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Rapid separation of soybean globulins by reversed-phase high-performance liquid chromatography

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Abstract

A rapid separation of the main soybean proteins (7S and 11S globulins) was carried out by reversed-phase high-performance liquid chromatography. For this purpose, a linear binary gradient acetonitrile–water–0.1% trifluoroacetic acid, at a flow-rate of 1 ml/min and 50°C temperature was designed. Under the experimental conditions of this work, it was possible to separate five peaks corresponding to the globulins from a soybean protein isolate in 9 min. The characterization of soybean proteins was accomplished by analyzing the 7S and 11S purified fractions obtained from a soybean protein isolate. The method was applied to the separation of soybean proteins from commercial foodstuffs: soybean flour, textured soybean and soybean milks.

Keywords: Soybean; Food analysis; Globulins; Proteins

1. Introduction

The increasing demand for animal proteins (milk and meat) around the world has promoted the search for new sources of proteins. Soybean proteins constitute an interesting alternative due to their high nutritional value and low cost. Soybean products contain important quantities of vegetable proteins with a low fat content [1]. In addition to proteins of nutritional interest (48–50%), soybean also contains lipids, minerals, vitamins and phosphorus [1–3]. However, the nutritional value of soybean is not the only factor enhancing its consumption. It plays an important role in preventing heart disease, obesity, blood cholesterol, cancer, diabetes, kidney disease and osteoporosis [4–9]. Furthermore, soybean proteins are also a suitable replacement for milk proteins from animal species, when individuals, especially infants, are allergic to these animal proteins [10].

Approximately 80–90% of the total soybean proteins are storage proteins. They are globulins and they precipitate at pH 4.5–4.8, for which they are often called acid-precipitable proteins. The major storage proteins in soybean are glycinin, and β- and γ-conglycinin which represent about 40, 28 and 3% (content determined immunologically) of the total storage proteins, respectively. Based on their sedimentation constants at pH 7.6 and an ionic strength

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of 0.5, they are designated as 11S (glycinin) and 7S (β- and γ-conglycinin). There are other less abundant storage proteins such as 9S, 15S, and 2S (α-conglycinin) which represents about 14% of soybean globulins [3,11].

Glycinin is a polymeric globulin of M_r 350 000–380 000 with an isoelectric point of 6.4. It is formed by the association of 6 acidic (M_r 20 000–22 000) and 6 basic (M_r 35 000–40 000) polypeptides. β-Conglycinin precipitates at pH 4.8 and has a polymeric structure [12]. Some authors have confirmed the existence of three, four or five 7S subunits [3]. On the other hand, α-conglycinin is a monomeric protein (M_r 26 000) with an isoelectric point of 4.5 [3,12].

There are different methods to fractionate the main globulins [13–15]. These procedures are based on the different globulin solubilities in certain conditions. The Thanh and Shibasaki [15] method seems to be the most widely used – being the least tedious – and allowing the simultaneous isolation of the main proteins present in soybean (whey, glycinin and β- and γ-conglycinin).

Upon their isolation, soybean proteins were characterized by using different analytical methods. This characterization was first performed by immunoelectrophoresis, disc electrophoresis, gel filtration chromatography, ion-exchange chromatography, and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [12,15–18]. More recently, high-performance liquid chromatography (HPLC) has also been used due to its versatility, short analysis times, high resolution, possibility of process automatization and diversity in operation modes. Reversed-phase (RP-HPLC) [19–22] and size-exclusion (HPSEC) [21,23,24] modes have been generally used. Characterization of soybean proteins, isolated generally from soybeans or soybean flour, has been performed and the methods have been applied to the development of a quantitative method with potential for assessing soybean cultivars based on protein content [21] and to the determination of soybean proteins in unheated meats [22] and dairy products [24]. Although gradient elution has been used in RP-HPLC, analysis times ranging from 35 to 90 min have generally been obtained.

