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Development of sandwich and competitive ELISA formats to determine β -conglycinin: evaluation of their performance to detect soy in processed food.

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Abstract

Two ELISA formats (sandwich and indirect competitive) were developed to quantify β -conglycinin, a major soy allergen. Their performance was evaluated using three model foods incurred with soy proteins. The sandwich format detects the addition of 0.005% and 0.05% soy proteins in pasteurized sausages and baked bread. However, the competitive format detects only 0.1 and 0.5%, respectively. β -conglycinin was not detected in sterilized pâté with any format. An industrial prototype of the sandwich ELISA was in-house validated, showing acceptable results of repeatability, reproducibility and robustness. Model foods spiked with β -conglycinin after processing showed recoveries between 93.3 and 138.7%. However, in model foods incurred with soy proteins before processing the recovery decreased with the increase of the severity of heat treatment applied. The sandwich format could differentiate most of the retail foods with soy declared or not as ingredient. The ELISA format and processing conditions greatly influence the determination of β -conglycinin in food.

Keywords

β -conglycinin, ELISA format, soy detection, allergen, model foods, test validation

45 1. Introduction

46 Soybean (*Glycine max*) proteins are one of the most important vegetable protein sources used in
47 food industry due to their high nutritional value and functional properties (Gandhi, 2009).

48 Furthermore, there is evidence that consumption of soybean has beneficial effects like lowering
49 plasma cholesterol, triglycerides and low-density lipoproteins (Duranti et al., 2004). For those
50 reasons, nowadays soybean proteins are widely used as ingredients in meat, dairy and bakery
51 products, edible spreads, cheese analogues, desserts, soups, etc. (Jideani, 2011).

52 However, soybean proteins have long been recognized as a source of dietary allergens for
53 humans. They can induce IgE-mediated reactions with cutaneous, respiratory and/or gastrointestinal
54 symptoms. Occasionally, soy proteins can cause anaphylaxis that may lead to death (Savage,
55 Kaeding, Matsui, & Wood, 2010). The prevalence rate of soy allergy is approximately 0.3-0.4% in
56 the general population, young children being more affected than adults (EFSA, 2014). The
57 threshold level of soy to trigger adverse reactions has been reported to range between 0.0013 mg
58 and 500 mg depending on the sensitivity of soy allergic individuals (L'Hocine & Boye, 2007).

59 Several studies were performed for the identification and characterization of specific allergenic
60 proteins from soy. Results have shown that a heterogeneous group of proteins has the ability to bind
61 to IgE from allergic soy individuals. Among them, β -conglycinin has been shown to be one of the
62 major soybean allergens (Holzhauser et al., 2009). It is a major storage protein of soy, which
63 accounts for about 30% of the total soy proteins. It is a trimeric protein with a molecular weight
64 ranging between 140 and 170 kDa. β -conglycinin is composed by three glycosylated subunits: α ,
65 α' and β having molecular weights of 76, 72 and 53 kDa, respectively (Morita, Fukase, Yamaguchi,
66 Fukuda, & Morita, 1996).

67 The extended use of soy proteins in the formulation of food products poses a serious problem for
68 soy allergic individuals. It is well established that the only effective treatment for food allergies is to
69 avoid the consumption of the offending food. At this respect, soy is included in the allergen
70 labelling regulation of the European Union and United States (Regulation EU 1169/2011 and Food

71 Allergen Labelling & Consumer Protection Act, FALCPA) and its presence must be obligatory
72 highlighted on the label when it is added as ingredient. However, the labelling of products which
73 may contain hidden allergens, which can be present due to cross-contamination is not mandatory. An
74 additional form of voluntary labelling (termed precautionary allergen labelling) has evolved on a
75 wide range of food products in an attempt by manufacturers to minimise the risk to allergic patients.
76 However, the indiscriminate use of precautionary labelling restricts consumer choice and supposes
77 a potential risk due to the lack of credibility (Cucu, Jaxsens, & Meulenaer, 2013; Allen et al., 2014;
78 Battisti et al., 2017).

79 In the recent study of Khuda et al. (2016), significant numbers of bakery and snack products
80 analyzed by commercial ELISA tests were positive for soy protein although it was not declared as
81 ingredient (25% and 11%, respectively). Likewise, 19% and 9% of bakery and snack products with
82 precautionary labelling also contained soy protein. These findings emphasize that suitable detection
83 techniques are necessary to be used by food industry for the implementation of an allergen risk
84 management and by international organizations to ensure the compliance with the current allergen
85 labelling regulations.

86 Several ELISA techniques have been developed to detect soy allergens due to their high
87 sensitivity and specificity, and technical simplicity. They are based on antibodies raised against
88 soluble soy proteins or single proteins, such as glycinin, β -conglycinin, Kunitz trypsin inhibitor and
89 p34 protein, among others (L'Hocine & Boye, 2007).

90 In previous reports, indirect competitive (You et al., 2008) and indirect sandwich (Hei, Li, Ma, &
91 He, 2012; Moriyama et al., 2005) ELISA techniques were designed to quantify β -conglycinin.
92 These techniques were applied to determine that protein in soy derived products (Moriyama et al.,
93 2005, You et al., 2008) and soy seeds from different origins (Hei et al., 2012). However, to our
94 knowledge, ELISA techniques to β -conglycinin for the detection of soy in model processed foods
95 prepared with incurred soy proteins and processed mimicking the actual industrial conditions have
96 not been previously reported.

97 In this study, two ELISA formats (sandwich and indirect competitive) to determine β -
98 conglycinin were developed. The performance of both assays was evaluated using sausage, bread
99 and pâté samples incurred with soy proteins as ingredient. A prototype of the sandwich ELISA was
100 validated and applied to survey a variety of retail foods.

