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Solid-phase extraction of soy isoflavones☆

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Abstract

An automated method using solid-phase extraction (SPE) for the concentration and clean-up of soy isoflavone extracts is proposed in this work. Using a standardized sample (0.1 g of a freeze dried soybean extract/25 mL of water); eight SPE cartridges with a wide range of sorbents (C_{18}, divinylbenzene and modified divinylbenzene) from different suppliers were evaluated and compared. A large variation on SPE cartridges performance was observed, especially regarding retention and breakthrough volume of isoflavones during sample load and washing steps. The most effective cartridges were the divinylbenzene based cartridges, especially Strata X (from Phenomenex) and HLB oasis (from Waters). Using Strata X cartridges, several extraction parameters, such as sample loading flow (5–15 mL min^{-1}), extracting solvent volume (2–6 mL of methanol), pH of the extracting solvent and the necessity of drying the sorbent before elution, were evaluated to provide a fast, specific, quantitative and reproducible SPE method. The optimized method consists of conditioning the cartridge with 10 mL of methanol and 10 mL of water (10 mL min^{-1}), loading 25 mL of the standardized extract onto the cartridges (5 mL min^{-1}), washing the cartridge with 10 mL of water (10 mL min^{-1}) and finally eluting with 4 mL of methanol (10 mL min^{-1}). Mean isoflavones recovery was 99.37% and mean intra- and inter-day reproducibility was higher than 98%. The developed sample clean-up/concentration (6.25:1) method takes less than 10 min and can be used in the analysis of isoflavones from soy extracts.

Keywords: Isoflavones; Solid-phase extraction; HPLC; Soy extracts

1. Introduction

Isoflavones are naturally occurring phenolic compounds found in soybeans. There are 12 main isoflavones in soybeans (Fig. 1): genistin, daidzin, glycitin and their respective malonyl, acetyl and aglucone forms [1]. The interest in soy isoflavones is increasing due to health claims related with the consumption of soybeans, such as prevention of certain cancers, reduction of cardiovascular disease risk and improvement of bone health. The isoflavones are thought to be related with these health protective effects, although the action mechanisms are yet to be identified [2,3].

Several extraction methods for these compounds have been proposed, from classical soxhlet and stirring to methods using new technologies, like ultrasound assisted extraction [4,5], supercritical fluid extraction [6,7] and pressurized liquid extraction [8,9]. However, a common drawback of these extraction methods is that additional clean-up procedures are often required before chromatographic analysis [10–12].

Solid-phase extraction (SPE) is a simple sample preparation technique that can be used to clean-up extracts as well as for extracting/concentrating soy isoflavones from liquid foods and extracts. Although SPE applications for the analysis of isoflavones from blood, plasma [13], urine [14] and serum [15] are relatively common, only a few works explored the SPE potential for the analysis of isoflavones from foods and most of them in the last 5 years.

Mitani et al. [16], for example, proposed an automated on-line in tube solid phase microextraction coupled to high-performance liquid chromatography (HPLC) for the determination of daidzein and genistein in soy foods. Also, an on-line SPE and HPLC–MS method was used by Doerge et al. [15], for the determination of isoflavone content of standardized

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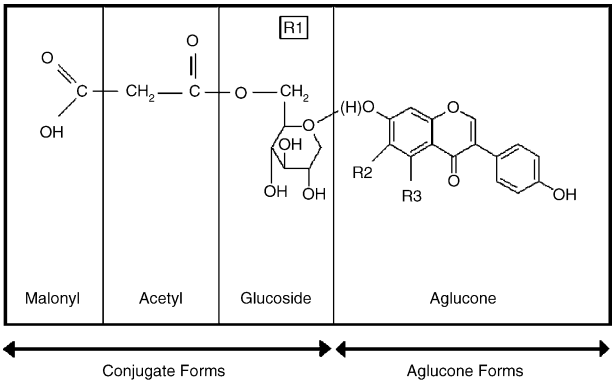

Isoflavone	Symbol	R1	R2	R3
Genistein	Ge	H	H	OH
Daidzein	De	H	H	H
Glycitein	Gle	H	OCH_3	H
Genistin	Gi	$C_6O_5H_{11}$	H	OH
Daidzin	Di	$C_6O_5H_{11}$	H	H
Glycitin	Gly	$C_6O_5H_{11}$	OCH_3	H
Acetyl-genistin	AGi	$C_6O_5H_{11}$+$COCH_3$	H	OH
Acetyl-daidzin	ADi	$C_6O_5H_{11}$+$COCH_3$	H	H
Acetyl-glycitin	AGly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

animal chow. Besides of these methods, a trace determination method for genistein and daidzein in infant formula powders using SPE was recently proposed [17].

