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Detection and quantitation of additions of soybean proteins in cured-meat products by perfusion reversed-phase high-performance liquid chromatography

Perfusion liquid chromatography has been applied in this work to the determination of soybean proteins in commercially available cured meat products, enabling the detection of additions of soybean proteins in cured meat products to which the addition of these vegetable proteins is forbidden and the quantitation of soybean proteins in cured meat products to which the addition of these proteins is allowed up to a certain limit. The analytical methodology is based on a sample treatment (fat extraction and soybean protein solubilization) prior to chromatographic analysis. Fat extraction with acetone and soybean protein solubilization with a buffer solution at basic pH (pH 10 or 9) were necessary to obtain selective and sensitive conditions. Use of water-acetonitrile-trifluoroacetic acid or water-tetrahydrofuran-trifluoroacetic acid linear binary gradients at a flow rate of 3 mL/min, a temperature of 50°C, and UV detection at 280 nm enabled chromatographic analysis of soybean proteins in cured meat products in less than 3 min.

Key Words: Soybean proteins; Cured-meat products; Perfusion reversed-phase high-performance liquid chromatography

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1 Introduction

The use of soybean proteins as meat extenders has significantly increased in recent years due to the interesting functional and nutritional properties of these vegetable proteins together with their low cost [1–4]. In fact, soybean proteins help to improve technological processes used in the manufacture of meat products, reduce their formulation cost, and can also be used as fat replacers enabling the manufacture of meat products with low fat content. However, maximum levels at which these proteins can be added to meat products are established by regulations which also control the accurate labelling of these products. Thus, the addition of soybean proteins is forbidden or allowed only up to a certain extent depending on the kind of meat product [5]. In the case of cured-meat products, the maximum allowance ranges from 1 to 5% (*w/w*) (relative to dry product) of non-meat proteins (milk proteins and/or vegetable proteins) depending on the quality of the product. In addition to this, the European Commission has amended the European Food Labelling Directive 2000/13/EC (Directive 2003/89/EC) to ensure that consumers are informed of the complete composition of foodstuffs [6]. Consequently, analytical methods for the detection of additions of soybean proteins in meat products are urgently required. However, the detection of soybean proteins in meat products is not an easy task since these food systems present a complex formulation. The main difficulties found in the determination of these vegetable proteins in meat products are related to the composition and the processing of meat products. In fact, the kind of meat used, its quality, the soybean protein source added, the presence of other non-meat proteins, or the processing conditions employed during the production of meat products are some of the main problems that can make the analysis of soybean proteins in these products very difficult. Despite this, different attempts have been made to determine these proteins in meat products. Most of the proposed methods, being based on electrophoretic (SDS-PAGE) [7] or immunochemical techniques [7–9], are generally tedious and time consuming. Moreover, interferences from other meat components made the measurement of soybean bands in electrophoretic methods quite difficult while the preparation of a suitable antibody against soybean proteins was the main limitation in immunochemical methods due to the different technological processing steps to which meat products are submitted. In spite of these drawbacks and due to the absence of more appropriate methodologies, the AOAC

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official method based on an Enzyme-Linked Immunosorbent Assay (ELISA) is nowadays the method most frequently employed [9]. Chromatographic methods have also been applied to the detection of soybean proteins in meat products [7, 10–12]. However, the methods proposed in the literature were used with fresh meats and no application was found to processed meat products. To this end, our research group has developed a perfusion high performance liquid chromatography (HPLC) method for the determination of soybean proteins in heat-processed meat products [13]. The use of perfusive stationary phases in HPLC permitted the chromatographic analysis of soybean proteins in these meat products in analysis times shorter than 3 min, providing a simple and inexpensive alternative to the ELISA method. While the products studied in our previous research work are submitted to thermal treatment during their production, there is also another kind of processed meat product which is not submitted to thermal treatment but to a ripening process (cured meat products). In addition to the complexity of these products, the ripening process may substantially alter the proteins present in the food system [14–17], making their determination even more difficult. Depending on the type of cured meat product, the addition of soybean proteins is forbidden or allowed up to a certain legal limit depending on the type of product.

The main goal of this work was to develop an analytical perfusion HPLC methodology enabling the detection of soybean proteins in cured meat products to which the addition of soybean proteins is forbidden and the quantitation of soybean proteins in cured meat products to which the addition of soybean proteins is allowed up to a certain limit. Because the elution of meat components from the chromatographic columns can be difficult, a comparative study of two organic modifiers in the mobile phase with different elution strength (acetonitrile and tetrahydrofuran) has been carried out in this work.