101

102 **2. Materials and methods**

103 *2.1. Isolation of β -conglycinin*

104 β -Conglycinin isolation was performed following the methods of Liu et al. (2007) and Mo,
105 Wang, & Sung (2001). Raw soybean seeds were soaked in distilled water, peeled and dried. Then,
106 seeds were ground and defatted with n-hexane. Proteins were extracted from defatted soy flour with
107 15 fold volumes of 0.03 M Tris-HCl buffer, pH 8.5 for 1 h at 37 °C. After centrifugation at 9000 x g
108 for 30 min at 4 °C, the supernatant was added with sodium metabisulfite (SBS) at a final
109 concentration of 0.01 M, the pH was adjusted to 6.4 and the mixture was incubated overnight at 4
110 °C and centrifuged. The supernatant was added with NaCl to a final concentration of 0.25 M and the
111 pH was adjusted to 5.5. Afterwards, the mixture was incubated for 30 min at room temperature
112 (RT) and centrifuged. The supernatant was diluted with distilled water (1:2, v/v), adjusted to pH 4.8
113 and centrifuged, collecting the pellet which contained β -conglycinin (Liu et al., 2007). Then, β -
114 conglycinin fraction was dissolved in 2.6 mM KH_2PO_4 , 32.5 mM K_2HPO_4 buffer pH 7.6 containing
115 0.4 M NaCl and 0.01 M SBS. The protein solution was added with ammonium sulfate to 75%
116 saturation, stirred in an ice water bath for 1 h at 4 °C and centrifuged. The precipitate was
117 centrifuged off and then ammonium sulfate was added to the supernatant to reach 100% saturation
118 (Mo et al., 2011). After centrifugation, the precipitate was collected and dialyzed against the same
119 phosphate buffer. The β -conglycinin fraction was applied onto a Sepharose 6B-CL column (85 x 1
120 cm) and fractions of 2 mL were collected and analyzed by SDS-PAGE. Purity of β -conglycinin was
121 determined by densitometry of stained gels.

122 2.2. *SDS-PAGE and Western-blotting*

123 SDS-PAGE under reducing conditions was performed according to Laemmli (1970) using 4-
124 20% precast polyacrylamide gels on a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories). Proteins
125 were stained with Coomassie blue. Western-blotting using anti- β -conglycinin antiserum was carried
126 out according to the procedure described by Benfeldt, Larsen, Rasmussen, Andreasen, & Petersen
127 (1995).

128 2.3. *Preparation and conjugation of antibodies*

129 Purified β -conglycinin was inoculated into rabbits to obtain antisera as previously described by
130 Wehbi et al. (2005). All procedures were performed under Project Licence 65/14 which was
131 approved by the Ethic Committee for Animal Experiments from the University of Zaragoza. The
132 care and use of animals were performed accordingly with the Spanish Policy for Animal Protection
133 RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used
134 for experimental and other scientific purposes.

135 Specific anti- β -conglycinin antibodies were purified by affinity chromatography using a HiTrap
136 NHS activated HP column (GE Healthcare, Fairfield, Connecticut, USA) previously coupled with β -
137 conglycinin. Antisera (10-15 mL) was applied onto the column and after washing with 1.5 mM
138 KH_2PO_4 , 8 mM Na_2HPO_4 , 0.14 mM KCl and 0.14 M NaCl, pH 7.4 (PBS), antibodies were eluted
139 with 0.1 M HCl-glycine buffer, pH 2.8 containing 0.5 M NaCl and immediately neutralized to pH
140 7.4 with 0.5 M Tris buffer, pH 8.0. Purified antibodies were conjugated with horseradish peroxidase
141 (HRP) using the Lighting-link HRP conjugation Kit (Innova Biosciences, Cambridge, UK).

142 2.4. *Indirect competitive ELISA*

143 Microtiter plates (Nunc, Roskilde, Denmark) were coated with β -conglycinin (5 $\mu\text{g}/\text{mL}$) in 0.05
144 M sodium carbonate buffer, pH 9.6 and incubated overnight under refrigeration. Afterwards, wells
145 were blocked with a 3% (w/p) ovalbumin solution in PBS at RT for 2 h. Before using, wells were
146 washed with PBS containing 0.05% of Tween-20 (PBST) and incubated 30 min at RT with

147 standards or samples (50 μL) and with 50 μL of anti- β -conglycinin antiserum. After washing wells
148 with PBST, 100 μL of antibodies against rabbit IgG labelled with peroxidase (Sigma, Poole, UK),
149 diluted 1/3000 in PBS, were added to wells and incubated for 30 min at RT. After washing again,
150 100 μL /well of tetramethylbenzidine (TMB) substrate were added and incubated for 30 min at RT.
151 Finally, 50 μL of 2 M H_2SO_4 were added to stop the enzyme reaction and absorbance was
152 determined at 450 nm using a microplate reader ELISA Multiskan MS (Labsystem, Helsinki,
153 Finland).

154 2.5. Sandwich ELISA

155 Wells were coated with anti- β -conglycinin antibodies (3 $\mu\text{g}/\text{mL}$) and the blocking and washing
156 steps were performed as previously described. Then, the wells were incubated with 100 μL per well
157 of standards or samples for 30 min at RT. After washing with PBST, wells were incubated during
158 30 min at RT with 100 μL of anti- β -conglycinin antibodies labelled with peroxidase diluted 1/20000
159 in PBS. Afterwards, incubation with the substrate, stopping of enzymatic reaction and absorbance
160 measurement of wells were performed as indicated above.

161 2.6. Preparation of model processed foods

162 Sausages, bread and pâté were elaborated at the pilot plant of the University of Zaragoza as
163 previously described (Segura-Gil et al., 2018). They were prepared by adding different percentages
164 (0.25, 0.5 and 1%, w/w) of a mixture of two commercial soy protein isolates. Lower percentages
165 were obtained by mixing samples containing 0.25% and 0% of soy.

166 Sausages were pasteurized at 95 $^{\circ}\text{C}$ for 12 min, bread dough was baked at 160 $^{\circ}\text{C}$ for 50 min, and
167 pâté was sterilised in an autoclave at 120 $^{\circ}\text{C}$ for 1 h (reaching internal temperatures of 90 $^{\circ}\text{C}$, 97 $^{\circ}\text{C}$
168 and 117 $^{\circ}\text{C}$, respectively).

169

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172 *2.7. Preparation of spiked samples*

173 A mixture of four commercial soy drinks was added to bovine UHT milk to obtain spiked
174 percentages of 0.0005-0.005%. Cookie samples were prepared by mixing commercial cookies with
175 and without soy to obtain spiked percentages of 0.005-0.1%

176 *2.8. Preparation of standards and food extracts*

177 β -conglycinin standards were prepared from a stock solution of pure protein (1 mg/mL in PBS)
178 determined by spectrophotometry at 280 nm using the extinction coefficient of 0.42. Standards were
179 prepared by dilution of the stock solution with PBS added with 0.25% bovine serum albumin.

180 Food samples were ground using a mixer. Then, an amount of 1.00 ± 0.01 g or 1.00 ± 0.01 mL
181 sample was added with 10 mL of PBS and incubated at 40 °C for 15 min. Afterwards the sample
182 was centrifuged at 3000 x g for 15 min and stored at -20 °C.

183 *2.9. In-house validation of sandwich ELISA test to β -conglycinin*

184 A prototype of the sandwich ELISA was prepared under industrial conditions. The prototype was
185 validated following standardized procedures established by the Association of Official Analytical
186 Chemists (AOAC) (Abbot et al., 2010; AOAC 2016 a, b) and the validation guidance of
187 EURACHEM (Magnusson, & Örnemark, 2014).

