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Fast analysis of isoflavones by high-performance liquid chromatography using a column packed with fused-core particles

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ABSTRACT

The recent development of fused-core technology in HPLC columns is enabling faster and highly efficient separations. This technology was evaluated for the development of an fast analysis method for the most relevant soy isoflavones. A step-by-step strategy was used to optimize temperature (25–50 °C), flow rate (1.2–2.7 mL/min), mobile phase composition and equilibration time (1–5 min). Optimized conditions provided a method for the separation of all isoflavones in less than 5.8 min and total analysis time (sample-to-sample) of 11.5 min. Evaluation of chromatographic performance revealed excellent reproducibility, resolution, selectivity, peak symmetry and low limits of detection and quantification levels. The use of a fused-core column allows highly efficient, sensitive, accurate and reproducible determination of isoflavones with an outstanding sample throughout and resolution. The developed method was validated with different soy samples with a total isoflavone concentration ranging from 1941.53 to 2460.84 $\mu g\,g^{-1}$ with the predominant isoflavones being isoflavone glucosides and malonyl derivatives.

1. Introduction

The primary role of diet is to provide sufficient nutrients to meet the nutritional requirements of an individual. However, nutritional sciences are advancing from the classical concepts of avoiding nutrient deficiencies to nutritional adequacy since there is increasing scientific evidence that consuming some foods and food components may have additional functional effects and may reduce the risk of disease and specifically contribute to maintain state of health and well being. Among such “health-enhancing” foods, soybeans and derived products (soy protein, soy milk, tofu, etc.) are attracting the attention of health professionals. Indeed, there are several epidemiological studies indicating that in Asian countries; where soybeans are consumed regularly in considerable quantities, present a lower incidence of certain diseases, such as cancer, cardiovascular diseases and osteoporosis, in relation to western countries [1–6]. Although there are plausible epidemiological indications that soy foods can potentially help reduce the risk of developing several diseases, the specific components occurring in these foods that are responsible for these potential beneficial health effects remain partially unknown. In the last decades, identification of soybeans phytochemicals that may be related to these possible positive health effects of soybeans has been, and continues to be, an active research area. In this context, several compounds were characterized in soybeans, including phytosterols, protease inhibitors, inositol hexaphosphate, saponins and isoflavones, among others [7–10].

Isoflavones are being extensively studied because of “in vitro” and “in vivo” biological activity consistent with the potential health effects associated with the consumption of soybeans. There is indication that isoflavones, at least in part and depending of several factors, may play a role on the effects of soy foods on improving health [4–6,11–16]. As a result, enormous efforts are being devoted worldwide to the evaluation of isoflavone composition in foods. However, in order to identify the potential health benefits associated with the consumption of isoflavones, it is of critical importance to have high-quality and comprehensive data. To this end, adequate analytical methodologies are essential for a reliable and exact identification as well as for quantification. Moreover, methodologies and techniques used need to keep up to date with technology to improve performance in terms of resolution, efficiency, precision, reproducibility and speed, allowing a proportionate increase in the amount and quality of information gathered [17,18].

For the determination of isoflavones in foods different techniques and methods have been developed, including gas

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chromatography, high-performance liquid chromatography (HPLC), capillary electromigration techniques, and immunoassays. In the last few years several reviews about the analysis of isoflavones using these techniques have been published [18–23].

Among these techniques, HPLC (with and without mass detectors) is the method of choice because it requires simple sample preparation, allows quantification of all isoflavone chemical forms in a timely fashion, is highly efficient and reproducible, widely available and has been extensively studied. The great majority of HPLC methods used for the separation of isoflavones are carried out by using reversed-phase columns with methanol or acetonitrile and water containing a small amount of acid (formic, acetic, phosphoric or trifluoroacidic acids) as mobile phase. Since all isoflavones exhibit maximum UV absorption in the range of 240–270 nm, UV and photo diode array are the most widely used detectors. Gradient elution is usually necessary in order to separate all main isoflavones since they are structurally close and isocratic elution has proven to be insufficient [18–23].

Without any doubt, the most important aspect of any chromatographic separation is the column. For the analysis of isoflavones this is also true. The great majority of methods use conventional 5 μm, C18 microparticulate columns of 250 mm and require approximately 1 h to completely separate all main isoflavones [18–23]. Nevertheless, in the last decade column technology has undergone constant and significant developments motivated by the need of higher sample throughput, analyte sensitivity, selectivity and resolution. In this aspect, there is an ever decreasing column packing particle size trend that is particularly relevant for the analysis of phytochemicals in foods, such as soy isoflavones. Although reduction of particle size results in improvement of efficiency, optimum lineal velocity and mass transfer, it also causes a significant increase in column back pressure. Particle size reaches the pressure capacity of “conventional” HPLC systems at 3–5 μm limiting both column length and flow rates to improve separation and reduce analysis time [24–28]. To explore very small particle size of column (<2 μm) and their potential to deliver higher efficiency and faster chromatographic analysis new systems capable of withstanding up to 1200 bar of pressure have been developed, with higher instrumentation and consumables costs.

Another alternative to improve separation efficiencies and speed without reducing particle size is the use of superficially porous particles, also termed fused-core particles. This technology generally consists of using techniques sol–gel to obtain a homogeneous porous layer on a solid core of silica. It has been suggested that the higher efficiencies of these type of particle resides on the smaller diffusion distance and improved mass transfer (smaller C term of van Deemter/Knox type equations), lower internal porosity (smaller B term) and narrower particle size distribution and better packing (smaller eddy diffusion or A term). Columns packed with fused-core particles at approximately 2.6 μm can provide speed and efficiency similar to columns packed with sub-2 μm particles. With reduced backpressures, it is possible to use columns packed with fused-core particles without updating HPLC instrumentation [27–33].

