**Electrochemical magnetoassay coupled to PCR as a quantitative approach to
detect the soybean transgenic event GTS40-3-2 in foods**

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26 **Abstract**

27 Simple, cost-effective and reliable tools for the quantification of genetically modified
28 organisms (GMO) in food and feed are highly demanded to enforce labelling
29 legislation in the EU. Herein, we report a novel method for quantitative analysis of
30 genetically modified soybean with the event GTS-40-3-2, also known as Roundup
31 Ready (RR) soybean, using magnetoassays with electrochemical detection, coupled to
32 DNA amplification by end-point polymerase chain reaction (PCR). For the proposed
33 work, two DNA sequences were targeted via hybridisation onto magnetic beads, one
34 specific for the transgenic event and the other for the taxon or species-specific *lectin*
35 gene. Enzymatic labelling was performed to obtain an electrochemically active
36 product measured by chronoamperometry. By optimising the number of PCR cycles,
37 among other parameters, two magnetoassays coupled to PCR were successfully
38 accomplished and linearity was obtained in the ranges of 53-4425 and 1093-88496
39 DNA copies for the event-specific and *lectin* sequences, respectively. The proposed
40 method provides accurate and precise RR soybean quantitative results, being
41 effectively compared to those obtained by real-time PCR, as the reference method.
42 These findings confirm the suitability of the method as an alternative tool for GMO
43 quantification.

44

45 **Keywords:** GMO, Roundup Ready soybean, electrochemical DNA detection, PCR,
46 biosensor.

47

48 **1. Introduction**

49 Recently, molecular biology techniques have been regarded as excellent tools for the
50 quality and safety assessment of foods. Within the sphere of DNA-based methods, the
51 polymerase chain reaction (PCR) has become a standard technique for food
52 authenticity and food control, being routinely applied to the detection/identification of
53 allergens, microorganisms, pathogens and genetically modified organisms (GMO) [1].

54 In the last decades, biotech crops have gained public attention, being the fastest
55 adopted crop technology in the history of modern agriculture [2]. Despite all the pros
56 and cons associated with the production and use of GM crops for food and feed, in
57 2014 the area of biotech crops continued to increase, totalising 181.5 million hectares.
58 Soybean is the most important GM crop, corresponding to 50% of the global biotech
59 area in 2014 [3]. Among the GM events cultivated worldwide, the soybean GTS 40-3-
60 2 event is one of the most representative, being commercially known as Roundup
61 Ready™ (RR) soybean since it was especially developed to be tolerant to the herbicide
62 with the same name.

63 In response to public demands, especial measures have been engaged to implement
64 labelling regulations for GMO in many countries, despite the lack of consensual
65 evidences on potential health risks [4, 5]. Accordingly, the European Union (EU)
66 issued the Regulation No. 1829/2003 that establishes the mandatory labelling of all
67 products consisting of, produced or containing authorised GMO above 0.9%. Below
68 this limit, GMO labelling is not required since its presence is considered adventitious
69 or technically unavoidable [6].

70 Currently, PCR-based methods are generally used for GMO screening and
71 identification, being real-time PCR the technique of choice for its quantification.
72 Methods based on the application of real-time PCR are specifically designed to

73 determine the relative content of GMO, commonly, by means of two parallel
74 reactions, in which one targets a GM-specific sequence (e.g. soybean Roundup
75 Ready) and the other targets the correspondent species-specific endogenous reference
76 gene (*lectin* for soybean). The quantification is further performed, either by
77 comparing the cycle threshold of the two amplified sequences (the ΔC_t method), or
78 via a standard curve [7]. In spite of the extensive application of real-time PCR assays
79 for the detection and quantification of GMO in raw and processed foods, both as
80 single or multi-target approaches, new strategies using different analytical
81 methodologies (e.g. biosensor platforms) have also been widely exploited for DNA
82 analysis.

