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Ultrasound-assisted extraction of isoflavones from soy beverages blended with fruit juices

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Abstract

A new method for the fast determination of isoflavones from soy beverages blended with fruit juices without the need of freeze-drying the sample was developed. During the method development, several parameters were studied: solvent (methanol and ethanol), sample:solvent ratio (5:1 to 0.2:1), temperature (10–60 °C) and extraction time (5–30 min). The most important parameter for the extraction of isoflavones from soy drinks was the sample:solvent ratio. The optimized method consists of extracting the sample with ethanol with a sample:solvent ratio of 0.2:1 on an ultrasound bath at 45 °C during 20 min. Also, samples were freeze-dried, extracted using conventional method and compared with the optimized method and no significant difference was observed on total and individual isoflavone concentration. The most representative samples from the Spanish market, with a wide variation of isoflavone concentration were analyzed using the optimized method. Differences between manufacturers reached an almost 10 times fold variation. Overall isoflavone concentration ranged from 6.7 to 58.2 mg L^{-1}.

Keywords: Isoflavones; Fast analysis; Ultrasound-assisted extraction; Soy beverages

1. Introduction

In the last decade, the interest in soybeans and soy-based products has grown significantly due to increasing evidence indicating that consumption of soy-containing foods is associated with protection against cardiovascular disease, reduced incidence of certain cancers and osteoporosis. Researchers have credited phytochemicals in soybeans, especially isoflavones, for some of these beneficial health effects [1–4].

There are 12 main isoflavones in soybeans (Fig. 1); 3 free aglycone isoflavones (genistein, daidzein and glycitein), and their respective glucosidic, malonyl and acetyl glucosidic conjugates [5,6]. Isoflavone content in soy-based foods depends of the specific product, its solids content and processing and storage conditions [7,8]. This implies careful monitoring of processing to maintain isoflavone concentration on standardized levels, which is especially important for products that express isoflavone concentration on the label.

Soy beverages blended with fruit juices are a new generation of soy products and are a convenient way to include soy in the regular diet [9]. In Spain, soy beverages sales are steadily increasing and several new products are available. However, there is no information of isoflavone concentration of most consumed soy beverages blended with fruit juices in Spain. Despite increased consumer acceptance and consumption of soy beverages, the industry is still faced with challenges due to limited analytical methodology for the determination of isoflavones on such products.

The presence of solid parts in soy beverages blended with fruit juices is the main difference between these beverages and other soy-derived drinks. Usually, extraction of isoflavones from solid samples is carried out using solid–liquid extraction techniques, like refluxing, magnetic stirring, ultrasound-assisted extraction, pressurized liquid extraction and supercritical fluid extraction [6,10–14]. Acetonitrile, ethanol and methanol mixed with certain amount of water are the most used solvents. High performance liquid chromatography (HPLC) using reversed-phase C18 stationary matrices, mostly with mixtures of methanol or acetonitrile, has proved to be the method of choice for the analysis of isoflavones [15–17].

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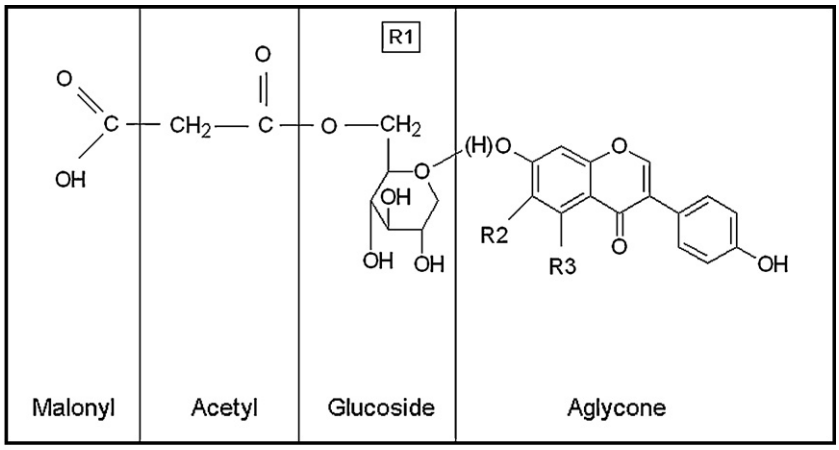

Isoflavone	Symbol	R1	R2	R3
Genistein	Ge	H	H	OH
Daidzein	De	H	H	H
Glycitein	Gle	H	OCH_3	H
Genistin	Gi	$C_6O_5H_{11}$	H	OH
Daidzin	Di	$C_6O_5H_{11}$	H	H
Glycitin	Gly	$C_6O_5H_{11}$	OCH_3	H
Acetyl-genistin	AGi	$C_6O_5H_{11}+COCH_3$	H	OH
Acetyl-daidzin	ADi	$C_6O_5H_{11}+COCH_3$	H	H
Acetyl-glycitin	AGly	$C_6O_5H_{11}+COCH_3$	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. Chemical structures of isoflavones and abbreviations.