2 Materials and methods

2.1 Chemicals and samples

Acetonitrile (ACN) (Merck, Germany), tetrahydrofuran (THF) (Scharlab, Barcelona, Spain), trifluoroacetic acid 99.5 atom% (TFA) (Sigma, St. Louis, MO, USA), and HPLC grade water (Milli-Q system, Millipore, Bedford, MA, USA) were used for the preparation of the mobile phases.

Sodium bicarbonate, tris-(hydroxymethyl)aminomethane (Tris), 2-mercaptoethanol, and urea (all of them from Merck, Darmstadt, Germany), dithiothreitol (ICN, Aurora, OH, USA), and sodium dodecylsulfate (SDS) (Fluka, Barcelona, Spain) were used for the solubilization of soybean proteins. Acetone (Merck, Darmstadt, Germany) was used to remove the fat of the meat products. Hydrochloric acid or sodium hydroxide (both from Panreac, Barcelona, Spain) were used to adjust the buffer pH.

A soybean protein isolate (SPI) with a protein content of 89.07% (standard deviation, $s = 0.17$, $n = 7$) was obtained from ICN (Aurora, OH, USA).

Three different types of cured meat products were used in this work: (i) a cured meat spread elaborated with pork meat and pork fat, paprika, salt, sugar, species, and additives, (ii) a dry-fermented sausage (Spanish *chorizo*) containing soybean proteins (pork meat, bacon, paprika, salt, lactose, dextrin, sugar, garlic, soybean proteins, and additives), and (iii) a high-quality dry-fermented sausage (Spanish *chorizo*) without soybean proteins (pork meat, salt, species, and additives). These cured meat products were subjected to a technological manufacturing process consisting of mixing of meat, fat, and additives, homogenization, stuffing into the casing, fermentation, and drying (17–30 days at 10 to 25°C) [18]. All meat products were purchased at local markets at Alcalá de Henares (Madrid, Spain).

Determination of the content of soybean proteins in the dry-fermented sausage (Spanish *chorizo*) by the ELISA method [9] was performed at the Laboratorio Arbitral of the Ministerio de Agricultura, Pesca y Alimentación (Spain).

2.2 Preparation of samples, standards, and solutions

Cured meat products were defatted before soybean protein solubilization. Fat extraction from 10 g of previously ground meat product was achieved by homogenization with acetone in an Ultraturrax mixer, mechanical stirring (13000 rpm) for 20 min, and centrifugation (3362 *g* for 60 min at 25°C). Fat extraction from cured meat products (10 g) was achieved with a 50 mL volume of acetone (one extraction) or three consecutive extractions with 25 mL of acetone, depending on the product. The resultant pellet was finally dried overnight at 60°C to give a defatted and dried residue.

Soybean protein solubilization was performed using a buffer at basic pH (0.05 M $NaHCO_3$ at pH 10.0 for the cured meat spread and 0.05 M Tris-HCl at pH 9.0 for the dry-fermented sausages) by homogenizing about 0.8 g of the defatted and dried residue obtained from the cured meat product in 25 mL of the buffer (single operation) or 10 mL of the buffer (three consecutive operations), stirring for 10 min at 50 °C, and sonicating for 5 min. After centrifugation (3362 *g* for 45 min at 25°C) the supernatants obtained were filtered (Whatman No. 1) prior to injection in the chromatographic system.

Preparation of SPI solutions was performed by weighing the required amount of SPI, dissolving it in the buffer solution, sonicating for 5 min, and centrifuging (3362 *g* for

45 min at 25°C). The resultant supernatant was directly injected in the chromatographic system.

Mobile phases used for the chromatographic separation contained 0.05 or 0.1% (*v/v*) TFA in Milli-Q water (mobile phase A) and in ACN or THF (mobile phase B). Mobile phase B was filtered in a solvent filtration system through a 0.45 μm membrane filter of PVDF.

2.3 Instrumentation

A Hewlett-Packard liquid chromatograph (Hewlett-Packard, Pittsburgh, PA, USA) equipped with a UV detector, an injection system (both of the series 1050), a degassing system, a quaternary pump, and a thermostated compartment for the column (all of the series 1100) were employed. Control and data acquisition were accomplished with HP-Chemstation software. A Hewlett-Packard 1100 Series liquid chromatograph (Hewlett-Packard, Pittsburgh, PA, USA) equipped with a diode array detector was used in order to obtain UV spectra of peaks. The injection volume was 20 μL and detection was carried out at 280 nm. The separation was accomplished with a reversed-phase perfusion column POROS R2/H (50 × 4.6 mm ID) from Perseptive Biosystems (Framingham, MA, USA), packed with polystyrene divinylbenzene beads (10 μm particle diameter).

