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# Development and validation of sensitive and rapid immunoassays to detect minute amounts of hazelnut in processed food and working surfaces

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ABSTRACT

Hazelnut (*Corylus avellana* L.) represents one of the most allergenic nuts and it can be found as a hidden allergen in processed food due to cross contamination. Therefore, sensitive and specific analytical techniques are in high demand to be used in allergen risk management plans at food industry. In this study, sandwich ELISA and Lateral Flow Immunoassay (LFIA) to detect hazelnut have been developed based on the determination of Cor a 9, one of the most abundant and allergenic proteins of hazelnut. Results showed that cross-reactivity was only found with walnut and Pecan nut, which was lower than 0.1%. When analyzing food spiked with a hazelnut extract or blended with hazelnut flour, ELISA and LFIA were able to detect 0.1 ppm and 0.5 ppm of hazelnut protein with a recovery from 82 to 110%. ELISA and LFIA could also detect 0.15 and 0.6 ppm of hazelnut protein in baked cookies incurred with ground hazelnut, respectively. Furthermore, LFIA could detect 1.25 μg of hazelnut protein in working surfaces of stainless steel and melamine. The sandwich ELISA was in-house validated, showing acceptable results of precision. Likewise, ELISA and LFIA showed to be robust tests. The combined use of both assays could improve the allergen risk management plans in food industry to monitor the presence of hazelnut traces in raw ingredients, processed food and working surfaces.

## 1. Introduction

Tree nuts are one of the most common foods causing allergic reactions. In Europe, the most common tree nut allergy is caused by hazelnut (17–100% of all tree nut allergies) and in the United States by walnut and cashew. Furthermore, allergy to tree nuts represents a great health concern due to the severity of the reactions, accounting for 18–40% of all food related deaths by anaphylaxis, and also due to the tend to last for the entire life (McWilliam et al., 2015).

Strict avoidance of offending food is the most effective treatment for food allergy. Therefore, availability of accurate labelling information of the relevant food allergens on processed food is needed. In Europe, the EU regulation 1169/2011 and in United States the Food Allergen Labelling and Consumer Protection Act of 2004 (FALCPA) require the mandatory labelling of substances or products causing allergies or intolerances listed in the corresponding regulations when they are added as ingredients, being tree nuts included in both of them.

Despite it, the number of allergen notifications by the Rapid Alert System for Food and Feed (RASFF) in the last years has increased notably, moving from 113 notifications in 2016 to 197 notifications in 2020, being milk, gluten, soy and tree nuts the most common allergens implicated.

On the other hand, these regulations do not cover the presence of allergenic components that may result from cross-contamination on the process line or during storage or shipping. In these cases, food manufacturers may apply voluntary precautionary allergen labelling (PAL), namely the "may contain" statement, in order to inform allergic consumers (Allen et al., 2014). This situation has resulted in the near ubiquitous utilization of precautionary allergen labels with subsequent confusion among allergic consumers. In this respect, the Voluntary Incidental Trace Allergen Labelling program (VITAL), a risk management methodology, was developed to be used by the food industry in assessing the impact of allergen cross-contamination to provide an appropriate PAL. A primary reason cited for the proliferation of PAL is that internationally recognized reference doses have been published only for a few allergenic foods. In the case of hazelnut, the VITAL 2.0 and

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3.0 reference dose as a safety limit for 95% of the susceptible population corresponds to 0.1 mg protein (Holzhauser et al., 2020). Recently, a WHO/FAO working group has established new reference doses for the most common food allergens, being for hazelnut 3 mg total protein from the allergenic source (Baumert et al., 2021).

Hazelnuts are consumed as natural, blanched and roasted, or their by-products (sliced, flour, butter, etc) as an ingredient to provide flavor in dairy, bakery, confectionary, candy, chocolates, ice creams or sauces products. Thus, the wide use of hazelnut represents a risk of finding it as a hidden ingredient or unintentional contaminant in a wide range of foods.

Cor a 9 is one of the most clinically important allergenic proteins of hazelnut. It has also been recognized of high diagnostic accuracy for hazelnut allergy in children (Nilsson et al., 2020). Cor a 9 is a major kernel storage protein and belongs to the 11S legumin-type globulins. It has a hexameric structure of 360 kDa composed by six subunits, each one constituted by an acid polypeptide (30–40 kDa) and a basic polypeptide (20 kDa) linked by a disulphide bond (Rigby et al., 2008).

Compliance with mandatory and/or precautionary labelling requires the development of reliable methods to be used in the analysis of raw food materials, food products and working surfaces to ensure consumer protection. Immunochemical assays such as enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFIA) are the most representative and widely used techniques for food allergen monitoring. ELISA technique could be applied to test the presence of allergens in final products and to validate the cleaning process, obtaining quantitative results. LFIA could be used to check raw materials, intermediary or final products and to verify routine cleaning processes on-site due to its easy-to-use and quick response (5–10 min), allowing immediate corrective actions.

Some ELISA techniques have been developed for hazelnut detection using antibodies against hazelnut total soluble proteins (Akkerdaas et al., 2004; Costa, Ansari, Mafra, Oliveira, & Baumgartner, 2015; Cucu et al., 2012; Drs et al., 2004; Koppelman et al., 1999) or the corylin fraction (Faeste, Holden, Plassen, & Almli, 2006; Holzhauser & Vieths, 1999). Besides, an ELISA to detect hazelnut using chicken egg yolk antibodies raised against Cor a 9 has been also reported (Trashin, Cucu, Adriaens & De Meulenaer, 2011). However, from our knowledge, only a LFIA technique using monoclonal antibodies against a hazelnut protein extract and labelled with carbon black nanoparticles has been reported (Ross, Bremer, Wichers, Van Amerongen, & Nielen, 2018).

