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Research Article

Development of a Competitive Enzyme Immunoassay Technique for the Detection of Peanut Traces in Gluten-free Products

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Abstract: The aim of this work was to develop a competititve enzymeimmunoassay technique, to detect the presence of traces of peanut in gluten-free products. Specific rabbit polyclonal antiserum against peanut was used as primary antibody. The optimal antigen concentration to be immobilized on the plate and the concentration of primary antibody to be used in competition was determined. The calibration curve was fitted using increasing concentrations of an extract of peanut product. The peanut product was extracted with Tris-HCl buffer 0.0625M with 3% sodium dodecylsulfate (SDS) and 2% sulphite (S) 0,1 M. All validation parameters studied were appropriate. Commercial samples of gluten-free products were analysed with this enzyme immunoassays and a commercial ELISA kit. Significant differences were observed in the quantitative results obtained with both methods; nevertheless the developed enzyme immunoassay could be used as screening method.

Keywords: ELISA, Allergens, Peanut Detection, Gluten-free Products

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1. Introduction

Food allergies are a growing problem in developing countries but also in emerging countries like Argentina. The main food groups that generate allergic reactions are the "big eight": milk, eggs, peanut, wheat, soy, nuts, fish and crustaceans [1]. However, the highest prevalence among allergic individuals occurs with milk, egg, soy, peanut and tree nut proteins.

In 2017, was incorporated into the Argentine Food Code the article 235 seventh that included declaration on food labelling of the allergens present in it [2].

Food manufacturers must be very carefully in allergen labelling. There is a need of methodology that enables the detection of allergenic proteins in products. The most common methodology for the analysis of these proteins is ELISA. There are no Latin American companies that produce ELISA kits and in Argentina these kits are very expensive.

The aim of this work was to develop a competitive enzyme immunoassay technique, to detect the presence of traces of peanut in gluten-free products. All validation parameters studied were appropriate. This competitive enzyme immunoassay could be used as screening method.

2. Materials and Methods

2.1. Samples analysed

2.1.1. Peanut

Ground peanut was used to prepare the model systems and it was used as antigen to be immobilized on the plate.

2.1.2. Model systems of rice flour and corn flour

Model systems of rice flour and/or corn flour with the addition of peanut were prepared in the laboratory. The model systems were: 50, 150 and 300 ppm of peanut in mixture with rice flour and/or corn flour.

2.1.3. Commercial gluten-free products

Sixteen gluten-free commercial products were selected by random sampling. A container of each product was randomly purchased from different markets in the Autonomous City of Buenos Aires, Argentina. Some products declared peanut in the ingredient list, others had a peanut warning statement, and others did not declare peanut. The gluten-free products analysed were 2 noodles, 2 breads, 2 pizzas, 7 sweet and salty cookies, 2 snacks and 1 cereal. A single batch of each product was analysed. All the content of the package was homogenized. Each commercial product was ground in a food processor.

Table 1 shows the product description, the list of ingredients and the allergen declaration of the sixteen commercial samples.

2.2. Protein extraction from peanut and model systems

Eighty mg of ground peanut and 200 mg of each model systems were weighed for the protein extraction.

Two mL of total protein extractive solution was added. This solution contains 0.0625 M Tris-HCL with 3% sodium dodecylsulfate (SDS) and 2% sulphite (S). The tubes were heated in a water bath at 100 °C for 5 minutes. After 2 minutes in the bath it was shaken with a rod. The contents of the extraction tubes were transferred to plastic tubes and centrifuged at 3000 rpm for 30 minutes. The supernatants were stored at -20 °C until analysis.

The method of Lowry [3] was used for the quantification of peanuts proteins in the extract and for the calculation of the recovery.

2.3. Optimization of the competitive enzyme immunoassay

The optimal concentration of antigen (peanut) to be immobilized on the plate and the optimal dilution of primary antibody (rabbit polyclonal antiserum specific for peanut proteins) to be used in competition was determined. Polyclonal antiserum obtained in rabbits immunized with peanut and obtained according to [4], was used.

