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Determination by perfusion reversed-phase high-performance liquid chromatography of the soybean protein content of commercial soybean products prepared directly from whole soybeans

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Abstract

The use of soybean flour as external standard for the determination of soybean proteins in soybean products directly prepared from whole soybeans is investigated. For that purpose a perfusion reversed-phase high-performance liquid chromatography method consisting of a linear binary gradient acetonitrile–water (both with 0.1% trifluoroacetic acid) in 3 min at a flow-rate of 3 ml/min, and a temperature of 60°C is used. Samples dissolved in water are directly injected in the chromatographic system. The method is validated by evaluating detection limits, precision, and accuracy and applied to the quantitation of soybean proteins in soybean products directly prepared from whole soybeans.

Keywords: Soybean; Food analysis; Perfusion chromatography; Proteins

1. Introduction

The nutritional interest of soybean proteins has promoted the appearance of a huge variety of commercial soybean products such as soybean flour, textured soybean, and soybean protein isolate [1]. Soybean flour and textured soybean are directly elaborated from whole soybeans following simple methods, while in the case of the soybean protein isolate, a more complex processing was followed, basically extracting soybean proteins from whole soybeans and precipitating them at their isoelectric pH. Thus, based on the raw material used for the preparation of soybean products, there are some soybean products prepared from soybean protein isolate (soybean milks (powdered and liquids), infant formulas, etc.) and other directly elaborated from whole soybeans such as soybean flour, textured soybean, certain powdered and liquid soybean milks, soybean shakes, etc. [1,2].

The great variety of soybean products commercially available and their growing use have prompted the development of analytical methods for their quality control. Among the different techniques used to separate soybean proteins [3–5], High-performance liquid chromatography (HPLC) has been the most employed recently. In the works found in literature, whenever HPLC techniques were applied to the separation of soybean proteins, analysis times ranged

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from 35 to 90 min (in reversed-phase chromatography, RP-HPLC) and 20–45 min [in size-exclusion (HPSEC) and ion-exchange (HPIEC) chromatography] [6–13]. Recently, these analysis times have been reduced to about 9 min using RP-HPLC [14].

On the other hand, the appearance of perfusion chromatography has enabled a major reduction of analysis times. This technique uses packing materials of cross linked polystyrene-divinylbenzene matrix having a bidisperse porous structure constituted by a macroporous region with 6000–8000 Å transecting pores (*through pores*) and a connected network of smaller size *diffusive pores* (800–1500 Å) that provides a large adsorption surface area [15–20]. This combination of large and diffusive pores in the perfusion particles maximizes intra-particle convection and pore diffusion of solutes and thus enhances and accelerates the mass transfer of large molecules such as proteins [17,19–21]. 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 [15,18].

This technique was recently applied for the first time by our research team to the quantitation of soybean proteins in commercial soybean products using a soybean protein isolate as external standard for calibration [22]. Products analyzed were dairy-like products (soybean milks and soybean infant formulas) made from soybean protein isolate. However, when the optimized method was applied to the quantitation of soybean proteins in products derived directly from whole soybeans using soybean protein isolate as external standard, results obtained were very different from those expected for these products.

For this reason, the primary goal of this work was to find an external standard for calibration to determine soybean proteins by perfusion RP-HPLC in soybean products obtained directly from whole soybeans.

2. Experimental

2.1. Chemicals and samples

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

Soybean milks [liquid (LM) and powdered (PM)] and textured soybeans (TS) used in this work were purchased from local markets in Alcalá de Henares, Madrid, Spain. Fifteen soybean commercial products corresponding to different lots of ten different trademarks were studied. Total protein content of a lot corresponding to every trademark of soybean products was determined by Kjeldahl analysis [23]. The soybean protein isolate (SPI) used initially as external standard in this work was obtained from ICN (Aurora, OH, USA) and its purity, determined by Kjeldahl analysis (7 replicates), was 92.99% [relative standard deviation (RSD), 2.86%] [22]. The standard proposed in this work to determine soybean proteins in soybean products made from whole soybeans was a soybean flour (SF) obtained from a local market (Alcalá de Henares, Madrid, Spain) and its purity, determined by Kjeldahl analysis (10 replicates), was 56.17% (RSD, 1.73%).