In this study, sandwich ELISA and LFIA techniques using antibodies against purified Cor a 9 were developed and tested to detect hazelnut traces. This is the first report on the development of a LFIA to detect hazelnut targeting Cor a 9. Results of LFIA derived by visual interpretation and using an electronic reader were also compared. The performance of both assays was evaluated using complex food matrixes spiked with a hazelnut extract as well as using foods blended with hazelnut flour and baked cookies incurred with minute amounts of ground hazelnut as ingredient. An in-house validation of both techniques was carried out following international standardized guidelines. Furthermore, both techniques have been applied to check the cleaning effectiveness in two types of working surfaces.

## 2. Materials and methods

### 2.1. Materials

Food commodities, processed food and shelled hazelnuts used in this study were acquired from local stores.

### 2.2. Purification of Cor a 9

Cor a 9 was purified following the method of Trashin, Cucu, Devreese, Adriaens, and De Meulenaer (2011) with some modifications. Briefly, hazelnut shells were removed and the seeds were ground and defatted three times using n-hexane at a ratio of 1/5 (w/v). The proteins were extracted from defatted flour with 50 mM Tris-HCl, 200 mM NaCl, pH 7.0 at a ratio 1/10 (w/v) by stirring for 1 h at 4 °C. After centrifugation, the supernatant was concentrated by ultrafiltration and loaded onto a gel filtration Sepharose CL-6B (GE Healthcare, Piscataway, NJ) column (84 × 1 cm). Fractions containing Cor a 9 were collected, dialyzed against 20 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 500 mM NaCl, pH 7.5 and loaded onto a Concanavalin A-Sepharose column (Merck, Darmstadt, Alemania). Then, after washing the column with 5 column volumes of the buffer, retained proteins were eluted with 5 column volumes of 400 mM methyl α-D mannopyranoside. Chromatographic fractions of 1.5 mL were collected and analyzed by SDS-PAGE.

### 2.3. Purification of specific antibodies

Antisera against Cor a 9 were obtained in rabbits by inoculating the purified protein as previously described (Civera et al., 2022). The correct use and care of the involved animals have been followed by EU Directive 2010/63 on protecting animals used for scientific purposes (Spanish policy RD53/2013). The Ethical Animal Experiment Committee at the University of Zaragoza approved all procedures framed on this work (License Project 30/19). Reactivity of antisera was determined by Western blotting.

Anti-Cor a 9 antibodies were isolated by affinity chromatography using an immunoadsorbent prepared by insolubilization of the pure protein in a HiTrap NHS-activated HP column (1 mL) (GE Healthcare, Piscataway, NJ) as previously described (Civera et al., 2022).

### 2.4. SDS-PAGE and western-blotting

SDS-PAGE under reducing conditions was carried out using 4–20% precast polyacrylamide gels and a Mini-PROTEAN Tetra Cell equipment (Bio-Rad Laboratories, Hercules, CA). Gels were stained using Coomassie Brilliant Blue R and the image was captured using an Image Scanner III (GE Healthcare, Chicago, IL). Western-blotting was performed using anti-Cor a 9 antiserum according to the procedure described by Benfeldt, Larsen, Rasmussen, Andreasen, and Petersen (1995).

### 2.5. Antibody labelling

Purified antibodies for the ELISA assays were conjugated with horseradish peroxidase using the Lightning-link Horseradish peroxidase conjugation Kit (Innova Biosciences, Cambridge, UK) following manufacturer instructions.

In the case of LFIA, purified anti-Cor a 9 antibodies and anti-internalin A antibodies were linked to red and blue carboxyl-modified dyed latex particles as previously described (Civera et al., 2022).

### 2.6. Preparation of standards

Standards of purified Cor a 9 or defatted hazelnut flour were obtained as indicated bellow. A stock solution of purified Cor a 9 was prepared and its concentration was determined by spectrophotometry, using the extinction coefficient at 280 nm for 1 mg/mL of 0.62 (Trashin et al., 2011). Standards of purified Cor a 9 to obtain calibration curves for sandwich ELISA or LFIA were prepared by diluting the stock solution in the extraction buffer.

The protein concentration in defatted and non-defatted hazelnut flour was determined by the nitrogen content according to the Kjeldahl method (AOAC Official Method 981.10, 1981) considering a conversion factor of 5.3 (Sharma, Su, Joshi, Roux, & Sathe, 2010). Protein values of 31.4% and 12.0% were obtained, respectively.

The hazelnut extract to prepare the calibration standards was obtained from the defatted hazelnut flour as follows: hazelnut flour was suspended at a concentration of 1 mg/mL in 10 mL extraction buffer.

Considering the 10-fold dilution factor, this solution contained 3140 ppm of hazelnut protein. Then, it was further diluted to obtain standards for ELISA or LFIA.

### 2.7. Preparation of spiked, blended and incurred foods

Spiked foods were prepared by adding different volumes of the hazelnut protein extract to the ground or liquid blank matrices to obtain the desired quantity of hazelnut protein.

Blended foods were prepared by adding directly defatted hazelnut flour to the ground or liquid blank matrices to obtain the desired quantity of hazelnut protein.

Model cookies (20 g, 7 cm diameter) incurred or not with ground hazelnut at a final concentration of 240 ppm hazelnut protein and baked at 205 °C for 10 min were prepared according to the method 10-50D of the American Association of Cereals Chemists (AACC, 2000). Cookies with lower level of hazelnut protein were produced by mixing the cookie containing hazelnut with the blank cookie to the desired concentration.

### 2.8. Preparation of food extracts

Ground samples (1.00 ± 0.01 g) or liquid samples (1.00 ± 0.01 mL) were added with 10 or 9 mL of extraction buffer, respectively, in a filter-plastic bag (Seward Stomacher®, Worthing, UK). After blending manually during 5 min, the filtered extract was collected.

