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Determination of soybean proteins in soybean–wheat and soybean–rice commercial products by perfusion reversed-phase high-performance liquid chromatography

A. Castro-Rubio, F. Castro-Rubio, M.C. García, M.L. Marina *

Departamento de Química Analítica, Facultad de Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km 33.600, 28871 Alcalá de Henares, Madrid, Spain

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Abstract

The determination of additions of soybean proteins in commercial bakery products containing soybean–wheat and soybean–rice binary mixtures has been achieved in this work by high-performance liquid chromatography using a perfusive column. Soybean proteins were solubilized in a 25:75 acetonitrile–water mixture containing 0.3% (v/v) acetic acid by ultrasonication for 10 min and centrifugation for 5 min. Soybean proteins were separated from rice and wheat proteins in less than 4 min using a linear binary gradient of acetonitrile–water containing 0.3% (v/v) acetic acid as ion-pairing agent. The proposed method was proved to be specific and sensitive making possible the detection and the quantitation of additions of about 0.10% (w/w) and 0.33% (w/w), respectively, of soybean proteins in soybean–wheat and soybean–rice products (related to 1 g of initial product). Precision and recoveries observed were also acceptable. The method was applied to the determination of soybean proteins in different commercial bakery products.

Keywords: Soybean proteins; Bakery products; Soybean–wheat and soybean–rice products; Perfusion liquid chromatography

1. Introduction

Cereals are the base of the human diet in most countries of the world. In fact, they provide most of the caloric energy and an important part of the proteins needed by human beings. Furthermore, there is evidence showing that healthy diets for humans should provide most of the calories as complex carbohydrates such as cereal starch (Dendy & Dobraszczyk, 2001). The most employed cereals in human feed are wheat and rice, although barley, rye, oat, and corn are also important.

Wheat flour has been extensively and widely used for the preparation of bakery products due to its inherent ability to form elastic doughs which retain gases and for being an important source of nutrients (Dhingra & Jood, 2002; Sliwinski, Kolster, & Van Vliet, 2004). Nevertheless, wheat is not suitable for gluten-intolerant people. Indeed, the mucous membrane of the small intestine of celiac people is damaged by gluten, resulting in poor absorption of nutrients. This has promoted the development of bakery products without gluten based on other cereals, such as rice (Ballesteros López, Guimaraes Pereira, & Gonçalves Junqueira, 2004).

Despite the benefits of cereals such as wheat and rice, they present a clear deficit in certain essential amino acids, such as lysine (Dhingra & Jood, 2001; Dhingra & Jood, 2002). In order to improve the quality of cereal-derived products, the addition of protein supplements rich in lysine is needed. Soybean proteins (rich in lysine) constitute a suitable and low cost complement to cereals (Erdman, O'Connor, Weingartner, Solomon, & Nelson, 1977). Consequently, the use of soybean flour and other soybean preparations for the supplementation of cereal products

* Corresponding author. Tel.: +34 91 8854935; fax: +34 91 8854971.
E-mail address: mluisa.marina@uah.es (M.L. Marina).

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has received considerable attention (Indrani, Savithri, & Venkateswara Rao, 1997; Mizrahi, Zimmermann, Berk, & Cogan, 1967).

Most numerous examples of cereal-based products fortified with soybean proteins are bread and biscuits. Fortifying wheat flour with full-fat soybean flour in bread manufacture results in a higher protein content and in a more balanced essential amino acid profile. Such fortification, however, can affect rheological properties and baking quality of wheat flour since it increases the water-absorption capacity of flour and decreases the loaf volume of bread (Urade et al., 2003). Regarding biscuits, there has been a trend to prepare high protein biscuits using wheat flour fortified with defatted soybean flour and soybean protein isolate. The addition of soybean in biscuits resulted in a drastic reduction in biscuit width (butter spread) and in an increase in biscuit thickness (height) (Chen, Weingartner, & Brewer, 2003).

