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Validation of End-point and Real-Time PCR methods for the rapid detection of soy allergen in processed products

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3 1 **Validation of End-point and Real-Time PCR methods for the rapid detection of soy**
4 2 **allergen in processed products**
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8 4 Montserrat Espiñeira, Beatriz Herrero, Juan M. Vieites and Francisco J. Santaclara
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15 8
16 9 **Abstract**

17
18 10 This work describes the development and validation of two PCR methods, End-point
19 11 and real-time PCR for the detection of presence of soy protein in a wide range of
20 12 foodstuffs. These techniques are reliable and sensitive, allowing detection of trace
21 13 amounts of soybean in processed products. The TaqMan real-time PCR had the
22 14 simplest, fastest testing process and the higher potential for automation, therefore
23 15 representing currently the most suitable method for screening. Moreover, in order to
24 16 verify the correct operation of the proposed methodology, the ELISA technique was
25 17 used for quantitative determination of the soy protein. Also, thirty-five meat, fish and
26 18 bakery processed products which potentially could contain soy which was not
27 19 announced on the label were tested for the presence of soy DNA by using the methods
28 20 proposed. The methodologies can be useful in questions regarding the presence of soy
29 21 protein in processed products, especially to verify labelling and security rules and to
30 22 protect consumer's rights.
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49 23 **KEYWORDS:** Soy; Soybean; TaqMan, real-time PCR; PCR; ELISA
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28 INTRODUCTION

29
30 The use of soy protein in the food industry has increased significantly in the last few
31 years, being one of the most important and frequently used vegetable protein additives.
32 Among its functional properties it is worth highlighting the emulsification capability
33 and water and fat absorption, slicing characteristics and organoleptic features such as
34 appearance and firmness (Belloque et al 2002). In the case of seafood, the water
35 absorption and retention properties of textured soy proteins can be used to bind moisture
36 in fish blocks and reduce fat and jelly deposits liberated during food canning, which
37 results in a firmer final product (Endres et al. 2001). The increase of water retention
38 gives rise to end-products of higher weight, which entails the use of soy protein for the
39 partial substitution of raw material, meat or fish, which is more expensive, making the
40 food products more cost-effective so that there is a considerable decrease in the final
41 cost of the product to the benefit of the manufacturers (MacedoSilva et al 2002).

42
43 On the other hand, food allergies represent an important health problem in industrialized
44 countries (Poms et al. 2004), affecting up to 2% of the adult population and up to 8% of
45 children (Woods et al 23002). Anaphylaxis to soy in particular has been reported in
46 several studies (Foucard et al. 1999; Sicherer et al. 2000). Soy is currently recognized as
47 one of the potential allergens that have to be labelled if they are present in foodstuffs,
48 according to the European Directives 2000/13/EC and 2003/89/EC (Directive
49 2000/13/EC; Directive 2003/89EC).

50
51 Due to the previously described arguments, methodology to detect soy protein in food
52 products is necessary in order to ensure food safety, consumer protection and, at the
53 same time, to guarantee fair competition between companies.

54
55 Soy detection is complex in processed products due to the fact that during processing,
56 the food undergoes high temperature treatments or enzymatic processes. This causes
57 alteration in the protein, which is denatured and forms ethanol-water insoluble
58 aggregates and only a small percentage of proteins can be extracted for subsequent
59 detection. For these reasons, the sensitivity obtained by means of immunological
60 methods, such as ELISA technique, is not sufficient to assure safety of food products
61 (Meyer et al 1996). DNA-based methods offer many advantages over protein-based

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3 62 methodologies, primarily that the target DNA is less degraded than the proteins from
4 63 food matrices (Poms et al. 2004). In addition, the ELISA technique is more expensive
5 64 and time consuming than PCR.
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10 65
11 66 Up to now, several PCR assays have been developed and applied to evaluate the
12 67 presence of genetically modified soy in food products (Abdullah et al. 2004; Brod et al.
13 68 2007; Kakihara et al. 2006; Moriuchi et al. 2007) allowing the detection and
14 69 quantification of different GMO's, among them some soy varieties, with total
15 70 reliability.
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20 71
21 72 In the present work two methodologies based on end-point and Real-Time PCR
22 73 techniques using specific primers were developed. These can be alternatively used to
23 74 detect the presence of soy protein in commercialized processed products by means of
24 75 the amplification of the lectin gene. The food samples analyzed included a wide range
25 76 of products that underwent different treatments in order to assess the use of soy protein
26 77 in the current food industry and check the labelling regarding its presence in foodstuffs.
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33 79 **MATERIALS AND METHODS**