Before analysis by HPLC, dry matter content of the SF and soybean samples were determined by drying at 130°C (for SF and TSs) or 102°C (for PMs) to a constant weight. The protocol for preparing sample and standard solutions was the following [22]: the sample was weighed and dissolved in water, then the mixture was sonicated for 3 min and centrifuged (3000 rpm, 5 min, 3°C) (Jouan, Saint Herblain, France) to remove the supernatants that were kept on ice until their injection in the chromatographic system. The concentration of standard solutions ranged from 0.10 to 0.72 mg/ml (as dry basis). The concentrations of sample solutions were as follows: 7–10 mg sample per milliliter for LMs, 1–2 mg sample per milliliter for PMs, and 1 mg sample per milliliter for TSs.

2.2. High-performance liquid chromatography

A Hewlett-Packard 1090 Series II liquid chromatograph (Hewlett-Packard, Pittsburgh, PA, USA) equipped with a diode array detector and an HP 9153C data acquisition system was used. The injection volume was 20 μl.

The separation of soybean proteins was accomplished with a Poros R2/H (PerSeptive Biosystems,

Framingham, MA, USA) 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 by using uracyl as non-retained solute. Proteins were detected by UV absorption at 254 nm.

Soybean proteins were eluted using a previously optimized RP-HPLC method at 3 ml/min (linear flow velocity of 1058 cm/h) and 60°C of temperature [22]. Mobile phases were as follows: mobile phase A, 0.1% TFA in water; mobile phase B, 0.1% TFA in ACN. A linear binary gradient in two steps (5–25% B in 1.7 min, 25–45% B in 1.3 min, followed by a linear reversed gradient from 45 to 5% B in 1 min, and a 1 min step at 5% B to re-equilibrate the column to the initial conditions between runs) was used. Mobile phases were filtered using 0.45 μm nylon filters (Gelman Sciences, USA) and degassed with helium before use.

2.3. Calibration

The overall system was daily calibrated by the external standard method. For this purpose, aqueous solutions of SF were injected into the chromatographic system. Peak areas corresponding to soybean proteins were integrated by setting the baseline from valley to valley, and the total peak area (calculated by addition of the individually integrated peak areas) was plotted against the injected concentration of SF. Content in protein of each standard solution was determined taking into account its purity and moisture. Using the standard curve, soybean protein content was quantitated in soybean dairy-like products and TSs from aqueous solutions of these samples. All solutions were injected three times, except in the studies of reproducibility and repeatability, for which ten replicates were made.

2.4. Data treatment

The linear model for calibration was obtained by least-squares regression analysis carried out with an Univariate Linear Calibration Program [24] and validated by means of the analysis of residuals and the analysis of the variance [25].

3. Results and discussion

In a previous work of our research team, soybean proteins were separated by perfusion RP-HPLC using a two-step linear binary gradient from 5 to 45%B in 3 min (13.33%/min) that allowed the separation of soybean proteins from soybean protein isolate in eight peaks [22]. In that work, soybean proteins were quantitated in different soybean products (all prepared from soybean protein isolate) using soybean protein isolate as external standard. Results found matched quite well those given by the manufacturer and those obtained by Kjeldahl analysis [22]. When soybean products, directly elaborated from whole soybeans, were quantitated using the optimized method and soybean protein isolate as external standard, huge differences between the values given by the manufacturer and the values experimentally found were obtained. In fact, the protein content determined by perfusion RP-HPLC was surprisingly much higher that the expected for these products. Fig. 1 shows the chromatograms corresponding to solutions of similar concentration of the soybean protein isolate and two products prepared directly from the whole soybeans (soybean flour and textured soybean). Soybean proteins in the soybean flour were separated into seven peaks corresponding to peaks 1–6 and 8 in the soybean protein isolate chromatogram, while textured soybean only presented peaks 1–6. In this figure it can be also observed that peaks obtained for soybean flour and textured soybean were much higher than those related to the soybean protein isolate, despite the protein content of the latter is almost twice the protein content of the first. In an attempt to explain the different behaviour observed between soybean protein isolate and those products directly prepared from whole soybeans, further studies were performed. When using sodium dodecyl sulphate–polyacryl-amide gel electrophoresis (SDS–PAGE) for the separation of soybean proteins [26] from different products prepared from soybean protein isolate and from whole soybeans, it was possible to observe that those products directly prepared from whole soybeans showed different electrophoretic patterns from those elaborated from soybean protein isolate. Indeed, soybean products from whole soybeans presented an additional electrophoretic band corresponding to high molecular weight compounds