Heterogeneous soy beverages are usually freeze-dried and treated as solid samples [18–20]. Freeze-drying is time consuming procedure that can take days and may, as well, increase variations on the determination of isoflavones, due to increased errors and degradation of the sample. Therefore, a fast and standardized analytical method to quantify these compounds in soy beverages without the necessity of freeze-drying the sample has become essential.

An extraction step can be used to extract the isoflavones and avoid freeze-drying the sample since most part of the isoflavones in this type of sample is in the suspended solids. There are already a few studies where the extraction was successfully used for the analysis of isoflavones from soy drinks and milks. Most authors used methanol (MeOH) or ethanol (EtOH) with a sample:solvent ratio ranging from 4:1 to 1.6:1 (v/v) and extraction by refluxing or shaking for 1–4 h [7,10,21,22].

Unfortunately, in these reports, the extraction method was not evaluated, very long extraction times were used or only a few isoflavones were studied. Also, the use of refluxing causes malonyl isoflavones to undergo degradation to the respective glucosides and aglycones, changing the isoflavone profile of the samples and limiting the information obtained.

Therefore, the objective of this work was to develop and evaluate a simple, fast, quantitative and reproducible method for the determination of all main chemical forms of isoflavones in soy beverages blended with fruit juices. The novelty of this work resides in its simplicity and rapidity when treating a troublesome liquid sample without the need of freeze-drying the

sample before extraction and at the same time shortening extraction time, measurement variation and degradation by the use of ultrasounds instead of refluxing or shaking. Also, an exhaustive optimization of the main extraction parameters for all main isoflavones present in the samples provide valuable information and ensure optimal extraction conditions.

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 Milli-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 and was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Samples

Several soy beverages blended with fruit juices were persuaded from a local supermarket and stored closed at 4–5 °C until used as sample. Their commercial information, abbreviations, soy source and amount of soy used are shown in Table 1.

2.3. Ultrasound-assisted extraction

Extractions were carried out on an ultrasonic bath (J.P. Selecta, Barcelona, Spain). The extractions were performed at constant temperature by means of a temperature controller coupled to the ultrasonic bath. The initial extraction protocol consisted of 25 mL of sample:solvent at different proportions (5:1 to 0.2:1) extracted at 10 °C during 10 min.

Several aliquots of the samples were freeze-dried. For the determination of isoflavone concentration of the freeze-dried samples, a reference method based on ultrasound-assisted extraction was used [11]. The extraction protocol consists of 0.25 g of sample extracted by 25 mL of 50% EtOH at 60 °C during 20 min on the ultrasonic bath.

After extraction, 0.5 mL of the internal standard was added to the extracts, which were centrifuged for 10 min. The internal standard was used for the correction of the extraction volume. Also, re-extractions of the samples were carried out to verify the achievement of quantitative recoveries. All samples were filtered through a 0.45 μm nylon syringe filter (Millex-HN, Ireland) before chromatographic analysis. Samples were analyzed within 12 h after extraction and stored at −32 °C to avoid degradation of malonyl isoflavones [23].

2.4. High-performance liquid chromatography

The HPLC-UV analysis was carried out on a Dionex system (Dionex Corp., Sunnyvale, CA, USA), consisting of an autosampler (ASI 100), pump (P680), chromatographic oven (TCC-100) and a photodiode array detector (PAD100). The analysis method was adapted from a previous work [17]. Analysis were performed on a 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. All isoflavones were resolved within 15 min. UV absorbance was monitored from 200 to 400 nm. Injection volume was 10 μL. 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 as well as by co-elution 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. Daily analysis ($n = 3$) of a reference standard mixture revealed a mean intra- and inter-day area and retention time relative standard deviations lower than 3%. A chromatogram of the reference standard mixture is given in Fig. 2.

Detection limits (DL) (mg L^{-1}) for malonyl daidzin (MDi), malonyl glycitin (MGly), malonyl genistin (MGi), acetyl daidzin

Table 1
Characteristics of commercial samples

Commercial brand	Abbreviation	Fruit juice source	Soy source
Hacendado	H-PiO	Pineapple (35%), orange (15%)	Soybeans (3.0%)
Hacendado	H-S	Strawberry	Soybeans (3.0%)
Hacendado	H-Pe	Peach	Soybeans (3.0%)
Juver	J-Pe	Peaches purée (30%), lemon (1%)	Isolated soy protein (0.8%)
Juver	J-Pi	Pineapple purée (30%), lemon (1%)	Isolated soy protein (0.8%)
Vive-Soy	VS-Pe	Concentrated peach juice (11%)	Soybeans (2.7%)
Vive-Soy	VS-Pi	Concentrated pineapple juice (11%)	Soybeans (2.7%)
Vive-Soy	VS-O	Concentrated orange juice (11%)	Soybeans (2.7%)
Don Simón	DS-Pi	Concentrated pineapple juice (11%)	Soybeans (3.0%)
Don Simón	DS-O	Concentrated orange juice (11%)	Soybeans (3.0%)
Don Simón	DS-Pe	Concentrated peach juice (11%)	Soybeans (3.0%)