83 Various types of hybridisation-based biosensors, including surface plasmon resonance
84 (SPR), quartz crystal microbalance or piezoelectric, thin-film optical, dry-reagent
85 dipstick-type and electrochemical have been introduced in GMO screening and/or
86 detection[8-10]. Electroanalytical techniques provide important advantages such as
87 the relative simplicity, low-cost of equipment and automatic on-line and portable
88 options [10, 11]. Currently, most of these devices require previous DNA amplification
89 by means of PCR in order to be applied to sample analysis [10, 12]. Despite their
90 remarkable sensitivity [13-16], the reported PCR-coupled devices have only been
91 used as confirmatory tools, whereas sensor-based quantitation of GMO has not yet
92 been reported. This has been attributed, in part, to the semi-quantitative kinetics of
93 PCR, compromising reliable quantification of DNA [12, 17,18]. Additionally, the
94 proposed sensors do not address the need for developing the detection of the
95 respective taxon-specific gene, which is required for establishing the relationship
96 between both measurements and enable a quantitative GMO analysis. To the best of
97 our knowledge, only screening/identification of GMO has been reported in food

98 samples using DNA sensors.

99 The aim of this work concerned the development of an alternative GMO quantitative
100 strategy, merging the advantages of both end-point PCR and electrochemical
101 approaches in terms of simplicity and low-cost to propose an accurate and robust new
102 method. Our strategy uses a versatile electromagnetic platform that couples the
103 hybridisation recognition reaction in a sandwich format on magnetic microparticles
104 with enzymatic labelling for electrochemical detection [19]. In this way, two
105 sequence-specific DNA probes, one targeting an event-specific sequence of RR
106 soybean and other the endogenous lectin gene, were both entrapped onto the surface
107 of magnetic beads. Sandwich hybridisation was performed for each PCR fragment in
108 order to indirectly tag the sequences with hapten molecules (fluorescein-
109 isothiocyanate - FITC, or digoxigenin - Dig). The enzyme horseradish peroxidase
110 (HPR) was used as reporter macromolecule, by affinity binding via anti-FITC or anti-
111 Dig Fab conjugation. Although FITC is frequently used as fluorescent dye in
112 biosassays, in this work it was used as intermediate tag to bind the DNA probe to an
113 enzymatic label. This type of conjugation offers strong and fast affinity binding with
114 minimum unspecific adsorption onto streptavidin-coated magnetic beads. FITC/Dig-
115 antiFITC/Dig-HRP conjugates have also been exploited in other biosensing platforms
116 [20, 21, 22]. Electrochemical readout was performed on the surface of disposable
117 carbon electrodes. By careful optimisation of the amplification-related parameters, we
118 demonstrate the utility of this approach for reliable quantification of GMO in foods.
119 The method was further validated by quantitative real-time PCR, using RR soybean as
120 the GMO target.

121

122 2. Experimental

123 2.1. *Samples*

124 Standards consisting of commercial certified reference material (CRM) developed by
125 the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) were
126 used. The CRM consisted of dried soybean flour (IRMM 410) containing 5, 2, 1, 0.5,
127 0.1 and 0% of Roundup Ready soybean expressed in mass percentage. One ground
128 soybean sample containing 1% of RR soybean, among other events, obtained from the
129 USDA/GIPSA (Grain Inspection, Packers, and Stockyards Administration)
130 Proficiency Program was also included in this study.

131 2.2. *DNA extraction*

132 DNA was extracted from 100 mg of soybean flour using the Wizard method as
133 described by Mafra et al. [23]. All extractions included a blank for the control of
134 reagents and potential contaminations during the extraction procedure.

135 Yield and purity of extracts were assessed by UV spectrophotometric DNA
136 quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments
137 Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was
138 determined using the nucleic acid quantification protocol with sample type defined for
139 double-strand DNA (1 absorbance unit corresponds to $50 \text{ ng}\mu\text{L}^{-1}$ of dsDNA) in the
140 Gen5 data analysis software version 2.01 (BioTek Instruments Inc., Vermont, USA).

141 The quality of extracted DNA was analysed by electrophoresis in a 1.0% agarose gel
142 carried out in $1\times$ of SGTB buffer (Grisp, Porto, Portugal) for 30 min at 200 V,
143 previously stained with $1\times$ of GelRed (Biotium Inc., Hayward, CA, USA). The
144 agarose gel was visualised with a UV light tray Gel Doc™ EZ System (BioRad
145 Laboratories, Hercules, CA, USA) and the digital image was obtained with Image Lab
146 software version 5.1 (BioRad Laboratories, Hercules, CA, USA).