As a consequence of the increasing use of soybean proteins in the preparation of bakery products, regulations have been established in order to forbid or limit the addition of soybean proteins to these products (Endres, 2001; Legislación alimentaria de aplicación en España, 2002). Depending on the kind of bakery product, the addition of soybean proteins can be forbidden, allowed up to a certain limit or not limited. FDA Standards of identity for enriched bread allow the use of up to 3% soybean flour as an optional ingredient. Nevertheless, there is no method for the reliable determination of the soybean protein content in bakery products. In fact, the analytical method normally used for the determination of the protein content in these products (Kjeldahl method) does not allow the discrimination between proteins, yielding only global amount of proteins. In order to apply the existent legislation, it is necessary the development of new analytical methods.

Soybean proteins have mostly been analyzed by HPLC with different purposes: compositional studies (Cole & Cousin, 1994; Morita, Fukase, Yamaguchi, Fukuda, & Morita, 1996), identification of soybean varieties (Buehler, Van Toai, & McDonald, 1988; Buehler, McDonald, Van Toai, & St. Martin, 1989; Mujoo, Trinh, & Ng, 2003; Oomah, Voldeng, & Fregeau-Reid, 1994; Peterson & Wolf, 1988, 1992; Peterson, Wolf, & Schaer, 1992), and detection and quantitation of soybean proteins in soybean products (García, Torre, & Marina, 1998), meat products (Ashoor & Stiles, 1987; Castro-Rubio, García, Rodríguez, & Marina, 2005a; Parris & Gillespie, 1988), and dairy products (Cattaneo, Feroldi, Toppino, & Olieman, 1994; Espeja, García, & Marina, 2001; García & Marina, in press; Hewedy & Smith, 1989). Nevertheless, to our knowledge, there is no analytical method for the reliable determination of soybean proteins in bakery products. Consequently, and despite the existence of legal limitations, it is not possible to control these products or the application of legal regulations. Therefore, the development of analytical methodologies enabling the determination of soybean proteins in this kind of product constitutes a paramount issue.

Recently, our research team has developed a perfusion high-performance liquid chromatography (HPLC) method to achieve, for the first time, the simultaneous separation of soybean and cereal proteins (Castro-Rubio, Castro-Rubio, Garcia, & Marina, 2005b). Major problems to overcome for the simultaneous separation of soybean and cereal proteins are their different solubilities. The main cereal proteins (glutelins and prolamins) (Belitz & Grosch, 1998) are characterized by their heterogeneity, unusual solubility, and aggregative tendencies, which make difficult the development of methodologies to analyze them. In fact, they have low solubility in aqueous media, the use of organic modifiers, acids or bases, denaturants or detergents being necessary to disrupt all non-covalent interactions (Bietz, 1990). On the other hand, soybean proteins, mainly constituted of 7S and 11S globulins, are soluble in aqueous solutions (García, Torre, Marina, & Laborda, 1997).

Therefore, the goal of this work was the application of a chromatographic method for the simultaneous separation of soybean and cereal proteins to evaluate the soybean protein content in soybean–wheat and soybean–rice bakery products. This work would constitute an important contribution to the application of established regulations that limit the soybean protein contents of some of these products.

2. Materials and methods

2.1. Chemicals and samples

HPLC grade acetonitrile (ACN) (Merck, Darmstadt, Germany) and Milli-Q water (purified by passing deionized water through a Millipore Milli-Q system (Millipore, Bedford, MA)) were used for the preparation of mobile phases and injection media. Acetic acid (Merck, Darmstadt, Germany) was used as ion-pairing agent.

Soybean flour (SF) (El Granero, Madrid, Spain) was used for the quantitation of soybean proteins. Its protein content, determined by Kjeldahl analysis (3 replicates), was 48.9% (relative standard deviation (RSD), 0.34%). Several commercial bakery products (breads, biscuits, doughnuts, and baby foods) containing soybean–wheat (15 products) and soybean–rice (5 products) binary mixtures were purchased in local markets in Madrid (Spain). All these products were prepared with wheat flour (wheat products) or rice flour (rice products) and contained soybean proteins from soybean flour, whole soybeans or soybean flakes. Moreover, in some cases, soybean proteins were also added, in addition to soybean flour. These bakery products were ground with an automatic miller before being used.