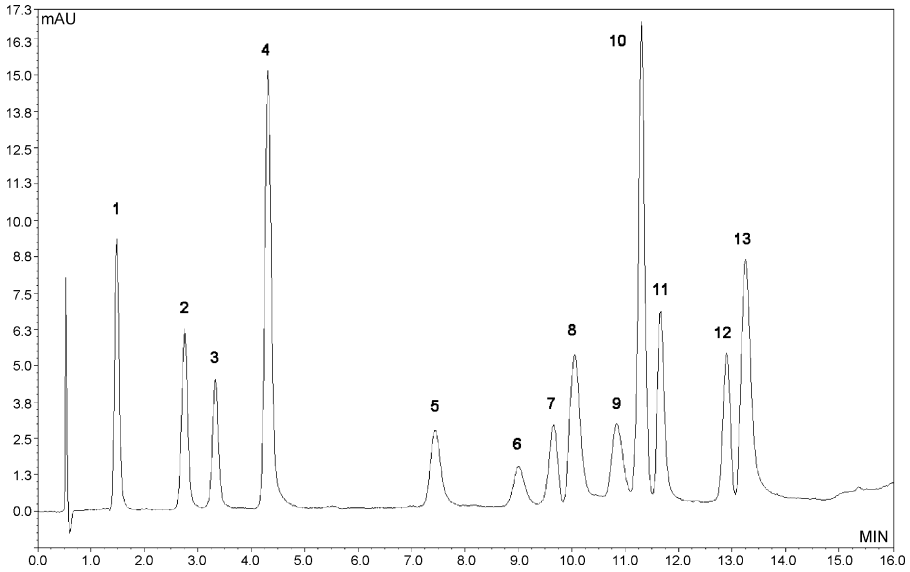

Fig. 2. Chromatogram of the reference standard mixture. (1) Internal standard (2,5-dihydroxybenzaldehyde), (2) Di, (3) Gly, (4) Gi, (5) MDi, (6) MGly, (7) ADi, (8) AGly, (9) MGi, (10) De, (11) Gle, (12) AGi and (13) Ge.

(ADi), acetyl glycitin (AGly), acetyl genistin (AGi), daidzin (Di), glycitin (Gly), genistin (Gi), daidzein (De), glycitein (Gle) and genistein (Ge) were 0.61, 0.62, 0.48, 0.50, 0.44, 0.50, 0.51, 0.50, 0.49, 0.40, 0.36 and 0.40, respectively. Quantification limits (QL) (mg L^{-1}) for MDi, MGly, MGi, ADi, AGly, AGi, Di, Gly, Gi, De, Gle and Ge were 1.83, 1.90, 1.79, 1.60, 1.62, 1.93, 1.59, 1.88, 1.94, 1.48, 2.05 and 1.96, respectively. DL and QL were calculated using ALAMIN software [24].

2.5. Statistical analysis

Results obtained during the method development were analyzed by one-way analysis of variance ($p < 0.05$) 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. For the comparison of the optimized method and the conventional method (freeze-drying the sample) a *t*-test was performed also using Excel XP software inbuilt features.

3. Results and discussion

3.1. Solvent and sample:solvent ratio

In order to determine the best solvent and sample:solvent ratio, several extractions were performed using MeOH and EtOH with different amounts of a random sample (HPi). The sample:solvent ratios ranged from 5:1 to 0.2:1 (v/v). The results are shown in Table 2.

For both MeOH and EtOH, gradually reducing the sample:solvent ratio from 5:1 to 0.2:1 increased the amount of all isoflavones determined in the sample. It is known that for effective extraction of isoflavones from soybeans, a certain amount of water in the extraction solvent is necessary [11,20]. In this case, the sample itself contributes the right amount of water in

Table 2
Effect of the sample:solvent ratio (Relative % ± R.S.D.) on the extraction of isoflavones from soy drinks

