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Fast analysis of soy isoflavones by high-performance liquid chromatography with monolithic columns

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Abstract

A fast method using high-performance liquid chromatography based on two monolithic columns has been developed for the simultaneous determination of isoflavones extracted from soybeans and derived foods. The 12 main isoflavones were resolved in 10 min in two coupled monolithic columns working at 35 °C using a elution gradient of acidified water (0.1% acetic acid) and methanol (0.1% acetic acid) at a flow rate of 5 mL min^{-1}. Retention time and relative area standard deviations were below 1% for all isoflavones. The method developed was successfully applied to several soy food samples and spiked samples. Total isoflavone concentration in sampled soy foods ranged from 34.28 mg L^{-1} to 4.29 mg g^{-1}.

Keywords: High-performance liquid chromatography; Monolithic columns; Isoflavones; Soybeans; Soy foods

1. Introduction

Soy isoflavones are a phytochemical group of interest since they may be associated with a variety of health-protection effects that include reduced risk of cardiovascular disease, lower rates of prostate, breast and colon cancers, and improved bone health [1–3]. There are 12 main isoflavones in soybeans: genistin, glycitin, daidzin and their respective acetyl, malonyl and aglycon forms [4,5].

Several methods for the analysis of isoflavones from soy and soy foods have been proposed in recent years (see Ref. [6] for a review). Separation of isoflavones is conventionally performed by high-performance liquid chromatography (HPLC), and different types of reverse-phase columns and elution systems have been used for analysis of these compounds in foods. Most of the analysis methods for all chemical forms of soy using conventional silica-based columns typically take between thirty minutes and one hour [4–6].

Fast methods for the determination of all chemical forms of soy isoflavones have recently been developed, achieving separation in less than 20 min [7,8]. Klejdus et al., studied the influence of several operational parameters (mobile phase composition, pH, gradient profile, flow rate and temperature) on the performance of a fast HPLC column (150 mm × 2.1 mm, 3.0 μm particle size). The developed method achieved separation of several isoflavones in less than 10 min and was successfully applied for the analysis soy bits extracts [9]. The same method was also employed for the determination of aglycon and glucoside distribution in soy plants and soybeans [10]. Klejdus et al. also used fast column HPLC for the analysis of isoflavones from soy bits and red clover extracts obtained by supercritical fluid extraction [11].

HPLC columns packed with monolithic supports are another alternative means of performing fast separations. Using monolithic columns, analysis time can be decreased by three to seven times, while maintaining a resolution comparable with typical 5 μm particle-packed 250 mm × 4.6 mm C_{18} columns [12–14]. Monolithic columns are increasingly being applied in phytochemical analysis, and many applications have been published recently (see Ref. [15] for a review).

There are already a few examples of the use of monolithic columns for the analysis of isoflavones. Rostagno et al. [16] used two monolithic columns in series for the analysis of soy isoflavones after solid phase extraction (SPE). Separation included all chemical forms of isoflavones and was achieved in 27 min. Shorter analysis times were not studied by the authors in that work, since its main objective concerned the SPE method, rather than the chromatographic separation of isoflavones.

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Apers et al. [17] proposed a fast HPLC method for quality control of soy extracts using monolithic columns. The method achieves separation of nine isoflavones (excluding the malonyl forms) in less than 20 min. The monolithic columns, apart from speeding up the analysis (2.4 times), provided an improvement in the efficiency of late-eluting isoflavones, as compared with conventional C_{18} columns. Further, Kim et al. [18] recently proposed another method using monolithic columns for the analysis of four isoflavones (genistin, genistein, daidzin and daidzein) from soybeans and soybean pastes in 7 min.

However, the reported methods does not include the malonyl forms of isoflavones, which predominate in soybeans and are present in large amounts in most soy foods, or take an excessively long time. On the other hand, these methods show the potential of the monolithic columns in the analysis of soy isoflavones and suggest that more research on isoflavone analysis using these columns could be productive.

Therefore, the objective of this work is to develop a fast HPLC method using monolithic columns for the analysis of all main soy isoflavones, including the malonyl derivatives, from several soy sources, in less than 10 min, in order to gain a significant reduction in the time needed for analysis as compared with previously reported methods.