With the aim of cleaning the sample, Genovese and Lajolo [18] employed a simple SPE method with a polyamide sorbent in the determination of isoflavone concentration in soy foods consumed in Brazil. Another simple method was used by Wang and Sporns [19] for the extraction of isoflavones from soy foods before matrix-assisted laser desorption/deonization time-of-flight mass spectrometry (MALDI–TOF MS) analysis. Rather than propose a SPE method, these authors employed the technique to provide a clean concentrated extract to be used in the chromatographic analysis. Unfortunately, the SPE methods were not evaluated.

Regarding the performance of SPE cartridges, Klejdus et al. [20] compared classical sorbents and a new polymeric sorbent (e.g. Water Oasis HLB extraction cartridges) for determination of isoflavones in red clover. The use of polymeric sorbents for sample pre-treatment enabled higher recoveries, higher reproducibility, and lower consumption of plant materials for the HPLC analysis than classical sorbents. The same authors [21] used a similar SPE method for identification of isoflavones conjugates in red clover extracts.

There are several new polymeric sorbents available that has not been evaluated for soy isoflavones. Selection of the most adequate solid phase is one of the most time consuming part of the method development. There is almost no information on the performance of these adsorbents for isoflavones. In this work, we have compared and evaluated several SPE cartridges to develop a fast, reliable, and automated SPE concentration and clean-up method for soy isoflavone extracts.

2. Materials and methods

2.1. Chemicals and solvents

Methanol (Merck, Darmstadt, Germany), and ethanol (Panreac, Barcelona, Spain) used were HPLC grade. Ultra pure water was supplied by a Mili-Q water purifier system from Millipore (Bedford, MA, USA). Isoflavones were purchased from LC Labs (Woburn, MA, USA) and stored at −32 °C. Purity of isoflavone glucosides and aglucones was higher than 99%, and purity of malonyl and acetyl glucosides was higher than 98%. Stock solutions were prepared in methanol and stored at −32 °C.

2.2. Sample

In order to evaluate the SPE method performance with accuracy, variation produced by the solid–liquid extraction of the isoflavones from the soybeans was eliminated by using a standardized extract with known isoflavone concentration. It was obtained by solid–liquid extraction of ground soybeans on an ultrasonic bath of 360 W (J.P. Selecta, Barcelona, Spain) using an adapted extraction protocol based on a previous work [5]. The analytical protocol was scaled up to obtain a large amount of extract and consists of extracting approximately 10 g of ground soybeans in 250 mL of 50% ethanol for 30 min at 60 °C. Extractions using this protocol provided 1 L of soy extract. The extract was centrifuged for 10 min, filtered through filter paper and freeze-dried. The resulting fine powder was stored at −32 °C. The standardized extract used throughout the development of the SPE method consists of 0.1 g of this freeze-dried extract dissolved in 25 mL of water. Concentrations of isoflavones in the freeze-dried extract are shown on Table 1.

2.3. Solid-phase extraction

The development of the SPE method was performed on a Zymark Rapid Trace (Caliper, Hopkinton, MA, USA) automated system. Selection of an adequate cartridge was based on evaluation and comparison of eight different cartridges from several suppliers. The cartridges sorbents ranged from conventional C_{18} based to new polymeric sorbents. Table 2 shows their main characteristics. An initial SPE protocol was used to evaluate all SPE cartridges: after cartridge conditioning with 10 mL of methanol and 10 mL

Table 1
Isoflavone concentration of the freeze-dried soy extract used for obtaining the standardized extract ($n = 6$)

Isoflavone concentration ($\mu g\,g^{-1}$)											
MDi	MGly	MGi	ADi	AGly	AGi	Di	Gly	Gi	De	Gle	Ge
5221 ± 80	1330 ± 20	5624 ± 91	268 ± 8	279 ± 8	285 ± 3	4626 ± 89	1194 ± 24	6721 ± 92	143 ± 5	190 ± 7	114 ± 4

of water (10 mL min^{-1}), 25 mL of the standardized extract were loaded onto the cartridges (5 mL min^{-1}); and washed with 10 mL of water (10 mL min^{-1}). To guarantee complete elution, two elution steps with 5 mL of methanol each (10 mL min^{-1}) were used. Sample and wash residues were collected and analyzed to evaluate losses during these steps.

