Detection of peanut (*Arachis hypogaea*) allergens in processed foods by immunoassay: influence of selected target protein and ELISA format applied.

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Abstract

Direct competitive and sandwich ELISA formats developed to determine Ara h1 and Ara h2 proteins were applied in the detection of peanut in model biscuits prepared with a commercial peanut butter as ingredient. The sandwich format for Ara h2 protein could detect the addition of 2.5% peanut butter, whereas the same format for Ara h1 could not detect 5% added peanut. Direct competitive formats for Ara h1 and Ara h2 proteins could detect the presence of 1% and 0.05% peanut butter, respectively. Therefore, competitive format for Ara h2 was selected to be evaluated by four laboratories, obtaining adequate results in term of repeatability and reproducibility. Results obtained indicate that processing decreased the level of extracted protein and underestimated the amount of Ara h1 and Ara h2 proteins, the effect being more severe for Ara h1. The selection of the target protein and the ELISA format applied greatly influence the detection of peanut in processed foods.

Keywords: Ara h1, Ara h2, allergen, peanut detection, processed foods, ELISA assay.

1. Introduction

Food allergy has emerged as a serious public health problem over recent years and its prevalence is rising, especially in industrialized countries. The reason appears to be related to changes in dietary habits as well as to the use of complex technological processes and ingredients in food industry (Nwaru et al., 2014; Sicherer & Sampson, 2010).

The estimated prevalence of peanut allergy in developed countries is between 0.6% and 1.0%. Peanut allergy deserves particular attention because very small amounts of peanut proteins can induce severe allergic reactions, it persists throughout life and it accounts for most of food-induced anaphylactic reactions (Al-Muhsen et al., 2003; Wen et al., 2007)

Until now, thirteen peanut proteins with allergenic capacity have been identified, and designated as Ara h1 to Ara h13 (Bublin & Breiteneder, 2014; Sáiz et al., 2013). Ara h1 and Ara h2 proteins are considered as the major allergens of peanut, more than 65% of peanut allergic individuals have specific IgE to Ara h1 and more than 71% to Ara h2. (Scurlock & Burks, 2004). They are both major proteins in peanut, as they account for 12 to 16% and 5.9 to 9.3% of the total seed protein content, respectively (Koppelman et al., 2001).

Ara h1 is a seed store glycoprotein that belongs to the vicilin family. It has a molecular mass of 63.5 kDa in its monomer form and an isoelectric point of 5.2. It exists as a trimer formed by three identical monomers stabilized mainly by hydrophobic interactions. Ara h2 is a glycoprotein of the conglutinin family with a molecular mass of 17.5 kDa and an isoelectric point of 4.6 (Wen et al., 2007). Both proteins have been found to maintain the IgE binding capacity after being exposed to thermal treatments or *in vitro* digestion with pepsin, chymotrypsin and trypsin (Lehmann et al., 2006; Maleki et al., 2000; Mondoulet et al., 2005).

The way to prevent peanut allergy is the strict avoidance of peanut consumption. However, contamination with hidden allergens can occur due to inefficient cleaning procedures of the production equipment or the use of contaminated raw ingredients, among others (Vierk et al., 2002). The implementation of a management plan in the food industry, the enforcement of labeling rules and its control by authorities are important strategies for protecting against allergic reactions.

Therefore, reliable methods to detect peanut are required to ensure compliance with the labeling legislation and to assist food manufacturers in order to improve consumer protection. Enzyme-linked immunosassay (ELISA) is the technique most widely used by food industries and official food control agencies for monitoring adventitious contamination of food products by allergenic ingredients because of its sensitivity and specificity (Monaci & Visconti, 2010). Several studies have been performed to develop ELISA techniques to detect peanut in foods. These studies include the design of one ELISA format (sandwich or competitive) and are based on the determination of one selected target (a mixture of peanut proteins or a specific peanut protein) (Holzhauser & Vieths, 1999; Kiening et al., 2005; Pomés et al., 2003; Stephan & Vieths, 2004).

It is worthwhile to remark that the determination of peanut proteins in foods can be impaired by their interaction with compounds of the complex food matrix and denaturation during processing. Consequently, protein extraction greatly decreases and protein recognition by antibodies is reduced (Chassaigne et al., 2007; Fu & Maks, 2013; Khuda et al., 2012).

Several recent studies have shown that results obtained by different ELISA tests give significantly varying results in quantitative assays when they are used to detect peanut in processed foods (Khuda et al., 2012; Poms et al., 2005). This variability may be explained by the fact that ELISA tests can use different antigens as targets, antibodies for antigen recognition and assay formats (Fu & Maks, 2013; Khuda et al., 2012; Montserrat et al., 2013; van Hengel et al., 2007).

In this work, four ELISA assays for the detection of peanut, based on the determination of Ara h1 or Ara h2 proteins (sandwich and direct competitive assay for each protein) have been developed. The performance of the four assays was evaluated using biscuits containing defined concentrations of a commercial peanut butter as ingredient. The ELISA format and the target protein that gave the best sensitivity was selected to determine peanut content in model biscuit the samples in blind duplicate by four laboratories. For clarity and explanation, this part of the study is called interlaboratory study, even though it did not involve the minimum number of laboratories requested by a full interlaboratory study as defined in the ISO 5725 standard (ISO, 1994).