188 The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were calculated as the
189 mean concentration of β -conglycinin from ten replicates of the zero standard plus 3 and 10 times
190 the standard deviation (SD), respectively.

191 Recovery was performed to determine the effect of matrix and processing on β -conglycinin
192 quantification. Recovery due to matrix effect was estimated as the ratio of β -conglycinin
193 concentration obtained in non-containing soy model foods and extraction buffer, both spiked with
194 the native protein at various levels (0.03, 0.1 and 0.15 $\mu\text{g/g}$). Recovery due to heat processing was
195 estimated as the ratio of β -conglycinin obtained in model foods containing 0.05% soy before and
196 after heat treatment.

197 Repeatability was estimated analyzing ten replicates of the same extract in one run. Intra-assay
198 reproducibility was estimated analyzing ten extracts of the same sample in one experiment. Inter-
199 assay reproducibility was determined assaying three extracts of the same sample in three different
200 days.

201 Robustness was determined applying small deliberate changes to the normal conditions in a
202 single experiment (Supplementary Table 1). A Youden matrix was designed, which makes use of a
203 fractional factorial design. The standard deviation of the differences (SDi) was calculated as
204 previously described (Karageorgou & Samanidou, 2014).

205

206 **3. Results and discussion**

207 *3.1. β -Conglycinin isolation*

208 Figure 1a shows the electrophoretic profile of soy protein extract and purified β -conglycinin. The
209 pure protein is composed by α , α' and β subunits with a degree of purity higher than 96% as
210 determined by densitometry.

211 Other methods have been widely applied in industry to isolate β -conglycinin, such as pH
212 adjustment and ultrafiltration membrane separation (Wu, Murphy, Johnson, Reuber, & Fratzke,
213 2000) and phytic acid enzyme degradation (Saito, Kohno, Tsumur, Kugimiya, & Kito, 2001).
214 However, the purity of β -conglycinin obtained with these methods is too low for immunoassay
215 applications. In this study β -conglycinin purified by ionic precipitation, salting-out and gel filtration
216 yielded protein purity good enough for the ELISA development.

217 *3.2. Titer and specificity of anti- β -conglycinin antisera*

218 An indirect non-competitive ELISA was used to determine the titer of anti- β -conglycinin
219 antisera, which gave values ranging between 1.10^5 and 5.10^5 depending on the bleeding.

220 The immunoreactivity of anti- β -conglycinin antisera was determined by Western-blotting (Fig.
221 1b). Antibodies mainly recognized α , α' and β subunits of β -conglycinin in samples of purified
222 protein as well as soy protein extract.

223 Moreover, standards of β -conglycinin and glycinin, the two major soy proteins, were assayed in
224 a concentration range of 10 and 3000 ng/mL by an indirect competitive ELISA using anti- β -
225 conglycinin antisera. The cross reactivity was calculated as the concentration of glycinin required to
226 produce 50% inhibition of antibody binding compared to β -conglycinin, this value being less than
227 3% (results not shown). These results indicate that antisera exhibited high specificity to detect β -
228 conglycinin (You et al., 2008).

229 3.3. Development and optimization of ELISA techniques to determine β -conglycinin

230 Calibration curves for the determination of β -conglycinin using the ELISA formats are shown in
231 Fig. 2. For the sandwich format, the best fit was obtained when representing the absorbance versus
232 the concentration of standards between 2 and 15 ng/mL which was adjusted to a polynomial curve.
233 For the competitive format, the best fit was obtained when plotting the ratio between absorbance of
234 the standards and the blank buffer (B/B_0) versus the logarithmic concentration of standards, being
235 linear between 10 and 3000 ng/mL. All assays gave coefficients of regression $r^2 \geq 0.985$. The β -
236 conglycinin concentration in food samples was calculated using the calibration curve of each assay.

237 The detection limit (LOD) of ELISA was 0.90 ng/mL for the sandwich and 30 ng/mL for the
238 indirect competitive format, respectively. The quantification limit (LOQ) was 2.1 ng/mL and 70
239 ng/mL, respectively.

240 The LOD of the developed sandwich ELISA test was similar and of the competitive format
241 higher than those previously reported for β -conglycinin. Thus, Hei et al (2012) reported LOD
242 values of 1.63 ng/mL for a sandwich format and 6.4 ng/mL for a competitive format, Moriyama et
243 al. (2005) of 2.0 ng/mL for a competitive format and You et al. (2008) of 5 ng/mL for a direct
244 ELISA. The lower LOD obtained for the sandwich compared to the competitive format is in
245 accordance with results reported when comparing both formats to quantify other food allergens, like

246 β -lactoglobulin, ovomucoid, glycinin and Ara h 1 protein (de Luis et al., 2008; Montserrat et al.,
247 2015; Segura-Gil et al., 2018).

248 3.4. Study of cross-reactivity

249 The specificity of the ELISA formats was assessed by testing undiluted extracts of 39 food
250 commodities including other legumes and nuts, and several ubiquitous food ingredients such as egg,
251 meat, fish and milk among others, as recommended by the AOAC for the validation of ELISA
252 methods (Abbot et al., 2012). In order to express results in μg of β -conglycinin/g food and
253 considering that 1 g or 1 mL of food was extracted with 10 mL of extraction buffer, values obtained
254 in ng/mL were divided by a factor of 100 (Table 1).

255 For the sandwich format, only the sample of chickpea gave an absorbance equivalent to a β -
256 conglycinin concentration of 0.06 $\mu\text{g/g}$, which is above the quantification limit of the test (0.02
257 $\mu\text{g/g}$). This value was negligible compared to that of soy protein isolates as these require diluting
258 the sample by 10^7 to obtain a similar level of detection.

259 For the indirect competitive ELISA, extracts of Brasil and pecan nut, walnut, chickpea, chicken
260 meat and cocoa gave concentrations of β -conglycinin higher than the LOQ of the test. This fact
261 would lead to false-positive results in food samples containing those commodities as ingredients. To
262 deal with this problem, a cut-off value was calculated above which a sample is considered positive
263 for soy presence. This value was estimated as the mean concentration of food commodities, with the
264 exception of those mentioned above, plus three times its SD, being of 1.0 $\mu\text{g/g}$.

265 The development of ELISA techniques to detect allergenic ingredients in food depends on the
266 selection of adequate antibodies, which should present high selectivity towards the target protein to
267 ensure specificity. It has been reported that antibodies raised against soy proteins might give cross-
268 reactivity with other legumes or nuts due to the homology of the proteins (Cucu, Devreese,
269 Kerkaert, & Meulenaer, 2012). In this study, insignificant reactivity was observed only with

270 chickpea proteins when using the sandwich format whereas considerable reactivity with several
271 foods were observed when using the competitive format.

