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Simultaneous and rapid determination of the anticarcinogenic proteins Bowman-Birk inhibitor and lectin in soybean crops by perfusion RP-HPLC

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ABSTRACT

There are numerous studies demonstrating a direct association between the ingestion of soybean and low cancer incidence. This fact has been related to the presence of Bowman-Birk inhibitor (BBI) and lectin in soybean. The simultaneous and fast determination of BBI and lectin in soybean is proposed, for the first time, in this work. Two different strategies were designed for the extraction of BBI and lectin: extraction of soybean proteins using a Tris–HCl buffer followed by isolation of BBI and lectin by the isoelectric precipitation of other soybean proteins (method I) or by the direct extraction of BBI and lectin using an acetate buffer (method II). The effect of the previous soybean defating on the extraction of BBI and lectin was also studied. Moreover, the possibility of using a high-intensity focalized ultrasonic probe for accelerating the extraction was explored and an optimization of the extraction time and ultrasound amplitude was performed. The extracts obtained were analysed by RP-HPLC–ESI-MS for the correct identification of BBI and lectin in soybean. Moreover, a fast chromatographic methodology using a perfusion column and UV detection was optimized for the rapid determination of BBI and lectin in soybean. After evaluating its analytical characteristics (linearity, precision, and recovery), the method was applied to the quantitation of BBI and lectin in different soybean varieties.

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1. Introduction

Soybean (*Glycine max*. (L.) Merr.) is a highly valuable legume and constitutes a cheap source of proteins (40–50%). Consumption of soybean has been linked to a healthy diet and with health benefits. Among others, soybean may reduce risk of osteoporosis, cholesterol, coronary heart disease, and cancer [1]. Indeed, there are numerous studies demonstrating a direct association between the ingestion of soybean and low cancer incidence, particularly breast, colon, and prostate cancer [2]. Despite the causes of this anticarcinogenicity are not totally clear, there are evidences associating this activity with the presence of Bowman-Birk inhibitor (BBI) and lectin [3].

Soybean BBI is a polypeptide of 71 aminoacids and a molecular weight of 7.8 kDa belonging to the serine-protease inhibitor family. This molecule is considered an antinutrient causing digestive disorders derived from their ability to inhibit trypsin and chymotrypsin enzymes. Nevertheless, BBI has also been demonstrated to inhibit proteases involved in the initiation and propagation of cancer and, consequently, has been considered by FDA as "investigational new drug" [4,5].

On the other hand, lectin (also called hemaglutinin or agglutinin) is a carbohydrate-binding protein present in vegetables and legumes such as soybean. Soybean lectin is a tetramer protein with a molecular weight of 110–120 kDa constituted by four subunits of 30 kDa each. Lectin is used as a therapeutic agent preferentially binding to cancer cell membranes or their receptors causing cytotoxicity and inhibition of tumor growth. Nevertheless, soybean lectin has also been associated with nutritional disorders. Indeed, lectin is resistant to digestion binding to its target glycoprotein receptors in the small intestinal surface causing disruption of the brush border membrane and interfering in the absorption of nutrients [4,6–8].

These premises make necessary the determination and control of BBI and lectin in soybean and soybean products. In fact, soybean breeders are developing programs for the genetic improvement of soybean promoting those varieties with higher contents in BBI and lectin. On the other hand, commercialization of soybean products for human consumption, especially those for infant consumption, requires a control of these proteins due to their antinutritional roles.

There are different works aimed to the determination of BBI and lectin contents in soybean products. Monoclonal antibodies in enzyme linked immunosorbent assays (ELISA) and sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western-Blot have been employed for the determination of BBI in soybean [3,9–11]. Moreover, capillary electrophoresis

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was used for studying the binding of BBI and enzymes but no determination of BBI was performed [12]. Soybean lectin has been determined by an agglutination assay using red blood cell, by immunoaffinity, and, more recently, by ELISA [11,13–15]. Despite the existence of these methodologies, the development of new analytical methods enabling faster and cheaper determinations are required for their implementation in routine analysis.

