A Perfusion Reversed-Phase Chromatographic Method for Ultrarapid Determination of Soybean Proteins in Soybean Infant Formulas and Soybean Milks: Method Development and Validation

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Abstract

Perfusion reversed-phase high-performance liquid chromatography (perfusion RP-HPLC) is applied for the first time to the determination of soybean proteins in commercial samples prepared from soybean protein isolate. A linear binary gradient of acetonitrile–water–0.1% trifluoroacetic acid at a flow rate of 3 mL/min and a temperature of 60°C is optimized to analyze soybean proteins in approximately 3 min. Direct injection of samples into the chromatographic system is possible by dissolving them in water. Quantitation of soybean proteins is achieved by the proposed method using a soybean protein isolate as an external standard. The method is validated and applied to the quantitation of soybean proteins in infant formulas and powdered and liquid soybean milks.

Introduction

Soybean derivatives currently constitute an important source of good quality vegetable proteins (1) and are considered an interesting alternative to the consumption of animal proteins (1–4). This has promoted the appearance of many products derived from soybean. Among these products, special attention should be paid to soybean dairy-like products such as infant formulas (IF), liquid milks (LM), and powdered milks (PM), because they are often the basis of the nourishment for individuals who are allergic to animal milk proteins (5).

Soybean milk can be prepared either from an aqueous extract of whole soybeans or by adding soybean protein isolate to hot water while agitating and then adding oil, sugar, and flavoring materials (6–8). Powdered soybean milk can be produced either directly from soybean protein isolates or by drying soybean milk (9). Soybean IFs are made with highly refined soybean protein isolates to avoid raffinose and stachyose which produce flatulence. In addition to soybean protein isolate, IFs also contain other nutrients (lipids, vitamins, minerals, and amino acids, generally methionine) to supply the deficiencies that a soybean protein isolate could produce in an infant (10). For these types of products, methodologies allowing protein analysis regardless of the amino acid content are necessary, because the determination of total nitrogen content in such methods as micro-Kjeldahl digestion is insufficient.

Analytical characterization of soybean proteins was initially performed using a variety of different techniques, the most frequently used in recent years being high-performance liquid chromatography (HPLC) because of the possibility of characterizing and determining soybean proteins in short analysis times and with high resolution (11–22). Among the different HPLC modes, reversed-phase HPLC (RP-HPLC) using gradient elution has been used the most (13–18). Although high analysis times (~ 90 min) have generally been required for the RP-HPLC separation of soybean proteins, this time has recently been reduced to about 9 min (21,22). In spite of the significance of this drastic decrease in analysis run time, shorter times are desirable for a method to be used for routine analysis. In this case, a change in the principle of the separation is recommended.

During the period from 1989 to 1991, a new HPLC technique characterized by rapid analysis was introduced: perfusion chromatography (23–26). This technique uses packing materials of a cross-linked polystyrene–divinylbenzene matrix having a bimodal porous structure constituting a macroporous region with 6000–8000-Å transecting pores (throughpores) and a connected network of smaller size 800–1500-Å diffusive pores that provide a large adsorption surface area (25,27,28). This peculiar structure of perfusion particles maximizes intraparticle convection and pore diffusion of solutes, thus favoring the mass transfer of large molecules such as proteins (25,27–29). Based on this structure, perfusion chromatography enables separations of biopolymers 10–100 times faster than traditional chromatographic packings without significant losses in resolution, efficiency, or capacity (23,26). This chromatographic technique has

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never been applied to the characterization and determination of soybean proteins.

The aim of this work is to take advantage of this promising technique by optimizing a gradient perfusion RP-HPLC method for the analysis of soybean proteins in some soybean dairy-like products derived from soybean protein isolate in analysis times short enough to be valid in routine analysis.

