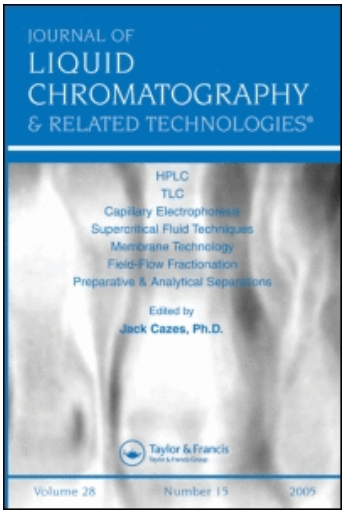

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DETERMINATION OF THE SOYBEAN PROTEIN CONTENT IN SOYBEAN LIQUID MILKS BY REVERSED-PHASE HPLC

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ABSTRACT

A reversed-phase high performance liquid chromatography method, previously optimized, was applied to determine the soybean protein content in commercial soybean liquid milks. Quantitation of soybean proteins was first performed by using soybean protein isolate as external standard. Although this standard seemed suitable for the quantitation of soybean proteins in some soybean liquid milks, for those soybean milks directly prepared from whole soybeans, a best estimation of the soybean protein content was obtained by using soybean flour as external standard.

INTRODUCTION

Soybean products constitute an interesting alternative for vegetarians and for those people suffering with allergies from animal whey proteins, hypercholesterolemia, obesity, etc.[1] In addition, the increasing demand for animal proteins has promoted the consumption of this cheap source of protein, especially in under developed countries.[2] The appearance of commercial soybean products for human consumption has opened a new line of research in the food field. Indeed, important efforts have being performed not only to produce new and

better soybean products but also to develop analytical methods to control the quality of these new products available for human consumption in the market.

Among soybean products, soybean milks and, more concretely, soybean liquid milks are the most consumed because it is possible to find a great variety of them. Soybean liquid milks can be prepared directly from whole soybeans, following a traditional method, or from a soybean protein isolate.[3,4]

The first effort to analyse commercial soybean products was accomplished by reversed-phase high performance liquid chromatography (RP-HPLC), using a conventional polymeric column.[5] However, despite the promising results obtained by this method, it was not used to quantitate soybean proteins in commercial soybean products.

At this time the only techniques used for the quantitative analysis of soybean proteins in commercial soybean products were perfusion chromatography[6,7] and capillary electrophoresis.[8]

Thus, the main goal of this work was to investigate the potential of a previously optimized RP-HPLC method developed with a conventional polymeric column[5] to determine the soybean protein content in commercial soybean liquid milks.

EXPERIMENTAL

Chemicals and Samples

HPLC-grade acetonitrile (ACN) (Sharlau, 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.

Soybean protein isolate (SPI) was obtained from ICN (Aurora, OH) and its protein content determined by Kjeldahl analysis (7 replicates) was 92.99% (relative standard deviation (RSD), 2.86%).[9] Soybean flour was obtained from a local market in Madrid (Spain) and its protein content determined by Kjeldahl analysis (10 replicates) was 56.17% (RSD, 1.73%).[9] Soybean liquid milks (seven different samples) were also purchased in local markets in Madrid (Spain) and their protein content determined by Kjeldahl analysis.[9]

Before analysis by HPLC, dry matter content of the SPI and soybean flour was determined by drying them at 130°C to a constant weight. The protocol for preparing all solutions was: samples were weighed, dissolved in distilled water, and sonicated for 15 min.

Prior to the injection in the chromatographic system, solutions were left reposing for another 15 min.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

A Hewlett-Packard (Pittsburgh, PA) liquid chromatograph consisting of an automatic degasser system, quaternary pump, and a thermostated column compartment, all of 1100 series, and an automatic injector system and a variable wavelength detector, both of 1050 series, were used. Peaks were integrated with an HP 3394 integrator. Proteins were monitored at 254 nm and injection volume was 20 μL.

The separation was accomplished with a PLRP-S column (150 x 4.6 mm I.D.) from Polymer Labs. (Church Stretton, UK), packed with polystyrene-divinylbenzene beads (300 Å, 8 μm particle size). The column's dead time, determined by injecting phenol with 7:1 ACN:water as mobile phase, was 1.94 min.