The chromatographic separations were achieved by perfusion RP-HPLC using the following linear binary gradients (i) 5–25% B in 0.8 min, 25–40% B in 0.8 min, 40–50% B in 1.1 min, and 50–5% B in 0.5 min where the composition of the mobile phases was 0.05% (*v/v*) TFA in water (mobile phase A) and 0.05% (*v/v*) TFA in ACN (mobile phase B), and (ii) 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 where the mobile phase A contained 0.05% (*v/v*) TFA in water and the mobile phase B contained 0.05% (*v/v*) TFA in THF. A flow rate of 3 mL/min, a temperature of 50°C, and UV detection at 280 nm were employed.

2.4 Calibration

Calibration was performed by the external standard and by the standard additions methods to investigate the existence of matrix interferences. Calibration by the external standard method was performed by injecting SPI solutions over the range 0.1–2.2 mg/mL. Calibration by the standard additions method was performed by adding known and increasing amounts of SPI to a cured meat product (dry-fermented sausage).

Peak integration was performed by setting the baseline from valley to valley.

2.5 Data treatment

Calibration curves obtained by least-squares regression analysis were validated as linear models by ANOVA ($\alpha = 5\%$). Limits of detection (LOD, 3 S_a/b) and quantitation (LOQ, 10 S_a/b) were determined from the standard error of the intercept (S_a) and the slope (b) of the calibration lines [19]. All the statistical analyses were carried out using Statgraphics Plus 5.0 (Statistical Graphics Corp.).

Experimental data were treated using Excel 7.0 (Microsoft) and graphs were plotted with Origin 7.0 (OriginLab Corporation) software.

3 Results and discussion

In order to develop an analytical method to determine soybean proteins in cured meat products, three different meat products were employed: two meat products to which the addition of soybean proteins is forbidden (a cured meat spread and a high quality dry-fermented sausage) and a meat product to which the addition of soybean proteins is allowed to a certain limit (1%) (a dry-fermented sausage). In all cases, the analysis of the meat products involved the following steps: (i) fat extraction from the meat product; (ii) solubilization of soybean proteins from the defatted and dried product; and (iii) chromatographic analysis. Fat extraction was achieved with acetone because this solvent was shown to have advantages over several other solvents investigated for extracting the fat from heat-processed meat products [13]. Using this solvent, the number of extractions needed to remove the high fat content of the products as well as the highly UV-absorbent components (i.e. paprika) [20] present in the cured meat products was studied. For that purpose, the fat of one cured meat spread and one dry-fermented sausage was extracted with acetone in a single step, in two successive steps, and in three successive steps. Three successive acetone extractions of the ground samples were needed to obtain a defatted, dry, and manageable powder from the cured meat spread due to its high fat and paprika contents. However, in the case of the dry-fermented sausages one extraction was enough to remove the fat. For soybean protein solubilization, different buffers at basic pHs with and without added denaturing or reducing agents were investigated in order to find the most adequate conditions for the solubilization of soybean proteins from cured meat products. Water as well as 0.05 M Tris-HCl buffer at pH 8.0 or 9.0 and 0.05 M $NaHCO_3$ buffer at pH 10.0 or 11.0 were used.

3.1 Detection and quantitation of soybean proteins in cured meat products using acetonitrile as organic modifier in the mobile phase

Initially, the chromatographic conditions previously optimized for the analysis of heat-processed meat products [13] were employed. These conditions consisted of a water-acetonitrile linear binary gradient from 5 to 25% B in

