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Microwave assisted extraction of soy isoflavones

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Abstract

A fast and reliable analytical method using microwave assisted extraction has been developed. Several extraction solvents (methanol (MeOH) and ethanol (EtOH), 30–70% in water and water), temperatures (50–150 °C), extraction solvent volume, as well as the sample size (1.0–0.1 g) and extraction time (5–30 min) were studied for the optimization of the extraction protocol. The optimized extraction conditions for quantitative recoveries were: 0.5 g of sample, 50 °C, 20 min and 50% ethanol as extracting solvent. No degradation of the isoflavones was observed using the developed extraction protocol and a high reproducibility was achieved (>95%).

Keywords: Microwave assisted extraction; Soybeans; Isoflavones

1. Introduction

Isoflavones are a subclass of flavanoids also denominated phytoestrogens. Isoflavones are attracting a lot of attention since several studies have demonstrated, both in vitro and in vivo, effects consistent with supposed health effects of soybean consumption, like estrogenic/antiestrogenic activity, antiproliferation, induction of cell-cycle arrest and apoptosis, prevention of oxidation, regulation of the host immune system, and changes in cellular signaling [1–4]. There are 12 main isoflavones in soybeans: genistin, daidzin, glycitin and their respective acetyl, malonyl and aglycone forms (Fig. 1). In soybeans, the conjugates of genistein, daidzein and glycitein are found in an approximate ratio of 6:3:1, respectively, although the isoflavone content is influenced by genetics, crop year, growth location, among other factors [5,6].

The analysis of soy isoflavones is usually performed by extracting the sample with aqueous organic solvents (methanol, ethanol or acetonitrile) and analyzing the extract by high performance liquid chromatography (HPLC) UV–vis detection [7,8]. For the extraction of isoflavones several extraction methods have been used, ranging from the classical soxhlet extraction [9] and magnetic stirring [10,11] to more modern sample preparation techniques, like supercritical fluid extraction, pressurized liquid extraction, solid phase extraction and ultrasound assisted extraction [12–18]. Using this last technique a total recovery of twelve main isoflavones in soybeans were achieved in 20 min [18].

Microwave-assisted extraction (MAE) has been used as an alternative to conventional methods in the extraction of organic compounds from plant materials and foods. It is based upon the selective and rapid localized heating of moisture in the sample by microwaves. Due to the localized heating, pressure builds up within the cells of the sample, leading to a fast transfer of the compounds from the cells into the extracting solvent, usually transparent to microwaves, then not heated by them. Additionally, by using closed vessels the extraction can be performed at elevated temperatures accelerating the mass transfer of target compounds from the sample matrix. Therefore, MAE can reduce solvent amount and/or enhance extraction efficiency [19,20]. Moreover, MAE can be applied to several samples simultaneously, therefore time extraction can be also reduced dramatically.

MAE is frequently applied for trace analysis of organic compounds in solid and liquid samples [20]. It has also been applied for the extraction of natural compounds from foodstuffs like flavanoids (puerarin from Radix puerariae), polyphenols compounds from tea, and from grape seeds and caffeine [21–23]. However, extraction of isoflavones from soybeans has not yet been reported. Therefore, the objective of this work was to develop a fast and reliable analytical protocol for the extraction of soy isoflavones using microwaves. The stability of isoflavones

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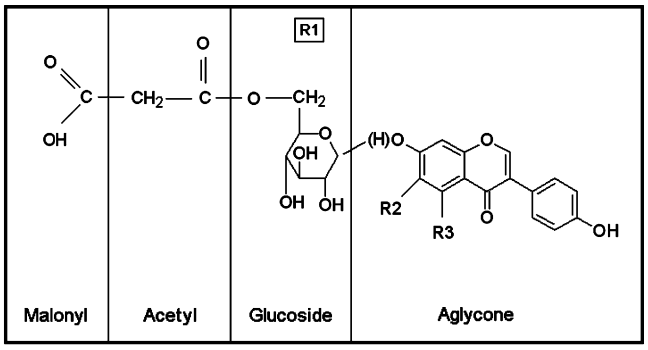

Isoflavone	Symbol	R1	R2	R3
Genistein	Ge	H	H	OH
Daidzein	De	H	H	H
Glycitein	Gle	H	OCH_3	H
Genistin	Gi	$C_6O_5H_{11}$	H	OH
Daidzin	Di	$C_6O_5H_{11}$	H	H
Glycitin	Gly	$C_6O_5H_{11}$	OCH_3	H
Acetyl-genistin	AGi	$C_6O_5H_{11}$+$COCH_3$	H	OH
Acetyl-daidzin	ADi	$C_6O_5H_{11}$+$COCH_3$	H	H
Acetyl-glycitin	AGly	$C_6O_5H_{11}$+$COCH_3$	OCH_3	H
Malonyl-genistin	MGi	$C_6O_5H_{11}$+$COCH_2COOH$	H	OH
Malonyl-daidzin	MDi	$C_6O_5H_{11}$+$COCH_2COOH$	H	H
Malonyl-glycitin	MGly	$C_6O_5H_{11}$+$COCH_2COOH$	OCH_3	H

Fig. 1. Isoflavone chemical structure and abbreviations.

during microwave assisted extraction was also evaluated during the method development to ensure that the isoflavone profile of the sample is not affected by degradation.

2. Material and methods

2.1. Chemicals and solvents

Methanol (Merck, Darmstadt, Germany), and ethanol (Panreac, Barcelona, Spain) used were HPLC grade. Ultra pure water was supplied by a Mili-Q water purifier system from Millipore (Bedford, MA, USA). Isoflavones were purchased from LC Labs (Woburn, MA, USA) and stored at −32 °C. Purity of isoflavone glucosides and aglycones was higher than 99%, and purity of malonyl and acetyl glucosides was higher than 98%. Stock solutions were prepared in 80% methanol in water (v/v) and stored at −32 °C. 2,5-dihydroxybenzaldehyde was used as internal standard for volume correction. It was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample

Soybeans were ground in a coffee grinder, freeze-dried and stored at −32 °C until used as sample.