2. Experimental

2.1. Samples

Soybean flour (SFL), texturized soy protein (TSP), soy fibre (SFI), powdered soy milk (PSM) and liquid soy drink (LSD) were purchased from a local supermarket and stored at −32 °C until used as samples.

2.2. Chemicals and solvents

The ethanol (EtOH), acetic acid (Panreac, Barcelona, Spain), acetonitrile (MeCN) and methanol (MeOH) (Merck, Darmstadt, Germany) used were HPLC grade. Water was supplied by a Milli-Q water purifier system from Millipore (Bedford, MA, USA). A solution of 1000 mg L^{-1} of 2,5-dihydroxybenzaldehyde. (Sigma, St. Louis, MO, USA) was used as internal standard for the extraction of isoflavones from soy foods. Isoflavone standards were obtained from LC labs (Woburn, MA, USA). Standards were prepared in 80% MeOH and stored at −32 °C. A mixture containing between 10 and 40 mg L^{-1} of all isoflavones was used in the development of the method.

2.3. Extraction of isoflavones

Extractions of soy foods were carried out in an ultrasonic bath of 360W (J.P. Selecta, Barcelona, Spain). Temperature was kept constant by means of a temperature controller coupled to the ultrasonic bath. Solid samples were ground into a fine powder in a coffee grinder. Approximately 0.25 g of solid samples were extracted under sonication with 25 mL of 50% EtOH (in water) for 20 min at 60 °C [19]. 10 mL of MeOH were added to 40 mL of liquid samples (LSD) before extraction under the same conditions. After extraction, 0.5 mL of the internal standard was added to the extracts which were then centrifuged for 10 min. The internal standard was used to correct the extraction volume.

In the case of the LSD extract, after centrifugation, 20 mL of sample supernatant was brought up to 50 mL with water and cleaned/concentrated using a previously developed solid phase extraction method [16]. The SPE method consists of a cartridge condition stage (10 mL of MeOH + 10 mL of DI water at 10 mL min^{-1}), a sample loading stage (50 mL at 5 mL min^{-1}), a washing stage (10 mL of DI water at 10 mL min^{-1}) and an elution stage with 4 mL of MeOH at 10 mL min^{-1}. After extraction 0.25 mL of the internal standard was added to the extract. 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 on intraday analysis [20].

2.4. High performance liquid chromatography

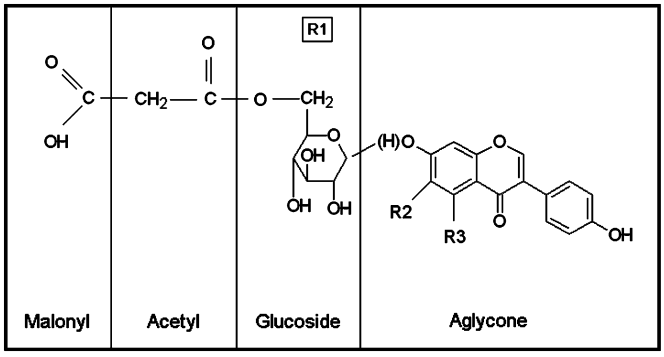
The HPLC-PDAD analysis was carried out in a Dionex system (Dionex Corp., Sunnyvale, CA, USA), consisting of automated sample injector (ASI-100), pump (P680), thermostatted column compartment (TCC-100) and photodiode array detector (PDA-100). Isoflavones were separated in monolithic columns (Chromolith RP-18e, Merck). The solvents used were: solvent A: water with 0.1% acetic acid (v/v); solvent B: methanol with 0.1% acetic acid (v/v). UV absorbance was monitored from 200 to 400 nm. Isoflavones and internal standard peak areas were quantified at 254 nm. Sample volume was 10 μL. The software used for control of the equipment and data acquisition was Chromeleon version 6.60. Identification of isoflavones was achieved by individual comparison of retention times and UV spectra, as well as by co-elution of separated compounds with authentic standards. Peak purity was evaluated using Chromeleon features. Evaluation of efficiency was made on the basis of retention time, peak width, capacity factor (k'), separation factor (α), resolution and peak asymmetry. Resolution and asymmetry were calculated using the European Pharmacopeia (EP) standard. Quantification was carried out by integration of the peak areas using the internal standardization method. The standard curve of each isoflavone was prepared by plotting the concentration against the area. Regression equations and correlation coefficient (r^2) were calculated using Microsoft Excel XP software. Detection and quantification limits (LOD and LOQ, respectively) were calculated using ALAMIN software [21].

