of

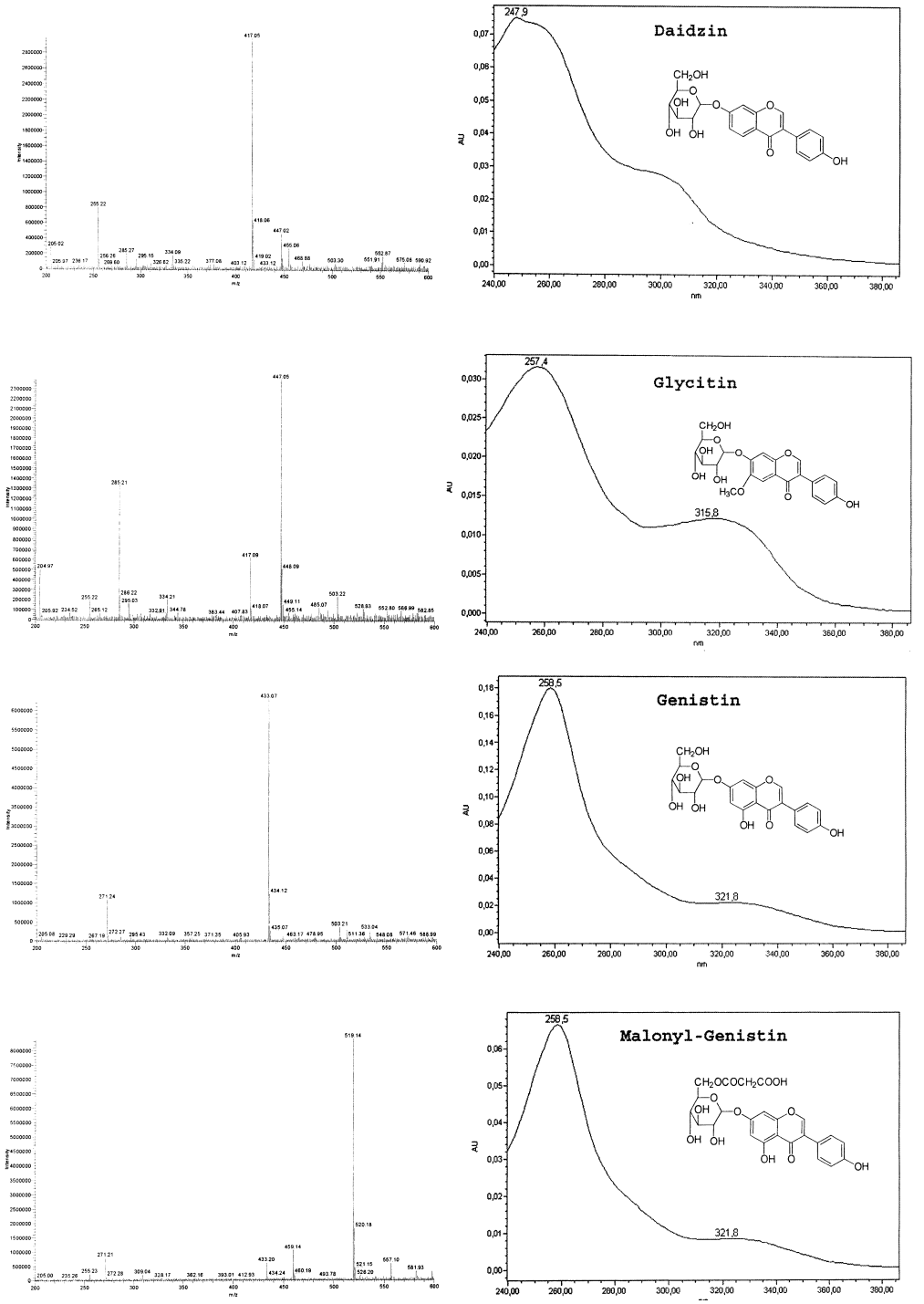


Fig. 1. Chemical structure, MS and UV-VIS spectra of analyzed isoflavones.