The RP-HPLC method used in this work was previously optimized by García et al.[5] The flow-rate used was 1 mL/min and the temperature was 50°C. The method consisted of a linear binary gradient in three steps: 20% B for 1 min, 20-35% B in 19 min, 35-46% B in 0.5 min, and finally, from 46 to 20% B in 0.5 min to re-equilibrate the column to the starting conditions. Mobile phases used were 0.1 % TFA in water as mobile phase A and 0.1% TFA in ACN as mobile phase B. ACN was filtered through 0.45 μm nylon filters.

Calibration

Quantitative analysis of soybean proteins was performed by the external standard method. The standard used for the determination of the soybean protein content in soybean liquid milks prepared from SPI was a SPI, and in soybean liquid milks directly prepared from whole soybeans, a soybean flour. Calibration curves were obtained by injecting aqueous solutions of SPI (over the range 1.0-2.5 mg/mL) or soybean flour (over the range 1.1-3.0 mg/mL).

Peak areas were integrated by setting the baseline from valley to valley. In all cases the integrated areas of certain peaks were added and plotted against the injected concentration of SPI or soybean flour (corrected for purity and moisture) to obtain the calibration curve. The content of soybean proteins in seven soybean milks was determined by interpolation of the area of the peaks chosen for quantitation in the calibration curve.

All solutions were injected three times except in the studies of repeatability, in which case at least ten injections were performed. The concentration of the solutions of soybean liquid milks injected was approximately of 20 mg/mL.

Data Treatment

The linearity of the calibration curve was obtained by least-squares regression analysis and the linear model was validated by means of the analysis of residuals and the analysis of variance (ANOVA) ($\alpha = 5\%$). All the statistical analyses were carried out using the Statgraphics Plus program.[10]

RESULTS AND DISCUSSION

The HPLC method used was optimized in a previous work by our research team and consisted of a linear binary gradient from 20 to 46% B in 20.5 min (20% B for 1 min; 20-35% B in 19 min, and 35-46% B in 0.5 min, being 0.1% TFA in water the mobile phase A and 0.1% TFA in ACN the mobile phase B). This method was applied to the separation of soybean proteins from different soybean products and enabled the differentiation between soybean dairy-like products (soybean milks and soybean infant formulas) and soybean basic products (SPI, soybean flour, and textured soybean).[5]

In the present work, this chromatographic method has been applied to the quantitation of soybean proteins in seven different commercial soybean liquid milks. Chromatograms obtained for soybean liquid milks were different from the chromatogram corresponding to the SPI, as it could be observed in Figure 1, which shows the chromatographic profile obtained for the SPI and for a commercial soybean liquid milk.

In the chromatogram corresponding to the SPI four peaks appeared corresponding to soybean proteins, peaks 3 and 4 did not appear in the soybean liquid milk chromatogram. This fact was also observed in the rest of soybean liquid milks studied.

In order to apply the RP-HPLC method to the determination of the soybean protein content of commercial soybean liquid milks, the SPI was used as standard for soybean proteins. The calibration was performed by the external standard method by injecting solutions of different concentrations of SPI in the chromatographic system.

The soybean protein content was determined by interpolation of the total area (corresponding to the area of peaks 1 and 2) in the calibration plot. The results obtained are grouped in Table 1 (column 4), together with the protein

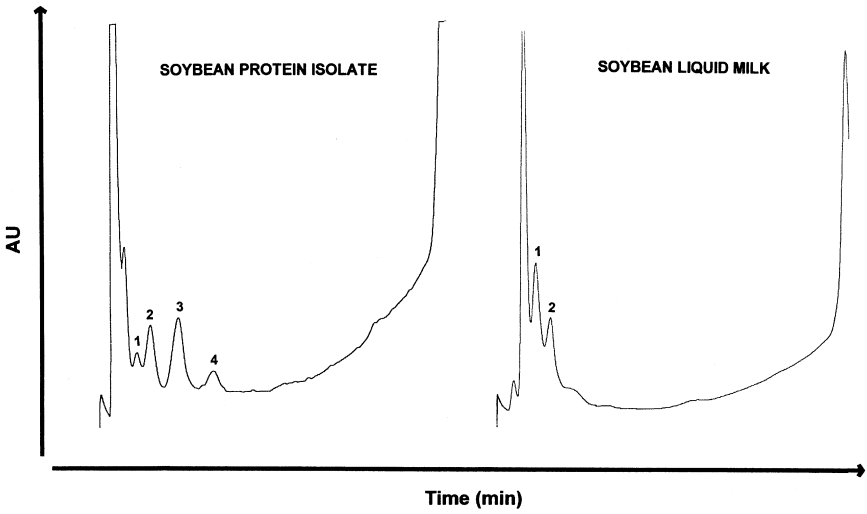

Figure 1. RP-HPLC separation of aqueous solutions of 1.45 mg/mL (as dry basis) of SPI and 24.82 mg/mL of a commercial soybean liquid milk from whole soybeans. Experimental conditions: flow-rate, 1 mL/min; temperature, 50°C; detection, 254 nm; gradient; 20% B for 1 min, 20-35% B in 19 min, and 35-46% B in 0.5 min; mobile phases: A, 0.1% (v/v) TFA in water; B, 0.1 % (v/v) TFA in ACN.