34 80

35 81 **1. Sample collection, storage and DNA extraction**

36 82 The samples used in the present work were the following: raw soybean; soy protein
37 83 isolate, concentrate and texturized; and soy flour. Also, 35 commercial processed
38 84 products of a type potentially containing soy protein were purchased from shops and
39 85 supermarkets in Spain (Table 1). The sampling included a wide range of foodstuffs with
40 86 different degrees of processing, some of them showing the presence of soy in the label
41 87 (meat, seafood and bakery products). Moreover other cereals were also obtained (wheat,
42 88 corn, oats, rice, rye, barley, triticale, lupin and kamut).
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52 90 Genomic DNA was extracted from 30 mg of the samples previously mentioned. For
53 91 meat, fish and bakery products the amount of sample used was 150 mg. In canned
54 92 products DNA extraction was performed, from 300 mg of different filtered coating
55 93 media (water, oil, tomato sauce, pickling brine...) and superficial scraping of the
56 94 products.
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3 95 Two different DNA extraction methods were evaluated: (1) a method based on silica gel
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5 96 columns using the *NucleoSpin Tissue kit (Macherey-Nagel)* following the supplier's
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7 97 protocol and (2) a CTAB method previously described (Roger and Bendich, 1988) with
8
9 98 slight modifications followed by purification with *NucleoSpin® Extract II* kit
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11 99 (*Macherey-Nagel*).

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13 100

14 101 The extracted DNA was loaded onto a 1% agarose gel containing 5 µg/mL of ethidium
15
16 102 bromide (*Sigma*), analysed by electrophoresis in 1X TBE buffer (*Sigma*) at 70 V for 50
17
18 103 min and visualized using the *Molecular Imager Gel Doc XR System* transilluminator
19
20 104 and the software *Quantity One® v 4.5.2 (Bio-Rad)*. The 100-1500 bp DNA ladder
21
22 105 (*Dominion, MBL*) was used as molecular weight marker.

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24 106

25 107 The DNA concentration was determined by measuring the absorbance at 260 nm and
26
27 108 the purity using the absorbance ratio of 260 and 280 nm (A_{260}/A_{280}). These measures
28
29 109 were carried out using a *NanoDrop™ 1000* spectrophotometer (*Thermo Scientific*).

30 110 DNA extracts were appropriately labelled and stored at -20 °C.

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

112 The primers Lec IF and Lec IR described by Zhang et al (2007) were used to amplify a
113 100 bp fragment of the soy lectin gene.

114 In all cases PCR reactions were carried out in a total reaction volume of 25 µL
115 containing 50 ng of DNA template, 0.8 mM of dNTP mix (*Bioline*), 2.5 µL of 10X
116 buffer, 2 mM of MgCl₂, 0.75 units of BioTaq™ DNA pol (*Bioline*), 0.8 µM of each
117 primer and molecular biology grade water (*Eppendorf*) up to adjust to the final volume.

118 PCR was performed in a thermal cycler *MyCycler*™ (*BIO-RAD*) under the following
119 conditions: a preheating step at 95 °C for 10 min, 38 cycles of amplification (95 °C for
120 30 s, 55-70 °C for 45 s, 65 °C for 45 s) and a final extension step of 65 °C for 5 min. The
121 reactions were run using a range of annealing temperatures between 55 and 70° C.

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

126

127 3. Confirmation of the identity of PCR products

128 3.a. DNA Sequencing

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

140 **3.b. RFLP methodology**

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

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

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

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

157 The theoretical specificity of the designed probes was first evaluated by a BLASTN
158 search in the GenBank database for sequences with 100% homology (Altschul et al.
159 1990).

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

167 The reactions were performed in a *iQ 96-well PCR plates* (*BIO- RAD*) covered with
168 *iCycler iQTM Optical Tape* (*BIO- RAD*) and run on *Bio-Rad iCycler iQTM Real Time*
169 *PCR instrument* with the following thermal cycling protocol: 95°C for 30 s followed by
170 45 cycles of 95°C for 5s and 55°C-60°C for 30 s.

171 **5. Specificity and sensitivity of the methods**

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

176

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

182 The second way used to establish the LOD was by adding soy protein powder to
183 sunflower/olive oil or water in percentages from 1% to 0.01% in tuna cans of 120 mL.
184 These products were autoclaved using a high-pressure steam sterilizer at 121 °C for 50
185 min. DNA extractions performed from filtered homogenates and superficial scraping of
186 these canned products as described previously were used to establish the detection limit.
187 Finally, corn flour samples containing amounts of soy flour from 10.000 to 10 ppm
188 were also analyzed. The PCR amplification was performed as described above.