Dry matter content of soybean flour was determined by drying at 130 °C to constant weight (AOAC method 925.10). The protocol for preparing the solutions was as follows: the sample was weighed (0.1–2.5 g) and dissolved in the appropriate media, ultrasonicated for 10 min, and, finally, centrifuged (3362*g*, 5 min, 25 °C) to remove the

supernatant. The medium used for the preparation of soybean flour solutions and bakery product solutions consisted of a 25:75 ACN–water mixture containing 0.3% (v/v) acetic acid as ion-pairing agent.

2.2. High-performance liquid chromatography

Chromatography was carried out with a Hewlett–Packard 1100 Series liquid chromatograph (Hewlett–Packard, Pittsburg, PA). It included a degasser system, a quaternary pump, a thermostatted column compartment, an automatic injector, and a diode array detector (all of the series 1100). The chromatograph was coupled to an HP Vectra Pentium computer with a HP-chemstation data acquisition and treatment programme. The separation was accomplished with a POROS R2/H perfusion column (50 mm × 4.6 mm i.d.) supplied by Perseptive Biosystems (Framingham, MA) and packed with 10 μm diameter polystyrene divinylbenzene beads. The separation of soybean proteins from wheat and rice proteins was carried out with a linear binary gradient, from 5% to 50% B in 3.8 min, and 50–5% B in 1 min, to equilibrate the column to initial conditions between runs. Mobile phases were: phase A, 0.3% (v/v) acetic acid in Milli-Q water; phase B, 0.3% (v/v) acetic acid in ACN. The flow-rate was 3 ml/min, temperature was 50 °C, injected volume was 20 μl, and UV detection was performed at 254 nm. The organic modifier was filtered through 0.45 μm nylon filters before use.

2.3. Calibration

Calibration was performed by the external standard and by the standard additions calibration methods, taking into account all peaks corresponding to soybean proteins. Calibration by the external standard method was carried out by injecting soybean flour solutions over the range 0.03–1.30 mg/ml of soybean proteins. The peak area integration was carried out by setting the baseline from valley to valley and the average of the total peak area, corresponding to three consecutive injections, was calculated. The soybean protein content in soybean–wheat and soybean–rice bakery products was determined by interpolation of the total area of the peaks corresponding to soybean proteins in the calibration curve. Bakery product solutions were prepared in order to obtain a signal to be interpolated in the middle part of the calibration plot (minimal error). Calibration by standard additions method was performed by spiking sample solutions obtained after solubilization of soybean proteins from soybean–wheat and soybean–rice bakery products with known and increasing amounts of soybean flour (five standard solutions, with additions ranging from 0 to 0.8 mg/ml of soybean proteins).

2.4. Data treatment

Total peak area corresponding to soybean proteins was plotted against injected concentrations. The linearity in this relationship was obtained by least-squares regression analysis. The linear model was validated by means of the analysis of residuals and variance when three replicates of every standard were injected in triplicate.

3. Results and discussion

3.1. Sample preparation

The chromatographic separation of binary mixtures of soybean–wheat and soybean–rice proteins was performed using an elution gradient previously optimized by our research team (Castro-Rubio et al., 2005b): from 5% to 50% B in 3.8 min, the mobile phase A being Milli-Q water containing 0.3% (v/v) acetic acid and the mobile phase B, ACN containing 0.3% (v/v) acetic acid. The use of a temperature of 50 °C and a wavelength of 254 nm to achieve the UV detection enabled the chromatographic separation of the above-mentioned binary mixtures of vegetable proteins in less than 4 min.

In that work the medium used to solubilize soybean and cereal proteins was also optimized and consisted of a mixture 25:75 ACN-water containing 0.3% (v/v) acetic acid. Sample solutions were prepared by dissolving the sample in this medium, shaking, and centrifuging for 10 min (3362*g*). Since no optimization on the sample preparation protocol was achieved in this previous work, the influence of some experimental parameters (shaking or centrifugation time) on the sample preparation was studied in the present work before quantitation.