2.4. High-performance liquid chromatography

A HPLC–UV Waters system, consisting of an autosampler (717 plus), pump controller (600S), pump (616), and a photodiode array detector (996) was used for chromatographic analyses. Isoflavones were separated on two coupled in series monolithic type columns (Chromolith TH Performance RP-18e, 4.6 mm, 100 mm, Merck, Darmstadt, Germany) 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 0.8 mL/min. The gradient was as follows: 0 min, 30% B, 7 min, 40% B, 15 min, 50%, 25 min, 50% B. UV absorbance was monitored from 200 to 400 nm. Injection volume was 10 μL. Samples were filtered through a 0.45 μm syringe filter (Millipore) before injection. The software for control of equipment and data acquisition was Millenium version 2.1.

Identification of isoflavones was achieved by comparison of retention times and UV spectra of separated compounds with authentic standards. Quantification was carried out by integration of the peak areas at 254 nm using the external standardization method. Response was linear between 0.1 and 50 mg L^{-1} (seven points curve) for all isoflavones and regression coefficients (r^2) were higher than 0.9998.

Quantification limits were calculated using ALAMIN software [22]. Quantification limits (mg L^{-1}) for daidzin(Di), glycitin (Gly), genistin (Gi), malonyl daidzin (MDi), malonyl glycitin (MGly), malonyl genistin (MGi), acetyl daidzin (ADi), acetyl glycitin (AGly), acetyl genistin (AGi), daidzein (De), glycitein (Gle) and genistein (Ge) were 2.40, 2.65, 2.76, 2.53, 2.40, 2.40, 2.80, 2.74, 2.87, 1.33, 3.14, 2.76, respectively. The standardized extract used as sample throughout the development of the SPE method was analyzed daily and all peak areas compared. Within the time frame of the work variation of peak areas of all isoflavones remained lower than 2%.

3. Results and discussion

3.1. Comparison of SPE cartridges

The first step in the development of the SPE method was the selection of the most appropriate SPE cartridge. Using the standardized extract and the initial SPE protocol (see Section 2.3), all cartridges were evaluated and compared. Relative isoflavone concentration on sample residue, wash residue and recoveries obtained with essayed SPE cartridges are shown in Table 3. These values are relative to the amount of each isoflavone in the standardized extract (100%).

In all eight cartridges, total isoflavone content could be accounted for, either on the sample and wash residues or in the final elution step.

The presence of isoflavones in the sample and wash residues is indication of an inadequate retention by the cartridge sorbent. As can be seen, there are great differences in the retention of isoflavones within essayed SPE cartridges. While some cartridges retained all isoflavones, others had severe isoflavones losses during sample loading and washing steps.

The cartridges EN and C18E, for example, presented a very low and variable retention capability. The EN cartridge retained between 60 and 80% of glucosides, between 18 and 24% of malonyl glucosides, between 19 and 30% of acetyl glucosides and between 27 and 57% of aglucones. The C18E cartridge retained between 71 and 85% of glucosides, between 30 and 40% of malonyl glucosides, between 22 and 64% of acetyl glucosides and between 67 and 98% of aglucones.

Table 2
Characteristics of evaluated SPE cartridges

Commercial brand	Abbreviation	Solid phase	Amount of solid phase (mg)	Supplier
Bond Elut C-18	VC18	Octadecyl silica	500	Varian
Bond Elut ENV	VEN	Styrene-divinylbenzene	200	Varian
Discovery DSC-18	DSC18	Octadecyl silica	500	Supelco
Strata C-18E	C-18E	Octadecyl silica	500	Phenomenex
Strata SDB-L	SDBL	Styrene-divinylbenzene	200	Phenomenex
Strata X	Strata X	Modified divinylbenzene	200	Phenomenex
LiChrolut EN	EN	Ethyl-vinyl-benzene styrene-divinylbenzene	200	Merck
Oasis HLB	HLB	Divinyl-benzene-co-*N*-vinylpyrrolidone	60	Waters

Table 3
Relative isoflavone concentration (% ± RSD) on sample and wash residues and recoveries obtained with essayed SPE cartridges ($n = 4$)