2. Materials and methods

2.1. Materials

Raw peanuts and peanut butter from the Spanish variety was provided by Chocolates Lacasa (Utebo, Spain). Peanut butter was prepared by roasting whole peanuts in a flame oven at 225 ºC for 27 min and afterwards, by grinding in a stone mill to obtain an emulsion with dark color. Horseradish peroxidase (HRP, 250-503 units/mg) and goat anti-rabbit IgG antibodies labelled with peroxidase were purchased from Sigma Chemical (Poole, UK). Tetramethylbenzidine (TMB) substrate (Reference ZE/TMB125) was obtained from ZEULAB (Zaragoza, Spain) and Maxisorp microtitration plates from Nunc (Roskilde, Denmark). The bicinchoninic acid (BCA) assay kit was from Pierce (Rockford, IL, USA).

2.2. Methods

2.2.1. Isolation of Ara h1 and Ara h2

Peanut proteins were extracted by stirring 20 g of ground raw peanut with 100 mL of 50 mM Tris-HCl buffer, pH 8.2. Proteins precipitated between 40 and 80% ammonium sulphate saturation was collected by centrifugation, suspended in Tris buffer and filtered. The extract was applied onto a Sephacryl S-200 column (90 x 2 cm). Fractions enriched in Ara h1 were applied onto a Q-Sepharose column (15 x 1.5 cm) as previously described (Montserrat et al., 2013) and fractions enriched in Ara h2 protein onto a Sephadex G-50 column (80 x 1 cm). The purity of isolated proteins, determined by SDS-PAGE was higher than 95%.

2.2.2. Preparation and conjugation of antibodies to Ara h1 and Ara h2

Antisera to Ara h1 and Ara h2 were obtained by immunization of rabbits as previously described (Wehbi et al., 2005). All procedures were approved by the Ethic Committee for Animal Experiments from the University of Zaragoza (Project Licence PI 48/10). The care and use of animals were performed following the Spanish Policy for

Animal Protection RD 1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes. Specificity of antisera against Ara h1 or Ara h2 proteins were assessed by Western blotting analysis (Franco et al., 2010).

Specific antibodies to Ara h1 or Ara h2 were purified by affinity chromatography using immunosorbents of the corresponding proteins as described by Montserrat et al. (2013). Antibodies were conjugated with HRP using the periodate method (Nakane & Kawaoi, 1974).

2.2.3. Sandwich and direct competitive ELISA assays for Ara h1 and Ara h2

For the sandwich ELISA, plates were coated with 120 µL per well of anti-Ara h1 or anti-Ara h2 antibodies (5 µg/mL), in 50 mM sodium carbonate buffer, pH 9.6 overnight at 4 °C. Then, wells were blocked with 300 µL of 2% (w/v) ovalbumin in 8 mM Na_2HPO_4, 3 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4 buffer, pH 7.4 (PBS) for 2 h at 37 °C and washed with PBS containing 0.5% Tween 20 (PBST). Afterwards, 100 µL of Ara h1 and Ara h2 standards or samples diluted in 0.1 M sodium borate buffer, pH 9.0 were added to the wells and incubated for 30 min at 37 °C. Then, wells were incubated with 100 µL of anti-Ara h1 or anti-Ara h2 antibodies HRP-conjugated diluted 1/6,000 and 1/10,000, respectively in the same buffer for 30 min at 37 °C. After washing with PBST, wells were incubated with 100 µL of TMB substrate for 20 min at room temperature. Finally, the enzymatic reaction was stopped by adding 50 µL of 2 M H_2SO_4 per well, and the absorbance determined at 450 nm using a microplate reader (Labsystem Multiskan, Helsinki, Finland).

Calibration curves for the sandwich assay of Ara h1 was obtained by plotting absorbance versus the concentration of standard solutions. For Ara h2, calibration curves were obtained using the relationship between the value of absorbance and the logarithm of the concentration of standard solutions. The concentration of Ara h1 and Ara h2 in the test samples was determined by interpolating absorbance data in the corresponding calibration curves.

For the direct competitive ELISA, plates were coated with 120 µL per well of Ara h1 or Ara h2 proteins (5 µg/mL) in 50 mM sodium carbonate buffer, pH 9.6. After overnight incubation at 4 °C, wells were washed and blocked with ovalbumin as indicated above. After washing with PBST, plates were incubated for 30 min at 37 °C with 50 µL of protein standards or samples diluted in 0.1 M borate buffer, pH 9.0 and 50 µL of HRP-labeled anti-Ara h1 or anti-Ara h2 antibodies diluted 1/30,000 and 1/40,000, respectively in the same buffer. Finally, after washing wells were incubated with TMB substrate and enzymatic reaction stopped with H_2SO_4 before measuring absorbance at 450 nm.

Calibration curves for direct competitive assays were obtained using the logit log model (Nix & Wild, 2000). The fraction bound ($r = B / B_0$), where B is the absorbance of each standard and B_0 the absorbance of the blank standard was calculated. A plot of logit (r) of standards against the $\log_{10}$ of the concentration, where logit (r) = ln [(1-r) / r] was obtained. The concentration of Ara h1 and Ara h2 in tests samples was determined from its fraction bound, which is the ratio between absorbance of the sample and absorbance of the blank standard (B_0).