This work has been focused to the development of an analytical method based on perfusion high-performance liquid chromatography for the rapid and simultaneous determination of BBI and lectin in soybean crops.

2. Experimental

2.1. Chemicals and samples

HPLC-grade acetonitrile (AcN), supragradient HPLC grade methanol (MeOH) (Scharlau, Barcelona, Spain), Milli-Q water (Milli-Q system, Millipore, Bedford, MA, USA), and trifluoroacetic acid (TFA) (Sigma, St. Louis, MO, USA) were employed for the preparation of mobile phases. Petroleum ether, *tris*-(hydroxymethyl) aminomethane (Tris), 2-mercaptoethanol (Merk, Darmstadt, Germany), hydrochloric acid (Panreac, Barcelona, Spain), sodium acetate (Fluka Biochemika, Steinheim, Switzerland), acetone, and ethanol (Scharlau) were employed for the extraction of BBI and lectin. Soybean trypsin-chymotrypsin inhibitor and lectin standards were from Sigma.

One commercial soybean purchased in a local market (Madrid, Spain) and seven different soybean varieties (Clark Fe, Clark E, Ape-18, and Clark Rji from USA, Fred from France, and Kita-musume and Shih-Shih from Japan), kindly donated by a Soybean Germplasm Bank (Centro de Recursos Fitogenéticos, CRF, Madrid, Spain), were employed.

All soybeans were ground with a domestic miller. Solutions were prepared by the direct extraction of 1–2 g of ground sample with 20 mL of 100 mM acetate buffer (pH 4.5) for 5 min using an ultrasonic probe (20% amplitude) followed by centrifugation at 3362 × *g* for 30 min at 20 °C. Samples were filtered through a 0.45-μm pore size regenerated cellulose filter membranes (Titan 2, Eatontown, NJ, USA).

2.2. High-performance liquid chromatography

An Agilent Technologies 1100 Series liquid chromatograph (Agilent Technologies, Pittsburg, PA, USA) and a Poros R2/H perfusion column (100 × 2.1 mm I. D.) (Perseptive Biosystems, Framingham, MA, USA) were employed for the separation of extracts. UV detection at 210 nm and mass spectrometry detection were employed. Chromatographic conditions used with UV detection were: elution gradient, 19% B for 0.5 min; 19–20% B in 3 min; 20–37% B in 0.5 min; 37–43% B in 5 min; 43–95% B in 0.5 min; flow-rate, 1.5 mL/min, temperature, 60 °C; injected volume, 10 μL; mobile phases, Milli-Q water 0.1% (v/v) TFA-AcN. Chromatographic conditions used with MS detection were: elution gradient, 5–14% B in 12 min; 14–16% B in 1 min; 16–20% B in 2 min; 20–28% B in 1 min; 28–40% B in 8 min; 40–45% B in 2 min, and 45–95% B in 0.5 min; flow-rate, 0.5 mL/min; temperature, 60 °C; mobile phases, Milli-Q water 0.05% (v/v) TFA-AcN; injected volume, 5 μL.

2.3. Mass spectrometry

HPLC was connected to an ion-trap mass spectrometer equipped with an orthogonal electrospray ionization interface (model 1100 from Agilent Technologies, Palo Alto, CA, USA). MS control, data acquisition, and data analysis were carried out using the LC/MSD Trap Software 5.2. The instrument was firstly tuned to get the MS conditions enabling the highest responses in the ion positive mode. These MS conditions were: capillary voltage, – 3000 V; end plate offset, – 500 V; skim 1 voltage, 45 V; capillary exit offset voltage, 50 V; octapole voltage, 3 V; octapole delta voltage, 0.5 V. Mass spectra were registered in the full-scan mode (*m*/*z*, 600–2000; the maximum accumulation time was 300 ms). The dry gas flow-rate was 10 L/min, the dry temperature was 350 °C, the target mass was 1500 *m*/*z*, and the nebulizer pressure was 40 psi.

Mass spectra deconvolution was performed with the LC1100/MSD Trap Deconvolution and Bioanalysis software from Agilent Technologies.

