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Simple and Inexpensive Method for the Reliable Determination of Additions of Soybean Proteins in Heat-Processed Meat Products: An Alternative to the AOAC Official Method

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Despite the existence of an AOAC official method based on an enzyme-linked immunosorbent assay (ELISA) for the determination of additions of soybean proteins in meat products, its use for quantitative assessment is limited. Accordingly, a simple and inexpensive method has been developed and validated in this work. The method involves defatting the meat samples with acetone, solubilization of soybean proteins in a 30 mM Tris−HCl buffer (pH 8) containing 0.5% (v/v) 2-mercaptoethanol, and the identification of two peaks from soybean proteins in the chromatogram obtained by perfusion reversed-phase chromatography and UV detection. Determination of soybean proteins by the proposed method did not suffer from matrix interferences, with a good linear correlation up to a concentration of 12.50 mg/mL soybean proteins being observed. The proposed method was proven to be specific, precise, accurate, robust, and sensitive, making possible the detection and the quantitation of additions of 0.07% (w/w) and 0.25% (w/w), respectively, of soybean proteins in meat products (related to 1 g of initial product). The method has been applied to the determination of the soybean protein content in commercial heat-processed meat products, obtaining results that were statistically similar to those obtained by the official ELISA method but with a higher reliability and simplicity and a lower cost and analysis time.

KEYWORDS: soybean proteins; meat product; perfusion chromatography; adulteration

INTRODUCTION

The addition of soybean proteins to processed meat products is a common practice (*1*, *2*). Processed meat products normally present high fat content, and their content in meat proteins is low. Since meat proteins play an important role as emulsifiers, preventing the coalescence of fat during heating, when the lean meat content (and meat protein content) is low, the addition of foreign proteins such as soybean proteins can supply the needed emulsion power (*2*). On the other hand, the demands of consumers for healthier and safer products have also promoted the use of soybean proteins in processed meat products as fat replacers (*3*−*5*).

Regarding safety, regulatory agencies are aware of illegal additions of soybean proteins in meat products and, consequently, regulations establishing maximum levels of soybean proteins and controlling the accurate labeling of these products have appeared (*3*, *6*). Obviously, the implementation of these regulations involves having an adequate methodology for monitoring the amounts of soybean protein added to processed meat products (*3*, *7*). Nevertheless, and despite the fact that many methods have been proposed for the identification of soybean proteins in meat products, to date, there is not a reliable one.

Methods developed for the determination of soybean proteins in meat products can be classified into two broad categories: (1) indirect methods involving the detection of substances accompanying soybean proteins such as some chemical and microscopic methods and (2) those methods focused on the examination of the protein itself. Chemical methods used in the detection of soybean proteins in meat products suffer from low specificity, and microscopic methods are effective only when the whole soybean is involved. Thus, direct methods are preferred as opposed to the indirect approaches (*8*).

Polyacrylamide gel electrophoresis has been the electrophoretic technique most applied for the detection of soybean proteins in processed meat products. However, these methods yield very crowded electropherograms, making it extremely difficult to detect the presence of bands originating from added soybean proteins, of minor intensity compared with bands originating from the meat proteins themselves (*2*, *9*).

Immunochemical methods have become very popular for the detection of soybean proteins in processed meat products,

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offering high specificity and sensitivity. In fact, the AOAC International granted an enzyme-linked immunosorbent assay (ELISA) as the AOAC Official Method (first action in 1988) for measuring soybean proteins in raw and heat-processed meat products (*10*). In this method, soybean proteins from a defatted meat product are submitted to denaturing conditions and then renaturing conditions and are finally analyzed by an inhibition mode of ELISA. In this immunoassay, soybean proteins are made to react with an appropriate antiserum in excess and the unreacted antibody is determined, after isolation, by its reaction with a second antibody conjugated with an enzyme. Capture enzyme activity is determined by adding a chromogenic substrate, yielding a product whose color intensity is measured at 405 nm (*10*). Despite the complexity, tediosity, and cost of this method, it continues to be applied by food control agencies in order to detect additions of soybean proteins in meat products. Furthermore, this method presents an additional limitation, since numerous factors can affect the quantitative results (*9*, *11*, *12*). In fact, the method has been considered as semiquantitative and, therefore, not reliable for the analysis of soybean proteins in meat products (*10*).