In order to have better control of shaking, the sample solutions (prepared as previously described) were submitted to different ultrasonication times ranging from 5 to 30 min. As examples, Fig. 1 shows the variation of the

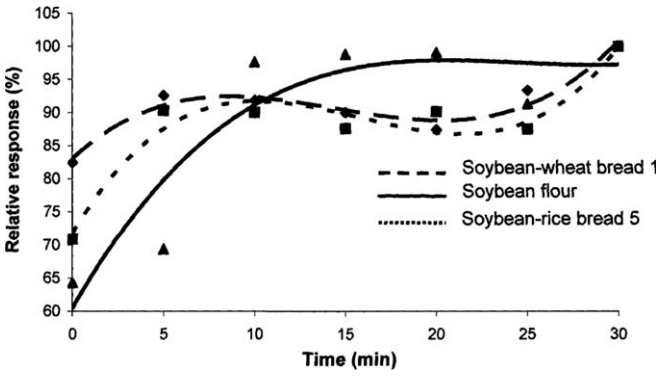

Fig. 1. Relative response (determined as the ratio between the total peak area for soybean proteins and the concentration injected and given as the percentage related to the maximum value of this ratio) for soybean proteins at different ultrasonication times, using standard solutions of approximately 3.5 mg/ml of soybean flour (about 2 mg/ml soybean proteins), soybean–wheat bread solutions of approximately 6 mg/ml of initial product and soybean–rice bread solutions of approximately 100 mg/ml of initial product. Chromatographic conditions: temperature, 50 °C; flow-rate, 3 ml/min; gradient: 5–50% B in 3.80 min; mobile phases: A, water–0.3%(v/v) acetic acid; B, ACN–0.3% (v/v) acetic acid; injected volume, 20 μl; detection, 254 nm.

relative responses obtained for soybean proteins as a function of the ultrasonication time in the soybean flour and in two commercial products containing soybean proteins (one soybean–wheat bread and one soybean–rice bread). The relative response for each protein was determined as the ratio between the total peak area obtained and the concentration injected for each protein. This ratio was expressed as the percentage related to the maximum value of this ratio. Soybean proteins, in soybean flour and in both breads, showed a similar variation with the ultrasonication time. Indeed, relative response increased significantly in the first few minutes and reached a plateau at ultrasonication times longer than 10 min. A value for the ultrasonication time of 10 min was chosen for further experiments.

In order to select the centrifugation time most adequate for maximizing the relative response for soybean proteins, this parameter was also varied from 5 to 30 min (at 3362*g* and 25 °C) (results not shown). Centrifugation time seemed not to significantly affect responses obtained for soybean proteins. The selected centrifugation time was 5 min.

3.2. Determination of soybean proteins in soybean–wheat and soybean–rice bakery products

The optimized conditions for the separation and preparation of sample solutions were applied to different commercial products containing soybean–wheat or soybean–rice proteins.

Chromatograms obtained for a representative soybean–wheat product (bread 3) and a representative soybean–rice product (biscuit 11) are shown in Fig. 2. These chromatograms were compared with that obtained from a soybean flour solution. Soybean flour showed five peaks (a–e) with the maximum signal for peak c and peaks a and b being overlapped. The comparison of this chromatogram with that of a soybean protein isolate (89.1% of proteins) revealed that these peaks corresponded to soybean proteins (Castro-Rubio et al., 2005b). Soybean–wheat bread and soybean–rice biscuit chromatograms showed profiles very similar to that of soybean flour with the maximum signal also for peak c. The soybean–wheat bread chromatogram showed four peaks corresponding to peaks a, c, d, and e, peak d being partially defolded. The soybean–rice biscuit chromatogram showed peaks a–e with an overlapping of peaks a and b, the defolding of peak c, and with peak d showing a shoulder. Wheat and rice proteins were eluted after soybean proteins as very small peaks, despite being present in higher proportions. This could be due to possible interactions between soybean and cereal proteins. In fact, soybean 11S globulin has been reported to interact with wheat gluten during the manufacture of baking products (Lampart-Szczapa & Jankiewicz, 1983; Ribotta, Edel León, Pérez, & Añón, 2005). Nevertheless, in our case, this interaction would not affect the content of soybean proteins determined since the peaks appearing in the chromatogram do not correspond to 11S globulin (García, Torre, & Marina, 2000).