2.4. Calibration

Calibration was performed by the external standard, the standard additions, and the single point calibration methods [16]. Calibration by the external standard method was carried out by injecting six BBI and lectin standard solutions in acetate buffer over the range of 0.025–0.500 mg/mL for BBI and lectin. Integration was carried out by setting the baseline from valley to valley, and the average of the area of three consecutive injections was calculated. BBI and lectin concentrations were calculated by interpolation of the area of the corresponding peaks in the calibration curve. Calibration by the standard additions method was performed by injecting soybean extracts spiked with known and increasing amounts of BBI and lectin (five standard solutions in the range 0–0.13 mg/mL for BBI and 0–0.07 mg/mL for lectin). Single point calibration was performed by the injection of two different sample solutions for every soybean sample: one soybean solution and an identical soybean solution spiked with known amounts of BBI and lectin. This kind of calibration was carried out after checking the intercept did not significantly differed from zero [16].

2.5. Data treatment

Peak areas were plotted against injected concentrations of BBI and lectin. The linearity in this relationship was obtained by least-squares regression analysis.

3. Results and discussion

3.1. Optimization of the extracting conditions

In order to prepare an extract containing both BBI and lectin, three different methodologies (methods I, II, and III) were tried. Method I was based on a previous method aimed to the fractionation of soybean proteins by isoelectric precipitation [17]. The method was modified for our purpose as follows: 1 g of ground sample was defatted by triplicate with 10 mL of petroleum ether with continuous agitation (30 min) and centrifugation at 3362 × *g* for 30 min (total time: 3 h), the resulting pellet was extracted with a 30 mM Tris–HCl buffer (pH 8.0) containing 10 mM 2-mercaptoethanol for 1 h with continuous agitation, and centrifuged again for 30 min. The supernatant containing soybean proteins was adjusted to pH 4.8 for precipitation of main storage proteins followed by centrifugation for 30 min (total time: 5 h). The resulting supernatant was injected into the chromatographic system for the detection of BBI and lectin. Preliminary chromatographic conditions employed with the selected perfusion column consisted of a binary gradient from 5 to 95% of mobile phase B (being mobile phase A, water with 0.1% (v/v) TFA and mobile phase B, MeOH with 0.1% (v/v) TFA) in 15 min at 0.5 mL/min of flow-rate and 25 °C of temperature (rest of conditions as stated in Section 2). On the other hand, method II was based on a method previously employed by Yeboah et al. [18] for the extraction of crude proteins for subsequent purification of soybean BBI. The method was modified as

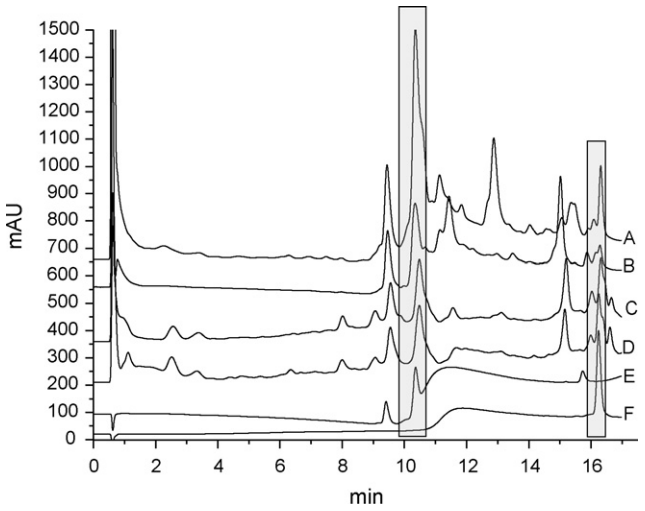

Fig. 1. Chromatograms corresponding to extracts obtained with the extracting method III (BBI fraction (A) and lectin fraction (B)), with the extracting method II (C) and with the extracting method I (D) and to the trypsin/chymotrypsin inhibitor (E) and lectin (F) standards. Chromatographic conditions: gradient, 5–95% B in 15 min; mobile phases, 0.1% (v/v) TFA in water (mobile phase A) and 0.1% (v/v) TFA in MeOH (mobile phase B); flow-rate, 0.5 mL/min; temperature, 25 °C; injection volume, 10 μL; UV detection, 210 nm.