2.4. Plate coating

Microplate plates (Maxisorp ®, NUNC, Denmark) were used. For this, 100 µL per well of two different concentrations of antigen were seeded. The concentrations were: 1 µg of peanut protein / 100 µL or 10 µg of peanut protein / 100 µL of carbonate / bicarbonate buffer, pH: 9, 6 (Buffer sodium carbonate 0.015M, 0.035M sodium bicarbonate, pH: 9.6). The plate was then incubated in a humid chamber, in the dark at 4 °C for 24 h. The plate was washed 5 times with wash solution (0.9% w/v NaCl and 0.0125% v/v Tween 20 in water). Two hundred µl of blocking solution (1% w/v bovine gelatin and 0.1% v/v Tween 20 in TBS) were seeded into each well. It was incubated for one hour in a humid chamber, in the dark at 37 °C, with shaking. The plate was washed 5 times with wash solution. Subsequently, 100 µL of different dilutions of the primary antibody diluted with TBS

buffer with 0.1% v/v Tween 20 and 3% polyethylene glycol were seeded. Primary antibody dilutions that were assayed were between 1/4000 and 1/128000. In the wells corresponding to the blank (blank 1 and blank 10) only the buffer used for the dilution of the primary antibody was seeded. It was incubated for one hour in a humid chamber, in the dark at 37 °C with shaking. The plate was washed 5 times with wash solution. One hundred µl of Bio-Rad alkaline phosphatase conjugated Anti-IgG secondary antibody (obtained in goats immunized with purified rabbit IgG) was seeded in the wells. The secondary antibody was diluted 1:3000 with TBS buffer with 0.1% v/v Tween 20 and 3% polyethylene glycol. It was incubated for one hour in a humid chamber, in the dark at 37 °C with shaking. The plate was washed 5 times with wash solution. Finally, 100 µl of a solution containing 1 mg/mL paranitrophenyl phosphate in a buffer containing 10% v/v diethanolamine and 0.01% magnesium chloride, pH: 9.8 were seeded. It was incubated 20 minutes in a humid chamber, in the dark at 37 °C with shaking. Absorbance was measured on an ELISA plate reader at 405nm. The absorbance values were corrected with the average absorbance corresponding to the blank. Corrected absorbance versus ln 1 / dilution of primary antibody curves were plotted, using a Microsoft Excel 2010 spreadsheet.

2.5. Validation of the competitive enzyme immunoassay for the detection / quantification of peanut traces in gluten-free products

2.5.1. Linearity

For the determination of the linearity of the method, increasing concentrations of a peanut product extract with 0.0625M Tris-HCL buffer with 3% SDS and 2% of S were used. Since an extraction buffer containing reducing and denaturing agents (S and SDS) that interfere with the antigen-antibody reaction is used in this assay, the dilution of the extractive solution was evaluated, as it will not affect the antigen-antibody binding. In this way the components of the extractive solution were diluted 1: 159 at all points of the curve. Dilutions were performed in carbonate / bicarbonate buffer, pH: 9.6. The curve had five points 0; 0.01; 0.03; 0.1 and 0.3µg peanut protein / mL carbonate / bicarbonate buffer, pH: 9.6. For each point of the curve a dilution of the original extract was performed but the SDS and S concentration remained constant.

Seventy five µL of the dilution of the primary antibody selected in the test optimization, and 75µL of each of the dilutions of the previously prepared curve points, were pre-incubated. In addition, two controls were prepared; a “non-specific control” (NS) containing 200 µL of the buffer used to dilute the primary antibody, and a "maximal binding" (M) control containing: 100 µL of the buffer used to dilute the primary antibody and 100 µL of the primary antibody selected in the test optimization. The preincubates were incubated at 4 °C in a humid chamber and in darkness for 24 h. Also, an ELISA plate was sensitized by sticking the concentration of antigen (peanut) that was previously selected in the test optimization. It was then incubated in a humid chamber, in the dark at 4 °C for 24 h. The plate was washed 5 times with wash solution. Two hundred µL of blocking solution were seeded into each well and incubated for one hour in a humid chamber, in the dark at 37 °C, with shaking. The plate was washed 5 times with wash solution. Subsequently, 100 µL of the preincubates were seeded. It was incubated for one hour in a humid chamber, in the dark at 37 °C with shaking. The plate was washed 5 times with wash solution. The protocol was followed as previously described in the plate coating item. The absorbance values were corrected with the mean absorbance corresponding to NS. An absorbance calibration curve was constructed, corrected absorbance versus ln µg of peanut protein / mL.

The tests used for the statistical analysis of the results were: Barlett method, for homogeneity of variances and linear regression analysis [5].