However, since fused-core columns have become commercially available only recently, there only a few reports and applications to real samples using this technology, mostly for pharmaceuticals and biological samples [34–38]. In this context, there are even fewer reports of the use of the new technology for natural products [39–43] and in the specific case of soy isoflavones there are apparently no reports to date. Therefore, the objective of this study was the development of an fast, rugged and sensitive analytical method for the determination of all main soy isoflavones.

2. Material and methods

2.1. Chemicals and solvents

HPLC grade ethanol, methanol and acetonitrile were purchased from VWR (West Chester, PA, USA). Acetic acid (96%) was obtained from Merck (Darmstadt, Germany). Ultra pure water was supplied by a Mili-Q water Advantage A10 purifier system from Millipore (Bedford, MA, USA). Isoflavones daidzin (Di), glycitin (Gly), genistin (Gi), Daidzein (De), glycitein (Gle) and genistein (Ge) were purchased from LC Labs (Woburn, MA, USA) while malonyl daidzin (MDi), malonyl glycitin (MGly), malonyl genistin (MGi), acetyl daidzin (ADi), acetyl glycitin (AGly), acetyl genistin (AGi), were purchased from Wako Chemicals (Neuss, Germany) stored at −32 °C until use. Their chemical structure and respective abbreviations used are shown in Fig. 1. Purity of isoflavone glucosides and aglycones was higher than 99%, while purity of malonyl and acetyl glucosides was higher than 90%. Stock solutions were prepared in methanol and stored at −80 °C.

2.2. Samples

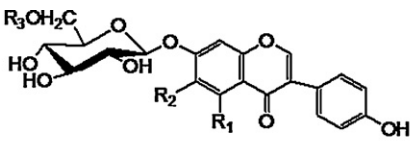
Two different yellow soybean and texturized soy protein samples were purchased from a local supermarket and stored at −32 °C until used as sample.

2.3. Sample preparation

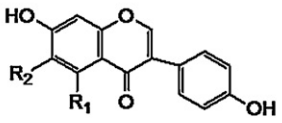
The protocol used for the extraction of isoflavones from the soy samples was based on the conditions optimized by Rostagno et al. [44]. Briefly, it consisted of extracting 0.5 g of sample with 25 mL of 50% ethanol during 20 min at 60 °C. Extractions were carried out on a multi-frequency (25 and 45 kHz) ultrasonic bath (Transsonic TH-I-55, Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) operating at 25 kHz at 100% intensity output. After the extraction, the samples were centrifuged at 10 °C for 10 min at 4000 rpm on a Universal 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). After the extraction, the sample was filtered through 0.2 μm nylon syringe filter (VWR, West Chester, PA, USA).

2.4. High-performance liquid chromatography

The HPLC-UV analysis was carried out on a Waters system (Waters Corp., Milford, Massachusetts), consisting of separation module (2695) with integrated column heater and auto-sampler and a photodiode array detector (2998). Analyses were performed on a fused-core type column (Kinetex™ C18, 2.6 μm, 100 Å, 100 mm × 4.6 mm, Phenomenex, Torrance, CA, USA). UV absorbance was monitored from 200 to 400 nm. Injection volume was 10 μL. The software for control of equipment and data acquisition was Empower 2 version 6.10.01.00. 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. The standard curve (7 points) of each isoflavone was prepared by plotting the concentration against the area. Regression equations and correlation coefficient (r^2) were calculated using Microsoft Excel 2007 software. Detection and quantification limits were determined by considering a value of 3 and 10 times the deviation of background noise obtained from blank samples ($n = 10$) dividing by the slope of the calibration curve line, respectively [45]. For the optimization of the method, different mobile phase compositions, temperatures (25–50 °C), flow rates (1.2–2.7 mL min^{-1}), and equilibration times (1–5 min) were tested using a standard mixture with all isoflavones. Concentration of isoflavones in the



Glucosides



Aglycones

Isoflavone	Abbrev.	R1	R2	R3
Malonyl Daidzin	MDi	H	H	$COCH_2COOH$
Malonyl Glycitin	MGly	H	OCH_3	$COCH_2COOH$
Malonyl Genistin	MGi	OH	H	$COCH_2COOH$
Acetyl Daidzin	ADi	H	H	$COCH_3$
Acetyl Glycitin	AGly	H	OCH_3	$COCH_3$
Acetyl Genistin	AGe	OH	H	$COCH_3$
Daidzin	Di	H	H	H
Glycitin	Gly	H	OCH_3	H
Genistin	Gi	OH	H	H

Isoflavone	Abbrev.	R1	R2
Daidzein	De	H	H
Glycitein	Gle	H	OCH_3
Genistein	Ge	OH	H

Fig. 1. Chemical structures of isoflavones and abbreviations.

Table 1
Concentration of isoflavones in the standard mixture used for the optimization of the chromatographic method.

Isoflavone ($mg\,L^{-1}$)											
Di	Gly	Gi	MDi	MGly	ADi	AGly	MGi	De	Gle	AGi	Ge
7.96	8.01	8.65	6.25	6.81	7.96	17.59	4.87	12.57	11.64	6.97	4.86

standard mixture is presented in Table 1. Column efficiency was evaluated on basis of retention time, peak width, capacity factor (K'), separation factor (α), resolution (R_s) and peak asymmetry (A). Resolution and peak asymmetry were calculated using the European Pharmacopeia (EP) standard.