Solid phase	Steps	Relative isoflavone concentration (% ± RSD)											
		MDi	MGly	MGi	ADi	AGly	AGi	Di	Gly	Gi	De	Gle	Ge
VC18	Sample residue	–	–	–	–	–	–	–	–	–	–	–	–
	Wash residue	11.3 ± 1.6	–	4.1 ± 0.8	–	–	–	–	–	–	–	–	–
	Recovery	88.8 ± 1.9	100.7 ± 1.2	95.5 ± 0.8	98.3 ± 2.6	98.6 ± 1.2	99.2 ± 1.3	100.0 ± 0.8	101.2 ± 1.2	99.8 ± 0.6	99.6 ± 2.1	100.8 ± 1.4	99.5 ± 1.1
DSC	Sample residue	–	–	–	–	–	–	–	–	–	–	–	–
	Wash residue	9.4 ± 4.3	–	4.3 ± 1.6	–	–	–	–	–	–	–	–	–
	Recovery	91.0 ± 4.2	100.5 ± 1.5	96.0 ± 2.7	99.4 ± 1.2	98.3 ± 0.5	100.1 ± 2.7	100.1 ± 1.0	100.8 ± 1.0	100.1 ± 0.4	99.7 ± 2.1	98.7 ± 3.4	100.3 ± 2.2
C18E	Sample residue	59.8 ± 3.4	54.6 ± 5.9	52.7 ± 0.3	34.9 ± 2.5	76.9 ± 9.3	53.2 ± 35.5	20.8 ± 3.1	27.2 ± 13.1	11.6 ± 5.0	31.4 ± 3.6	–	–
	Wash residue	10.2 ± 4.7	5.1 ± 5.0	8.0 ± 3.8	–	–	–	2.6 ± 3.1	–	1.0 ± 1.0	–	–	–
	Recovery	29.5 ± 2.0	39.7 ± 6.1	38.1 ± 4.1	64.2 ± 2.4	22.0 ± 8.4	46.5 ± 34.1	74.7 ± 4.0	71.8 ± 11.3	85.7 ± 6.8	67.3 ± 3.5	98.9 ± 0.9	98.7 ± 1.5
Strata X	Sample residue	–	–	–	–	–	–	–	–	–	–	–	–
	Wash residue	–	–	–	–	–	–	–	–	–	–	–	–
	Recovery	100.4 ± 0.3	100.4 ± 0.4	99.2 ± 0.6	99.2 ± 1.0	99.8 ± 0.6	99.8 ± 0.6	100.1 ± 1.6	100.7 ± 1.8	100.5 ± 0.6	100.2 ± 1.0	100.5 ± 1.1	99.3 ± 0.8
HLB OASIS	Sample residue	–	–	–	–	–	–	–	–	–	–	–	–
	Wash residue	–	–	–	–	–	–	–	–	–	–	–	–
	Recovery	99.8 ± 1.5	100.7 ± 0.8	100.2 ± 0.2	100.2 ± 0.8	99.8 ± 0.6	100.9 ± 0.6	98.9 ± 1.6	99.0 ± 1.0	99.5 ± 0.9	100.8 ± 0.2	98.1 ± 1.2	99.3 ± 2.6
EN	Sample residue	80.3 ± 3.4	75.8 ± 17.4	78.0 ± 5.1	79.6 ± 1.0	65.7 ± 16.3	69.4 ± 1.0	22.5 ± 3.4	38.9 ± 6.2	18.9 ± 4.1	50.0 ± 2.5	97.8 ± 3.3	42.0 ± 3.2
	Wash residue	1.4 ± 2.9	–	0.8 ± 0.4	–	–	–	–	–	–	–	–	–
	Recovery	18.6 ± 5.4	23.9 ± 17.4	20.2 ± 6.7	19.1 ± 2.6	31.7 ± 17.8	27.9 ± 0.7	76.5 ± 3.1	61.0 ± 5.5	80.1 ± 4.3	49.5 ± 2.8	–	57.1 ± 2.2
VEN	Sample residue	–	–	–	–	–	–	–	–	–	–	–	–
	Wash residue	–	–	–	–	–	–	–	–	–	–	–	–
	Recovery	97.2 ± 4.4	99.3 ± 0.9	99.5 ± 0.8	98.9 ± 0.9	99.0 ± 0.5	98.6 ± 1.3	99.6 ± 0.3	99.7 ± 1.0	99.2 ± 1.4	100.1 ± 0.8	99.8 ± 0.5	98.2 ± 1.1
SDBL	Sample residue	–	–	–	–	–	–	–	–	–	–	–	–
	Wash residue	9.4 ± 3.6	–	.43 ± 1.5	–	–	–	–	–	–	–	–	–
	Recovery	91.0 ± 4.2	100.5 ± 1.4	96.0 ± 3.6	99.4 ± 1.3	98.3 ± 1.4	100.1 ± 0.6	100.1 ± 1.2	100.8 ± 1.4	100.1 ± 0.8	99.7 ± 1.4	98.7 ± 0.8	100.3 ± 1.3

Steps: sample: 25 mL of standardized extract; wash: 10 mL water; recovery: 5 mL MeOH; SPE 2: 5 mL MeOH (isoflavones were not detected on SPE 2); –: not detected.