The primary objectives of this work were initially to develop a rapid RP-HPLC method for the separation of soybean proteins by considerably reducing the analysis time with respect to the previous HPLC methods presented in literature, and secondly, to apply this method to the characterization of soybean proteins isolated from different soybean products such as textured soybean and soybean flour and others previously not described in the literature such as soybean milks and soybean infant formulae.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (ACN) (HPLC grade; Scharlau, Barcelona, Spain), trifluoroacetic acid (TFA) (HPLC, Spectro grade; Pierce Europe, Oud-Beijerland, Netherlands), and HPLC-grade water (Milli-Q system; Millipore, Bedford, MA, USA) were used in the preparation of the mobile phases.

Tris(hydroxymethyl)aminomethane (Tris) and 2-mercaptoethanol (analysis grade, Merck, Darmstadt, Germany) were used for fractionating soybean globulins.

The soybean protein isolate was obtained from ICN (Aurora, OH, USA), and textured soybean, soybean flour, soybean milks and soybean infant formulae were purchased from a local market in Alcalá de Henares, Madrid, Spain. Total protein content of these samples (Table 1) was determined by an AOAC (1990) method [25].

The 11S and 7S globulins were obtained by an isoelectric precipitation with 0.30 mol/l Tris–HCl buffer, containing 0.01 mol/l 2-mercaptoethanol, of

Table 1
Protein content and relative standard deviation (R.S.D.) of different products from soybean

Soybean	Protein (%)	R.S.D. (%)(n=5)
Protein isolate	79.88	0.93
Flour	46.49	1.96
Textured	46.70	0.84
Milk	4.51	0.40
Powder milk	34.90	0.55
Infant formula		
A	14.44	1.81
B	13.10	3.24
C	14.04	0.48
D	14.09	0.23
E	15.78	1.63

the 11S globulin (at pH 6.4) and 7S globulin (at pH 4.8), as described by Thanh and Shibasaki [15]. Both samples were isolated from the supernatant by centrifugation (10 000 rpm, for 20 min).

Samples were dissolved in water and filtered through 0.22-μm disposable sterile polysulfone filters (Alltech Associates, Deerfield, IL, USA) before injection.

All samples and standards were stored at 3°C or frozen when appropriate. Sample solutions were prepared on the day of analysis and kept on ice until use.

2.2. High-performance liquid chromatography

A Hewlett-Packard 1090 Series II liquid chromatograph (Hewlett-Packard, PA, USA) equipped with a diode array detector and a HP 9153C data acquisition system was used. A 20-μl sample was injected in the chromatograph every time.

The separation was carried out with a PLRP-S column (150×4.6 mm I.D.) from Polymer Labs. (Church Stretton, UK). It was packed with poly-(styrene–divinylbenzene) beads (300 Å, 8 μm particle size). The column's dead time (1.75 min) was determined by using uracil as non-retained solute. Proteins were detected by UV absorption at 254 nm.

The weak mobile phase A was 0.1% TFA in water and the strong mobile phase B was 0.1% TFA in ACN. Mobile phases were filtered through 0.45-μm nylon filters and degassed by sparigng with helium.

After optimizing the experimental conditions (as described in Section 3), a flow-rate of 1 ml/min and 50°C temperature was selected. For a suitable elution of soybean proteins, a linear binary gradient of four steps was used: 20% B for 1 min, 20–35% B in 19 min, and 35–46% B in 0.5 min, followed by a linear reversed gradient 46–20% B in 0.5 min to re-equilibrate the column to starting conditions between runs.

3. Results and discussion

3.1. Optimization of chromatographic conditions

3.1.1. Gradient

Nineteen linear binary gradients were tested to obtain a suitable separation of soybean proteins. For this study, aqueous solutions of the soybean protein isolate were used. These separations were performed using a flow-rate of 1 ml/min, and a temperature of 40°C. All gradients lasted long enough (23.5 min) to ensure that all soybean proteins were eluted.

Fig. 1 shows four of the gradients tried. The first one was 40–46% B in 20 min (gradient range, 0.3%/min) and no separation took place under these conditions (Fig. 1a). Based on this experiment, the gradient was started at a lower percentage of mobile phase B, increasing the gradient range (1.3%/min). The resulting separation showed several peaks, which were better resolved (Fig. 1b). Then, this gradient was divided into two steps to improve band spacing. As shown in Fig. 1c, six peaks could be separated in less than 9 min using this gradient. Further increase in the gradient range deteriorated the separation (Fig. 1d). From these results, the gradient corresponding to Fig. 1c was chosen for the separation of soybean globulins.