Isoflavone	Sample:solvent ratio (v/v)									
	5:1		2:1		1:1		0.5:1		0.2:1	
	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH
Di	3.0 ± 4.6^{h}	2.9 ± 4.6^{h}	7.4 ± 4.4^{g}	7.4 ± 4.5^{g}	12.0 ± 3.4^{f}	14.1 ± 4.3^{e}	27.2 ± 2.8^{d}	30.5 ± 2.6^{c}	64.1 ± 2.7^{b}	70.8 ± 3.4^{a}
Gly	10.4 ± 4.0^{g}	10.4 ± 5.1^{g}	15.6 ± 3.0^{f}	14.6 ± 3.7^{f}	20.4 ± 4.5^{e}	22.8 ± 3.5^{e}	40.3 ± 3.6^{d}	43.9 ± 3.6^{c}	77.7 ± 4.0^{b}	86.4 ± 2.7^{a}
Gi	4.1 ± 5.0^{h}	4.2 ± 3.2^{h}	8.0 ± 3.6^{g}	7.9 ± 3.9^{g}	12.4 ± 3.2^{f}	14.6 ± 3.6^{e}	23.6 ± 2.6^{d}	30.0 ± 2.8^{c}	33.0 ± 2.2^{b}	53.1 ± 3.7^{a}
Mdi	3.8 ± 4.9^{h}	4.0 ± 5.0^{h}	7.3 ± 4.2^{g}	7.4 ± 3.7^{g}	14.7 ± 3.6^{f}	17.8 ± 4.0^{e}	24.8 ± 4.5^{d}	29.6 ± 2.3^{c}	48.1 ± 2.6^{b}	60.7 ± 2.8^{a}
Mgly	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.4 ± 4.7^{b}	27.0 ± 4.0^{a}
Mgi	n.d.	n.d.	1.3 ± 4.1^{g}	1.1 ± 4.4^{g}	8.2 ± 4.2^{f}	12.4 ± 3.4^{e}	14.2 ± 3.5^{c}	22.1 ± 4.2^{b}	13.0 ± 3.2^{d}	39.1 ± 3.5^{a}
Agi	47.5 ± 3.7^{d}	47.3 ± 3.2^{d}	49.1 ± 3.6^{cd}	49.1 ± 3.9^{cd}	52.0 ± 3.2^{c}	54.6 ± 4.3^{b}	54.0 ± 3.7^{b}	57.5 ± 4.0^{b}	59.0 ± 2.3^{b}	65.8 ± 3.4^{a}
Ge	37.7 ± 3.4^{ns}	37.8 ± 3.6^{ns}	38.5 ± 3.7^{ns}	39.4 ± 3.0^{ns}	38.6 ± 3.2^{ns}	40.7 ± 4.2^{ns}	40.0 ± 3.5^{ns}	39.7 ± 3.4^{ns}	39.5 ± 4.5^{ns}	41.3 ± 4.3^{ns}
Total	4.9 ± 3.8^{h}	3.9 ± 3.7^{h}	7.4 ± 3.7^{g}	7.3 ± 2.9^{g}	12.7 ± 3.3^{f}	15.4 ± 3.8^{e}	24.0 ± 2.6^{d}	29.2 ± 2.5^{c}	43.8 ± 1.3^{b}	58.5 ± 2.5^{a}

Extraction temperature, 10 °C; length, 10 min; total volume, 25 mL (sample + solvent), $n = 3$. Values are relative to the total amount present in the sample determined by the reference method (100%). Means followed by different superscripts are statistically different ($p < 0.05$). n.d: no detected.

the extracting solvent, i.e. with the sample:solvent ratio of 0.2:1, the water content of the extracting solvent is approximately 20%, on other words, 80% EtOH and 80% MeOH, which have been demonstrated, in some cases, as the most effective extraction solvents for isoflavones [11,20].

Another important aspect observed using different sample:solvent ratio was the detection of some isoflavones in some cases. With high sample:solvent ratios, the isoflavones MGly and MGi were not detected and with decreasing ratios the amount of these isoflavones detected increased, being the highest amount detected with a sample:solvent ratio of 0.2:1.

When using sample:solvent ratios higher than 1:1, there was no significant difference between MeOH and EtOH ($p < 0.05$). Most likely, it is due to the low amount of solvent, which has less influence in the extraction efficiency, and to the low amount of isoflavones extracted itself. However, when using smaller sample:solvent ratios than 1:1, there is an increasing difference between both solvents. Using a sample:solvent ratio of 0.2:1, EtOH extracts approximately 15% more isoflavones than MeOH. These differences are indications that there is an extraction process taking place and that direct injection of soy drinks can seriously underestimate isoflavone concentration in the samples. Also, it can be drawn from the results that the most part of isoflavones (>95%) are present on the solids in the juices (possibly linked to proteins) and only very small amounts in the liquid.

Therefore, it is feasible to avoid the time consuming freeze-drying step by a simple solid–liquid extraction with the appropriate solvents at the appropriate proportions. Based on these results, EtOH and the sample:solvent ratio of 0.2:1 were selected and used for further optimization of extraction conditions.

3.2. Extraction temperature

With the aim of improving extraction efficiency, extraction temperature was increased from 10 to 15, 30, 45 and 60 °C.