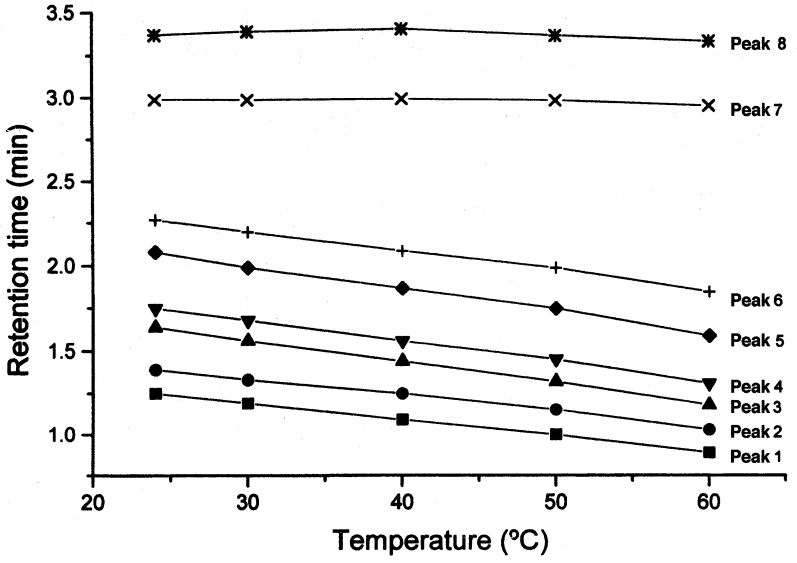

Figure 1. Variation of the retention time of peaks 1–8 of an SPI with temperature. Conditions: aqueous solution of SPI, 0.4758 mg/mL (as dry basis); flow rate, 2.5 mL/min; gradient, 5–25% B in 2 min, 25–45% B in 1.5 min; mobile phase A, 0.1% TFA in water; mobile phase B, 0.1% TFA in ACN; injection volume, 20 µL; detection, 254 nm. The number to the right of every line corresponds to the peak number in Table I.

Experimental

Chemicals and samples

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

Analysis-grade tris(hydroxymethyl)aminomethane (Tris), 2-mercaptoethanol (Merck, Darmstadt, Germany), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and sodium chloride (Merck) were employed for the optimization of the sample injection.

The soybean protein isolate (SPI) standard used in this work was obtained from ICN (Aurora, OH). The protein content of this protein isolate, determined by Kjeldahl analysis (7 replicates), was 92.99% (relative standard deviation [RSD] 2.86%) (30). Soybean IFs and soybean liquid (LM) and powdered (PM) milks (all derived from soybean protein isolate) were purchased from chemists and local markets of Alcalá de Henares, Madrid,

Table I. Retention Time and RSD of Peaks 1–8 for the SPI as a Function of the TFA Percentage*

TFA (%)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5		Peak 6		Peak 7		Peak 8	
	t_R†	RSD‡ (%)	t_R	RSD (%)	t_R	RSD (%)	t_R	RSD (%)	t_R	RSD (%)	t_R	RSD (%)	t_R	RSD (%)	t_R	RSD (%)
0.05	0.98	0.77	1.10	0.87	1.30	0.69	1.43	0.74	1.72	0.73	1.96	0.68	2.85	0.32	3.24	0.12
0.1	0.98	0.44	1.14	0.80	1.30	0.25	1.42	0.07	1.71	0.07	1.96	0.01	2.98	0.13	3.36	0.13
0.2	0.99	0.69	1.14	0.87	1.31	0.34	1.44	0.12	1.73	0.12	1.97	0.15	3.04	0.08	3.44	0.06
0.3	0.94	0.12	1.11	0.47	1.26	0.09	1.39	0.11	1.69	0.03	1.94	0.14	3.07	0.13	3.47	0.15

* Three consecutive runs for every TFA percentage, aqueous solution of 0.4728 mg/mL SPI (dry basis).
† Retention time.
‡ Relative standard deviation.

Table II. Characteristics of the Chromatograms for SPI Injected in Different Solvents*

Solvent	Number of peaks	Total peak area	RSD (%)
Water (HPLC grade)	8	24.51	3.59
0.010 mol/L 2-Mercaptoethanol	9†	26.65	1.96
0.400 mol/L Sodium chloride	7	15.34	2.99
0.027 mol/L Phosphate buffer (pH 7)	8	17.03	8.24
0.027 mol/L Phosphate buffer (pH 8)	8	19.77	1.78
0.027 mol/L Phosphate buffer + 0.400 mol/L sodium chloride (pH 7)	7	15.70	6.37
0.035 mol/L Tris-HCl buffer (pH 9)	8	21.17	5.02
0.035 mol/L Tris-HCl buffer (pH 10)	9†	26.84	0.10
0.035 mol/L Tris-HCl buffer + 0.400 mol/L sodium chloride (pH 10)	9†	16.71	2.02
0.035 mol/L Tris-HCl buffer + 0.010 mol/L 2-mercaptoethanol (pH 10)	9†	31.47	1.60

* Experimental conditions are the same as in Figure 2. SPI concentration (dry basis) in the sample injected is approximately 0.5 mg/mL.
† Peak 8 splits into two poorly resolved peaks.