272 Antiserum to β -conglycinin was used to develop the competitive format and immunoaffinity
273 purified antibodies from the same antiserum to develop the sandwich format. Our results suggest
274 that the purification process allows the selection of antibodies which show a high selectivity for the
275 target protein. Furthermore, the higher specificity of the sandwich format could be attributed to the
276 requirement of two primary antibodies reacting with different epitopes of β -conglycinin, whereas in
277 the competitive format only one primary antibody must reacts with the target protein. The lower
278 specificity of the competitive format could be due in part to the matrix effect by the presence of
279 interfering compounds in some food commodities.

280 The reactivity of cocoa in ELISA assays has already been reported by other authors and was
281 attributed to interferences produced by polyphenols, and it could be reduced by adding to the
282 extraction buffer some blocking agents like gelatine or milk powder (Poms , Klein, & Anklam,
283 2004).

284 *3.5. Analysis of incurred model foods*

285 Sandwich and competitive ELISA formats have been developed to determine allergenic proteins
286 but only a few studies have been carried out to compare their performance to detect allergens in
287 foods (de Luis et al., 2008; Montserrat et al., 2015; Segura-Gil et al., 2018).

288 Furthermore, the use of incurred foods in which allergenic ingredients are incorporated into the
289 formulation of various representative food matrices and then, processed in a similar manner that in
290 food industry, allows determining the effect of processing on the target protein and thus, it could
291 reveal some of the limitations of ELISA formats to detect allergens (Cucu et al., 2013; Taylor,
292 Nordlee, Niemann, & Lambretch., 2009). The Food Allergy Research and Resource Program
293 (FARRP) strongly recommends the use of the so-called incurred samples in the assessment of
294 ELISA tests for the detection of allergenic proteins in processed foods.

295 In previous studies to quantify β -conglycinin, indirect competitive, indirect sandwich and direct
296 ELISA techniques were developed and used to determine this protein in soy from different sources
297 and soybean products such as meal, protein isolates and concentrates, extruded and fermented
298 soybean, among others (Hei et al., 2012; Moriyama et al., 2005; You et al., 2008). However, from
299 our knowledge, the performance of competitive and sandwich ELISA formats using antibodies
300 against β -conglycinin to evaluate incurred model foods subjected to heat treatments has not been
301 previously reported.

302 When analyzing incurred model foods (sausage, bread and pâté) using competitive and sandwich
303 formats we observed that the blank food samples gave β -conglycinin concentration values below
304 the LOQ of the sandwich format and below the cut-off value established for the competitive format,
305 indicating that no false-positives were found (Fig. 3).

306 For the sandwich format, samples were found to be positive at percentages of incurred soy
307 proteins of 0.005% and 0.05% for sausages and bread, respectively (Fig. 3a) whereas for the
308 competitive format, samples were positive at higher percentages of soy protein, 0.1% and 0.5% for
309 the same model foods, respectively (Fig. 3b). For pâté samples, both ELISA formats failed to detect
310 β -conglycinin even at 1% of added soy. These results are in accordance with those we previously
311 obtained to determine glycinin by a sandwich ELISA (Segura-Gil et al., 2018), in spite of glycinin
312 is more thermostable than β -conglycinin (Hermansson, 1986).

313 Our results are also in agreement with those reported for the detection of β -lactoglobulin or
314 glycinin in model processed foods in which the sandwich format resulted in an assay with better
315 sensitivity and specificity than the competitive format (de Luis et al., 2008; Segura-Gil et al., 2018).

316 Results derived from our study indicate that thermal processing has a great influence on the
317 determination of β -conglycinin. These results are in line with those obtained by Hei et al. (2012) in
318 which β -conglycinin determined in soybean products like soybean meal, extruded soybean meal
319 and soybean protein concentrate showed much lower concentrations than in unprocessed soy seeds.

320 In order to know the effect of processing on the decrease of β -conglycinin immunoreactivity,
321 samples incurred with 0.05% soy proteins were analyzed in raw and processed foods. The
322 concentration of β -conglycinin in sausage subjected to pasteurization treatment was similar to that
323 of raw sausage ($109.0 \pm 4.2\%$) whereas in baked bread decreased to $37.9 \pm 0.6\%$ and it could not be
324 detected in pâté subjected to sterilization treatment. When comparing with the data reported for
325 glycinin sandwich ELISA, a similar recovery value was reported for sausage ($106.0 \pm 4.1\%$),
326 whereas a higher recovery value was obtained for bread ($54.5 \pm 0.5\%$) and glycinin could not be
327 detected in pâté (Segura-Gil et al., 2018).

328 The interest on the effect of thermal treatment on the detection of allergenic proteins has
329 increased in the last few years. However, only a few studies have investigated the effect of
330 processing on soy allergen quantitation in complex food matrices. At this respect, Cucu et al. (2012)
331 using an indirect competitive ELISA test to denatured soy proteins (treated at $70\text{ }^{\circ}\text{C}$ for 48 h),
332 obtained recoveries from 1.5% to 7% in cookies incurred with a soybean protein extract prepared
333 with sucrose as ingredient and baked at $205\text{ }^{\circ}\text{C}$ for 10 min. When lactose was added to the dough,
334 an increase in the recovery of soybean proteins up to 24% was observed, beside a more brown color
335 of cookies as a result of the ongoing Maillard reaction. In the same study, using an ELISA to the
336 native Kunitz trypsin inhibitor (KTI), recoveries between 15% and 31% were obtained when
337 cookies were prepared with sucrose whereas KTI could not be detected when lactose was added to
338 the cookies. These findings were attributed to the modifications taking place on the target proteins,
339 which produced an enhanced interaction of soy proteins with antibodies raised to modified soy
340 proteins but a lower binding of native KTI with its specific antibodies raised to the native form of
341 the protein. Gomaa & Boye (2013) found that the baking time and the cooking size had a great
342 effect on recoveries of soy proteins in cookies baked at $177\text{ }^{\circ}\text{C}$ or 10, 15 and 25 min determined by
343 using two commercial ELISA tests, recoveries ranging between 0% and 33%.

344

345 In contrast, Morishita et al. (2008) using a sandwich ELISA to soy p34 protein (Gly m Bd 30k)
346 found recoveries ranging between 87.7 and 98.7 in five kind of model processed foods spiked with
347 defatted soybean protein and processed at 80 °C for 20 min, 100 °C for 10 min or 121 °C for 10 min.
348 However, these treatments are of much lower intensity than the baking and sterilization treatments
349 applied in our study.

