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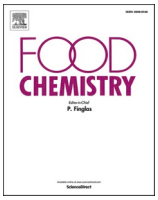

Sensitive ELISA and lateral flow immunoassay for the detection of walnut traces in processed food and working surfaces

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ABSTRACT

Walnut represents one of the most allergenic nuts that can be found as a hidden allergen. In this study, sandwich ELISA and lateral flow immunoassay (LFIA), based on the determination of Jug r 1, were developed to detect walnut. Cross-reactivity was only found with Pecan nut among a panel of 88 food ingredients tested. ELISA and LFIA could detect 0.25 and 0.5 μg/g of walnut protein in complex food matrices spiked with walnut extract, respectively. Furthermore, walnut was detected in blended (chocolate) and incurred foods (ice cream and bread) added with ground walnut at levels of 0.5 and 1.5 μg protein/g by ELISA and LFIA, respectively. LFIA could also detect 0.1 μg of walnut protein in working surfaces. ELISA displayed acceptable precision and high recovery (71–97 %) and both tests were robust. This study shows that developed ELISA and LFIA are reliable tools to be applied in allergen control programs.

1. Introduction

Tree nuts are valuable foods as they constitute a good source of proteins, unsaturated fatty acids, vitamins, minerals and antioxidants (Ros, 2010), and their consumption has increased in recent years due to their beneficial health properties. However, tree nuts are considered a potent source of food allergens representing a risk for sensitized individuals. The prevalence of nut allergy worldwide has been reported to range between 0.05 % and 4.9 %, varying among particular nuts and regions and it has increased in recent years (McWilliam et al., 2015; Motosue et al., 2017; Miles et al., 2020). Likewise, allergy to tree nuts has particular relevance as it often induces an anaphylactic reaction (Clark and Ewan, 2005).

The most effective way to prevent allergic reactions for sensitized consumers is to avoid the consumption of the offending food. For that reason, the regulatory authorities of numerous countries make mandatory to declare nuts on the food label when they are added as ingredients to inform the consumers about their presence. However, despite the labelling regulations, the wide use of nuts in the food industry makes that they are often present as hidden allergens due to cross-contact during food processing mainly by sharing production lines, which possess a risk to allergic consumers. For these situations, voluntary precautionary allergen labelling (PAL) has evolved as an essential tool to communicate this risk. However, the indiscriminate use of PAL leads limiting food choices and induces risk behaviours in allergic consumers. Therefore, it should only be used when after performing a thorough risk assessment, a genuine risk of allergen cross-contact within the supply chain is identified that cannot be eliminated (Holzhauser et al., 2020).

The Allergen Bureau's VITAL (Voluntary Incidental Trace Allergen Labelling) program was developed to provide a risk-based methodology for food producers to use in assessing the impact of allergen cross-contact and provide appropriate PAL (https://allergen bureau.net/vital/) (Brooke-Taylor et al., 2018). More recently, the report published by FAO/WHO about "Risk Assessment of Food Allergens" established the threshold levels in foods for priority allergens (FAO/WHO, 2022). Both programs have established different protein action levels for several allergenic ingredients considering the reference doses reported for them in relation to the usual serving sizes (from 5 to 500 g). Therefore, any analytical technique to determine those allergenic ingredients requires having enough sensitivity to detect or quantify the established levels to allow effective management of the unintended presence of allergens.

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Walnut (*Juglans regia*) is a popular nut that belongs to the Juglandaceae family and is cultivated all over the world, mainly in temperate climate areas. It is estimated that about 3 % of European adults are sensitized to walnuts, ranging between 0.1 % in Iceland and 8 % in Switzerland and Spain. Furthermore, walnut is the most elicitor nut in the United States (37–48 % of nut allergy) and Israel (74.6 % of suspected nut allergy) (Borres et al., 2022).

Currently, eight allergenic proteins (Jug r 1–8) have been identified in walnut and appear in the WHO/IUIS (2023) allergen database. Jug r 1 is a member of the 2S albumin protein family that seems to have an active role during germination providing nitrogen and sulphur to the seed and also showing antifungal properties (Hauser et al., 2008). It has been reported that about 75 % of allergic patients to walnut have IgE against it (Teuber et al., 1998). Furthermore, Jug r 1 has been recognized as the best forecaster in the diagnosis of walnut allergy (Borres et al., 2022) and it is associated with severe symptoms in sensitized individuals.

Jug r 1 is characterized by its small size (139 aminoacids) having an estimated molecular weight of about 14 kDa. It is composed of two polypeptide chains of 3.5 kDa and 8 kDa (light and heavy chains, respectively) that are held together by two interchain disulphide bridges and noncovalent bonds. Besides, the heavy chain of the protein contains two more intrachain disulphide bonds that contribute to its globular conformation. The compact structure of Jug r 1 makes the protein to have high stability to enzymatic degradation as well as to heat denaturation, maintaining its conformation after being heated at 90 °C (Moreno & Clemente, 2008; Sordet et al., 2009).