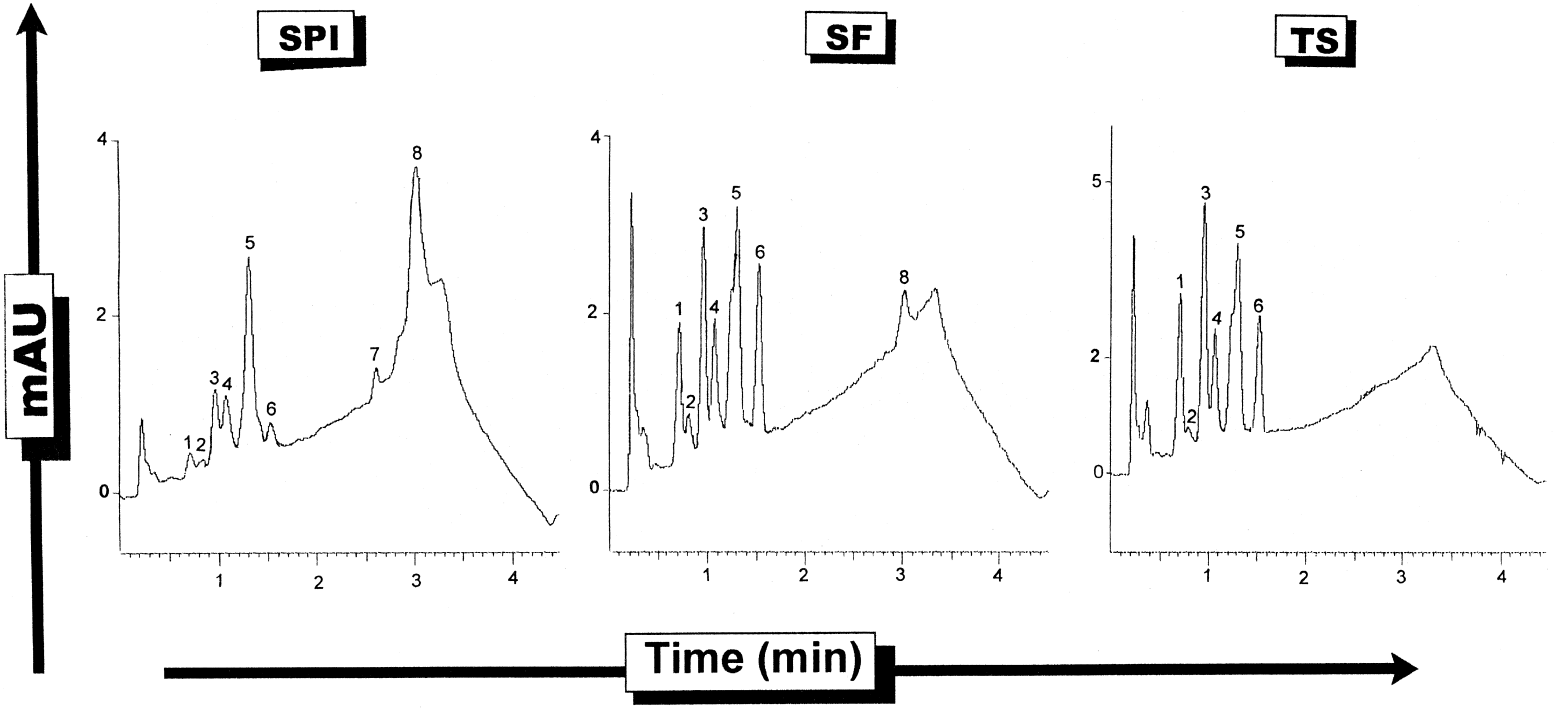

Fig. 1. Chromatograms corresponding to aqueous solutions (as dry basis) of soybean protein isolate (SPI) (0.88 mg/ml), soybean flour (SF) (0.90 mg/ml) and textured soybean (TS) (0.90 mg/ml). Conditions: flow-rate, 3 ml/min; temperature, 60°C; gradient: 5–25% B in 1.70 min, 25–45% B in 1.30 min; mobile phase A, 0.10% TFA in water, mobile phase B, 0.10% TFA in ACN; injection volume, 20 μl; detection at 254 nm.

which could not pass through the gel maybe due to aggregates formed during the processing of these products.

On the other hand, when using diode-array detection, spectra and corresponding derivatives for every chromatographic peak obtained by perfusion RP-HPLC from products prepared from soybean protein isolate and directly from whole soybeans could be compared. As an example, Fig. 2 shows spectra and second derivatives obtained for peak 5 (which is one of the majority peaks in the three soybean products studied) in the soybean protein isolate, soybean flour, and textured soybean, being possible to observe that spectra relating to soybean flour and textured soybean were similar but different from that corresponding to the soybean protein isolate. This fact was corroborated when comparing first and, especially, second derivatives. These differences could be attributed to the different procedures to which soybean proteins are submitted during the preparation of these products. Soybean flour and textured soybean used to be prepared directly from whole soybeans by grinding, while in the case of the soybean protein isolate, soybean proteins used to be extracted at basic pH and precipitated at isoelectric pH (4.5) from whole soybeans.

For all these reasons and because soybean protein isolate did not seem to be a suitable standard for calibration to determine soybean proteins in products prepared directly from whole soybeans, another standard was tested. Since the use of whole soybeans as standard involves further treatment for preparing the standard solutions, soybean flour was tested as external standard for evaluating the concentration of soybean proteins in liquid milks, powdered milks, and textured soybean, all of them prepared from whole soybeans.

3.1. Method validation

Prior the quantitative analysis of soybean proteins in soybean commercial products, validation of the proposed method when using soybean flour as external standard for calibration is important. For this purpose, the characteristics of the calibration plots were obtained and precision (repeatability and reproducibility) and accuracy were evaluated.