Spain. The total protein content of these samples was also measured by Kjeldahl analysis. All samples were stored at 3°C. Before analysis by HPLC, dry matter content of the SPI and soybean samples was determined by drying them at 130°C (for SPI) or 102°C (for samples) to a constant weight.

The protocol for preparing either the solutions of SPI or of dairy-like soybean products was as follows: the sample was weighed and dissolved in the appropriate solvent (distilled water), sonicated for 3 min and then centrifuged (3000 rpm, 5 min, 3°C) to remove the supernatant, which was then kept on ice until its injection into the chromatographic system.

HPLC

A Hewlett-Packard (Pittsburgh, PA) 1090 series II liquid chromatograph equipped with a diode array detector and an HP 9153C data acquisition system was used. After optimizing the wavelength for soybean protein detection (described in Results and Discussion), the absorbance of these proteins was monitored at 254 nm. The injection volume was 20 µL.

The separation was accomplished with a POROS R2/H (PerSeptive Biosystems, Framingham, MA) perfusion column (50 × 4.6-mm i.d.) packed with crosslinked polystyrene–divinylbenzene beads (10-µm particle size). The column's dead time (0.234 min) and efficiency (1281 plates/m) were determined using uracil as nonretained solute.

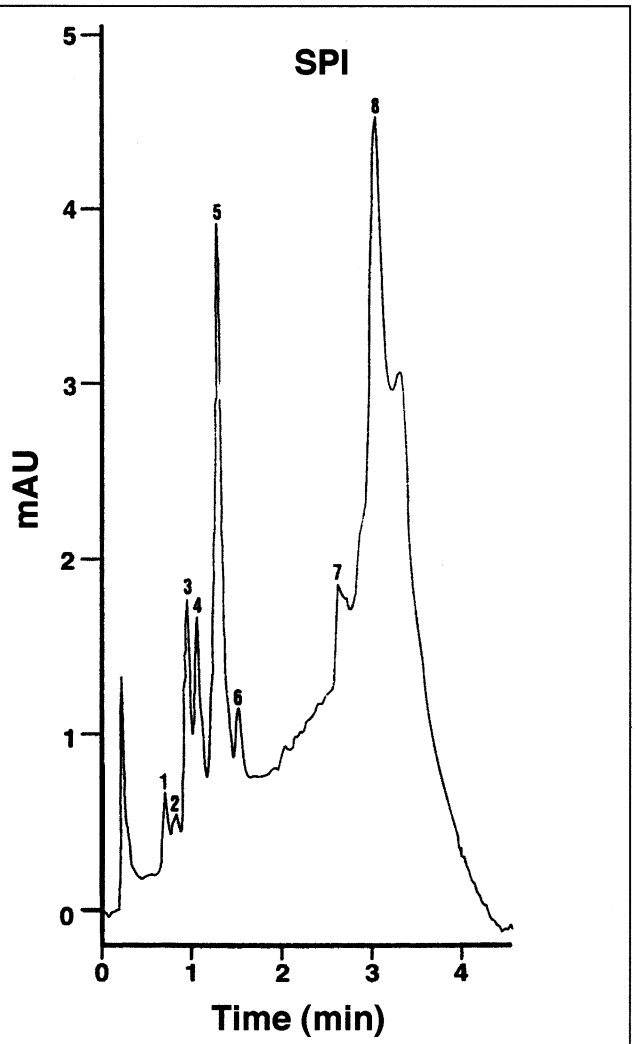

Figure 2. Separation of soybean proteins from an SPI. Conditions: aqueous solution of SPI, 1.2026 mg/mL (as dry basis); flow rate, 3 mL/min; temperature, 60°C; gradient: 5–25% B in 1.7 min, 25–45% B in 1.3 min; mobile phase A, 0.1% TFA in water; mobile phase B, 0.1% TFA in ACN; injection volume, 20 µL; detection, 254 nm.