### 2.9. Sandwich ELISA

A volume of 120 μL/well of anti-Cor a 9 antibodies at 5 μg/mL in 50 mM carbonate buffer, pH 9.6 was added for coating wells (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. Wells were washed three times with 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 3 mM KCl and 140 mM NaCl, pH 7.4 (PBS) containing 0.05% Tween 20 (PBST) and then incubated with a blocking solution composed by 3% ovalbumin in PBS for 2h at RT. After a washing step with PBST (3 times), the plates were stored at 4 °C. Before using, wells were washed with PBST, incubated with of standards or samples (100 μL/well) for 30 min at RT and washed again 3 times with PBST. Then, wells were incubated with 100 μL/well of a solution of peroxidase labelled anti-Cor a 9 antibodies (1.3 mg/mL) diluted 1/25,000 in PBS for 30 min at RT. After washing, wells were incubated with 100 μL/well of 3,3′,5,5′-tetramethylbenzidine substrate for 30 min. Finally, the enzymatic reaction was stopped by adding 50 μL/well of sulfuric acid 2 M and the absorbance of the wells were read at 450 nm on a microplate reader ELISA Multiscan MS (Labsystem, Helsinki, Finland).

### 2.10. Lateral flow immunoassay

Beads conjugated with anti-Cor a 9 and anti-Internalin A antibodies were mixed at a ratio of 1:1 and dispensed over the conjugated pad composed of glass fiber membrane (GE Healthcare) using the ZX 1010 Dispenser (Bio-Dot, Irvine, USA). Specific antibodies against internalin A (control line) and Cor a 9 (test line) were dispensed in two independents lines over a nitrocellulose membrane at a concentration of 0.5 mg/mL. An adhesive backing card was used to assemble the nitrocellulose membrane, conjugate and adsorbent pads, with an overlapping among them of 2 mm. Finally, strips of 4 mm width were cut with a Guillotine Cutter CM4000 (Bio-Dot, Irvine, USA) and stored at room temperature until used. The test procedure was performed by dipping the strip into 150 μL of standards or samples and incubating for 10 min. Results were gathered in two ways: with naked eye interpretation and using an electronic strip reader (IRIS, ZEULAB, Zaragoza, Spain). The electronic device contains embedded software that calculates the signal value of control and test lines of the test.

### 2.11. In-house validation

Validation of ELISA and LFIA was performed following the standardized guidelines of the Association of Official Analytical Chemists (AOAC) (Abbott et al., 2010; AOAC International, 2016). Specificity was determined by assaying 74 basic ingredients including, different food categories like tree nuts, legumes, seeds, cereals, animal food, spices, fruits and vegetables (Table 1) after the extraction as it was previously described. All samples were assayed undiluted unless spices that were diluted 1/10.

#### 2.11.1. Validation of ELISA test

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated as the mean concentration of the blank standard (ten replicates) plus 3.3 and 10 times the standard deviation, respectively.

Precision parameters were determined using a blended chocolate soy drink and model incurred cookies, both at different levels of hazelnut protein. Extracts from eight independent extractions of the sample were analyzed in one assay to determine the repeatability of the method.

**Table 1**
Food commodities analyzed in the cross-reactivity study by ELISA and LFIA tests.

| Tree nuts | Legumes | Seeds | Fruits and Vegetables | Animal foods | Spices |
|---|---|---|---|---|---|
| Almond | Beans | Poppy | Apple | Beef | Aniseed |
| Brazil nut | Chick-peas | Pumpkin | Apricot | Chicken | Basil |
| Cashew | Lentils | Sesame | Banana | Egg | Caraway |
| Chestnut | Lupine | Sunflower | Carrot | Hake | Cardamom |
| Macadamia | Pea | Linseed | Celery | Ham | Chili |
| Pecan nut | Peanut | | Cocoa | Cow milk | Cinnamon |
| Pinions | Red beans | | Coconut | Pork | Cloves |
| Pistachio | Soy | | Grapes | Prawn | Coriander |
| Walnut | Soy lecithin | | Kiwi | Trout | Curry |
| | White beans | | Melon | Tuna | Garlic |
| | | | Orange | | Ginger |
| **Cereals** | | | Peach | | Nutmeg |
| Barley | | | | | Oregano |
| Buck-wheat | | | | | Paprika |
| Corn | | | | | Parsley |
| Oats | | | | | Black Pepper |
| Rice | | | | | White Pepper |
| Rye | | | | | Rosemary |
| Wheat | | | | | Spearmint |
| | | | | | Sumac |
| | | | | | Turmeric |

Three independent extractions from the same sample in three different days were also analyzed to know the intermediate precision, also called within-laboratory reproducibility (Magnusson & Örnemark, 2014).

The matrix effect was evaluated in different foods (bovine milk, orange juice, apple vinegar, chocolate soy drink, salad dressing, coffee liquor and cheese) at two spiked levels of hazelnut protein. Recovery was calculated as the ratio between protein concentrations determined by ELISA respect to the concentration of protein added based on Kjeldahl determination.

Robustness was estimated according to a Youden matrix design in which seven variables were selected (Table S1) for the fractional factorial design (Karageorgou & Samanidou, 2014). The chocolate soy drink at two levels of hazelnut protein addition was tested to obtain the standard deviation of the differences (SDi), a factor that allows to estimate the robustness of the method.

#### 2.11.2. Validation of LFIA test

A number between 20 and 80 independent samples of purified Cor a 9 or hazelnut protein extract at different protein levels, were used to estimate the Probability of Detection (POD). POD was estimated as the ratio between the number of positive results and the number of total samples analyzed at each protein level, the POD value must be above 0.95 to ensure a level with at least 95% confidence (AOAC International, 2014; Wehling, LaBudde, Brunelle, & Nelson, 2011). The limit of detection was confirmed by assaying different spiked, blended and incurred food matrixes.