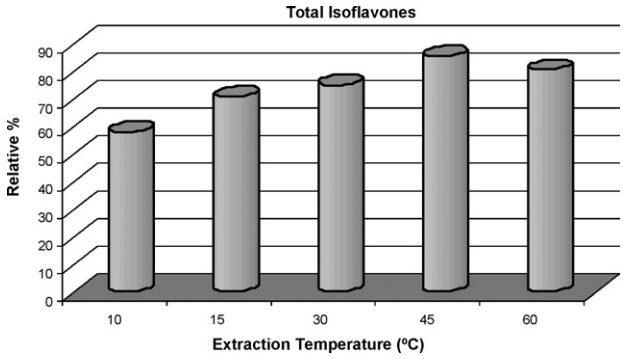

Fig. 3. Effect of the temperature on the extraction of total isoflavones from soy drink sample. Sample:EtOH ratio, 0.2:1; total volume, 25 mL (sample + EtOH); length, 10 min. Values are relative to the total amount present in the sample determined by the reference method (100%).

The results are shown in Fig. 3 (total isoflavones) and Fig. 4 (isoflavone derivatives).

As can be seen in Fig. 3, the effect of the extraction temperature is straightforward, gradually increasing the extraction temperature until 45 °C increased the amount of isoflavones extracted. However, higher temperatures (60 °C) extracted lower amounts of isoflavones ($p < 0.05$) possibly due to degradation of malonyl glucosides. It can be observed in Fig. 4 that higher amounts of glucosides and lower amounts of malonyl glucosides were found in the extract using 60 °C when compared to 45 °C, which can be interpreted as indication of degradation.

On a previous report [11], we did not observe degradation of isoflavones extracted from soy flour using 60 °C during 20 min under sonication and the observed variation might be caused by higher enzymatic activity in the soy–fruit beverage than in soy flour from the previous study. Also, pH may be playing a role since the medium may be acidic due to the high proportion of fruit juice and is probable that acidic hydrolysis of isoflavone glycosides is taking place at higher temperatures (60 °C).

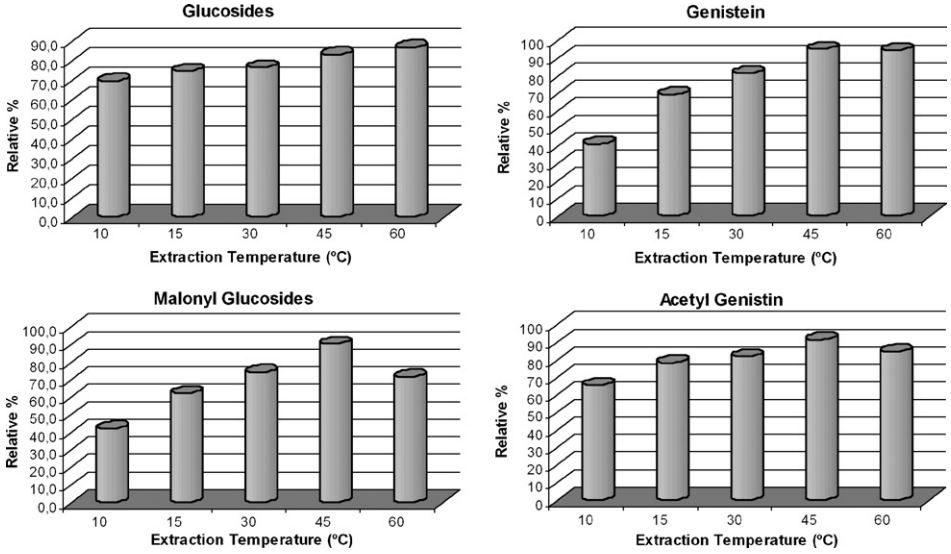

Fig. 4. Effect of the temperature on the extraction of isoflavones derivatives present in the sample. Sample:EtOH ratio, 0.2:1; total volume, 25 mL (sample + EtOH); length, 10 min. Values are relative to the total amount present in the sample determined by the reference method (100%).

Table 3
Effect of the extraction time (Relative % ± R.S.D.) on the extraction of isoflavones from soy drinks