After optimizing the experimental conditions (described in Results and Discussion), the RP-HPLC method was performed at a flow rate of 3 mL/min at 60°C using a linear binary gradient in two steps: 5–25% B in 1.7 min, 25–45% B in 1.3 min. The column was re-equilibrated between runs with a linear gradient from 45 to 5% B (1 min) followed by a 1 min step at 5% B. Mobile phases for the gradient were as follows: mobile phase A, 0.1% TFA in water; mobile phase B, 0.1% TFA in ACN. The mobile phases were filtered using 0.45-µm nylon filters and degassed with helium before use.

Calibration

The chromatographic system was calibrated using the external standard method with aqueous solutions of SPI over the range of 0.4–1.5 mg/mL. The peak areas corresponding to soybean proteins were integrated by setting the baseline from valley to valley, and the average of the total area of three injections was calculated. To obtain the calibration curve, the total peak area was plotted against the injected SPI concentration (corrected for purity and moisture). Using the standard curve, the soybean content of dairy-like soybean products was quantitated from aqueous solutions of these samples. The concentrations of these solutions were as follows: 3–6 mg sample per milliliter (PMs and IFs) and 12–15 mg sample per milliliter for LMs.

Data treatment

The linearity in this relationship was obtained by least-squares regression analysis carried out with an Univariate Linear Calibration Program (31) and by the procedure described by Dorschel et al. (32). The linear model was validated by means of the analysis of residuals and the analysis of variance (33).

Results and Discussion

Optimization of the chromatographic separation of soybean proteins

All experiments conducted to develop a perfusion RP-HPLC method for separating soybean proteins were carried out with solutions of approximately 0.5 mg/mL SPI. The effect of certain gradient parameters, such as the variation in the volume fraction of mobile phase B during the gradient ($\Delta\Phi$), gradient time (t_g), flow-rate (F), and gradient slope (% B/min) on the separation of soybean globulins were investigated. In addition, other chromatographic conditions (i.e., the percentage of TFA in the mobile phase, temperature, ultraviolet wavelength for protein detection, and the solvent to prepare the sample solutions for injection in the chromatographic system) were also studied.

First, 16 linear binary gradients with an increasing percentage of mobile phase B in one step were tested for the elution of soybean globulins using a flow rate of 2.5 mL/min at 50°C. In these gradients, $\Delta\Phi$ was varied from 0.40 to 0.77 for different initial and

final mobile phase compositions within the range of 5–85% mobile phase B. Gradient times of 2, 3, 4, and 5 min were tested for each one of the gradient ranges. Using these conditions, the gradient slope was varied between 8 and 38.5%/min. All of these gradients were followed by a reversed linear gradient in 1 min (column re-equilibration to initial mobile phase composition) and maintained at this composition for 1 min. As shown in these experiments, the gradient elution conditions that enabled better separation of soybean globulins were 5–45% mobile phase B in 3.5 min. The resulting separation showed eight peaks which were better resolved when the gradient was divided into two steps to improve band spacing: 5–25% mobile phase B in 2 min (10%/min) and 25–45% mobile phase B in 1.5 min (13.33%/min).

With this linear gradient of 2.5 mL/min at 50°C, the influence of the TFA percentage in mobile phases on the separation of soybean proteins was investigated. TFA was chosen as the mobile phase additive because of its low pH, protein denaturing properties, and ion-pairing characteristics (34). Table I groups the values of retention time and RSD corresponding to the eight peaks in the chromatogram of soybean proteins. Nearly identical retention times for the peaks of globulins were seen when the percentage of TFA in mobile phases A and B was varied from 0.05 to 0.3%. In addition, 0.05% TFA deteriorated peak resolution. For these reasons, the lower percentage of additive (0.1% TFA) was chosen for all ensuing work, enabling an optimum separation of soybean proteins.

The effect of temperature was studied in the range 24–60°C using the aforementioned optimized conditions for the chromatographic separation of soybean globulins. As illustrated in Figure 1, retention times for the various globulin peaks generally decreased as the column temperature was increased from 24 to 60°C, this effect being less significant for the most-retained proteins. This behavior (in which a different variation of retention time with temperature is observed for chromatographic peaks) could be expected for proteins (35,36). Furthermore, improved resolution, decreased bandwidth, and lower backpressure were noticed at higher temperatures. From these results, a temperature of 60°C was selected as suitable to carry out the separation of soybean proteins without reducing the lifetime of the column.

