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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 pâtè 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

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1. Introduction

45

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

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

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

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

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

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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 ºC. After centrifugation at 9000 x g

108   for 30 min at 4 ºC, 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   ºC 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 ºC 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.

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2.2. SDS-PAGE and Western-blotting

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

2.3. Preparation and conjugation of antibodies

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

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

2.4. Indirect competitive ELISA

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

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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 µg/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 ºC for 12 min, bread dough was baked at 160 °C for 50 min, and

167   pâté was sterilised in an autoclave at 120 °C for 1 h (reaching internal temperatures of 90 ºC, 97 ºC

168   and 117 ºC, respectively).

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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 ºC for 15 min. Afterwards the sample

182 was centrifuged at 3000 x g for 15 min and stored at -20 ºC.

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 µg/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.

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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.

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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

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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 μg/g, which is above the quantification limit of the test (0.02

257 μg/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 μg/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

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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.

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

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

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

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

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

13

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 °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 ℃ 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 ℃ 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 ℃ for 20 min, 100 ℃ for 10 min or 121 ℃ 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 μg/g. Twelve of 18 foods (67%) declaring soy proteins or derivatives

374   on label contained more than 0.02 μg/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,

16

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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18

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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, ■; bread, ▨ and

568 pâté, □) incurred with different percentages of soy protein isolate and determined by sandwich (a) or

569 indirect competitive (b) ELISA. Values are the mean ± 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 ± 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 μg/g (■), 0.02-0.1 μg/g (▨) and >0.1 μg/g (□).

24

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 (μg/g food) from two different extractions analyzed in three assays.

	β-conglycinin (μg/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 (μg/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 (µg/g)	CV (%)	Mean (µg/g)	CV (%)	Mean (µg/g)	CV (%)	Mean (µg/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

(a)

$y = -0.0026x^2 + 0.1932x + 0.0559$
$R^2 = 0.9929$

Absorbance 450 nm

β-conglycinin (ng/mL)

(b)

