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Abstract

This work describes the development and validation of two PCR methods, End-point and real-time PCR for the detection of presence of soy protein in a wide rage of foodstuffs. These techniques are reliable and sensitive, allowing detection of trace amounts of soybean in processed products. The TaqMan real-time PCR had the simplest, fastest testing process and the higher potential for automation, therefore representing currently the most suitable method for screening. Moreover, in order to verify the correct operation of the proposed methodology, the ELISA technique was used for quantitative determination of the soy protein. Also, thirty-five meat, fish and bakery processed products which potentially could contain soy which was not announced on the label were tested for the presence of soy DNA by using the methods proposed. The methodologies can be useful in questions regarding the presence of soy protein in processed products, especially to verify labelling and security rules and to protect consumer's rights.

KEYWORDS: Soy; Soybean; TaqMan, real-time PCR; PCR; ELISA

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INTRODUCTION

The use of soy protein in the food industry has increased significantly in the last few years, being one of the most important and frequently used vegetable protein additives. Among its functional properties it is worth highlighting the emulsification capability and water and fat absorption, slicing characteristics and organoleptic features such as appearance and firmness (Belloque et al 2002). In the case of seafood, the water absorption and retention properties of textured soy proteins can be used to bind moisture in fish blocks and reduce fat and jelly deposits liberated during food canning, which results in a firmer final product (Endres et al. 2001). The increase of water retention gives rise to end-products of higher weight, which entails the use of soy protein for the partial substitution of raw material, meat or fish, which is more expensive, making the food products more cost-effective so that there is a considerable decrease in the final cost of the product to the benefit of the manufacturers (MacedoSilva et al 2002).

On the other hand, food allergies represent an important health problem in industrialized countries (Poms et al. 2004), affecting up to 2% of the adult population and up to 8% of children (Woods et al 23002). Anaphylaxis to soy in particular has been reported in several studies (Foucard et al. 1999; Sicherer et al. 2000). Soy is currently recognized as one of the potential allergens that have to be labelled if they are present in foodstuffs, according to the European Directives 2000/13/EC and 2003/89/EC (Directive 2000/13/EC; Directive 2003/89EC).

Due to the previously described arguments, methodology to detect soy protein in food products is necessary in order to ensure food safety, consumer protection and, at the same time, to guarantee fair competition between companies.

Soy detection is complex in processed products due to the fact that during processing, the food undergoes high temperature treatments or enzymatic processes. This causes alteration in the protein, which is denatured and forms ethanol-water insoluble aggregates and only a small percentage of proteins can be extracted for subsequent detection. For these reasons, the sensitivity obtained by means of immunological methods, such as ELISA technique, is not sufficient to assure safety of food products (Meyer et al 1996). DNA-based methods offer many advantages over protein-based

methodologies, primarily that the target DNA is less degradated than the proteins from food matrices (Poms et al. 2004). In addition, the ELISA technique is more expensive and time consuming than PCR.

Up to now, several PCR assays have been developed and applied to evaluate the presence of genetically modified soy in food products (Abdullah et al. 2004; Brod et al. 2007; Kakihara et al. 2006; Moriuchi et al. 2007) allowing the detection and quantification of different GMO's, among them some soy varieties, with total reliability.

In the present work two methodologies based on end-point and Real-Time PCR techniques using specific primers were developed. These can be alternatively used to detect the presence of soy protein in commercialized processed products by means of the amplification of the lectin gene. The food samples analyzed included a wide range of products that underwent different treatments in order to assess the use of soy protein in the current food industry and check the labelling regarding its presence in foodstuffs.

MATERIALS AND METHODS

1. Sample collection, storage and DNA extraction

The samples used in the present work were the following: raw soybean; soy protein isolate, concentrate and texturized; and soy flour. Also, 35 commercial processed products of a type potentially containing soy protein were purchased from shops and supermarkets in Spain (Table 1). The sampling included a wide range of foodstuffs with different degrees of processing, some of them showing the presence of soy in the label (meat, seafood and bakery products). Moreover other cereals were also obtained (wheat, corn, oats, rice, rye, barley, triticale, lupin and kamut).