The low backpressure enabled an increase in the flow rate to 3 mL/min with a corresponding adjustment of gradient time to 3 min (at constant gradient volume, $F \times t_g \sim 9$ mL). Under these conditions, the eight peaks in the chromatogram could be well resolved in less than 3.5 min (total analysis time of 5 min).

Finally, the effect of monitoring the absorbance of soybean globulins at wavelengths other than 254 nm on the final chromatogram of these proteins was investigated. The wavelengths considered for this study were those of greater absorption sensitivity for the major amino acids present in soybean proteins: 210–220 nm (peptide bonds), 228 nm (histidine), 240 nm (cystine), 254 nm (phenylalanine), and 280 nm (tryptophan and tyrosine) (35). In comparison with 254 nm, the chromatograms of proteins detected at 280 nm showed less sensitivity. When the protein absorbance was measured at 228, 240, and especially at 210 nm, chromatograms showed a baseline shift much higher than that observed at 254 nm. In view of these results, the need for detection at 254 nm for this work was obvious.

Figure 2 shows the resulting chromatogram for the separation of soybean proteins from SPI under optimized conditions. This chromatogram proved the ability of the newly developed perfusion RP-HPLC method to carry out a high-speed separation of soybean

Table III. Calibration by the External Standard Method of Soybean Proteins by Perfusion RP-HPLC*

Linear concentration range (mg/mL)	Slope†	Intercept†	Standard error	r^2‡
0.4089–1.1183 (4)§	38.54 (3.54)	0.102 (2.73)	0.4266	0.9990
0.4314–1.0932 (5)	35.10 (2.65)	1.635 (2.18)	0.4491	0.9984
0.4401–1.3153 (5)	36.44 (3.54)	1.444 (3.45)	0.7493	0.9972
0.4388–1.3141 (6)	34.46 (4.33)	0.873 (4.03)	1.1430	0.9918
0.4379–1.3025 (4)	32.64 (2.07)	1.994 (1.95)	0.3433	0.9996
0.4381–1.1410 (5)	33.80 (2.46)	1.536 (2.03)	0.4285	0.9984
0.4382–1.3070 (6)	38.95 (3.62)	−0.416 (3.36)	0.9526	0.9956

* Experimental conditions as in Figure 2. Calibration lines obtained on seven different days within a two-month period.
† Errors in the slope and intercept of the regression line are given in parentheses.
‡ Squared correlation coefficient.
§ Number of points considered for the regression. Each point represents the average of three consecutive injections of each standard solution.

Table IV. Precision Expressed as RSD (%) for the Results in Peak Area and in Concentration Corresponding to the Analysis of a Soybean Protein Isolate and Some Soybean Dairy-Like Produts Analyzed by a Perfusion RP-HPLC Method*

SPI				Soybean milk		
Repeatability ($n = 10$)†		Inter-day reproducibility in peak area ($n = 4$)‡		reproducibility in concentration ($n = 10$)§		
Peak area	Concentration	0.4365 mg/mL	1.1298 mg/mL	IF	PM	LM
3.10	3.11	3.96	2.79	5.00	2.65	2.00

* Experimental conditions are the same as in Figure 2.
† Number of injections of an SPI solution of 0.7659 mg/mL (as dry basis and corrected by the purity of the standard).
‡ Analysis was performed on four different days; each standard solution was injected three times per day.
§ Analysis of ten individual samples of the following concentrations as dry basis (mean value ± SD): 5.2893 ± 0.1183 mg/mL (IF), 1.4113 ± 0.0050 mg/mL (PM), 9.7626 ± 0.4712 (LM).

proteins. The analysis of these eight peaks by the diode array scanning technique showed typical spectra obtained for proteins.

Sample injection

To optimize the appropriate solvent for preparing protein solutions before their chromatographic analysis, different solvents were used: water, 0.010 mol/L 2-mercaptoethanol, 0.400 mol/L sodium chloride, 0.027 mol/L phosphate buffers at pH 7 and 8 with and without 0.400 mol/L NaCl, 0.035 mol/L Tris-HCl solutions at pH 9 and 10 with and without 0.400 mol/L NaCl, and a 0.035 mol/L Tris-HCl solution with 0.010 mol/L 2-mercaptoethanol.