Reliable methods for the determination of walnut are necessary to ensure appropriate food labelling and to protect the health of allergic consumers. Different methodological approaches have been developed to detect walnut in food products based on the analysis of proteins by immunoassays or mass spectrometry and on the analysis of DNA using the polymerase chain reaction (PCR) technique. These techniques have been the object of some reviews (Costa et al., 2014, Downs et al., 2016, Luparelli et al., 2022). Immunoassays are the most widely used methods in the food industry for allergen detection because they present a high sensitivity and specificity and are relatively simple. ELISA techniques developed to detect walnut based on the use of polyclonal or recombinant antibodies raised against soluble proteins (Niemann et al., 2009; Madrid et al., 2018) or walnut 2S albumin Jug r 1 (Doi et al., 2008; Wang et al., 2014) as the target proteins have been reported. These ELISA tests, which present limits of detection below 1 mg/kg and good recoveries in different food matrices, could be applied to detect walnut as a hidden allergenic ingredient in processed foods. Due to the duration of the ELISA tests and the need of an equipped laboratory to perform the assays, their use, in general, is restricted to analytical services and official control laboratories. Food industry needs immediate results for making decisions so LFIA tests are the best choice because they are very easy to use and rapid.

The aim of this study has been to develop ELISA and LFIA tests using Jug r 1 as the target protein to detect traces of walnut. Although some commercial LFIA tests are available, like those from Romer Labs, Neogen and Morinaga, to our knowledge, this is the first published manuscript on the development of LFIA to determine Jug r 1. The “in house” validation of ELISA and LFIA tests has been performed following international guidelines using commercial complex matrices spiked with a walnut protein extract as well as blended food and incurred food with minute levels of ground walnut as ingredient.

2. Materials and methods

2.1. Materials

Shelled walnuts, basic ingredients and commercial processed foods were acquired from different local markets. HiTrap NHS-activated HP, HiTrap Phenyl HP and HiTrap SP HP were purchased from Cytiva (Uppsala, Sweden) and ELISA maxisorp flat bottom wells from Nunc (Roskilde, Denmark). The TMB substrate containing 3,3́’,5,5́’-tetramethylbenzidine was provided by ZEULAB S.L. (Zaragoza, Spain) and the Lightning-link Horseradish Peroxidase conjugation Kit by Innova Biosciences (Cambridge, UK). Red and blue carboxyl-modified dyed latex particles were acquired from Estapor (Merck, Darmstadt, Germany) and the conjugate pad of glass fiber membrane from Cytiva (Uppsala, Sweden). SDS-PAGE gels were acquired from Bio-Rad (Berkeley, CA, EEUU), PageRuler™ Prestained Protein Ladder molecular weight marker from Thermo Scientific (Waltham, EEUU) and Stomacher Biomaster 80 Closure Bags from Seward (Worthing, UK).

2.2. Purification of Jug r 1

The shells of the walnuts were removed and the seeds were ground in a Cucina HR7633 grinder (Phillips, EEUU). Jug r 1 was purified as described by Doi et al. (2008) with some modifications as follows. The proteins were extracted from ground walnut with 25 mM Tris-HCl buffer, pH 8.2 containing 1 M NaCl, 0.1 % PVPP, 1 mM EDTA and 0.02 % sodium azide at a proportion of 1/10 (w/v). After 1 h of stirring at 4 °C, the mixture was centrifuged at 3000 x *g* for 30 min. The supernatant was filtered through paper filter and a saline precipitation was carried out by adding ammonium sulphate to reach 90 % saturation. The mixture was stirred at 4 °C for 1 h and centrifuged at 9000 x *g* for 30 min. The pellet was collected and suspended in 0.05 M sodium phosphate buffer, pH 7.0 containing 1.5 M ammonium sulphate and filtered through 0.45 μm. Then, the extract was loaded onto a HiTrap Phenyl Sepharose HP (1 mL) column. After washing, retained proteins were eluted with the same buffer without ammonium sulphate. Fractions containing Jug r 1 were dialyzed against 0.05 M sodium phosphate, pH 8 and applied onto a HiTrap SP Sepharose HP column (1 mL). After washing the column, retained proteins were eluted with a gradient of sodium chloride (0–0.4 M). Chromatographic fractions of 2 mL were collected and analyzed by SDS-PAGE under reducing and non-reducing conditions.

2.3. Purification of specific antibodies for capture and detection

The purification of anti-Jug r 1 antibodies was carried out as described by Civera et al., 2022. Previously, purified Jug r 1 was inoculated into rabbits to obtain antisera in compliance with the Spanish policy RD53/2013 for the correct use and care of animals, which meets the European Union’s requirements (EU Directive 2010/63). The protocol was assessed within the Project License 30/19 approved by the Ethics Committee for Animals Experiments from the University of Zaragoza.

Antisera (15 mL) were loaded on an affinity HiTrap NHS activated HP column, which was previously coupled with Jug r 1. After washing the column with 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl and 140 mM NaCl, pH 7.4 (PBS), anti-Jug r 1 antibodies were eluted with 0.1 M glycine, 0.5 M NaCl buffer, pH 2.8 and immediately neutralized with 0.5 M Tris buffer, pH 8.0. These purified antibodies were used as capture antibodies in ELISA and LFIA assays. Besides, aliquots of the specific antibodies were used to prepare detection antibodies. For ELISA assays, anti-Jug r 1 antibodies were conjugated with horseradish peroxidase using a commercial kit. For the LFIA assay, anti-Jug r 1 antibodies were linked to red carboxyl-modified latex particles following manufacturer’s indications.

2.4. SDS-PAGE electrophoresis and western-blotting

SDS-PAGE was performed under reducing and non-reducing conditions using precast 16.5 % Tris-Tricine or 4–20 % polyacrylamide gels on a Mini-Protean Tetra Cell (BioRad Laboratories, Hercules, CA). Proteins were stained using Coomassie Brilliant Blue R. The gels were processed in an Image Scanner III (GE Healthcare, Chicago, IL).