Genomic DNA was extracted from 30 mg of the samples previously mentioned. For meat, fish and bakery products the amount of sample used was 150 mg. In canned products DNA extraction was performed, from 300 mg of different filtered coating media (water, oil, tomato sauce, pickling brine…) and superficial scraping of the products.

Two different DNA extraction methods were evaluated: (1) a method based on silica gel columns using the *NucleoSpin Tissue kit (Macherey-Nagel)* following the supplier's protocol and (2) a CTAB method previously described (Roger and Bendich, 1988) with slight modifications followed by purification with *NucleoSpin© Extract II* kit *(Macherey-Nagel)*.

The extracted DNA was loaded onto a 1% agarose gel containing 5 µg/mL of ethidium bromide *(Sigma)*, analysed by electrophoresis in 1X TBE buffer *(Sigma)* at 70 V for 50 min and visualized using the *Molecular Imager Gel Doc XR System* transilluminator and the software *Quantity One® v 4.5.2 (Bio-Rad).* The 100-1500 bp DNA ladder (*Dominion, MBL*) was used as molecular weight marker.

The DNA concentration was determined by measuring the absorbance at 260 nm and the purity using the absorbance ratio of 260 and 280 nm (A_{260}/A_{280}). These measures were carried out using a *NanoDrop™ 1000* spectrophotometer *(Thermo Scientific)*. DNA extracts were appropriately labelled and stored at -20 ºC.

2. End-point PCR assay to detect the presence of soy

The primers Lec IF and Lec IR described by Zhang et al (2007) were used to amplify a 100 bp fragment of the soy lectin gene.

In all cases PCR reactions were carried out in a total reaction volume of 25 µL containing 50 ng of DNA template, 0.8 mM of dNTP mix *(Bioline),* 2.5 µL of 10X buffer, 2 mM of $MgCl_2$, 0.75 units of BioTaq™ DNA pol (*Bioline*), 0.8 µM of each primer and molecular biology grade water *(Eppendorf)* up to adjust to the final volume.

PCR was performed in a thermal cycler *MyCycler™ (BIO-RAD)* under the following conditions: a preheating step at 95 ºC for 10 min, 38 cycles of amplification (95 ºC for 30 s, 55-70 ºC for 45 s, 65 ºC for 45 s) and a final extension step of 65 ºC for 5 min. The reactions were run using a range of annealing temperatures between 55 and 70º C.

The amplified fragments were analyzed by electrophoresis on a 2% agarose gel *(Sigma)* in 1X TBE buffer *(Sigma)* with 0.3 µg/mL of ethidium bromide *(Sigma)* and were visualized as described above. The 50 bp DNA ladder (*GE Healthcare*) was used as molecular weight marker.

3. Confirmation of the identity of PCR products

3.a. DNA Sequencing

In order to confirm the identity of the amplified PCR products, these were sequenced. For that, double-stranded DNA products were purified using the *NucleoSpin© Extract II (Macherey-Nagel)* according to the manufacturer's instructions. The concentration and purity were measured by means of a *NanoDrop™ 1000* spectrophotometer *(Thermo Scientific).* Subsequently, both DNA extracts were sequenced on an ABI Prism 3130 (*Applied Biosystems*) using BigDye Terminator Cycle Sequencing Ready Reaction v1.1 (*Applied Biosystems*). Next, these sequences were analyzed with *Sequencing Analysis Software v5.3.1. (Applied Biosystems)* and aligned with *Clustal W* (Thomson et al 1997) available in the program *BioEdit 7.0 (Hall, 1999).* The nucleotide sequences obtained were submitted to the *GeneBank* database of the *National Centre for Biotechnology Information (NCBI).*

3.b. RFLP methodology

Restriction maps of the DNA sequences obtained were generated using the software *Webcutter 2.0 (Heiman, 1997).* The enzyme H*inf* I was selected for its ability to generate a characteristic restriction profile with band sizes easily distinguishable on agarose gels. About 100 ng of PCR products were digested with 2 units of this enzyme. Digestive reaction mixes were incubated at 37ºC for 2 hours. The results of PCR-RFLP analysis were carried out as described above, except that the electrophoresis was performed on 3% low melting agarose gels (*Pronadisa*) at 70 V for 110 min. Sizes of fragments were estimated from a 50 bp DNA ladder (*GE Healthcare*).