147 2.3. Target gene selection and oligonucleotides

148 In this work, two sequences of each selected DNA region, namely a taxon-specific
149 gene (*lectin*) and RR soybean-specific (event or construct) were targeted. In both
150 cases, primers were selected from validated real-time PCR methods [24, 25]. Primers
151 Lec-F/Lec-R and RRS-Fm/RRS-Rm were used for amplification by end-point PCR to
152 produce fragments to be detected by the magnetoassay (Table 1). The primers RRS-
153 Fm/RRS-Rm, targeting the recombination region between the transgenic insert and
154 the plant genome, were used as event-specific, being slightly modified in order to
155 increase the melting temperature (Table 1). Other primers and probes were used in
156 quantitative real-time PCR to validate the method (Table 1). The primers and probes
157 were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

158 2.4. End-point PCR

159 The PCR amplifications were carried out in 25 µL of total reaction volume containing
160 2 µL of templateDNA, 1× buffer (67 mM of Tris-HCl (pH 8.8), 16 mM of
161 (NH₄)₂SO₄, 0.01% of Tween 20), 200 µM of each dNTP (Grisp, Porto, Portugal), 1.0
162 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 2.0
163 mM of MgCl₂ and 480 nM or 280 nM of each set of primers, Lec-F/Lec-R and RRS-
164 Fm/RRS-Rm, respectively (Table 1). The PCR amplifications were performed in a MJ
165 Mini thermal cycler (BioRadLaboratories, Hercules, CA, USA) using the following
166 programme of temperatures: 95 °C for 5 min; 30 or 35 cycles of 95 °C for 30 s, 66 or
167 60 °C for 30 s for primers Lec-F/Lec-R or RRS-Fm/RRS-Rm, respectively, and 72 °C
168 for 30 s; and a final extension at 72 °C for 5 min. The amplified fragments were
169 analysed by electrophoresis in a 1.5% agarose gel stained with GelRed and carried out
170 in 1× SGTB for 30 min at 200 V. The agarose gel was visualised with a UV light tray
171 Gel Doc™ EZ System (BioRad Laboratories, Hercules, CA, USA) and the digital

172 image was obtained with Image Lab software version 5.1 (BioRad Laboratories,
173 Hercules, CA, USA).

174

175 2.5. *Real-time PCR*

176 The amplifications by real-time PCR were performed in 20 μ L total reaction volume
177 containing 2 μ L of DNA template, 1 $\times$ iQTM Supermix (Bio-Rad Laboratories,
178 Hercules, CA, USA), 600 nM or 900 nM of each primer Lectin-F/Lectin-Ror RRS-
179 F/RRS-R, and 200 nM or 100 nM of each probe Lectin-TMPor RRS-TMP (Table 1),
180 respectively for lectin gene and event-specific region. Parallel reactions were prepared
181 for each target sequence. The assays were performed on a fluorometric thermal cycler
182 CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA,
183 USA) using the following temperature conditions: 95 $^{\circ}$ C for 5 min, 45 cycles at 95 $^{\circ}$ C
184 for 30 s and 60 $^{\circ}$ C for 1 min, with collection of fluorescence signal at the end of each
185 cycle. Data were collected and processed using the BioRad CFX Manager software
186 version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR trials were
187 repeated in two independent assays using three replicates for each unknown or blind
188 sample and two replicates for each standard.

189 2.6. *Electrochemical magnetoassays*

190 The experimental procedures were based on single assays described in our previous
191 work [19]. In Fig. 1, a general overview of the magnetoassays coupled to PCR is
192 illustrated and the oligonucleotide probes used are presented in Table 1.
193 Oligonucleotides were obtained as lyophilised desalted salts from Sigma-Life
194 Sciences.

195 2.6.1. *Biomodification of the magnetic beads*

196 Streptavidin-coated magnetic beads (MB) Dynabeads MyOne streptavidin C1 (1- μ m
197 diameter - 10 mgmL⁻¹) were acquired from Life Technologies (Spain). The
198 modification of the MB, the hybridisation and labelling assays were performed in a
199 12-tube mixing wheel (DynaL MX1) and magnetic separations were carried out with a
200 magnet DynaMag2, both from Life Technologies (Spain). Following a procedure
201 described in previous work [19], the commercial bead suspension was mixed with a
202 tween-containing phosphate buffer. After washing steps, 1 μ M of biotinylated capture
203 probe was added to the beads and incubated for 30 min at room temperature in the
204 mixing wheel. According to manufacturer recommendations, there is a known
205 relationship between the amount of biotinylated ssDNA and the amount of magnetic
206 beads (approximately 500pmol ssDNAmg⁻¹ MB). An excess of capture probewas
207 used to ensure all beads were biomodified.