3. Results and discussion

3.1. Method development

3.1.1. Initial conditions

Using a standard mixture with all isoflavones (Fig. 1), a series of isocratic conditions (80:20 to 65:35 (% A:% B) (v/v) in 2.5%



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 structure and abbreviations of analysed soy isoflavones.

increments of the amount of solvent B) in one monolithic column were assayed at 25 °C with a flow rate of 6 mL min^{-1}. Flow rate was adjusted in order not to exceed the maximum pressure limitation of the column (3000 psi).

The best separation, where most isoflavone peaks were resolved, was achieved using 70:30 A:B. Under these conditions the isoflavones separated were: genistin, malonyl daidzin, malonyl glycitin, acetyl daidzin, daidzein, glycitein, acetyl genistin and genistein and the unresolved peaks were daidzin/glycitin and malonyl genistin/acetyl glycitin/daidzein. Analysis time was less than 5 min and can be expressed as the retention time of the last eluting isoflavone, genistein (RT = 4.50 min). Mean retention time, peak width, k', α, resolution and asymmetry of separated isoflavones with one column were 2.07 min, 0.22 min, 5.88, 1.26, 2.39 and 1.20, respectively.

From the results it can be inferred that the most difficult isoflavones to separate from each other are daidzin/glycitin and malonyl genistin/acetyl glycitin/daidzein and that when developing a method for the analysis isoflavones, special attention should be given to the separation of these peaks. Moreover, the isocratic condition of 70:30 A:B, 25 °C and 6 mL min^{-1} are appropriate for use as initial conditions for further optimization of the analysis method.

3.1.2. Column length

The use of a second column with the initial conditions (70:30 A:B, 25 °C, 6 mL min^{-1}) was evaluated. The most important effect observed from increasing the column length was the improvement of separation of most isoflavones, especially malonyl genistin which was separated from acetyl glycitin. This will be very useful since malonyl genistin is among the isoflavones that were unresolved using one column.

Increasing the column length increased mean selectivity, peak width (48%), α and the resolution (8%). However, it also increased peak asymmetry (4%) and retention time (51%) and reduced k'. Mean retention time, peak width, k', α, resolution and asymmetry of separated isoflavones with two columns were 4.21 min, 0.42 min, 5.01, 1.31, 2.58 and 1.24, respectively.

Using two columns, resolution improved mainly for early eluting isoflavones while late eluting isoflavones had values similar to those obtained using one column. The k' and α values with two columns were similar to those with one column. Asymmetry presented a slight increase, especially for late eluting isoflavones, possibly due to increased diffusion caused by extended analysis time.

However, the main disadvantage of using two columns is the longer analysis time (RT of genistein = 9.42 min) and increased peak width. Analysis time is more than doubled when using one column and peak width increased in similar proportion. On the other hand, time lost with the additional length (i.e. column) is gained in selectivity and resolution, which needs to be improved in the case of daidzin/glycitin and malonyl genistin/acetyl glycitin/daidzein.

High speed is achieved by making the column as short as possible consistent with the resolution requirement. Since there are several unresolved peaks, it was considered advisable to increase resolution at the expense of the column length. Improved peak selectivity and resolution are indications that the use of two columns may provide better results than with one column. Thus, the method was optimised using two monolithic columns.

3.1.3. Temperature

The effect of increasing temperature from 25 to 35 °C and 45 °C was evaluated using two columns with the initial conditions (70:30 A:B, 6 mL min^{-1}). Increasing temperature to 35 °C had several positive effects on the analysis. It reduced average peak width (25.1%), k' (15.8%), peak asymmetry (19.6%) and retention time (7.3%) and increased selectivity, α (2.0%) and resolution (16.2%). Using 35 °C, mean retention time, peak width, k', α, resolution and asymmetry of separated isoflavones were 3.65 min, 0.32 min, 4.22, 1.34, 3.08 and 1.00, respectively.