3. Results and discussion

3.1. Initial conditions

The mobile phase selection was based on a previous series of experiments using water (solvent A) and methanol or acetonitrile (solvent B) with different amounts of acetic acid (0–2%) in both solvents (A and B). Using pure acetonitrile, the system back pressure was 38.8% lower than with pure methanol ($1.2\,mL\,min^{-1}$ and 25 °C). A similar difference in pressure (36.79%) was also obtained with different proportions of these solvents and water during the gradient, although at different proportions. The highest pressure using mixtures of methanol or acetonitrile and water was obtained with 40% of methanol (4547 psi) and 20% of acetonitrile (2874 psi). Therefore, acetonitrile was selected as mobile phase B in order to take advantage of the lower back pressure generated in the later stages of the optimization process to increase flow rate and reduce analysis time. On the other hand, the amount of acetic acid in the mobile phase also influenced separation and the mobile phase composition where a significant gain in resolution was observed using acidified water (1% acetic acid) (solvent A) and pure acetonitrile (solvent B). For the separation of all isoflavone by the above mentioned solvents, several different gradients were tested with a flow rate of $1.2\,mL\,min^{-1}$, 25 °C and an equilibration time of 5 min. Initial gradient conditions were based on the method previously reported by Chang et al. [45] using a conventional C_{18} particle column.

After adjusting the conditions to flow rate, column dimensions and several trial-and-error experiments to optimize the gradient, separation of all isoflavones was achieved in approximately 18.42 min using the following gradient: 0 min, 8% B; 2.33 min, 15% B; 8.0 min, 17% B; 15,33 min, 24% B; 20.0 min, 50% B; 23.0 min; 100% B; 28.0 min, 100% B; 30.0 min, 8% B. A good separation of all isoflavones was achieved with very narrow and symmetric peaks. Retention time of all isoflavones ranged from 4.46 min (Di) to 18.42 min (Ge), peak width from 15.2 (Gly) to 31 s. (MGi) and mean peak separation was 1.27 min. During the optimization of the gradient, isoflavones in the center of the chromatogram, ADi, AGly and MGi, were the most difficult to resolve from each other, followed by the two first peaks (Di and Gly). Rostagno et al. [47] also reported similar findings when studying the separation of soy isoflavones using a monolithic column and suggested that separation of these compounds is critical to achieve fast separations.

3.2. Column temperature

Once methodological conditions allowed separation of all isoflavones, a strategy to reduce analysis time was adopted based on higher temperatures and flow rates. The use of higher temperatures is a useful tool to reduce analysis time since mobile phase viscosity is significantly reduced which in turn decreases the pressure drop across the column allowing higher linear velocities of the mobile phase. Also, as known by the Strokes–Einstein relationship, the diffusion coefficient is directly proportional to the absolute temperature and inversely proportional to the viscosity. The lower viscosity and higher diffusivity of a mobile phase at high temperatures produce much lower mass transfer resistance, thereby decreasing the peak width and leading to flatter van Deemter curves. A flatter van Deemter curve allows the use higher linear velocities without affecting column efficiency [28]. There-

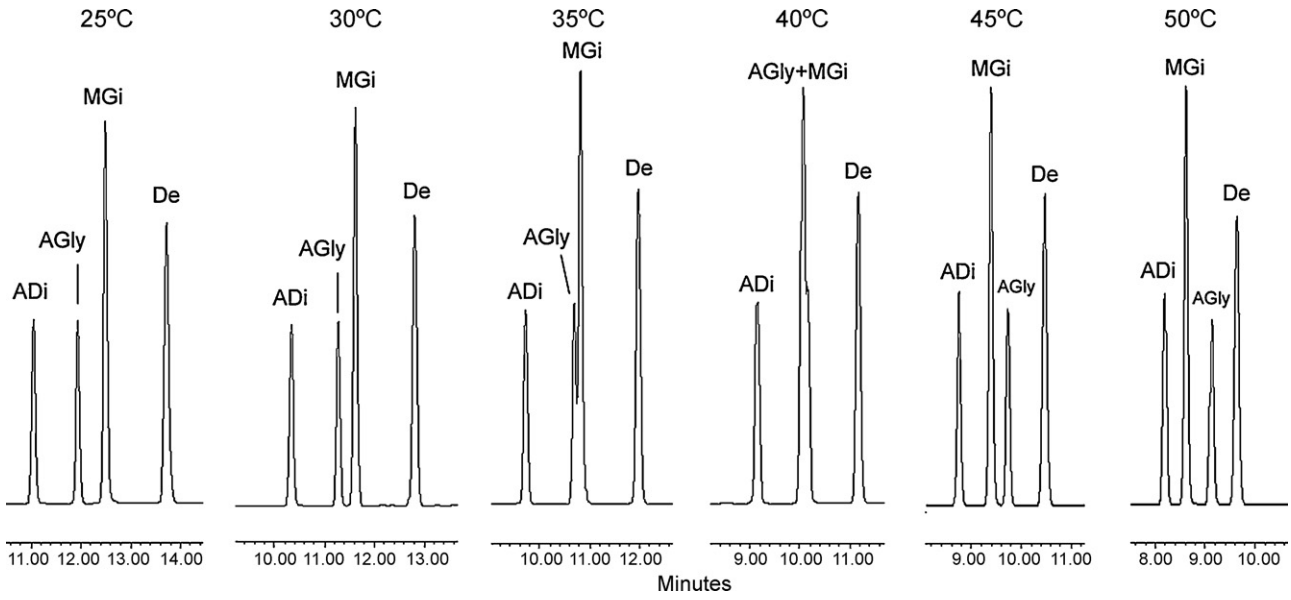


Fig. 2. Effect of column temperature in elution order of isoflavones.

fore, by increasing column temperature there is an improvement of analyte resolution through an increased diffusion coefficient of the mobile phase and a lower mass transfer resistance. However, it is also important not to exceed the column maximum operating temperature (60 °C) since it may significantly reduce expected column life. Therefore 50 °C was selected as the maximum working temperature.

Based on these principles and limitations, column temperature was gradually increased from 25 to 50 °C, in 5 °C intervals. Increasing column temperature to 30, 35, 40, 45 and 50 °C from 25 °C resulted in a mean reduction of retention time of all analytes of 8.08, 13.23, 18.12, 21.87 and 26.77%, respectively. The retention time for last eluting peak (Ge) in these temperatures was 17.93, 17.33, 16.50, 15.64 and 14.66 min, respectively. In this context, the smallest reduction of retention time caused by the increase of the temperature was observed for Ge, while the highest reduction was observed for MDi. This behavior can be explained not only by the effect of the temperature on viscosity of mobile phase but also on the solubility of the specific isoflavones on the mobile phase and to the coupled effect of reduction of retention time and relative concentration of organic solvent in the mobile phase in the gradient.