2.2.4. Preparation of model biscuits

Biscuits were prepared at the pilot plant of the University of Zaragoza following standard manufacturing processes. They were made by mixing 6 hen eggs (55-65 g), 120 g butter, 300 g wheat flour, 150 g sugar and peanut butter to obtain final concentrations of 0, 0.25, 0.5, 1.0, 2.5 and 5.0%, (w/w). The ingredients were kneaded for 30 min using a bread and dough maker (Deluxe: Bread and Dough Maker, Oster, USA) equipped with a blade type "pigtail". Then, 40 g of homogenized material was placed in a baking mould (10 cm diameter) and pressed to obtain round cookies of 1 cm height. Then, biscuits were introduced into an oven and cooked at 160 °C for 12 min.

2.2.5. Extraction procedure

Food samples purchased from local retailers and model biscuits were ground into fine powder with a mincer. An amount of 3.00 ± 0.01 g of ground samples were extracted in 30 mL of 0.1 M sodium borate buffer, pH 9.0 and incubated in a shaking water bath at

30 ºC for 15 min. Extracts were clarified by centrifugation at 3,000 x g for 15 min, and the supernatants stored in aliquots at -20 ºC until use. Supernatants were directly assayed in the ELISA plates.

2.2.6. Evaluation of direct competitive ELISA for Ara h2

The evaluation study was performed following the procedure previously described (Abbot et al., 2010; AOAC, 2012). Four laboratories with ELISA experience participated in this study to evaluate the direct competitive ELISA for Ara h2 protein to detect peanut in model biscuits. The study was coordinated by the group of the University of Zaragoza.

The samples to be sent to the participants were prepared as follows. Biscuits containing 0, 0.25, 0.5, 1.0 and 2.5% peanut were ground and 3.00 ± 0.01 g was weighted into 50 mL plastic tubes. Biscuits with peanut concentrations of 0.01, 0.05 and 0.1% were prepared by mixing appropriate quantities of the ground 0.25% samples with the blank sample into plastic tubes to give a total weight of 3.00 + 0.01 g. Extraction of test samples was performed as indicated above.

The coordinator provided two sets of 8 pre-weighed test samples, randomly coded, and ZEULAB provided the ELISA kits containing plates, reagents, standards and instructions. Each set of samples was extracted once in different days and analyzed in triplicate in the ELISA assay. Absorbance data of calibration standards and blind samples of each set were sent to the coordinator. Calibration curves were obtained for each ELISA assay using the logit log model. Determination of repeatability and reproducibility data were calculated according to ISO 5725.

3. Results

3.1. Specificity of antisera to Ara h1 and Ara h2

The specificity of antisera against Ara h1 and Ara h2 proteins were assessed by Western blotting (Figure 1). Results showed that antibodies to Ara h1 only reacts with Ara h1 and antibodies to Ara h2 only bind to Ara h2. In both cases, no reaction was observed with any other protein from crude peanut extract demonstrating that antisera obtained were specific for each protein.

3.2. *Development of sandwich and direct competitive ELISA for Ara h1 and Ara h2*

Immunoassay formats for Ara h1 and Ara h2 were optimized to choose the assay conditions which gave the highest sensitivity, that were chosen for the validation and the interlaboratory study. The relationship found was linear within the range of concentrations between 20 ng/mL and 2 µg/mL for direct competitive assays and for the sandwich format of Ara h2, and curvilinear between 20 ng/mL and 800 µg/mL for the sandwich format of Ara h1 protein. All assays gave regression coefficients $r^2 \geq 0.985$ (Figure 2). The detection limit (LOD) of the immunoassays tests was determined as the mean concentration of Ara h1 and Ara h2 corresponding to the absorbance of eight replicates of the blank standard plus 3.3 times the standard deviation (Miller et al., 2006) (Table 1).

3.3. Determination of peanut in model biscuits

Results obtained in the analysis of model biscuits which contained different amounts of peanut butter using sandwich and direct competitive assays to determine Ara h1 and Ara h2 proteins are shown in Figure 3. Biscuit samples were extracted in three different days and assayed by triplicate. Previously, a cut-off value was established to consider a sample as positive for peanut addition for each ELISA test. This value was estimated as the average concentration of the blank biscuit plus 3.3 times the value of its standard deviation (Lexmaulová et al., 2013) (Table 1). The assumption of this value ensures that interference caused by the matrix effect in each assay is minimized.

In this study, biscuit samples without added peanut gave a concentration value below the cut-off calculated for each format assay. The sandwich format based on Ara h2 protein could detect the addition of 2.5% peanut, whereas the same format for Ara h1 could not detect samples containing 5.0% peanut. Direct competitive assays for Ara h1 and Ara h2 proteins could detect biscuits samples containing 1.0% and 0.05% of peanut addition, respectively. Biscuit samples which contained a lower percentage of peanut than those indicated above gave false-negative results in the corresponding assays and those which contained higher percentages gave a concentration of Ara h1 and Ara h2 that increased gradually.

On the other hand, the concentration of soluble proteins, estimated by the bicinchoninic acid, and of Ara h1 and Ara h2 was determined in peanut butter and in raw dough of biscuits. The protein concentration in the peanut butter extract was of 8.1 ± 0.4% (w/w) and the concentration of Ara h1 and Ara h2 proteins, estimated using the direct competitive assays was 1,000 ± 20 and 2,750 ± 13 mg/kg, respectively. Samples of raw peanut from the same variety were also analyzed and a protein content of 16.2 ± 0.4% (w/w) and concentrations of Ara h1 and Ara h2 of 20,244 ± 68 and 5,873 ± 87 mg/kg respectively, were obtained. When these proteins were determined in biscuits added with 1.0 and 5.0% peanut butter, the concentration of Ara h1 and Ara h2 was found to be about 1% and 45% of that in the raw dough before the baking treatment.