208 2.6.2. Sandwich hybridisation with PCR amplicons

209 A sandwich-type hybridisation assay was carried out with 25 μ L of PCR product,
210 undiluted for RR soybean or diluted for *lectin* (50-fold or 100-fold), and 10 nM of the
211 corresponding signalling probe (SP), fluorescein isothiocyanate (FITC-SP) for RR
212 soybean or digoxigenin (Dig-SP) for *lectin*. The mixture was homogenised and
213 subsequently subjected to a thermal cycle, 5 min at 98 °C and 5 min in ice, to
214 facilitate the denaturation of the double-stranded amplicon and to allow homogeneous
215 hybridisation. After resting for 25 min at room temperature, the mixtures were added
216 to the microparticles in order to proceed with heterogeneous hybridisation between
217 the partial duplex and the capture probes. This step took place for 1 h at room
218 temperature under rotation. The hybrid-conjugated beads were then washed three
219 timeswith phosphate buffer and casein buffer.

220 2.6.3. Enzymatic labelling

221 An anti-fluorescein-peroxidase (anti-FITC-POD) or anti-digoxigenin-peroxidase
222 (anti-Dig-POD) conjugate (Roche Diagnostics GmbH, Mannheim, Germany) were
223 added at a final concentration of $0.25 \text{ } \mu\text{mL}^{-1}$ to the beads and incubated for 30 min
224 under rotation. After two washing steps, the beads were finally re-suspended in
225 phosphate buffer.

226 2.6.4. *Electrochemical detection*

227 The electrochemical detection of the enzymatic products was performed onto the
228 surface of a disposable carbon electrode, by means of chronoamperometry. Fifteen
229 microliters of the fully modified beads were magnetically captured onto the working
230 electrode with a magnet placed under it. After 2 min, the liquid was carefully
231 removed. Finally, $45 \text{ } \mu\text{L}$ of the enzymatic substrate, tetramethylbenzidine (TMB) in a
232 ready-to-use reagent format (K-blue enhanced activity substrate, also containing
233 H_2O_2 , obtained from Sigma-Aldrich, Spain), was placed on the disposable screen-
234 printed carbon electrodes (SPCE, DropSens-110, Spain), covering the three
235 electrodes. Chronoamperometric measurements were performed after 60 s of
236 enzymatic reaction using a potential pulse to 0 V, and sampling the current during
237 60s. Electrochemical measurements were performed using a computer-controlled
238 $\mu\text{AutoLab}$ type II potentiostat with GPES 4.9 (EcoChemie, The Netherlands). Before
239 carrying out the measurement step, the SPCE were washed with ethanol and deionised
240 water, and dried with nitrogen.

241

242 3. Results and discussion

243 3.1. *Optimisation of end-point PCR*

244 For the effective application of PCR, a critical step to overcome in the case of

245 complex and processed food matrices is the DNA extraction and purification. DNA
246 extracts presented, generally, high concentration ($355\pm 71\text{ ng}\mu\text{L}^{-1}$) and purity
247 (2.0 ± 0.1), as determined by UV spectrophotometry. The evaluation of extracts by
248 agarose gel electrophoresis evidenced high molecular weight genomic DNA ($\sim 10\text{ kb}$),
249 with only little sheared DNA (data not shown). The tested sample and CRM amplified
250 positively by end-point PCR with soybean taxon-specific primers, confirming that all
251 DNA extracts presented the adequate quality, integrity and purity for PCR
252 amplification. These results were in good accordance with other reports for certified
253 reference materials and flours, as those are basically submitted to mechanical
254 processing [26].

255 Despite using primers from a validated method, it was necessary to optimise the end-
256 point PCR conditions, for both *lectin* gene and soybean RR specific region. Besides,
257 the PCR conditions were particularly optimised to obtain high sensitivity, but also
258 relatively low band saturation to improve linearity and range of application with the
259 proposed magnetoassay. After testing several parameters (e.g. number of
260 amplification cycles, temperatures of annealing, magnesium concentration), the final
261 conditions for *lectin* and RR soybean amplification were set: annealing temperature of
262 $66\text{ }^{\circ}\text{C}$ or $60\text{ }^{\circ}\text{C}$ with 30 or 35 cycles for lectin or RR soybean, respectively, using 2.0 mM
263 of magnesium chloride. For both targets, the PCR assays allowed positive
264 amplifications down to 1.23 ng of template DNA (data not shown).