Chromatographic methods have also been applied for the detection of soybean proteins in meat products. Some of these methods were based on amino acid composition and sequence and others on the direct detection of the soybean proteins themselves. Chromatographic methods have been developed for the analysis of soybean proteins by the detection of certain peptides in trypsin hydrolysates. Although these methods were useful for qualitative assessment, quantitative results were not accurate. Total hydrolysis and analysis of the amino acidic pattern of soybean proteins has also been tried, although the sensitivity was very low and similarities between soybean and meat amino acidic compositions yielded interferences (*2*, *9*). The few attempts for the direct determination of soybean proteins in meats by high-performance liquid chromatography (HPLC) have been focused on raw meats and were never applied to commercial meat products but to synthetic meat−soybean blends (*13*−*15*).

Thus, despite the efforts carried out, all the methods proposed suffered for nonreliability or were very tedious and difficult to standardize. A method capable of yielding the soybean protein content added to a heat-processed meat product with suitable accuracy, sensitivity, simplicity, and speed and at a low cost so as to fulfill quality control requirements still did not exist. Therefore, the aim of this work has been the development of a simple and inexpensive method for the reliable determination of additions of soybean proteins in heat-processed meat products. For that purpose, the ordinary analytical technique HPLC has been used with perfusion columns, enabling the drastic reduction of analysis times (*16*).

EXPERIMENTAL PROCEDURES

Chemicals and Samples. HPLC grade acetonitrile (ACN) (Merck, Darmstadt, Germany), trifluoroacetic acid (TFA) (Sigma, St. Louis, MO), and water obtained from a Milli-Q system (Millipore, Bedford, MA) were used in the preparation of mobile phases. Tris(hydroxymethyl)aminomethane (Tris), 2-mercaptoethanol, and urea from Merck (Darmstadt, Germany), ditiothreitol from ICN (Aurora, OH), sodium dodecyl sulfate (SDS) from Fluka (Barcelona, Spain), and sodium hydrogen carbonate and sodium hydrogen phosphate from Panreac (Barcelona, Spain) were used for the solubilization of proteins. Hexane and acetone (Merck, Darmstadt, Germany) and ethanol and petroleum ether (Panreac, Barcelona, Spain) were employed for fat extraction. Thirty commercial heat-processed meat products (chopped pork meats) containing pork meat, water, soybean proteins, and additives (meat products A−M), that were purchased in local markets in Madrid, Spain, or supplied by a meat company (Campofrío Alimentación S. A., Spain), were used. Moreover, model meat products with the same composition as the previous processed meats but without soybean proteins (model meat product 1) and with the same composition as the previous processed meats (including soybean proteins) but not submitted to any heat processing (model meat product 2) were also supplied by the meat company. All meat products were defatted before their analysis by the following procedure: 10 g of meat was ground with an automatic miller, homogenized with 25 mL of acetone in an Ultraturrax mixer (3 min), submitted to agitation for 15 min, and centrifuged (3362 g, 30 min, 25 °C). The supernatant was removed, and the pellet was extracted again with another 25 mL of acetone following the same procedure. Finally, the pellet was dried overnight at 60 °C to remove the remaining acetone. Meat solutions with concentrations ranging from 70 to 190 mg/mL (related to initial product) from meat products containing 1−2% (w/w) soybean proteins were used in this work. These solutions were prepared by weighing the appropriate amount of the defatted and dried meat product (0.3−0.7 g), solubilizing it in 30 mM Tris−HCl buffer (pH 8) containing 0.5% (v/v) 2-mercaptoethanol with ultrasonic agitation for 10 min at 50 °C, and centrifuging it at 3362*g* for 20 min to inject the supernatant in the chromatographic system. The soybean protein content in meat samples was also determined by the ELISA procedure described in the AOAC method 988.10 (*10*). The soybean protein isolate (SPI) Supro 500E (Anvisa, Madrid, Spain) was used as the standard of soybean proteins. Its protein content, determined by Kjeldahl analysis (six replicates), was 90.93% (relative standard deviation (RSD), 0.73%). The dry matter content of the SPI was determined by drying at 130 °C to constant weight. The protocol for preparing standard solutions consisted of weighing and dissolving the standard in 30 mM Tris−HCl buffer (pH 8) containing 0.5% (v/v) 2-mercaptoethanol, sonicating for 5 min, and centrifuging at 3362*g* for 10 min.

