Summary

The addition of soybean proteins to raw and processed meat products is a common practice. In the case of raw meats, soybean proteins can be mixed with minced meats or pump injected or massaged (tumble technology) into large pieces of muscle tissue. The reasons for this addition are generally related with the improvement of meat characteristics, the reduction of meat cost or the reduction of meat fat content. In addition, processed meat products normally have a 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. On the other hand, the demands of consumers for healthier and safer products have also promoted the addition of soybean proteins in processed meat products as fat replacer. Moreover, soybean proteins have very interesting nutritional properties and there are also well known health benefits associated with the consumption of soybean.

The increasing use of foreign proteins in the manufacture of meat products has been parallel to the demands for regulations controlling this practice. Nowadays, all developed countries have elaborated regulations limiting or, even, forbidding the use of foreign proteins in the meat industry. These laws not only regulate the addition of these proteins but they also control the adequate labelling of meat products. The need for these regulations is reasonable. In addition to possible economical advantages derived from the substitution of meat proteins by cheaper ones (such as soybean proteins), most of the foreign proteins used by meat industry are allergens and their undeclared addition could result in terrible consequences for sensitive people. For example, in the case of soybean proteins, different allergens have been detected.

The implementation of regulations establishing maximum levels for soybean proteins and controlling the accurate labelling of meat products involves having an adequate analytical methodology for monitoring the amounts of soybean protein added to meat products. However, this is not an easy task. In fact, the determination of soybean proteins in meat products poses several limitations, the most important being that these proteins are present in meat products in a very low proportion in comparison with meat proteins and that soybean proteins could become altered during the processing of meat products. All these facts difficult and even make not possible the detection of soybean proteins. Electrophoretic, immunological and chromatographic methods have been developed for the determination of soybean proteins in meat products but most of them presented important limitations, especially in the case of heat-processed meat products.

Due to the absence of reliable analytical methods, in this work, new analytical methodologies were developed and validated to determine soybean proteins in raw and processed meat products using HPLC with perfusive stationary phases.

The developed methodologies involved defatting the meat samples with acetone, solubilization of soybean proteins in an extracting saline medium at basic pH and the analysis of such protein extract by Perfusion Reversed-Phase High Peformance Liquid Chromatography (Perfusion RP-HPLC) with UV detection and gradient elution. Optimization of the extraction and separation steps was also performed.

The best protein extraction conditions were related to the use of a Tris-HCl buffer solution at pH 8.0 (which could contain in some cases a modifier) with ultrasonic agitation and centrifugation to obtain a supernatant to be directly injected in the chromatographic system.

The Perfusion RP-HPLC method chosen 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, being

mobile phases A and B, 0.05 % (v/v) trifluoroacetic acid in water and 0.05 % (v/v) trifluoroacetic acid in acetonitrile, respectively. A perfusion reversed-phase column (50 x 4.6 mm d.i.) packed with 10 µm diameter polystyrene divinylbenzene beads, a 3 mL/min flow rate, a temperature of 50 ºC, and UV diode-array detection at 280 nm were also employed. Under these conditions, chromatographic separation of protein extracts was achieved in less than 3 min.

The developed methodologies enabled the selection of a "marker" peak for the determination of soybean proteins in meat products and were proven to be specific, precise, accurate, robust, and sensitive, making possible the detection and quantitation of additions of ~ 0.08 % (m/m) and ~ 0.3 % (m/m), respectively, of soybean proteins in meat products. Validation of the developed methodologies was achieved for raw and processed meat products with different composition (elaborated with pork, turkey, chicken, and beef meats or mixtures of them) showing that the presence of milk proteins in the meat products did not interfere.

Quantitation of soybean proteins in a variety of commercial heat-processed meat products with different composition (elaborated with meats of different species that could also contain milk proteins) was carried out using a soybean protein isolate as standard for soybean proteins. Results obtained showed that soybean contents determined in commercial meat products were within the limits authorized by the Spanish law (3 % soybean proteins referred to the meat product as is basis).