follows: 1 g of ground sample was defatted with petroleum ether as previously (see method I) and extracted using 10 mL of a 100 mM acetate buffer (pH 4.5) for 1 h followed by centrifugation for 30 min (total time: 4.5 h) [18]. The extracts obtained with methods I and II, enabling the simultaneous fractionation of BBI and lectin (both in the same fraction), were compared with the extracts obtained with method III. This method was proposed by Pusztai et al. [19] for the individual fractionation of BBI and lectin (every one in a separated fraction) from soybean. Shortly, the method consisted of extracting the sample with 60% aqueous ethanol for 30 min by duplicate followed by centrifugation. The combined supernatants were extracted with acetone, left overnight, centrifuged, and stored as BBI. The pellet obtained from the ethanol extraction was treated again with 70% aqueous ethanol to remove oligosaccharides and glycosines and centrifuged. The resulting pellet was dissolved in an acetate buffer (pH 5.8) and centrifuged again. The supernatant was stored as lectin.

Fig. 1 shows the separations obtained for the extracts resulting from the three methods along with BBI and lectin standards. Methods I and II (Fig. 1C and D) yielded similar separations. Lectin standard (Fig. 1F) showed a peak at the end of the chromatogram whose retention time corresponded to one peak observed in all the extracts. Soybean trypsin-chymotrypsin inhibitor standard (Fig. 1E) eluted in three peaks also observed in all the extracts. Regarding method III, the fraction stored as BBI (Fig. 1A) resulted in different peaks some of them observed in the trypsin/chymotrypsin inhibitor standard. It is interesting to point out the size of the peak appearing at 10.4 min that was also observed in the chromatogram of the soybean trypsin/chymotrypsin inhibitor standard and that could correspond to BBI. The fraction labelled as lectin (Fig. 1B) showed different peaks but only one of the smallest matched with the peak appearing in the lectin standard (Fig. 1F). From these results, it could be possible to confirm that the peak appearing at 10.4 min in the extracts obtained from methods I and II is likely to be BBI. Nevertheless, in the case of lectin, a further confirmation was needed. Therefore, a RP-HPLC–ESI-MS analysis of the extracts and the trypsin-chymotrypsin inhibitor and lectin standards was carried out. For that purpose, the same chromatographic column and similar chromatographic conditions were employed. Resulting total ion chromatograms (TICs) and mass spectra corresponding to the extract obtained from method I and the standards are grouped in Fig. 2. The new gradient enabled the separation of lectin standard into two main peaks and a small one between them whose retention times and mass spectra corresponded to peaks observed in the extract TIC. Mass spectra analysis of lectin standard showed that main peaks corresponded to lectin subunits of 28.6 and 29.4 kDa. Inhibitor standard yielded several peaks. Analysis of mass spectra enabled to observe that BBI (7.8 kDa) eluted in the peak appearing at 17.5 min and to confirm the presence of BBI in the extract.

Methods I and II were also employed with the previous removing of the fat extraction step observing it was not necessary in any case (results not shown). This constituted a significant reduction of extraction time since this step took 3 h. Moreover, Fig. 3 shows the separations obtained when extractions following methods I and II were performed with magnetic agitation for 1 h and using an ultrasonic probe at different extraction times (from 5 min to 1 h). In both cases, ultrasonic extraction for 5 min was enough to extract BBI and lectin. Finally, since method II was simpler, shorter, and cheaper than method I, the first one was selected for the simultaneous extraction of BBI and lectin in soybean cultivars.