$y = -0.2488x + 1.1759$
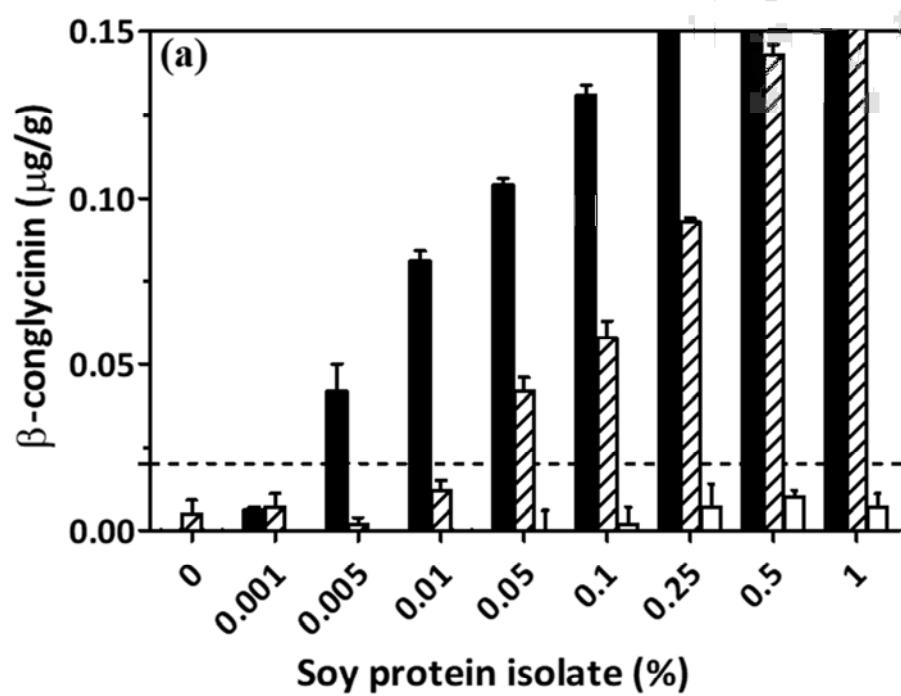$R^2 = 0.997$

B/B0

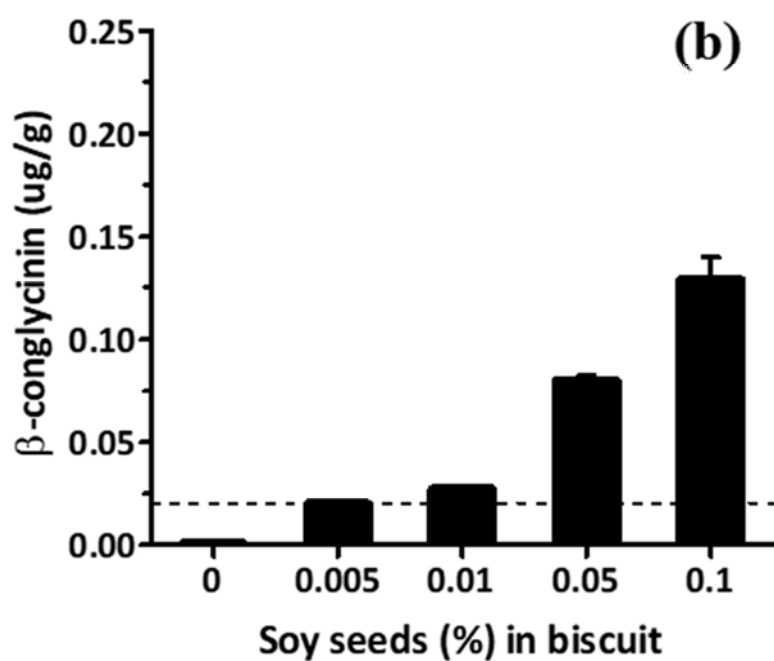