The robustness of the method was tested by establishing four predetermined deliberated changes in the assay protocol and estimating the effects on the results (Table S1).

### 2.12. Surface and rinsing water testing

Surfaces of stainless steel and melamine were included in this study. Different levels of hazelnut protein were spread using the swab over a 10 × 10 cm surface and let dry at RT as recommended by FoodDrinkEurope (2013). Swabs were previously dipped into 0.5 mL buffer and rubbed over the delimited surface in all possible directions. Afterwards, it was dipped again in the buffer and shaked for a few seconds manually. Then, the same extracted sample was analyzed by both ELISA and LFIA tests. Recovery ratios on ELISA test was calculated as the ratio of hazelnut protein measured in the extraction buffer after rubbing and shaking compared to the amount spread on the surface.

Rinsing water studies were carried out using solutions containing different concentration of HCl and NaOH (0.1, 0.25, 0.5 and 1 N) with or without hazelnut protein.

## 3. Results and discussion

### 3.1. Cor a 9 and antibody characterization

The electrophoretic profile of purified Cor a 9 is shown in Fig. S1a. It mainly contains intensive bands of 20–25 kDa and 35–38 kDa that correspond to basic and acidic subunits of the protein. The profile also contains some minor groups of light polypeptides (<20 kDa) that probably correspond to peptides formed by partial hydrolysis of Cor a 9, as it was also mentioned previously (Rigby et al., 2008; Trashin et al., 2011). The degree of purity of the protein was above 90% as determined by densitometry.

The titer of antisera, which corresponds to the inverse of the log dilution that yields half of the maximum absorbance, was determined by a non-competitive indirect ELISA using wells coated with Cor a 9. The titers ranged between 4.5 and 5.6 depending of animal and extraction. The specificity of the antibodies by Western-blotting showed the recognition of both acidic and basic polypeptides of Cor a 9 (Fig. S1b).

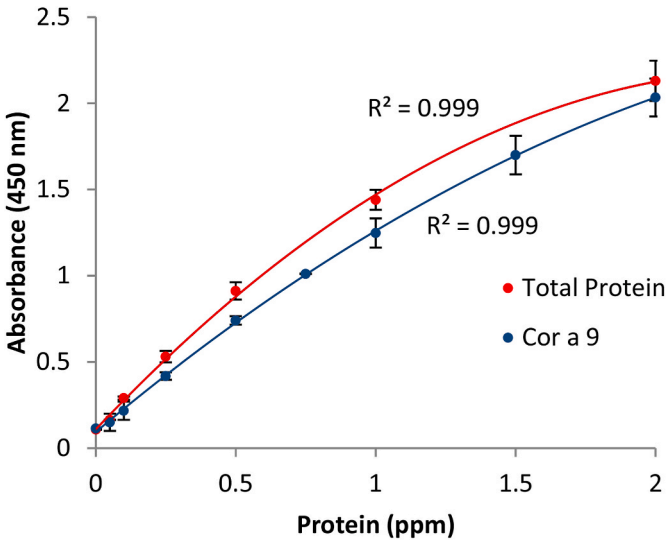


**Fig. 1.** Calibration curves obtained with purified Cor a 9 and hazelnut extract by sandwich ELISA test. Error bars indicate the standard deviations of the absorbance values (n = 10).

### 3.2. Sandwich ELISA assay

The development of sandwich ELISA included optimizing different conditions, mainly the concentration of capture and detection antibodies.

Calibration curves for purified Cor a 9 and hazelnut protein extracts showed a reliable concentration range of 5–200 ng/mL of protein for both curves, which correspond to 0.05–2 ppm, taking into account the extraction buffer dilution, which was 1/10 (Fig. 1). Both curves fitted on polynomial curves that gave regression coefficients ($R^2$) above 0.99. The calibration curves allow estimating the concentration of hazelnut protein in samples by interpolating their absorbance values.

The test using both calibration curves showed LOD and LOQ values of 0.02 and 0.05 ppm of hazelnut protein, which correspond to 2.1 and 4.6 ng/mL of Cor a 9, respectively. Calibration curves were linear from 0.05 to 2 ppm. The similar concentration range obtained for Cor a 9 and hazelnut extract could be due to the extraction buffer solubilized mainly the target protein in the hazelnut extract.

The LOD and LOQ obtained in this study are slightly lower than those previously reported by Trashin et al. (2011) using sandwich ELISA and antibodies against Cor a 9 which was 4 and 10 ng/mL.

### 3.3. Lateral flow immunoassay

Lateral flow immunoassay was optimized by testing different levels of antibody dosed on the nitrocellulose membrane and the ratio of antibody to latex microparticles. A positive result is considered when both blue and red lines appear and a negative result is when only the blue line is visualized. The control line ensures that the test had been carried out correctly so as if the blue line does not appear, the test must be considered invalid.

Results were interpreted by the naked eye and with a strip reader. When using the reader, a threshold value of 2.2 arbitrary units of color intensity was set to consider a sample as positive. This cut-off value was calculated as the mean signal from negative food commodities ingredients (n = 74) plus 3.3 times their standard deviation (Abbott et al., 2010).