Isoflavone	Extraction time (min)					
	5	10	15	20	25	30
Di	66.0 ± 3.1[d]	82.4 ± 2.7[c]	90.6 ± 2.9[b]	97.4 ± 2.8[a]	101.3 ± 3.5[a]	101.3 ± 3.4[a]
Gly	76.5 ± 4.8[c]	91.0 ± 2.2[b]	92.6 ± 3.0[b]	98.2 ± 2.7[ab]	100.9 ± 3.7[a]	101.0 ± 3.6[a]
Gi	63.4 ± 3.4[d]	77.2 ± 3.9[c]	90.1 ± 4.4[b]	100.2 ± 3.3[a]	100.4 ± 4.2[a]	99.4 ± 3.3[a]
Mdi	59.7 ± 4.8[c]	96.4 ± 3.7[ab]	94.0 ± 2.2[b]	102.9 ± 1.2[a]	97.9 ± 4.3[ab]	99.2 ± 4.8[a]
Mgly	63.4 ± 4.7[c]	82.0 ± 4.0[b]	100.4 ± 4.9[a]	100.4 ± 2.9[a]	99.7 ± 3.1[a]	99.9 ± 2.4[a]
Mgi	58.4 ± 4.3[b]	93.6 ± 2.6[a]	94.7 ± 4.4[a]	100.6 ± 3.2[a]	99.8 ± 4.7[a]	99.6 ± 3.7[a]
Agi	70.6 ± 3.4[c]	91.8 ± 3.1[b]	92.8 ± 5.0[ab]	98.0 ± 3.3[ab]	101.0 ± 3.3[a]	101.0 ± 3.3[a]
Ge	58.2 ± 4.1[b]	95.4 ± 4.5[a]	96.6 ± 4.7[a]	100.6 ± 3.9[a]	99.8 ± 3.8[a]	99.6 ± 4.8[a]
Total	63.0 ± 1.8[d]	85.9 ± 2.9[c]	92.0 ± 3.1[b]	99.7 ± 1.5[a]	100.2 ± 1.8[a]	100.1 ± 1.6[a]

Sample:EtOH ratio, 0.2:1; total volume, 25 mL (sample + EtOH); extraction temperature, 45 °C. Values are relative to the total amount present in the sample determined by the reference method (100%). Means followed by different superscript letters are statistically different ($p < 0.05$), $n = 3$.

Based on the results, the extraction temperature of 45 °C was selected to be used for the optimization of method since it improves extraction efficiency and does not promote changes in the sample isoflavone profile.

3.3. Extraction time

In order to determine the extraction kinetics, extractions were carried out using extraction times ranging from 5 to 30 min. The results are shown in Table 3. Increasing the extraction time from 5 to 20 min gradually increased the amount of isoflavones extracted and extractions longer than 20 min did not increase isoflavone extraction ($p < 0.05$). This is an indication that quantitative recoveries were achieved and that extending the extraction time beyond 20 min is unnecessary and also may increase variation and degradation of malonyl isoflavones. To ensure the achievement of quantitative extractions, re-extractions of the solid was carried out using the reference procedure using UAE as for the freeze-dried samples (see Section 2.3) and no isoflavones were detected in the extracts. Also, it can be observed that the major part of isoflavones present in the sample (>85%) were extracted in the first 10 min of extraction and that 20 min are needed to extract more than 95% of isoflavones in the sample.

3.4. Reproducibility

To evaluate the method reproducibility, a series of extractions in two consecutive days ($n = 12$) were carried out. Mean R.S.D. for determination of total isoflavones using the developed method is 2.8%. It was observed that Mgi has the lowest reproducibility (R.S.D. = 4.0%) and Adi the highest one (R.S.D. = 2.6%).

3.5. Recovery of isoflavones

The recovery of isoflavones added to the sample was determined using the developed method. One milliliter of a standard mixture containing 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 98.3, 98.4, 99.0, 98.1, 98.8, 97.9, 99.1, 100.5, 101.3, 99.0, 100.8 and 102.6, respectively. Values are relative to the amount added to the sample.

3.6. Samples

The optimized method was used for the determination of isoflavones from 11 soy beverages blended with fruit juices available in the Spanish market (Table 4), $n = 5$. The chromatogram of a peach derived sample is shown in Fig. 5. For comparison, all samples were freeze-dried and extracted using the reference method. The results are shown in Table 5 ($n = 5$). The mean difference in the total amount of isoflavones for all samples determined by the developed method and the reference method (freeze-drying the sample) is 1.8%. This is evidence that the optimized method provide precise results and that it can be used to replace the conventional method that is time consuming due to the freeze-drying step.

When comparing essayed samples, however, huge differences were observed on isoflavone concentration, reaching an almost 10 times fold differences in some cases. Overall isoflavone concentration ranged from 6.7 to 58.2 mg L^{-1}.

The samples with lowest soy amount (J-Pe and J-Pi, both with 0.8% of isolated soy protein as soy source) presented the lowest isoflavone concentration (7.8 and 6.7 mg L^{-1}, respectively). However, the samples with the highest amount of soy (DS-Pi, DS-Pe, DS-O, H-S, H-Pe and H-Pi, all with 3.0% of soybeans) did not present the highest isoflavone concentration. The sample with highest isoflavone concentration was VS-Pe with 58.2 mg L^{-1} (2.7% of soybeans). Therefore, higher amount of soy used in the product does not necessarily means higher isoflavone concentration. Samples with the same soy source and amount (3.0% soybeans) from different manufacturers had different isoflavone levels. Raw materials and the different type and intensity of processing used by different manufacturers may be responsible for these differences. Also, differences due to the fruit component, like enzymes, phenolic profile and pH may also be acting during storage affecting the stability of isoflavone glucoside derivatives.