4. Design of a specific RT-PCR method to detect the presence of soy

From sequences of lectin gene obtained with the primers described above, a specific *Taqman*® probe was designed for the development of a Real-Time PCR method to detect the presence of soy.

The *Lectin probe* 5' (FAM) CAC ATG CAG GTT ATC TTG GTC 3' (TAMRA) was labeled with the fluorescent reporter dye 6-carboxy-fluorescein FAM at the 5' end and with the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end.

The theoretical specificity of the designed probes was first evaluated by a BLASTN search in the GenBank database for sequences with 100% homology (Altschul et al. 1990).

In all cases the RT-PCR reactions were carried out in a total volume of 25 µL with the composition previously described, except the amount of primers and probe that were optimized. Optimal amount of primers and probe were evaluated by preparing dilution series. A common range of working stock concentrations of 50, 300 and 900 nM of each primer and 50, 100 and 250 nM of the Taqman® probe were used to determine the optimal concentrations. Molecular biology grade water *(Eppendorf)* was used to adjust to the final volume.

The reactions were performed in a *iQ 96-well PCR plates (BIO- RAD)* covered with *iCycler iQ™ Optical Tape (BIO- RAD)* and run on *B*io-Rad *iCycler iQ™* Real Time PCR instrument with the following thermal cycling protocol: 95ºC for 30 s followed by 45 cycles of 95ºC for 5s and 55ºC-60ºC for 30 s.

5. Specificity and sensitivity of the methods

The specificity of the two assays was evaluated by testing the amplification of DNA from cereals (wheat, corn, oats, rice, rye, barley, triticale, lupin and kamut) and different fish and land animals (tuna, cod, hake, salmon, squid, chicken, pig, cow, rabbit and turkey) .

The sensitivity of the proposed methods was assessed in 3 ways. The first one was established from DNA dilutions. The range of concentration was obtained from raw soy with DNA amounts ranging between 150 ng and 1 pg. The dilutions were prepared by adding DNA from different cereals and animal species to the soy DNA until completing the final amount.

The second way used to establish the LOD was by adding soy protein powder to sunflower/olive oil or water in percentages from 1% to 0.01% in tuna cans of 120 mL. These products were autoclaved using a high-pressure steam sterilizer at 121 ºC for 50 min. DNA extractions performed from filtered homogenates and superficial scraping of these canned products as described previously were used to establish the detection limit.

Finally, corn flour samples containing amounts of soy flour from 10.000 to 10 ppm were also analyzed. The PCR amplification was performed as described above.

6. Quantification of the developed methodology using the ELISA technique

In order to verify the correct operation of the methodology proposed in the present work, the *Soya protein assay kit* (*Tepnel Biosystems*) was used for the quantitative determination of soy protein, according to the manufacturer's protocol.

7. Methodological validation

In order to evaluate the correct operation of the methodologies proposed in the present work, different food products were elaborated in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology) adding different types of soy protein (Table 2). The most extreme treatment applied to them was sterilization by horizontal retort steel-air at 115 ºC for 50 min and 1.2 bars of overpressure.

The amount of soy added to the samples employed in the methodological validation corresponded to the minimum amount detected during development of the methodology. Subsequently, the samples were analysed with the methodologies developed in the present work.

The aim of the methodological validation was to check whether the manufacturing process which processed food underwent had no influence on the detection of soy protein with the proposed methods.

8. Application to commercial products

After the validation of the methods developed in the present work, these were applied to 35 processed products in order to determine if soy protein was added during the manufacturing process (Table 1). These products were acquired from Spanish supermarkets, and the purpose of the analysis was to evaluate the current status of labelling regarding soy content of foodstuffs on the Spanish market.