2.5.2. Limit of detection and Limit of quantification

To determine the limits of detection and quantification of the method, a sample of rice or corn flour without analyte (peanut) was used. It was extracted five times as described above. Each extract was analysed in duplicate, as described above, by pre-incubating the dilutions 1:159 with carbonate/bicarbonate buffer, pH: 9.6. The analyte concentration in each sample was calculated according to the formula (1). The mean value of the analyte for the flour sample without analyte and the corresponding standard deviation were calculated. The detection limit was calculated as the mean value plus three times the standard deviation. The quantification limit was calculated as the mean value plus ten times the standard deviation.

The amount of peanut protein in μg / 1000 mg of rice or corn flour is calculated according to the following formula:

$$\text{Amount of peanut protein in the rice of corn flour-}\mu\text{g} = \frac{\text{amount of peanut prot-}\mu\text{g}(1)\times V\text{-}\mu L(2)1\times 1000\text{-}mg(3)}{6.3\text{-}\mu L(4)\times P\text{-mg}(5)} \quad (1)$$

(1) μg of peanut protein interpolated in the calibration curve.
(2) Volume of supernatant obtained when extracting rice or corn flour with extractive solution of total proteins: 600 μL (rice flour) and 800 μL (corn flour).
(3) 1000 mg: to express the content in 1000 mg of rice or corn flour.
(4) 6.3-μL. It is the volume of extract that is taken from the supernatant and diluted 1: 159. Then 6.3 μL are brought to 1000 μL with Buffer Carbonate / Bicarbonate; pH 9.6.
(5) P: 200 mg. It is the weight of gluten-free products that is extracted with extractive solution of total proteins.

2.5.3. Precision

To evaluate the intraday precision of the method, three samples of rice or corn flour containing equal amount of analyte (50 ppm of peanut protein and 150 ppm of peanut protein, respectively) were analysed. Each sample was extracted as described above (n=3). Each extract was analysed with the competitive enzyme immunoassay in duplicate as described above, by performing the 1: 159 dilution of each of the samples prior to the preparation of the preincubates. The analyte concentration in each sample analysed was determined according to the formula (1).

For the statistical processing, the analyte values of the three samples were averaged, standard deviation and coefficient of variation (CV) were calculated. This CV corresponds to the precision of the method in the day.

In order to evaluate interdays precision, the same procedure as previously described in the intraday test was performed on three different days (n=9). For statistical processing, the mean, standard deviation and coefficient of variation of the nine values obtained were calculated. The CV corresponds to the precision of the method between days.

It was adopted as an acceptance criterion that the CV of the intraday precision and the CV of interdays precision did not exceed 15% [6].

2.5.4. Recovery

Three model systems of rice and corn flour mixed with 50, 150 and 300 of peanut protein were analysed to evaluate the recovery of the method.

The model systems were extracted in triplicate as described above. They were analysed in duplicate as described above, by performing the 1: 159 dilution of each of the samples prior to the preparation of the preincubates. The analyte concentration in each

sample analysed was determined according to the formula (1). For each model system the three analyte values were averaged.

The percent recovery was calculated by the formula described below.

% Recovery = obtained value x 100 /real value

- Obtained value: concentration of peanut protein obtained when applying the enzyme immunoassay for SM of 50, 150, 300 ppm of peanut protein.
- Real value: 50, 150, 300 ppm of peanut protein.
- The recoveries of the three model systems were then averaged. Recovery values between 70-130% were considerate adequate values [7].

2.6. Commercial ELISA kit

The commercial gluten-free products were analysed with the competitive enzyme immunoassay and also with the ELISA Veratox ® for Peanut Allergen from Neogen. All samples were assayed in duplicate following the protocols of this kit. [8].

The quantitation limit (QL) for this kit is: 2.5 ppm peanut with a quantification range of 2.5-25.0 ppm peanut. Protein conversion: Test kit results are expressed as total peanut. To express results as protein, the result is multiply by 0.258.

3. Results

3.1. Quantification of peanut proteins in the extract

The concentration of peanut proteins obtained in the extract of total proteins was 7.9 mg of peanut protein / mL of extractive solution. The real concentration in that extract was 8.4 mg of peanut protein / mL of extractive solution. The recovery rate was 94%, it means that 94% of the peanut proteins were soluble in the extractive solution of total proteins.