2.3. Ultrasound assisted extraction

In order to develop the MAE method the isoflavone concentration in the soy sample was determined ($n = 6$) by a reference method based on ultrasound assisted extraction (UAE) [14]. The extractions were carried out on a high intensity ultrasonic probe system of 200 W and 24 kHz (Dr. Hielscher, model UP 200S, Teltow, Germany) equipped with a 2 mm microtip. The extraction protocol consists of 0.1 g of sample extracted by 25 mL of 50% EtOH at 60 °C during 20 min at full ultrasonic power (100% of nominal power) applying one cycle s^{-1}. The concentration (mg g^{-1}) of malonyl daidzin (MDi), malonyl glycitin (MGly), malonyl genistin (MGi), acetyl daidzin (ADi), acetyl glycitin (AGly), acetyl genistin (AGi), daidzin (Di), glycitin (Gly), genistin (Gi), daidzein (De), glycitein (Gle) and genistein (Ge) are 0.44, 0.20, 0.84, 0.30, 0.17, 0.60, 0.56, 0.27, 1.04, 0.28, 0.16 and 0.52, respectively. The standard deviations for all isoflavones were below 5%.

2.4. Microwave assisted extraction

The development of the MAE method was performed on a microwave extractor ETHOS 1600 (Milestone, Sorisole, Italy) Extractions were performed at 500 W using magnetic stirring at 50% of nominal power, using four vessels in a batch (one reference with the temperature probe and three samples). Two different solvent systems (EtOH or MeOH, with several water percentages (30–70%) and temperatures (50–150 °C) were evaluated for the extraction of soy isoflavones. The initial extraction protocol used 0.5 g of 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 internal standard was used to correct the extraction volume. The extracts were then filtered through a 0.45 μm nylon syringe filter (Millex-HN, Millipore, Bedford, MA, USA) before chromatographic analysis.

2.5. Stability of isoflavones during MAE

In order to evaluate the performance of different extraction conditions with accuracy, stability of the isoflavones during the extraction was determined prior the method development. The stability study was performed using a standardized extract obtained by solid–liquid extraction of ground soybeans on an ultrasonic bath of 360 W (J.P. Selecta, Barcelona, Spain) using an adapted extraction protocol based on the reference UAE method [15]. The analytical protocol was basically scaled up to obtain a large amount of extract and consists of extracting approximately 10 g of ground soybeans in 250 mL of 50% ethanol for 30 min at 60 °C. Four extractions using this protocol provided 1 L of soy extract. The extract was centrifuged for 10 min, filtered through filter paper and stored at −32 °C. The standardized UAE extract was analyzed ($n = 6$) daily and all peak areas compared. The concentration (mg L^{-1}) of MDi, MGly, MGi, ADi, AGly, AGi, Di, Gly, Gi, De, Gle and Ge were 2.38, 1.34, 4.42, 2.36, 1.33, 4.39, 2.48, 1.36, 4.57, 2.34, 1.32 and

4.35, respectively. Within the time frame of the work variation of peak areas of all isoflavones remained lower than 2%.

2.6. High-performance liquid chromatography

The HPLC–UV analysis was carried out on a Dionex system (Dionex, Sunnyvale, CA, USA), consisting of an autosampler (ASI 100), pump (P680), chromatographic oven (TCC-100) and a photodiode array detector (PAD100). Isoflavones were separated on one monolithic type column (Chromolith TH Performance RP-18e, 4.6 mm, 100 mm, Merck) using a mobile phase of acidified water (0.1% acetic acid) (solvent A) and acidified methanol (0.1% acetic acid) (solvent B) with a flow-rate of 3.0 mL min^{-1}. The gradient was as follows: 0 min, 20% B; 3 min, 35% B; 8 min, 35% B; 11 min, 40% B and 15 min, 100% B. UV absorbance was monitored from 200 to 400 nm. Injection volume was 10 μL. Samples were filtered through a 0.45 μm syringe filter (Millipore) before injection. The software for control of equipment and data acquisition was Chromeleon version 6.60.

Identification of isoflavones was achieved by comparison of retention times and UV spectra of separated compounds with authentic standards. Quantification was carried out by integration of the peak areas at 254 nm using the external standardization method. Response was linear between 0.1 and 100 mg L^{-1} (six points curve) for all isoflavones and regression coefficients (r^2) were higher than 0.9998. Quantification limits were calculated using ALAMIN software [24]. Quantification limits (mg L^{-1}) of MDi, MGly, MGi, ADi, AGly, AGi, Di, Gly, Gi, De, Gle and Ge were 1.8, 1.9, 1.8, 1.6, 1.6, 1.9, 1.6, 1.9, 1.9, 1.5, 2.1 and 2.0, respectively.

2.7. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and Duncan's multiple range test (MRT) for comparing means. The ANOVA was performed using Excel XP software (Microsoft Co., Redmond, WA, USA) inbuilt features and the MRT, using a calculation table created with the same software.

3. Results and discussion

3.1. Isoflavone stability

Based on previous studies [12,14,15], the selected temperature range is between 50 and 150 °C. On the other hand, extraction time when handling large number of samples should be inferior to 30 min in order to laboratories to be able to process a large number of samples. When using high temperatures it is advisable to access the stability of target compounds, and in the case of isoflavones, especially the malonyl forms. Therefore, the first aspect to be evaluated was the isoflavone stability under extraction conditions for 30 min using different temperatures. This will allow the selection of an adequate extraction temperature for an analytical method that improves extraction efficiency without affecting the isoflavone profile on the sample.