3.3. Method validation

The method has been proven to be useful for soybean protein detection in bakery products. In order to apply the method for quality control purposes, a validation procedure was used. The possibilities of the method were examined, following a standardized validation procedure for food chemistry laboratories (Feinberg & Raguènès, 1999). The quantitation of soybean proteins in these samples presents the difficulty of the selection of a suitable standard. Indeed, there is no certified reference material that could be used as a standard of soybean proteins for the determination of these proteins in bakery products. Therefore, and since soybean flour has been the raw material in the preparation of the studied products, a commercial soybean flour was used as standard in the determination of soybean proteins in soybean–wheat and soybean–rice products.

The parameters evaluated for the validation of the method were linearity of the calibration plot, detection and quantitation limits, existence of matrix interferences, specificity, precision, and accuracy. Table 1 groups the results obtained in the determination of all these parameters. A good linear correlation coefficient ($r^2 > 0.99$) was observed between the total peak area for soybean proteins (sum of the areas of peaks a–e in the chromatogram of

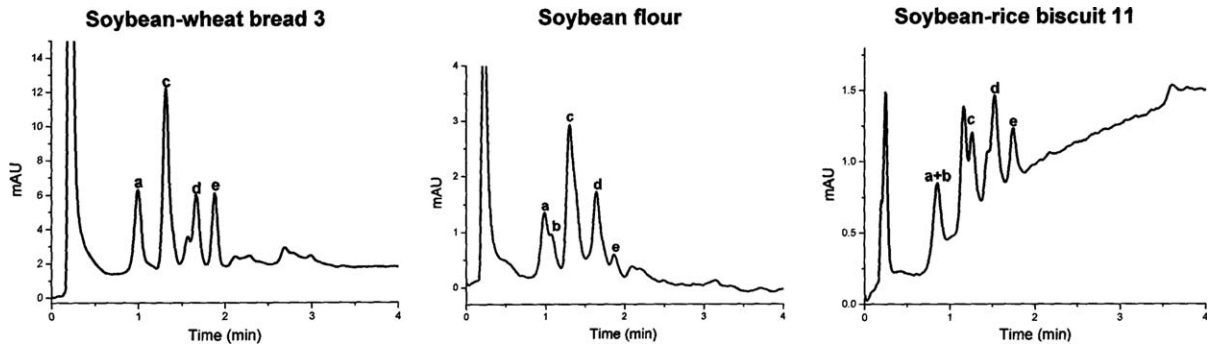

Fig. 2. Chromatograms corresponding to solutions obtained from a commercial soybean–wheat bread (30 mg/ml), a solution of soybean flour (1.42 mg/ml), and a commercial soybean–rice biscuit (1.62 mg/ml). Chromatographic conditions as in Fig. 1.

Table 1
Characteristics of the perfusion RP-HPLC method for the analysis of soybean proteins in soybean–wheat and soybean–rice bakery products