3.2. Optimization of the competitive enzyme immunoassay

Figure 1 shows the two curves obtained for the determination of the optimum concentration of peanut antigen and the primary antibody dilution to be used in the final assay.

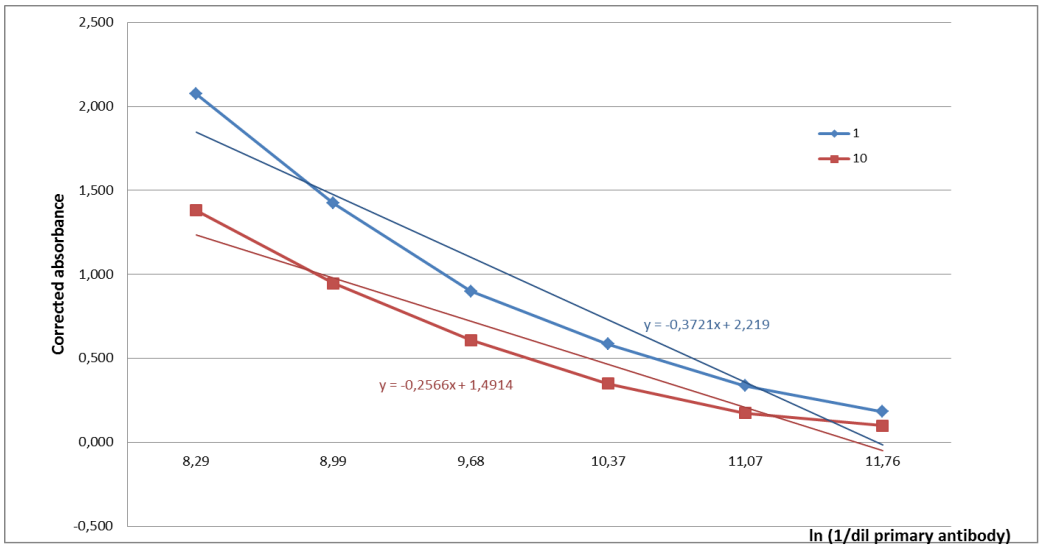

Figure 1. Curves obtained for the determination of the optimal concentration of peanut antigen and the dilution of primary antibody to be used in the final competitive enzyme immunoassay.

Figure 1 shows the curves corresponding to 1 µg of peanut protein / 100 µl of carbonate / bicarbonate buffer, pH: 9.6 and 10 µg of peanut protein / 100 µl of carbonate / bicarbonate, pH: 9.6 which were obtained in the test optimization.

The curve with the highest slope was chosen for the selection of the optimal concentration of antigen to be used in the test (1µg of peanut protein / 100µL of buffer).

In order to obtain a method with adequate sensitivity, the optimal dilution of primary antibody to be used in the competition was chosen in the area most sensitive to changes in the curve of 1µg of peanut protein / 100µL of buffer. The dilution of primary antibody selected for use in the competition was 1/16000. (Ln 1 / primary antibody dilution: 9.68).

3.3. Validation

3.3.1. Linearity

Five points were used to establish linearity 0; 0.01; 0.03; 0.1 and 0.3 µg peanut protein / mL (Figure 2).

A test of homogeneity of variances was applied to the values of corrected absorbances obtained for each level of concentration and no significant differences were found between the variances of the different levels analysed.