When a hazelnut extract was tested at different dilutions to evaluate the sensitivity of the LFIA test, the lowest level obtained with a Probability of Detection (POD) higher than 0.98 was 0.5 ppm with the naked eye and 1 ppm with the strip reader (Table 2). The different level of detection obtained by naked eye and strip reader could be due to the

**Table 2**
Limit of detection of LFIA test determined in hazelnut extracts using naked eye and strip reader. N: number of replicates analyzed. x: number of positive results. POD: probability of detection. LCL: lower control limit (95% CI). UCL: upper control limit (95% CI). CI: confidence level. SD: standard deviation of strip reader signal.

| | Hazelnut protein (ppm) | Mean | SD | N | x | POD | LCL | UCL |
|---|---|---|---|---|---|---|---|---|
| **Naked eye** | 160000 | | | 6 | 0 | 0.00 | 0.00 | 0.39 |
| | 80000 | | | 6 | 3 | 0.50 | 0.19 | 0.81 |
| | 40000 | | | 6 | 6 | 1.00 | 0.61 | 1.00 |
| | 10 | | | 6 | 6 | 1.00 | 0.61 | 1.00 |
| | 5 | | | 6 | 6 | 1.00 | 0.61 | 1.00 |
| | 1 | | | 42 | 42 | 1.00 | 0.92 | 1.00 |
| | 0.50 | | | 42 | 41 | 0.98 | 0.88 | 1.00 |
| | 0.10 | | | 20 | 0 | 0.00 | 0.00 | 0.16 |
| **Strip reader** | 160000 | 1.0 | 0.6 | 6 | 0 | 0.00 | 0.00 | 0.39 |
| | 80000 | 2.8 | 1.6 | 6 | 3 | 0.50 | 0.19 | 0.81 |
| | 40000 | 8.8 | 5.2 | 6 | 6 | 1.00 | 0.61 | 1.00 |
| | 10 | 22.6 | 4.3 | 6 | 6 | 1.00 | 0.61 | 1.00 |
| | 5 | 16.9 | 4.2 | 6 | 6 | 1.00 | 0.61 | 1.00 |
| | 1 | 4.0 | 1.5 | 42 | 42 | 1.00 | 0.92 | 1.00 |
| | 0.50 | 2.1 | 0.9 | 42 | 26 | 0.62 | 0.47 | 0.75 |
| | 0.10 | 0.4 | 0.3 | 20 | 0 | 0.00 | 0.00 | 0.16 |

faint lines could be visually observed but the signal intensities from the reader were under the threshold value. This fact was also evidenced in a previous study performed with a LFIA test to detect almond (Civera et al., 2022). When the purified Cor a 9 was tested at different dilutions, the lowest level obtained with a POD higher than 0.98 was 100 ng/mL with both reading methods (Table S2).

An important aspect to be evaluated in sandwich LFIA is the hook effect, occurring when samples contain a high concentration of the analyte, as it causes a decrease in color intensity in the test line giving a false negative result (Galan-Malo et al., 2019; Ross, Filippini, Nielen, & Salentijn, 2020). As shown in Table 2, this effect appeared at hazelnut protein concentrations higher than 40,000 ppm. It is expected that the samples to be analyzed do not contain such a big amount of hazelnut protein as the test is designed to detect the presence of low levels of hazelnut due to cross-contamination. The concentration of hazelnut protein to produce the hook effect in this study is much higher than that reported by Ross, Salentijn, and Nielen (2019) using a sandwich LFIA with monoclonal antibodies that indicated levels of 100 ppm of total hazelnut protein.

### 3.4. Cross-reactivity

The specificity of the ELISA and LFIA was tested using extracts of various nuts and several ubiquitous food ingredients (Table 1). These studies are highly recommended in international guidelines (Abbott et al., 2010) for assuring the applicability of the tests on different types of matrixes. Samples were assayed undiluted unless for spices, which were tested 10-fold diluted because they are normally added in the range between 0.5 and 1% (Kaefer & Milner, 2008). Cross-reactivity was calculated as the concentration ratio respect to a hazelnut extract (100%). In both tests, no significant cross reactivity was found for the ingredients assayed with the exception of walnut and Pecan nut that showed reactivity below 0.04% and 0.09% respectively when assayed by ELISA and below 0.05% and 0.1% when assayed by LFIA. Almond, coconut, oat, pumpkin, poppy and sunflower seeds gave percentages of cross-reaction, below 0.001% for LFIA and ELISA but such levels does not seem relevant from a practical point of view.

The presence of cross-reactivity has been also found with Pecan nut, coconut, Macadamia nut and white and red beans using an ELISA to Cor a 9 (Trashin et al., 2011). In other studies based on ELISA tests to detect soluble hazelnut proteins, cross-reactivity has been also reported with ingredients such as peanut, walnut, almond and Pecan nut (Akkerdaas et al., 2004; Cucu et al., 2012).

### 3.5. Analysis of spiked food

The food matrix can influence the results obtained by immunochemical techniques and studies to evaluate this effect are usually based on the analysis of spiked food. In the present study, seven types of food matrixes (bovine milk, orange juice, coffee liqueur, chocolate soy drink, salad dressing, apple vinegar and goat cheese) labelled as hazelnut-free were spiked at different levels of hazelnut protein and tested by ELISA and LFIA (Table 3). Commercial food selected to determine the matrix effect included products in which the presence of components like tannins or polyphenols or the acidic pH could hamper the interaction of the target protein with antibodies. All matrixes gave a negative result by ELISA and LFIA when no hazelnut was added.

Recoveries from 83 to 110% were obtained by ELISA with samples spiked at 0.1, 0.5 and 1 ppm of hazelnut protein (CV<25%). These results are within acceptance levels established by the AOAC to quantify food allergens (Abbott et al., 2010). Recovery results obtained in this study are similar to those reported by Trashin et al. (2011) using extracts of blank cookies spiked with a hazelnut extract. In other studies in which ELISA is targeting hazelnut soluble proteins, detection levels of 1 ppm of hazelnut protein has been reported (Akkerdaas et al., 2004; Costa et al., 2015; Faeste et al., 2006; Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005). The lowest level in which hazelnut protein could be detected by ELISA was reported by Blais, Gaudreault, and Phillippe (2003), with 0.1 ppm. However, this level was only reached for chocolate ice cream while for the other types of food the detection limit was 1 ppm.