3.4.Cross-reactivity study

The specificity of anti-Ara h1 and Ara h2 antibodies was also examined by testing its cross-reactivity with other food ingredients such as, tree nuts (almond, cashew nut, pistachio, walnut and hazelnut), legumes (chick pea, soya, green pea and lentil), and ingredients used in the elaboration of biscuits (wheat, milk, egg and sugar). Extracts of all ingredients and peanuts were prepared following the extraction protocol and tested undiluted. Protein concentration of extracts assayed ranged from 0 to 32 mg/kg. All ingredients gave a small decrease (in competitive format) or increase (in sandwich format) of the absorbance value compared to the blank standard indicating a certain degree of interference (results not shown). Concentration values of Ara h1 and Ara h2 determined in these ingredients were below the cut-off established for each ELISA assay to consider a sample as positive for peanut protein.

3.5.Evaluation of direct competitive ELISA for Ara h2

The direct competitive ELISA test to determine Ara h2 protein was evaluated by four laboratories for the detection of peanut in the model biscuits. Concentration of Ara h2 in two set of blind biscuit samples prepared with peanut butter were determined.

Using the standards of Ara h2 indicated in Table 1, calibration curves were obtained for every ELISA plate using the logit log model, obtaining regression coefficients higher than 0.976. The concentration of Ara h2 in test samples was calculated as indicated above. The mean concentration of Ara h2 obtained for each set of samples by each laboratory is shown in Table 2.

The cut-off value for the interlaboratorial study was determined as 3.3 times the reproducibility (S_R) of the blank biscuit (Lexmaulová et al., 2013), obtaining a value of 0.81 mg/Kg.

The four laboratories obtained concentrations of Ara h2 in the blank biscuit samples below the cut-off established for interlaboratory study to consider a sample as positive, indicating that no false-positive samples were found. For all laboratories, Ara h2 was detected in samples with a percentage equal or higher than 0.05% of peanut butter. At 0.01% of peanut addition, the concentration of Ara h2 was below the cut-off with the exception of one laboratory. At higher percentages, concentration of Ara h2 increased for all laboratories. Results and performance characteristics (repeatability and reproducibility data) of the interlaboratory study are summarized in Table 3. Values of repeatibility RSD (RSD_r) ranged between 15.83 and 44.07% and values of reproducibility RSD (RSD_R) between 30.18 and 111.13%.

4. Discussion

The search for the selection of an immunoassay format and a target protein to detect peanut in processed foods led us to develop direct competitive and sandwich ELISA formats to determine Ara h1 and Ara h2 proteins, the two major peanut allergens.

The optimum conditions led to the development of sandwich and direct competitive ELISA tests with sensitivities comparable to those previously obtained for Ara h1 and Ara h2 proteins (Pomés et al., 2003; Schmitt et al., 2004).

Certain degree of interference was observed between Ara h1 and Ara h2 with basic food ingredients when they were analyzed using competitive ELISA tests. The existence of cross-reactivity between Ara h1 and other vicilin storage proteins of legumes such as soya, green pea and beans have been reported (Beardslee et al., 2000; Sicherer et al., 2000). These proteins have some 30-45% of amino acids in common with peanuts and a similar folding. However, homology at surface residues requires a higher degree of amino acid identity (Pomés et al., 2003). In this study, we did not observe a higher level of interference when analyzing legumes compared to other foods. Thus, it is assumed that interference could be produced by non-specific interaction between components of the food matrix and antibodies.

Model biscuits containing several different percentages of peanut butter as ingredient were analyzed using developed ELISA assays. We selected this processed material to prepare biscuits because it is commonly used in the elaboration of nougats, confectionery products, seasoning blends, bakery mixes, frostings, fillings, chocolate, creams and cereal bars. Results obtained indicated that the processing of peanut to obtain butter caused a decrease in the level of extracted proteins of about 50% and a loss of immunoreactive proteins of about 95% and 53% for Ara h1 and Ara h2, respectively.

Our results are in good agreement with those previously reported on the effect of thermal processing of peanut on protein solubility and detectability by ELISA techniques (Chassaigne et al., 2007; Fu & Maks, 2013; Schmitt et al., 2010). Thus, Chassaigne et al. (2007) found that roasting of peanuts under mild or strong conditions decreased extraction efficiency of proteins by 75% and 82%, respectively. In the same study, the concentration of Ara h1 and Ara h2 proteins under mild and strong roasting of peanuts, determined by ELISA kits, were reported to be about 15% and 8% of that of the raw peanut extract for Ara h1 and 59% and 47% for Ara h2, respectively. Fu & Maks (2013) studied the effect of heat treatment of peanut flour on the solubility of proteins and compared the performance of two commercial ELISA test kits targeting whole peanut proteins or Ara h1 for quantitation of residual peanut. They found that dry heating at 232 and 260 ºC for 10 min caused an approximately 49.9% and 85.7% decrease in the amount of proteins extracted, respectively. Likewise, the two ELISA kits underestimated the level of proteins in the samples, the degree of immunoreactivity loss being greater for the kit targeted to Ara h1 than for the kit targeted to whole peanut proteins, about 62.7% and 75.0% at 232 ºC and 98.5% and 99.4% at 260 ºC for kits targeted whole peanut proteins and Ara h1, respectively.