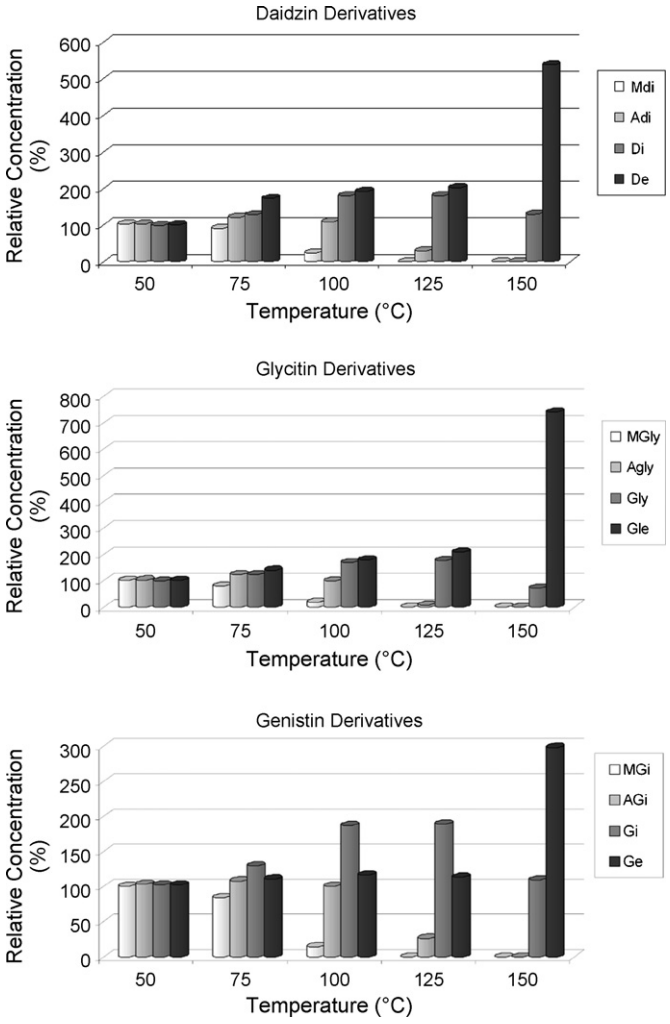

Fig. 2. Stability of isoflavone derivatives during extraction at different temperatures. Extraction conditions: 25 mL of sample, 500 W, magnetic stirring 50% of nominal power, 30 min.

The individual relative concentration of isoflavones in the standardized extract (obtained previously by UAE) submitted to different temperatures under extraction conditions for 30 min is shown in Fig. 2. The values are relative to the initial isoflavone concentration in the standardized extract (100%). Extraction temperature has a clear effect on isoflavone concentration and can be divided in parts. Extractions performed at 50 °C do not affect isoflavone concentration on the extract, whilst extractions performed at higher temperatures expose isoflavones to degradation. Extractions performed between 75 and 100 °C affects mainly malonyl isoflavones; between 100 and 125 °C also affects acetyl isoflavones and higher temperatures sharply increase degradation of glucosides. There is no evident degradation of aglycones in the temperature range essayed. From the results we can infer that 50 °C is a safe temperature used for the development of a reliable extraction method.

The information obtained about the stability of isoflavones at different temperatures is very interesting and can be used for the development of selective hydrolytic extraction methods with the

Table 1
Amount of isoflavones extracted using different solvents ($n = 3$)

Isoflavone	Recovery (% ± R.S.D.)				
	Extraction solvent				
	MeOH	50% MeOH	EtOH	50% EtOH	Water
MDi	51.5 ± 2.5^{c}	76.0 ± 2.1^{a}	1.9 ± 1.4^{e}	71.0 ± 2.4^{b}	40.1 ± 2.4^{d}
MGly	45.3 ± 2.0^{b}	73.7 ± 2.0^{a}	2.9 ± 2.1^{c}	76.2 ± 2.3^{a}	45.1 ± 2.9^{b}
MGi	46.7 ± 2.5^{c}	71.8 ± 3.0^{b}	1.6 ± 2.7^{e}	77.0 ± 2.2^{a}	22.7 ± 3.2^{d}
ADi	72.0 ± 2.6^{b}	74.2 ± 2.4^{b}	19.0 ± 2.6^{d}	86.3 ± 2.4^{a}	31.0 ± 2.7^{c}
AGly	99.9 ± 2.4^{a}	83.4 ± 2.7^{b}	26.1 ± 2.3^{d}	87.0 ± 2.4^{b}	43.3 ± 2.6^{c}
AGi	82.5 ± 2.3^{b}	70.1 ± 2.8^{c}	23.2 ± 2.7^{d}	87.2 ± 2.3^{a}	–
Di	66.3 ± 2.2^{b}	75.7 ± 2.5^{a}	15.9 ± 2.0^{d}	74.2 ± 2.2^{a}	36.2 ± 2.7^{c}
Gly	66.7 ± 3.2^{b}	65.3 ± 2.2^{b}	15.4 ± 2.7^{d}	73.1 ± 2.1^{a}	41.8 ± 3.1^{c}
Gi	66.3 ± 3.8^{ab}	64.1 ± 2.4^{b}	16.1 ± 2.5^{c}	71.8 ± 2.4^{a}	12.5 ± 3.5^{c}
De	73.7 ± 3.0^{a}	35.5 ± 2.9^{b}	23.8 ± 3.1^{c}	70.7 ± 2.3^{a}	–
Gle	79.2 ± 2.0^{b}	101.7 ± 3.1^{a}	40.4 ± 2.1^{c}	103.1 ± 2.1^{a}	44.4 ± 3.1^{c}
Ge	85.1 ± 2.3^{a}	13.7 ± 2.6^{d}	30.7 ± 2.6^{c}	70.1 ± 2.3^{b}	–
Total	63.0 ± 2.7^{b}	68.0 ± 2.5^{b}	12.9 ± 2.4^{d}	75.2 ± 2.3^{a}	23.1 ± 2.9^{c}

Extraction conditions: 0.5 g of sample, 25 mL of extracting solvent, 50 °C, 500 W, magnetic stirring 50% of nominal power, 10 min. Means followed by different superscripts are statistically different ($P < 0.05$).

aim of obtaining extracts on which specific types of isoflavones predominate.