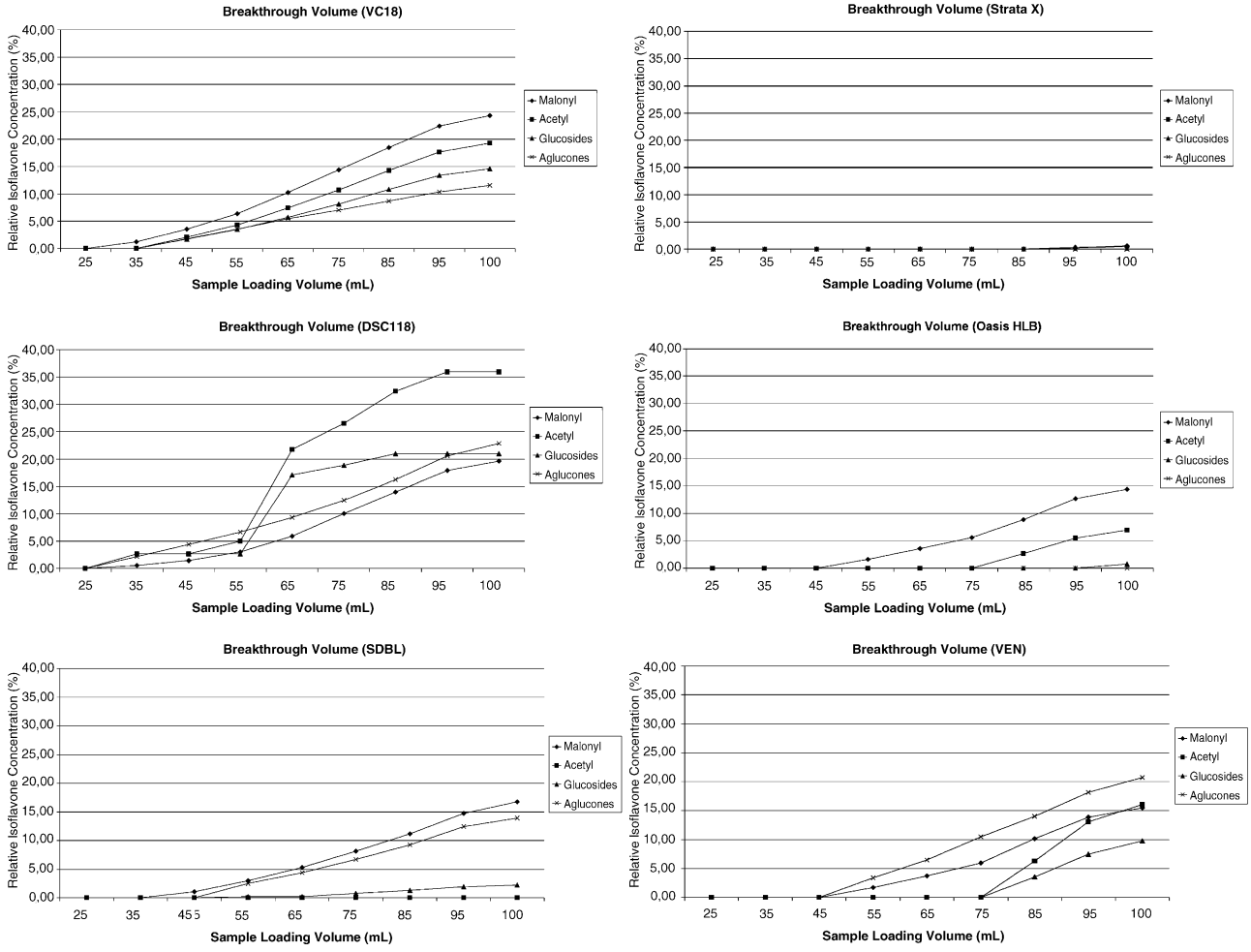

Fig. 2. Losses of isoflavones detected in the sample residue during sample loading with selected SPE cartridges ($n = 3$). Malonyl (mean relative concentration of MDi + MGly + MGi), acetyl (mean relative concentration of ADi + AGly + AGi), glucosides (mean relative concentration of Di + Gly + Gi) and aglucones (mean relative concentration of De + Gle + Ge).