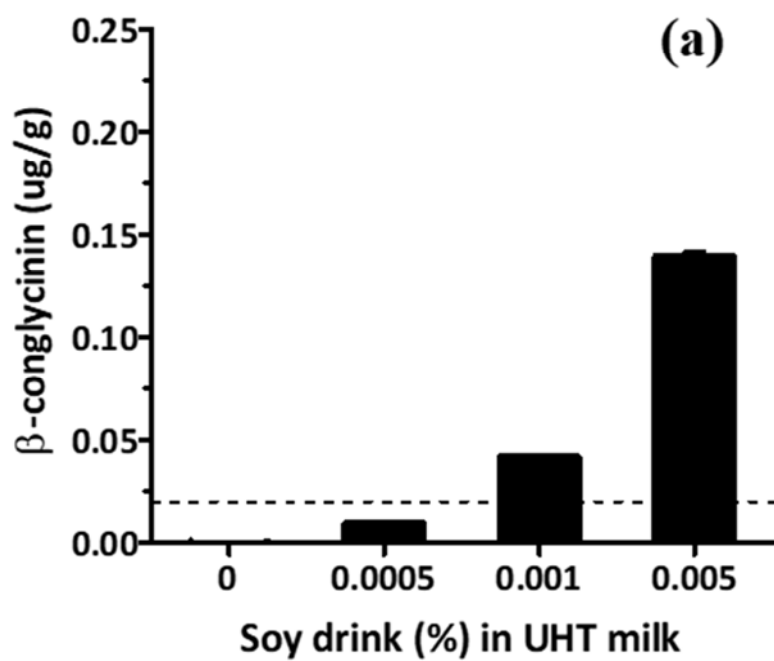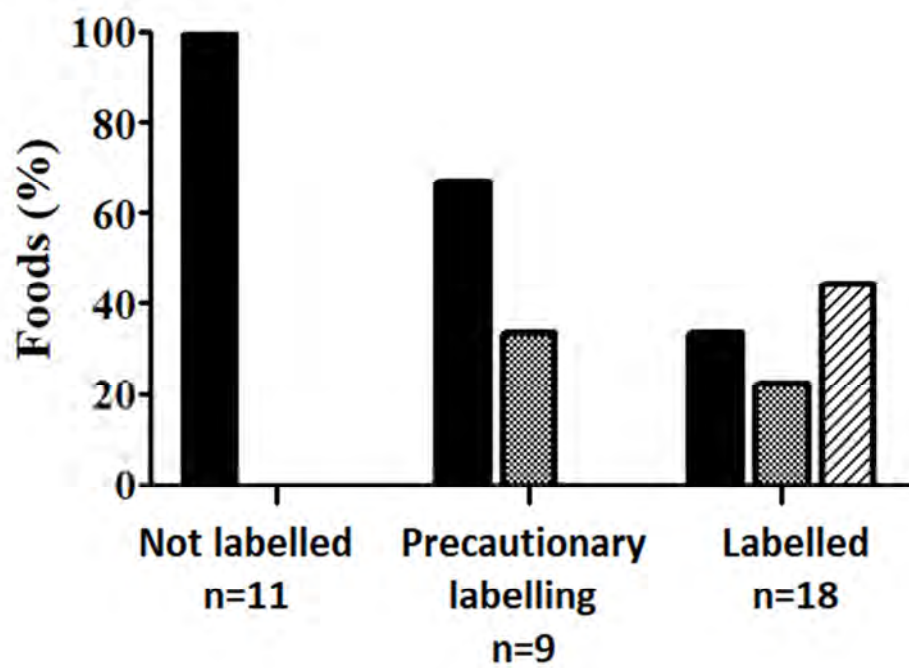Log β-conglycinin (ng/mL)
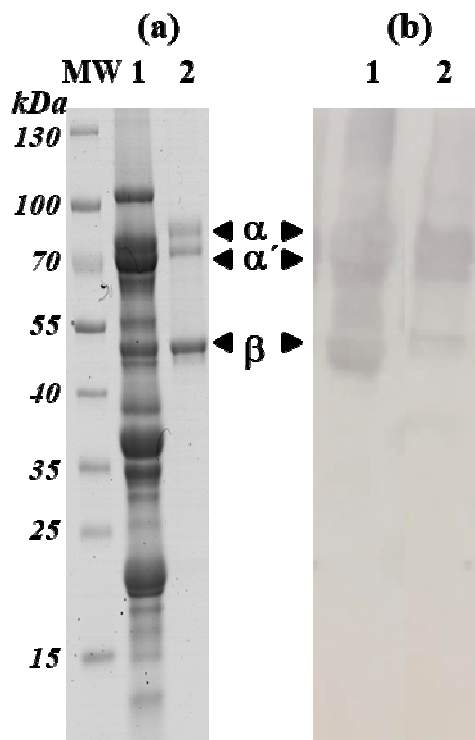