189

190 **6. Quantification of the developed methodology using the ELISA technique**

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

194

195 **7. Methodological validation**

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

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

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3 205 The aim of the methodological validation was to check whether the manufacturing
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5 206 process which processed food underwent had no influence on the detection of soy
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7 207 protein with the proposed methods.
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10 209 **8. Application to commercial products**

11 210 After the validation of the methods developed in the present work, these were applied to
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13 211 35 processed products in order to determine if soy protein was added during the
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15 212 manufacturing process (Table 1). These products were acquired from Spanish
16
17 213 supermarkets, and the purpose of the analysis was to evaluate the current status of
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19 214 labelling regarding soy content of foodstuffs on the Spanish market.
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215 RESULTS AND DISCUSSION

216 217 1. DNA extraction

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

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

231 232 2. Confirmation of identity of PCR products

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

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

239 240 2.a. DNA Sequencing and Blast analysis

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

247 **2.b. RFLP methodology**

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

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

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

269

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

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

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

280 **4. Specificity and sensitivity of the methods**

281 Assay specificity was assessed by means of conventional and Real-Time PCR methods.

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

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

293 The second way to establish the LOD was through the minimum percentage of soybean
294 powder added to cans of fish of 120 mL. The detection limit thus determined was
295 0.0625% and 0.05% for end-point and Real-Time PCR methods respectively.

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

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

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

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3 311 **5. Quantification of the results obtained by the developed methodology using the**
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5 312 **ELISA technique**

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7 313 The kit *Soya protein assay kit (Tepnel Biosystems)* employs the principle of enzyme
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9 314 immunoassay and is a sensitive and specific assay for soy protein in the presence of
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11 315 vegetable, meat and other proteins.

12 316 The kit used for the quantitative determination of soy protein provides a detection limit
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14 317 of 5000 mg/kg, far from the detection limit of 10 mg/kg that has been obtained by
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16 318 means of RT-PCR when corn flour and soy flour mixtures are analyzed.

17 319 However, both conventional and Real-Time PCR developed in this work may be used
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19 320 as screening methodologies at the first step. If the results are negative it may be
20
21 321 concluded that soy presence in the sample is below 10 mg/kg. When a positive result is
22
23 322 obtained, the ELISA technique may be applied, but only if quantification is necessary.
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25 323 This technique has lower sensitivity, is more expensive, laborious and time consuming
26
27 324 than genetic methodologies and may give a negative response if the quantity in the
28
29 325 sample does not exceed 5000 mg/kg. Therefore both techniques (ELISA and PCR) may
30
31 326 be combined. The PCR alternatives herein developed can be used for screening and
32
33 327 ELISA for soy protein quantification in case of positive results, allowing the detection
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35 328 and quantification of soy in a particular sample.

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39 330 **6. Methodological validation**

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41 331 The aim of the validation step was to evaluate the correct operation of the proposed
42
43 332 methodology. The products elaborated in the pilot plant of CECOPESCA were analyzed
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45 333 by the proposed molecular methodologies. The different treatments they were subjected
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47 334 to during the elaboration process allowed us to evaluate the correct DNA amplification
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49 335 in the most extreme cases of degradation (canned products). The soy was detected in all
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51 336 these samples by the methodological approaches developed in the present study.
52
53 337 Therefore, it might be applied to canned, frozen, fried, cooked, and battered products.

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57 339 **7. Application to commercial products**

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59 340 Thirty-five meat, fish and bakery processed products were tested for the presence of soy
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341 DNA by using the methods proposed in the present work (Table 1). Extracted DNA
342 showed a good yield and purity optimal in all products analyzed (evaluated by means of
343 optical measurements). Seventeen samples showed a positive result for the presence of
344 soy using the developed methodologies (Table 1). The results obtained for processed

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3 345 food products demonstrate the wide use of soy protein in the food industry for products
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5 346 commercially available in Spain.
6
7 347 Altogether, this work describes the development and validation of two PCR methods to
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9 348 detect the presence of soy protein in a wide range of foodstuffs. Among the advantages of
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11 349 these techniques it is worth highlighting that these are reliable and sensitive, allowing to
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13 350 detect trace amounts of soybean in processed products. Moreover it is quick and cost-
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15 351 saving. Therefore it can be useful in questions regarding the presence of soy protein in
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17 352 processed products, especially to verify the fulfilment of the labelling rules and to
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19 353 protect consumers' rights.
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453 **FIGURE CAPTION**