When the flow-rate was increased two-fold, decreasing the gradient time by the same factor, the resolution for the early eluting peaks decreased (results not shown).

All gradient profiles were confirmed by adding acetone (5%, v/v) to the acetonitrile phase and recording the signal at 254 nm as a function of time.

3.1.2. Temperature

The influence of temperature on protein separation, using the previously optimized gradient, can be deduced from Fig. 2. As shown in this figure, retention times decreased with increasing temperature in the range 30–60°C, especially for the most retained peaks. However, resolution between peaks 4 and 5 decreases when increasing the temperature. This fact together with some distortion observed for the baseline at 60°C, prompted us to select a 50°C temperature as suitable to carry out a rapid separation without reducing the lifetime of the column.

3.1.3. Mobile phase

The effect of the TFA percentage in mobile phases A and B on protein separation was also studied using the previously optimized gradient. The TFA concentration ranged from 0.1 to 0.4% (v/v).

The lowest concentration (0.1%) gave better results in terms of peak heights, baseline (flat), and reduction in the time required to accomplish the

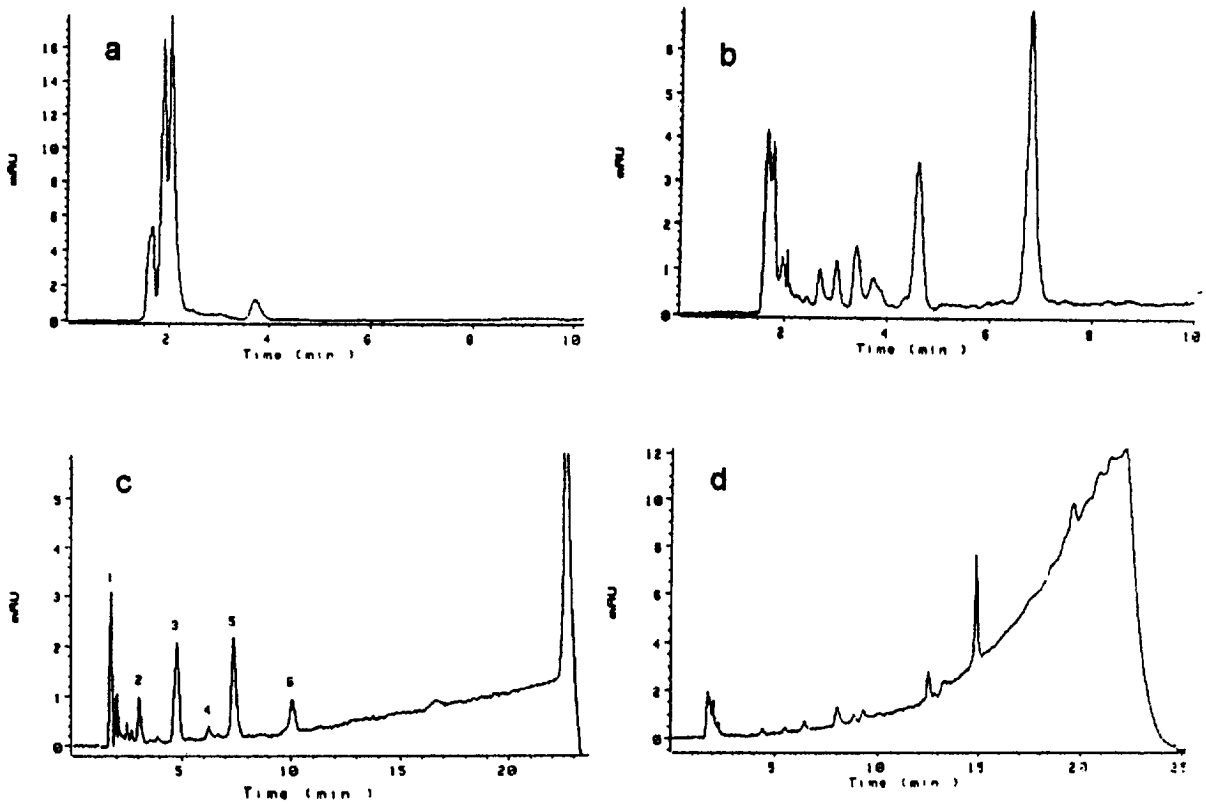

Fig. 1. Chromatograms corresponding to four tested gradients to separate globulins from samples of water diluted soybean protein isolate under the following conditions: temperature, 40°C; flow-rate, 1 ml/min. (a) 40% B, 1 min; 40–46% B in 19 min; 3.1250 mg/ml sample; (b) 20% B, 1 min; 20–46% B in 19 min; 3.1250 mg/ml sample; (c) 20% B, 1 min; 20–35% B in 19 min; 35–46% B in 0.5 min; 1.0034 mg/ml sample; (d) 10% B, 1 min; 10–20% B in 9 min; 20–46% B in 10 min; 1.5485 mg/ml sample. For composition of A and B see Section 2.