Table 4
Isoflavone concentration (mg L^{-1} ± R.S.D.) of essayed samples determined by the optimized method

Isoflavone	Samples (optimized method) (mg L^{-1} ± R.S.D.)										
	J-Pi	J-Pe	DS-Pi	DS-O	DS-Pe	VS-Pe	VS-Pi	VS-O	H-S	H-Pe	H-Pi
Di	2.0 ± 2.4	2.0 ± 2.8	16.8 ± 2.8	17.9 ± 3.5	14.7 ± 2.5	20.4 ± 3.5	19.3 ± 3.1	20.2 ± 3.8	7.7 ± 2.6	7.3 ± 2.8	7.1 ± 2.4
Gly	0.4 ± 2.3	n.d	1.2 ± 2.4	1.1 ± 2.9	1.3 ± 2.2	1.0 ± 2.8	1.6 ± 2.7	1.5 ± 3.3	0.6 ± 4.7	0.4 ± 3.3	0.6 ± 4.2
Gi	2.7 ± 2.3	3.2 ± 2.6	12.8 ± 2.7	16.0 ± 3.5	14.2 ± 2.4	22.7 ± 3.4	20.0 ± 3.0	19.6 ± 3.7	8.9 ± 2.5	7.4 ± 2.7	7.5 ± 2.3
Mdi	n.d	1.0 ± 2.3	1.8 ± 2.4	0.6 ± 3.0	4.1 ± 2.4	4.7 ± 3.3	4.7 ± 2.9	6.1 ± 3.7	2.3 ± 2.4	2.2 ± 2.6	1.9 ± 2.1
Mgly	n.d	n.d	n.d	n.d	n.d	0.5 ± 3.1	n.d	n.d	n.d	n.d	n.d
Mgi	n.d	n.d	0.8 ± 6.4	3.0 ± 4.8	4.0 ± 3.2	5.5 ± 4.2	6.0 ± 3.6	7.4 ± 4.4	2.2 ± 3.9	2.1 ± 4.4	1.7 ± 4.0
Adi	n.d	n.d	0.7 ± 1.8	n.d	n.d	0.8 ± 2.4	0.8 ± 2.0	0.9 ± 2.7	n.d	n.d	n.d
Agly	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Agi	1.1 ± 2.0	1.2 ± 2.3	1.1 ± 3.8	1.3 ± 4.4	1.2 ± 2.9	1.6 ± 2.4	1.5 ± 2.3	1.5 ± 2.0	0.0	1.2 ± 2.8	1.1 ± 2.8
De	n.d	n.d	n.d	n.d	0.6 ± 4.0	0.4 ± 2.8	0.7 ± 2.9	0.5 ± 3.2	n.d	n.d	n.d
Gle	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Ge	0.4 ± 3.0	0.4 ± 4.0	0.6 ± 3.5	0.5 ± 5.5	0.9 ± 3.8	0.5 ± 2.1	0.7 ± 2.2	0.5 ± 2.3	0.6 ± 2.5	0.6 ± 2.5	0.5 ± 4.5
Total	6.7 ± 2.3	7.8 ± 1.7	35.9 ± 2.8	40.3 ± 3.6	41.1 ± 2.6	58.2 ± 3.4	55.3 ± 3.0	58.2 ± 3.7	22.4 ± 2.7	21.1 ± 2.6	20.4 ± 2.0

Sample:EtOH ratio, 0.2:1; total volume, 25 mL (sample + EtOH); extraction temperature, 45 °C; extraction length, 20 min; $n = 5$. n.d: no detected.