3.2. Optimization of the chromatographic separation

Chromatographic method was modified in order to reduce analysis time and improve separation of proteins by optimizing temperature, organic modifier in the mobile phase, elution gradient, flow-rate, and UV detection. Temperatures ranging from 25 to 60 °C were employed selecting 60 °C for enabling a better separation. Different gradients were tried using MeOH in the mobile phase. Nevertheless, it was not possible to obtain a suitable separation of BBI and lectin in any case. Therefore, the organic modifier in the mobile phase was replaced by AcN being possible a better separation of BBI and lectin. Moreover, the increase in temperature enabled an increase in the flow-rate from 0.5 mL/min to 1.5 mL/min. Finally, the selected gradient for the separation of BBI and lectin in soybean was: 19% B in 0.5 min; 19–20% B in 3 min; 20–37% B in 0.5 min; 37–43% B in 5 min; 43–95% B in 0.5 min. Fig. 4 shows the separation obtained with the optimized conditions for a soybean sample extracted with method II (Fig. 4A) and for the BBI (Fig. 4B) and lectin (Fig. 4C) standards. The optimized chromatographic method enabled the simultaneous separation of BBI and lectin in less than 9 min observing three peaks for lectin that corresponded to lectin subunits. The effect of the variation of the detection wavelength (210, 254, and 280 nm) was also studied observing no improvement in sensitivity (results not shown) when using 254 and 280 nm.

3.3. Method characterization

The developed methodology was characterized by the evaluation of linearity of the calibration plot, detection and quantitation limits, presence of matrix interferences, precision, and recovery. Table 1 groups the results obtained.

Good linear correlation ($r^2 > 0.99$) was observed between the signal and the concentration of BBI and lectin in the working range from 0.025 to 0.500 mg/mL for BBI and lectin. The lowest concentrations of BBI and lectin detected were 5.0 μg/100 mg and 7.5 μg/100 mg, respectively (calculated based on the calibration curve (following ICH guidelines) as the concentration corresponding to a signal equal to the intercept plus 3.3 times the standard error) [16]. The lowest concentrations of BBI and lectin that could be determined were 22.5 μg/100 mg and 24.9 μg/100 mg for BBI and lectin, respectively (calculated based on the calibration curve (following ICH guidelines) as the concentration corresponding to a signal equal to the intercept plus 10 times the standard error