Moreover, increasing column temperature resulted in some particularly interesting findings. The increase of temperatures affected retention time of some isoflavones more than others to a point that at 35 °C some peaks overlapped and above this temperature changes in elution order were observed. As can be seen in Fig. 2, at 25 and 30 °C, MGi, eluted after AGly, between 35 and 40 °C peaks were overlapped and above 40 °C MGi elute before AGly. During the temperature increase, it was observed that mean reduction of retention time for MGi is 23.6% higher than for AGly. This effect can also be attributed to an increased solubility of MGi in the mobile phase when compared to AGly. These observations can have some practical aspects for the analysis of isoflavones. As previously mentioned, separation of ADi, AGly and MGi can be troublesome [47], and to solve selectivity problems, especially for the separation of these critical pairs of isoflavones, increasing column temperature can be an attractive alternative. It also evident that depending of chromatographical conditions, elution order may change and therefore researchers should be careful when adjusting literature information to identify peaks.

Regarding peak width and height there was a variable response depending of specific isoflavones and temperatures and no clear trend was observed. In general, higher temperatures reduced peak width and increased peak heights of more polar isoflavones, while the less polar ones saw a lower effect, especially at higher temperatures. However, it is noteworthy that the gradient used was kept constant and therefore an increase in mass transfer rates and reduction of retention time and peak width (and indirectly peak height) caused by the increase in the temperature can be, to some extent, undermined by the lower concentration of the organic solvent by the time the analytes are released from the stationary phase. This produces an increase of the diffusion coefficient and a higher mass transfer resistance due to the lower relative polarity of the mobile phase. The higher analyte diffusion and mass transfer resistance is translated in wider peaks and lower peak heights. This outcome is especially important for the less polar isoflavones and can be clearly observed for Ge peak. In this case, Ge peak width increases and height decrease as higher temperatures are used and reduces retention time. This negative effect on late eluting peaks can be potentially solved by adjusting the gradient proportionally to the reduction of retention time caused by the increase in the temperature.

Another obvious observed effect of increasing column temperature was the reduction of peak separation (separation in minutes between peak pairs). Increasing temperature from 25–30, 35, 40, 45 and 50 °C resulted in a mean reduction of peak separation of 6.52, 6.67, 8.98, 20.91 and 22.88%, respectively. These arrays refer only to resolved peaks in temperatures between 30 and 50 °C. This finding was expected since as total analysis time is reduced, separation between peak pairs is smaller. However, the effect of temperature on separation between peak pairs was variable and depended of specific compounds. In general, separation between the least polar isoflavones, De, Gle, AGi and Ge, increased with the increase in the temperature, while there was a reduction of separation of early eluting isoflavones. This tendency may be, at least in part, explained by the constant gradient maintained with different temperatures. As retention of compounds is reduced by the increasing temperature, there is a lower concentration of organic solvent in the mobile phase when each peak eluted from the stationary phase. The less polar is each isoflavone, the higher is the influence of the composition of the mobile phase on its retention and therefore, separation

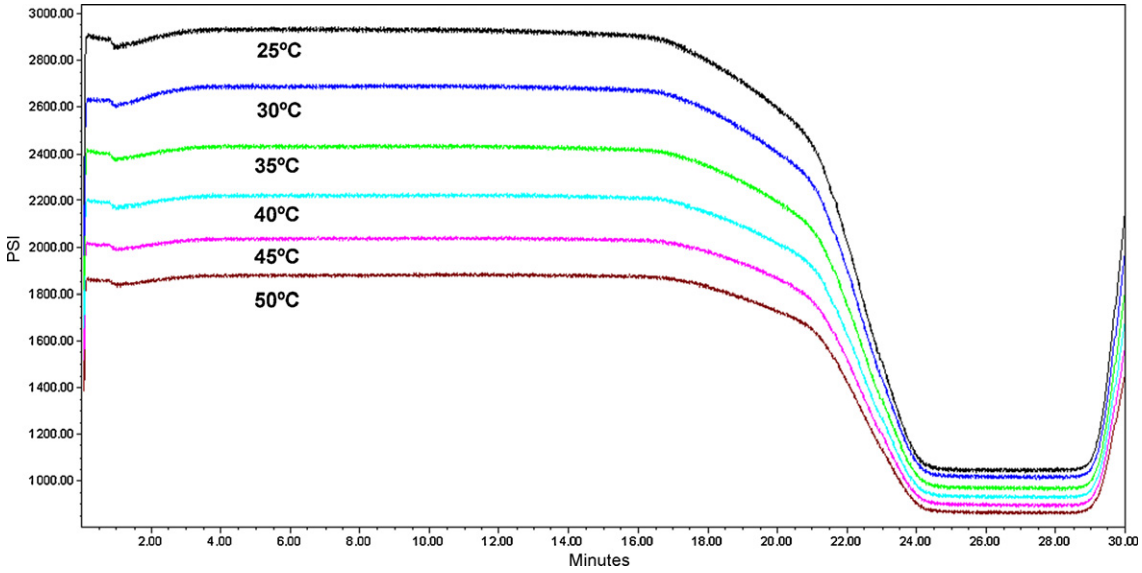


Fig. 3. System back pressure using different column temperatures.

between peaks increases. However, since the reduction effect of the temperature on separation of the more polar isoflavones is more pronounced than on the separation of less polar, mean separation among all peak is lower, which is reflected in the above mean values.