Western blotting was performed as described by Benfeldt et al. (1995) using anti-Jug r 1 antiserum as primary antibody and goat anti-rabbit IgG as secondary antibody.

2.5. Preparation of Jug r 1 standards and food extracts

The concentration of a stock solution of Jug r 1 was determined by spectrophotometry at 280 nm using the estimated extinction coefficient for 1 mg/mL of 0.64. Standards of Jug r 1 were prepared by diluting the stock solution in the extraction buffer.

An extract of ground walnut was obtained by weighing 1 g of walnut and adding 10 mL of extraction buffer. The protein concentration of the extract was assumed considering the protein content of ground walnut previously determined by Kjeldahl (AOAC, 2002), using a nitrogen conversion factor of 5.3 (Sharma et al., 2010), obtaining 14.9 % of protein.

For the extraction of food samples, solid food was ground using a mixer. Then, 1 g (or 1 mL for liquid samples) was weighed into a filter plastic bag and 10 mL (or 9 mL) of extraction buffer were added. After blending the mixture manually for 5 min, the filtered extract was taken for analysis.

2.6. Spiked, blended and incurred food preparation

Commercial foods were spiked with a walnut extract and blended and incurred foods were added with ground walnut. In both cases, the amount of walnut protein was estimated considering the protein content determined in ground walnut by Kjeldahl (14.9 %).

Several commercial liquid or ground solid foods were spiked with the walnut extract to obtain 0.25, 0.5, 1 and 2.0 μg/g of walnut protein.

Blended food (chocolate bar) was prepared by adding walnut flour to ground chocolate to obtain the desired quantity of walnut protein. The chocolate bar containing 90 % of cocoa was melted in a water bath at 60 °C under continuous stirring. Then, ground walnut was added and after stirring the mixture for 45 min at 45 °C, it was poured into a mold and kept at room temperature for half an hour and stored at 4 °C.

Model incurred foods (bread and ice cream) were prepared by adding ground walnut as an ingredient to obtain 100 μg/g of walnut protein and then processed.

Lower levels (0.25, 0.5, 1.0, 1.5, 5.0 and 10 μg/g) in blended chocolate and incurred bread and ice cream were obtained by mixing the food samples containing 100 μg/g of walnut protein with the corresponding blank model foods prepared without walnut.

White bread was elaborated in a DoughMaker device (Oster, MN, USA) using as ingredients 480 g of wheat flour, 205 mL of water, 20 g of sugar, 10 g of butter, 8.8 g of fresh yeast, 5 g of salt and ground walnut. The program selected 'Traditional White Bread' included a kneading step for 30 min, a fermentation step for 2.5 h at 25 °C and a baking step at 160 °C for 40 min.

Ice cream was elaborated in an Ice System equipment Model BTM5 A (STAFF, Italy) by mixing 1 L of UHT whole milk, 250 mL of UHT whipping cream, 160 g of sugar and ground walnut. The mixture was kept stirring for 15 min and then subjected to a freezing process at −18 °C for 24 h.

All blended and model foods were also elaborated without walnut and considered as blank samples.

2.7. Sandwich ELISA

A volume of 120 μL of capture antibodies at a concentration of 3 μg/mL was added into wells, and afterwards, wells were blocked with ovalbumin at 3 % in PBS for 2 h and washed three times with PBS. For the assay, 100 μL of the standards or samples were added to wells and after incubation for 30 min, wells were washed five times with PBS containing 0.05 % Tween-20 (PBST). Then, wells were incubated with 100 μL of an appropriate dilution of the conjugated detector antibody in PBS for 30 min. After washing five times, wells were added with a colorimetric substrate containing TMB (100 μL) and after 30 min of incubation, the reaction was stopped by adding 50 μL sulfuric acid 2 M. The absorbance of wells was read at 450 nm in a microplate ELISA reader (Multiscan MS, Labystem). All the ELISA procedure was carried out at room temperature.

2.8. Lateral flow immunoassay

Anti-Jug r 1 antibodies and Internalin A were dispensed as two independent lines over a nitrocellulose membrane at 0.5 mg/mL for the test and control line, respectively, using a ZX 1010 Dispenser (Bio-Dot, Irvine, USA). Anti-Jug r 1 and anti-internalin A antibodies conjugated with red and blue latex beads were dispensed over a pad of glass fiber membrane at a ratio of 1:1.

All the components of the strip (nitrocellulose membrane, conjugate pad and adsorbent pads) were assembled using an adhesive backing card keeping an overlapped among them of 2 mm. Strips of 4 mm width were

Table 1
Food commodities (88) used in the cross-reactivity study analyzed by ELISA and LFIA.