265 3.2. *PCR coupled to the magnetoassay: GMO calibration strategies*

266 For GMO quantification, the method using two standard curves, one that targets the
267 GM specific-region and other the correspondent taxon-specific gene, is normally
268 elected as the most adequate [26]. Based on this approach, in the present work, it was
269 intended the development of a double calibration curve method by means of two

270 PCRcoupled to the magnetoassays for each target sequence of *lectin* gene and RR
271 soybean.

272 A preliminary test of the PCRcoupled to the magnetoassay consisted on the
273 measurement of the electrochemical current related to the RR soybean target
274 amplified from all CRM levels (0, 0.1, 0.5, 1, 2, 5% of RR soybean). This test was
275 carried out aiming at evaluating if the electrochemical response was directly
276 proportional to the amount of RR soybean present in the sample. Additionally, it was
277 also intended to verify whether the 0% RRsoybean CRM together with the remaining
278 PCR reagents (enzyme, dNTP, primers) could interfere in the blank response.

279 Therefore, 200 ng of total DNA from the 6 different CRM levels were submitted to
280 PCR amplification using 38 cycles targeting the RR soybean specific region. After
281 amplification, products were hybridised with signalling and capture probes and the
282 responses were registered as described in the Experimental section. From the results in
283 Fig. 2A, two main issues could be observed. Firstly, the lowest tested level (0.1% RR
284 soybean) could not be accurately detected, and secondly, the system reached a
285 saturation point at 5% of RR soybean. Moreover, using the referred amplification
286 conditions (38 cycles), the level 0.1% could not be differentiated from the level 0.5%
287 of RR soybean. The logarithm of electrochemical responses versus the logarithm of
288 GMO content confirms the non-proportional correlation for the 5% CRM (Fig.
289 2B). When PCR reaches later cycles, the reaction enters in its plateau phase, making
290 inaccurate the quantification of end-point products [27]. With 38 cycles, amplicons are
291 most likely to be 'levelled-off', thus disabling their correct quantification.

292 Accordingly, new PCR conditions had to be tested, lowering the number of
293 amplification cycles until reaching linearity. It is also important to point out that the
294 0% RR soybean gave a similar response to the one from the blank of synthetic

oligonucleotides, suggesting that the background genomic DNA and the PCR components do not interfere with the proposed magnetoassay.

3.3. Calibration models for absolute determination of *lectin* and RR soybean DNA: cycle number optimisation

The evaluation of the electrochemical response of the proposed magnetoassay was performed for PCR products of five serial dilution (3-fold) levels of 5% RR soybean CRM, starting from 100 ng of total template DNA (88,496 copies) until 1.2 ng (1,093 copies), which corresponded to 5.0 ng of RR soybean DNA (4,425 copies) until 61.7 pg (55 copies), respectively. DNA copies were calculated according to the prime estimate value of the available soybean genome size (1.13 pg) from the Plant DNA C-value database [28] and assuming that the targeted sequences are single copy genes. The electrochemical responses of RR soybean or *lectin* PCR products were assayed using 35, 36 and 38, or 30, 32 and 35 cycles of amplification, respectively. In each assay, the total volume of PCR products (25 μ L) was added to the hybridisation mix containing 10 nM of signalling probe, as described in the Experimental section. In the case of *lectin*, the PCR product was 50-fold diluted before hybridisation. After enzymatic labelling of the target-signalling probe hybrids, the electrochemical readout took place. Chronoamperometry results were plotted against the initial DNA copy numbers of *lectin* or RR soybean sequences. The negative control, which was always included in the assay, presented a very similar response to the ordinary blank obtained with the magnetoassay (all components except the target, submitted to the same treatment of the complete method).

In Fig. 3, it can be noted that as expected the electrochemical signals decreased considerably by lowering the number of PCR cycles. With 36 and 38 cycles, the electrochemical responses showed a slight plateau above ~500 DNA copies of RR