It was observed that the degree of the temperature effect is directly dependent on the particular isoflavone and its retention time. This can be observed when comparing the reduction of the retention time of the first and the last eluting isoflavones, daidzin and genistein respectively. The reduction of daidzin retention time obtained was 0.05 min while reduction of genistein retention time was 1.38 min.

One of the most important aspects of increasing the temperature to 35 °C was the improvement of malonyl genistin/acetyl glycitin/daidzein separation. However, the temperature increase

reduced separation of daidzin/glycitin. Higher temperatures than 35 °C had a negative effect on separation. Increasing the temperature to 45 °C reduced retention time which in turn reduced separation. Therefore, further comparison of conditions was made with two monolithic columns at 35 °C.

3.1.4. Flow rate

Since improved separation between malonyl genistin/acetyl glycitin/daidzein was needed, lower flow rates (5, 4.5 and 4 mL min^{-1}) were evaluated using two coupled columns (70:30 A:B, 35 °C). Using 5 mL min^{-1} increased peak width (30.7%), peak asymmetry (19.4%) and retention time, and more important, increased selectivity and resolution. Mean RT, peak width, k', α, resolution, and asymmetry of separated isoflavones were 4.96 min, 0.47 min, 6.09, 1.26, 3.02 and 1.24, respectively.

Reducing the flow rate to 4.5 mL min^{-1} increased peak widths and reduced the separation of malonyl genistin/acetyl glycitin/daidzein. Overall selectivity decreased even more when the flow rate was lowered to 4 mL min^{-1} and therefore the optimization of the method that follows was carried out using two coupled monolithic columns, at 35 °C and flow rate of 5 mL min^{-1}.

3.1.5. Gradient

Since the previous results with isocratic conditions (i.e. 80:20 A:B) indicate that a simple gradient might separate daidzin from glycitin, a gradient from 0% B to 30% B in 2 min was introduced at the start of the program while maintaining the rest of the program in isocratic conditions (70:30 A:B).

The implemented gradient completely separated daidzin from glycitin and improved malonyl genistin/acetyl glycitin/daidzein separation. Increasing the amount of methanol on the mobile phase to 31% in one minute and maintaining these conditions until the end of the analysis improved separation of malonyl genistin/acetyl glycitin/daidzein by providing peaks with less width. By adding another step from 31% B to 35% B in one minute and maintaining these conditions until the end of the analysis separation of malonyl genistin/acetyl glycitin/daidzein was improved and all isoflavones were completely separated in less than 15 min. Since the last three eluting peaks (glycitein, acetyl genistin and genistein) were adequately separated from each other, a final step gradient from 35 to 100% in 1.5 min was added, with the aim of reducing total analysis time, improving peak shape and cleaning the column.

Finally, the optimised chromatographic conditions for the separation of all isoflavones are: two monolithic columns, 35 °C, 5 mL min^{-1}, with the gradient shown on Fig. 2.

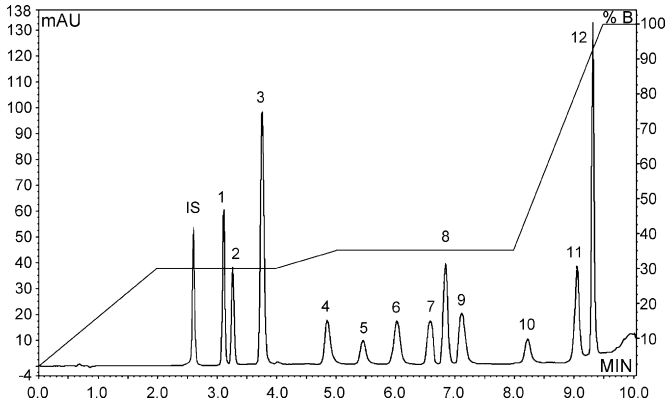


Fig. 2. HPLC chromatogram of isoflavone standards using the optimised method. IS: internal standard—2,5-dihydroxybenzaldehyde. (1) Di, (2) Gly, (3) Gi, (4) MDi, (5) MGly, (6) ADi, (7) MGi, (8) AGly, (9) De, (10) Gle, (11) AGi and (12) Ge. HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), sample volume (10 μL), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B). Identity of isoflavones as Fig. 1.