In the case of LFIA, the test could detect all the foods spiked with 0.5 ppm of hazelnut protein except for bovine UHT milk, which was detected at 1 ppm. This fact could be attributed to the presence of interferences with this specific matrix. These results confirm the LOD value of 0.5 ppm of hazelnut protein determined for LFIA using the naked eye lecture.

The LOD value obtained in our study is similar to that reported by Ross et al. (2019) by LFIA with antibodies against hazelnut soluble proteins as they obtained a positive result when testing biscuits spiked with 0.5 ppm of hazelnut protein.

Although the same antibodies were used in ELISA and LFIA developed in this study, the LOD of LFIA is about one order of magnitude higher than that obtained for ELISA. This fact could be explained by the limitation of reagents and/or the short incubation time used in LFIA compared to ELISA tests. These differences in sensitivity have also been reported for other allergenic proteins, such as beta-lactoglobulin or amandin, when using both immunoassays (Civera et al., 2022; Galan-Malo et al., 2019).

### 3.6. Analysis of blended and incurred food

Blended foods are another alternative to evaluate the recovery of allergens from a sample when incurred foods are not available. Blended foods are prepared by adding the allergenic ingredient, instead of an extract of the allergenic ingredient, to the processed blank food.

Furthermore, according to recommendations established by international guidelines (Abbott et al., 2010; AOAC International, 2014), validation of an immunoassay should include testing incurred foods with known levels of allergens. Incurred foods are prepared using allergenic ingredients and subjected to processing conditions equivalent to those used in the food industry. Incurred standards allow verify the effects of processing on the recovery and detection of the target protein (Taylor, Nordlee, Niemann, & Lambrecht, 2009).

In our study, blended samples of chocolate soy drink and incurred samples of cookies, containing different levels of hazelnut protein were tested by ELISA and LFIA tests.

Results showed that blank food samples of soy drink and cookies were below the limit of detection of ELISA and LFIA tests, indicating the

**Table 3**
Detection and recovery of hazelnut protein in food matrixes spiked with hazelnut extract and analyzed by ELISA and LFIA. P: percentage of positive results. The number of independent extraction analyzed by ELISA and LFIA were n = 2. ND, Not detected. NA, Not analyzed. CV: coefficient of variation.

| | | LFIA | | ELISA | |
|---|---|---|---|---|---|
| Food matrix | Hazelnut protein (ppm) | P (%) Naked eye | P (%) Strip reader | Mean recovery (%) | CV (%) |
| Chocolate soy drink | 0 | 0 | 0 | ND | |
| | 0.1 | NA | NA | 109.4 | 6.6 |
| | 0.5 | 100 | 100 | 108.7 | 3.0 |
| | 1.0 | 100 | 100 | 109.8 | 18.5 |
| Coffee liquor | 0 | 0 | 0 | ND | |
| | 0.1 | NA | NA | 83.5 | 16.4 |
| | 0.5 | 100 | 100 | 93.6 | 10.7 |
| | 1.0 | 100 | 100 | 82.9 | 14.1 |
| Goat cheese | 0 | 0 | 0 | ND | |
| | 0.1 | NA | NA | 84.4 | 5.1 |
| | 0.5 | 100 | 100 | 105.0 | 7.9 |
| | 1.0 | 100 | 100 | 99.4 | 21.3 |
| Bovine UHT Milk | 0 | 0 | 0 | ND | |
| | 0.1 | NA | NA | 92.0 | 8.3 |
| | 0.5 | 50 | 0 | 107.4 | 5.0 |
| | 1.0 | 100 | 100 | 96.9 | 11.3 |
| Orange juice | 0 | 0 | 0 | ND | |
| | 0.1 | NA | NA | 105.4 | 7.8 |
| | 0.5 | 100 | 100 | 109.9 | 25.3 |
| | 1.0 | 100 | 100 | 106.8 | 2.2 |
| Salad dressing | 0 | 0 | 0 | ND | |
| | 0.1 | NA | NA | 84.6 | 11.1 |
| | 0.5 | 100 | 100 | 94.9 | 3.4 |
| | 1.0 | 100 | 100 | 83.3 | 6.3 |

Recovery was calculated as the ratio between protein concentrations determined by ELISA respect to the concentration of protein added based on Kjeldahl determination.

absence of interference with these food matrices (Fig. 2). When using the ELISA test, samples of blended chocolate soy drink containing 0.10 ppm and incurred cookies containing 0.15 ppm of hazelnut protein could be quantified by the ELISA test. The concentration of hazelnut protein increased with the level of added hazelnut protein in both type of samples.

When the same food samples were analyzed by LFIA, cookies containing 0.30 ppm of hazelnut protein and soy drink samples added with 0.25 ppm of hazelnut protein could be not detected. At 0.5 ppm (chocolate soy drink) and 0.6 (incurred cookies) of addition, samples of both foods were positive when the lecture was performed by naked eye (Table S3). The same level of hazelnut protein addition was detected in incurred cookies when using the strip reader. However, blended soy drink added with 0.5 ppm gave a POD of 0.8 and at least a level of 1 ppm addition was necessary to obtain a positive result in all samples analyzed (Table S3).