Tree nuts	Legumes	Seeds	Fruits and Vegetables	Animal food	Spices
Almond	Beans	Quinoa	Apple	Beef	Aniseed
Brazil nut	Chickpeas	Poppy	Apricot	Chicken	Basil
Cashew	Lentils	Pumpkin	Banana	Cow milk	Caraway
Chestnut	Lupine	Sesame	Carrot	Egg	Cardamom
Hazelnut	Pea	Sunflower	Celery	Hake	Chili
Macadamia nut	Peanut	Linseed	Cocoa	Ham	Cinnamon
Pecan nut	Red beans		Coconut	Lamb	Cloves
Pinions	Soy		Grapes	Pork	Coriander
Pistachio	Soy lecithin		Kiwi	Prawn	Curry
	White beans		Lemon	Trout	Garlic
			Mushrooms	Tuna	Ginger
Cereals	**Others**		Melon		Nutmeg
Barley	Coffee		Nectarine		Oregano
Buckwheat	Olive Oil		Orange		Paprika
Corn	Salt		Peach		Parsley
Oats	Sugar		Pineapple		Black pepper
Rice	Tea		Watermelon		White pepper
Rye	Vinegar				Rosemary
Wheat	Wine				Spearmint
					Sumac
					Turmeric

cut using a Guillotine Cutter CM4000 (Bio-Dot, Irvine, USA). For the assay, the strip was dipped into a volume of 150 μL of standards or samples and incubated for 10 min. The results were gathered with both visual interpretations using the naked eye or using an optical strip reader that calculated the intensity signal of the control and test lines (IRIS, ZEULAB, Zaragoza, Spain).

2.9. ELISA validation

Different parameters including sensitivity, selectivity, precision and robustness were determined according to AOAC guidelines (Abbott et al., 2010; AOAC, 2016).

For the selectivity study, 88 food commodities belonging to different categories including tree nuts, legumes, seeds, cereals, animal food, fruits, vegetables and spices were tested (Table 1). Two extractions by duplicate were analyzed for all ingredients.

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as the mean concentration obtained in ten extracts by duplicate of the blank model foods (ice cream, bread and chocolate bar) plus 3.3 or 10 times its standard deviation (Abbott et al., 2010).

Blended and incurred foods were used to determine the precision parameters. Three extracts of incurred foods (blank and three levels of added walnut protein) per operator were analyzed by duplicate using the ELISA test. Repeatability was calculated as the coefficient of variation of samples determined by the same operator and intermediate precision as the coefficient of variation of samples determined by two operators which performed the assay in different days.

Spiked food (vinegar, red wine, orange juice, soy drink, chocolate milkshake, cupcake and sauce) at three levels of walnut protein addition were tested to evaluate the matrix effect. The recovery was calculated as the ratio of the walnut protein calculated by ELISA and the actual concentration determined by Kjeldahl.

Slight variations in the experimental conditions (temperature, volume, washing steps and assay time) were tested to determine the robustness of the ELISA test. A Youden matrix design that applies a fractional factorial design which combines seven variables was applied (Supplementary Table 1). Samples of model ice cream at two levels of walnut protein addition were tested to obtain the standard deviations of the differences (SDi) between values obtained with modified and established conditions of the ELISA (Karageorgou & Samanidou, 2014).

2.10. LFIA validation

Parameters of selectivity, sensitivity and robustness were determined following AOAC guidelines (2014) to validate the LFIA test.

A panel of undiluted extracts from 88 different ingredients were tested to check the cross-reactivity of the test, unless spices which were assayed at 1/10 dilution.

Extracts of ground walnut and purified Jug r 1 at different protein levels were tested to determine the sensitivity of the test. At least 30 independent samples were assayed at a concentration which was near the limit of detection to calculate the LOD through the probability of detection (POD) method. The POD value was estimated as the ratio between the number of positive samples and the total number of samples analyzed. A POD value above 0.95 ensures a positive sample with at least 95 % confidence (AOAC, 2014; Wehling et al., 2011).

Commercial foods spiked with walnut protein were also tested to confirm the LOD. In addition, incurred foods with different levels of added walnut protein were analyzed to evaluate the impact of food processing on the LOD.

The robustness of the test was determined using ice cream at a level of 1 μg/g of added walnut protein. Deliberated changes in the time, temperature, volume of the extract and weight of the sample were included to estimate the effect on the results (Supplementary Table 1).

2.11. Walnut detection on surfaces

Working surfaces of stainless steel and melamine were chosen due to their wide use in the food industry. Different concentrations of walnut protein were prepared and a volume of 50 μL was spread over a square surface of 10 cm side and allowed to air dry overnight as indicated by FoodDrinkEurope (2013). Then, a polystyrene swab was immersed in 0.5 mL of extraction buffer and rubbed over the square surface. Afterwards, the swab was dipped again in the extraction buffer and discarded and the same buffer was analyzed by ELISA and LFIA. Recovery of walnut protein from the surface was estimated by ELISA considering the amount of walnut protein in the extraction buffer after rubbing and shaking respect to the spread amount.

3. Results and discussion

3.1. Characterization of Jug r 1 and anti-Jug r 1 antibodies

Purified Jug r 1 was characterized by SDS-PAGE under reducing and non-reducing conditions (Supplementary Fig. 1 A and C). Under non-reducing conditions, a single band of about 14 kDa is observed whereas under reducing conditions two bands of about 7 and 5 kDa are visualized, which correspond to heavy and light polypeptide chains of the protein that are bound by disulfide bonds. The profile of Jug r 1 is similar to that obtained in other studies (Doi et al., 2008, Downs et al., 2014, Sordet et al., 2009). The analysis by densitometry showed that the purity degree of Jug r 1 was greater than 90 %. Besides, the analysis of purified Jug r 1 by Western blotting showed that antibodies recognized the protein (Supplementary Fig. 1B).

3.2. Sandwich ELISA

The optimization of capture and detection anti-Jug r 1 antibodies showed the best results when using concentrations of 3 μg/mL for coating wells and 10 ng/mL for the conjugate antibody solution.