In contrast, cartridges DCS, VC18 and SDBL retained approximately 100% of all isoflavones, except for MDi and MGi (90 and 95%, respectively). Losses of these isoflavones took place only during washing. The observed losses are an indication that malonyl isoflavones are not properly retained in some sorbents, and therefore, high sample load and wash flow rates could increase losses during these steps.

The cartridges Strata X, HLB Oasis and VEN, had an excelent performance, retaining approximately 100% of all isoflavones with no observable losses. Based on the losses of isoflavones during sample loading, EN and C18-E cartridges were discarded as option to be used in the method.

The next step in the method development was the determination of the breakthrough volume of the selected cartridges. This was achieved by increasing the sample amount (from 25 mL of the standardized extract to 100 mL) to an extent that losses can be observed, indicating saturation of the sorbent bed.

A high breakthrough volume will guarantee that no losses will take place during sample loading and washing steps as observed for some cartridges. The breakthrough volumes in

Table 4
Effect of sample loading flow on sample residue isoflavone concentration ($n = 4$)

Sample loading flow (mL min^{-1})	Relative isoflavone concentration on sample residue (% ± RSD)				
	MDi	MGly	MGi	Di	Gi
5	–	–	–	–	–
10	7.5 ± 1.2	–	5.6 ± 1.3	–	–
15	15.9 ± 2.0	1.92 ± 1.0	11.5 ± 1.0	2.6 ± 1.6	3.5 ± 0.3

Solid phase: Strata X; sample: 25 mL of standardized extract; isoflavones ADi, AGly, AGi, Gly, De, Gle and Ge were not detected on the sample residue with all sample loading flow rates, –: not detected.

terms of concentration in the sample residue of the selected SPE cartridges are shown in Fig. 2. The values are relative to the total isoflavones in the standardized extract (100%).

The C_{18} based sorbents (VC18 and DSC) presented a low breakthrough volume (35 mL). The breakthrough volume for polymeric sorbents VEN, SDBL and HLB Oasis was 55 mL. The HLB Oasis cartridge had a surprisingly high breakthrough volume since it has the lowest sorbent bed mass (60 mg) compared to another cartridges (200–500 mg). However, the best cartridge was without doubt Strata X (breakthrough volume = 95 mL). Even with such high sample volume, only small malonyl forms losses (<1%) were observed.

Higher losses of malonyl isoflavones may be due to its higher concentration and molecular mass or more possibly to a combination of these two factors. The small pore size of the sorbents makes it less accessible to large molecules such as the malonyl isoflavones. They are not properly retained and consequently washed out by the sample residue or by the water of the washing step. Based on these results, the Strata X cartridge was selected to be used in the extraction parameters optimization.

3.2. Sample loading flow

In order to reduce the duration of the method, one option is to increase the sample loading flow. However, previous results indicate that losses may take place during sample loading. With that in mind, the sample loading flow was increased from 5 to 10 mL min^{-1} and 15 mL min^{-1}. The concentration of isoflavones in the sample residue is shown in Table 4. Values are relative to the total amount of each isoflavone in the standardized extract.

Increasing the sample loading flow rate from 5 to 10 mL min^{-1} resulted in losses of MDi and MGi, which increased with the increase of the flow rate up to 15 mL min^{-1}. With a sample loading flow rate of 15 mL min^{-1} other isoflavones were also lost (Di, Gi and MGly).

The results confirm the observations from the comparison of SPE cartridges that malonyl forms are the most susceptible forms to be lost during the sample loading.

Therefore, the selected sample load flow was 5 mL min^{-1}. Lower sample loading flows were not tested since it would extend the duration of the method.

3.3. Amount of eluting solvent

Before fixing the amount of eluting solvent, extractions with and without a 10 min drying step with nitrogen were compared. The comparison was based on the concentration of isoflavones in three different elution steps with 2 mL of methanol. The differences observed between the two procedures are not significant in all three eluting steps and both procedures recovered approximately 100% of all isoflavones With both procedures, mean isoflavone recovery with the first 2 mL of methanol was approximately 87% and with the following 2 mL of methanol approximately 13%. Isoflavones

Table 5
Effect of eluting solvent pH on isoflavone recovery ($n = 3$)