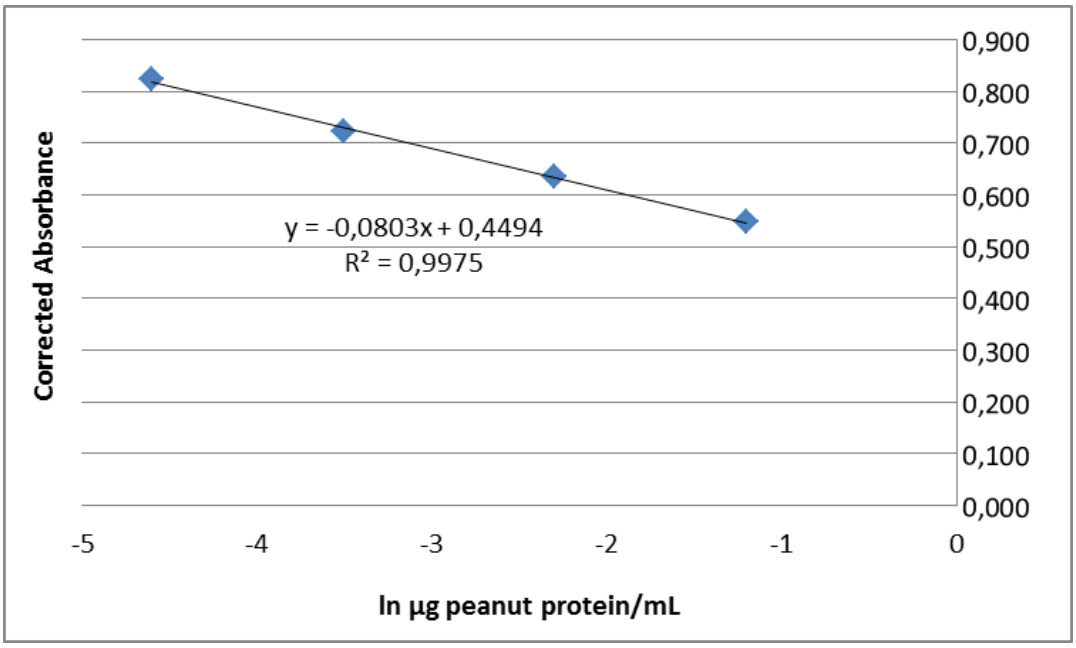

Figure 2. Calibration curve: Corrected Absorbance vs. ln µg peanut protein /mL.

The linearity test was performed using the profesional Infostat version 2004d.1 developed by the National University of Córdoba. A value of F = 1.46 (CM linearity deviation / CM pure error) and p = 0.2880 was obtained. It was concluded that the range 0.01; 0.03; 0.1 and 0.3 µg of peanut protein / mL showed lineal. The obtained line had a slope of -0.0803 with a lower limit 95% (LL) of -0.09 and an upper limit 95% (UL) of -0.07, intercept 0.4494 with LL: 0.43 and UL: 0.49 and a correlation coefficient of 0.9975. The formula (1) presented above was applied to calculate the upper limit values of the calibrations curves (0.3 µg of peanut protein / mL), in order to calculate the working range for the peanut protein in rice or corn flour. The lower limit values are the quantification limits for rice or corn flour assay. The working range was 32-143 ppm of peanut proteins in rice flour, and 36- 190 ppm of peanut proteins in corn flour.

3.3.2. Detection and Quantification limits

The values of the detection and quantification limits were 14.0 and 32.0 ppm of peanut protein in rice flour, and 16.0 and 36.0 ppm of peanut protein in corn flour, respectively.

3.3.3. Precision

The intraday and interdays precision of the method expressed as coefficient of variation (CV) were 13 (n = 3) and 12 (n = 9) in peanut protein in rice flour and 3 (n = 3) and 9 (n = 9) in peanut protein and in corn flour, respectively. These precision values are adequate.

3.3.4. Recovery

Model systems of rice and corn flour with 50, 100 and 300 ppm peanut protein were analysed. The recovery of the method was calculated, the result was for model systems of rice flour 101,5% and for model systems of corn flour was 87.6%. These recoveries were considerate adequate values.

3.4. Analysis of commercial gluten-free products

Table 1 presents the results obtained in the determination of peanut protein in commercial gluten-free products, using the Neogen kit and the competitive enzyme immunoassay developed.

Table 1. Results obtained in the determination of peanut protein in commercial gluten-free products, using the Neogen kt and the competitive enzyme immunoassay developed.