Fig. 1 shows the calibration curve obtained with walnut extract and Supplementary Figure 2 the calibration curve obtained with purified Jug r 1. The concentration of walnut protein of the standards is expressed in μg/g considering the concentration of protein in ground walnut determined by the Kjeldahl method and the 10-fold dilution necessary to make the extraction. The concentration of Jug r 1 standards is expressed in ng/mL. Linear ranges of those curves were from 0.05 to 2.5 μg/g for walnut protein and from 0.25 to 30 ng/mL for Jug r 1. The model that best described the relationship between absorbance and concentration of standards of walnut protein or Jug r 1 was a second-degree polynomial curve, which gave coefficients of regression higher than 0.99 in both cases. The concentration of Jug r 1 or walnut protein in samples

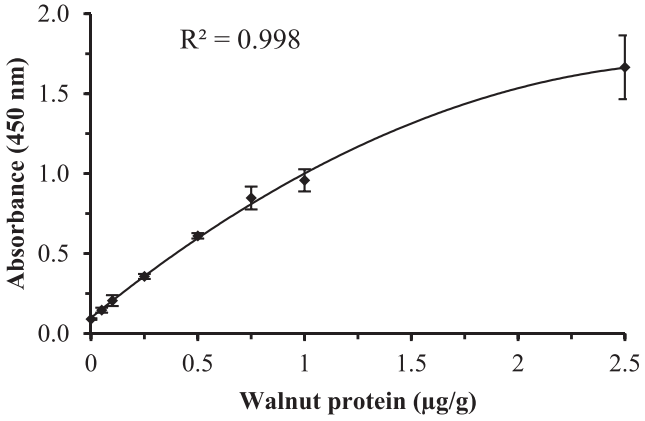

Fig. 1. Calibration curve obtained with walnut extract by ELISA test. Error bars indicate the standard deviations of the absorbance values.

was calculated by interpolating the absorbance values into the calibration curve obtained in each assay.

To estimate the relationship between the concentration of Jug r 1 and walnut protein, the concentration of Jug r 1 was determined in a series of walnut extracts, prepared from ground walnut as previously described and results showed that Jug r 1 represents about 5.2 % of the walnut protein.

The values of LOD and LOQ obtained for the ELISA test using the walnut protein curve or the Jug r 1 curve were 0.04 and 0.11 μg/g of walnut protein (0.2 and 0.5 ng/mL of Jug r 1), respectively. These values are slightly better than those reported by other authors using anti-Jug r 1 antibodies, which reported LOD and LOQ values of 0.16 and 0.31 μg/g (Doi et al., 2008) and 0.22 and 0.44 μg/g walnut protein, respectively (Wang et al., 2014).

3.3. Lateral flow immunoassay

The concentration of anti-Jug r 1 antibodies dispensed on the membrane as well as the ratio between dyed latex microparticles and antibodies were optimized to minimize the background signal in negative samples and maximize the signal in positive samples. When both blue and red lines appeared, the result is considered positive and if only the blue line appears, the test is considered negative. The blue control line verifies that the test was performed correctly, thus if the blue line does not appear, the test is considered invalid.

The visual interpretation of results in the LFIA test may be subjective when a low intensity is displayed. For that reason, in this study, we incorporated an objective read that allows establishing a cut-off value above which a sample is considered positive for walnut presence. This value was estimated considering the mean signal obtained from the negative food commodities analyzed, unless Pecan nut, in the cross-reactivity study (*Section 3.4*) plus 3.3 times the SD, obtaining a signal LOD value of 2.4 arbitrary units (a.u.) (Abbott et al., 2010).

When the walnut extract was analyzed at a wide range of concentrations, the LOD value was set at 0.5 μg/g of walnut protein, which gave a POD value of 1, for both the naked eye and strip reader (Table 2). Furthermore, high concentration levels of walnut protein were also assayed to study the hook effect, which occurs when the analyte of the sample saturates the binding sites of the antibodies linked to the latex microparticles and those immobilized in the test line, giving a false negative result (Galan-Malo et al., 2019). Results showed that although there is a decrease in the signal intensity of the test line when walnut protein concentration was higher than 10 μg/g, the test line could be observed even at 148800 μg/g (1 g walnut /10 mL) giving a value of 55.7 a. u. in the strip reader.

When different concentrations of pure Jug r 1 were assayed (Table 2), the lowest level obtained with a POD value of 1.00 was 5 ng/ml in both naked eye and strip reader. The higher LOD of LFIA test for Jug r 1 or walnut protein compared to those obtained by ELISA is probably due to the shorter assay time of LFIA, which, on the other hand, is the main advantage of this test. This fact has also been evidenced in other studies that developed ELISA and LFIA to detect other allergenic proteins such as Pru du 6 or Cor a 9 (Civera et al., 2022; Civera et al., 2023).

3.4. Cross-reactivity

One important parameter to determine in immunoassays is the possible cross-reactivity of antibodies with other food ingredients, because it could give false positive results. Following AOAC recommendations, a wide variety of ingredients of different categories including nuts, legumes, seeds, cereals, spices, animal-origin food, vegetables and fruits were tested. Except for the spices that were diluted 10 times as they are normally used for seasoning (Kaefer & Milner, 2008), the rest of the ingredients were tested undiluted and if certain reactivity was observed, they were also assayed diluted several times. The percentage of cross-reactivity was calculated as the ratio of the concentration of walnut protein obtained in the ingredient and in the walnut extract (100 %). In the case of the ELISA test, all the 88 tested food ingredients, including eight tree nuts, gave a concentration below the LOQ except the Pecan nut (*Carya illinoinensis*) which gave a cross-reactivity of about 0.001 %. Similarly, for the LFIA test, only the Pecan nut gave a positive signal showing a cross-reactivity of 0.3 %.