3.1.6. Performance

Average results obtained with the analysis of the same standard mixture on different days ($n = 12$) are shown on Table 1. It also illustrates the performance of the optimised method. All isoflavones were separated in less than 10 min (RT of genistein = 9.37 min).

Table 1
Properties of the method developed ($n = 12$)

Isoflavones	RT (min)	RT (R.S.D.)	Area (R.S.D.)	Width (min)	k'	α	Resolution	Asymmetry	LOD (mg L^{-1})	LOQ (mg L^{-1})
Di	3.15	0.1	0.6	0.08	3.50	1.07	3.06	1.10	0.34	1.15
Gly	3.32	0.1	0.7	0.08	3.74	1.20	4.74	1.17	0.38	1.27
Gi	3.85	0.1	0.8	0.11	4.50	1.33	11.39	1.24	0.38	1.32
MDi	4.88	0.1	0.7	0.18	5.98	1.13	4.51	1.23	0.43	1.44
MGly	5.42	0.1	0.7	0.18	6.74	1.13	2.08	1.21	0.43	1.45
ADi	6.03	0.1	0.9	0.20	7.61	1.09	1.06	1.09	0.38	1.25
MGi	6.52	0.1	0.8	0.26	8.31	1.04	1.32	0.98	0.36	1.19
AGly	6.77	0.1	0.9	0.27	8.67	1.05	1.47	1.05	0.59	1.96
De	7.05	0.1	0.9	0.30	9.07	1.18	3.51	1.20	0.24	0.80
Gle	8.18	0.1	0.8	0.38	10.69	1.12	3.72	1.39	0.25	0.84
AGi	9.08	0.1	0.7	0.13	11.98	1.03	1.11	1.03	0.49	1.64
Ge	9.37	0.1	0.7	0.08	12.38	0.63	23.22	1.38	0.40	1.32
Mean	6.13	0.1	0.8	0.19	7.76	1.08	5.06	1.17	0.39	1.03

HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), sample volume (10 μL), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B). RT: retention time; R.S.D.: relative standard deviation; LOD: limits of detection; LOQ: limits of quantification. Identity of isoflavones as Fig. 1

Using the optimised gradient, the mean retention time, peak width, k', α, resolution and asymmetry of the optimized method were 6.13 min, 0.19 min, 7.76, 1.08, 5.06 and 1.17, respectively. Mean retention time and area reproducibility were 99.89 and 99.33%, respectively.

The high reproducibility observed can be attributed, in part, to the thermostatted column compartment, which ensured a constant temperature. As demonstrated, changes in the temperature can affect retention time and resolution. In such short runs at elevated flow rates with a precise gradient designed to separate specific compounds, temperature oscillations can reduce resolution and separation performance by small variations in the retention time of the compounds in question.

Calibration curves were calculated for each isoflavone and response was linear between 0.1 and 100 mg L^{-1} for all isoflavones; regression coefficients (r^2) were higher than 0.9998. The LOD and LOQ (mg L^{-1}) for each isoflavone are shown in Table 1.

3.1.7. Robustness

3.1.7.1. Effect of injection volume. To evaluate the effect of the injection volume on peak resolution and asymmetry, a series of injections with different volumes (10–50 μL) of the standard mixture were performed. The effect of injection volume is shown in Table 2. As can be seen, there are small differences in the performance of the method when larger injection volumes are used, thereby offering alternatives to increase LOD and LOQ if necessary.

3.1.7.2. Sample solvent. Most isoflavone extraction methods use 80% MeOH as solvent. In some cases after extracting with acidified water/MeCN mixtures, the solvent is removed and the residue is dissolved in aqueous MeOH. One reason for using MeOH as extraction solvent is to avoid peak distortion caused by injecting samples containing high concentrations of MeCN onto columns equilibrated with a low MeCN concentration. This problem can be prevented by limiting the sample size, as shown by Griffith et al. [8].