Eluting solvent	Steps	Relative isoflavone concentration (% ± RSD)											
		MDi	MGly	MGi	ADi	AGly	AGi	Di	Gly	Gi	De	Gle	Ge
MeOH	SPE 1 (2 mL)	85.6 ± 0.5	88.2 ± 0.4	80.3 ± 0.8	81.8 ± 1.1	95.7 ± 0.7	90.7 ± 0.7	80.8 ± 1.1	90.7 ± 1.4	82.2 ± 0.7	91.4 ± 1.2	88.8 ± 1.0	89.5 ± 1.0
	SPE 2 (2 mL)	14.7 ± 0.4	11.8 ± 0.3	20.4 ± 0.4	18.5 ± 0.7	4.8 ± 0.3	8.9 ± 0.5	19.2 ± 0.9	9.5 ± 0.5	18.0 ± 0.4	8.1 ± 0.9	11.0 ± 0.6	10.3 ± 0.4
	Recovery	100.3 ± 0.4	100.1 ± 0.3	100.7 ± 0.7	100.4 ± 1.0	100.4 ± 0.6	99.6 ± 0.5	100.0 ± 1.1	100.2 ± 1.2	100.2 ± 0.4	99.5 ± 1.2	99.8 ± 0.8	99.78 ± 0.87
MeOH + 2.8 mg L^{-1} NaOH	SPE 1 (2 mL)	85.1 ± 0.6	88.3 ± 0.6	79.7 ± 0.9	82.2 ± 1.3	95.4 ± 0.8	91.0 ± 1.0	80.9 ± 1.1	91.1 ± 1.4	81.9 ± 0.6	92.05 ± 1.2	89.4 ± 0.9	90.0 ± 1.0
	SPE 2 (2 mL)	14.3 ± 0.2	11.8 ± 0.1	20.6 ± 0.3	18.2 ± 0.8	4.9 ± 0.2	8.7 ± 0.4	19.0 ± 0.9	9.5 ± 0.5	18.1 ± 0.2	8.6 ± 1.0	11.4 ± 0.5	10.4 ± 0.3
	Recovery	99.5 ± 0.5	100.2 ± 0.5	100.4 ± 1.0	100.4 ± 1.2	100.3 ± 0.8	99.7 ± 0.9	99.9 ± 1.1	100.5 ± 1.1	100.1 ± 0.5	100.6 ± 1.2	100.8 ± 0.98	100.4 ± 0.9
MeOH + 8.4 mg L^{-1} NaOH	SPE 1 (2 mL)	85.6 ± 0.4	87.7 ± 0.5	78.7 ± 0.7	81.5 ± 1.2	95.4 ± 0.6	91.7 ± 0.7	81.7 ± 1.0	90.3 ± 1.4	82.3 ± 0.6	91.0 ± 1.1	88.6 ± 1.0	89.0 ± 0.9
	SPE 2 (2 mL)	14.5 ± 0.2	11.9 ± 0.3	20.8 ± 0.4	17.9 ± 0.8	5.0 ± 0.3	8.7 ± 0.4	18.1 ± 0.9	10.0 ± 0.5	17.9 ± 0.3	8.7 ± 0.7	11.8 ± 0.5	10.6 ± 0.4
	Recovery	100.1 ± 0.4	99.6 ± 0.4	99.5 ± 0.5	99.3 ± 1.1	100.5 ± 0.5	100.4 ± 0.6	99.8 ± 1.0	100.3 ± 1.3	100.2 ± 0.5	99.7 ± 1.0	100.4 ± 0.7	99.5 ± 0.9

Solid phase: Strata X; sample: 25 mL; SPE 1: 2 mL MeOH; SPE 2: 2 mL MeOH; SPE 3: 2 mL MeOH, no isoflavone detected in all cases.

Table 6
Reproducibility of the optimized extraction method ($n = 9$)

Isoflavone concentration (% ± RSD)											
MDi	MGly	MGi	ADi	AGly	AGi	Di	Gly	Gi	De	Gle	Ge
99.6 ± 1.4	99.0 ± 1.4	99.4 ± 1.9	99.2 ± 1.3	99.3 ± 1.8	99.7 ± 1.7	99.5 ± 1.2	99.4 ± 1.6	99.7 ± 1.5	99.1 ± 1.5	99.2 ± 1.6	99.4 ± 1.5

were not detected in the third elution step with 2 mL of methanol. From this information we can infer that is feasible to skip the drying step, which is time consuming (it almost doubles the method duration). Moreover, 4 mL of MeOH are needed to recover the retained isoflavones from the cartridge.