Previous studies performed to detect hazelnut based on the determination of the corylin fraction or Cor a 9 have mainly assayed commercial foods and processed foods spiked with a hazelnut protein extract (Faeste et al., 2006; Holzhauser & Vieths, 1999). From our knowledge, only Trashin et al. (2011) included incurred foods to determine hazelnut protein by sandwich ELISA using antibodies against Cor a 9. In that study, cookies incurred with either a hazelnut extract or a defatted hazelnut flour, were assayed and the test could detect the addition of 1 ppm hazelnut protein in both types of samples. In the study of Costa et al. (2015) performed with model chocolate prepared by mixing melted chocolate at 40 °C with ground hazelnut, a detection level of 0.0005% hazelnut added was found which is in accordance with our

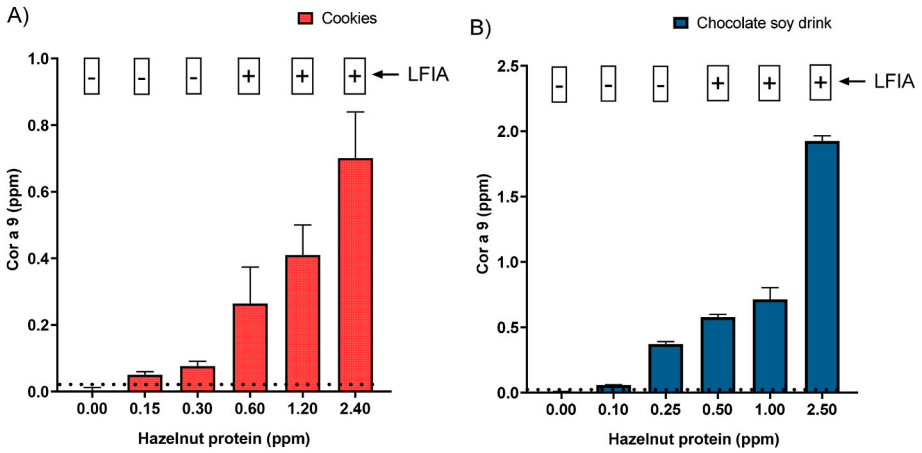


**Fig. 2.** Concentration of immunorective Cor a 9 in model cookies incurred with ground hazelnut (A) and chocolate soy drink blended with defatted hazelnut flour (B) determined by sandwich ELISA. Squares-boxes indicate results obtained by LFIA test using naked eye lecture. Values are the mean ± SD of two independent extractions analyzed in at least three assays. The dotted line corresponds to the LOQ of the ELISA test.

**Table 4**
Results of the precision study obtained by the ELISA test in cookies incurred with ground hazelnut and chocolate soy drink blended with hazelnut flour. Mean values correspond to the concentration of Cor a 9 (ppm). CV: coefficient of variation.

| Hazelnut protein (ppm) | Cookies | | | | Chocolate soy drink | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.5 | | 1.0 | | 0.1 | | 0.4 | |
| | Mean | CV (%) | Mean | CV (%) | Mean | CV (%) | Mean | CV (%) |
| **Repeatability** | 0.22 | 19.15 | 0.41 | 16.70 | 0.10 | 16,64 | 0.33 | 8.35 |
| **Intermediate precision** | 0.19 | 11.04 | 0.43 | 12.15 | 0.11 | 25.43 | 0.43 | 19.75 |
| Day 1 | 0.22 | | 0.50 | | 0.14 | | 0.36 | |
| Day 2 | 0.17 | | 0.40 | | 0.09 | | 0.40 | |
| Day 3 | 0.19 | | 0.43 | | 0.10 | | 0.52 | |

results using model cookies (0.0002% hazelnut added).

### 3.7. Precision of ELISA

Precision of an analytical method may be evaluated by testing different extractions of a sample in one assay (repeatability) or in different days or by different analysts (intermediate precision). Precision of the ELISA test were determined at two levels using incurred cookies (0.5 and 1 ppm hazelnut protein) and blended chocolate soy drink (0.1 and 0.4 ppm hazelnut protein). Repeatability ranged between 8% and 19% and intermediate precision between 11% and 25%, as shown in Table 4. These values are within the acceptable range established by AOAC guidelines and for ELISA methods to quantify food allergens (Abbott et al., 2010; AOAC International, 2016).

### 3.8. Robustness

The robustness of the ELISA test was determined using the blended chocolate soy drink at two levels of hazelnut addition (0.1 and 0.4 ppm), making slight variations in experimental conditions such as the volume, the temperature or the time used in different steps of the assay. Results showed no differences in the determination of the target protein within the range of 22–32 °C, which corresponds to the temperature usually found in a laboratory. The value of the standard deviation of the differences (SDi) obtained for each level was 0.026 and 0.080, respectively (Table S4). These values are lower than those determined for the intermediate precision (0.028 and 0.085) indicating that the ELISA test is robust (Karageorgou & Samanidou, 2014).

For LFIA, the robustness was also determined by assaying small changes in the standard procedure. Experiments were performed at two levels of hazelnut protein (0.5 and 1 ppm) using the blended chocolate soy drink (Table S5).

An increase of 10% in the volume of the extraction buffer or a decrease of 20% in the sample portion affected the limit of detection of the assay, giving negative results at 0.5 ppm of hazelnut protein. However, these results were not altered at 1 ppm of addition.

Concerning the deviations in the analysis procedure, only the assay time at both levels showed influence on the detection, with negative results at 2 and 5 min, whereas at 10 and 15 min the test was positive. These results remark the relevance of maintaining an incubation time of the strip for at least 10 min. Likewise, the sample portion and the volume of the extraction buffer are also critical factors to obtain reliable results when using the LFIA test to detect hazelnut protein in foods.

### 3.9. Effect of food processing

Food contamination usually occurs by using contaminated raw ingredients or shared equipment lines before processing. In the present study, the raw dough was prepared by adding the ground hazelnut as an ingredient before the heat processing treatment to determine the effect of the baking on the detection of hazelnut.

The raw dough and baked cookies at a level of 240 ppm hazelnut protein were extracted and diluted to 0.5 ppm to fit into the calibration curve of ELISA. Results showed that the concentration of immunoreactive Cor a 9 in baked cookies decreased by about 49.6 ± 3.0% (n = 4) with respect to the raw dough.