To evaluate the effect of the sample solvent on isoflavone peaks a series of extractions of 0.25 g of the soy flour using different solvents (50% EtOH, 50% MeOH and 50% MeCN) were performed following the extraction protocol (Section 2.3).

Table 3 shows the effect of the extraction solvent on the method performance. The differences between the various extraction solvents employed for k', α, resolution and asymmetry

Table 2
Effect of injection volume on analysis of isoflavone peaks

Sample volume (μL)	Width (min)	k'	α	Resolution	Asymmetry
10	0.19	7.76	1.08	5.12	1.16
20	0.20	7.76	1.08	5.14	1.16
30	0.20	7.76	1.08	5.16	1.11
40	0.22	7.76	1.08	5.10	1.11
50	0.22	7.73	1.08	5.09	1.21

Results are relative to mean values of all isoflavones. HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B).

Table 3
Effect of sample solvent on analysis of isoflavone peaks

Extraction solvent	Width (min)	k'	α	Resolution	Asymmetry
80% MeOH	0.19 b	7.76	1.08	5.18	1.17
50% MeOH	0.16 a	7.76	1.08	5.68	1.03
50% MeCN	0.20 b	7.77	1.08	5.72	1.09
50% EtOH	0.27 c	7.77	1.08	5.59	1.08

Results are relative to mean values of all isoflavones. For extraction conditions see Section 2.3. HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), sample volume (10 μL), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B). Means followed by different letters are statistically different ($P < 0.05$).

Table 4
Recovery of standards from the spiked samples of texturised soy protein

Spiking level	Added (mg L^{-1})				Found (mg L^{-1})				Recovery (%)				
	1	2	3	4	1	2	3	4	1	2	3	4	Mean
Di	4.0	7.1	9.7	11.9	3.9	7.1	9.8	11.9	97.6	99.7	100.7	100.1	99.5 ± 1.3
Gly	1.7	3.1	4.2	5.2	1.8	3.1	4.4	5.3	102.6	100.0	103.7	102.3	102.2 ± 1.5
Gi	5.7	10.2	13.9	17.0	5.7	10.2	13.8	16.9	101.1	99.8	99.7	99.9	100.1 ± 0.7
MDi	5.7	10.3	14.0	17.1	5.6	10.3	13.9	17.1	98.5	100.2	99.2	99.6	99.4 ± 0.7
MGly	2.4	4.3	5.8	7.1	2.4	4.2	5.9	7.1	101.1	98.7	101.9	100.3	100.5 ± 1.2
ADi	4.9	8.8	12.0	14.7	4.9	9.1	12.0	14.8	100.7	103.0	100.2	101.3	101.3 ± 1.6
MGi	5.2	9.4	12.8	15.6	5.3	9.3	12.9	15.5	102.5	99.0	100.8	99.6	100.5 ± 2.1
AGly	19.6	35.2	48.0	58.7	20.4	35.2	48.2	58.6	104.3	100.0	100.5	99.9	101.2 ± 0.6
De	2.6	4.6	6.3	7.7	2.6	4.7	6.3	7.7	100.7	101.6	100.3	100.5	100.8 ± 2.7
Gle	11.0	19.8	27.0	33.0	11.0	19.0	26.2	33.7	99.6	96.1	97.1	102.0	98.7 ± 2.7
AGi	2.3	4.2	5.7	7.0	2.4	4.2	5.7	7.0	102.6	100.3	100.2	99.8	100.7 ± 1.3
Ge	5.9	10.6	14.5	17.7	6.0	10.5	14.4	17.8	102.1	98.8	99.6	100.5	100.3 ± 1.4

For extraction conditions see Section 2.3. HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), sample volume (10 μL), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B). Identity of isoflavones as Fig. 1.