On the other hand, a direct correlation was observed between the increase of the temperature and column back pressure (Fig. 3). Increasing temperature from 25 to 30, 35, 40, 45 and 50 °C resulted on a reduction of column back pressure of 8.47, 16.95, 24.58, 30.51 and 35.59%, respectively. These reductions were not linear and differences were greater at lower temperatures, between 25 and 30 °C and between 30 and 35 °C. However, it could be considered that each temperature degree increased results on a mean pressure reduction of 1.42%. The importance of the pressure reduction caused by temperature is that it allows using a higher linear mobile phase velocity, which can be explored to reduce analysis time. Therefore, considering the reduction of retention time, peak width (and consequent increase of peak heights), and pressure while maintaining an excellent separation of all isoflavones and peak symmetry, higher temperatures (i.e. 50 °C) can be considered as the most appropriate for the development of fast analysis methods.

3.3. Flow rate

Once optimum temperature was selected, the reduced column back pressure allowed exploring flow rate in order to shorten analysis time. Consequently, flow rate was step-by-step increased from 1.2 to 2.7 mL min^{-1}. Maximum flow rate was determined by the system pressure's limitation, which was 5000 psi. As flow rate was increased, a proportional reduction of the gradient was applied in order to maintain separation of all peaks. For example, if flow rate was doubled, the gradient time was reduced to half while maintaining the same percentage of solvents of the mobile phase. The increases of flow rate and consequent increase of pressure and adjustment of gradient are graphically represented in Fig. 4. The pressure profile represented includes the adjusted gradient and the time required to clean the column (1 min). A proportional adjustment of the gradient to the increase of flow rate maintained the separation between compounds although separation between peaks was smaller, caused by the shorter analysis time. When compared to initial conditions (50 °C, 1.2 mL min^{-1}), mean retention time, peak width and separation time reduction were 51.76, 38.48 and 55.24%, respectively, at the highest flow rate (2.7 mL min^{-1}). Retention time of the last eluting isoflavone (Ge) was 6.15 min, equivalent to 2.38 times less than initial conditions, which is proportional to an increase of 2.25 times of flow rate (1.2–2.7 mL min^{-1}). However, since the last eluting peak was far from the preceding peak (AGi), the final part of gradient was adjusted in order to reduce analysis time. This process was accomplished by a step increase of the solvent B percentage at the retention time of the previous peak. However, a too steep increase in the concentration mobile phase caused baseline to drift and therefore we used an intermediate option, where analysis time was reduced without seriously affecting baseline. Therefore, the optimized gradient was 0 min, 8% B; 1.04 min, 15% B; 3.56 min, 17% B; 4.56 min 19.2% B, 5.50 min, 50% B; 6.50 min% B, 7.50 min% B; 8.50 min% B. The gradient includes 1 min at 100% of mobile phase B for column clean-up (6.50–7.50 min) and 1 min to return to initial conditions (7.50–8.50 min).

3.4. Re-equilibration time and reproducibility

Total analysis time is the amount of time from injection to injection and includes both the run time, column clean-up and re-equilibration time. Re-equilibration time is necessary in gradient HPLC in order to ensure that the column environment has returned to initial stable conditions. This condition is particularly important when using gradient elution since the difference between initial and final organic composition of the mobile phase is significant. The importance of equilibration time is even greater since the failure to optimize re-equilibration time can lead to unnecessary overextension of analysis time, with the increased cost and reduced sample throughput associated. Usually, equilibration time is recommended by manufacturers on basis of the column volume and flow rate. Standard recommendation is approximately 10 times the column volume, although it depends of the applications and more importantly the mobile phases and gradient used. In this study, all previous sets of experiments were carried out using 5 min between runs, which is equivalent to approximately 40% of the total method duration (including elution, clean-up and re-equilibration times) and equivalent to 14.8 volumes of the column. Therefore, in order to optimize this important and time consuming component of the overall method duration, shorter re-equilibration times (1–4 min)

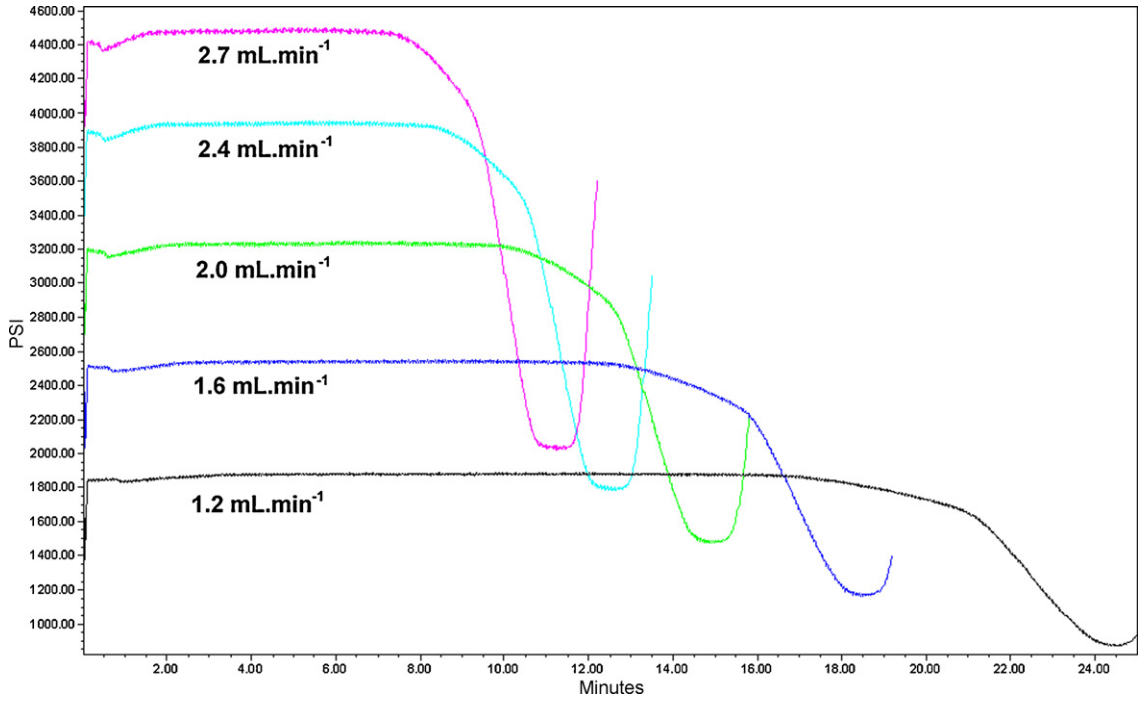


Fig. 4. System back pressure using different flow rates.