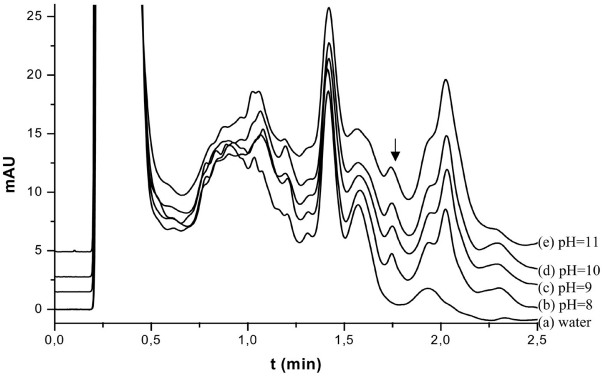

Figure 1. Chromatogram corresponding to 46 mg/mL of a cured-meat product to spread (≈1.5 mg/mL of the defatted and dried meat product) spiked with a 0.4% (*w/w*) of SPI referred to initial product (10 g of the meat product was spiked with 40 mg of soybean proteins). The peak chosen to detect soybean proteins is indicated by an arrow. The media employed for soybean protein solubilization were the following: (a) water, (b) 0.05 M Tris-HCl buffer at pH 8.0 (c) 0.05 M Tris-HCl buffer at pH 9.0, (d) 0.05 M $NaHCO_3$ buffer at pH 10.0, and (e) 0.05 M $NaHCO_3$ buffer at pH 11.0. 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, and 42–50% B in 0.6 min, followed by 50–5% B in 0.5 min. Mobile phases: A, water with 0.05% TFA and B, ACN with 0.05% TFA.

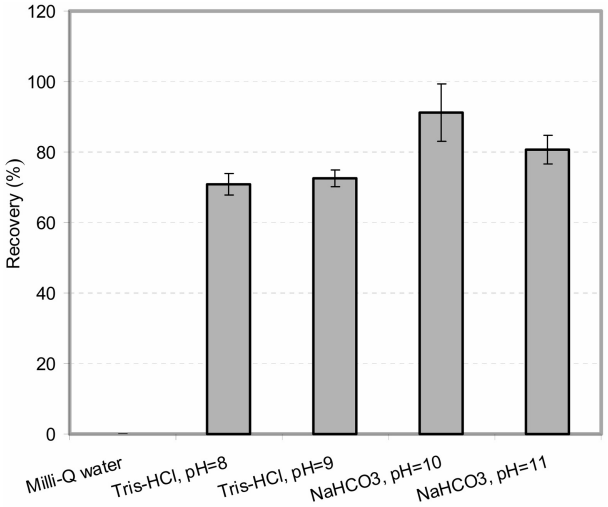

Figure 2. Recovery (%) of soybean proteins from a solution of 46 mg/mL of a cured-meat spread (≈1.5 mg/mL of the defatted and dried meat product) spiked with a 0.4% (*w/w*) of SPI referred to initial product (10 g of meat product was spiked with 40 mg of SPI), using: water, 0.05 M Tris-HCl buffer at pH 8.0 or 9.0, and 0.05 M $NaHCO_3$ buffer at pH 10.0 or 11.0 for soybean protein solubilization. Chromatographic conditions as in Figure 1.

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 reversed gradient from 50 to 5% B in 0.5 min to return to the initial conditions at a flow rate of 3 mL/min, 50°C as working temperature, and UV detection at 280 nm (mobile phase A: 0.05% TFA in water, mobile phase B: 0.05% TFA in acetonitrile). The chromatograms obtained under these conditions for a cured meat spread spiked with SPI when water and buffer solutions at four different pHs were used to solubilize soybean proteins are shown in **Figure 1**. There was a chromatographic peak at 1.7 min which had the same retention time as one of the peaks corresponding to SPI and that did not appear in the chromatogram obtained for the same cured product without added SPI. In addition, the UV spectra and first and second derivatives obtained for this peak in the SPI and the cured meat product spiked with SPI were identical. This peak (1.7 min) was chosen to calculate the recovery obtained for soybean proteins when using the above-mentioned solutions as solubilizing media for these proteins. This recovery was calculated by dividing the peak area measured for the selected chromatographic peak in the chromatogram obtained for the cured meat product by the peak area measured for the same peak in the chromatogram obtained for SPI (under the same experimental conditions and at the same concentration as in the meat product), and expressed as a percentage, that is, $A_{product}/A_{SPI} \times 100$. Results (average of three determinations) indicate that the use of a 0.05 M $NaHCO_3$ buffer at pH 10.0 gave the highest recovery for soybean proteins (≈91%) (see **Figure 2**) from the cured meat spread. Then, this buffer solution was selected to investigate the effect of the addition of denaturing or reducing agents on the recovery of soybean proteins. However, the addition of these agents led to recoveries higher than 100%, suggesting that solubilization of meat components occurs together with that of the soybean proteins. As a consequence, a 0.05 M $NaHCO_3$ buffer at pH 10.0 was employed and successive extractions were performed to increase the solubilization of soybean proteins. The highest recovery (≈96%) was obtained when three consecutive extractions were performed. However, in the case of dry-fermented sausages, only one step was necessary for solubilization of soybean proteins and the highest recoveries together with the best selectivity (peak purity) were obtained for a 0.05 M Tris-HCl buffer at pH 9.0.