Table II shows the results concerning the number of peaks in the chromatogram obtained and the total peak area for soybean proteins. These results showed that when 2-mercaptoethanol was used (alone or mixed with Tris-HCl buffer) instead of water, soybean proteins unfold as a result of the reduction of disulfide bonds; consequently, the number and size of peaks increase. In contrast, the presence of sodium chloride in the solvent stabilizes the structure of the soybean proteins, making it necessary to raise temperatures to denature them; as a consequence, a reduction in the number and size of the peaks was observed at neutral pH. However, the use of sodium chloride at pH 10 increases the number of peaks (from 7 to 9) and the total area. This effect of increasing total area and number of peaks with increasing the pH may be considered a general effect, regardless of the additive used. This could be caused by the denaturation of soybean proteins produced by repulsions among the negatively charged groups that appear at these pH values. These results suggest that water, in addition to being the simplest solvent, is the most appropriate for preparing soybean protein solutions before their analysis by perfusion RP-HPLC because it provides a good resolution between peaks and a high value of total peak area.

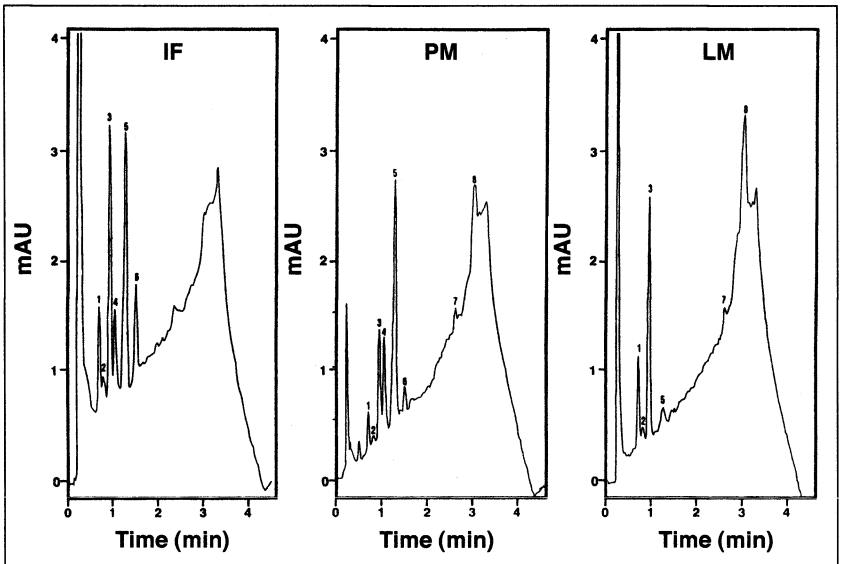

Figure 3. Perfusion RP-HPLC separation of aqueous solutions (as dry basis) of 5.7314 mg/mL IF 2 (A), 1.6748 mg/mL PM 1 (B), and 15.0560 mg/mL LM 1 (C). Experimental conditions are the same as in Figure 2.

Method validation

In order to use the optimized perfusion RP-HPLC method to quantitate soybean proteins in real samples, the overall system was validated by evaluating its linearity, precision, robustness, and recovery of soybean protein in spiked soybean samples.

Table III compiles the linear concentration range and the values of the slope, intercept, standard error, and correlation coefficient for the equations of calibration plots (external standard) obtained on seven different days within a two-month period. Good linear correlation (correlation coefficient $r^2 > 0.99$) was found between the

Table V. Recovery for Some Dairy-Like Soybean Products Found by the Optimized Perfusion RP-HPLC Method*

Sample	Concentration of sample solution (mg/mL)†	Amount of SPI added to the sample (mg)‡	Total protein concentration (mg/mL)	Protein concentration found using perfusion RP-HPLC (mg/mL)	Recovery (%)
IF (11.02 mg/100 mg,	5.3061 ± 0.0925	0.4201	0.6476	0.5945 ± 0.0330	91.80
SD = 0.55)§		1.1883	0.7896	0.7389 ± 0.0300	93.58
		1.6022	0.8484	0.8176 ± 0.0300	96.37
Mean value					93.92 ± 2.30
PM (35.49 mg/100 mg,	1.4144 ± 0.0319	0.3700	0.5608	0.5229 ± 0.0670	93.24
SD = 0.94)		1.1768	0.6887	0.6511 ± 0.0550	94.54
		1.5319	0.7637	0.7027 ± 0.0530	92.01
Mean value					93.26 ± 1.26
LM (4.31 mg/100 mg,	10.5171 ± 0.8290	0.3285	0.4563	0.4440 ± 0.0630	97.30
SD = 0.09)		0.9697	0.5452	0.5547 ± 0.0570	101.74
		1.2850	0.5985	0.5534 ± 0.0570	92.46
Mean value					97.17 ± 4.64