	Soybean–wheat		Soybean–rice
Linear concentration range	Up to 1.30 mg/ml soybean proteins		
Detection Limit	0.04 mg/ml soybean proteins (0.10% (w/w))[a]		
Quantitation limit	0.13 mg/ml soybean proteins (0.33% (w/w))[a]		
Existence of matrix interferences			
– Slope by the external standard method	57.1 ± 4.2 ($n = 8$)		
– Slope by the standard additions method	61.0 ± 2.3 ($n = 5$)		54.9 ± 2.5 ($n = 5$)
Specificity[b]	$Y = -0.01\ (0.02) + 1.04\ (0.03)$		
Repeatability (RSD, %) ($n = 10$)[c]	Soybean–wheat sample	Standard	Soybean–rice sample
– Retention time	0.36	0.29	0.33
– Peak area	0.39	0.44	0.17
Intermediate precision[d] (RSD, %)	Standard (0.03 mg/ml soybean protein)		Standard (1.00 mg/ml soybean protein)
– Retention time	1.63		1.03
– Peak area	4.53		8.95
– Slope	9.20		
Recovery (%)[e]			
0.20 mg/ml	99.7		102
0.40 mg/ml	96.5		105
0.60 mg/ml	97.9		103
0.80 mg/ml	98.1		104

[a] Limits of detection and quantitation in percentage (w/w) were determined related to 1.0 g of initial product.
[b] The *t*-tests for the verification of slope and intercept indicated that they were statistically equal unity and zero, respectively. Standard deviations of slope and intercept are given in parentheses.
[c] Number of injections of a solution of 1.10 mg/ml of soybean flour and of the solutions obtained after solubilization of soybean proteins from a soybean–wheat biscuit and a soybean–rice bread (40.0 mg/ml of initial product).
[d] Analysis performed by the external standard method in 7 days (for retention time and peak area) or in 13 days (for slope) during a period of 7 months.
[e] Recovery obtained for soybean proteins when different amounts of soybean flour were added to the solutions obtained after solubilization of soybean proteins from a soybean–wheat bread and a soybean–rice bread.

soybean flour) and the concentration of soybean proteins up to 1.30 mg/ml. Moreover, the linear model was successfully validated in the working concentration range (0.03–1.30 mg/ml of soybean proteins) by means of the analysis of the residuals and variance (Miller & Miller, 2000).

The detection limit for soybean proteins was 0.04 mg/ml (calculated from the calibration plot as the concentration corresponding to a signal equal to the intercept plus three times the standard deviation of the regression line) which means that it is possible the detection of an addition of 0.10% (w/w) of soybean proteins in a soybean–wheat/rice bakery product (related to 1 g of initial product). The quantitation limit for soybean proteins detected by this method was 0.13 mg/ml (calculated from the calibration plot as the concentration corresponding to a signal equal to the intercept plus 10 times the standard deviation of the regression line) which means that it is possible to quantitate an addition of 0.33 % (w/w) of soybean proteins in a soybean–wheat/rice bakery product (related to 1.0 g of initial product).

Comparison of the slopes of the calibration plots obtained by the external and the standard additions calibration methods allowed study of the existence of matrix interferences. The comparison was performed by using the Statgraphics programme (Statgraphics Plus version 5.0). Results suggested that the proposed method did not suffer from matrix interferences, either for soybean–wheat bakery products or for soybean–rice bakery products ($P < 0.05$).

The specificity of the method was verified by adjusting a straight line between added and recovered concentrations of soybean proteins in soybean–wheat and soybean–rice bakery products. For that purpose, 10 soybean–wheat and soybean–rice bakery products were analyzed. For each sample, additions from 0.2 to 0.8 mg/ml of soybean proteins (as soybean flour) were made. Specificity of the method was considered acceptable, since the slope and the intercept of the previous straight line did not significantly differ from unity and zero, respectively (*t*-test, $P < 0.05$) (see Table 1).

Precision of the method was determined by means of the repeatability and intermediate precision and results obtained are also shown in Table 1. Repeatability, expressed as RSD (%) for 10 consecutive injections of a soybean flour solution or solutions obtained from a soybean–wheat and a soybean–rice bakery product, was better than 0.5% in peak area and in retention time. The intermediate precision, expressed as RSD, was obtained by injecting two solutions of soybean flour (corresponding to the lowest and the highest concentrations of the calibration plot (maximum error)) in seven different days in a period of 7 months. The RSD values observed were good (below 9% in peak area and below 2% in retention time), taking into account the period of 7 months used for the experiment and the higher imprecision expected in these parts of the calibration plots. Furthermore, the precision in the slope of the calibration lines obtained in the same period (7 months) was about 9% (RSD, %).