were evaluated. Equilibration time was implemented as a delay after the mobile phase composition returned to initial conditions (i.e. 8.50 min), after which a new sample (mixture of standards) was injected into the column.

Using 5 min to re-equilibrate the column between runs provided a mean (intra and interday) area and retention time variability lower than 0.272 and 0.221%, respectively. Reducing re-equilibration time to 4, 3, 2 and 1 min resulted on a mean area variability lower than 0.283, 0.425, 0.382 and 0.807%, and mean retention time variability lower than 0.339, 0.300, 0.411 and 0.376%, respectively. On the other hand, there was no clear trend regarding the isoflavones with higher and lower variability. Using 5 min, for instance, the highest retention time variability was observed for Gi and the lowest for AGly, while using 4 min they were observed for Gly and Ge, using 3 min they were observed for ADi and Ge, using 2 min they were observed for MDi and Ge while using 1 min they were observed for Di and Gle. These results indicate that this variability can be attributable to the chromatographic system capability of delivering a reproducible solvent mixture at relatively high flow rates (2.7 mL min^{-1}) rather to the intrinsic variability of the stationary phase itself. The chromatographic system manufacturer (Waters) states that normal solvent delivery accuracy (including solvent mixture and flow rate) range between 0.5 and 1% (RSD). Of course this outcome will depend of flow rate and pump stroke volume (set to 100 μL in this case), but the observed variability is reasonable within normal reproducibility range of the chromatographic system. Although using very short re-equilibration times variability was within the normal range, a

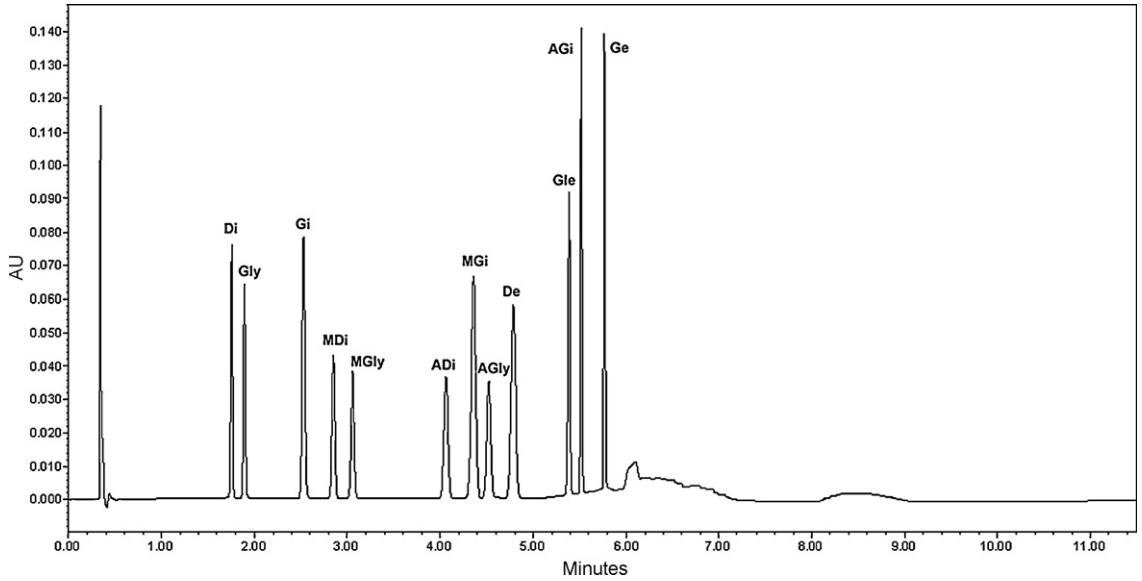


Fig. 5. Representative chromatogram of the developed method.

Table 2
Chromatographic properties of the optimized method.

Isoflavone	Retention time (min)	RSD tr	Theoretical plates/meter (Nm^{-1})	Asymmetry A	Retention factor, K'	Selectivity factor, α	Resolution, R_s	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
Di	1.75	0.33	101.46	1.08	4.14			23.13	77.08
Gly	1.89	0.34	109.96	1.04	4.56	1.10	3.82	22.58	75.27
Gi	2.53	0.28	96.38	1.01	6.46	1.42	14.00	25.00	83.34
MDi	2.85	0.26	102.27	1.04	7.40	1.15	5.65	12.63	42.1
MGly	3.07	0.23	103.05	0.98	8.04	1.09	3.51	31.84	106.13
ADi	4.07	0.19	109.84	0.96	11.03	1.37	13.95	239.07	796.89
MGi	4.36	0.18	106.68	0.96	11.88	1.08	3.37	25.25	84.16
AGly	4.52	0.16	146.51	0.94	12.36	1.04	1.98	5.95	19.83
De	4.78	0.12	116.77	0.94	13.11	1.06	2.95	11.9	39.66
Gle	5.39	0.06	590.47	0.95	14.87	1.13	8.52	17.27	57.57
AGi	5.52	0.07	1951.44	1.06	15.26	1.03	3.65	3.34	11.14
Ge	5.76	0.07	2082.49	1.04	15.98	1.05	9.22	60.3	200.99

slight higher reproducibility for some isoflavones was observed using equilibration times higher than 2 min, and therefore 3 min can be considered as the most appropriate in order to achieve the highest possible reproducibility while not overextending total run time. This equilibration time is equivalent to 8.90 times the column's volume and slightly lower than the recommended. However, depending of the application and the reproducibility requirements of the analysis at hand, it is possible to increase sample throughout by using re-equilibration times between 1 and 3 min achieving good quality data.