* Experimental conditions are the same as in Figure 2.
† Expressed as dry basis.
‡ Expressed as dry basis and corrected by the purity of the standard.
§ Mean value of protein concentration, expressed as dry basis, found by analyzing ten individual samples by the optimized perfusion RP-HPLC method. SD, standard deviation.

total peak area of soybean proteins and the concentration of SPI injected into the chromatographic system by least-squares regression analysis. The linearity of the calibrations was also checked by plotting sensitivities (detector response/concentration) versus the logarithm of concentrations, such as those in the procedure described by Dorschel et al. (32). In all cases, calibration lines show linear plots within a tolerance less than ± 5%. The slope of the straight line was quite reproducible (interday RSD = 6.67 %), and in all the calibration lines found by this method, the intercept on the ordinate did not significantly differed from zero (t-test, $P < 0.05$). These results accounted for the good quality and robustness of the optimized perfusion chromatographic method. In addition, detection limits were about 70 µg soybean proteins per milliliter.

Figure 3 shows the chromatograms corresponding to a soybean IF, PM, and LM. The chromatogram of PM shows the same eight peaks of the SPI chromatogram (Figure 2). The other two chromatograms do not contain all the peaks of the SPI. The lack of certain peaks in the chromatograms obtained for IF and LM could be due to the different manufacturing conditions these soybean products have been subjected to (temperature, hydrolysis, etc.).

Precision of the perfusion RP-HPLC method was evaluated using both the SPI and some representative samples of soybean dairy-like products (IF, PM, and LM). Results obtained in this study are grouped in Table IV. Repeatability, calculated as the RSD (in peak area and in concentration) for ten injections of 0.7659 mg/mL SPI, was about 3%. The retention times for the eight peaks obtained for SPI in the same experiment (results not included in

Table VI. Protein Content in Dairy-Like Soybean Products Determined by the Optimized Perfusion RP-HPLC Method*

		Protein concentration (mg /100 mg sample)†		
Soybean product	Lot	Kjeldahl method‡	Label	Perfusion RP-HPLC method§
IF 1	1**	13.79 (0.54)	14.00	7.04 (0.35)
	2**			4.75 (0.04)
	3			4.96 (0.30)
IF 2	1††,**	14.86 (0.44)	14.60	11.02 (0.55)‡‡
	2**			12.99 (0.02)
IF 3	1	14.12 (1.19)	14.30	9.94 (0.48)
IF 4	1	15.44 (0.17)	15.30	9.02 (0.18)
IF 5	1††,**	14.86 (0.33)	14.70	13.54 (0.69)
	2**			12.82 (0.07)
	3			7.23 (0.03)
IF 6	1††,**	13.21 (0.15)	15.17	9.33 (0.15)
	2**			9.28 (0.61)
	3			10.38 (0.20)
IF 7	1††,**	14.15 (0.21)	14.20	8.34 (0.08)
	2			8.30 (0.08)
IF 8	1**	15.60 (0.47)	15.60	11.57 (0.30)
	2			7.15 (2.31)
IF 9	1**	13.27 (0.56)	13.70	6.44 (0.03)
	2††			9.57 (0.81)
IF 10	1	14.30 (0.70)	15.00	15.37 (0.83)
	2††,**			16.75 (0.25)
	3**			16.01 (0.21)
PM 1	1††,**	22.31 (0.70)	21.30	27.87 (0.57)
PM 2	1**	34.81 (1.63)	35.00	35.49 (0.94)‡‡
LM 1	1	2.82 (0.12)	3.10	2.82 (0.04)
	2**			2.11 (0.01)
	3**			2.84 (0.03)
LM 2	1	2.89 (0.15)	3.30	4.31 (0.09)‡‡
	2††,**			3.43 (0.04)
	3**			3.96 (0.03)

* Experimental conditions as in Figure 2.
† Results expressed as dry basis.
‡ Six replicates. Standard deviation given in parentheses.
§ Mean of two individual determinations (SD). Standard deviation given in parentheses.
** Samples were opened some days prior to their analysis.
†† Expired samples.
‡‡ Ten replicates.