SAMPLE	PRODUCT DESCRIPTION	INGREDIENTS	ALLERGEN DECLARATION	COMPETITIVE ENZYME IMMUNOASSAY (ppm peanut protein)	NEOGEN (ppm PEANUT *0,258 = ppm peanut protein)
1	Spaghetti noodles	Corn flour, rice flour, emulsifiers: INS 471, INS 401.	May contain soy	<36	>25
2	Sliced bread	Rice flour, corn starch, cassava starch, potato starch, dehydrated egg white powder, high oleic sunflower oil, emulsifiers: methylcellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, salt, brewer's yeast, preservative: calcium propionate , acidifier: citric acid, acidity regulators: sodium acetate.	Contains milk and egg derivatives	<32	<2.5
3	White bread	Corn starch, rice flour, vegetable fiber, sunflower oil, soy protein, yeast, salt, citrus fiber, sugar, ESP (INS 464).	Contains soy derivatives	<32	<2.5
4	Pizza	Corn starch, cassava starch, vegetable oil, rice flour, whole milk powder, instant dry yeast, egg powder, sugar, salt, CONS (INS 282), EMU (INS 412, INS 415), mozzarella cheese, sauce of tomato.	Contains milk, egg and milk derivatives. May contain nuts and soy derivatives	<32	<2.5
5	Rice toast	Rice flour, sugar, skimmed milk powder, fine salt, emulsifier; INS 322 and INS 471	Contains milk and soy derivatives	<32	<2.5
6	Pizza without cheese	Water, corn starch, rice flour, cassava starch, tomato sauce, salt, sugar, high oleic sunflower oil, preservative: calcium propionate, acidifier: citric acid, acidity regulator: sodium acetate, yeast.	Main contain milk and egg	<32	<2.5
7	Vegetable mix noodles	Corn flour, rice flour, pea flour, chickpea flour, bell pepper powder, vitamin A, vitamin B1, vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin B9, vitamin B12, ferrous fumarate, iron oxide zinc, emulsifiers: INS 471 and INS 401.	May contain soy	<36	<2.5
8	Cheese scones	Cassava starch, corn flakes, rice flour, soy flour, margarine, whole egg powder, whole milk powder, fine salt, sugar, chemical raising agents: ammonium bicarbonate, monocalcium phosphate, sodium bicarbonate, Flavouring: aroma artificial cheddar cheese.	-	<32	<2.5
9	Almonds cookies	Corn starch, maltitol-based blend, polydextrose, sucralose (18 mg/100 g), acesulfame potassium (8 mg/100 g), sodium saccharin (5 mg/100 g), almonds, high oleic sunflower oil, water, rice flour, soybean flour, sesame seeds, lactose-based powder, milk powder, maltodextrin, anhydrous milk fat, soy lecithin and lactase, salt, emulsifier for pastry and confectionery industrial food use based on : INS 1520, INS 471, INS 433, INS 422, RAI: INS 500ii, INS 503ii, CONS: INS: 282, EMU: INS 322, ARO: cereal and almond essence.	Contains milk, almonds, soy derivatives. May contain eggs, nuts, cashew nuts and peanuts.	<32	<2.5
10	Vanilla cookies	Corn starch, sugar, high oleic sunflower oil, rice flour, glucose syrup, powder based on: (lactose, powdered milk, maltodextrin, anhydrous milk fat, soy lecithin and lactase), interesterified vegetable oil, water, soy flour, salt, EMU: INS 132, CONS: INS 282, RAI: INS 500ii-503ii, ARO: vanilla essence and three milk essence. Fat matter 15%.	Contains milk and soy derivatives. May contain eggs, almonds, walnuts, cashew nuts and peanut.	<32	<2.5
11	Rice "cubanitos" (type of cookie)	Peanut praline felling (sugar, roasted peanut paste, vegetable fat, milk solids, emulsifier INS322, aromatizing/flavouring). White fantasy pastry coating (hydrogenated vegetable oil, sugar, whole milk powder, skimmed milk powder, flavouring, ethyl vanillin, INJS322). "Cubanito" (corn flour, rice flour, sugar, whole milk powder, trehalose, salt, stabilizer, calcium carbonate, emulsifier INS 322)	Contains milk, peanut and soy derivatives. May contain hazelnuts.	>190	>25
12	Peanut and cocoa bar	Roasted peanut halves without salt, muscovado sugar, honey, chia seeds, whole sesame and cocoa powder.	Contains peanuts. May contain sulphite, almonds, cashews and walnuts.	>143	>25
13	Sweet cookies	Corn starch, palm fat, cane sugar, corn flour, soybean meal, soybean bran, buckwheat flour, beet syrup, modified tapioca starch. salt, RAI (INS503ii and INS 500ii)	-	<36	<2.5
14	Toast	Corn starch, rice flour, high oleic sunflower oil, sugar, vegetable fiber, rice syrup, buckwheat flour, lentil flour, pea protein, yeast, salt, ESP (INS 464), ARO (natural to vanilla)	-	<32	<2.5
15	Sugared shortbread coconut flavor	Corn starch, rice flour, sugar, high oleic sunflower oil, water, glucose, soy flour, salt, ARO: coconut, COL: INS 100i, EMU: INS 322, EST: INS 500ii, RAI: INS 503ii, CONS : INS 282, INS 202.	Contains soy derivatives. May contain traces of milk, egg, almonds, walnuts, cashews and peanuts.	<32	<2.5
16	Granola	Cornflakes, Sugar, Puffed Rice, Sunflower Seeds, Blueberries, Water, Almonds, Flaxseeds, Vegetable Oil, Chia Seeds, Salt, Antioxidants: Mixed Tocopherols	Contains almonds. May contain sulphites, milk, peanuts and soy derivatives.	<32	>25