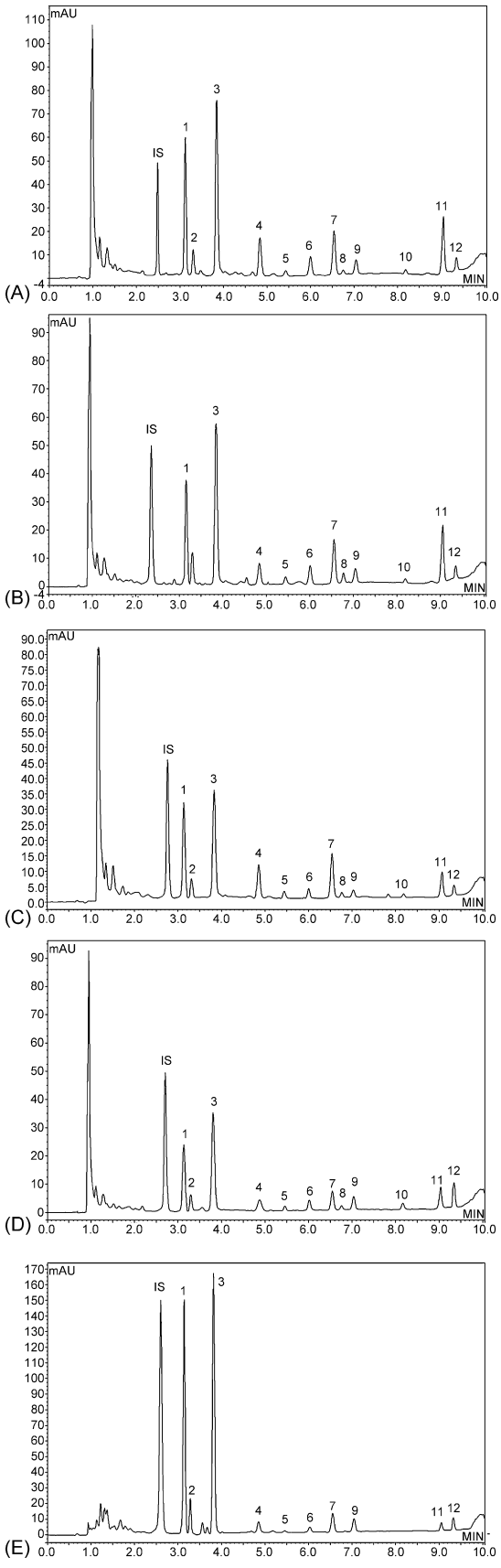


Fig. 3. Chromatograms of soy food samples. (A) Soy flour, (B) texturised soy protein, (C) soy fibre, (D) powdered soy milk and (E) liquid soy drink. HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), sample volume (10 μL), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B). IS: Internal standard—2,5-dihydroxybenzaldehyde. (1) Di, (2) Gly, (3) Gi, (4) MDi, (5) MGly, (6) ADi, (7) MGi, (8) AGly, (9) De, (10) Gle, (11) AGi and (12) Ge. Identity of isoflavones as Fig. 1.

were not significant ($P < 0.05$). There were, however, significant differences in peak width ($P < 0.05$) depending of the extracting solvent employed. Using 50% MeOH as extracting solvent, the peaks had less width, while using 80% MeOH and 50% MeCN widths were intermediate. The samples extracted using 50% EtOH presented the most peak width.

Despite differences in peak width, all these solvents can be used for the extraction of isoflavones without major impact on the HPLC analysis, since k', α, resolution and asymmetry are similar for all the solvents assayed. This will allow flexibility when choosing an extracting solvent with the advantage of eliminating the troublesome evaporation and dissolution step commonly used by several authors for the extraction of isoflavones. The results observed are, in part, due to the high flow rate used (5 mL min^{-1}).

In addition, the texturised soy protein (TSP) extract was spiked with different amounts of the standards mixture. The sample was spiked with isoflavones one hour before analysis in order to ensure interaction of standards with the extract. Recovery was obtained by dividing the concentrations added by the isoflavone concentration in the TSP. The results for each isoflavone are shown in Table 4. Mean recovery for all isoflavones with the sample spiked at different levels was 100.4 ± 1.6%. The highest recovery was found for acetyl glycitin (104.3%), and the lowest for glycitein (96.1%).