RESULTS AND DISCUSSION

1. DNA extraction

Two methods for DNA extraction were tested. The best results in terms of DNA yield and purity were obtained with the CTAB method followed by purification on a *NucleoSpin© Extract II kit (Macherey-Nagel)*. Good performances using CTAB-based DNA extraction methods were obtained in previous works for soybean products (Gryson et al. 2004; Olexová et al. 2004). On the other hand, Mafra *et al* (2008) obtained best results for DNA extraction from soybean flours and protein isolates using the second method evaluated in the present work, the *NucleoSpin Tissue kit (Macherey-Nagel)* that showed the best results for DNA extractions, although the CTAB method was generally well suited to all kinds of food matrices tested (Mafra et al. 2008).

The purity of DNA extractions was adequate in all cases ($A_{260}/A_{280} > 1.80$). This parameter is important to guarantee high-quality results in PCR amplification, both in end-point and RT-PCR.

2. Confirmation of identity of PCR products

PCR amplification of a 100 bp fragment of the lectin gene using the primer set Lec IF/IR was successfully achieved in all kind of samples (raw soybean; soy protein isolate concentrate and texturized; and soy flour).

This amplicon was analyzed by means of sequencing and RFLP in order to asses the identity of the PCR products. This approximation allows verifying the specificity of the primers in the conditions of cycling described.

2.a. DNA Sequencing and Blast analysis

Sequencing followed by Blast analysis is a powerful technique to confirm the identity of PCR products. It is used in laboratories devoted to food control, although it has the drawback of high cost. The results of this approach showed that the obtained sequences were identical to those available in NCBI for soy, achieving a homology score of 100% with the Megablast algorithm in all cases. These sequences were deposited in the NCBI database (accession numbers FJ876983-FJ876987).

2.b. RFLP methodology

RFLP is an alternative technique to sequencing and BLAST analysis to confirm the identity of a particular PCR product. PCR followed by digestion of amplification products using informative restriction enzymes was used in several previous works in order to confirm the identity of a PCR product, since it presents several advantages in comparison to sequencing (Ge et al. 2008; von Buren et al. 2001). For instance, the low cost, and the fact that it is fast and easy. However, some authors advise against using RFLP for forensic identification when there are moderate levels of intraspecific variability, because this could make the RFLP unstable and lead to misidentifications (Santaclara et al. 2007). The intraspecific variability can be due to the species or the molecular markers. The housekeeping genes, as Lectin in soy, show very little variability. In this study intraspecific variability was not found in the soy samples analyzed as previously reported (Simon et al 2003).

In accordance with the sequence data, the restrictase selected was H*inf* I that generated two DNA fragments of 71 and 29 bp, the 71 bp fragment being easily distinguishable on agarose gels (Figure 1). This restriction profile was obtained for all samples analyzed with this technique. Therefore, RFLP represents a suitable alternative technique to sequencing for the assessment of PCR products' identity, since it permits obtaining the characteristic restriction profile.

Both RFLP and sequencing techniques are used for confirmation of results. In routine assays for soy detection their application would not be necessary although it is advisable and important throughout the methodological development stage.

3. Design of method RT-PCR assay specific to detect the presence of soybean

Among the advantages of the real-time PCR technique it is worth highlighting its specificity, sensitivity, reproducibility and rapidity. This technique allows verification of the functioning of the PCR while it is running, saving the time from secondary visualization or identification techniques of the PCR products. The optimization of the PCR conditions allows granting highest level of sensitivity while maintaining the specificity of the technique.

The conditions that led to the best results were established by means of primers and probe matrix. The concentrations of 900 nM for both primers and 250 nM for the probe yielded the best results in terms of specificity and sensitivity.

4. Specificity and sensitivity of the methods

Assay specificity was assessed by means of conventional and Real-Time PCR methods. The specificity of the primer set LEC IF/LEC IR for soybean was confirmed by PCR amplification using DNA extractions from wheat, corn, oats, rice, rye, barley, triticale, lupin and kamut. No cross-reactivity was detected with any of the samples tested. In this way the optimal annealing conditions were established at 54 ºC and 58 ºC for end-point and Real-Time PCR methods respectively, in order to ensure the higher specificity of the developed methodologies.