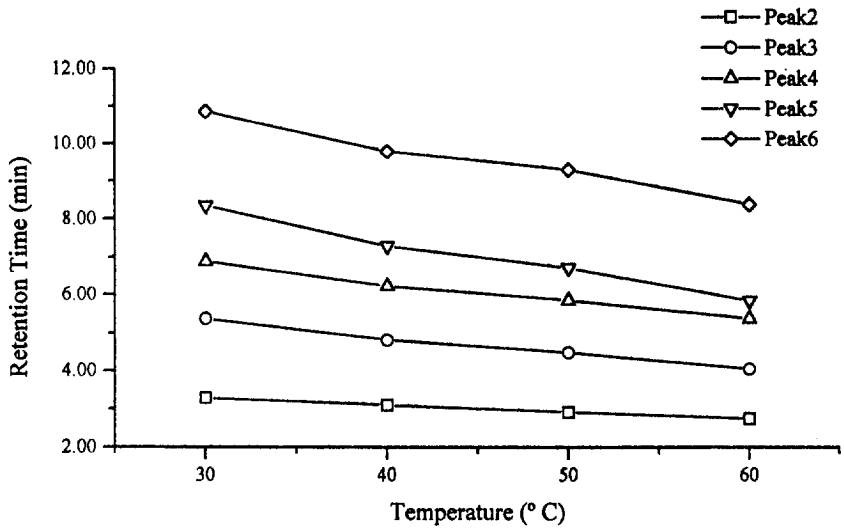

Fig. 2. Variation of the retention times of peaks 2–6 with temperature in a sample of soybean protein isolate. Flow-rate: 1 ml/min. Gradient: 20% B, 1 min; 20–35% B in 19 min; 35–46% B in 0.5 min; 1.0034 mg/ml of soybean protein isolate. For composition of A and B see Section 2.

separation of all proteins per sample (results not shown).

3.2. Chromatographic behaviour of soybean proteins

In order to study the chromatographic behaviour of soybean proteins, aqueous solutions of the soybean protein isolate and its fractionated globulins (7S and 11S) were analysed under the optimized chromatographic conditions. The whey fraction was also injected into the chromatographic system to test the existence of 7S or 11S globulins remaining from the fractionation procedure.

The chromatograms corresponding to these samples are shown in Fig. 3a–d. Fig. 3a shows the appearance of six peaks for the soybean protein isolated, one of them is a minority peak (peak 4). This peak as well as peak 1 disappears when the 7S and 11S fractions are injected. In fact, Fig. 3b and c show that only peaks 2, 3, 5 and 6 are obtained for these fractions and that the two chromatograms are very similar. This similarity could be explained whether by a non-quantitative separation of 7S and 11S fractions in the fractionation method used or by assuming these proteins could be continuously suffering a conversion from one globulin into another ($7S \rightleftarrows 11S$) as it was reported in literature [14,26]. Fig. 3d shows that whey fraction did not contain either 7S or 11S globulins.

The precision, expressed as relative standard deviation, obtained by this method for the measurement of the retention times (Table 2) was less than 3.5%, run to run. In addition, the repeatability between days, was less than 2.1%.