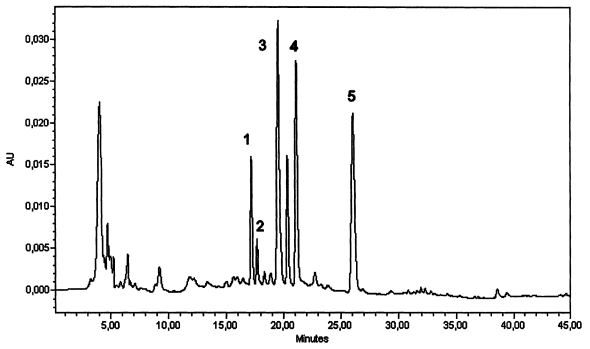


Fig. 2. UV chromatogram of a typical soy isoflavone extract obtained using 0.5 g of sample and 70% EtOH at 60 °C for 10 min. 1—Daidzin, 2—glycitin, 3—internal standard (2,5-dihydroxybenzaldehyde), 4—genistin, 5—malonyl genistin.

ultrasonic waves in aqueous solvents. The reduction of extraction efficiency common to all the three solvents with high amounts of water (>60%), can reasonably depend on an increased production of radicals from the ultrasound dissociation of water. In the presence of these high-energy species, oxidative reactions can cohabit with the extraction reactions when water is higher than 50%, decreasing the amount of target compounds available in the food matrix for extraction, consequently decreasing the extraction efficiency [15].

It also worth noting that in this soy sample, malonyl genistin and genistin were found in significantly higher amounts than other isoflavones, and glycitin was the isoflavone glucoside with the lowest levels.

Since no significant difference was observed between 50% EtOH, 50% MeOH and 40% MeCN at 60 °C, MeCN was discarded as a solvent option, since it is potentially more harmful to the environment and also more toxic and expensive than EtOH and MeOH.

In order to evaluate the effect of the temperature on the extraction efficiency of the isoflavone glucosides, several extractions were performed at 10 °C using both UAE and mix-stirring methods. Figs. 3 and 4 also show the yields (μg/g) of individual and total isoflavones obtained, with different extraction methods and extracting solvents, at 10 °C, for 10 min.

As can be seen, temperature has a great impact on the extraction of isoflavones. Increasing the temperature from 10 to 60 °C significantly increased the extraction efficiency using both UAE and mix-stirring. Extractions at 60 °C increased extraction efficiency of all isoflavones when compared to extractions performed at 10 °C. The higher yields in the extractions at 60 °C may be partially attributed to the increase in the number of cavitation bubbles formed and to enhanced mass transfer rates. Temperature seems to affect EtOH and MeOH the same way, since similar behavior of the solvents at 10 and 60 °C was observed. That is, the best recoveries were obtained using the same extraction solvent at both 10 and 60 °C.

The necessity for water (40–60%) in the extraction solvent is even more evident when the extraction is performed at lower temperatures. Very low recoveries of all the isoflavones tested were obtained at 10 °C using pure solvents, especially with EtOH.

For both UAE and mix-stirring methods, at 10 and 60 °C, 50% EtOH appears to be the superior extraction solvent for extracting total and individual isoflavones from ground freeze–dried soybeans. Although the differences in the total and individual isoflavone recoveries (except for glycitin) are not significant, EtOH offers several advantages when compared to the other solvents, like lower cost, lower toxicity, and environmental compatibility. On that basis, 50% EtOH and 60 °C were chosen for further study. Because UAE produced higher recoveries than mix-stirring extraction for all the extraction conditions, the following optimization process was carried out only for UAE.

The extraction time and the amount of sample must be adjusted to obtain quantitative recoveries. To establish the kinetics of the extraction, the quantity of sample used was reduced from 0.5 to 0.25, 0.1 and 0.05 g (Table 1) and the time of the extraction was extended to 15, 20 and 30 min (Table 2).

Decreasing the sample size and maintaining the extracting solvent volume (25 ml) increases the sample:solvent ratio favouring the extraction efficiency. This also reduces the amount of isoflavones available to extract allowing one to determine when quantitative recoveries are achieved.