The detection limit was determined in 3 ways. The first of them defined the minimum amount of DNA necessary to yield a visible band on agarose gel after amplification or a positive fluorescence signal in the end-point and RT-PCR respectively. The detection limit obtained for raw soy DNA extractions was 40 pg and 10 pg for end-point and Real-Time PCR methods respectively (Figure 2 and 3).

The second way to establish the LOD was through the minimum percentage of soybean powder added to cans of fish of 120 mL. The detection limit thus determined was 0.0625% and 0.05% for end-point and Real-Time PCR methods respectively.

The last way to establish the LOD from corn flour containing soy flour in variable amounts (10000, 1000, 100 and 10 mg/kg), allowed establishing a detection limit of 100 mg/kg and 10 mg/kg for end-point and Real-Time PCR methods respectively.

In the case of RT-PCR an important parameter reflecting specificity and sensitivity of this metodology is the threshold cycle (Ct). It is necessary to find the lowest value of Ct that allows maintaining high fluorescence levels together with lowest possible LOD for the technique. In all the cases positive Ct values were between 21 and 38.

The results from the specificity and sensitivity evaluation of the developed methodology clearly indicate RT-PCR as the best alternative for soy detection. Besides displaying lower LOD values it is highly specific since this parameter is directly proportional to the specificity of the set primers-probe. The results of this study demonstrated that the developed primers and probe are capable of efficiently amplifying the lectin gene using a real-time fluorescence detector. The assay is rapid, the entire procedure can be completed within 4 h. Moreover, application of the real-time PCR method to food samples has demonstrated the specificity of the method.

5. Quantification of the results obtained by the developed methodology using the ELISA technique

The kit *Soya protein assay kit* (*Tepnel Biosystems)* employs the principle of enzyme immunoassay and is a sensitive and specific assay for soy protein in the presence of vegetable, meat and other proteins.

The kit used for the quantitative determination of soy protein provides a detection limit of 5000 mg/kg, far from the detection limit of 10 mg/kg that has been obtained by means of RT-PCR when corn flour and soy flour mixtures are analyzed.

However, both conventional and Real-Time PCR developed in this work may be used as screening methodologies at the first step. If the results are negative it may be concluded that soy presence in the sample is below 10 mg/kg. When a positive result is obtained, the ELISA technique may be applied, but only if quantification is necessary. This technique has lower sensitivity, is more expensive, laborious and time consuming than genetic methodologies and may give a negative response if the quantity in the sample does not exceed 5000 mg/kg. Therefore both techniques (ELISA and PCR) may be combined. The PCR alternatives herein developed can be used for screening and ELISA for soy protein quantification in case of positive results, allowing the detection and quantification of soy in a particular sample.

6. Methodological validation

The aim of the validation step was to evaluate the correct operation of the proposed methodology. The products elaborated in the pilot plant of CECOPESCA were analyzed by the proposed molecular methodologies. The different treatments they were subjected to during the elaboration process allowed us to evaluate the correct DNA amplification in the most extreme cases of degradation (canned products). The soy was detected in all these samples by the methodological approaches developed in the present study. Therefore, it might be applied to canned, frozen, fried, cooked, and battered products.

7. Application to commercial products

Thirty-five meat, fish and bakery processed products were tested for the presence of soy DNA by using the methods proposed in the present work (Table 1). Extracted DNA showed a good yield and purity optimal in all products analyzed (evaluated by means of optical measurements). Seventeen samples showed a positive result for the presence of soy using the developed methodologies (Table 1). The results obtained for processed

food products demonstrate the wide use of soy protein in the food industry for products commercially available in Spain.

Altogether, this work describes the development and validation of two PCR methods to detect the presence of soy protein in a wide rage of foodstuffs. Among the advantages of these techniques it is worth highlighting that these are reliable and sensitive, allowing to detect trace amounts of soybean in processed products. Moreover it is quick and cost-saving. Therefore it can be useful in questions regarding the presence of soy protein in processed products, especially to verify the fulfilment of the labelling rules and to protect consumers' rights.

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FIGURE CAPTION

Figure 1. RFLP analysis of PCR products obtained with the LEC primer set and DNA template from soybean. Lane PCR: PCR products; Lane 1: PCR product; Lane 2: PCR products after digestion with H*inf* I; L-50: molecular size marker (50 bp DNA ladder, *GE Healthcare*).