Table IV) yielded RSDs ≈ 1.2 % for each peak. Day-to-day reproducibility was calculated as the RSD value obtained for the injection of two standards whose protein concentration corresponded to the lowest and highest concentrations of the linear range (maximum error range) on four different days. A value of RSD ≈ 4% was obtained in peak area for both standards. Moreover, Table IV shows that the reproducibility obtained for three real samples from soybean (one IF, one PM, one LM), expressed as the RSD in concentration corresponding to the injection of ten individual samples of each product, was always less than 5%.

The recovery (%) of soybean proteins when a known quantity of the SPI was added to a real sample of a soybean product was also determined. Data in Table V show the results obtained when quantities corresponding to 10, 30, or 40% of the protein content of the sample measured by the perfusion RP-HPLC method (mean of ten determinations) were added to each of the three samples of soybean dairy-like products previously mentioned. Recoveries obtained (mean of the three determinations) were higher than 93% for the three samples. The best recoveries were obtained for LM, though the values corresponding to IF and PM were also considered acceptable.

Results shown in Table V also allowed for the comparison of the slopes of the regression lines obtained in these experiments with those obtained for the previously performed calibration using the SPI as external standard. These slopes did not differ significantly (*F*- and *t*-tests to compare variances of the regression lines, $P < 0.05$), demonstrating that the perfusion RP-HPLC method optimized in this work did not suffer from matrix interferences.

Quantitative analysis of soybean proteins in soybean IFs and soybean milks

Quantitative analysis of soybean proteins in 14 different commercial samples of soybean dairy-like products were performed using the optimized method. The samples consisted of 10 IFs, 2 PMs, and 2 LMs. Different lots of each commercial sample were also analyzed to investigate if there were differences in the soybean protein content in lots corresponding to different dates of manufacture. The results obtained are grouped in Table VI, which lists the protein content for each sample determined using perfusion RP-HPLC, using the Kjeldahl method, and given by the manufacturer (on the label). It can be observed that the protein content found using the Kjeldahl method generally agrees with the protein content on the label, which in turn agrees quite well with the protein content obtained using our perfusion method for PM and LM but not for IF. For IF, the perfusion method very often provided a soybean protein content lower than that determined using the Kjeldahl method, probably because free amino acids and vitamins were added in the formulation of these products which increased the content of total nitrogen obtained using the Kjeldahl method. In these cases, the perfusion method presented in this work enabled the rapid determination of the real content of soybean proteins, differentiating them from the free-amino-acid content. Concerning the results obtained for different lots of the same commercial sample, Table VI shows that for some samples analyzed, the content in soybean protein was similar regardless of the lot studied (IF 2, 6, 7, and 10 and LM 1 and 2), suggesting a higher homogeneity in the manufacturing. In contrast, some IFs (1, 5, 8, and 9) furnished soybean protein contents that were statistically different (*t*-test, $P < 0.05$).

Finally, it should be noted that the robustness of this method of protein analysis may be confirmed by taking into account the reproducibility (RSD) between results of protein content found for some IFs studied on two different days within a two-month period: 1, lot 1 = 10.6%; 5, lot 1 = 7.30%, lot 2 = 5.26%; 6, lot 1 = 3.18%, lot 2 = 1.46%; 7, lot 1 = 4.04%; 8, lot 1 = 6.20%; 9, lot 1 = 7.80%; and 10, lot 3 = 6.30%.

Conclusion

A new perfusion RP-HPLC method is presented that allows the determination of soybean proteins in soybean infant formulas and soybean milks (powdered and liquid). This method consists of a linear binary gradient of 0.1% TFA water–0.1% TFA acetonitrile that allows the characterization and determination of soybean proteins in an analysis time close to 3 min (eight peaks). The robustness, precision, and recovery of the method were evaluated and considered adequate to achieve the rapid quantitation of soybean proteins in real samples such as soybean dairy-like products (infant formulas and powdered and liquid milks). The method enables the determination of the real soybean protein content when the sample contains free amino acids or other additives (which increases the value of the content of total nitrogen furnished by the Kjeldahl method). These features, together with the simple sample preparation before its analysis in the chromatographic system (samples can be injected dissolved in water), increased the interest in this method for routine analysis.

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