3.5. Optimized method

A representative chromatogram is presented in Fig. 5 and the properties of the developed method are reported in Table 2. Retention time of main soy isoflavones ranged from 1.75 (Di) to 5.76 min (Ge), representing an almost ten times reduction of analysis time when compared to conventional methods. Illustratively, retention time of Ge of a recently proposed AOAC method is approximately 51 min [48]. On the other hand, when comparing the performance of the developed method with the fastest method reported to date [18,46] obtained using a monolithic column, reduction of analysis time was almost 2 times. Moreover, total analysis time (sample-to-sample), including clean-up, returning of initial conditions and re-equilibration time, is only 11.5 min, with very low mean retention time and area variability (0.33 and 0.69%, respectively), narrow (mean peak width = 7.48 s) and symmetric peaks (mean asymmetry = 1.00). Peak width ranged from 5.07 (AGi) to 10.93 s (AGly), with a variability lower than 3.90%. It was also observed that early and late eluting isoflavones (Di, Gly, AGi, Ge) are narrower and more symmetric than isoflavones in the middle of the chromatogram (ADi, AGly, MGi and De) which may, at least in part, explain the difficulty for their separation. In general, the developed method presented good chromatographical performance, with high plate count, retention factors, selectivity and resolution. Mean values of these chromatographic parameters were 468.12, 10.42, 1.14 and 6.42, respectively. Finally, response was linear between 0.05 and 50 $mg\,L^{-1}$ (seven points curve) for all isoflavones and regression coefficients (r^2) were higher than 0.9955. Also, very low limits of detection and quantification were achieved, ranging from 3.34 (AGi) to 239.07 (ADi) and from 11.14 (AGi) to 796.89 $\mu g\,L^{-1}$ (ADi), respectively.

Although the developed method allows an fast separation of all major soy isoflavones with excellent chromatographical performance, there is still potential to be explored to further reduce total analysis time in future research. Temperature used in the proposed method is 10 °C below the maximum temperature recommended by the manufacturer (60 °C). Based on the results presented in this work, increasing 10 °C can reduce retention time by 10.7% approximately and column back pressure by 14.20%. The reduction of pressure can be used to increase flow rate and reduce total analysis time, and thus combining these two strategies (higher temperature and flow rate) it is feasible to expect a combined reduction of analysis time of 24.9% (8.64 min). However, this reduction of the analysis time would come at the expense of column life, since it is at the limit of the supplier's recommended temperature. Another aspect regarding the flow rate used in the proposed method is that it was limited by the maximum pressure allowance of the chromatographic system used in this study (5000 psi). If a different conventional HPLC system, with a higher allowance is used (i.e. 8700 psi), higher linear velocities can be implemented and it is expected that it can lead to at least 1.5 times reduction of analysis time (5.76 min). This would be possible because our system pressure limitation was well below the column's maximum recommended pressure (8700 psi). Obviously, any increase in the flow rate will require a proportional adjustment of the gradient to maintain separation.

3.6. Real samples

Since the developed method was optimized using a standard mixture, it is of critical importance that it is validated with different real samples. The importance of this validation underlies in that real samples are complex matrices and extraction methods are often not selective and other compounds may co-elute or interfere with separated isoflavones using the optimized method. Therefore, extracts of two soybean (soybean sample 1 (SS1) and 2 (SS2)) and two texturized soy protein (TSP1 and TSP2) were analyzed with the developed method. Chromatograms of the samples SS1, SS2, TSP1 and TSP2 are illustrated in Fig. 6(A–D), respectively and the individual isoflavone concentration of each sample is shown in Table 3. Retention time and UV spectra of separated isoflavones coincided with the respective standards. Peak purity analysis indicates that other compounds are not co-eluted with isoflavone peaks despite the variety of the samples. Total isoflavone concentration in the samples ranged from 1941.53 (SS1) to 2460.84 $\mu g\,g^{-1}$ (SP2). It can be observed in Table 3 that in all samples, malonyl and glucosides as well as genistin derivatives were the predominant isoflavones, and in some cases, the only detected chemical forms. Acetyl isoflavones were present in high concentration in both texturized soy protein samples and in particular high concentration in TSP2, indicating that both samples were submitted to a high dry temperature treatment during processing [18]. Also, the highest mean variability was observed for the texturized soy protein samples, although the last eluting peaks (AGi and Ge) of the soybean sample 2 (SS2) showed the highest variability of all isoflavones. In all samples, concentration variability of individual isoflavones was below 5%.