The recoveries obtained for soybean proteins when different amounts of soybean flour were added to the solutions obtained after solubilization of soybean proteins from a soybean–wheat bakery product and from a soybean–rice bakery product were also determined. Table 1 shows, as examples, the results obtained for a soybean–wheat bread (bread 1) and for a soybean–rice bread (bread 5). The recoveries were in all cases close to 100 %. Similar recoveries (98–106%) were observed when other kinds of soybean–wheat products (doughnuts) prepared following a different cooking procedure (frying instead of baking), were studied. Furthermore, the recovery of soybean proteins was also determined by spiking (at origin) different bakery products, not containing soybean, with known amounts of soybean flour. The recoveries obtained in these cases ranged from 90% to 100%.

3.4. Application to edible soybean–wheat and soybean–rice bakery products

The optimized method was applied to the determination of the soybean protein content in soybean–wheat and soybean–rice bakery products. For that purpose, the extracts obtained from these samples, under the optimized conditions, were injected into the chromatographic system and the concentration of soybean proteins was calculated by interpolating the total peak area for soybean proteins in the calibration plot obtained by the external standard method, using soybean flour as standard for soybean proteins. Since, for those products regulated, limitations are given as percentages related to initial product, the soybean protein contents given are expressed as percentages as the basis. Tables 2 and 3 show the contents obtained for 15 soybean–wheat products and 5 soybean–rice products by the external standard method. Moreover, these Tables also include the information given on the label of each product regarding the soybean source used in the preparation of the product and regarding the (approximate) proportion of these raw materials added in bulk during the preparation of the product (when given). From these estimated contents of each raw material, and taking into account that whole soybeans can contain from 20% to 50% of soybean proteins and soybean flour and flakes can be from 40% to 55% in soybean proteins, it is possible to get an estimation of the soybean protein content in these products. These indicated soybean protein contents were in good agreement with those obtained with the optimized method.

Two kinds of commercial products were found in the case of soybean–wheat: (i) soybean bakery products containing wheat proteins (breads 1–4 and biscuits 1–7), in which soybean appeared as subtitled, contiguous to the product name, or as part of the descriptive name, and (ii) wheat bakery products containing soybean proteins as ingredient (biscuit 8 and doughnuts 1–3). In this last kind of products, the soybean protein content is limited by regulations and should be below 3% of soybean flour related to the initial product (1.5% soybean protein). Regarding soybean–rice products, the baby food was the only product sold as a rice product and containing soybean as ingredient. Bread 5 and biscuits 9–11 were sold as products intended for gluten-free diets. In these products, soybean and rice appeared in the ingredient list but were not a part of the descriptive name of the product.

Table 2
Soybean protein content determined in soybean–wheat bakery products by the external standard and the standard additions calibration methods

Soybean–wheat bakery product	Declared content of raw material indicated in the label of the product	Soybean protein content (mg/100 mg) obtained by the proposed perfusion RP-HPLC method	
		External standard[a,b]	Standard additions[a,b]
Bread 1[c]	Soybean flour	5.00 (0.45)	5.40 (0.48)
Bread 2[c]	Soybean flour	1.99 (0.11)	1.97 (0.12)
Bread 3[c]	Soybean flour (5.8%); whole soybeans (7.6%)	4.71 (0.11)	–
Bread 4[c]	Whole soybeans (8%)	2.19 (0.31)	2.07 (0.25)
Biscuit 1[c]	Soybean flakes (13.3%)	7.69 (0.65)	7.26 (0.19)
Biscuit 2[c]	Whole soybean (18%)	9.79 (0.26)	–
Biscuit 3[c]	Soybean flour (5%); soybean protein isolate (4%); whole soybean (2.3%)	3.21 (0.74)	–
Biscuit 4[c]	Whole soybean (9%); soybean flour (6%)	2.79 (0.42)	–
Biscuit 5[c]	Soybean flour (8.2%)	3.42 (0.21)	–
Biscuit 6[c]	Soybean flour (24%)	5.72[e]	–
Biscuit 7[c]	Soybean flour (4.5%)	0.99 (0.08)	–
Biscuit 8[d]	Soybean flour	0.17[e]	0.21[e]
Doughnut 1[d]	Soybean flour	<Q.L.[f]	<Q.L.
Doughnut 2[d]	Soybean flour	< Q.L.	< Q.L.
Doughnut 3[d]	Soybean flour	0.18 (0.03)	0.22 (0.07)