Figure 2. Limit of detection of the End-point PCR assay. Lane L-50: 50 bp DNA Ladder, *(GE Healthcare);* Lane 1-6: 1 ng, 500 pg, 250 pg, 100 pg, 50 pg and 40 pgof DNA extracted from soybean used as template in the PCR.

Figure 3. Limit of detection of the RT-PCR assay. A: 100 ng, B: 10 ng, C: 1 ng, D: 100 pg, E: 10 pg of total DNA extracted from soybean used as template in the PCR.

TABLES

Table 1. Processed products susceptible of containing soy protein as ingredient, which has not been indicated on commercial label. Analyses carried out with the herein DNA-based methodology.

Type of food	Product	Result
Meat products		
	Boiled ham	+
	Mincemeat	+
	Ham croquettes	+
	Beef hamburgers	+
	Chicken hamburgers	+
	Chicken nuggets	-
	Chicken pasties	-
	Breaded ham and cheese	-
	Beef sausage	+
	Pork sausage	+
Seafood products		
	Surimi	+
	Tuna pate	-
	Smoked salmon pate	-
	Canned tuna in oil	-
	Canned tuna in pickled marinade	-
	Canned tuna in tomato sauce	-
	Canned tuna in water	+
	Tuna parties	-
	Tuna snacks	+
	Tuna hamburger	+
	Salmon nuggets	-
	Hake steaks	-
	Cod croquettes	-
	Tuna croquettes	-
	Fish cake	-
	Breaded hake fillets	+
	Breaded haddock fillets	+
	Battered cod fillets	-
	Battered squid rings	-
	Battered prawns	+
Bakery products		
	Sliced bread	+
	Toasted bread	+
	Cookies	+
	Cereals	-
	Cake	-

Table 2. Processed products elaborated and types of soy protein added to them

Type of soy protein	Product	Samples
Soy protein isolate		
	Canned tuna in oil	3
	Canned tuna in tomato sauce	3
	Tuna hamburger	2
	Chicken hamburgers	2
Soy protein concentrate		
	Pork sausage	2
	Chicken nuggets	2
	Salmon nuggets	2
	Breaded ham and cheese	2
Textured soy protein		
	Surimi	2
	Tuna pate	2
	Smoked salmon pate	2

FIGURE 1

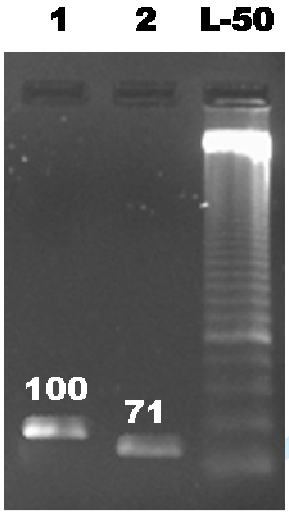

FIGURE 2

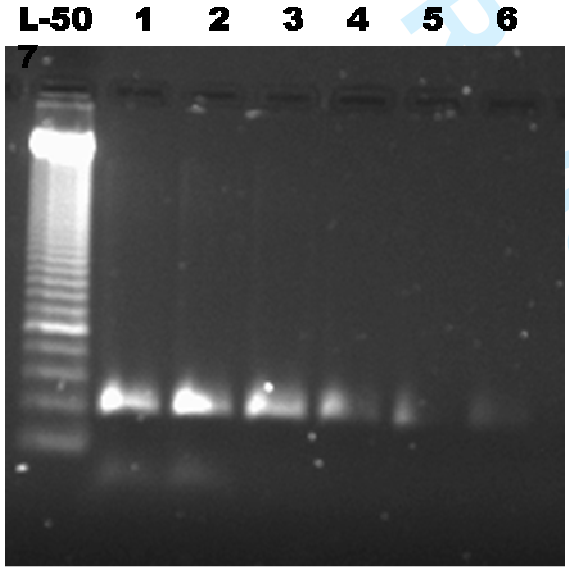

FIGURE 3