High-Performance Liquid Chromatography. A Hewlett-Packard 1100 Series liquid chromatograph (Hewlett-Packard, Pittsburgh, PA) equipped with a diode array detector was used. The injected volume was 20 μL, and the detection was performed at 280 nm. The separation was accomplished with a POROS R2/H column (50 × 4.6 mm i.d.) from Perseptive Biosystems (Framingham, MA) packed with 10 μm diameter polystyrene divinylbenzene beads. The reversed-phase HPLC (RP-HPLC) method consisted of a linear binary gradient in three steps: 5−25% B in 0.8 min, 25−42% B in 0.8 min, 42−50% B in 0.6 min, and finally 50−5% B in 0.5 min to equilibrate the column to initial conditions between runs. The flow rate was 3 mL/min, and the temperature was 50 °C. The mobile phases were the following: phase A, 0.05% TFA (v/v) in Milli-Q water; phase B, 0.05% TFA (v/v) in ACN. The organic modifier was filtered through 0.45 μm nylon filters before use.

Calibration. Calibration was performed by the external standard and standard additions methods. Calibration by the external standard method was performed by injecting (seven) SPI solutions over the range 0.5−6.0 mg/mL. Integration was carried out by setting the baseline from valley to valley, and the average of the area corresponding to three consecutive injections was calculated. The soybean protein content in heat-processed meat products was determined by interpolation of the area of the peaks corresponding to soybean proteins in the calibration curve. Meat solutions were prepared (see the Chemicals and Samples section) by taking into account that the signal was interpolated in the middle part (less error) of the calibration plot. Calibration by the standard additions method was performed by injecting extracts of the meat products spiked with known and increasing amounts of the SPI (five standard solutions in the range 0−5.0 mg/mL).

Data Treatment. The peak area corresponding to soybean proteins was plotted against the 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 by triplicate.

RESULTS AND DISCUSSION

The development of a method for the determination of soybean proteins added to processed meat products involves

distinguishing these proteins from the meat proteins. This is not expected to be an easy task, since the samples under study are very complex (insoluble mixture of denatured cross-linked proteins and other components), the soybean proteins are present in a minor proportion compared to the meat proteins, and the soybean proteins could become altered during the processing of these meat products. Taking into account these premises, the proposal of this work was to develop a chromatographic method enabling the separation of soybean proteins from the rest of meat components from a meat extract. For that purpose, denaturing conditions were used for both the preparation of meat extracts and the chromatographic separation in order to have soybean proteins in the same random-coil conformation.

Chromatographic Separation. The knowledge accumulated by our research team in the determination of soybean proteins in foodstuffs helped to establish some initial separation conditions for the determination of soybean proteins in heat-processed meat products. Thus, the separation of soybean proteins from meat components was first tried with a previously optimized gradient used to separate soybean proteins: 5−25% B in 1.7 min (11.8%/min) and 25−45% B in 1.3 min (18%/min).[17] For that purpose, proteic extracts prepared from a heat-processed meat product (processed meat product M) containing the SPI and an identical meat product without the SPI (model meat product 1) were employed. The extraction conditions initially applied were selected from the information obtained in a previous bibliographic review.[2] The preparation of the sample solution consisted of (i) fat extraction with acetone followed by (ii) protein solubilization with a Tris−HCl buffer (pH 9) in a thermostated bath at 60 °C for 30 min and centrifugation at 3362*g* for 30 min. Although the initial gradient conditions did not enable the separation of soybean proteins in the heat-processed meat product, they were quite useful for the selection of the final gradient conditions. Thus, the gradient was modified by decreasing the gradient range and among the different gradients attempted, that from 5 to 25% B in 0.8 min, from 25 to 42% B in 0.8 min, and from 42 to 50% B in 0.6 min, followed by a linear gradient from 50 to 5% B in 0.5 min was chosen. The chromatograms obtained when applying this gradient to extracts obtained from heat-processed meat products with and without the SPI and from the SPI itself are shown in **Figure 1**. The chromatograms corresponding to meat products with and without the SPI were clearly different in two peaks at approximately 1.7 and 2 min. These two peaks appeared at retention times identical to the last two peaks of the SPI. When the UV spectra and first and second derivatives obtained for these two peaks in the SPI and the meat product containing the SPI were compared, it was observed that only those corresponding to the peak at 1.7 min in the meat product containing the SPI and the SPI were identical.

The effect of the variation of other parameters such as the percentage of TFA in the mobile phase (0.025−0.2% (v/v)) and the detection wavelength (254 and 280 nm) was also studied, with no significant improvement being observed in the sensitivity of the method when trying values different from the initially selected ones (0.05% (v/v) TFA and 280 nm) (results not shown).