Table 1 compiles the linear concentration ranges

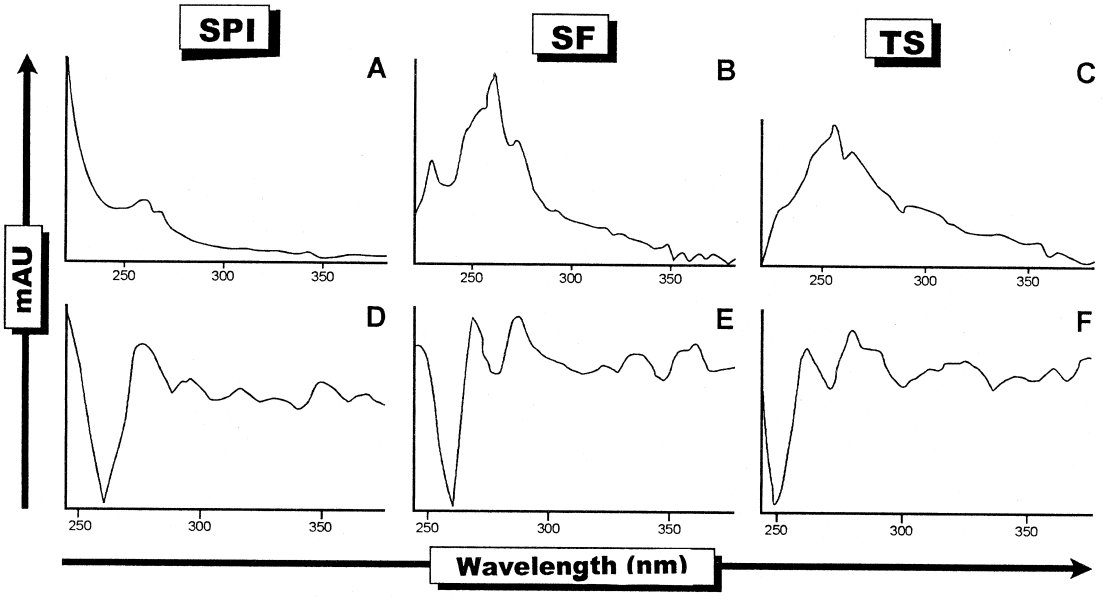

Fig. 2. Absorption espectra (A, B, and C) and second derivatives (D, E, and F) corresponding to peak 5 in the soybean protein isolate (SPI), soybean flour (SF) and textured soybean (TS).

Table 1
Characteristics of the calibration straight lines obtained by perfusion RP-HPLC using soybean flour as external standard[a,b]

Linear concentration range (mg/ml)	Slope[c]	Intercept[c]	Standard error	r^{2}[d]
0.10–0.69 (6)[e]	80.72 (1.20)	0.30 (0.52)	0.61	0.999
0.10–0.70 (6)	79.07 (2.52)	3.47 (1.25)	1.42	0.996
0.20–0.72 (6)	85.40 (2.07)	1.84 (1.02)	0.91	0.998
0.21–0.72 (6)	96.34 (1.77)	1.25 (0.88)	0.76	0.999
0.21–0.62 (5)	90.89 (2.58)	1.55 (1.13)	0.84	0.998
0.21–0.71 (6)	95.44 (0.42)	0.53 (0.21)	0.18	1.000

[a] Experimental conditions as in Fig. 1.
[b] Calibration lines obtained in six different days within a one-month period.
[c] Errors in the slope and intercept of the regression line are given in parentheses.
[d] Squared correlation coefficient.
[e] Number of points considered for the regression. Each point represents the average of three consecutive injections of each standard solution.

and the values of the slope, intercept, standard error, and correlation coefficient for the equations of the calibration plots obtained in six different days within a one-month period. As it can be observed, a good linear correlation ($r^2>0.99$) was found between the total peak area measured for soybean proteins and the concentration of soybean flour injected into the chromatographic system. The slope of the straight line was reproducible inter-days (RSD, 8.39%) and in all the calibration lines found by this method the intercept did not significantly differ from zero (*t*-test, $P<0.05$). The average detection limit obtained (defined as the concentration calculated from the calibration curve corresponding to a signal equal to the intercept of the regression line plus three times its standard error) was 50 μg of soybean proteins/ml which corresponded to 5–7 mg of soybean proteins/ml of soybean liquid milk, 25–50 mg of soybean proteins/g of soybean powdered milk, and 50 mg of soybean proteins/g of textured soybean.

Precision of the perfusion RP-HPLC method was evaluated by using both soybean flour and some representative samples of liquid milk, powdered milk, and textured soybean. Table 2 groups the RSD values found for the repeatability and reproducibility. Repeatability (in peak area and in concentration) for ten injections of a solution of 0.51 mg/ml of soybean flour, was about 1%. Inter-day reproducibility was evaluated as the RSD value obtained for the injection in four different days of two standards whose protein content corresponded to the lowest and highest concentrations of the linear range (maximum error range). A value of RSD≤6.2% was obtained in peak area for both standards. The RSD in

Table 2
Precision expressed as RSD (%) for the peak area and concentration corresponding to the analysis of the standard (soybean flour) and some soybean products by perfusion RP-HPLC[a]

Soybean flour				Soybean products		
Repeatability ($n=10$)[b]		Inter-day reproducibility in peak area ($n=4$)[c]		Reproducibility in concentration ($n=10$)[d]		
Peak area	Concentration	0.21 mg/ml	0.62 mg/ml	LM	PM	TS
1.04	0.99	6.20	5.08	5.58	3.73	3.38