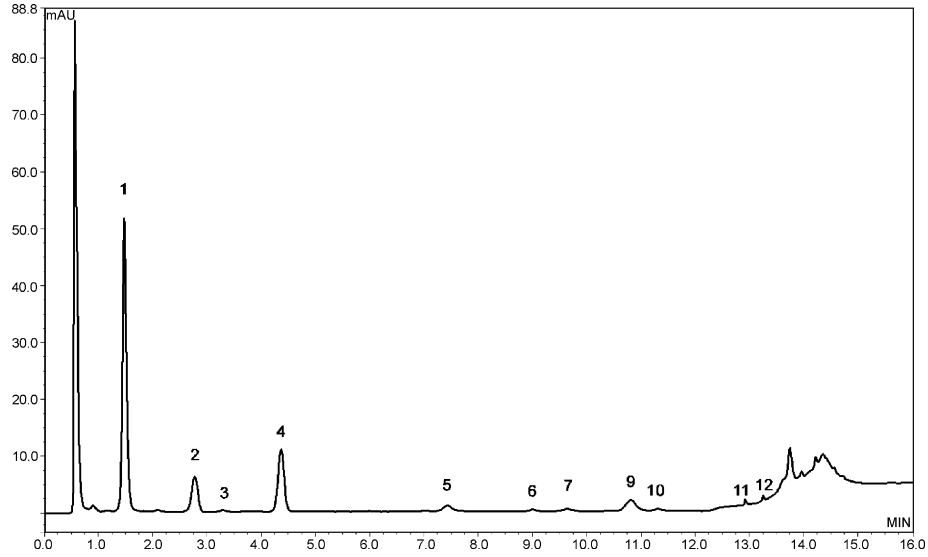

Fig. 5. Chromatogram of a representative peach-derived juice (VS-Pe). (1) Internal standard (2,5-dihydroxybenzaldehyde), (2) Di, (3) Gly, (4) Gi, (5) MDi, (6) MGly, (7) ADi, (8) AGly, (9) MGi, (10) De, (11) Gle, (12) AGi and (13) Ge.

Table 5
Isoflavone concentration (mg L^{-1} ± R.S.D.) of essayed samples determined by the conventional method

Isoflavone	Samples (freeze-dried) (mg L^{-1} ± R.S.D.)										
	J-Pi	J-Pe	DS-Pi	DS-O	DS-Pe	VS-Pe	VS-Pi	VS-O	H-S	H-Pe	H-Pi
Di	1.9 ± 3.4	1.9 ± 4.2	17.2 ± 3.7	17.3 ± 3.8	14.1 ± 4.5	20.5 ± 3.8	19.4 ± 4.3	19.9 ± 2.7	7.8 ± 4.5	7.4 ± 4.5	7.1 ± 4.3
Gly	0.5 ± 4.8	0.0	1.3 ± 2.7	1.0 ± 3.8	1.2 ± 3.8	1.0 ± 4.8	1.5 ± 3.3	1.3 ± 4.5	0.7 ± 4.6	0.6 ± 5.0	0.7 ± 4.2
Gi	2.6 ± 3.0	3.3 ± 3.3	12.6 ± 3.6	15.3 ± 3.5	14.9 ± 4.1	22.6 ± 4.2	19.8 ± 3.1	19.3 ± 3.7	8.7 ± 3.7	7.1 ± 3.1	7.2 ± 4.9
Mdi	n.d	0.9 ± 2.5	1.5 ± 3.0	0.6 ± 2.8	4.3 ± 3.8	4.1 ± 4.1	4.7 ± 4.7	6.4 ± 4.7	2.2 ± 4.8	2.3 ± 3.5	2.1 ± 4.4
Mgly	n.d	n.d	n.d	n.d	n.d	0.4 ± 4.4	n.d	n.d	n.d	n.d	n.d
Mgi	n.d	n.d	0.9 ± 3.4	2.8 ± 4.3	4.1 ± 3.8	5.3 ± 3.4	6.5 ± 4.9	7.1 ± 4.5	2.1 ± 4.0	2.0 ± 4.7	1.9 ± 4.6
Adi	n.d	n.d	0.8 ± 4.7	n.d	n.d	0.8 ± 4.5	0.7 ± 3.5	0.9 ± 3.6	n.d	n.d	n.d
Agly	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Agi	1.0 ± 3.1	1.0 ± 3.1	1.1 ± 3.9	1.3 ± 4.3	0.5 ± 3.3	1.5 ± 3.1	1.4 ± 3.7	1.6 ± 4.0	n.d	1.2 ± 3.4	1.2 ± 4.4
De	n.d	n.d	n.d	n.d	0.5 ± 3.1	0.4 ± 3.9	0.7 ± 3.7	0.6 ± 3.7	n.d	n.d	n.d
Gle	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Ge	0.5 ± 3.0	0.5 ± 4.7	0.7 ± 3.7	0.6 ± 2.5	1.0 ± 3.3	0.7 ± 3.8	0.8 ± 3.6	0.7 ± 4.1	0.7 ± 4.9	0.8 ± 4.4	0.7 ± 3.4
Total	6.4 ± 3.0	7.5 ± 2.0	36.0 ± 4.9	39.0 ± 3.8	40.5 ± 4.9	57.2 ± 3.4	55.4 ± 4.2	57.8 ± 3.5	22.2 ± 4.1	21.3 ± 4.0	20.9 ± 3.8

0.25 g of freeze-dried sample, 25 mL of 50% EtOH, 60 °C, 20 min, $n = 5$.

4. Conclusions

Using the ultrasound-assisted extraction for isoflavone extraction from soy beverages blended with fruit juices, these compounds can be extracted in a simple and reproducible way. The optimized method consists of extracting the sample with ethanol with a sample:solvent ratio of 0.2:1 on an ultrasound bath at 45 °C during 20 min. Total and individual isoflavone concentration obtained with the optimized method are not significantly different ($p < 0.05$) from those was obtained with the conventional method. Therefore, the freeze-drying step could be avoided.

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