Sample Preparation Optimization. The initial conditions used for the preparation of the sample were modified in order to increase the sensitivity of the method. For that purpose, different media (buffers at different pHs and concentrations or containing denaturing agents) for protein solubilization, different protein extraction conditions (extraction time, temperature, and number of extractions), and different fat extraction conditions

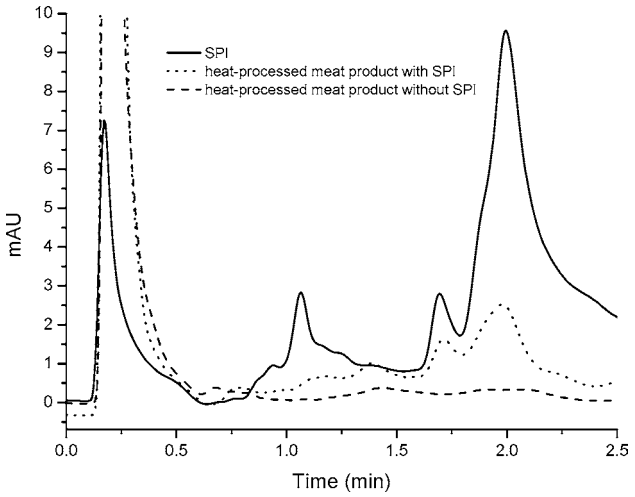

Figure 1. Chromatograms corresponding to a heat-processed meat product containing the SPI, a heat-processed meat product without the SPI, and the SPI itself. Chromatographic conditions: temperature, 50 °C; flow rate, 3 mL/min; gradient, 5–25% B in 0.8 min, 25–42% B in 0.8 min, 42–50% B in 0.6 min, and 50–5% B in 0.5 min; mobile phases, (phase A) 0.05% (v/v) TFA in water and (phase B) 0.05% (v/v) TFA in ACN; injected volume, 20 μL; detection, 280 nm. Sample preparation: fat extraction with acetone followed by protein solubilization in 50 mM Tris–HCl buffer (pH 9) for 30 min.

Table 1. Relative Response (%) of Soybean Proteins with the Proposed Method When Using Different Media for Protein Solubilization

solvent	pH	concn (mM)	relative response[a] (%)
Milli-Q water	6.4		3.6
phosphate buffer	7.0	50	22.1
Tris–HCl buffer	8.0	50	26.0
Tris–HCl buffer	9.0	50	22.1
bicarbonate buffer	10.0	50	22.1
bicarbonate buffer	11.0	50	10.3
Tris–HCl buffer + 8 M urea	8.0	50	
Tris–HCl buffer + 0.5% SDS	8.0	50	
Tris–HCl buffer + 0.1% ditiothreitol	8.0	50	33.5
Tris–HCl buffer + 0.1% 2-mercaptoethanol	8.0	50	33.2
Tris–HCl buffer + 0.5% 2-mercaptoethanol	8.0	50	50.2
Tris–HCl buffer + 1% 2-mercaptoethanol	8.0	50	46.8
Tris–HCl buffer + 0.5% 2-mercaptoethanol	8.0	10	90.6
Tris–HCl buffer + 0.5% 2-mercaptoethanol	8.0	20	97.6
Tris–HCl buffer + 0.5% 2-mercaptoethanol	8.0	30	100.0
Tris–HCl buffer + 0.5% 2-mercaptoethanol	8.0	40	94.6
Tris–HCl buffer + 0.5% 2-mercaptoethanol	8.0	100	54.4

[a] Determined as the ratio (area of the peak at 1.7 min/concentration of processed meat product) expressed as the percentage related to the highest value of this parameter.

(the solvent used and number of extractions) were tried when using one heat-processed meat product containing the SPI and one heat-processed meat product without the SPI (model meat product 1) for control purposes. Different media for protein solubilization with pHs ranging from 6.4 to 11.0 were tried. The relative responses (%) of soybean proteins when using these media for protein solubilization are grouped in **Table 1**. Relative responses were calculated as the ratio (area of the peak at 1.7 min/concentration of processed meat product) expressed as the percentage related to the highest value of this parameter. The highest response was observed when solubilizing proteins at pH 8 with a Tris−HCl buffer. Using this buffer, the effect of the addition of different denaturing agents (urea, SDS, ditio-