[a] Experimental conditions as in Fig. 1.
[b] Number of injections of a soybean flour solution of 0.51 mg/ml (as dry basis and corrected by the purity of the standard).
[c] Analysis performed in four different days; each standard solution was injected three times per day.
[d] Analysis of ten individual samples (each individual sample injected by triplicate) of the following concentrations as dry basis (mean value±SD): 9.85±0.27 (liquid milk, LM), 1.14±0.00 mg/ml (powdered milk, PM), 0.91±0.01 mg/ml (textured soybean, TS).

the concentrations obtained for three real samples (one liquid milk, one powdered milk and one textured soybean) and corresponding to the injection of ten individual samples of each product was always minor than 5.6%.

As for retention time, repeatability obtained when injecting ten solutions of about 0.51 mg/ml of soybean flour was approximately 0.3% and reproducibility in two consecutive days better than 1.2% (results not included in tables).

The accuracy of the method was determined as the recovery (%) of soybean proteins obtained when a known quantity of soybean flour was added to a real sample of a soybean product. Table 3 shows the results obtained when quantities corresponding to 10, 20, 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 soybean products considered representative of every kind of product: one liquid milk, one powdered milk, and one textured soybean. Recoveries obtained (mean of four determinations) ranged from 95% to 106%.

The presence of relative and fixed bias was investigated by plotting the amount of soybean protein recovered in the previous study versus the real protein content added which corresponds to the expected content if the recovery had been of 100% [25]. From this plot, a regression line was obtained characterized by a slope and an intercept, the confidence intervals of which included the slope and the intercept expected if the recovery had been of 100% (slope=1; intercept=0): intercept: -0.01 ± 0.23; slope: 1.03 ± 0.40; correlation coefficient: 0.88. These results demonstrated the absence of systematic errors when determining the protein content in these soybean products.

One way of testing the presence of matrix interferences in an analysis is comparing the slopes of the regression lines obtained by the standard additions method and by the external standard method. When using soybean flour as standard, slopes of the regression lines obtained by both methods of calibration did not differ significantly (F-test to compare variances and t-test to compare slopes, $P<0.05$), demonstrating that the quantitative method used in

Table 3
Recovery (%) of soybean proteins for some soybean products derived from whole soybeans found by perfusion RP-HPLC[a]

Sample	Concentration of sample solution (mg/ml)[b]	Amount of SF added to the sample (mg)[c]	Total protein concentration (mg/ml)	Protein concentration found using perfusion RP-HPLC (mg/ml)	Recovery (%)
LM (4.86 mg/100 mg, SD=0.27)[d]	8.18±0.15	0.38	0.44	0.48±0.02	109
		0.77	0.49	0.53±0.02	107
		1.15	0.55	0.57±0.02	104
		1.52	0.58	0.62±0.02	106
				Mean value: 106±2	
PM (45.72 mg/100 mg, SD=1.71)[d]	1.11±0.06	0.36	0.58	0.58±0.01	100
		0.62	0.57	0.52±0.01	91
		0.95	0.59	0.55±0.01	93
		1.30	0.62	0.59±0.01	95
				Mean value: 95±4	
TS (67.50 mg/100 mg, SD=2.28)[d]	0.76±0.03	0.42	0.57	0.59±0.03	103
		0.84	0.58	0.59±0.03	101
		1.28	0.62	0.64±0.03	103
		1.87	0.71	0.79±0.04	111
				Mean value: 104±4	

[a] Experimental conditions as in Fig. 1.

[b] Expressed as dry basis.

[c] Expressed as dry basis and corrected by the purity of the standard.

[d] Mean value of protein concentration, expressed as dry basis, found by analyzing ten individual samples by perfusion RP-HPLC. SD, standard deviation.

this work was not affected by matrix interferences when soybean flour was used as external standard for calibration [25].