Although these initial chromatographic conditions enabled optimization of the solubilization media for soybean proteins, as observed in Figure 1 there was a partial overlapping of the peak of interest (1.7 min) and peaks corresponding to meat components. For this reason, the chromatographic separation conditions used to analyze cured meat products were further optimized in order to increase the resolution. Thus, six different gradients with different slopes were tested. From the results obtained on using these gradients, a gradient slightly longer and with lower slope than the initial gradient was chosen: from 5 to 25%

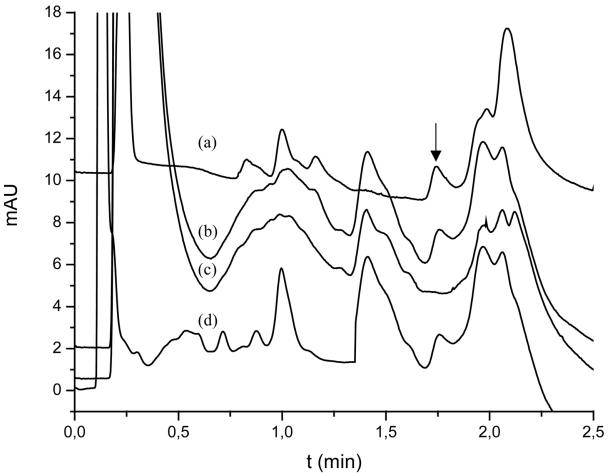

Figure 3. Chromatograms corresponding to: (a) a solution of 1.5 mg/mL of SPI, (b) a solution of 46 mg/mL of a cured meat product to spread (≈1.5 mg/mL of the defatted and dried meat product) spiked with a 0.34% (*w/w*) of SPI (10 g of meat product was spiked with 34 mg of SPI), (c) a solution of 46 mg/mL of a cured meat product to spread (≈1.5 mg/mL of the defatted and dried meat product) without soybean proteins, and (d) a solution of 46 mg/mL of a cured meat product (dry-fermented sausage) (≈0.8 mg/mL of the defatted and dried meat product) with soybean proteins in its composition. Chromatographic conditions: temperature, 50°C; flow rate, 3 mL/min; gradient: 5–25% B in 0.8 min, 25–40% B in 0.8 min, and 40–50% B in 1.1 min, followed with 50–5% B in 0.5 min. Mobile phases: A, water with 0.05% TFA and B, ACN with 0.05% TFA.

B in 0.8 min, from 25 to 40% B in 0.8 min, and from 40 to 50% B in 1.1 min followed by a reversed gradient from 50 to 5% B in 0.5 min to return to the initial conditions (mobile phase A: 0.05% TFA in water, mobile phase B: 0.05% TFA in acetonitrile). In addition, a study of the influence of the percentage of TFA in the mobile phase on the separation of soybean proteins and meat components revealed that a 0.05% concentration of TFA was the most appropriate value of this parameter. **Figure 3** shows the chromatograms corresponding to SPI, the cured meat spread, this same product spiked with SPI, and a dry-fermented sausage produced with SPI and then subjected to the curing process. It is shown that the peak chosen to determine soybean proteins (peak at 1.7 min) permitted the detection of soybean proteins in cured meat products to which the addition of soybean proteins is forbidden even after they have been subjected to the production process (case of the dry-fermented sausage).

Once we had demonstrated the applicability of the developed method to detect potential adulterations of cured meat products by fraudulent addition of soybean proteins to meat products to which the addition of soybean proteins is forbidden, the characteristics of the method for quantitative analysis of soybean proteins in cured products were studied. The quantitation of soybean proteins in cured meat products would enable the determination of the level of adulteration in cured meat products in which the addition of soybean proteins is forbidden or the quality control of those products to which the addition of soybean proteins is allowed up to a certain limit.

Linearity, limits of detection and quantitation, precision, and accuracy were determined. **Table 1** shows the results obtained for a dry-fermented sausage. The linear working concentration range used was from 0.2 to 2.2 mg/mL of soybean proteins, with a linear relationship between signal and soybean protein concentration being observed up to 6 mg/mL. The limit of detection (calculated as the concentration corresponding to a signal equal to the intercept plus three times the standard error of the calibration plot) was 0.03 mg/mL of soybean proteins, which means that it is possible to detect up to 0.04 g of soybean protein per 100 g of product. The limit of quantitation (calculated as the concentration corresponding to a signal equal to the intercept plus ten times the standard error of the calibration plot) was 0.10 mg/mL of soybean proteins, that is, 0.13 g of soybean protein per 100 g of product may be quantified.