3.2. Solvent selection

Initial extractions performed to determine the best solvent choice were carried out using 0.5 g of sample and 25 mL of solvent at 50 °C for 10 min. The essayed solvents were: methanol, 50% methanol in water (v/v), ethanol, 50% ethanol in water (v/v) and pure water. The relative amount of each isoflavone extracted with the different essayed solvents is shown in Table 1. The values are relative to the isoflavone concentration in the sample determined by the reference method (100%).

The solvent which extracted the highest amount of isoflavones was 50% EtOH, followed by 50% MeOH. Within pure solvents essayed, MeOH stood out and extracted high amounts of isoflavones, with no significant difference on total isoflavones extracted by 50% MeOH. Water and EtOH were the solvents which extracted the lowest amounts of isoflavones. EtOH extracted very low amounts of every isoflavones reaching approximately only 13% of the total isoflavone content of the sample. Methanol was the solvent which extracted the highest amount of AGly and Ge. 50% MeOH was the most efficient solvent for MDi, while 50% EtOH extracted the highest amount of MGi, ADi, AGi and Gly. There was no difference between 50% MeOH and 50% EtOH for the extraction of MGly, Di and Gle. MeOH and 50% EtOH were the best solvents for the extraction of Gi and De.

The use of 50% EtOH has several advantages to the use of 50% MeOH or pure MeOH, like higher extraction efficiency, environmental compatibility and lower toxicity and cost, suggesting the use of this solvent in the extraction method being developed. However, it is still needed to check if using different water percentages in ethanol (30–70%) is possible to increase extraction efficiency. Therefore, extractions were carried out using different water percentages in ethanol using the same previous conditions. The relative amount of each isoflavone extracted using different water percentages on the solvent are shown in Table 2.

It can be observed that a clear effect on extraction efficiency of most isoflavone derivatives depends on the amount of water in the extraction solvent. For the malonyl derivatives, a general trend of higher extraction efficiency with higher water content (between 50 and 70%) in the extraction solvent can be observed. In most cases, water content lower than 50% significantly decreased extraction efficiency.

Regarding the acetyl derivatives, the extraction solvent water content has an inverse effect on extraction efficiency, i.e. significantly higher extraction efficiency is achieved with low water content (≤50%). There are, however, differences depending on the isoflavone. For ADi and AGly, most effective extraction solvents have intermediate water content (50–60%) while for AGi require lower water content (<40%) to achieve better results. A similar trend was observed for aglycone derivatives. For De and Ge, very low amounts of water (<30%) were necessary to achieve the best recoveries, while for Gle intermediate amounts of water (50%) were necessary.

In contrast, the amount of water on the extraction solvent does not significantly affect the extraction of the main glucosidic isoflavones (Di and Gi) and only low amounts of water (70% EtOH) decreased extraction efficiency of Gly. Therefore, it is clear that for most isoflavones the main extraction variable is the polarity of the extraction solvent, i.e. extraction efficiency for most isoflavones is linked to its polarity and solubility on the extraction solvent.

Since differences on the total amount of isoflavones extracted were not significant ($P < 0.05$), the selection of the best solvent was based on the overall extraction efficiency for isoflavones individually. To balance the effectiveness of the essayed solvents for the extraction of all isoflavones, grades were assigned to the classification based on the amount extracted. Grades of 1.0, 0.75, 0.5, 0.25 and 0.1 were assigned to solvent classification a, b, c,

Table 2
Effect of the extraction solvent water percentage on isoflavone extraction ($n = 3$)

Isoflavone	Recovery (% ± R.S.D.)				
	Extraction solvent				
	30% EtOH	40% EtOH	50% EtOH	60% EtOH	70% EtOH
MDi	77.0 ± 2.3^{a}	72.9 ± 2.3^{ab}	71.0 ± 2.4^{b}	67.2 ± 2.4^{b}	62.2 ± 2.3^{c}
MGly	79.4 ± 2.4^{a}	77.3 ± 2.4^{a}	76.2 ± 2.3^{a}	66.9 ± 2.3^{b}	58.0 ± 2.4^{c}
MGi	73.2 ± 2.2^{a}	76.3 ± 2.1^{a}	77.0 ± 2.2^{a}	69.5 ± 2.2^{b}	67.8 ± 2.2^{b}
ADi	73.5 ± 2.3^{d}	81.0 ± 2.4^{c}	86.3 ± 2.4^{b}	91.5 ± 2.3^{a}	80.6 ± 2.3^{c}
AGly	38.0 ± 2.3^{d}	58.2 ± 2.4^{b}	87.0 ± 2.4^{a}	58.6 ± 2.3^{b}	43.2 ± 2.3^{c}
AGi	64.8 ± 2.2^{d}	83.0 ± 2.4^{c}	87.2 ± 2.3^{c}	96.4 ± 2.3^{b}	102.9 ± 2.2^{a}
Di	74.8 ± 2.3^{ns}	72.7 ± 2.3^{ns}	74.2 ± 2.2^{ns}	70.6 ± 2.2^{ns}	68.5 ± 2.2^{ns}
Gly	73.9 ± 2.1^{a}	71.8 ± 2.2^{a}	73.1 ± 2.1^{a}	68.8 ± 2.2^{a}	58.5 ± 2.2^{b}
Gi	68.2 ± 2.4^{ns}	73.3 ± 2.4^{ns}	71.8 ± 2.4^{ns}	71.0 ± 2.4^{ns}	70.1 ± 2.4^{ns}
De	32.4 ± 2.4^{d}	62.5 ± 2.3^{c}	70.7 ± 2.3^{b}	74.3 ± 2.4^{b}	79.0 ± 2.31^{a}
Gle	83.7 ± 2.0^{c}	97.1 ± 2.1^{b}	103.1 ± 2.1^{a}	57.7 ± 2.2^{d}	48.2 ± 2.1^{e}
Ge	28.6 ± 2.2^{d}	45.4 ± 2.3^{c}	70.1 ± 2.3^{b}	72.2 ± 2.2^{b}	76.8 ± 2.2^{a}
Total	69.4 ± 2.3^{ns}	73.6 ± 2.4^{ns}	75.2 ± 2.3^{ns}	72.3 ± 2.3^{ns}	70.2 ± 2.2^{ns}
EF	5.6	6.75	8.5	6.0	4.35