3.2. Quantitative analysis of soybean proteins in soybean products directly prepared from whole soybeans

Soybean proteins from fifteen soybean commercial products corresponding to ten different trademarks were quantitated by using the perfusion method: eleven liquid milks (corresponding to six different trademarks), two powdered milks (corresponding to two different trademarks), and two textured soybeans (corresponding to two different trademarks). Fig. 3 shows the chromatograms corresponding to a powdered milk and a liquid milk prepared from whole soybeans. The powdered milk presents a chromatogram in which only the first block of peaks of the soybean protein isolate chromatogram appears, while the liquid milk shows the eight peaks that appeared in the soybean protein isolate chromatogram. Table 4 groups the results obtained by the perfusion RP-HPLC method when using both soybean flour and soybean protein isolate as external standards. The protein content for each sample determined by our research team by the Kjeldahl method, and the protein content given by the manufacturer (label) were also included in this table. In most cases, there was a good agreement between the values of protein content given by Kjeldahl analysis and those indicated on the label of every product by the manufacturer. When using soybean protein isolate as external standard for the quantitation of soybean proteins in products prepared directly from whole soybeans, huge differences were found between the protein content given by the manufacturer and the Kjeldahl method and that furnished by the perfusion method. The protein content obtained by the perfusion method when the soybean flour was used as external

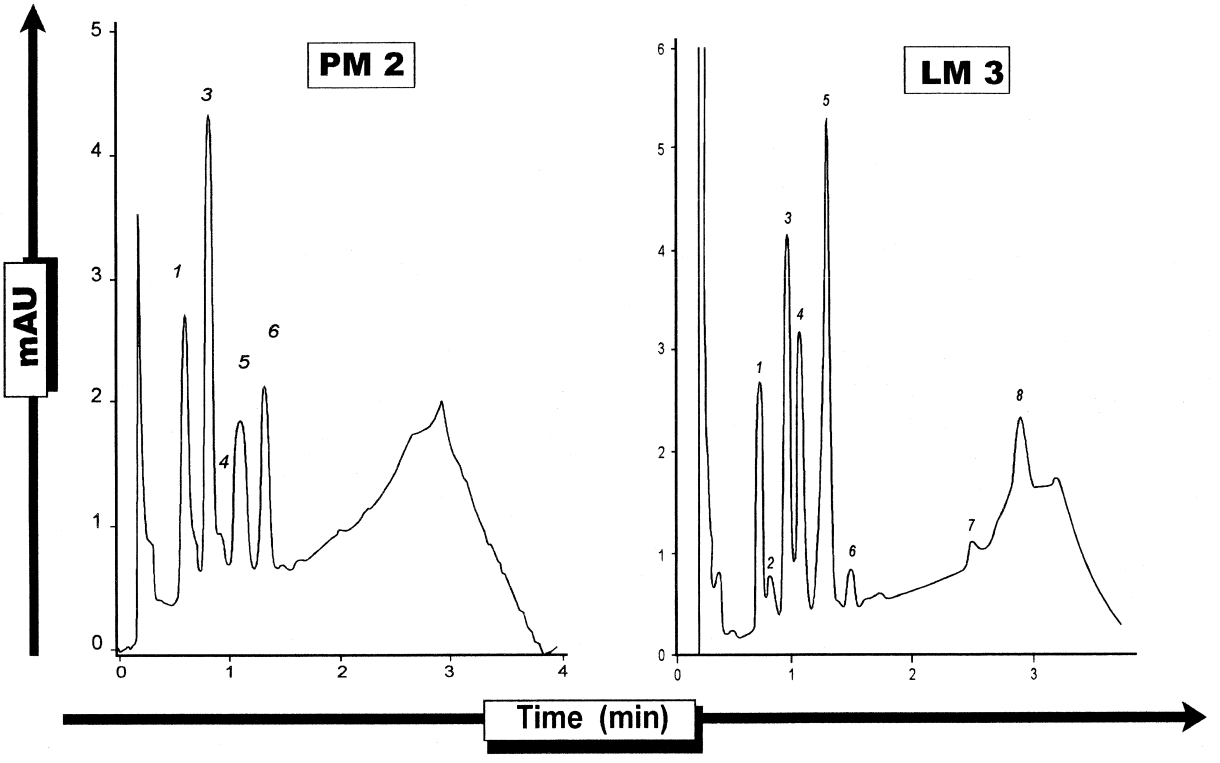

Fig. 3. Perfusion RP-HPLC separation of aqueous solutions (as dry basis) of a powdered milk (PM 2) (1.20 mg/ml) and a liquid milk (LM 3) (9.97 mg/ml). Experimental conditions as in Fig. 1.