Our study confirms that thermal processing of peanuts decreases solubility of peanut proteins as well as immunoreactivity of Ara h1 and Ara h2 proteins, the effect being more marked for Ara h1. This fact could be attributed to a higher degree of denaturation and/or aggregation of Ara h1 compared to Ara h2, which causes a higher loss of epitopes recognized by antibodies and a higher reduction of its solubility. Our results and those obtained by other authors (Chassaigne et al., 2007; Schmitt et al., 2010) support the previously reported good thermal stability of Ara h2 (Owusu-Apenten, 2002) and suggest that Ara h2 would be a better target than Ara h1 when immunoassays are going to be used for the detection of peanut in processed foods.

Results obtained in the analysis of model biscuits which contained different amounts of peanut butter indicate that direct competitive formats have a higher sensitivity to detect added peanut butter than the sandwich formats. Differences in the recognition of antigen by competitive and sandwich ELISAs could be due to the former requires only one site of interaction with the antibodies whereas the later requires two binding sites. It should be also considered that the way that specific antibodies are presented to its target protein is different depending on the ELISA format. In the sandwich format, capture antibodies are coated on the wells whereas in the competitive format antibodies are in solution and thus, the accessibility of adsorbed antibodies may differ from the antibodies in solution.

Our results are in accordance with those reported by de Luis et al. (2008) using competitive and sandwich ELISA assays based on the determination of ovomucoid to detect egg in model foods. In that study, both formats performed well to detect egg added to pasteurized sausages and baked bread whereas only the competitive format could detect egg in high heat treated foods such as sterilized pâté.

Our results also show that sandwich and direct competitive assays based on the determination of Ara h2 protein are able to detect lower percentages of added peanut compared to their counterparts for Ara h1. These findings can be attributed to a more severe denaturation and/or aggregation for Ara h1 than for Ara h2 induced by the baking process, which result in a lower level of extracted Ara h1 and/or in a lower recognition of this protein by their specific antibodies, as indicated above.

Pomés et al. (2003) developed a sandwich ELISA for Ara h1 to monitor peanut allergen in foods that could detect peanut in cookies and pancake mix spiked with 0.2% of ground peanut. They observed that the recovery of Ara h1 progressively decreased when lower amounts of peanut were added to those foods, obtaining recoveries in biscuits of 86% and 6% at spiked levels of 16% and 0.2%, respectively. This fact indicates that compounds of the matrix impaired recognition of Ara h1 by its specific antibodies. Peng et al. (2013) developed a monoclonal-antibody sandwich ELISA for Ara h1 that could detect milk samples spiked with pure Ara h1 at levels between 60 and 240 ng/mL, obtaining recoveries ranging from 95.45 to 105.18%.

The performance of the assays developed in our work to detect peanut addition is difficult to compare with other studies (Peng et al., 2013; Pomés et al., 2003). Although the standards used are composed in all these studies of Ara h1, we used food samples, in which a commercial peanut butter was added at the ingredient stage and afterwards subjected to processing, whereas in the others, food products analyzed were spiked with pure Ara h1 (Peng et al., 2013) or with a raw peanut extract (Pomés et al., 2003). The use of spiked foods is useful to determine the effect of food matrix but they do not provide information about the effect of processing on assay performance. In the last few years, the potential effects of processing on the quantitation of proteins by ELISA have become recognized. The use of incurred samples, in which the allergenic food is added as ingredient and afterwards, processed in a manner mimicking as closely as possible the actual conditions under which the sample matrix would normally be manufactured, allows evaluating the actual effect of processing on the detection efficiency of an immunoassay (Khuda et al., 2012; Taylor et al., 2009). Although incurred samples are considered difficult and costly to obtain, some regulatory bodies may be unwilling to consider approval of validation data without the inclusion of data generated with incurred samples prepared with material for the allergen being targeted (AOAC, 2012).

Recently, Khuda et al. (2012) performed a study to establish the effect of food processing on peanut detection by five commercial ELISA kits using cookie dough prepared with defatted light-roasted peanut flour before baking. These authors obtained that recovery was drastically reduced after baking at 190 ºC for 30 min, being less than 18% at all added levels.

Our study and others demonstrates that ELISA tests could not give accurate results when they are used to determine allergenic proteins present in thermal processed foods due to changes in solubility and immunoreactivity of the target proteins (Fu & Maks, 2013; Khuda et al., 2012). Therefore, an understanding of the effects of processing on allergen structure in a specific matrix, as it relates to immunoreactivity and solubility, is necessary to evaluate the performance of ELISA methods to detect allergens in processed foods. The limitations of immunoassays should be considered when they are going to be applied in the evaluation of food allergen control programs.

Performance characteristic of direct competitive ELISA for Ara h2 were determined within the interlaboratorial study. This ELISA test could detect percentages of peanut butter addition higher than 0.05% and false-negative results were found at 0.01% addition. It has been shown that relatively low values of RSD_R from 30.18 to 53.47% for model biscuits can be achieved at 0.05-5% peanut addition, obtaining the highest value at the lowest levels of peanut addition (0.01%), in which sample Ara h2 could not be detected.