Reducing the quantity of sample and maintaining the extraction period (10 min) did not improve the

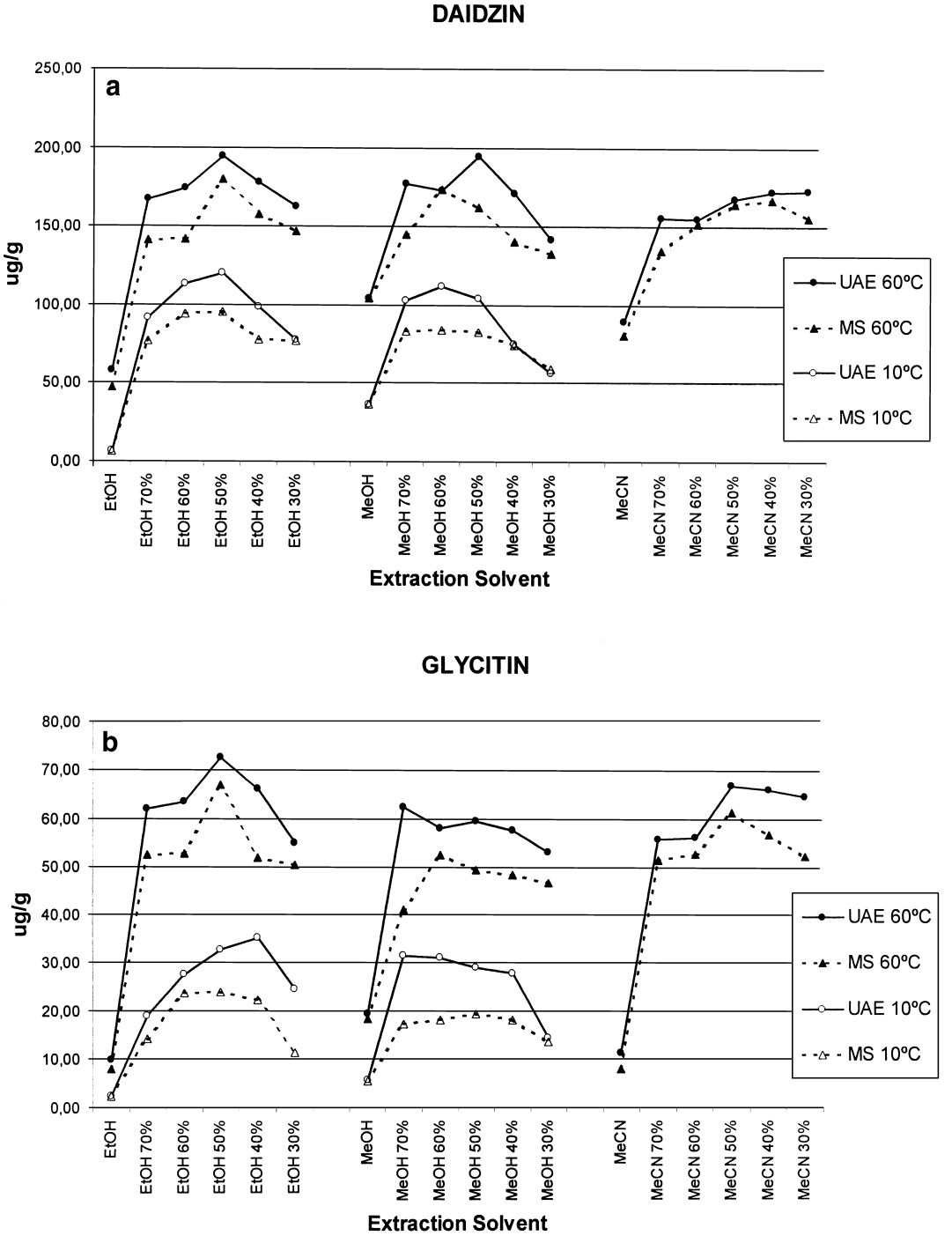
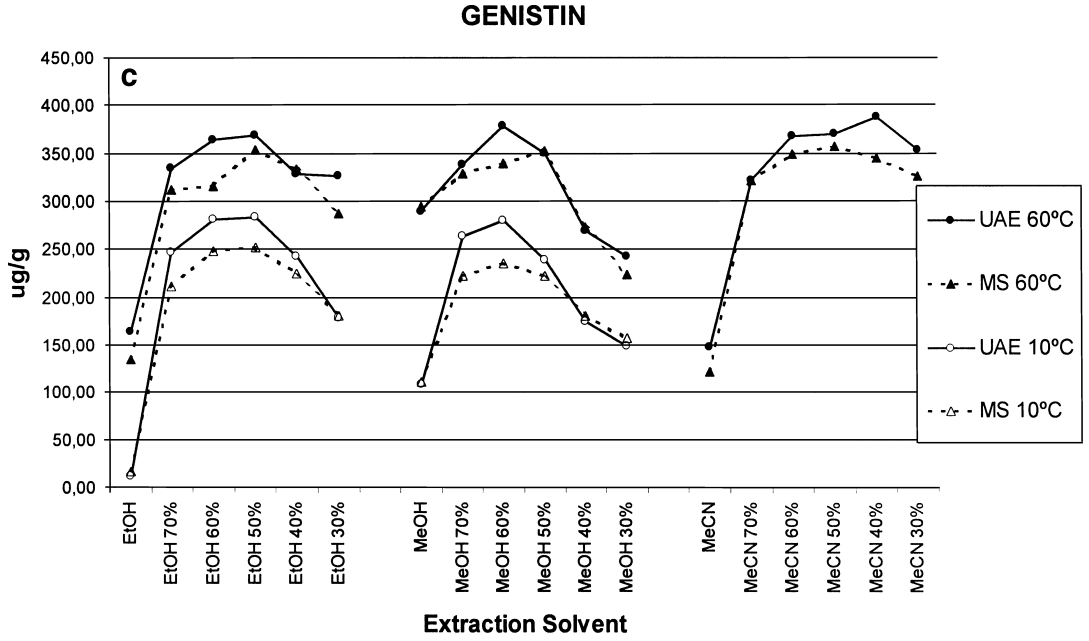