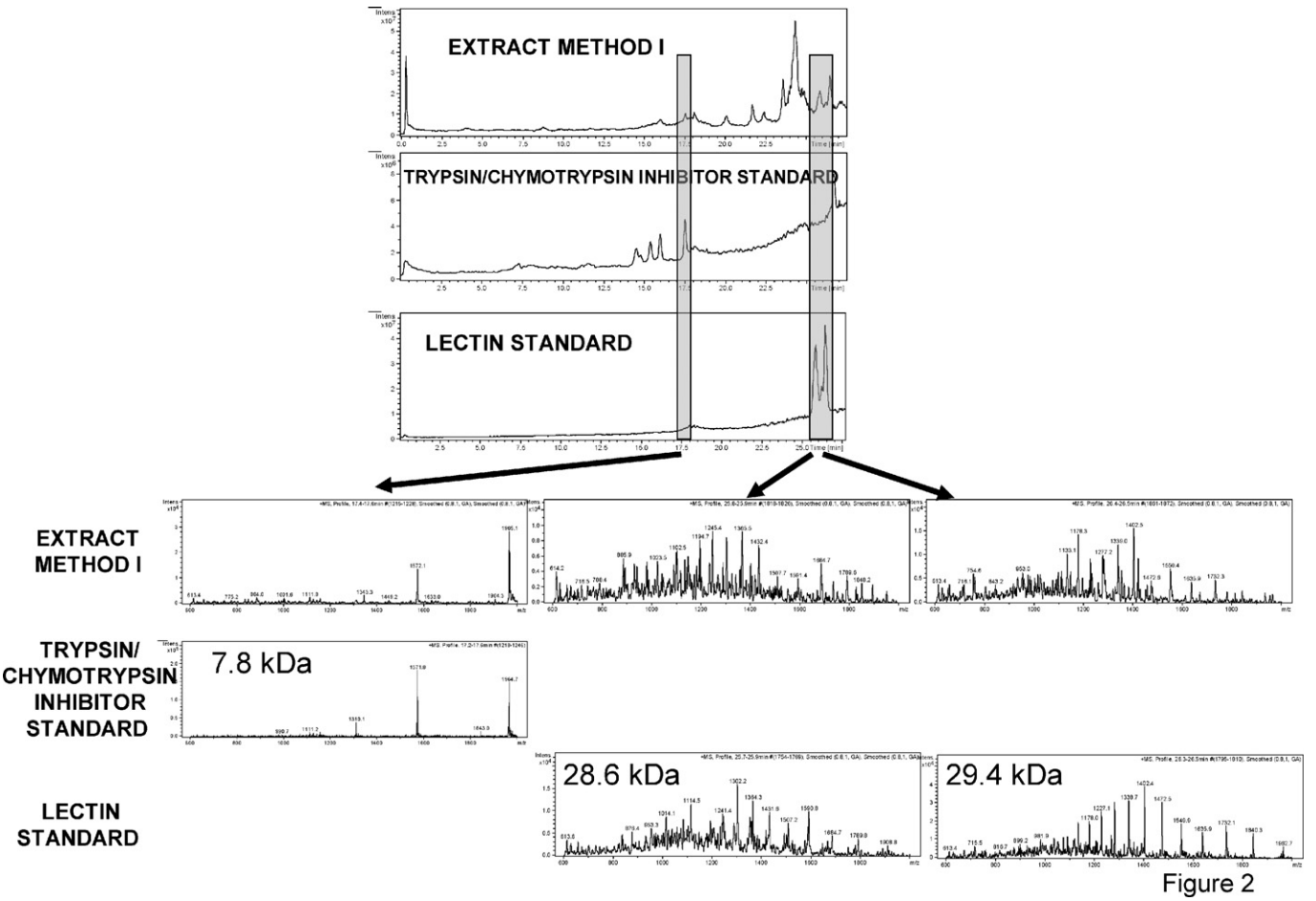

Fig. 2. Total ion chromatograms and spectra corresponding to an extract obtained with the extracting method I and to the trypsin/chymotrypsin inhibitor and lectin standards. Chromatographic conditions: gradient, 5–14% B in 12 min; 14–16% B in 1 min; 16–20% B in 2 min; 20–28% B in 1 min; 28–40% B in 8 min; 40–45% B in 2 min, and 45–95% B in 0.5 min; mobile phases, 0.05% (v/v) TFA in water (mobile phase A) and 0.05% (v/v) TFA in AcN (mobile phase B); flow-rate, 0.5 mL/min; temperature, 60 °C; injected volume, 5 μL; MS detection.

of the calibration plot) [14]. The precision of the method was determined by the evaluation of repeatability and intermediate precision. Repeatability (expressed as relative standard deviation (RSD, %)) in five consecutive injections of two standard solutions (corresponding to one of the lowest and the highest concentrations of the working linear range) was better than 4% for BBI and 1.5% for lectin in retention time and peak area. Intermediate precision was determined by injecting two standard solutions (corresponding to one of the lowest and the highest concentrations of the working linear range) in five different days within a period of one month. RSDs obtained in peak area were acceptable. Recovery was also evaluated observing values close to 100%. Specificity was demonstrated by comparing the UV spectra and first and second derivatives obtained with the standards and with the samples. The comparison of the slope obtained by the external standard and the standard addition calibrations enabled to