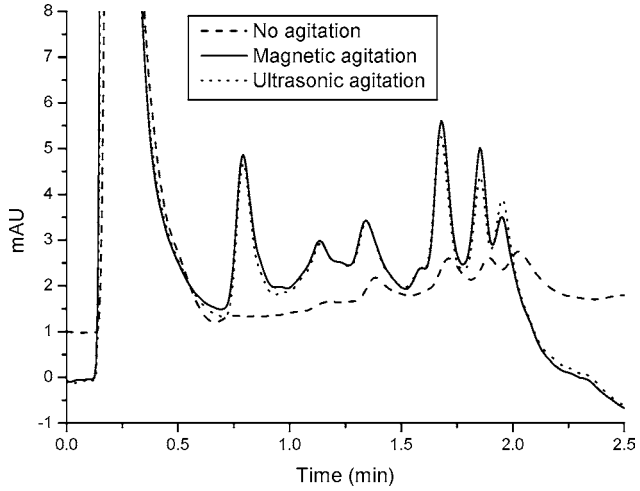

Figure 2. Chromatograms corresponding to a heat-processed meat product when not using agitation and when using magnetic or ultrasonic agitation for protein solubilization for 10 min. The chromatographic conditions are the same as those in **Figure 1**. Sample preparation: fat extraction with acetone followed by protein solubilization in 30 mM Tris–HCl buffer (pH 8) containing 0.5% (v/v) 2-mercaptoethanol.

threitol, and 2-mercaptoethanol) at concentrations normally used was studied. Regarding urea and SDS, the calculation of the relative response was not possible, since the peak of interest overlapped with the peak at 2 min. Ditiothreitol and 2-mercaptoethanol enabled an increase in the area of the peak at 1.7 min in comparison with the area obtained without these agents (26.0). Moreover, it was also observed that the peak at 2 min defolded when using these agents. Three different concentrations of 2-mercaptoethanol were also tried; it was observed that the increase in the concentration of this agent enabled a significant improvement in peak area. Other experiments using combinations of denaturing agents were also performed, although no increase in peak area was observed (results not shown). Finally, the concentration of the buffer itself was modified from 10 to 100 mM, observing that concentrations higher than 30 mM presented a negative effect on the area of the peak of interest. From these studies, a 30 mM Tris−HCl buffer (pH 8) with 0.5% (v/v) 2-mercaptoethanol was chosen for the solubilization of soybean proteins in heat-processed meat products.

The solubilization conditions were also optimized to get a further increase in sensitivity. As observed in **Figure 2**, the use of magnetic agitation or ultrasonication for the solubilization of soybean proteins allowed a significant increase in peak area as well as the appearance of a tiny peak close to the peak at 1.7 min, both in standard and sample solutions, that was also taken into account from now in the determination of the soybean protein content. Extractions for more than 10 min and temperatures higher than 50 °C did not result in an increase in protein extraction percentages, as observed in **Figures 3** and **4**, respectively. Finally, successive extractions of soybean proteins with the optimized conditions were also tried, but better results than those obtained with just one extraction were not observed.

Fat extraction solvents different from acetone were tried (hexane, petroleum ether, and ethanol), and the results are compared in **Figure 5** with those obtained with acetone. Acetone was the solvent yielding the highest peak area. Moreover, extracting more than twice with acetone did not result in a significant increase in responses.

Method Validation. Once the suitability of the method for the detection of soybean proteins in heat-processed meat products was proven, the method was validated for its application as a quality control method. For that purpose, the SPI Supro 500E was used as the standard of soybean proteins. Method validation was mainly performed following a standardized validation procedure for quantitative methods for food chemistry laboratories (*18*). The parameters evaluated were the linearity of the calibration plot, detection and quantitation limits, presence of matrix interferences, specificity, precision, robustness, and accuracy. **Table 2** groups the results obtained in the determination of all of these parameters.