320 soybean, whereas at 35 cycles the responses presented a close linear profile (Fig.
 321 3A). To support these findings, real-time PCR assay targeting the RR construct-
 322 specific region was performed, using 3-fold serial dilutions of 5% RR soybean DNA
 323 (10 ng down to 41.1 pg). In Fig. 4, the red arrow signals the stage of amplification of
 324 each DNA concentration when reaching 35 cycles. The highest amount (10 ng – 8,850
 325 DNA copies of RR soybean) is located just before the beginning of the plateau phase,
 326 whereas the following DNA dilutions are all within the exponential phase of PCR.
 327 Therefore, the results from real-time PCR suggested that 35 cycles of end-point PCR
 328 amplification ensure linearity in the 3-fold diluted range of concentrations (from 5.0
 329 ng down to 61.7 pg of RR soybean DNA). The electrochemical measurements
 330 performed at end-point PCR using such range of concentrations and 35 cycles result
 331 in the curve plot shown in Fig. 5A. By fitting the plot to a logarithmic model, linearity
 332 was obtained between 53 and 4,425 DNA copies of RR soybean (Fig. 5B), with a
 333 corresponding regression equation of $\text{Log (I)} = 0.645(\pm 0.006) \text{ Log (DNA copies)} -$
 334 $2.077(\pm 0.018)$ and a correlation coefficient of 0.9997. A similar behaviour was
 335 previously verified using the band intensities to develop quantitative models based on
 336 end-point PCR, in which the number of amplification cycles had to be optimised to
 337 reach linear log-log models [29, 30]. The absolute LOD of 53 copies of RR soybean
 338 DNA, which corresponds to an estimated relative LOD of 0.06%, shows that the
 339 proposed method reaches a sensitivity comparable with the one reported in the real-
 340 time PCR assay using the same primers (0.045%) [24].
 341 In the case of *lectin* gene amplification, a close linear profile of electrochemical
 342 responses was verified with 30 cycles, while with 32 and 35 cycles a plateau was
 343 reached above ~10,000 DNA copies (Fig. 3B). Earlier saturation is observed for *lectin*
 344 gene, which can be explained by the high quantity in which is present in the amplified

345 material, compared to RR (5:100 ratio). The electrochemical response presented the
346 best results using 30 cycles (Fig 5C), but it was necessary to perform a 50-fold
347 dilution of PCR products in order to obtain a linear log-log adjusted fit with the
348 magnetoassay (Fig. 5D). With these conditions, the magnetoassay allowed linearity
349 between 1,093 and 88,496 copies of soybean *lectin* gene, with a corresponding
350 regression equation of $\text{Log (I)} = 0.631(\pm 0.023) \text{ Log (DNA copies)} - 2.338(\pm 0.094)$ and
351 a correlation coefficient of 0.9959.

352 In both systems, adequate linearity parameters were obtained using 30 and 35 cycles
353 of amplification for *lectin* and RR soybean products, respectively, as evidenced by
354 their adequate correlation coefficients and response factors (Fig. 5B, 5D). The
355 respective limits of quantification corresponded to the first points of the calibration
356 curves (53 copies for RR and 1093 copies for *lectin*). For the same conditions of PCR,
357 the magnetoassay enabled achieving sensitivities 3- or 9-times higher for RR soybean
358 (61.7 pg) and lectin (1.2 ng), respectively, than the conventional electrophoretic
359 methods (185 pg for RR soybean and 11.1 ng of lectin). These findings emphasise the
360 high sensitivity of electrochemical detection over traditional electrophoretic
361 techniques, with special focus on its use for quantitative determinations.

362 3.4. Validation of magnetoassays with quantitative real-time PCR

363 Once the magnetoassays were developed and optimised for lectin and RR soybean
364 quantitative detection, validation tests were carried out using 1% CRM of RR soybean
365 and soybean flour (1%) obtained from the USDA/GIPSA Proficiency Program as
366 blind samples. Parallel quantitative assays were performed by real-time PCR and by
367 the proposed PCR coupled to magnetoassays in order to assess the analytical
368 performance of the latter. The performance parameters of real-time PCR were in good
369 agreement with the acceptance criteria defined for this technique [31], namely

370 correlation coefficient >0.98 , slope between -3.5 and -3.1 that corresponded to PCR
371 efficiency between 90 and 110%. For real-time PCR targeting RR soybean, PCR
372 efficiency was 97.6%, slope -3.380 and a correlation coefficient of 0.982. The real-
373 time PCR performance parameters targeting *lectin* gene were 94.5%, -3.461 and
374 0.992, respectively for PCR efficiency, slope and correlation coefficient. With respect
375 to the proposed PCR coupled to the magnetoassays, the correlation coefficients were
376 >0.99 , with response factors (slopes) >0.63 in both amplified targets. Although the
377 response factor should be the closest possible to unity to enable accurate
378 determinations, an adequate compromise among linearity, response factor and
379 sensitivity was accomplished [31].