Fig. 3. Yields (μg/g) of daidzin (a), glycitin (b), genistin (c) and malonyl genistin (d) obtained with different extraction methods using extracting solvents at 10 and 60 °C for 10 min.



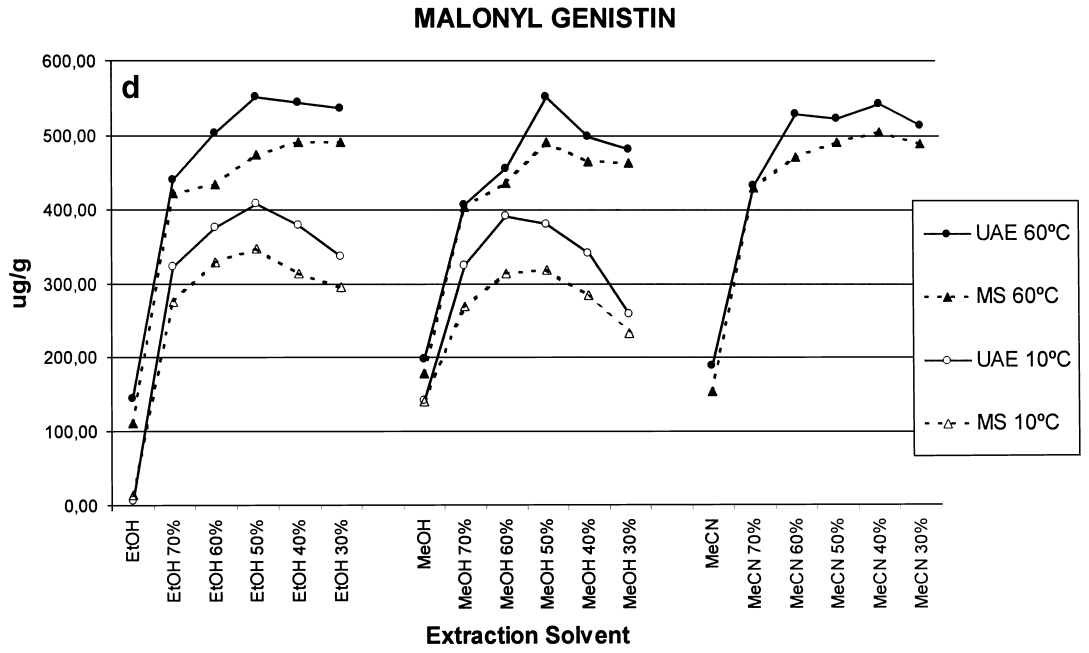


Fig. 3. (*continued*)

extraction efficiency for daidzin, glycitin and malonyl genistin (Table 1). Only the amount of genistin extracted showed a small increase (1.7–12.5%) with the reduction of the sample size. So, it can be concluded that any ratio of sample mass to extraction solvent volume between 0.5 g/25 ml to