4. Discussion

4.1. Validation

In the present study, the detection and quantification limits of the developed assays are different for the analysed gluten-free flours (rice and corn). Food matrix can influence on the results of enzyme-linked immunosorbent assays. In previous works it has been observed that the different matrices can interfere with the ELISA results, giving different detection and quantification limits [9]. It is also important to know that assay performance and quantification of the kits can vary in samples from different food sources, due to interference from matrix effects and the susceptibility of affected proteins during processing. Some studies show that the extraction solution and the composition of the matrix have a great impact on the extraction efficiency and the detection limit of allergenic proteins [10].

The different values of detection and quantification limits of the competitive enzyme immunoassay developed, are high compared to the quantification limits presented by commercial kits. The usefulness of this test only lies in the possibility of confirming the presence of peanuts in those samples that present positive results.

In relation to the intraday and interday precision of the competitive enzyme immunoassays to detect peanut proteins in mixture with the two gluten-free flours used, it is observed that they present equal to or less than 15%, a value taken as acceptance criterion [6].

In relation to the recovery values of the competitive enzyme immunoassays to detect peanut proteins in mixture with the gluten-free flours studied, they complied with the values considered as reference 70 - 130% [7].

4.2. Analysis of commercial gluten-free products

It has been observed in previous studies that the results between different commercial ELISAs may be different.

Although Elisa method is accepted as standard method for allergen measurement, these results seemed to vary from manufacturer to manufacturer. This variation could be due to the lack of standardization of the method, the calibration material used, the extraction solutions used or the antibody specificity [11]. Therefore the quantitative results between the competitive enzyme immunoassay and Neogen kit cannot be compared.

In sample 1 that do not contain or declared peanut the result obtained with Neogen kit exceeds the upper limit of the calibration curve whereas the competitive enzyme immunoassay did not detect peanut, in this case the result was lower than the test quantification limit (36 ppm of peanut protein). This value corresponds to the test applied to corn flour. The sample declares corn flour as the first ingredient in its list of ingredients.

According to previous studies, there are numerous foods that have a high risk of containing undeclared allergens and can lead to a risk potential for the health of allergic consumers. Many studies have shown a high prevalence of undeclared food allergens with or without phrases of warning. The presence of undeclared proteins is a risk for allergic consumers [12, 13].

In samples 2-8, 13 and 14 that do not contain or do not declared peanut even enzyme immunoassay and Neogen kit do not detect peanut. In sample 7 and 13 the result was lower than the test quantification limit of 36 ppm of peanut protein, a value corresponding to the test applied to corn flour. The sample declares corn flour as ingredient in its ingredient lists. In the rest of the samples (2-6, 8 and 14), the result was lower than the test quantification limit of 32 ppm of peanut protein, a value corresponding to the test applied to rice flour. The samples declare rice flour as an ingredient in its ingredient lists.

In samples 9, 10 and 15 that declared “may contain peanut” none of the ELISAs detect peanut protein. The result was lower than the test quantification limit of 32 ppm of peanut

protein, a value corresponding to the test applied to rice flour. The samples declare rice flour as an ingredient in its ingredient lists.

In 11 and 12 samples the enzyme immunoassay and Neogen kit detect peanut, exceeded the uppers limits of the calibration curves. These samples declare peanut.

In sample 16 the enzyme immunoassay do not detect peanut and Neogen kit detect peanut, exceeded the upper limit of the calibration curve. In the enzyme immunoassay the result was lower than the test quantification limit of 36 ppm of peanut protein, a value corresponding to the test applied to corn flour. The sample declares corn flakes as an ingredient in its ingredient lists.

These results indicate that if a sample shows a positive result with the competitive enzyme immunoassay, it is not necessary to use a commercial ELISA kit because the sample contains peanut (example: 11, 12). The enzyme immunoassay developed has a considerably lower cost than commercial kits. Therefore, this could be used as screening method, to analyze samples in which a possible cross-contact with peanut is suspected. If negative results are obtained with this methodology, it should be confirmed with a commercial ELISA kit of adequate sensitivity, to ensure the absence of peanut (example: 1 and 16).