380 The application of the two proposed magnetoassay models was successful for the
381 estimation of both DNA targets by interpolation, which enabled to determine the
382 relative content of RR soybean of two soybean materials (Table 2). The results
383 showed that both values were estimated with high accuracy since bias was lower than
384 10% in both samples. It is important to note that the GIPSA material is not a CRM, so
385 we consider as reference value for comparison the average result of 0.86% obtained
386 from the Proficiency Program. The high accurate estimates obtained by real-time PCR
387 confirm and validate the magnetoassay results, particularly in the GIPSA sample.
388 Besides the high accuracy of the proposed quantitative magnetoassay, a high
389 precision was also obtained as evidenced by relative standard deviations lower than
390 20%. It should be stressed that the reproducibility of the assays is owed mostly to the
391 high level of monodispersity of the magnetic microparticles (coefficient variation of
392 less than 1%) and their stability throughout the different stages of biorecognition
393 events and multiple washing steps.

394 4. Conclusions

395 To the best of our knowledge, this is the first effective attempt of developing a
396 biosensing approach for GMO quantification in real food samples since methods
397 described in the literature rely on synthetic oligonucleotides, lacking true
398 applicability. In this work, we have arduously focused on applying the method for
399 GMO quantification by successfully establishing a correlation between the
400 electrochemical response and the initial amount of template DNA. The proposed
401 method relies on a two target approach, in which *lectin* and RR event-specific
402 sequences are both targeted. Reliable quantification of DNA with end-point PCR has
403 been established by decreasing the number of amplification cycles owing to the
404 increased magnetoassay sensitivity, it is possible to obtain linear calibration
405 approaches able to quantify the GMO proportion in flour samples. Further studies are
406 required to evaluate the applicability of the PCR-magnetoassay approach on more
407 complex/processed food matrices. Furthermore, concomitant studies will be carried
408 out with streptavidin-coated magnetic nanoparticles, in order to improve the
409 sensitivity of our current system for analysis of more complex samples.
410 Considering that simplicity and economy are two of the most desirable features in
411 GMO analysis, this work represents an attractive and reliable alternative for the
412 quantification of RR soybean in commercial foods without requiring real-time PCR
413 instrumentation.

414

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527

Table 1

Sequence of primers and probes used to target the endogenous reference gene (*lectin*) and RR soybean by end-point PCR and real-time PCR amplifications, and oligonucleotides used in the magnetoassay.

Target region	Name/description	Sequence (5' → 3')	Amplicon (bp)	Reference
End-Point PCR <i>Lectin</i> gene	Lec-F	CCA GCT TCG CCG CTT CCT TC	74	[24]
	Lec-R	GAA GGC AAG CCC ATC TGC AAG CC		
	RRS-Fm	TTC ATT CAA AAT AAG ATC ATA CAT ACA GG	84	[24]
	RRS-Rm	GGC ATT TGT AGG AGC CAC CTT C		
Real-time PCR <i>Lectin</i> gene	Lectin-F	TCC ACC CCC ATC CAC ATT T	81	[25]
	Lectin-R	GGC ATA GAA GGT GAA GTT GAA GGA		
	Lectin-TMP	FAM-AAC CGG TAG CGT TGC CAG CTT CG-BHQ1	83	[25]
	RRS-F	GCC ATG TTG TTA ATT TGT GCC AT		
	RRS-R	GAA GTT CAT TTC ATT TGG AGA GGA C		
CTP/35S junction	RRS-TMP	FAM-CTT GAA AGA TCT GCT AGA GTC AGC TTG TCA GCG-BHQ1		
Magnetoassay <i>Lectin</i> gene	Taxon-specific target	CCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCCCTGACACAAAAAGGCTTGCAGAT74 GGGCTTGCCTTC		[19]
	Signalling probe-Dig	Dig-GAAGGCAAGCCCATCTGCAAGCCTTTTGTGTCAAGGGGCATAGAAAGGTG		
	Capture probe	AAGTTGAAGGAAGCGGCGAAGCTGG-Biotin	84	[19]
	Transgenic target	TTCATTCAAAAATAAGATCATACATACAGGTAAATAAACATAGGGAACCCAAATGGAAAA		
	Signalling probe-FITC	GGAAGGTGGCTCCTACAAATGCC		
	Capture probe	TTCCATTGGGTTCCCTATGTTATTTTAAACCTGTATGATGATCTTATTTGAATGAA-FITC Biotin- GGCATTGTAGGAGCCACCTTCCTT		