3.3. Separation of soybean proteins in a soybean flour, textured soybean, soybean milks and soybean infant formulae

The method described above has been applied for the characterization of several products derived from soybean, which are commercially available for human consumption: soybean flour, textured soybean, soybean milk (both liquid and powder) and five samples of soybean infant formulae. Also, the 7S and 11S fractions obtained from soybean flour and textured soybean were injected into the chromatographic system.

When the fractionation of the globulins from soybean flour and textured soybean was carried out and the fractions (7S and 11S) were injected in the chromatographic system in the same conditions used previously, it could be observed (results not shown) that the chromatograms obtained for these fractions were quite similar to those obtained for soybean flour and textured soybean samples shown in Fig. 4. The only difference was that peak 1 did not appear in 7S and 11S fractions (as in the case of the fractions obtained from the soybean protein isolated). This allows us to think that peak 1 does not correspond to any of these globulins because it appears only when the non-treated samples or the soybean protein isolate were injected. However, when a fractionation procedure is achieved to isolate the globulins, peak 1 disappears. Peak 4, however, appeared in the chromatograms of fractions 7S and 11S obtained from soybean flour and textured soybean and indicated that this peak should correspond to a globulin. The fact that this peak does not appear in the chromatograms of these same fractions when they were obtained from the soybean protein isolate (Fig. 3b and c), leads us to think that peak 4 can be useful in the characterization of different products derived from soybean that have had a different processing or have been mixed with different additives such as vitamins, amino acids, etc., as is the case of milks and other dairy products derived from soybean [27]. In fact, chromatograms shown in Fig. 4 for a soybean milk and a powder soybean milk show that for these products peak 4 did not appear in the chromatogram. The same was obtained for the five samples analysed of soybean infant formulae (chromatograms not shown).

Table 3 groups the area percentage and the standard deviation of every peak obtained for the soybean samples studied. Peak 4 is absent in every soybean milk product. Peak 3 presents the highest area percentage in all samples while peak 4 (when present) and peak 6 are minority peaks. Peak 5 presented the maximum variability in area percentage among the soybean dairy products and also between soybean non-dairy products. Thus peak 4 characterizes non-dairy soybean products and peak 5 allowed the differentiation among products of the same group.