454

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

459

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

463

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

466

467 **TABLES**

468

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

472

473

Type of food	Product	Result
Meat products		
	Boiled ham	+
	Mince meat	+
	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	-

474

475

476 **Table 2.** Processed products elaborated and types of soy protein added to them
 477

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

478

FIGURE 1

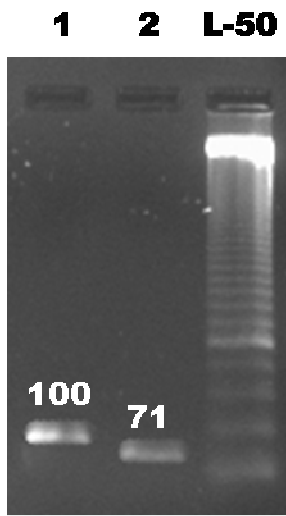
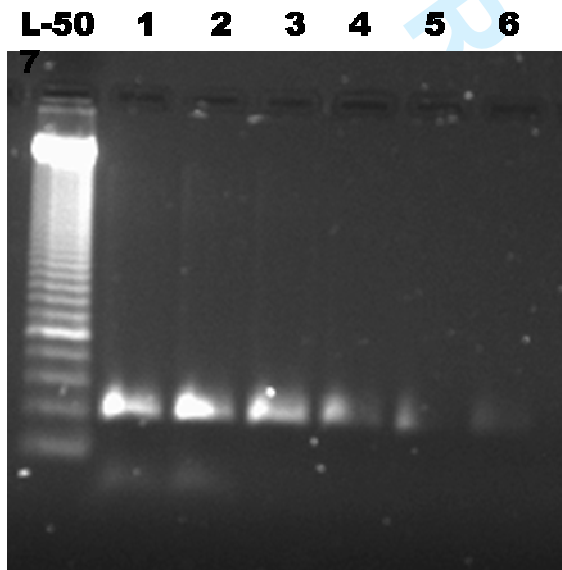
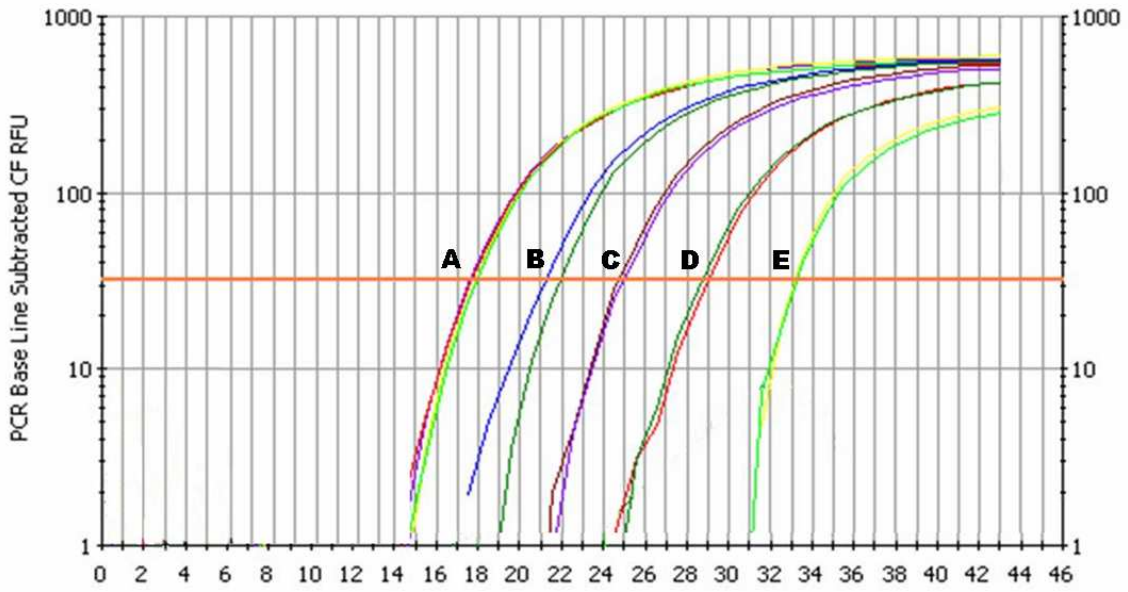


FIGURE 2



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FIGURE 3



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