Although we are aware of the difficulties of developing a commercial ELISA kit with adequate sensitivity, its availability is necessary in order to unify and obtain an official method for each allergen and each matrix. As a result, it will be possible to determine a threshold or cut-off point from which companies must declare allergens, as was done in Japan [14]. This would be an indispensable tool for use by health authorities and by different companies.

5. Conclusions

This competitive enzyme immunoassay could be used as screening method. If in a sample the result is positive with these competitive enzyme immunoassays, the presence of peanut can be confirmed. However, if the result obtained is negative (less than the limit of quantification of these methods) it is necessary to confirm the result with a commercial ELISA peanut kit of adequate sensitivity. The development of methods for the detection of allergens in food is considered important both for the health authorities that control the labels of commercial products, as well as for industries and consumers. If it is necessary to detect traces of peanut proteins in gluten-free products, the use of the developed enzyme immunoassays would be a suitable option as a screening method.

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References

[1] Ward R. Chapter 1: Introduction to food allergy. In Flanagan S, Handbook of Food Allergen Detection and Control. 2014. Woodhead Publishing. Cambridge, UK.

[2] Argentine Food Code. Available online: https://www.argentina.gob.ar/sites/default/files/anmat_capitulo_v_rotulacion_actualiz_2021-08.pdf, (accessed on 01 09 2022).

[3] Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin Phenol reagent. J Biol Chem. 1951. 193: 265–275. doi: 10.1016/S0021-9258(19)52451-6

[4] Rozenfeld P, Docena G, Añón M, Fossati C. Detection and identification of a SP component that cross reacts with caseins from cow milk. Clin Exp Immunol. **2002**. 130(1): 49-58. doi: 10.1046/j.1365-2249.2002.t01-1-01935.x

[5] Box G, Hunter W, Stuart Hunter J. Statistics for researchers. Introduction to the design of experiments, data analysis and model construction. 1999. Editorial Reverté S.A. Mexico D.F.

[6] Huber L. Validation of Analytical Methods. 2010. Agilent Technologies. Germany.

[7] Gatti M y Ferretti C. Chapter 17: Soy Allergen Detection. In, Popping B, Diaz Amigo C, Hoenicke K, Molecular Biological and immunological techniques and applications for food chemists. 2010. John Wiley & Sons, Inc., Canada.
[8] Neogen Veratox® for Peanut Allergen. 2016. Available online: https://www.neogen.com/globalassets/pim/assets/original/10001/8430_pro.pdf (accessed on: 01 08 2022).
[9] Cellerino Karina, Lopez Laura Beatriz. Soy protein detection in raw and cooked meat products using different ELISA kits, Journal of Food and Nutrition Sciences**.** 2016. 4(6): 170-174. doi: 10.11648/j.jfns.20160406.15
[10] FASTER, Food Allergy Safety, Treatment, Education and Research Law. 2021. Available online: https://www.congress.gov/117/plaws/publ11/PLAW-117publ11.pdf (acceded on 03 10 2022).
[11] Diaz Amigo C. Towards a comprehensive validation of ELISA kits for food allergens. Case 2- Milk. Food Analytical Methods. 2010. 3: 351-356. doi: : 10.1007/s12161-009-9095-y
[12] Do AB, Khuda SE, Sharma MG. Undeclared food allergens and gluten in commercial food products analysed by ELISA. J AOAC Int. 2018. 101 (Pt 1): 23-35. doi: 10.5740/jaoacint.17-0384
[13] Cellerino Karina, Cagnasso Carolina, Greco Carola, Docena Guillermo, Polenta Gustavo, Ferreyra Verónica, López Laura Beatriz. Protein Ingredients Control in Gluten Free Products Using SDS-PAGE, Developed Competitive Enzyme Immunoassays and Commercial ELISA Kits. World Journal of Food Science and Technology. 2018**.** 2, (1): 12-18. doi: 10.11648/j.wjfst.20180201.12
[14] Hiroshi Akiyama, Reiko Adachi. Japanese food allergy-labeling system and comparison with the international experience; detection and thresholds. Food Safety. **2021.** 9 (4): 101–116. doi: 10.14252/foodsafetyfscj.D-21-00008