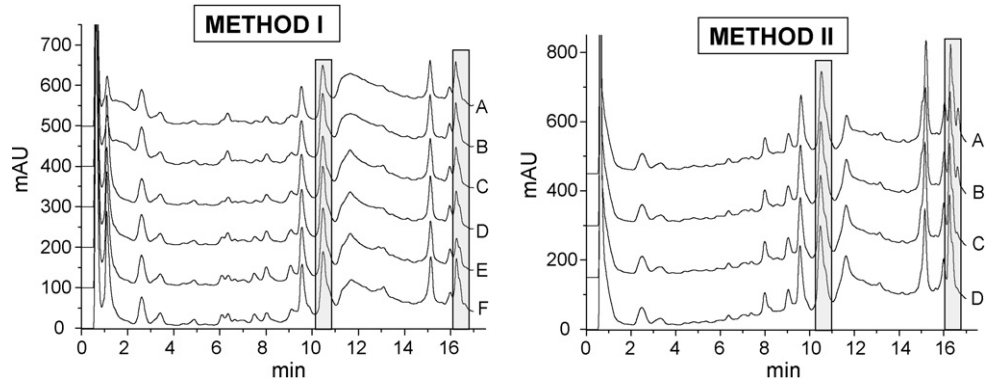

Fig. 3. Separations obtained with extracting methods I and II using different extracting conditions: 5 min agitation with ultrasonic probe (A), 10 min agitation with ultrasonic probe (B), 15 min agitation with ultrasonic probe (C), 30 min agitation with ultrasonic probe (D), 1 h agitation with ultrasonic probe (E), and 1 h magnetic agitation. Chromatographic conditions as in Fig. 1.

Table 1
Characteristics of the perfusion RP-HPLC method for the determination of BBI and lectin in soybean.

	BBI		Lectin					
Working linear concentration range	0.025–0.500 mg/mL							
Detection limit	5.0 μg/mL (5.0 μg/100 mg)[a]		7.5 μg/mL (7.5 μg/100 mg)[a]					
Quantitation limit	22.5 μg/mL[a]		24.9 μg/mL[a]					
Slope by the external standard method[b]	2597.8 (RSD (*n* = 6), 9.2%)		2521.7 (RSD (*n* = 3), 21.0%)					
Repeatability (RSD, %) (*n* = 5)	0.098 mg/mL	0.245 mg/mL	0.050 mg/mL				0.149 mg/mL	
Retention time	3.53	2.93	0.12[c]	0.29[c]	0.17[c]	0.13[c]	0.14[c]	0.10[c]
Peak area	3.82	1.15	1.18[d]				1.45[d]	
Intermediate precision (RSD,%) (*n* = 5)	0.049 mg/mL	0.246 mg/mL	0.025 mg/mL				0.199 mg/mL	
Peak area	15.61	11.56	12.94				8.41	
Recovery (%)								
Spiked solution A[e]	101.0		100.5					
Spiked solution B[f]	92.2		106.0					

[a] Detection and quantitation limits expressed as mg/100 mg units were determined relative to 2 g of sample.
[b] Mean slopes obtained in a period of 1 month.
[c] RSD obtained for the retention time in the three peaks corresponding to lectin.
[d] RSD of the total area obtained by the addition of the areas corresponding to the three peaks of lectin.
[e] Solution containing 0.1439 mg/mL of BBI and 0.1060 mg/mL of lectin spiked with 0.0959 mg/mL of BBI and 0.2121 mg/mL of lectin.
[f] Solution containing 0.3358 mg/mL of BBI and 0.5302 mg/mL of lectin spiked with 0.1439 mg/mL of BBI and 0.2121 mg/mL of lectin.

conclude that the sample matrix affected to the instrument's response.

3.4. Quantitation of BBI and lectin in soybean crops

The method was applied to the simultaneous quantitation of BBI and lectin in eight different soybean cultivars. For that purpose, a single point standard calibration was carried out once established that the response was linear and that the intercept did not significantly differed from zero ($P > 0.05$) [16]. Table 2 shows the concentrations observed in every case for lectin and BBI. Lectin concentrations ranged from 0.28 to 1.03 mg/100 mg of sample while BBI ranged from 0.22 to 0.62 mg/100 mg of sample. Clark cultivars presented the highest contents in lectin and BBI. These contents were in accordance with those given in literature and highlight the interest of this kind of methodologies to explore which varieties are more advantageous for their anticarcinogenic activity [3,9–15].