content given by the manufacturer in the label of every product (column 3) and the protein content determined by Kjeldahl analysis (column 2).[6,7]

As observed, the protein content obtained by the RP-HPLC method was much higher than the protein content given by the manufacturer which, in turn, was very similar to that value found by the Kjeldahl method. However, it was observed that while peak 1 in soybean liquid milks was the majority, this peak in the SPI was minority (Figure 1). This could be because peak 1 in soybean milks not only elute soybean proteins, but also other compounds present in these soybean liquid milks, such as carbohydrates. As a consequence, we would take as protein something which is not protein and the protein content of these soybean milks would be much higher than the real content. Thereby, the calibration plot was established, again, without taking into account peak 1 neither in the standards nor in the soybean liquid milks.

Table 2 compiles the linear concentration range and the values of slope, intercept, standard error, and correlation coefficient for the equations of calibration plots obtained in four different days when using SPI as external standard. As observed, there is a linear correlation between the total peak area (cor-

Table 1

Quantitative Analysis of Soybean Proteins in Commercial Soybean Liquid Milks by RP-HPLC[a]

Soybean Product	Protein Content (mg/100 mg Sample)[b] Kjeldahl Analysis[c,d]	Label	RP-HPLC Method[e]	RP-HPLC Method[d,f]	RP-HPLC Method[d,g]
1[g]	2.82 (0.12)	3.1	6.00	4.63 (1.75)	----
2[g]	2.89 (0.15)	3.3	6.45	4.70 (2.42)	----
3[h]	4.23 (0.08)	3.8	18.87	13.41	4.40 (0.54)
4[h]	3.08 (0.08)	3.5	9.72	6.68	2.47 (0.68)
5[h]	3.38 (0.03)	3.5	16.30	12.70	4.51 (0.43)
6[h]	3.70 (0.05)	3.6	10.81	5.96	3.46 (0.52)
7[i]	4.13 (0.21)	3.8	26.22	20.46	9.15 (0.29)

[a] Experimental conditions as in Figure 1. [b] Results expressed as dry basis. [c] Six replicates. [d] Standard deviation given in parentheses. [e] When using the SPI as external standard and taking into account peaks 1-4 in the SPI and peaks 1-2 in the soybean liquid milks. [f] When using the SPI as external standard and taking into account peaks 2-4 in the SPI and peak 2 in the soybean liquid milks. [g] When using the soybean flour as external standard and taking into account peaks 2-4 in the soybean flour and peak 2 in the soybean liquid milks. [h] Soybean liquid milks directly prepared from whole soybeans. [i] Soybean liquid milk prepared from a proteic extract obtained from an ecological soybean cultivar.

responding to peaks 2-4) and the concentration of SPI injected into the chromatographic system.

The slope of straight line was reproducible inter-days (RSD, 9.3%) and the average detection limit (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)[11] was 0.40 mg of soybean proteins/mL.

The results obtained in the quantitative analysis of soybean liquid milks (with the area of peak 2) using a calibration plot obtained with the areas of peaks 2-4 for SPI are shown in Table 1 (column 5). As observed, results get better when calibration is performed without peak 1 for soybean liquid milks 1 and 2, although for the rest of soybean milks,[3-7] the protein content given by the RP-HPLC method kept on being very different than the value expected.