[a] Mean of three individual determinations (every solution injected in triplicate).
[b] Standard deviations given in parentheses.
[c] Soybean bakery products containing wheat flour.
[d] Bakery products with soybean proteins as ingredient.
[e] One determination (solution injected by triplicate).
[f] Soybean protein content below the quantitation limit of the method.

Table 3
Soybean protein content determined in soybean–rice bakery products by the external standard and the standard additions calibration methods

Soybean–rice bakery product	Declared content of raw material indicated in the label of the product	Soybean protein content (mg/100 mg) obtained by the proposed perfusion RP-HPLC method	
		External standard[a,b]	Standard additions[a,b]
Bread 5	Soybean flour	2.19 (0.48)	2.04 (0.52)
Biscuit 9	Soybean flour and soybean protein isolate	5.28 (0.49)	–
Biscuit 10	Soybean flour and soybean protein isolate	1.50 (0.26)	1.71 (0.53)
Biscuit 11	Soybean flour	20.92 (2.09)	–
Baby food	Whole soybean (8%)	1.19 (0.14)	1.06 (0.15)

[a] Mean of three individual determinations (every solution injected in triplicate).
[b] Standard deviations given in parentheses.

As expected, the soybean protein content observed in soybean bakery products containing wheat were much higher than those observed for wheat or rice bakery products containing soybean proteins. Indeed, the soybean protein content in the first group of products ranged from 1.0% to 10.0%. In the case of bread, these contents agreed with the difference between the usual content in proteins of an ordinary white bread (8–9%) and the total content in proteins of soybean–wheat breads (usually ranging from 13% to 14 %) (Endres, 2001).

Wheat and rice bakery products containing soybean as ingredient (biscuit 8, doughnuts 1–3, and all soybean–rice products) had soybean protein contents ranging from 0.2% to 5.3% with the exception of the biscuit 11. In general, the soybean protein content in rice bakery products was higher than those in wheat bakery products. The levels of soybean proteins found in wheat bakery products with soybean were always below the maximum allowance specified by Spanish regulations and FDA for fortified breads (3% soybean flour, approximately 1.5% soybean proteins).

In order to confirm the reliability of these data, some of the results obtained were compared with those obtained when the standard additions method was employed, using also soybean flour as standard. The contents obtained for soybean proteins by the external standard and the standard additions calibration method, in all wheat and rice samples, did not present statistically significant differences.

4. Conclusions

An analytical method is proposed, for the first time, for the determination of the soybean protein content in soybean–wheat and soybean–rice bakery products. The method consisted of the preparation of the sample solution and the chromatographic separation of that solution in less than 4 min. The quantitative analysis of these products using, soybean flour as standard of soybean proteins, was carried out by the external standard calibration method. The method has been successfully validated, following a standardized procedure for food chemistry laboratories. The method enabled the detection and the quantitation of additions of 0.10% (w/w) and 0.33% (w/w) of soybean proteins, respectively, in soybean–wheat and soybean–rice bakery products (related to 1 g of initial product). The study of the precision and accuracy of the method gave good results and the method was proved to be specific and did not suffer from matrix interferences. The method was applied to the determination of the soybean protein content in commercial bakery products, giving values in accordance with those expected for these samples. The method constitutes a suitable tool for the quality control of these bakery products, enabling the application of established regulations, even when the soybean flour used in the elaboration of bakery products was unknown and there is no certified reference material. Moreover, the method would constitute an alternative to the Kjeldahl method that yields total protein contents and does not allow differentiation between different kinds of proteins.

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