Poms et al. (2005) carried out an interlaboratory validation of five commercial ELISA test kits for the determination of peanut in two food matrices (biscuits and dark chocolate) at four levels of peanut contamination. They found that variance of results between laboratories (RSD_R) for biscuits for the different concentration levels ranged between 23.4 and 127.0%. Matsuda et al. (2006) evaluated the analytical performance of two ELISA kits to detect peanut in an interlaboratory study and found RSD_R values of 14% and 9% for cookies added with peanut proteins at a level of 10 μg/g of food. Lexmaulová et al. (2013) performed a collaborative study to validate an ELISA method for the quantitative determination of peanut protein in foods. They used six real foods with peanut declared in the ingredient list and obtained variation coefficient of reproducibility between 31.4 and 59.4% depending on the sample. Thus, RSD_R values obtained in our study are in the range of those reported in other studies.

5. Conclusions

In this study, direct competitive and sandwich ELISA formats to determine Ara h1 and Ara h2 proteins were developed and assayed in model biscuits prepared with a commercial peanut butter as ingredient. Direct competitive formats could detect lower levels of peanut butter in biscuits compared to sandwich formats. Moreover, ELISA assays based on the determination of Ara h2 protein were able to detect lower percentages of peanut than their counterparts for Ara h1. Therefore, direct competitive format for Ara h2 were selected to be evaluated by four laboratories, obtaining adequate results in term of repeatability and reproducibility.

Results obtained revealed that detected levels of Ara h1 and Ara h2 were drastically reduced after the roasting of peanuts to obtain the peanut butter used as ingredient and also after the baking of biscuits, the effect being more marked in the case of Ara h1. This is an important point, as these proteins that are underestimated by ELISA have been reported to retain or even to increase their allergenicity after processing in sensitized individuals.

These findings underline the fact that the determination of allergenic proteins is greatly affected by the nature of the immunoassay format, the target protein and the food processing conditions. The limitations of each allergen assay should be considered before applying ELISA assays for evaluation of food allergen control programs and to assess allergen risk management studies.

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FIGURE LEGENDS

Figure 1: SDS-PAGE (a) and Western-blotting against rabbit antiserum to Ara h1 (b) and Ara h2 (c) of raw peanut extract.

Figure 2: Calibration curves obtained for sandwich (a, b) and direct competitive (c, d) ELISA formats for determination of Ara h1 (a, c) and Ara h2 (b, d) concentration in standard solutions of pure proteins.

Figure 3: Concentration of immunoreactive Ara h1 (a, c) and Ara h2 (b, d) in model biscuits added with different amounts of peanut butter. Sandwich (a, b) and direct competitive (c, d) ELISA. Values are the mean + SD of three sample extractions assayed by triplicate expressed in mg/kg.

Lines indicate the cut-off value above which biscuits are considered positive for peanut butter addition, and were calculated as the mean value + 3.3 SD of the blank biscuit.

Figure 1

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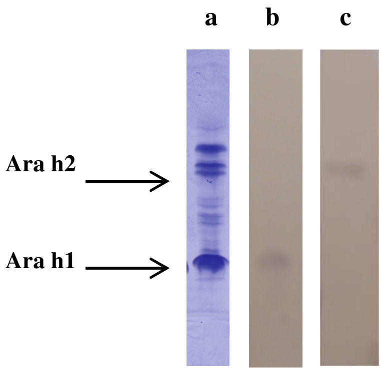