3.1.7.3. Application to real samples. Since solid–liquid extractions are, in general, not selective and co-extracted compounds can interfere with the analysis of isoflavones, it is essential that the optimised method is validated with different real samples. Therefore, extracts of soybean flour (SFL), texturized soy protein (TSP), soy fibre (SFI), powdered soy milk (PSM) and liquid soy drink (LSD) were analysed with the optimised method. Chromatograms of SFL, TSP, SFI, PSM and LSD are shown in Fig. 3(A), (B), (C), (D), and (E), respectively. and the individual isoflavone concentration of each sample is shown in Table 5.

Total isoflavone concentration in the samples of soy foods ranged from 34.28 mg L^{-1} (in LSD) to 4.29 mg g^{-1} (in TSP). In all samples, malonyl and glucosides as well as genistin derivatives were the predominant isoflavones; this clearly shows the importance of analysing the malonyl forms of isoflavones in soy foods. Peak purity analysis indicates that other compounds are not co-eluted with any isoflavone despite the variety of the samples. Retention time and UV spectra of separated isoflavones coincided with the respective standards.

Repeat analyses of the same TSP sample ($n = 12$) resulted in an overall inter-day and intra-day repeatability higher than 98.18% for area and higher than 99.98% for retention time.

Table 5
Isoflavone concentration in the samples analyzed with the optimized method ($n = 3$)

Isoflavone	Concentration (mg g^{-1} ± R.S.D.): soy sample				
	SFL	TSP	SFI	PSM	LSD (mg L^{-1})
Di	411.3 ± 2.1	628.0 ± 2.0	234.9 ± 2.6	306.8 ± 2.4	12.4 ± 2.1
Gly	128.5 ± 2.1	113.7 ± 2.3	56.9 ± 2.4	53.1 ± 2.5	2.0 ± 2.3
Gi	612.7 ± 2.1	799.5 ± 1.9	333.9 ± 2.5	351.9 ± 2.1	13.7 ± 2.4
MDi	239.1 ± 2.3	584.3 ± 2.3	118.6 ± 2.6	267.5 ± 2.4	1.2 ± 2.2
MGly	64.7 ± 2.2	89.4 ± 2.0	17.4 ± 2.7	41.8 ± 2.5	0.2 ± 2.7
ADi	274.4 ± 2.4	388.9 ± 2.1	101.8 ± 2.6	104.4 ± 2.4	0.5 ± 2.5
MGi	423.6 ± 2.1	690.0 ± 2.6	145.9 ± 2.7	315.5 ± 2.4	1.3 ± 2.2
AGly	263.1 ± 2.5	139.2 ± 2.4	68.4 ± 2.5	62.7 ± 2.3	0.3 ± 2.2
De	41.0 ± 2.6	49.2 ± 2.5	63.6 ± 2.3	19.9 ± 2.3	0.5 ± 2.2
Gle	163.9 ± 2.4	77.7 ± 2.6	93.8 ± 2.3	32.1 ± 2.5	0.6 ± 2.6
AGi	477.4 ± 2.3	559.5 ± 2.1	172.4 ± 2.5	116.1 ± 2.4	0.7 ± 2.5
Ge	151.8 ± 2.8	170.3 ± 2.5	195.02.4	101.9 ± 2.3	1.2 ± 2.2
Total	3251.5	4289.8	1602.5	1773.8	34.3

SFL: soy flour; TSP: texturized soy protein; SFI, soy fiber; PSM: powdered soy milk; LSD: liquid soy drink. For extraction conditions see Section 2.3. HPLC parameters: two monolithic columns, flow rate (5.0 mL min^{-1}), temperature (35 °C), sample volume (10 μL), gradient of water (0.1% acetic acid) and methanol (0.1% acetic acid): 0 min (0% B), 2.0 min (31% B), 4.0 min (31% B), 5.0 min (35% B), 8.0 min (35% B), 9.5 min (100% B). Identity of isoflavones as Fig. 1.

4. Conclusions

A very fast method employing monolithic columns has been developed for the analysis of all the main soy isoflavones, including the malonyl derivatives, in less than 10 min. Given the high chromatographic resolution (>1.06), the high reproducibility (R.S.D. < 0.9 %) and the low LOQ (0.80–1.96 mg L^{-1}) obtained, the method can be applied to the determination of isoflavones in several soy-derived foods.

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