Cross-reactivity to Pecan nut and hazelnut was also reported by other authors when using ELISA assays based on Jug r 1 (Doi et al., 2008; Wang et al., 2014; Sakai et al., 2010). The existence of certain reactivity with Pecan nut could be due to similarities of the allergenic 2S albumin protein as both species belong to the hickory family of Juglandaceae (Atanasov et al., 2018). In fact, several clinical studies confirm that most of the walnut allergic patients also show an allergy to Pecan nut and vice

Table 2
Limit of detection of walnut protein determined in walnut extracts and pure Jug r 1 solutions by LFIA using naked eye and strip reader.

Naked eye				Strip reader					
	units	N	X	X	Mean	SD	POD	LCL *(95 %CI)*	ULC *(95 %CI)*
Walnut protein	0.1	20	0	0	0.9	0.8	0.00	0.00	0.16
(μg/g)	0.5	40	40	40	6.2	2.8	1.00	0.91	1.00
	1.0	40	40	40	12.0	6.7	1.00	0.91	1.00
	5	40	40	40	48.2	29.1	1.00	0.91	1.00
	10	6	6	6	89.6	25.4	1.00	0.61	1.00
	100	6	6	6	73.4	8.1	1.00	0.61	1.00
	1000	6	6	6	71.9	5.3	1.00	0.61	1.00
	10,000	6	6	6	62.6	6.4	1.00	0.61	1.00
	100,000	6	6	6	61.6	9.8	1.00	0.61	1.00
	148,800	6	6	6	55.7	5.2	1.00	0.61	1.00
Jug r 1	2.5	30	16	10	2.1	1.7	0.33	0.19	0.51
(ng/mL)	5	30	30	30	6.9	2.4	1.00	0.89	1.00
	10	30	30	30	13.9	4.6	1.00	0.89	1.00
	25	6	6	6	41.9	8.0	1.00	0.61	1.00
	50	6	6	6	58.5	16.1	1.00	0.61	1.00
	100	6	6	6	97.6	7.4	1.00	0.61	1.00
	500	6	6	6	72.0	5.9	1.00	0.61	1.00
	1000	6	6	6	65.0	7.9	1.00	0.61	1.00

N: number of replicates assayed. X: number of positive results. POD: Probability of detection. LCL: lower control limit. UCL: upper control limit. CI: confidence level. SD: standard deviation of strip reader signal.

Table 3
Level of detection and recovery of walnut protein in food matrices spiked with walnut extract at different levels of walnut protein analyzed by ELISA and LFIA. Two extracts were analyzed by duplicate.

		LFIA				ELISA	
		Naked eye	Strip reader				
Food Matrix	Spiked level μg/g	P (%)	P (%)	Mean	SD	Recovery (%)	CV (%)
Chocolate drink	**Blank**	0	0	0.3	0.3	nd	
	0.25	na	na	–	–	89.6	18.4
	0.5	100	100	3.1	0.3	90.5	15.7
	1.0	100	100	6.8	2.9	91.1	17.7
	2.0	100	100	15.7	7.0	na	
Orange juice	**Blank**	0	0	0.4	0.7	nd	
	0.25	na	na	–	–	96.9	16.2
	0.50	100	100	4.0	0.7	82.4	11.4
	1.0	100	100	10.6	6.7	78.3	10.5
	2.0	100	100	26.6	15.3	na	
Milk	**Blank**	0	0	0.7	0.4	nd	
	0.25	na	na	–	–	93.4	12.0
	0.5	100	100	3.5	0.2	86.2	10.2
	1.0	100	100	7.7	2.2	77.1	9.1
	2.0	100	100	31.2	13.4	na	
Muffin	**Blank**	0	0	1.1	0.0	nd	
	0.25	na	na	–	–	85.2	19.4
	0.5	100	100	4.1	0.8	96.7	18.3
	1.0	100	100	9.0	2.9	71.5	9.3
	2.0	100	100	32.7	11.3	na	–
Romesco Sauce	**Blank**	0	0	0.2	0.7	nd	
	0.25	na	na	–	–	85.3	9.7
	0.5	100	100	3.4	0.4	81.2	12.5
	1.0	100	100	7.4	3.3	85.2	17.3
	2.0	100	100	22.0	1.8	na	
Vinegar	**Blank**	0	0	0.2	0.2	nd	
	0.25	na	na	–	–	79.2	12.9
	0.50	100	100	3.0	0.1	74.9	12.5
	1.0	100	100	7.0	2.7	80.9	18.1
	2.0	100	100	27.2	21.1	na	

P: percentage of positive results. nd: not detected. na; not analyzed. CV: coefficient of variation. SD: standard deviation of strip reader signal.

versa (Dreskin et al., 2021). This fact has been mainly attributed to 2S albumins which correspond to Jug r 1 (English walnut) and Car i 1 (Pecan nut), which cause symptoms in most allergic individuals.

3.5. Analysis of spiked food

To determine the existence of possible interferences with particular matrices that could hamper the interaction of antibodies with the target protein such as acidic foods or foods containing tannins or polyphenols, samples of orange juice, vinegar, sauce and chocolate drink were tested.