350 The low recovery of soy proteins obtained after food processing in this and other studies is
351 probably due to denaturation of the target protein, which leads to modification of the
352 conformational or/and lineal epitopes recognized by antibodies, and also to aggregation which
353 decreases the solubility of the target protein and mask the epitopes that interact with antibodies.

354 Therefore, the choice of the target protein to be used in immunoassays has a great relevance. It
355 seems logical that soy proteins that are abundant in the seed should be selected as target proteins as
356 proteins that are less abundantly present will be more difficult to detect and consequently the
357 detection limit will be high. On the other hand, the chosen protein should be stable during food
358 processing or otherwise false-negative results might occur.

359 *3.6. Analysis of spiked foods*

360 As the intended use of the sandwich ELISA is to detect soy in bovine milk and cookies, UHT
361 milk and cookies spiked with soy, prepared as indicated above, were analyzed. Results of spiked
362 samples showed that β -conglycinin could be detected in UHT milk added with 0.001% of soy
363 drinks and in cookie added with a final concentration of 0.01% soy seeds (Fig. 4).

364 *3.7. Survey of retail foods*

365 Forty five retail foods purchased from local stores were analyzed using the sandwich format
366 (Fig. 5). Foods were classified in three groups depending on the label indications: samples with soy
367 protein or derivatives included or not in the list of ingredients and samples with the precautionary
368 labelling “may contain traces of soy. Three levels of concentration of β -conglycinin were
369 established for each group.

370 Samples were considered positive if β -conglycinin concentration was higher than the LOQ value
371 of the test. β -Conglycinin was not detected in the samples not labelled with soy ($n = 16$). Three of
372 the 9 foods (33%) with “may contain traces of soy” on the label, contained β -conglycinin in the
373 range between 0.02 and 0.1 $\mu\text{g/g}$. Twelve of 18 foods (67%) declaring soy proteins or derivatives
374 on label contained more than 0.02 $\mu\text{g/g}$ of β -conglycinin. However, β -conglycinin could not be
375 detected in 6 of those samples (33%).

376 Results of the survey suggest that a correct labelling has been applied to those products in which
377 soy is not included in the ingredient list. These results are in contrast to those previously reported to
378 address the frequency of soy allergens in bakery and snack products with no soy disclosed on the
379 label (Khuda et al., 2016). In that study, it was found that a significant number of those products
380 (25% and 11%, respectively) were positive for soy protein, and this fact was attributed to the co-
381 mingling of soy in wheat ingredients used in many bakery and snack supermarket products. Respect
382 to the foods with a soy precautionary statement, results of our study are in good agreement with
383 those indicated by Khuda et al. (2016), who found that 19% of bakery samples and 11% of snack
384 samples with a precautionary statement contained soy proteins. Foods with warning messages
385 though with no detectable β -conglycinin were probably labelled because cross-contamination could
386 not be excluded.

387 The declared presence of soy proteins was not confirmed in samples of potato snacks, canned
388 meatballs, pâtè, pizza, soysprouts and refined soy oil. These results are in accordance with those
389 reported in other studies in which soy proteins in foods with soy declared on the label were not
390 detected (Pedersen et al., 2008; Segura-Gil et al., 2018). These false-negative results could be due to
391 the amount of β -conglycinin present is beyond the limit of detection of the test or to the complete
392 absence of soy protein. The possibility that β -conglycinin could not be detected due to denaturation
393 by heat processing applied should be also considered. The failure to detect β -conglycinin in
394 soysprouts could due to the degradation of storage proteins that takes place during the germination
395 by proteases present in seed (Kim, Choi, Ryu, Lee, & Kwon, 2011). In the case of refined soy oil,

396 the absence of β -conglycinin was expected since proteins have not been detected in this type of oils
397 (Martín-Hernández, Benet & Obert, 2008).

398 3.8. *In-house validation of the sandwich ELISA*

399 The sandwich ELISA test was produced under industrial conditions to obtain a prototype that
400 was validated to determine recovery, precision and robustness. Recoveries obtained in the analysis
401 of the three blank foods (sausage, bread and pâté) spiked with three different β -conglycinin amounts
402 before the extraction are shown in Table 2. Values correspond to the ratio between β -conglycinin
403 determined in spiked blank model foods respect to the buffer spiked at the same levels of protein.
404 Results showed that recoveries were independent from the type of matrix in all samples, ranging
405 between 111.7% and 119.2% for sausage, between 130.3% and 138.7% for bread and between
406 93.3% and 119.9% for pâté.

407 Precision of the test was determined using model sausage incurred with 0.01% and 0.05% of soy
408 proteins, cow's milk spiked with 0.0025% of soy drink and cookie with 0.05% of soy seeds. Results
409 obtained are summarized in Table 3. Repeatability resulted in variation coefficients ranging
410 between 8.7% and 10.9%, the intra-assay reproducibility between 4.9% and 14.9% and the inter-
411 assay precision between 9.6% and 11.5%. Values of repeatability and reproducibility are within the
412 limits recommended by the AOAC for food allergens (AOAC, 2016a).

413 To determine the robustness of the sandwich ELISA, sausages incurred with 0.01% and 0.05%
414 soy proteins were assayed. We performed experiments in which parameter conditions were slightly
415 changed to determine their impact on the results. Therefore, the comparison of the average values of
416 the capital letters with the average values of their corresponding small letters allows evaluating the
417 effect of a certain factor (Supplementary Table 2). When the value of the standard deviation of the
418 differences SDi were calculated, we obtained values of 0.007918 and 0.005041, for sausages with
419 0.01% and 0.05% of added soy proteins, respectively. These values are significantly smaller than

420 the standard deviation of the inter-assay reproducibility (0.008805 and 0.006941, respectively),
421 suggesting that the sandwich test is robust (Karageorgou & Samanidou, 2014).

422

423 **4. Conclusions**

424 In this study, the performance of two ELISA formats to determine β -conglycinin was evaluated
425 using spiked and incurred model foods with added soy proteins. The sandwich format resulted more
426 sensitive and specific than the competitive format. Moreover, the sandwich format was able to
427 detect much lower percentages of soy than the competitive assay in sausage and bread samples
428 subjected to pasteurization and baking processing, respectively. However, any of the assays could
429 detect soy added in sterilized pâté, which was subjected to severe heating conditions. In addition,
430 the sandwich ELISA could differentiate retail foods with declared or not soy on the label, although
431 β -conglycinin could not be detected in some products subjected to severe heat treatment or in
432 soysprouts. The recoveries of β -conglycinin in model food spiked after processing determined by
433 the sandwich ELISA ranged between 93.3% and 138.7%. However, results obtained in model food
434 spiked before processing indicate that thermal treatments decreases detectability and leads to
435 underestimate the amount of β -conglycinin present in foods. The particularities of each ELISA
436 format have a considerable influence on the determination of β -conglycinin in food, as it has been
437 demonstrated in this study. These aspects should be considered when ELISA tests are going to be
438 applied in risk assessment of potential exposure to food allergens as low estimation of thermal
439 processed allergens do not necessarily implies a decreased allergenicity.