Table 2

Accuracy assessment of RR soybean by the electrochemical magnetosassays in comparison to real-time PCR results

Assay	Actual RR soybean (%)	Lectin ^a		RR soybean ^a		Estimated RR soybean (%)	Bias (%)
		Signal (Ct or μ A)	DNA copies	Signal (Ct or μ A)	DNA copies		
Real-time PCR	1.0	22.17 $\pm$ 0.23	297375 $\pm$ 53385	29.44 $\pm$ 0.06	2824 $\pm$ 129	0.95	-5.0
Electrochemical magnetosassay	1.0	4.59 $\pm$ 0.14	56236 $\pm$ 2784	0.468 $\pm$ 0.018	511.1 $\pm$ 29.9	0.91	-9.0
Real-time PCR	0.86 ^d	22.19 $\pm$ 0.14	291711 $\pm$ 28707	29.60 $\pm$ 0.10	2439 $\pm$ 177	0.84	-1.7
Electrochemical magnetosassay	0.86 ^d	7.00 $\pm$ 0.98	110204 $\pm$ 24361	0.653 $\pm$ 0.003	856.5 $\pm$ 5.8	0.78	-6.9

^a Values are mean $\pm$ standard deviation; ^b certified reference material (flour) containing 1.0% of RR soybean; ^c ground soybean sample from the USDA/GIPSA Proficiency Program with 1.0% RR soybean fortification; ^d value is the average result from the USDA/GIPSA Proficiency Program.

538 **Figure captions**

539 **Fig. 1.** Scheme of the electrochemical magnetoassays coupled to PCR: (A)
540 after DNA extraction, PCR is carried out and respective fragments are submitted to a
541 thermal-ice cycle in order to denature the dsDNA and perform the hybridisation with the
542 signalling probe (SP); (B) in a separate tube, the attachment of capture probe(s) (CP) to
543 the surface of magnetic beads (MB) takes place, after which the SP-target complex is
544 added to the MB and heterogeneous hybridisation with the capture probe occurs; being
545 followed by the addition of the Fab-enzyme conjugate to allow enzymatic labelling; (C)
546 biomodified MB are entrapped onto the working area of a screen printed carbon electrode
547 via a small magnet placed under it, followed by the addition of the enzymatic substrate
548 (TMB), which undergoes enzymatic oxidation. Subsequently, TMB reduction is recorded
549 at the electrode surface by chronoamperometry.

550

551 **Fig. 2.** Electrochemical responses obtained with the magnetoassay applied to CRM
552 materials (0, 0.1, 0.5, 1, 2 and 5% of RR soybean) after 38 cycles of PCR amplification
553 targeting RR soybean. A, electrochemical responses vs. RR soybean relative content; B,
554 logarithm of electrochemical responses vs. logarithm of RR soybean relative content.

555

556 **Fig. 3.** Electrochemical responses obtained with the magnetoassays coupled to PCR
557 fragments using different amplification cycles from a 3-fold serially diluted DNA extract
558 (5% RR soybean CRM). A, RR soybean amplified products (5.0 ng of RR soybean DNA
559 (4,425 copies) until 61.7 pg (55 copies)); B, *Lectin* gene amplified products (100 ng of
560 total genomic DNA (88,496 copies) until 1.2 ng (1,093 copies)), but to decrease signal
561 saturation, further dilution of amplicons was required prior to hybridisation: 100-fold for
562 the assay with 35 cycles and 50-fold for the other assays.

563

564 **Fig. 4.** Real-time PCR amplification curves and respective calibration curve targeting a
565 RR soybean sequence from a serially diluted soybean DNA containing 10 ng to 0.0137ng
566 of RR soybean DNA (5% RR soybean CRM).

567

568 **Fig. 5.** Calibration plots (A, C) and the corresponding linear log-log models (B, D) of
569 electrochemical responses obtained with the magnetoassays coupled toPCR fragments for
570 RR soybean with 35 cycles (A, B) and *lectin* with 30 cycles (C, D).

571

572

573 **Fig. 1**

Fig 2

Fig. 3

582

583 **Fig. 4**

584

Fig. 5

C. Lorena Manzanares-Palenzuela is a PhD student at Complutense University of Madrid since 2012. During her master's degree and PhD studies she has worked with electrochemical biosensors applied to food-related DNA sequences. Currently, her work is focused on the quantification of transgenic varieties of soybean by novel electrochemical approaches.

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