In addition, other matrices such as soy drinks, milk or muffin were also tested, since possible cross-contact could occur when sharing the same equipment or facilities with similar foods in which walnut would commonly be used. Results showed that all these foods gave a negative result by both methods (ELISA and LFIA) before adding the walnut extract.

In the case of ELISA test, concentrations of 0.25, 0.5 and 1 μg/g of added walnut protein were tested for each matrix (Table 3). Recovery values ranged from 71 % to 97 % depending on the matrix with variation coefficients from 9 % to 26 %. These results are considered acceptable according to the recovery levels reported by the AOAC for the quantification of food allergens (Abbott et al., 2010).

Niemann et al., (2009) using a sandwich ELISA to soluble walnut proteins reported a limit of quantification of 1 μg/g in butter cookies, ice cream, muffins and milk chocolate spiked with ground walnut and a recovery ranging between 72 % and 119 % in the analysis of chocolate samples incurred with walnut. Also, a recovery study using spiked matrices with walnut powder was performed by Wang et al. (2014) at 10 μg/g with recoveries ranging from 86 to 112 %.

For the LFIA test, all spiked foods tested at 0.5, 1 and 2 μg/g of added walnut protein were found positive using the naked eye and optical reader. These results confirm the LOD value of 0.5 μg/g previously established with the walnut protein extracts (Table 3).

3.6. Analysis of blended and incurred food

In this study, a blended food (chocolate) and two model incurred foods (bread and ice cream) were prepared at the Pilot Plant in which known concentrations of ground walnut were added as an ingredient and then processed. The use of blended and mainly incurred food is a more realistic approach to real food that can reveal some of the limitations of the immunoassays in the detection of allergenic food residues (Taylor et al., 2009).

Naturally incurred food allows the evaluation of the effects of processing on the target protein in the milieu of a food matrix. These effects include chemical modification induced by Maillard reaction, shear effects on protein structure by homogenization, emulsion formation or pH effects, among others. Some regulatory bodies may be unwilling to consider the approval of validation studies if they do not include results obtained with incurred samples (AOAC, 2014; Taylor et al., 2009).

Table 4
Level of detection of walnut protein in blended (chocolate bar) and model incurred (bread and ice cream) foods prepared with ground walnut at different levels of walnut protein analyzed by LFIA.

			Naked eye	Strip reader					
	Walnut Protein (μg/g)	N	X	X	Mean	SD	POD	LCL *(95 %CI)*	UCL *(95 %CI)*
Ice Cream	0	12	0	0	0.2	0.2	0.00	0.00	0.24
	0.5	42	11	4	1.3	0.7	0.10	0.04	0.23
	1.0	40	40	40	5.3	1.2	1.00	0.91	1.00
	1.5	40	40	40	16.5	5.6	1.00	0.91	1.00
	5.0	6	6	6	72.3	11.7	1.00	0.61	1.00
	10	6	6	6	85.4	11.3	1.00	0.61	1.00
	100	6	6	6	59.0	28.4	1.00	0.61	1.00
Bread	0	12	0	0	0.3	0.3	0.00	0.00	0.24
	0.5	40	30	29	3.9	1.1	0.73	0.57	0.84
	1.0	40	40	40	8.5	3.8	1.00	0.91	1.00
	1.5	40	40	40	11.3	2.4	1.00	0.91	1.00
	5.0	6	6	6	73.3	5.2	1.00	0.61	1.00
	10	6	6	6	110.2	10.2	1.00	0.61	1.00
	100	6	6	6	75.5	9.4	1.00	0.61	1.00
Chocolate bar	0	12	0	0	0.6	0.5	0.00	0.00	0.24
	1.0	40	25	22	2.8	1.4	0.55	0.40	0.69
	1.5	40	40	40	8.9	5.0	1.00	0.91	1.00
	2.0	40	40	40	13.4	3.1	1.00	0.91	1.00
	5.0	6	6	6	52.5	12.1	1.00	0.61	1.00
	10	6	6	6	70.9	6.3	1.00	0.61	1.00
	100	6	6	6	50.3	8.4	1.00	0.61	1.00

N: number of replicates assayed. X: number of positive results. POD: Probability of detection. LCL: lower control limit. UCL: upper control limit. CI: confidence level. SD: standard deviation of optical reader signal.

Table 5
Results of the precision study obtained by the ELISA test in blended (chocolate bar) and incurred model (bread and ice cream) foods prepared with ground walnut at different levels of walnut protein. Mean values correspond to the concentration of walnut protein (μg/g). CV: coefficient of variation.

	Walnut protein (μg/g)	Repeatability		Intermediate precision	
		Mean (μg/g)	CV (%)	Mean (μg/g)	CV (%)
Ice cream	0.25	0.22	10.1	0.18	24.8
	0.50	0.49	8.5	0.49	18.0
	1.00	0.94	5.6	0.85	11.2
Bread	0.50	0.28	11.0	0.26	25.8
	1.00	0.53	8.6	0.46	17.8
	1.50	0.73	16.4	0.68	17.7
Chocolate bar	0.50	0.14	12.1	0.15	19.6
	1.00	0.53	18.0	0.45	16.6
	1.50	0.61	15.4	0.58	14.9

The blended food (chocolate) and model foods included in this study (ice cream and bread) were selected as they are complex matrices in which there is a potential risk to find traces of walnut due to cross contact from the use of shared equipment.