440

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- 556

557 **Figure legends**

558 Fig. 1. SDS-PAGE in 4-20% acrylamide gel (a) and Western-blotting against rabbit antiserum to β -
559 conglycinin (b). MW, molecular weight marker. Lane 1, Raw soy extract. Lane 2, purified β -
560 conglycinin.

561

562 Fig. 2. Calibration curves obtained for the determination of β -conglycinin by sandwich (a) and
563 indirect competitive (b) ELISA formats. Standards were prepared with purified β -conglycinin. B
564 and B_0 correspond to the absorbance of the standards and the blank buffer, respectively. Each data
565 point represents the mean of ten measurements of the absorbance at 450 nm.

566

567 Fig. 3. Concentration of immunoreactive β -conglycinin in model foods (sausage, \blacksquare ; bread, \square and
568 pâté, \square) incurred with different percentages of soy protein isolate and determined by sandwich (a) or
569 indirect competitive (b) ELISA. Values are the mean \pm SD of two sample extractions analyzed in at
570 least three assays and are expressed in μg β -conglycinin/g food. The discontinuous lines correspond
571 to the LOQ of the sandwich format and the cut-off of the competitive format, and the continuous
572 line to the upper limit of quantification of the sandwich format.

573

574 Fig. 4. Concentration of immunoreactive β -conglycinin in UHT milk spiked with soy drink (a) and
575 cookies spiked with cookies containing soy seeds (b), determined by sandwich ELISA. Values are
576 the mean \pm SD of two sample extractions analyzed in at least three assays and are expressed in μg β -
577 conglycinin/g food. The lines correspond to the LOQ of the sandwich test.

578

579 Fig. 5. Survey of commercial foods analyzed by sandwich ELISA. Foods were classified into three
580 groups according to their labelling: foods with soy or soy derivatives declared or not on label and
581 foods with the precautionary label “may contain traces of soy”. Each group was divided in three β -
582 conglycinin concentration ranges: <0.02 $\mu\text{g/g}$ (\blacksquare), 0.02 - 0.1 $\mu\text{g/g}$ (\square) and >0.1 $\mu\text{g/g}$ (\square).

Table 1. Study of cross-reactivity using food commodities, analyzed by sandwich and competitive ELISAs to determine β -conglycinin. Results correspond to the average concentration of β -conglycinin ($\mu\text{g/g}$ food) from two different extractions analyzed in three assays.

	β -conglycinin ($\mu\text{g/g}$)	
	Sandwich	Competitive
Cashew	<LOQ	<LOQ
Hazelnut	<LOQ	<LOQ
Macadamian nut	<LOQ	<LOQ
Walnut	<LOQ	0.92
Brazil nut	<LOQ	1.05
Pecan nut	<LOQ	2.67
Pine kernel	<LOQ	<LOQ
Peanut	<LOQ	<LOQ
Beans	<LOQ	<LOQ
White beans	<LOQ	<LOQ
Lupine	<LOQ	<LOQ
Red beans	<LOQ	<LOQ
Pea	<LOQ	<LOQ
Chickpea	0.06	0.83
Lentils	<LOQ	<LOQ
Sesame seed	<LOQ	<LOQ
Linseed	<LOQ	<LOQ
Poppy seed	<LOQ	<LOQ
Pumpkin seed	<LOQ	<LOQ
Sunflower seed	<LOQ	<LOQ
Chicken meat	<LOQ	0.95
Beef meat	<LOQ	<LOQ
Pork meat	<LOQ	<LOQ
Buckwheat	<LOQ	<LOQ
Wheat	<LOQ	<LOQ
Corn	<LOQ	<LOQ
Rice	<LOQ	<LOQ
Barley	<LOQ	<LOQ
Rye	<LOQ	<LOQ
Oat	<LOQ	<LOQ
Crustacean	<LOQ	<LOQ
Hake	<LOQ	<LOQ
Tuna	<LOQ	<LOQ
Carrot	<LOQ	<LOQ
Coconut	<LOQ	<LOQ
Kiwi	<LOQ	<LOQ
Milk	<LOQ	<LOQ
Egg	<LOQ	<LOQ
Cocoa	<LOQ	1.95

Table 2. Recovery of β -conglycinin in spiked blank model food by the sandwich ELISA prototype. Values are the mean of two sample extractions analyzed in at least two assays.

Spiked level ($\mu\text{g/g}$)	Sausage		Bread		Pâté	
	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
0.03	111.7	4.4	130.3	1.7	100.0	4.8
0.10	116.4	3.5	138.7	7.5	119.9	3.5
0.15	119.2	13.3	136.5	8.0	93.3	20.1

Table 3. Results of the precision study performed with the sandwich ELISA prototype for the determination of β -conglycinin in soy incurred sausage and in soy spiked milk and cookie.

	Sausage 0.01%		Sausage 0.05%		Milk 0.0025%		Cookie 0.05%	
	Mean ($\mu\text{g/g}$)	CV (%)	Mean ($\mu\text{g/g}$)	CV (%)	Mean ($\mu\text{g/g}$)	CV (%)	Mean ($\mu\text{g/g}$)	CV (%)
Repeatability	0.053	10.9	0.14	9.7	0.091	8.7	0.048	13.6
Reproducibility intra-assay	0.045	14.9	0.15	4.9	0.135	8.4	0.070	10.0
Reproducibility inter-assay	0.050	11.5	0.148	10.6	0.096	9.6	0.083	16.7
Day 1	0.050		0.13		0.11		0.098	
Day 2	0.045		0.15		0.089		0.065	
Day 3	0.057		0.16		0.091		0.086	