Extraction conditions: 0.5 g of sample, 25 mL of extracting solvent, 50 °C, 500 W, magnetic stirring 50% of nominal power, 10 min. Means followed by different superscripts are statistically different ($P < 0.05$). EF: effectiveness factor.

d and e, respectively. Using these grades, an effectiveness factor (EF) is calculated by adding the grades of effectiveness of all isoflavones in each solvent. For example, the EF of EtOH is 8.5 ($0.75 + 1 + 1 + 0.75 + 1 + 0.5 + 0 + 1 + 0 + 0.75 + 1 + 0.75 = 8.5$). The EF has been used for the selection of the best overall solvent. The results are also shown in Table 2. As can be seen, the highest effectiveness factor was obtained by 50% EtOH (8.5), followed by 40% (6.75), 60% EtOH (6.0), 30% EtOH (5.6) and 70% (4.35). Therefore, it can be assumed that 50% is the best water:ethanol proportion for the extraction of isoflavones using microwaves, since it achieves the highest overall effectiveness for all derivatives. Based on the results of total isoflavones extracted, the differences observed between isoflavone extractability, which is dependent on the water content of the solvent and on the EF, 50% EtOH will be used as the extracting solvent for further optimization of extraction conditions.

3.3. Solvent volume

To evaluate the effect of the sample volume on extraction efficiency of isoflavones from soybeans, a series of extraction using the same sample mass (0.5 g) and different solvent volumes (15–35 mL) were carried out at 50 °C for 10 min. The extracted amount of each isoflavone is presented in Table 3. For most isoflavone derivatives, and therefore total isoflavones,

Table 3
Effect of the extraction solvent volume on isoflavone extraction ($n = 3$)

Isoflavone	Recovery (% ± R.S.D.)				
	Solvent volume (mL)				
	15	20	25	30	35
MDi	61.4 ± 2.2^{b}	70.0 ± 2.2^{a}	71.0 ± 2.4^{a}	74.0 ± 2.6^{a}	75.8 ± 3.06^{a}
MGly	59.6 ± 2.0^{d}	70.7 ± 2.1^{b}	76.2 ± 2.3^{a}	68.9 ± 2.7^{bc}	64.5 ± 3.0^{cd}
MGi	72.8 ± 2.0^{ns}	74.8 ± 2.1^{ns}	77.0 ± 2.2^{ns}	76.5 ± 2.5^{ns}	72.2 ± 2.9^{ns}
ADi	63.4 ± 2.1^{bc}	67.8 ± 2.2^{bc}	87.2 ± 2.3^{a}	62.3 ± 2.7^{bc}	57.6 ± 3.3^{c}
AGly	20.3 ± 2.1^{e}	49.9 ± 2.2^{b}	86.3 ± 2.4^{a}	40.6 ± 2.7^{c}	28.0 ± 3.3^{d}
AGi	61.1 ± 2.0^{bc}	64.5 ± 2.1^{b}	87.0 ± 2.4^{a}	66.0 ± 2.7^{b}	58.2 ± 3.5^{c}
Di	70.5 ± 2.0^{bc}	71.3 ± 2.1^{bc}	74.2 ± 2.2^{a}	69.5 ± 2.5^{bc}	65.7 ± 3.0^{c}
Gly	63.6 ± 2.1^{b}	66.9 ± 2.0^{b}	73.1 ± 2.1^{a}	65.6 ± 2.7^{b}	66.8 ± 3.1^{b}
Gi	65.4 ± 2.1^{ab}	67.2 ± 2.2^{ab}	71.8 ± 2.4^{a}	67.6 ± 2.7^{b}	63.7 ± 3.0^{b}
De	82.3 ± 2.1^{a}	85.2 ± 2.2^{a}	70.7 ± 2.3^{b}	69.0 ± 2.6^{b}	67.4 ± 3.1^{b}
Gle	32.1 ± 2.0^{d}	63.6 ± 2.1^{b}	103.1 ± 2.1^{a}	43.3 ± 2.6^{c}	25.4 ± 3.4^{e}
Ge	63.2 ± 2.0^{b}	$65.3 \pm 2.1b$	70.1 ± 2.3^{b}	83.8 ± 2.6^{a}	65.7 ± 3.5^{a}
Total	66.1 ± 2.3^{b}	69.6 ± 2.3^{ab}	75.2 ± 2.3^{a}	70.0 ± 2.8^{ab}	66.0 ± 3.3^{b}
EF	7.1	9.0	10.5	8.5	6.4

Extraction conditions: 0.5 g of sample, 50% EtOH, 50 °C, 500 W, magnetic stirring 50% of nominal power, 10 min. Means followed by different superscripts are statistically different ($P < 0.05$). EF: effectiveness factor.

highest extraction efficiency is achieved using between 20 and 30 mL of solvent. As can be seen, the total amount of isoflavones extracted increased with the increase on solvent volume from 15 to 20 mL. No significant difference was observed between 20, 25 and 30 mL of extracting solvent and higher solvent volumes extracted lower amounts of total isoflavones.