Good linear correlation ($r^2 > 0.99$) was observed between the signal and the concentration of soybean proteins up to 12.50 mg/mL soybean proteins. Moreover, the linear model was successfully validated in the working concentration range (0.50−6.0 mg/mL) by means of the analysis of residuals and variance (p-value, 0.28 ($P < 0.05$)) (*19*). The lowest concentration of

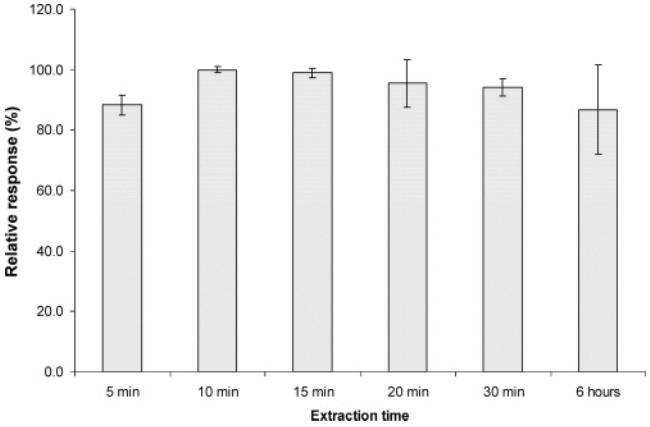

Figure 3. Effect of the extraction time used for the solubilization of soybean proteins on the relative response of soybean proteins determined as the ratio (area/concentration of processed meat product). The chromatographic and sample preparation conditions are the same as those in **Figure 2**.

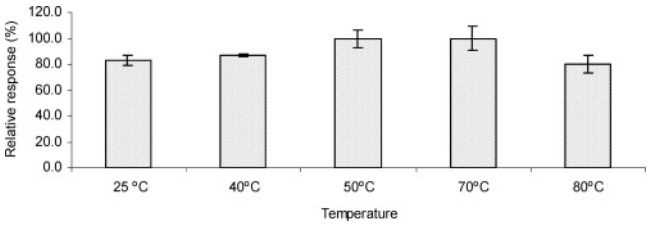

Figure 4. Influence of the temperature used for the solubilization of soybean proteins on the relative response of soybean proteins determined as the ratio (area/concentration of processed meat product). The chromatographic and sample preparation conditions are the same as those in **Figure 2**.

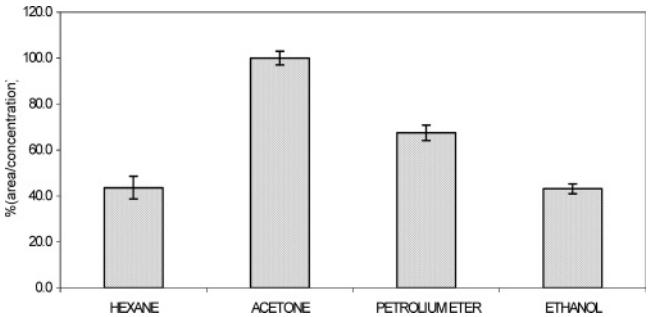

Figure 5. Relative response of soybean proteins determined as the ratio (area/concentration of processed meat product) obtained when using different solvents for fat extraction. The chromatographic conditions and the rest of the sample preparation conditions are the same as those in **Figure 2**.

Table 2. Characteristics of the Perfusion RP-HPLC Method for the Analysis of Soybean Protein in Processed Meat Products

linear concentration range	up to 12.50 mg/mL		
detection limit	0.20 mg/mL (0.07% (w/w))[a]		
quantitation limit	0.68 mg/mL (0.25% (w/w))[a]		
existence of matrix interferences[b]			
slope by the external standard method	8.09 ± 0.08 ($n = 7$)		
slope by the standard additions method	8.04 ± 0.36 ($n = 5$)		
specificity[c]	$y = 0.924(0.093)x + 0.057(0.156)$		
repeatability[d] (RSD, %) ($n = 10$)			
retention time	0.16		
peak area	3.95		
intermediate precision[e] (RSD, %) ($n = 7$)	sample[f]		standard[g]
retention time	0.61		0.26
peak area	4.74		1.71
concentration	10.76		
slope	0.93		
internal reproducibility[h] (RSD, %) ($n = 4$)			
retention time	1.79		
peak area	4.49		
robustness[i]	conventional parameters		modified parameters
column lot	1.07 ± 0.14 ($n = 7$)		1.14 ± 0.12 ($n = 7$)
buffer concentration	1.07 ± 0.14 ($n = 7$)		1.24 ± 0.11 ($n = 2$)
detection wavelength	1.14 ± 0.12 ($n = 7$)		1.11 ± 0.11 ($n = 7$)
recovery[j] (%)	heat-processed meat		raw meat
0.90 mg/mL soybean proteins	91.6 ± 4.6		93.8 ± 3.2
1.65 mg/mL soybean proteins	97.4 ± 4.4		92.4 ± 3.6
2.53 mg/mL soybean proteins	98.0 ± 1.9		94.1 ± 1.4
3.40 mg/mL soybean proteins	98.4 ± 2.4		92.4 ± 1.2
absolute recovery[k] (%)			
processed meat spiked with 2.04% soybean proteins	94.9 ± 2.4		