Fig. 1

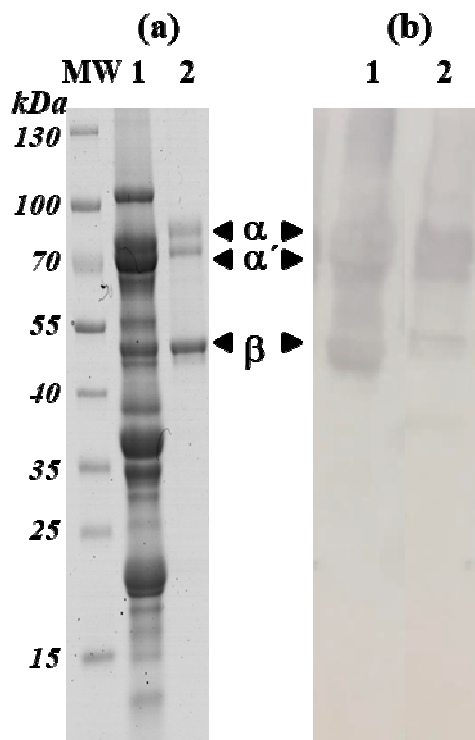


Fig. 2

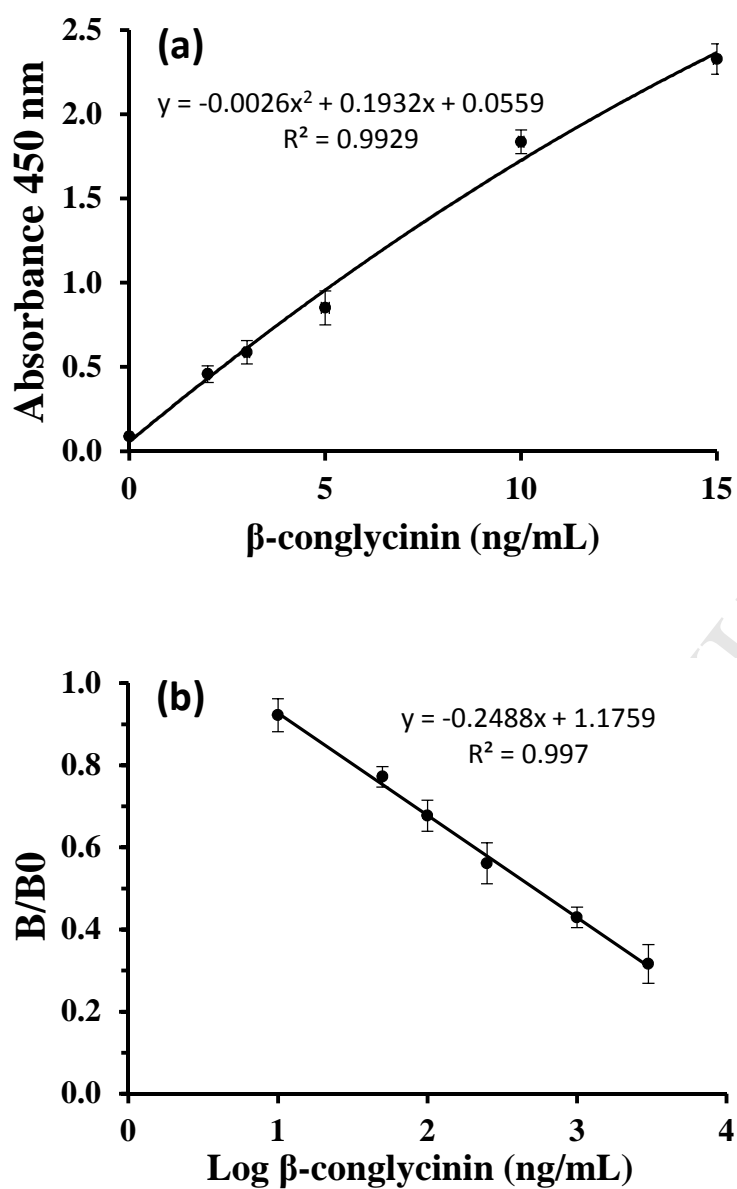


Fig. 3

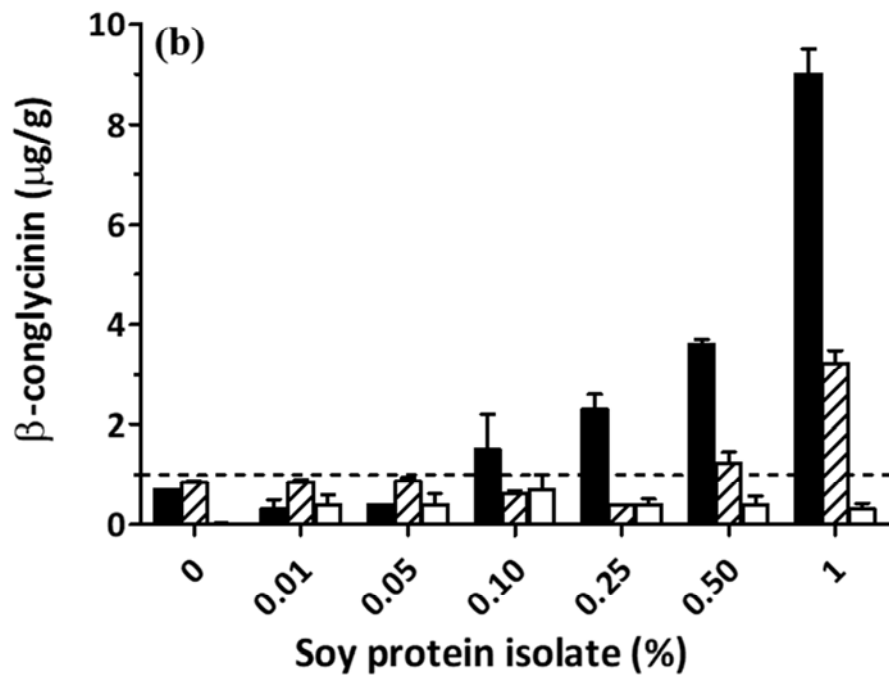
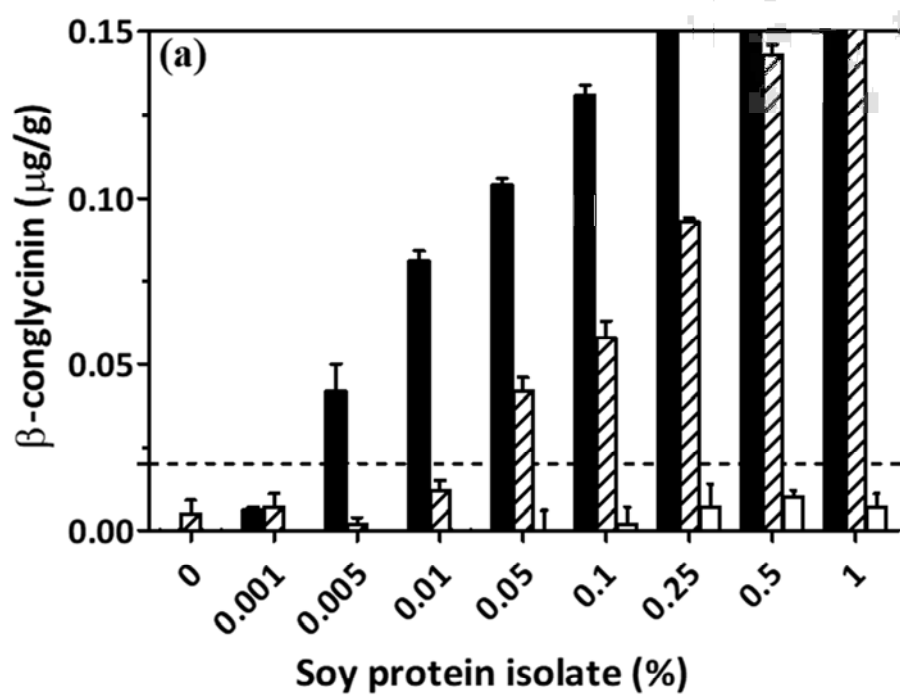