Since there was no significant difference between 20, 25 and 30 mL, the EF was calculated and is presented in Table 3. When comparing the effectiveness of each of these solvent volumes on individual recovery of each isoflavone, it is clear that 25 mL is the most efficient volume for most isoflavones (EF = 10.5), followed by 20 mL (EF = 9.0) and 30 mL (EF = 8.5). Based on these observations, 25 mL is the most adequate solvent volume using samples of 0.5 g, and therefore will be used for further optimization of extraction conditions.

3.4. Sample amount

Once the solvent volume has been optimized, sample amount can be optimized to improve extraction efficiency. However, the effect of the sample mass:solvent volume ratio can affect the microwave assisted extraction differently than it would affect other extraction techniques, and therefore not only smaller samples, but also larger sample amounts than the one being used till now (0.5 g) were evaluated. The effect of sample size on extraction efficiency of each isoflavone derivative and on total isoflavone is shown in Table 4.

The sample amount has a clear effect on the extraction efficiency which increases approximately 20% with the increase of the sample mass from 0.1 to 0.5 g. For most isoflavones, best extraction efficiency is achieved using samples of 0.5 g and larger samples do not improve extraction of total isoflavones ($P < 0.05$) maintaining similar extraction efficiencies. There are, however, significant differences on the extraction of individual isoflavones depending on the sample size.

For malonyl derivatives, extraction efficiency is maximized with samples larger than 0.25 g, and in the case of MGly larger than 0.5 g. A similar trend is observed for glucosidic derivatives, which are more easily extracted using samples larger than 0.5 g. On the other hand, acetyl derivatives are more sensible to the sample size and are better extracted using samples of 0.5 g. Higher or lower sample amounts negatively affected extraction efficiency of these derivatives. Regarding aglycone derivatives, a variable behavior was observed and the best sample size will depend on the derivative. While there is no significant difference in the extraction for De in the sample range essayed, Gle is better extracted using smaller samples (<0.5 g) and Ge is better extracted using larger samples (>0.5 g). The inconvenience of smaller samples (i.e. 0.1 g) is that concentration of some isoflavones (AGly and Gle) does not allow its detection and also increased the standard deviation. Samples of 0.1 g produced more than the double of the standard deviation observed for samples of 0.5 g or larger. Samples larger than 0.5 g did not seriously affect standard deviation.

The observed results with the extractions using different sample amounts indicates that a sample:solvent ratio of 0.5 g:25 mL is an adequate ratio since it maximizes the extraction efficiency keeping that sample as small as possible. However, these are surprising results since previous reports of the best sample mass:solvent volume ratio is 0.1:25 mL [14,15], and an opposite effect of the sample mass on the extraction efficiency was reported. During the optimization of these extraction methods the extraction efficiency improves with lower sample amounts while we have found that MAE extraction efficiency improves with higher sample amounts up to 0.5 g. Guo et al. [21] observed similar trend on the extraction of puerarin (another isoflavone) from pueraria radix. Better results were obtained with sample mass:solvent ratio of 1:5 while increasing solvent volume to ratios of 1:50 negatively affected extraction efficiency. However, this effect is not exclusive to isoflavones. Mattina et

Table 4
Effect of sample amount on isoflavone extraction ($n = 3$)

Isoflavones	Recovery (% ± R.S.D.)				
	Sample amount (g)				
	0.1	0.25	0.5	0.75	1.0
MDi	43.0 ± 4.6^{b}	68.9 ± 3.7^{a}	71.0 ± 2.4^{a}	74.7 ± 2.2^{a}	74.2 ± 2.0^{a}
MGly	23.7 ± 4.1^{c}	47.6 ± 3.4^{b}	76.2 ± 2.3^{a}	77.4 ± 2.2^{a}	72.3 ± 2.1^{a}
MGi	59.7 ± 4.7^{b}	70.5 ± 3.3^{a}	75.0 ± 2.2^{a}	74.7 ± 2.1^{a}	74.8 ± 2.1^{a}
ADi	48.4 ± 4.5^{d}	54.7 ± 3.1^{c}	86.3 ± 2.4^{a}	68.1 ± 2.2^{b}	60.1 ± 2.1^{c}
AGly	–	37.7 ± 3.5^{b}	87.0 ± 2.4^{a}	23.3 ± 2.1^{c}	10.4 ± 2.0^{d}
AGi	53.0 ± 4.4^{d}	63.1 ± 3.4^{c}	87.2 ± 2.3^{a}	73.5 ± 2.1^{b}	67.9 ± 2.0^{bc}
Di	64.0 ± 4.1^{b}	66.4 ± 3.3^{ab}	74.2 ± 2.2^{a}	71.4 ± 2.0^{ab}	68.4 ± 2.1^{ab}
Gly	50.1 ± 4.9^{b}	54.8 ± 3.1^{b}	73.1 ± 2.1^{a}	72.7 ± 2.2^{a}	75.9 ± 2.0^{a}
Gi	59.1 ± 4.8^{c}	65.6 ± 3.1^{b}	71.8 ± 2.4^{ab}	71.3 ± 2.1^{ab}	75.0 ± 2.1^{a}
De	65.6 ± 4.7^{ns}	67.7 ± 3.1^{ns}	70.7 ± 2.3^{ns}	69.2 ± 2.2^{ns}	68.8 ± 2.1^{ns}
Gle	–	103.7 ± 3.3^{a}	103.1 ± 2.1^{a}	28.9 ± 2.0^{b}	12.3 ± 2.0^{c}
Ge	53.9 ± 4.8^{c}	67.0 ± 3.7^{b}	70.1 ± 2.3^{b}	97.4 ± 2.0^{a}	101.8 ± 2.0^{a}
Total	55.0 ± 5.0^{c}	65.6 ± 3.5^{b}	75.2 ± 2.3^{a}	72.6 ± 2.2^{ab}	72.4 ± 2.1^{ab}

Extraction conditions: 25 mL of 50% EtOH, 50 °C, 500 W, magnetic stirring 50% of nominal power, 10 min. Means followed by different superscripts are statistically different ($P < 0.05$).