[a] Limits of detection and quantitation expressed as w/w units were determined relative to 1 g of sample. [b] An *F*-test for the comparison of variances and *t*-test for the comparison of slopes were employed. [c] The *t*-tests for the verification of slope and intercept were statistically equal to the unit and zero, respectively. The standard deviations of the slope and intercept are given in parentheses. [d] Number of injections of a solution of 190 mg/mL heat-processed meat product. [e] Analysis performed by the external standard method in 7 different days in a period of 12 days. [f] Injection of a solution of 70 mg/mL heat-processed meat product. [g] Injection of a 3.33 mg/mL SPI solution. [h] Analysis of four individual samples of 120 mg/mL heat-processed meat product in the same day. [i] Concentration of soybean proteins determined when varying the column lot, buffer concentration (30 and 50 mM Tris–HCl buffer (pH 8)), or detection wavelength (280 and 254 nm). [j] Recovery of soybean proteins when different amounts of the SPI were added to the extract obtained from a heat-processed meat product. [k] Recovery of soybean proteins when different amounts of the SPI were initially added to heat-processed meat products.

soybean proteins detected by this method was 0.20 mg/mL (calculated from the calibration plot as the concentration corresponding to a signal equal to the intercept plus 3 times the standard error) which means that the method enabled the detection of an addition of 0.07% (w/w) soybean proteins in a meat product (related to 1 g of initial product). The lowest concentration of soybean proteins that could be reliably determined by the proposed method was 0.68 mg/mL (calculated as the concentration corresponding to a signal equal to the intercept plus 10 times the standard error of the calibration plot) and corresponded to an addition of 0.25% (w/w) soybean proteins in a meat product (related to 1 g of initial product). Moreover, the slopes of the calibration plots obtained by the external and standard additions calibration methods were compared in order to detect the existence of matrix interferences. The comparison of these slopes by *t*- and *F*-tests ($P < 0.05$) (*17*) suggested the proposed method did not suffer from matrix interferences. For control of the specificity of the analytical method, 10 processed meat products were used. For each sample, one standard addition covering the range from 2.00 to 4.00 mg/mL was made. The specificity was verified by adjusting a straight line between added and recovered concentrations of soybean proteins in these samples. The specificity of the method was considered acceptable, since the slope and the intercept did not significantly differ from the unit and zero (*t*-test, $P < 0.05$), respectively.

The precision of the method was determined by the evaluation of repeatability, intermediate precision, and internal reproducibility (**Table 2**). The repeatability (expressed as the relative standard deviation (RSD, %)) in 10 consecutive injections of a solution obtained from a heat-processed meat product was better than 4.0% in peak area and retention time. Intermediate precision was determined by injecting one meat solution and one standard solution in 7 days during a period of 12 days, and a RSD better than 5% in peak area and retention time for both samples was observed. In the case of the meat solution, the variability in the soybean protein content determined seven times in a 12-day period was 10.76%. Another parameter evaluated was the slope reproducibility, yielding a RSD lower than 1% in 12 days. Internal reproducibility was determined by the injection of four individual solutions of a heat-processed meat product and was better than 5% in peak area and retention time.

The robustness of the method was evaluated by the deliberated and systematic variation of three method parameters: column lot, buffer concentration (30 and 50 mM Tris−HCl buffer), and detection wavelength (254 and 280 nm). Since the soybean protein contents determined for one processed meat product when varying these parameters did not differ significantly (*F*- and *t*-tests ($P < 0.05$)) from the values observed with the optimized conditions, the method was supposed to be robust enough for its utilization as a routine method for the quality control of meat products.