Maximum selectivity and recovery could also be obtained by a precise adjustment of pH values in the final elution step. Therefore, by increasing the pH of the elution solvent, isoflavones retained in the sorbent would be more easily removed allowing reduction of the elution solvent volume. With that in view, the pH of the eluting solvent (MeOH) was increased by adding sodium hydroxide (2.8 and 8.4 mg L^{-1}).

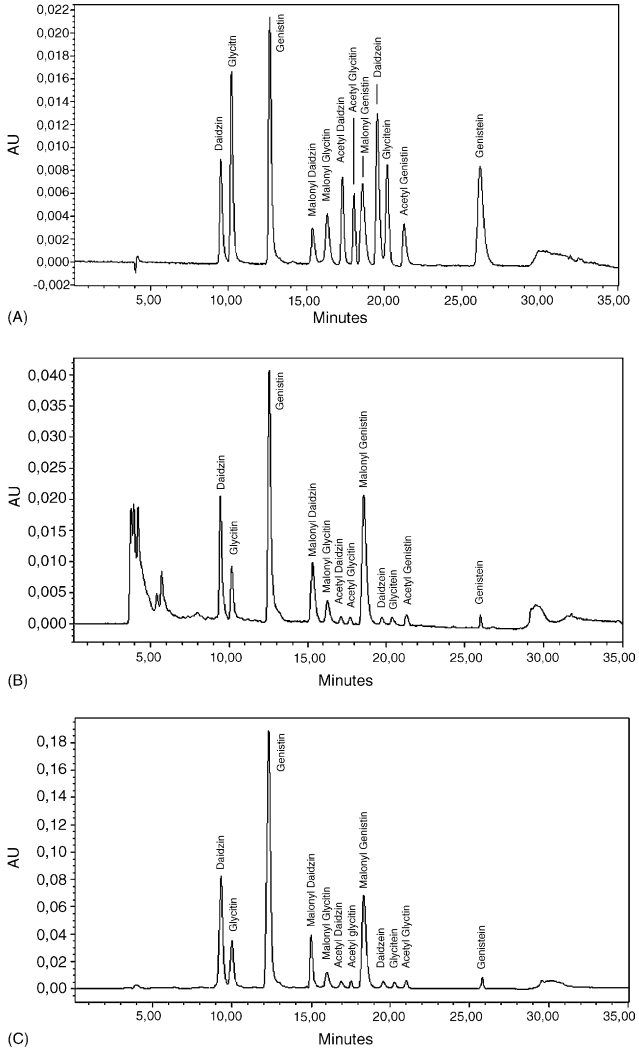

Fig. 3. Chromatograms of isoflavone standards (A), freeze-dried soy extract before (B) and after (C) applying the optimized SPE protocol.

Despite of increasing the pH (Table 5) of the eluting solvent no differences were observed in recovery of isoflavones in the pH range essayed. Even combining higher pH and dry procedures it was not possible to reduce the elution solvent volume and complete elution of all isoflavones was obtained with 4 mL (2 + 2 mL). Therefore, the elution solvent volume selected was 4 mL.

3.4. Sample clean up

One of the most important aspects of the proposed SPE method is that it is feasible to clean-up the sample. It is evident when comparing the chromatograms of the sample before and after application of the method (Fig. 3). The optimized method provides a much cleaner extract, which is less detrimental to the chromatographic column than the original sample.

3.5. Reproducibility

The repeatability and inter-day precision were determined by running the SPE developed method on three different days with the standardized extract. The standard deviation and coefficient variation were calculated for each day. The RSDs for isoflavone recoveries were always below 2%. A mean recovery (Table 6) ($n = 9$) of 99.37% (RSD = 1.58) for all isoflavones indicates that the optimized method provides reliable and accurate results.

4. Conclusions

There was a large variation in the retention of isoflavones within the essayed SPE cartridges. Best retention of isoflavones was achieved with the Strata X cartridge, and therefore, it was used for the SPE method development. The optimized method consists of conditioning the cartridge using 10 mL of methanol and 10 mL of water (10 mL min^{-1}), loading 25 mL of the standardized extract onto the cartridges (5 mL min^{-1}), washing the cartridge with 10 mL of water (10 mL min^{-1}) and finally eluting the isoflavones with 4 mL of methanol (10 mL min^{-1}). The developed SPE method is fast (less than 10 min), extremely accurate and reproducible, achieving high isoflavone recoveries (>98%). The optimized method also concentrates the sample (25–4 mL) allowing measurement of isoflavones at low concentrations and provides cleaner extracts, which are less detrimental to column life than original samples.

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