Table 5
Amount of isoflavones extracted using different extraction times ($n = 3$)

Isoflavone	Recovery (% ± R.S.D.)				
	Extraction time (min)				
	10	15	20	25	30
MDi	71.0 ± 2.4^{c}	79.3 ± 2.6^{b}	98.1 ± 2.7^{a}	100.2 ± 2.7^{a}	97.6 ± 3.4^{a}
MGly	76.2 ± 2.3^{c}	85.6 ± 1.3^{b}	102.4 ± 1.4^{a}	102.0 ± 1.5^{a}	101.7 ± 2.0^{a}
MGi	77.0 ± 2.2^{c}	84.8 ± 2.3^{b}	103.0 ± 2.5^{a}	103.7 ± 3.0^{a}	104.9 ± 3.5^{a}
ADi	86.3 ± 2.4^{c}	94.2 ± 2.3^{b}	97.1 ± 2.5^{a}	101.5 ± 2.7^{a}	100.2 ± 2.9^{a}
AGly	87.0 ± 2.4^{b}	101.4 ± 2.4^{a}	102.8 ± 2.6^{a}	102.1 ± 2.8^{a}	101.2 ± 3.1^{a}
AGi	87.2 ± 2.3^{b}	97.4 ± 2.6^{a}	100.6 ± 3.0^{a}	98.4 ± 3.1^{a}	100.0 ± 3.3^{a}
Di	74.2 ± 2.2^{c}	93.7 ± 2.3^{b}	100.9 ± 2.4^{a}	100.5 ± 2.8^{a}	98.8 ± 3.0^{a}
Gly	73.1 ± 2.1^{c}	88.3 ± 2.1^{b}	102.1 ± 2.1^{a}	100.1 ± 2.4^{a}	97.4 ± 2.8^{a}
Gi	71.8 ± 2.4^{c}	87.3 ± 2.4^{b}	102.7 ± 2.4^{a}	102.0 ± 2.8^{a}	103.6 ± 3.3^{a}
De	70.7 ± 2.3^{c}	87.4 ± 2.3^{b}	99.7 ± 1.4^{a}	101.1 ± 1.8^{a}	101.6 ± 2.0^{a}
Gle	103.1 ± 2.1^{ns}	103.0 ± 2.4^{ns}	103.4 ± 2.8^{ns}	102.6 ± 3.0^{ns}	100.9 ± 3.4^{ns}
Ge	70.1 ± 2.3^{b}	98.5 ± 2.4^{a}	100.8 ± 2.8^{a}	99.9 ± 3.0^{a}	99.7 ± 3.4^{a}
Total	75.2 ± 2.3^{c}	88.6 ± 2.4^{b}	101.5 ± 2.5^{a}	101.4 ± 2.7^{a}	101.5 ± 3.1^{a}

Extraction conditions: 0.5 g of sample, 25 mL of 50% EtOH, 50 °C, 500 W, magnetic stirring 50% of nominal power, 10 min. Means followed by different superscripts are statistically different ($P < 0.05$).

al. [25], for example, reported slight increase on the recovery of taxanes by increasing the sample amount from 0.12 to 0.24 g. In contrast, for other compounds, like antrazine, Xiong et al. [26], observed that the sample amount:solvent volume ratio is not an important parameter for recovering this compound from soils.

3.5. Extraction time

To achieve quantitative recoveries, the extraction time was increased from 10 to 15, 20, 25 and 30 min using the optimized conditions until the moment (0.5 g of sample, 25 mL of EtOH 50% at 50 °C). Table 5 presents the total amount of isoflavones extracted using different extraction times. As can be seen, most part of the isoflavones present in the sample are extracted in 10 min (approximately 75%) and increasing extraction time from 10 to 15 min increases 13% of the total amount of isoflavones extracted. A similar increase (12.9%) on total isoflavones extracted was observed with the increase inf the extraction time from 15 to 20 min, where the total amount of isoflavones is compatible with those obtained with reference method. Using longer extraction times (25 and 30 min) achieved the same recovery as with 20 min, indicating the quantitative extraction of isoflavones from the sample.

The effect of extraction time on the amount extracted of all isoflavone derivatives is similar to the one observed for total isoflavones: the amount extracted increases with extraction time until 20 min and longer extractions recovers the same amount as 20 min. However, for some isoflavones (AGly and Gle) quantitative recoveries are achieved in 15 min. This is due, in part, to their low concentration on the sample, favoring the extraction. It can be concluded that quantitative extraction of all isoflavones derivatives is achieved in 20 min, and extractions of 30 min do not affect the results.

3.6. Reproducibility

For the determination of the isoflavone concentration in the sample and to evaluate the method reproducibility, a series of extractions in two consecutive days ($n = 12$) were carried out. A typical chromatogram obtained from the soy sample is shown in Fig. 3. The determined concentration (mg g^{-1}) of MDi, MGly, MGi, ADi, AGly, AGi, Di, Gly, Gi, De, Gle and Ge are 0.43, 0.19, 0.84, 0.29, 0.16, 0.57, 0.56, 0.26, 1.04, 0.27, 0.15 and 0.50, respectively. Mean R.S.D. for determination of all isoflavones using the developed method is 2.43%. It was also observed that AGi has the lowest reproducibility (R.S.D. = 2.99%) and that MGly has the highest (R.S.D. = 1.27%).