Table 4
Protein content in soybean products directly prepared from whole soybeans by perfusion RP-HPLC[a]

Protein concentration (mg/100 mg sample)[b]					
Soybean product	Lot	Kjeldahl method[c,d]	Label	Perfusion RP-HPLC using SF as external standard[d,e]	Perfusion RP-HPLC using SPI as external standard[e]
LM 1	1	4.23 (0.08)	3.80	7.31 (0.04)[f]	18.83
LM 2	1	3.70 (0.05)	3.60	4.43[g]	10.51
	2			3.27[g]	7.97
	3			5.68 (0.06)[f]	15.40
LM 3	1	3.08 (0.08)	3.50	5.79[g]	13.39
	2			4.86 (0.27)[f]	14.34
	3			4.24 (0.06)[h]	11.51
LM 4	1	3.76 (0.06)	3.70	2.29[g]	5.76
LM 5	1	3.38 (0.03)	3.50	4.97[g]	11.91
	2			5.23 (0.01)[f]	14.21
LM 6	1	4.13 (0.21)	3.80	7.38 (0.02)[f]	19.26
PM 1	1[i]	27.58 (0.88)	28.00	28.13 (4.41)[f]	68.24
PM 2	1[i]	32.81 (1.16)	40.00	45.72 (1.71)[h]	111.26
TS 1	1	55.65 (1.00)	54.00	52.73 (0.52)[f]	135.91
TS 2	1	52.90 (0.50)	54.00	67.72 (3.37)[f]	176.14

[a] Experimental conditions as in Fig. 1.
[b] Results expressed as dry basis.
[c] Six replicates.
[d] Standard deviation given in parentheses.
[e] Protein concentrations determined using SF and SPI as standards, respectively.
[f] Mean of two individual determinations (every sample injected by triplicate).
[g] One determination injected by triplicate.
[h] Ten replicates (every sample injected by triplicate).
[i] Samples were opened some days prior to their analysis.

standard enabled a better estimation of the protein content in these samples. In fact, when plotting the protein content determined by the perfusion method using soybean flour as standard for calibration versus that obtained by the Kjeldahl method or by the manufacturer, regression lines obtained presented slopes and intercepts that were statistically similar to 1 and 0, respectively (test-*t*, $P<0.05$). These results were corroborated by the Mann–Whitney test [25].

When different lots were tested for a same soybean liquid milk it was observed that the protein content varied with the lot. This difference could be due to some lack of homogeneity in the manufacturing. It is also important to highlight that LM 6 was not prepared with ordinary whole soybeans but with a soybean extract from an ecologic cultivation such as the manufacturer indicated in the label. Thus, in this case results could be improved if a standard similar to the material used in its manufacturing had been used. Finally, the different protein content obtained for textured soybean (TS) 2 by the perfusion method with respect to the value corresponding to the Kjeldahl analysis could be explained taking into account that textured soybean 2 has a higher granulation than textured soybean 1 in whose case the protein content obtained by the perfusion method matched that expected for this sample.

4. Conclusions

The use of soybean flour as external standard for the quantitation of soybean proteins in products prepared directly from whole soybeans by perfusion RP-HPLC enables a better estimation of this content than that obtained when using soybean protein isolate, being possible to detect up to 5–7 mg of soybean proteins/ml of soybean liquid milk, 25–50 mg of soybean proteins/ml of soybean powdered milk, and 50 mg of soybean proteins/ml of textured soybean. In addition, precision and accuracy of the perfusion method using soybean flour as standard

can be considered adequate to achieve rapid quantitation of soybean proteins in this kind of samples. So, the use of soybean flour as external standard for the rapid determination of soybean proteins by perfusion RP-HPLC could be considered a suitable alternative to the tedious Kjeldahl method normally used for this purpose.

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