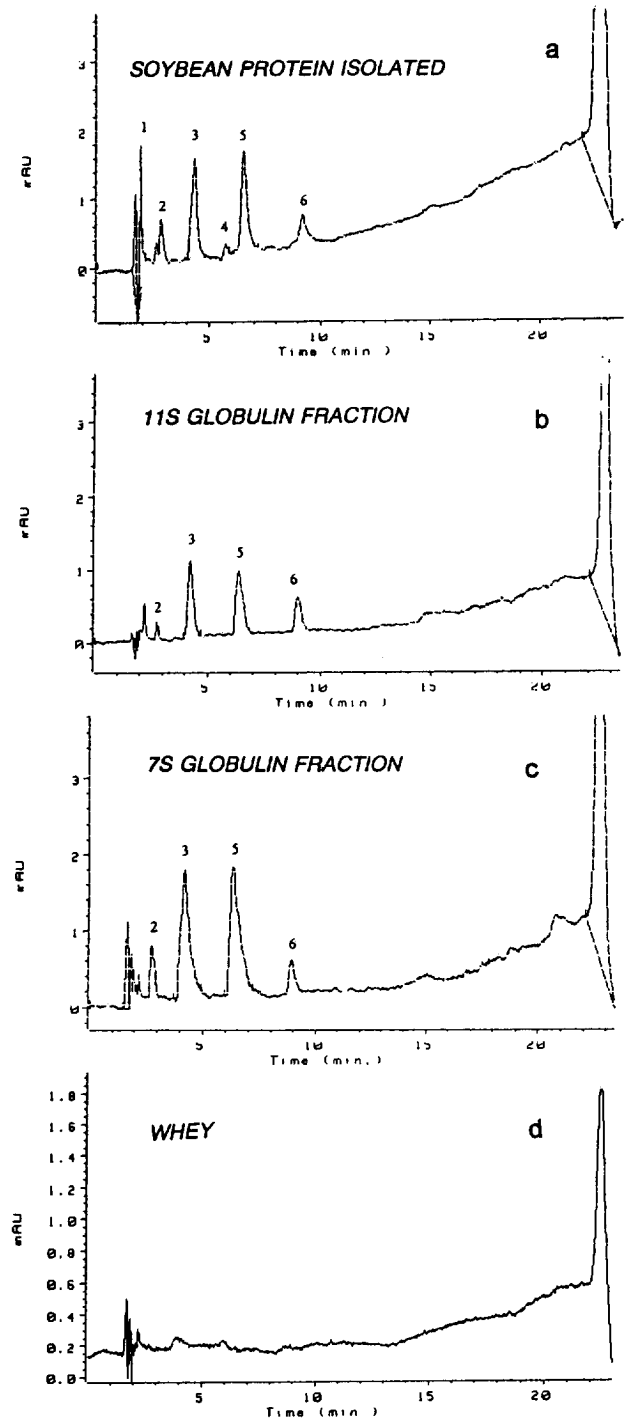

Fig. 3. Chromatograms corresponding to aqueous solutions of (a) 0.9768 mg/ml soybean protein isolate; (b) 22.8273 mg/ml 11S globulin fraction; (c) 9.8048 mg/ml 7S globulin fraction; (d) 2.9977 mg/ml whey fraction which came from the fractionation of 50 g of commercialized soyabean protein isolate. Experimental conditions optimized in this work.

Table 2
Retention times (t_R) and relative standard deviation (R.S.D.) of peaks 1–6 in the soybean protein isolate, and in the 11S and 7S isolated fractions

Sample	n	Concentration (mg/ml)	Peak											
			1		2		3		4		5		6	
			t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)
Soybean protein isolate	7	0.9768	1.98	2.11	2.82	1.99	4.23	2.29	5.63	2.20	6.30	3.45	8.87	3.41
11S Fraction	3	22.8273	–	–	2.79	1.37	4.22	0.80	–	–	6.37	0.64	9.00	0.36
7S Fraction	3	9.8048	–	–	2.74	0.04	4.16	0.22	–	–	6.29	0.16	8.93	0.25

n: Number of consecutive runs in the same day.