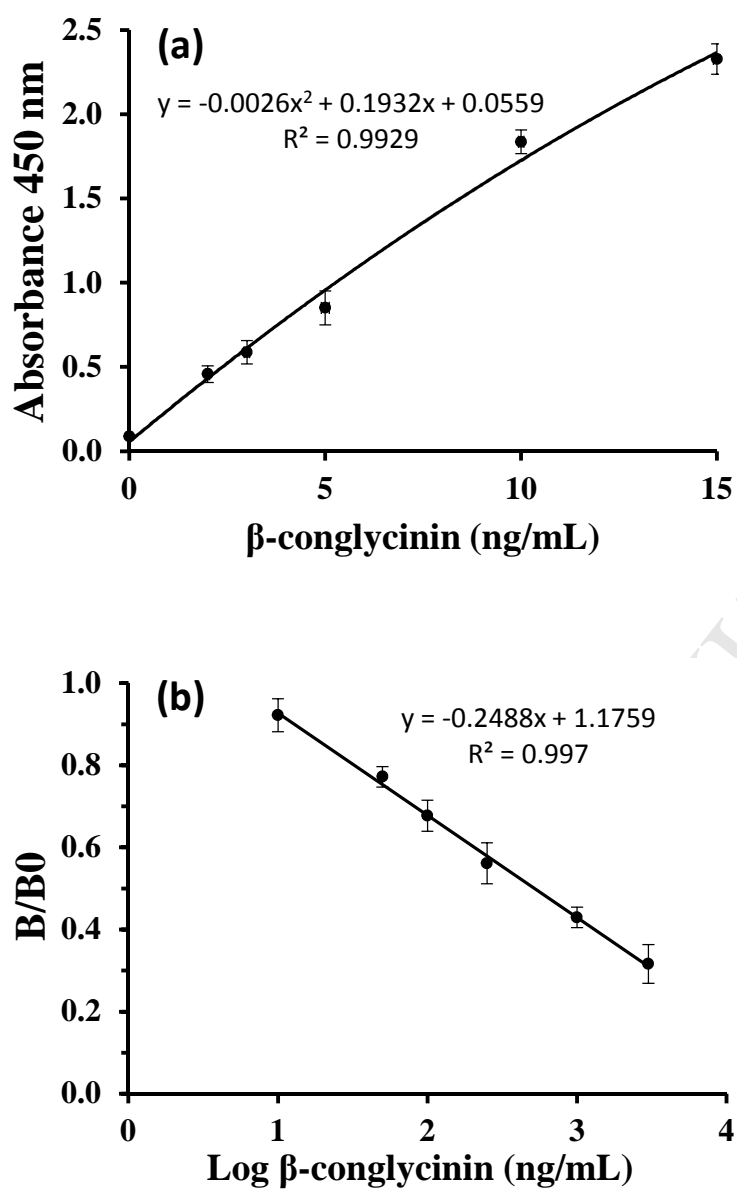
Fig. 3
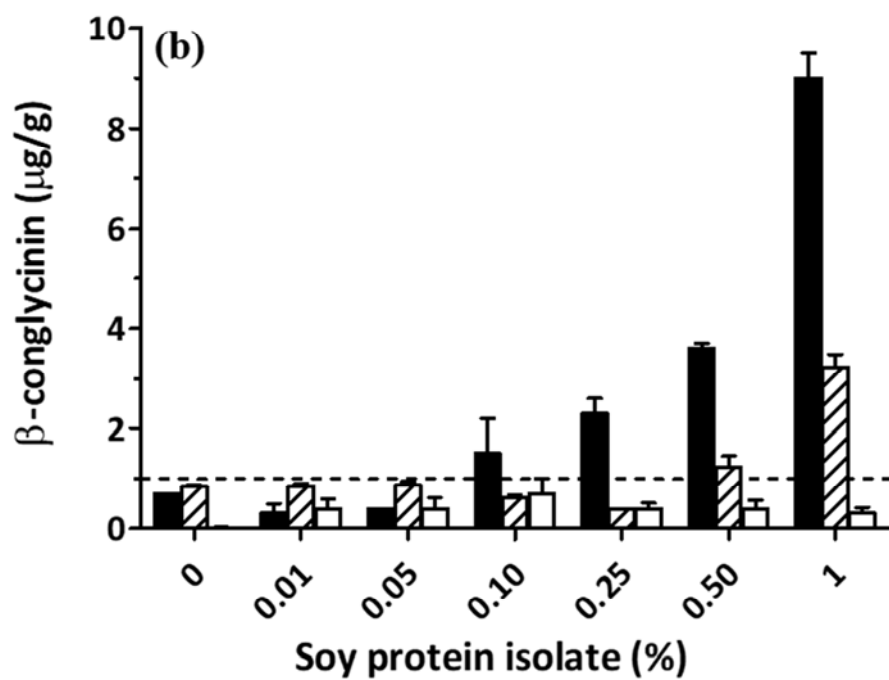
Fig. 4

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.