Figure 2

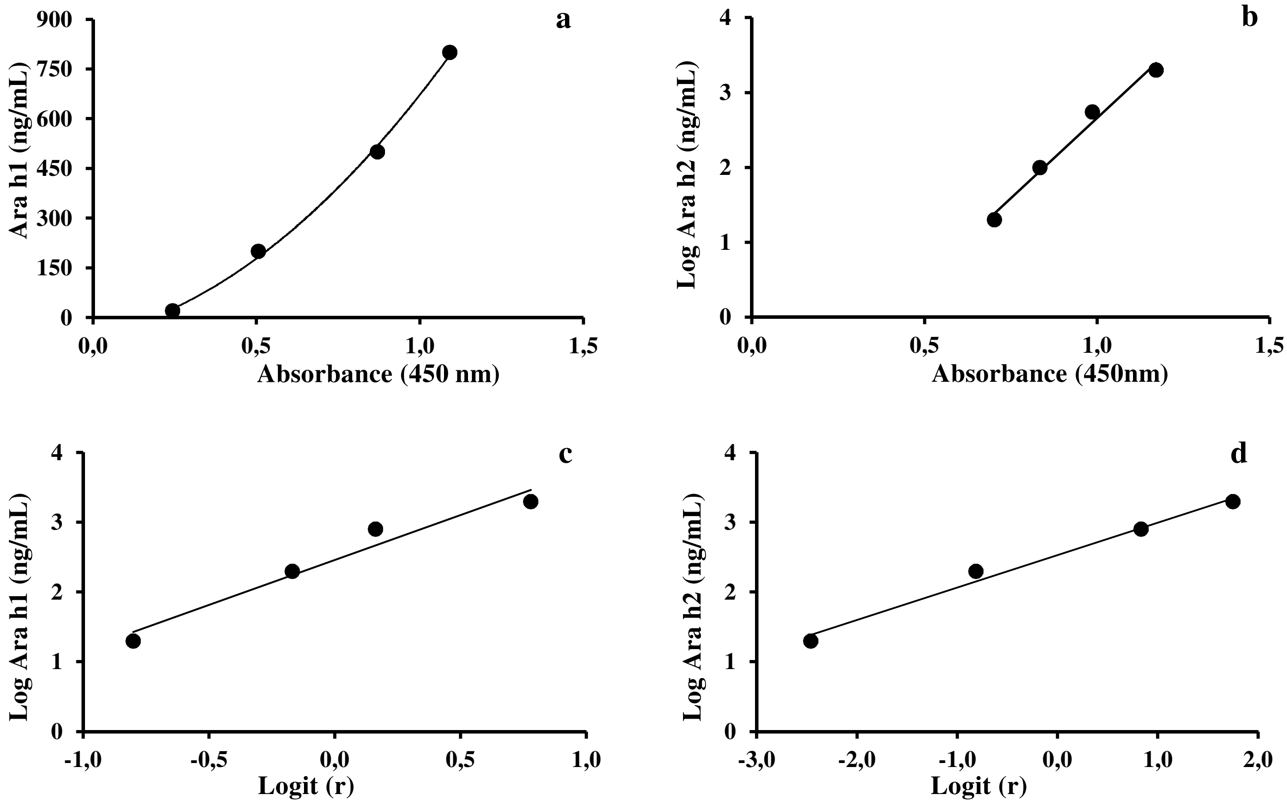
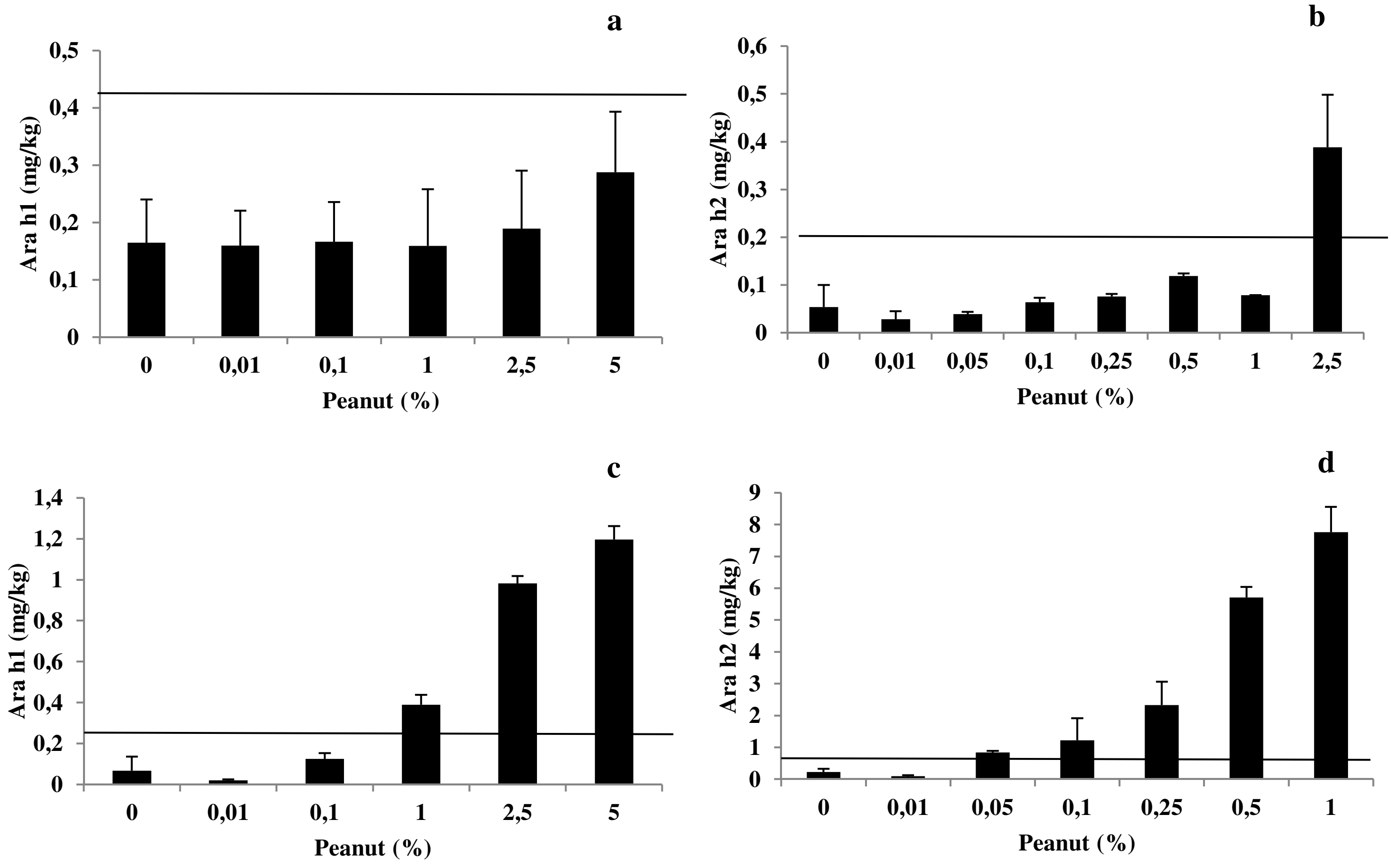

a
Ara h1 (ng/mL)
0
150
300
450
600
750
900
0,0
0,5
1,0
1,5
Absorbance (450 nm)
b
Log Ara h2 (ng/mL)
0
1
2
3
4
0,0
0,5
1,0
1,5
Absorbance (450nm)
c
Log Ara h1 (ng/mL)
0
1
2
3
4
-1,0
-0,5
0,0
0,5
1,0
Logit (r)
d
Log Ara h2 (ng/mL)
0
1
2
3
4
-3,0
-2,0
-1,0
0,0
1,0
2,0
Logit (r)