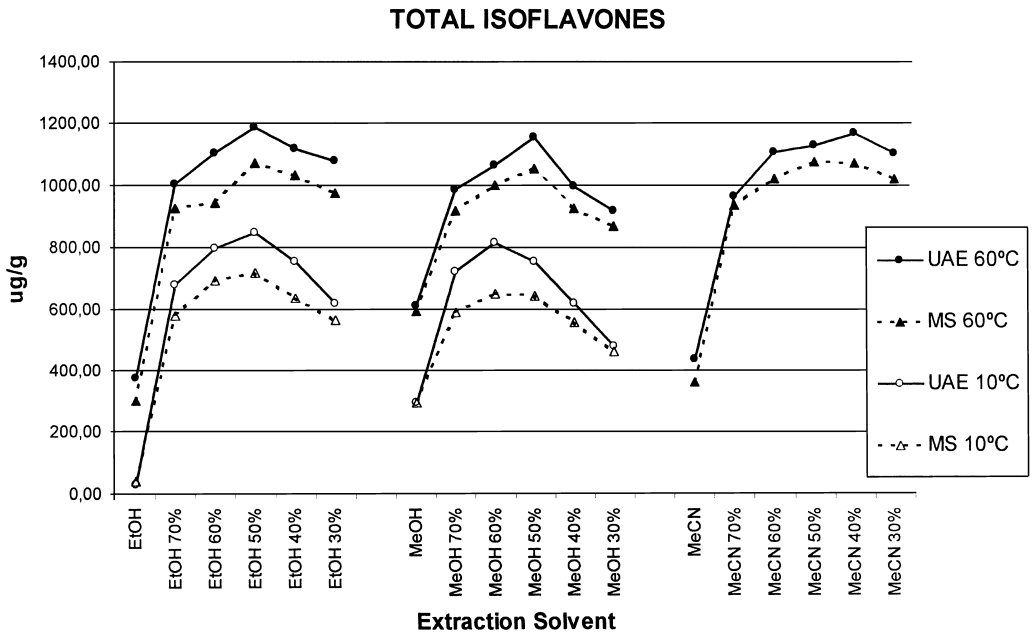


Fig. 4. Yields (μg/g) of total isoflavones obtained with different extraction methods using extracting solvents at 10 and 60 °C for 10 min.

Table 1
Amount of isoflavones (μg/g) extracted using 50% EtOH at 60 °C for 10 min and different sample quantities

Sample (g)	Daidzin	Glycitin	Genistin	Malonyl genistin	Total
0.5	194.72±6.99	72.58±3.29	369.00±17.43	550.45±22.14	1186.75
0.25	183.60±7.84	78.37±3.12	411.81±17.11	523.77±23.95	1197.55
0.1	185.69±8.45	78.37±3.04	453.83±18.56	529.64±24.35	1247.53
0.05	180.32±9.65	75.70±3.61	455.36±21.74	539.07±25.74	1250.45

0.05 g/25 ml could be used, and would give similar extraction efficiency. As stated, it was observed that only genistin extraction efficiency increased, indicating that still there was genistin in the sample. Consequently the extraction time was extended.