Table 2

Calibration by the External Standard Method of Soybean Proteins when Using SPI and Soybean Flour as Standards by RP-HPLC[a]

Standard[a]	Linear Conc. Range[b] (mg/mL)	Slope[c]	Intercept[c]	Standard Error	r
SPI	1.150-1.862 (6)[d]	936667 (93376)	-515973 (160233)	72860	0.98
SPI	1.132-2.505 (4)	797022 (75981)	-468927 (136545)	90194	0.99
SPI	1.096-2.567 (5)	761349 (41019)	-276393 (76131)	49869	0.99
SPI	1.096-2.567 (5)	806017 (193974)	-405602 (360017)	235829	0.92
Soybean flour	1.175-3017 (5)	1529360 (15499)	-47264 (32740)	23637	1.00
Soybean flour	1.175-3.017 (5)	1291330 (25331)	468570 (54948)	35673	1.00
Soybean flour	1.175-2.269 (6)	1377020 (66196)	-3826 (140356)	103297	0.99

[a] Experimental conditions as in Figure 1. [b] Taking into account peaks 2-4. [c] Errors in the slope and intercept of the regression line are given in parentheses. [d] Number of points considered for the regression.

Curiously, soybean milks for which the protein content obtained by the RP-HPLC method was close to the value given by the manufacturer[1,2] were prepared from SPI and soybean liquid milks[3-7] for which the protein content given by the RP-HPLC method was very much higher than the value expected, were all prepared directly from whole soybeans. This different behaviour between soybean milks prepared from SPI and those directly prepared from whole soybeans was also shown when characterising soybean proteins in commercial soybean products by SDS-PAGE.[12]

In fact, products directly prepared from whole soybeans showed electrophoretic patterns which presented a thick electrophoretic band corresponding to high molecular weight compounds which could not pass through the gel. This band, which did not appear in products prepared from SPI, was attributed to aggregates formed during the processing of products directly prepared from whole soybeans.[12] As SPI did not seem to be a suitable standard for the calibration of soybean proteins in soybean liquid milks directly prepared from whole soybeans, another soybean product was used as standard.

Whole soybeans could be an interesting candidate as external standard, although its use involves a treatment for the preparation of the standard solu-

tions consisting of dehulling, defatting, etc. Another possibility is to use a product prepared directly from whole soybeans by a very simple process as in the case of soybean flour.

Table 2 shows the linear concentration range and the values of slope, intercept, standard error, and correlation coefficient for the equations of calibration plots obtained in three different days when using soybean flour as external standard. The slope of the straight line was reproducible inter-days (RSD, 8.6%) and the average detection limit determined for the SPI was 0.12 mg of soybean proteins/mL.

The protein contents determined in the soybean milks directly prepared from whole soybeans when using the soybean flour as standard were closer to the expected value than when using SPI as standard (see Table 1, column 6). The only exception was the soybean milk 7, which was prepared from a protein extract prepared from an ecological soybean cultivar. In this case, results would probably have improved if we had used a protein extract prepared from an ecological soybean cultivar as a standard of soybean proteins.

Precision of the RP-HPLC method was evaluated by determining the repeatability with both standards, SPI and soybean flour, and the reproducibility with a commercial soybean liquid milk for the peak area, concentration, and

Table 3

Precision Expressed as RSD (%) for the Peak Area, Concentration, Retention Time Corresponding to the Standards (SPI and Soybean Flour) and a Commercial Soybean Liquid Milk by RP-HPLC[a]

	Sample	Peak Area	Concentration[b]	Retention Time
Repeatability	SPI (12)[c]	4.60	3.11	0.35
	Soybean flour (10)[c]	0.860	0.85	1.23
Reproducibility	Soybean milk (10)[d]	4.41	4.61	0.77

[a] Experimental conditions as in Figure 1. [b] Corresponding to peak 2. [c] Number of injections of the standard solution: 1.76 mg/mL for the SPI and 2.27 mg/mL for the soybean flour (both as dry basis and corrected by the purity of the standard). [d] Analysis of ten individual samples (each individual sample injected by triplicate) of 20.69 mg/mL of a soybean liquid milk.

retention time corresponding to peak 2. Table 3 groups the RSD values obtained. Repeatability in peak area and concentration when using the SPI as external standard was always worse than the repeatability obtained when using soybean flour.

Reproducibility obtained in the same day in peak area and in concentration when injecting ten individual solutions of a soybean liquid milk was about 4.5%. As for retention time, repeatability for SPI and soybean liquid milk was always better than 0.8% and for the soybean flour was about 1.2%.

CONCLUSIONS

This RP-HPLC method enables the estimation of the soybean protein content in commercial soybean liquid milks. For that purpose, the SPI can be used as external standard for the quantitation of soybean proteins in soybean milks prepared from SPI. Nevertheless, this standard is not suitable for the quantitation of soybean proteins in soybean liquid milks directly prepared from whole soybeans. In this case, the use of soybean flour as external standard provides better results.

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