The accuracy of the method was determined in two different ways: by spiking meat extracts or the meat product directly with known amounts of the SPI (absolute recovery) or by comparing the soybean protein content determined by the HPLC method with that obtained by the official method of analysis (*10*). The recoveries of soybean proteins when extracts obtained from a heat-processed meat product containing soybean proteins were spiked at different levels with the SPI ranged from 97 to

Table 3. Soybean Protein Content Determined in Five Meat Products by the Official ELISA Method and the Proposed HPLC Method

meat product	protein concn[a] (mg/100 mg of sample)	
	ELISA[b]	perfusion HPLC[c]
meat product A[d]	1.04	1.10 (0.20)[e]
meat product B[d]	1.67	1.67 (0.22)[e]
model meat product 2[f]	1.44	1.15
meat product C[d]	1.80	1.42 (0.06)[e]
meat product D[d]	0.85	0.96 (0.11)[e]

[a] Results expressed as is basis. [b] Determined following the official AOAC method 998.10.[10] [c] Determined by the external standard calibration method following the proposed method. [d] Heat-processed meat products. [e] Mean of two individual determinations. Standard deviation given in parentheses. [f] Raw meat product. One determination.

Table 4. Soybean Protein Content Determined in Different Commercial Heat-Processed Meat Products by the Perfusion HPLC Method

processed meat product	protein concn[a] (mg/100 mg of sample)
A	1.10
B	1.67
C	1.42
D	0.96
E	1.60
F	1.46
G	1.71
H	1.67
I	1.33
J	1.85
K	1.30
L	1.73
M	1.14

[a] Results expressed as is basis.

99% with the exception of the smallest addition in which case a recovery of 91.6% was observed. These recoveries were compared with those obtained with a meat product (model meat product 2) not submitted to any heat treatment, with similar recoveries (92−95%) being observed. These results suggested that the reliability of the proposed analytical method seemed not to be affected by the heating process to which the meat product was submitted. Absolute recovery, obtained when one heat-processed meat product not containing soybean proteins was initially spiked with the SPI, was also close to 100%. For the comparison of the proposed method with the official ELISA method (*10*), four commercial heat-processed meat products (meat products A−D) and one raw meat product (model meat product 2) were analyzed by both methods, and the results obtained are shown in **Table 3**. No statistical difference between the contents determined by both methods were detected when applying a paired *t*-test ($P < 0.05$).

Application to Edible Samples. The optimized method has proven successful in the determination of soybean proteins in heat-processed meat products, which is useful for controlling these samples' full legal limitations and also to prevent potential frauds. Thus, the method described has been applied to the determination of the soybean protein content in 30 heat-processed meat products commercially available in spanish markets by injecting extracts obtained from these samples into the chromatographic system. The concentration of soybean proteins was calculated by interpolating in calibration plots obtained by the external standard method using the SPI Supro 500E as the standard of soybean proteins. As observed in **Table 4**, these contents were below the maximum allowance by the spanish regulations, 3% soybean proteins in the meat product as is basis (*6*).

A final comment from the results obtained can also be extracted. One difficulty found when developing a method of these characteristics is the election of a suitable standard. Unfortunately, there is not any certified reference material for the determination of soybean proteins in meat products and all optimization and validation results shown in this work have been obtained when using a commercial SPI (Supro 500E from Anvisa, Madrid, Spain) as the soybean protein standard. As stated in the Experimental Procedures, this SPI had been employed in the elaboration of some of the meat products used in this work but the rest of the commercial meat products studied were prepared with an unknown SPI. The results obtained in this work indicated no difference between the contents obtained for processed meat products prepared with the SPI Supro 500E and the others, concluding that the method enabled the determination of the soybean protein content even when the SPI used in the elaboration of the meat product was unknown. This supposed an additional advantage of the proposed method regarding the official ELISA method in which case the relative responses of the immunoassay varied depending on the soybean protein source used (*9*, *12*).

Conclusions. An alternative to the official ELISA method for the determination of soybean proteins in commercial heat-processed meat products has been developed. The proposed method involved the preparation of an extract from the processed meat product and its injection in a chromatographic system. In comparison with the AOAC method, this method not only enabled a significant reduction in analysis time and price but also the complexity of the process itself was reduced. The proposed method has been validated following a standardized procedure for food chemistry laboratories. The method enabled the detection of up to 0.07% (w/w) soybean proteins and the quantitation of up to 0.25% (w/w) soybean proteins in meat products, which is more sensitive than the official method. Method specificity has been successfully proven. The precision of the method was also evaluated, with reproducible results being observed when varying analysis time and sample solution. The results obtained when varying different parameters of the method (column lot, buffer concentration, and detection wavelength) enabled us to ensure the robustness of the method. The trueness of the method was verified when spiking meat products, with recoveries close to 100% being observed. When comparing the soybean protein content in different meat products obtained by the proposed and official methods, no significant differences were found. All these results suggested that the proposed method provided a convenient alternative for the quantitative estimation of soybean proteins in food products.

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