The longer the extraction times, the higher the extraction efficiency of isoflavones, up to 20 min (Table 2). Between 20 and 30 min, the amount extracted of all isoflavones tested decreased slightly. It is possible that oxidation may be taking place after 20 min when submitted to sonication. From these data we can infer that, by using extractions of

Table 2
Amount of isoflavones (μg/g) extracted using 50% EtOH at 60 °C for 10 min and different extraction times

Extraction time (min)	Daidzin	Glycitin	Genistin	Malonyl genistin	Total
5	146.44±5.14	46.89±3.01	347.17±15.35	479.17±19.31	1019.66
10	194.55±6.99	72.58±3.29	369.00±17.43	550.45±22.14	1186.75
15	202.54±6.32	79.28±3.59	407.73±16.18	595.76±18.44	1285.30
20	216.77±7.67	84.24±3.38	433.00±14.32	619.46±23.89	1353.47
30	210.76±6.71	79.28±3.47	424.53±15.78	606.75±22.11	1321.32

Table 3
Four extractions (4×25 ml) of the same sample (0.5 g) using 50% EtOH at 60 °C for 10 min (nd—not detected)

Extraction	Daidzin	Glycitin	Genistin	Malonyl genistin	Total
1	196.11	72.65	368.28	546.59	1183.63
2	28.47	8.72	31.61	85.30	154.10
3	nd	nd	5.75	13.29	19.04
4	nd	nd	nd	nd	nd
Total	224.58	81.37	405.64	645.18	1356.77

10 min, it is possible to extract a large proportion of the isoflavones (80–90%) present in a soy flour sample. If quantitative extractions are the objective, extractions of 20 min should be used.

In order to verify the amount of isoflavones present in the sample and to be sure that quantitative extraction are obtained using extractions of 20 min, extractions (4×25 ml) of the same sample (0.5 g), using 50% EtOH at 60 °C for 10 min, were performed (Table 3). The total amount of isoflavones present in the sample obtained with extractions of 20 min (1353.47 μg/g) was comparable to that obtained with extractions using 50% EtOH (1356.77 μg/g). Therefore, isoflavone contents (μg/g) of the soy sample were: daidzin: 216.77±7.67, glycitin: 84.24±3.38, genistin: 433.00±14.32, malonyl genistin: 619.46±23. Isoflavone aglucons were below the detection limits in the soy extracts.

It was also interesting to determine if an ultrasonic bath can give similar extraction efficiency to the probe horn system. For analytical purposes, which require the processing of many small volume samples, an ultrasonic bath may be more desirable than the probe horn for the extraction. The ultrasonic bath is more widely available and many samples can be processed at the same time, in contrast to only one at a time with the probe horn. Also, sonication with the bath is non-intrusive to the sample, which will eliminate possible contamination and loss of the extract [16].

The yields (μg/g) of individual and total isoflavones obtained using the ultrasonic probe and bath are shown in Table 4. Using 50% EtOH at 60 °C, there was little or no difference in the extraction efficiency. Using 50%, MeOH the difference between the ultrasonic probe and bath was more evident, especially for malonyl genistin. Although some differences were observed, both systems can be used on the analytical scale.

To evaluate the repeatability of the extraction procedure, a series of five replicated extractions were performed. The results obtained [mean (μg/g)±SD] for daidzin (184.27±7.36), glycitin (66.89±3.14), genistin (381.16±6.28) and malonyl genistin (552.80±9.69) revealed RSDs lower than 5%.

4. Conclusions

UAE was found to be fast and reliable, and gave better results than the mix-stirring technique for the extraction of isoflavone glucosides. Ethanol (50%) seems to be the best choice to extract these compounds from ground soybeans, for its high efficiency,

Table 4
Yields (μg/g) of individual and total isoflavones obtained using an ultrasonic probe and an ultrasonic bath

		Daidzin	Glycitin	Genistin	Malonyl genistin	Total
Probe	50% EtOH	194.72±3.66	72.58±0.43	369.00±8.41	550.45±14.57	1186.75
	50% MeOH	194.34±8.29	59.39±6.18	349.24±11.33	551.63±16.19	1154.60
Bath	50% EtOH	193.69±12.22	70.37±3.58	373.78±17.65	516.47±36.95	1154.31
	50% MeOH	183.74±1.43	58.90±1.34	326.35±1.18	493.38±5.17	1062.37

low cost, low toxicity and environmental compatibility. Large amounts of isoflavones are extracted in 10 min at 60 °C and quantitative recoveries are obtained after 20 min. An ultrasonic bath can be used as an alternative to the probe horn since it gave similar results.

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