In order to investigate the existence of matrix interferences, the slope of the calibration lines obtained by the external standard method and the standard additions method were compared (t-test). Results obtained for the comparison of the slopes revealed that the developed method did not suffer from matrix interferences.

The precision of the method was evaluated by determining the repeatability, reproducibility, and intermediate precision (see results in Table 1). Repeatability was calculated by injecting ten consecutive times a solution of ≈2.8 mg/mL of the defatted and dried residue obtained from the dry-fermented sausage studied, obtaining a RSD value for the retention time of 0.1% and for peak area of 3.4%. Reproducibility was determined by injecting six solutions of ≈1.5 mg/mL of the defatted and dried residue obtained from the dry-fermented sausage on different days. The RSD value for retention time was 0.4% and for peak area was 10%. Intermediate precision was determined as the RSD of the slopes of the straight lines obtained by the external standard method on four different days. Good intermediate precision was obtained (RSD values about 1.6%).

Finally, the accuracy of the method was evaluated in two ways: (i) by spiking the dry-fermented sausage with known amounts of SPI (absolute recovery) or (ii) by comparison of the soybean content determined by this method with the content obtained by the ELISA AOAC official method [9]. The absolute recovery of soybean proteins when the ground cured meat product was spiked with increasing amounts of SPI ranging from 95 to 99% (see

Table 1. Characteristics of the perfusion RP-HPLC method using ACN as organic modifier for the analysis of soybean proteins in cured-meat products containing soybean proteins.

Linearity[a)]	Up to 6 mg/mL
Detection limit[a)]	0.03 mg/mL (0.04% (*w/w*))[b)]
Quantitation limit[a)]	0.10 mg/mL (0.13% (*w/w*))[b)]
Existence of matrix interferences[c)]	
– Slope by the external standard method	3.65 ± 0.24 ($n = 5$)
– Slope by the standard additions method	3.41 ± 0.11 ($n = 5$)
Precision	
Repeatability (RSD,%) ($n = 10$)[d)]	
– Retention time	0.1
– Peak area	3.4
Internal reproducibility (RSD,%) ($n = 6$)[e)]	
– Retention time	0.4
– Peak area	10.0
Intermediate precision (RSD,%)[f)]	
– Slope (external standard method, $n = 5$)	6.6
Accuracy	
– Absolute recovery[g)]	97 ± 2

a) Linearity and detection and quantitation limits relative to mg/mL of soybean proteins.
b) Limits of detection and quantitation expressed as *w/w* units were determined related to 1 g of sample.
c) An F-test for the comparison of variances and an t-test for the comparison of slopes were employed.
d) Number of injections of a solution of 2.8 mg/mL of a defatted and dried cured-meat product.
e) Analysis of six solutions of 1.5 mg/mL of a defatted and dried cured-meat product on different days.
f) Analysis performed on six different days.
g) Calculated from the soybean protein content predicted by the HPLC method and the theoretical content considering the SPI added (from 0.14 to 0.23 mg/mL) and the soybean proteins contained in the product (0.79% (*w/w*) relative to dried product).

Table 1). On the other hand, the soybean protein content determined by the HPLC method in the dry-fermented sausage containing soybean proteins (whose chromatogram appears in Figure 3.d) was 0.79 ± 0.03% (*w/w*) relative to the dried product (initial product corrected with its moisture). This soybean protein content was in good agreement with the content determined by the ELISA AOAC official method [9]: 0.74% (*w/w*) relative to the dried product. In addition, these values were within the limits authorized by law for this product, a 1% (*w/w*) of non-meat proteins (milk proteins and/or vegetal proteins) relative to dried product.

From the results obtained in this work, it can be stated that the analytical method developed is reliable for detecting and quantifying soybean proteins in cured meat products.

The only drawback of the developed method was that periodic washing of the column was necessary in order to elute meat components retained on the stationary phase when using a mobile phase containing acetonitrile as organic modifier. For this reason, a different organic modifier with a higher elution strength such as tetrahydrofuran has also been investigated in this work to achieve the determination of soybean proteins in cured meat products.