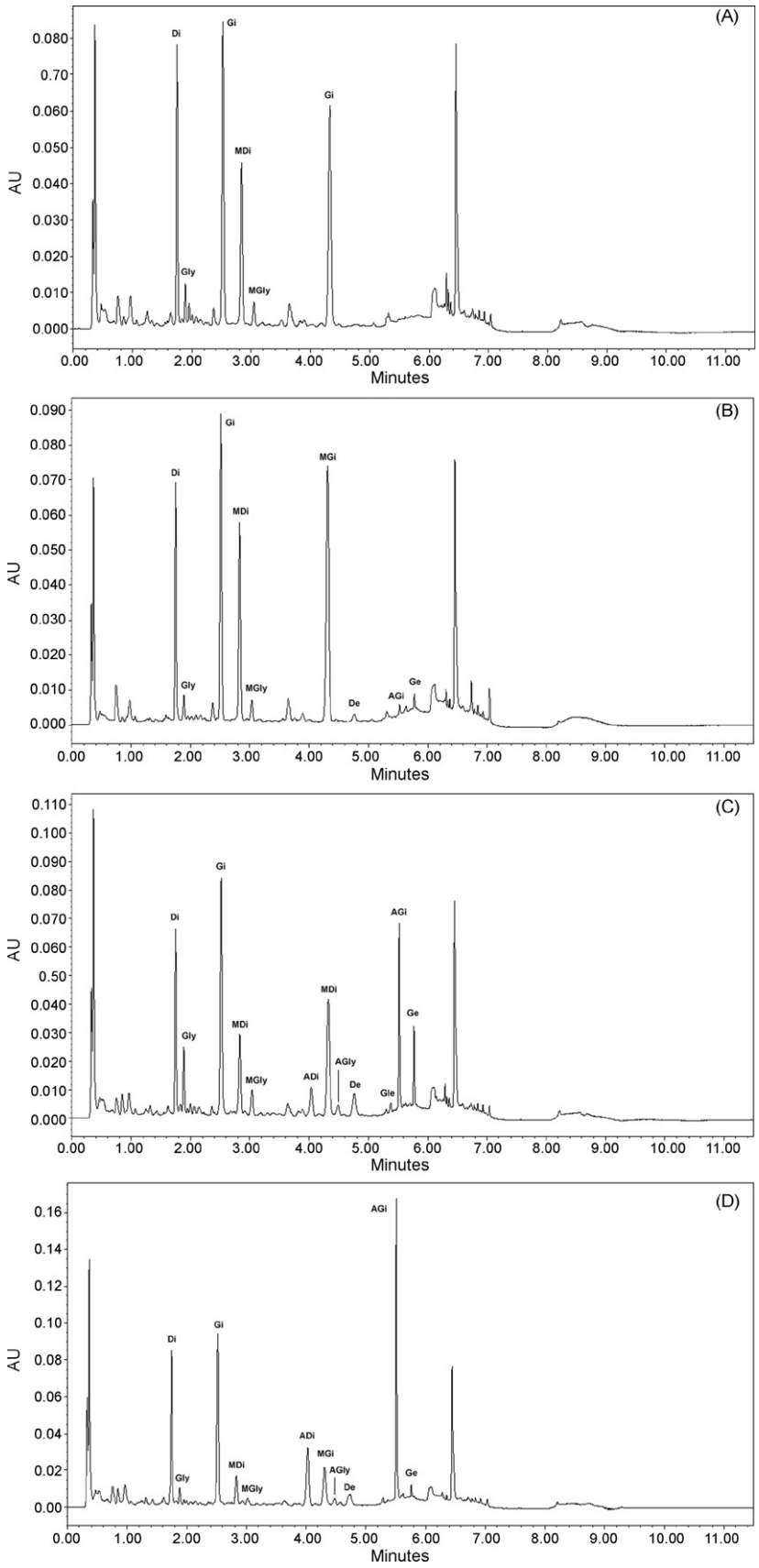


Fig. 6. Chromatograms of the samples obtained with the optimized method. Soybean sample 1 (A), soybean sample 2 (B), texturized soy protein sample 1 (C) and texturized soy protein sample 2 (D).

Table 3
Isoflavone concentration in the samples analyzed with the optimized method ($n = 2$). SS1: soybean sample 1; SS2: soybean sample 2; TSP1: texturized soy protein sample 1; TSP2: texturized soy protein sample 2.

Isoflavones	Samples							
	SS1		SS2		TSP1		TSP2	
	$\mu g\,g^{-1}$	RSD	$\mu g\,g^{-1}$	RSD	$\mu g\,g^{-1}$	RSD	$\mu g\,g^{-1}$	RSD
Di	458.28	0.71	404.90	0.09	404.24	2.88	500.81	1.03
Gly	99.07	3.05	70.98	0.87	176.84	2.41	87.46	0.89
Gi	484.37	0.75	483.50	0.02	492.26	3.74	539.67	1.85
MDi	340.59	0.14	407.94	0.19	211.09	2.50	137.48	1.01
MGly	83.12	0.08	76.08	0.39	105.53	3.04	64.83	1.52
ADi	n.d.	n.d.	n.d.	n.d.	136.06	1.18	349.37	0.82
MGi	476.11	0.40	537.55	0.07	328.75	3.92	163.29	1.40
AGly	n.d.	n.d.	n.d.	n.d.	72.45	0.76	61.42	2.88
De	n.d.	n.d.	20.07	1.20	96.56	3.17	72.20	3.60
Gle	n.d.	n.d.	n.d.	n.d.	35.61	2.74	n.d.	n.d.
AGi	n.d.	n.d.	13.52	4.35	174.21	3.53	451.69	1.22
Ge	n.d.	n.d.	29.36	4.14	65.64	2.42	32.63	0.89
Total	1941.53		2043.92		2299.23		2460.84	

4. Conclusions

In the present study, a step-by-step optimization strategy of chromatographical parameters (mobile phase composition, temperature, flow rate, gradient and re-equilibration time) was used to develop an fast and reproducible analysis method for the determination of the 12 main soy isoflavones. Separation of all compounds was achieved in about 5.8 min and total analysis time, including column clean-up and re-equilibration time, was completed in less than 12 min. The optimized method showed an excellent chromatographic performance in terms of resolution, peak symmetry, reproducibility, quantification and detection levels and was successfully used for the analysis of different real samples with similar performance. The combination of state-of-the art column technology and optimized conditions significantly increased sample throughout in standard chromatographic systems when compared to conventional methods. Based on the results gathered during the method development, it is clear that fused-core column technology still has a great potential to deliver faster and more sensitive methods for the analysis of isoflavones and other natural products and that it shows a promising future in this field. Considering the excellent performance of fused-core columns, it can be expected that once explored for different analytes from a wide range of matrices, the number of applications will exponentially increase in the near future.

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