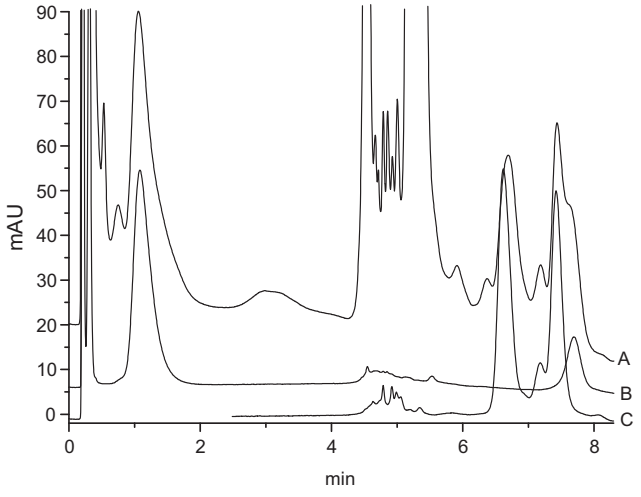

Fig. 4. Chromatographic separation obtained with a Clark Rji soybean extracted using method II (A) and with the trypsin/chymotrypsin inhibitor (B) and lectin (C) standards. Chromatographic conditions: gradient, 19% B in 0.5 min; 19–20% B in 3 min; 20–37% B in 0.5 min; 37–43% B in 5 min; 43–95% B in 0.5 min; mobile phases, 0.1% (v/v) TFA in water (mobile phase A) and 0.1% (v/v) TFA in AcN (mobile phase B); flow-rate, 1.5 mL/min; temperature, 60 °C; injected volume, 10 μL; UV detection, 210 nm.

Table 2
BBI and lectin contents in different soybean varieties determined by the optimized perfusion RP-HPLC method.

Soybean variety	Origin	Lectin concentrations (mg/100 mg of sample)[a]	BBI concentrations (mg/100 mg of sample)[a]
Clark Fe	USA	1.03 (0.16)[b]	0.62 (0.14)[b]
Clark Rji	USA	0.97 (0.06)[b]	0.57 (0.02)[b]
Kita-musume	Japan	0.61 (0.02)[b]	0.25 (0.04)[b]
Ap-18	USA	0.42 (0.02)[c]	0.22 (0.02)[c]
Clark E	USA	0.91 (0.02)[c]	0.60 (0.07)[c]
Fred	France	0.51 (0.02)[c]	0.36 (0.01)[c]
Shih-Shih	Japan	0.28 (0.02)[c]	0.22 (0.01)[c]
Commercial[d]	Unknown	0.57 (0.02)[c]	0.42 (0.06)[b]

[a] Results expressed as is basis.
[b] Mean of two individual determinations every one injected by triplicate. Standard deviation is given in parenthesis.
[c] Mean of three consecutive injections of the same sample solution. Standard deviation is given in parenthesis.
[d] Commercial soybean purchased in a local market in Madrid (Spain).

4. Conclusions

A fast chromatographic methodology has been developed for the simultaneous determination of two proteins with anticarcinogenic activity in soybean: Bowman-Birk inhibitor and lectin. For that purpose, soybean was directly extracted with a sodium acetate buffer for 5 min using direct ultrasonication and injected into the chromatographic system for its separation in less than 9 min. The extract resulted in different peaks being possible the detection of one peak corresponded to Bowman-Birk inhibitor and three peaks corresponding to lectin subunits. Identification of peaks was possible by the comparison of retention times of BBI and lectin standards with the observed in the extracts and by examination of UV spectra, first and second derivatives, mass spectra. The method enabled the detection of 5.0 μg/100 mg of BBI and 7.5 μg/100 mg of lectin. Recovery and reproducibility were also evaluated observing acceptable values for reproducibility and recoveries close to 100%. The method was applied to the determination of BBI and lectin in different soybean varieties observing values ranging from 0.28 to 1.03 mg/100 mg of sample for lectin and from 0.22 to 0.62 mg/100 mg of sample for BBI. These values demonstrated that anticarcinogenic activity of soybean cultivars was not the same in all varieties being possible to suggest certain varieties for their anticarcinogenic activity.

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