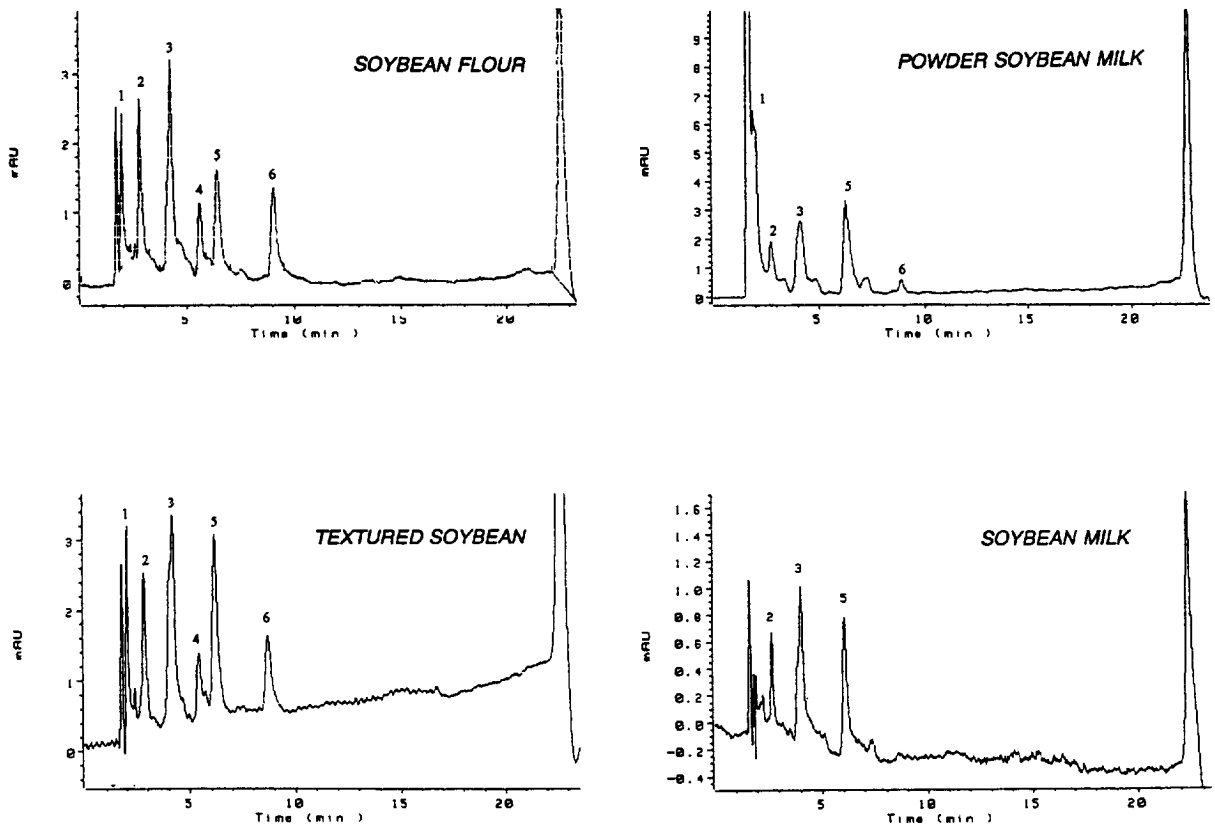

Fig. 4. RP-HPLC of aqueous samples of soybean flour (0.9926 mg/ml), textured soybean (0.9977 mg/ml), soybean milk (4.0665 mg/ml), and powder soybean milk (4.0934 mg/ml). Experimental conditions optimized in this work.

Table 3
Area percentage (*A*) and standard deviation (S.D.) of peaks 2–6 in different soybean products

Sample	Concentration (mg/ml)	Peaks									
		2		3		4		5		6	
		A (%)	S.D.	*A* (%)	S.D.	*A* (%)	S.D.	*A* (%)	S.D.	*A* (%)	S.D.
Soybean protein isolate	0.9768	13.28	1.55	37.23	1.47	3.52	0.32	37.85	2.85	9.09	1.88
Soybean flour	0.9926	21.12	2.50	34.22	2.52	8.41	0.91	16.84	0.83	15.20	0.99
Textured soybean	0.9977	17.53	0.39	35.28	1.50	7.63	0.97	31.70	2.53	11.07	1.03
Soybean milk	4.0665	13.16	0.67	46.36	2.76	–	–	40.48	2.68	–	–
Powder soybean milk	3.2877	10.27	0.55	42.21	0.56	–	–	43.51	0.58	4.04	0.43
Soybean infant formula A	4.0170	16.62	1.68	55.41	2.07	–	–	25.95	0.98	–	–
Soybean infant formula B	3.6858	9.32	0.86	46.52	0.37	–	–	35.90	2.18	8.15	0.80
Soybean infant formula C	3.7282	18.45	0.86	69.31	1.22	–	–	12.23	0.37	–	–
Soybean infant formula D	3.7858	5.91	0.55	39.31	2.61	–	–	48.14	2.10	11.32	0.18
Soybean infant formula E	3.8846	10.16	0.78	47.64	2.50	–	–	35.25	1.07	6.95	1.06

4. Conclusions

The proposed RP-HPLC method, provides a good and simple separation of soybean proteins in less than 9 min. Chromatograms obtained for a soybean protein isolate showed six peaks, five of which were identified as corresponding to 7S and 11S globulins.

The method was applied to the separation of peaks for the 7S and 11S fractions, not detecting any difference between chromatograms of both fractions. This chromatographic behaviour may be attributed to a continuous conversion of one globulin into another as some authors have suggested or to the fractionation method not being quantitative.

The method was suitable to characterize different commercial products derived from soybean. One of the peaks of globulins (peak 4) characterizes non-dairy soybean products since it is absent in soybean milk samples. Peak 5 exhibits the maximum variability in the percentage area among different soybean products of the same group.

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