With the aim of characterizing the chromatographic peaks obtained with the developed methodologies for soybean proteins, Perfusion RP-HPLC was coupled to a mass spectrometer for the analysis, for the first time, of soybean intact proteins in different kinds of soybean cultivars. Separations were carried out using a perfusion reversed-phase column (100 x 2.1 mm d.i.) at a flow rate of 0.5 mL/min, a temperature of 60 ºC, and an elution gradient from 5 to 14 % B in 12 min, 14-16 % B in 1 min, 16-20 % B in 2 min, 20-28 % B in 1 min, 28-40 % in 8 min, 40-45 % B in 2 min, and 45-95 % B in 0.5 min. Mobile phases A and B consisted of 0.05 % (v/v) trifluoroacetic acid in water

and 0.05 % (v/v) trifluoroacetic acid in acetonitrile, respectively. A step-by-step procedure was used for the optimization of electrospray-ion trap-mass spectrometry parameters enabling the most sensitive detection. The influence of the capillary voltage, octapole voltage, octapole delta voltage, capillary exit offset voltage, and the skim voltage on the signal obtained was investigated. The use of a capillary voltage of 3 kV enabled to observe the highest signal not obtaining any significant improvement when using higher voltages. The selected octapole voltage was 4 V for the sensitive detection of soybean proteins and the octapole delta voltage showing the best results was 0.5 V. The lowest capillary exit offset voltage tried (50 V) resulted to yield the highest response. Regarding the skim voltage, the highest responses were observed at intermediate values selecting 45 kV.

Perfusion RP-HPLC-ESI-MS analysis of soybean cultivars showed that the chromatographic peak obtained for soybean cultivars at the same retention time as the "marker" peak for soybean proteins in meat products corresponded to 11S soybean proteins. Besides, another interesting application was derived from this study.

The similarities and differences between yellow soybeans (the most usual soybeans) and other beans with different pigmentation (green, red, and black) commercialized as soybean were investigated using the developed Perfusion RP-HPLC-ESI-MS method. Red beans commercialized as *azuki* that are frequently sold as red soybean were also analyzed. Moreover, the main soybean proteins (11S and 7S globulins) obtained by a fractionation procedure were also analyzed.

Beans presenting identical pigmentation presented identical TIC, regardless they were sold as soybean or not (*azuki*). In addition, yellow soybeans presented a very different TIC from those observed for the other seeds studied (red, green, and black) with the exception of one black soybean. In fact, some peaks corresponding to yellow soybeans were assigned to some proteins characteristic of soybean while this assignation was not possible for the other pigmented beans. Fractionation of main soybean proteins (7S and 11S globulins) was achieved for all beans studied enabling

their Perfusion RP-HPLC-ESI-MS analysis to confirm the differences found among yellow soybeans and the other pigmented beans. An interesting application was derived from this work related with the differentation of soybean from other similar cultivars (*azuki or mungbean*).

Finally, due to the difficulties related to the characterization of the "marker" peak for soybean proteins in meat products by the above Perfusion RP-HPLC-ESI-MS method, a new methodology using multidimensional liquid chromatography coupled to tandem mass spectrometry was also developed. Chromatographic prefractionation on the protein level by Perfusion RP-HPLC was employed to isolate peaks of interest from extracts of soybean protein isolate (SPI) and of meat products elaborated with SPI. After enzymatic digestion using trypsin, the collected fractions were analyzed by nanoflow liquid chromatography-tandem mass spectrometry. Results obtained enabled the identification of marker soybean proteins for the detection of adulterations of meat products with these vegetable proteins. In fact, different glycinin A subunits could be identified from the peak discriminating between meat products with and without soybean proteins added. Among these, glycinin G4 subunit A4 was consistenly found in all samples. Consequently, this protein (subunit) can be used as a target for new analytical techniques in the course of identifying the addition of soybean protein to meat products.