3.2 Detection and quantitation of soybean proteins in cured meat products using tetrahydrofuran as organic modifier in the mobile phase

Mobile phases consisting of tetrahydrofuran-water-0.05% TFA were also employed in this work. In order to optimize the elution gradient, different linear binary gradients with different gradient slopes (% of mobile phase B/min) and different gradient times were tested. From these experiments, a linear binary gradient from 5 to 25% B in 0.8 min, 25 to 42% B in 0.8 min, and 42 to 50% B in 0.6 min followed by a reversed gradient from 50 to 5% B in 0.5 min to return to the initial conditions was chosen (mobile phase A: 0.05% TFA in water, mobile phase B: 0.05% TFA in tetrahydrofuran). A flow rate of 3 mL/min, a working temperature of 50°C, and UV detection at 280 nm were used as before.

Table 2. Characteristics of the perfusion RP-HPLC method using THF as organic modifier for the analysis of soybean proteins in cured-meat products containing soybean proteins.

Linearity[a)]	Up to 6 mg/mL
Detection limit[a)]	0.05 mg/mL (0.06% (*w/w*))[b)]
Quantitation limit[a)]	0.17 mg/mL (0.22% (*w/w*))[b)]
Existence of matrix interferences[c)]	
– Slope by the external standard method	7.01 ± 0.16 (n = 5)
– Slope by the standard additions method	7.63 ± 1.10 (n = 3)
Precision	
Repeatability (RSD,%) (n = 10)[d)]	
– Retention time	0.1
– Peak area	1.5
Internal reproducibility (RSD,%) (n = 4)[e)]	
– Retention time	0.9
– Peak area	3.3
Intermediate precision (RSD,%)[f)]	
– Slope (external standard method, n = 5)	2.3
Accuracy	
– Absolute recovery[g)]	99 ± 3

a) Linearity and detection and quantitation limits relative to mg/mL of soybean proteins.
b) Limits of detection and quantitation expressed as *w/w* units were determined relative to 1 g of sample.
c) An F-test for the comparison of variances and an t-test for the comparison of slopes were employed.
d) Number of injections of a solution of 0.8 mg/mL of a defatted and dried cured-meat product.
e) Analysis of six solutions of 2.3 mg/mL of a defatted and dried cured-meat product on different days.
f) Analysis performed on five different days.
g) Calculated from the soybean protein content predicted by the HPLC method and the theoretical content considering the SPI added (from 0.14 to 0.22 mg/mL) and the soybean proteins contained in the product (0.56% (*w/w*) relative to dried product).

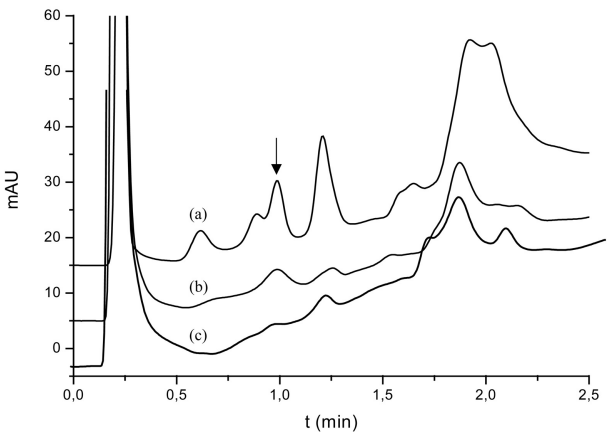

Figure 4. Chromatograms corresponding to: (a) a solution of 6 mg/mL of SPI, (b) a solution of 46 mg/mL (≈0.8 mg/mL of the defatted and dried meat product) of a dry-fermented sausage with soybean proteins in its composition, and (c) a solution of 46 mg/mL (≈0.8 mg/mL of the defatted and dried meat product) of a dry-fermented sausage without soybean proteins in its composition. Chromatographic conditions: temperature, 50°C; flow rate, 3 L/min; gradient: 5–25% B in 0.8 min, 25–42% B in 0.8 min, and 42–50% B in 0.6 min, followed by 50–5% B in 0.5 min. Mobile phases: A, water with 0.05% TFA and B, THF with 0.05% TFA.

Figure 4 shows the chromatograms obtained for the SPI and for the dry-fermented sausages with and without soybean proteins when solubilization of the proteins was performed with a 0.05 M Tris-HCl buffer at pH 9.0. It can be observed that there is a chromatographic peak corresponding to SPI eluting at 1.0 min which appears in the chromatogram corresponding to the dry-fermented sausage containing soybean proteins but does not appear in the dry-fermented sausage without soybean proteins (a high quality dry-fermented sausage to which the addition of soybean proteins is forbidden). In addition, the UV spectra and the first and second derivatives obtained for this peak in the chromatogram of SPI and in that of the dry-fermented sausage containing soybean proteins were very similar, indicating that this peak could be used for the detection and quantitation of soybean proteins in cured meat products when employing tetrahydrofuran as organic modifier in the mobile phase.