Our results are in accordance with those obtained by Trashin et al. (2011) and Cucu et al. (2012), as they found a decrease of immunoreactivity of 23–44% and 10–18% respectively, when comparing the concentration of hazelnut protein in raw dough respect to that determined in cookies after baking at 205 °C for 10 min.

The recovery values obtained in our and other studies were expected due to denaturation of the target protein and/or self-aggregation or interaction with other components of the matrix induced by the heat treatment applied.

**Table 5**
Recovery of hazelnut protein from stainless steel and melamine surfaces with ELISA and LFIA test. Results of LFIA are shown as percentage of positives. N: number of replicates analyzed. x: number of positives. POD: probability of detection. LCL: lower control limit (95% CI); UCL: upper control limit (95% CI). CI: confidence level. CV: coefficient of variation.

| | | LFIA | | | | | ELISA | |
|---|---|---|---|---|---|---|---|---|
| | Hazelnut protein (μg) | N | x | POD | LCL | UCL | Recovery (%) | CV (%) |
| **Stainless steel** | 5000 | 5 | 5 | 1.0 | 0.6 | 1.0 | 37 | 14 |
| | 62.5 | 5 | 5 | 1.0 | 0.6 | 1.0 | 23 | 6 |
| | 12.5 | 5 | 5 | 1.0 | 0.6 | 1.0 | 19 | 13 |
| | 2.5 | 20 | 20 | 1.0 | 0.8 | 1.0 | 24 | 28 |
| | 1.25 | 20 | 20 | 1.0 | 0.8 | 1.0 | 13 | 53 |
| | 0.625 | 10 | 0 | 0.0 | 0.0 | 0.3 | 17 | 49 |
| **Melamine** | 5000 | 5 | 5 | 1.0 | 0.6 | 1.0 | 26 | 28 |
| | 62.5 | 5 | 5 | 1.0 | 0.6 | 1.0 | 37 | 36 |
| | 12.5 | 5 | 5 | 1.0 | 0.6 | 1.0 | 24 | 13 |
| | 2.5 | 20 | 20 | 1.0 | 0.8 | 1.0 | 30 | 26 |
| | 1.25 | 20 | 20 | 1.0 | 0.8 | 1.0 | 32 | 33 |
| | 0.625 | 20 | 20 | 1.0 | 0.8 | 1.0 | 16 | 27 |
| | 0.3125 | 10 | 0 | 0.0 | 0.0 | 0.3 | 12 | 39 |

Recovery was calculated as the ratio of hazelnut protein concentration in the extracted sampled respect to the initial amount of protein.

### 3.10. Working surface testing

The removal of allergens from equipment or processing lines through cleaning has been recognized as one of the key points for effective allergen control (Jackson et al., 2008). Therefore, cleaning procedures must be validated and routinely verified using appropriate analytical methods.

In this study, the detection of hazelnut proteins in working surfaces was evaluated by ELISA and LFIA using stainless steel and melamine. ELISA could detect at least 0.6 and 0.3 μg in stainless steel and melamin, respectively. However, when using LFIA, the lower amount of hazelnut protein detected was 1.2 and 0.6 μg in stainless steel and melamin, respectively (Table 5). The recovery obtained by ELISA at these levels was similar in both surfaces about 13% and 16%, respectively. Recovery values obtained are slightly higher than that determined for almond protein (Civera et al., 2022) but lower than that determined for egg and milk proteins (Galan-Malo et al., 2017) in stainless steel surfaces. The rinsing water obtained after cleaning could be also assayed to verify the completeness of the cleaning process (Jackson et al., 2008). The cleaning in place (CIP) systems commonly consists of two cleaning cycles, alternating acid and basic solutions. That could affect hazelnut detection. Results obtained in the analysis of water with different concentrations of sodium hydroxide or hydrochloric acid showed no interference at concentrations of 0.5 N or lower for both substances (Table S6).

## 4. Conclusions

This study includes the development and validation of sandwich ELISA and LFIA tests for the detection of hazelnut based on the determination of Cor a 9, being the first LFIA report using that protein as the target. Both techniques showed a high sensitivity and absence of cross-reaction with a wide number of food commodities. The in house validation following international guidelines proved the precision of ELISA and the robustness of both tests in most challenging situations. The performance of both techniques was tested on complex food matrices, whose composition or pH could hamper the detection of the allergenic target protein, that were spiked with an extract of hazelnut proteins, giving good recoveries by ELISA and confirming the sensitivity of LFIA. Furthermore, ELISA and LFIA could detect hazelnut in food matrices blended with hazelnut flour at levels of 0.10 ppm and 0.50 ppm of hazelnut protein or in cookies incurred with ground hazelnut at levels of 0.15 ppm and 0.60 ppm of hazelnut protein, respectively, demonstrating the valuable properties of developed immunoassays. Both tests could be also applied to check out whether the cleaning procedures have been correctly performed. ELISA should be applied to carry out an initial validation of cleaning procedures or when significant changes are included in the process whereas LFIA could be used as a routine analysis during manufacturing process for on-site decision-making. Besides, the lecture of LFIA by an electronic strip reader would allow making an objective measurement of the results avoiding misinterpretation. The suitable application of these tests in food industry could improve the allergen risk management plans based on analytical data reducing the indiscriminate use of the precautionary labelling.

## CRediT authorship contribution statement

**Alba Civera:** Investigation, Formal analysis, Writing – original draft, Validation. **Patricia Galan-Malo:** Methodology, Investigation, Writing – review & editing. **Luis Mata:** Conceptualization, Writing – review & editing. **Ana P. Tobajas:** Investigation. **Lourdes Sánchez:** Visualization. **María D. Pérez:** Conceptualization, Supervision, Project administration.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109868.

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