Fig. 4

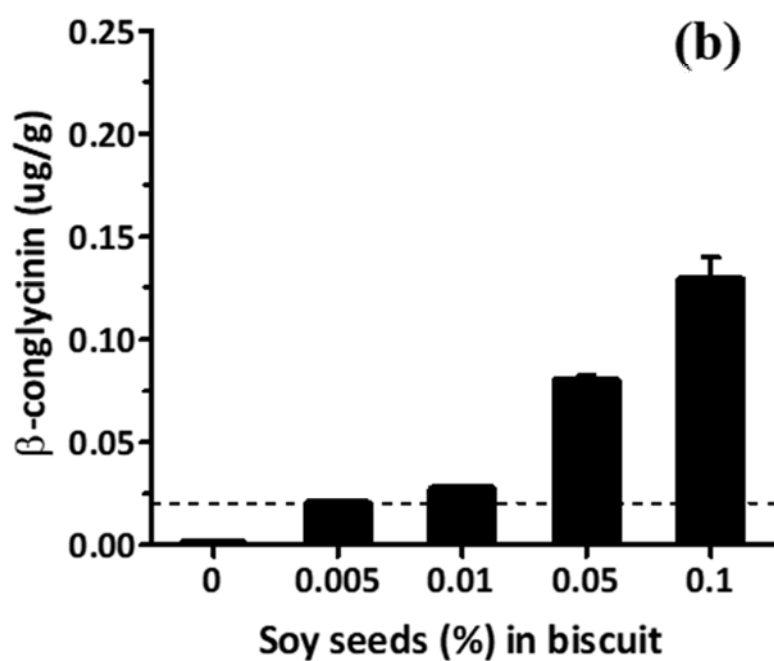
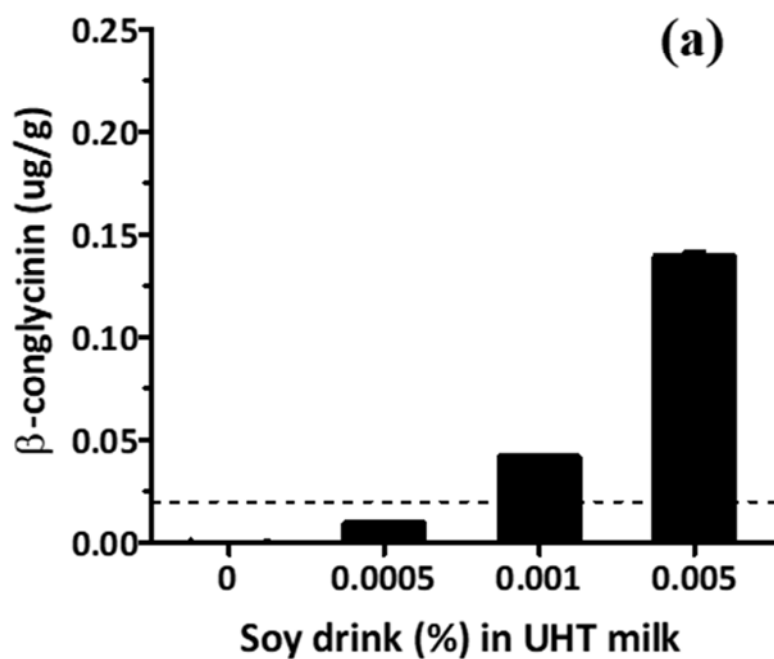
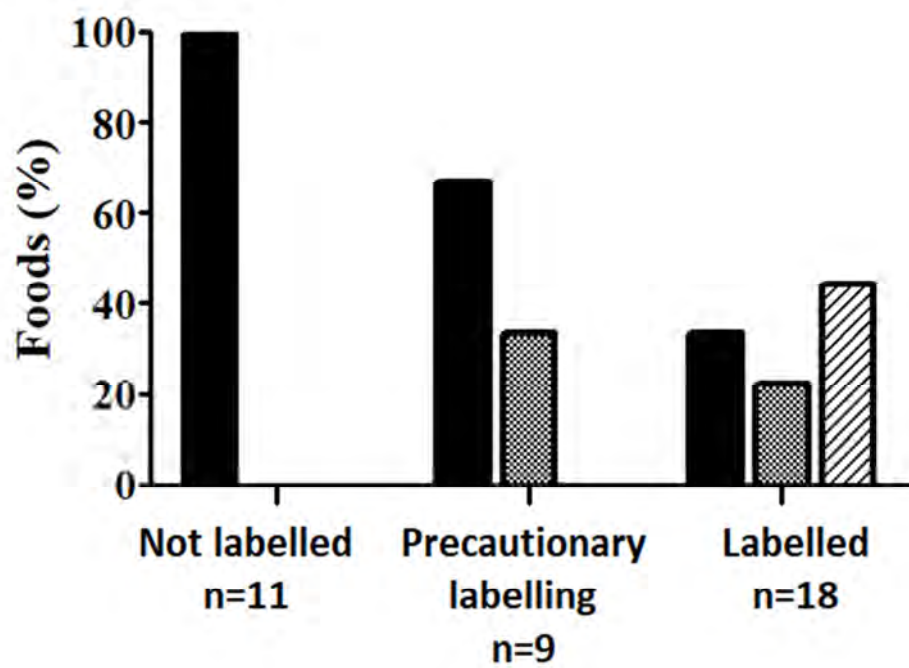


Fig. 5



Highlights

Competitive and sandwich ELISA for β -conglycinin were developed to detect soy.
The ELISA format influences the detection of soy in model processed food.
Sandwich ELISA showed higher sensitivity and specificity than competitive format.
Heat processing influences the recovery of β -conglycinin by ELISA.
The sandwich ELISA test gives reproducible results and is robust.