Using the standardized UAE extract (Section 2.5) no degradation of the isoflavones in the sample was observed. The mean difference in the total amount of isoflavones determined by the developed method and the reference method is 2.02%. The mean difference between the methods in the determination of Di, Gly and Gi derivatives is 1.74, 5.15 and 2.00%, respectively. The higher difference of Gly derivatives between both methods is possibly due to its lower concentration in the sample and the lower sample amount used in the reference method, or more possibly due to a combination of these two factors.

3.7. Recovery of isoflavones

The recovery of isoflavones added to the sample was determined using the developed method ($n = 3$). One milliliter of a standard mixture containing approximately 50 mg L^{-1} of all isoflavones was added to the sample 1 h before being submitted to extraction conditions. This aging time was performed to allow the standards to interact with the sample matrix. The recoveries (%) obtained for MDi, MGly, MGi, ADi, AGly, AGi, Di, Gly, Gi, De, Gle and Ge were 99.5, 98.7, 99.4, 97.6, 101.6, 104.3, 102.6, 97.8, 103.7, 102.4, 101.5 and 103.6, respectively. Val-

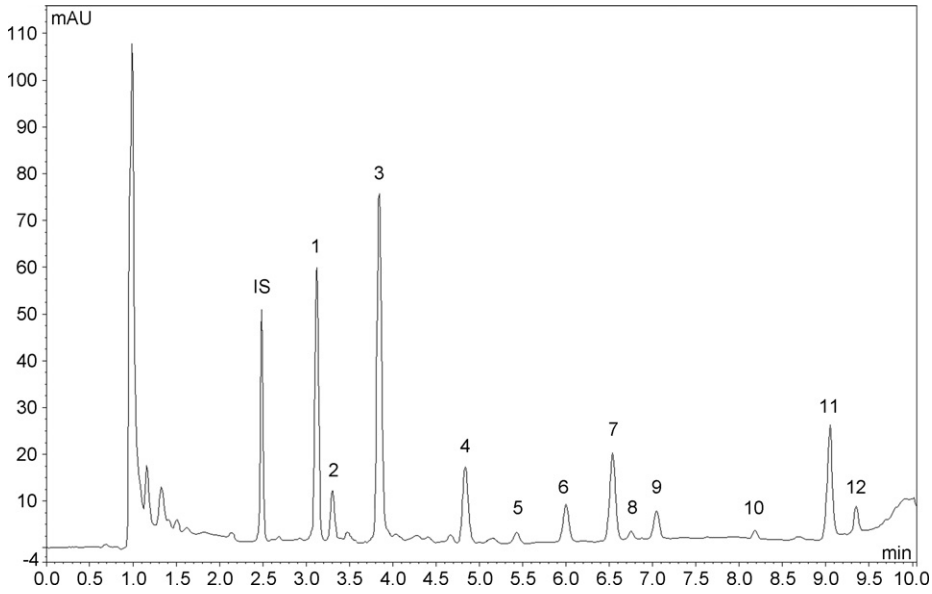

Fig. 3. Typical chromatogram a soy sample extract after optimized extraction. 1: Internal standard, 2: Di, 3: Gly, 4: Gi, 5: MDi, 6: MGly, 7: ADi, 8: AGly, 9: MGi, 10: De, 11: Gle, 12: AGi, 13: Ge. For chromatographic conditions see Section 2.6.

ues are relative to the amount present in the sample determined by the optimized method and the amount added to the sample. Relative standard deviation for all samples was below 5%. Mean isoflavone recovery was 101.1 ± 4.53% and the isoflavone with lower recovery was Adi (97.6%), while the isoflavone with highest recovery was Gly (104.3%).

3.8. Comparison with ultrasound assisted extraction method

The resulting recoveries using the MAE method were compared with those obtained using the previously developed ultrasound assisted extraction method ($n = 12$). The UAE method has been proved as a useful method for routine isoflavone analysis in terms of reproducibility and speed [15]. Table 6 shows the resulting recoveries using both extraction methods. No significant differences were found for any isoflavone. It has to be noted that using the new method, up to 10 samples can be processed simultaneously obtaining the same recovery than using the UAE method.

Table 6
Amount of isoflavones ($\mu g\ g^{-1}$) extracted using the microwave assisted extraction method and the ultrasound assisted extraction method ($n = 6$)

Isoflavone	Extraction method ($\mu g\ g^{-1} \pm$ S.D.)	
	MAE	UAE
MDi	434.3 ± 11.6	439.0 ± 20.9
MGly	194.4 ± 2.4	200.2 ± 9.2
MGi	843.2 ± 24.8	845.1 ± 34.4
ADi	294.9 ± 7.7	302.1 ± 12.45
AGly	164.3 ± 4.8	171.0 ± 7.9
AGi	574.2 ± 17.1	602.2 ± 29.4
Di	563.4 ± 13.5	561.3 ± 16.9
Gly	264.7 ± 5.0	269.4 ± 11.5
Gi	104.3 ± 2.3	104.9 ± 3.9
De	274.3 ± 3.3	279.5 ± 10.8
Gle	154.1 ± 3.8	162.2 ± 6.8
Ge	504.2 ± 12.7	517.3 ± 20.7
Total	5281 ± 126.7	5374 ± 241.8

No significant differences were found for any isoflavone.

4. Conclusions

A fast (20 min) and quantitative method has been developed for the microwave-assisted extraction of isoflavones from soybeans. During the method development, several parameters were evaluated such as the extraction temperature, solvent, solvent volume, sample size and extraction time. The optimized extraction conditions were: 0.5 g of sample extracted by 25 mL of 50% EtOH at 50 °C for 20 min. Using these conditions approximately 75% of total isoflavones are extracted in 10 min and achieved quantitative recoveries in 20 min. Degradation of isoflavones was not detected and high reproducibility was observed (>95%).

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