In order to show the possibilities of the developed method for quantitation of soybean proteins in cured meat products, the analytical characteristics of the method were studied. **Table 2** shows the linearity, limits of detection and quantitation, precision, and accuracy determined for

the dry-fermented sausage containing soybean proteins under the above chromatographic conditions using tetrahydrofuran as organic modifier in the mobile phase. A linear relationship was observed between the signal and the soybean protein concentration up to 6 mg/mL, with the working concentration range lying between 0.1 and 2.2 mg/mL of soybean proteins. The method enabled us to detect up to 0.05 mg/mL of soybean proteins (0.06 g of soybean proteins per 100 g of cured meat product) and to quantitate up to 0.17 mg/mL of soybean proteins (0.22 g of soybean proteins per 100 g of cured meat product).

Comparison of the values of the slope of the calibration lines obtained by the external standard method and the standard additions method (t-test) for the dry-fermented sausage revealed the absence of matrix interferences.

As it can be seen in Table 2, the precision of the method was evaluated by determining the repeatability, reproducibility, and intermediate precision. Repeatability was calculated by injecting ten consecutive times a solution of ≈0.8 mg/mL of the defatted and dried residue obtained from the dry-fermented sausage, giving a RSD value of 0.1% for retention time and of 1.5% for peak area. Internal reproducibility was determined by injecting four solutions of ≈2.3 mg/mL of the defatted and dried residue obtained from the dry-fermented sausage on different days. The RSD value was 0.9% for retention time and 3.3% for peak area. Intermediate precision determined as the RSD of the slopes of the straight lines obtained by the external standard method on five different days was 2.3%.

The accuracy of the method was calculated by determining the absolute recovery obtained for soybean proteins when spiking the dry-fermented sausage with known amounts of SPI. This recovery ranged from 96 to 102% (see Table 2). In addition, the soybean protein content determined in the dry-fermented sausage (whose chromatogram appears in Figure 4.b) was 0.56 ± 0.04% (*w/w*) relative to the dried product (initial product corrected by its moisture) which can be considered close to the content determined by the ELISA method for a different batch of the same product (0.74% (*w/w*) relative to the dried product). Again the soybean protein content was within the limit authorized by law for this product, i.e. 1% (*w/w*) of non-meat proteins based on dried product. Therefore, this alternative analytical method is also able to determine the amount of soybean proteins contained in cured meat products such as the dry-fermented sausage studied. Although this analytical methodology gave satisfactory results favouring the elution of proteins from the stationary phase and avoiding the need for periodic washing, the use of tetrahydrofuran as organic modifier decreased the lifetime of the chromatographic columns due to its higher elution strength, which can be considered the only drawback of this methodology compared to the use of acetonitrile as organic modifier in the mobile phase.

4 Concluding remarks

An initial approach enabling the detection and quantitation by perfusion HPLC of soybean proteins in commercial cured meat products where the addition of these vegetable proteins is forbidden or allowed up to a certain limit has been achieved in this work. The analytical methodology developed (fat extraction with acetone and protein solubilization with a buffer at basic pH before the chromatographic analysis) has enabled the detection of soybean proteins not only in spiked cured meat products (a cured meat spread to which the addition of soybean proteins is forbidden), but also in products containing soybean proteins, a dry-fermented sausage (Spanish *chorizo*) where the vegetable proteins were added before the ripening process showing that the technological processing of the meat product did not affect the analytical methodology. Water-acetonitrile-trifluoroacetic acid or water-tetrahydrofuran-trifluoroacetic acid linear binary gradients at a flow rate of 3 mL/min, a temperature of 50°C, and UV detection at 280 nm could be used in order to achieve the chromatographic analysis of soybean proteins with reversed-phase perfusive stationary phases in less than 3 min. The use of tetrahydrofuran as organic modifier in the mobile phase avoided the need for a periodic washing of the column due to the difficult elution from the stationary phase of some meat components although it decreased the lifetime of the column due to its higher elution strength. Good performance for the developed analytical methods was observed, permitting the quantitation of soybean proteins in a cured meat product which proved to have a soybean content below the legal limit for this product (1% (*w/w*) relative to dry product of non-meat proteins (milk proteins and/or vegetable proteins)).

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