Figure 3:


a
Ara h1 (mg/kg)
0,5
0,4
0,3
0,2
0,1
0
0
0,01
0,1
1
2,5
5
Peanut (%)
b
Ara h2 (mg/kg)
0,6
0,5
0,4
0,3
0,2
0,1
0
0
0,01
0,05
0,1
0,25
0,5
1
2,5
Peanut (%)
c
Ara h1 (mg/kg)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
0,01
0,1
1
2,5
5
Peanut (%)
d
Ara h2 (mg/kg)
9
8
7
6
5
4
3
2
1
0
0
0,01
0,05
0,1
0,25
0,5
1
Peanut (%)

.

Table 1: Limit of detection (LOD) of the ELISA tests for Ara h1 and Ara h2 and cut-off establish for the ELISA tests to determine a biscuit sample as positive for peanut addition. Calibration points correspond to the protein concentration of standards used in each ELISA tests. Mean value + SD are given in brackets.

Test format	**Target protein**	**LOD (mg/kg)**	**Cut-off (mg/kg)**	**Calibration points (mg/kg)**
Sandwich	Ara h1	0.10 (0.04 ± 0.02)	0.42 (0.16 ± 0.08)	0-0.2-2.0-5.0-8.0
Sandwich	Ara h2	0.13 (0.11 ± 0.01)	0.20 (0.05 ± 0.05)	0-0.2-1.0-5.5-20.0
Competitive	Ara h1	0.19 (0.10 ± 0.03)	0.30 (0.07 ± 0.07)	0-0.2-2.0-8.0-20.0
Competitive	Ara h2	0.06 (0.02±0.011)	0.64 (0.24 ± 0.12)	0-0.2-1.0-5.5-20.0

Table 2: Results obtained by the four participating laboratories for the determination of Ara h2 (mg/kg) in model biscuits added with different percentages of peanut butter, using the direct competitive ELISA format.

Peanut Butter (%)	Assay 1				Assay 2			
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 1	Lab 2	Lab 3	Lab 4
0	0.18 ± 0.16*	0.53 ± 0.11*	0.60 ± 0.09*	0.38 ± 0.25*	0.30 ± 0.03*	0.61 ± 0.22*	0.19 ± 0.09*	0.60 ± 0.29*
0.01	0.12 ± 0.02*	0.81 ± 0.34	0.73 ± 0.21*	0.42 ± 0.36*	0.16 ± 0.13*	1.48 ± 0.31	0.48 ± 0.07*	0.40 ± 0.25*
0.05	0.95 ± 0.50	1.87 ± 0.11	1.38 ± 0.33	1.72 ± 0.46	1.20 ± 0.18	1.70 ± 0.20	0.98 ± 0.09	1.35 ± 0.28
0.10	1.03 ± 0.21	2.69 ± 0.59	2.31 ± 0.19	3.10 ± 0.11	1.76 ± 0.57	2.27 ± 0.42	1.04 ± 0.24	1.74 ± 0.21
0.25	1.82 ± 0.24	4.02 ± 0.52	3.06 ± 0.29	6.02 ± 1.13	2.76 ± 0.29	3.75 ± 0.47	2.60 ± 0.38	3.86 ± 1.27
0.50	6.10 ± 1.45	9.91 ± 0.93	5.69 ± 0.60	7.11 ± 0.65	5.53 ± 0.88	5.62 ± 1.18	4.65 ± 0.41	6.97 ± 0.74
1.00	7.93 ± 3.48	20.53 ± 2.11	14.33 ± 2.28	15.56 ± 1.03	8.33 ± 0.47	9.69 ± 0.37	6.58 ± 1.46	15.16 ± 2.00
2.50	62.75 ± 9.38	51.43 ± 20.5[illegible]	21.87 ± 1.53	21.45 ± 7.69	49.32 ± 6.42	27.15 ± 5.09	44.55 ± 5.22	43.75 ± 2.21

*Food samples with concentration values below the cut-off established for the interlaboratory study.

Table 3: Results of the interlaboratory study. Performance criteria (repeatability and reproducibility data)

Performance characteristics	Abbreviation	Peanut Butter (%)							
		0.00	0.01	0.05	0.10	0.25	0.50	1.00	2.50
Total number of laboratories	P	4	4	4	4	4	4	4	4
Total number of replicates	n	8	8	8	8	8	8	8	8
Mean value	$\overline{X}$	0.42	0.57	1.39	1.99	3.49	6.45	12.26	40.28
Repeatability SD	S_r	0.169	0.253	0.221	0.721	0.856	1.572	4.714	14.924
Reproducibility SD	S_R	0.247	0.638	0.506	0.907	1.864	1.946	5.964	17.755
Repeatability RSD	RSD_r	39.91	44.07	15.83	39.19	24.56	24.38	38.44	37.05
Reproducibility RSD	RSD_R	58.32	111.13	36.39	45.55	53.47	30.18	48.62	44.07
Repeatability limit	r	0.473	0.708	0.618	2.018	2.397	4.401	13.199	41.788
Reproducibility limit	R	0.691	1.787	1.416	2.540	5.220	5.449	16.698	49.713