Results showed that blank blended and model foods were negative when analyzed by ELISA and LFIA, indicating the absence of interferences with those matrices (Supplementary Figs. 3 and 4 and Table 4). For food with added walnut, ELISA test could determine the addition of 0.25 μg/g of walnut protein in ice cream and 0.5 μg/g in chocolate bar and bread. The detection of lower levels observed with ice cream compared to baked bread could be attributed to partial denaturation and/or aggregation of the target protein by the baking processing. Furthermore, it should be considered that chocolate usually induces interferences in immunoassays due to the interaction of polyphenolic compounds with antibodies. The lower level of Jug r 1 estimated by ELISA in bread and chocolate concerning that obtained in ice cream is evidenced at all levels of walnut protein addition as can be seen in Supplementary Figure 3. When the same blended and model foods were analyzed by LFIA using the naked eye and optical reader, ice cream and bread were positive at lower levels of added walnut (1 μg/g) than chocolate (1.5 μg/g) (Table 4). This fact could be caused by a fluidic problem due to the viscosity of chocolate samples.

Doi et al. (2008) using an ELISA based on Jug r 1 found that the test could detect walnut in several model foods (porridge, meatballs, bread, cake, biscuits, jelly and orange juice) incurred with 10 μg/g of walnut but no lower levels of added walnut were tested. Thus, it cannot be assumed that the ELISA test would determine trace levels of walnut that could represent a cross-contaminated food, as we tested in our study.

Although the action levels for walnut have not been established in the Vital 3.0 program, if considering those indicated for hazelnut (Holzhauser et al., 2020), the developed ELISA and LFIA tests have shown enough sensitivity to detect walnut even for a serving size of 100 g, which requires a level of detection of 1 μg/g of protein to ensure the protection for walnut allergic consumers. Recently, in the report

published by the FAO/WHO (2022), a higher level of 10 μg/g of walnut protein per 100 g of serving size has been reported for walnut.

3.7. Precision of ELISA test

The precision of the ELISA test was determined by analyzing three independent extracts by duplicate of the blank samples and three levels of walnut protein for each blended or model incurred food. The analysis was performed in one assay by one analyst (repeatability) or by two analysts in different days (intermediate precision) (Table 5). Results showed that the variation coefficients ranged from 5.6 to 18.0 % for the repeatability and from 11.2 to 25.8 % for the intermediate precision, being both acceptable regarding the guidelines of the AOAC for ELISA methods to quantify food allergens (AOAC, 2016).

3.8. Robustness of ELISA and LFIA tests

The robustness of the ELISA test was determined using model ice cream with 0.5 and 1 μg/g of added walnut protein. Slight modifications of the experimental conditions (assay temperature, time, volume and number of washes) were applied and the standard deviation of the differences (SDi) for each added level was calculated. The higher variation was obtained when the assay temperature was changed (Supplementary Table 2). Nevertheless, taking into account that the SDi of the robustness (0.0207 and 0.379 at 0.5 and 1 μg/g walnut protein addition) was lower than the standard deviation of intermediate precision, the ELISA test could be considered robust (Karageorgou & Samanidou, 2014).

The robustness of LFIA was also determined using model ice cream added with 1 μg/g of walnut protein (Supplementary Table 3). Results showed that a variation of 10 % in the weight of the sample portion or the volume of the extraction buffer as well as in the volume of the assay does not affect the sensitivity of the test. Likewise, the increase of the assay time to 15 min did not change results whereas the reduction to 5 or 2 min gave a negative result. Results obtained indicated that the incubation time of the assay is essential to obtain reliable results when using the LFIA test.

3.9. Working surface testing

Cross-contact of food with allergens is often caused by inadequate cleaning of shared equipment or processing lines. Thus, the allergen removal through cleaning is considered one of the critical points for effective allergen control (Jackson et al., 2008). A regular monitoring of the working surfaces using analytical techniques should be implemented in the food industry to ensure allergen removal. In this study, the detection of walnut protein in surfaces of stainless steel and melamine, which are commonly used in the food industry, was evaluated by ELISA and LFIA. In both surfaces, the lower level detected by ELISA was 0.025 μg of walnut protein with recoveries ranging from 46 to 64 %. When using LFIA, the lower level detected with a POD of 1 was 0.1 μg in both surfaces (Supplementary Table 4). The level of detection of walnut protein in surfaces obtained by ELISA ad LFIA is better than that reported for other nuts like almond and hazelnut (Civera et al., 2022; Civera et al., 2023). This fact suggests that the walnut target protein is more efficiently recovered during the rubbing of the surface and the dipping on the extraction buffer than the target proteins used in those studies.

4. Conclusion

In this study, ELISA and LFIA tests were developed and used to determine the presence of traces of walnut protein in spiked, blended and incurred foods subjected to different processing. Although some commercial LFIA tests are available, this is the first manuscript of a LFIA test based on Jug r 1 as the target protein to detect walnut residues. The inclusion of an optical strip reader has the great advantage of giving an objective measurement, avoiding confusion when determining results and mainly when faint bands are obtained. The in-house validation of the tests showed that they have a high sensitivity and specificity as well as acceptable recovery, precision and robustness. ELISA test could be applied to quantify the level of walnut in ingredients and final products and to map contaminations on working surfaces when the time to obtain results is not limited. However, LFIA test could be useful to carry out analysis during the manufacturing process that requires making quick decisions due to its simplicity and rapid response. LFIA is also a useful tool to verify proper cleaning of utensils and work contact surfaces between manufactured batches. It should be remarked that ELISA and LFIA tests have enough sensitivity to verify the established action levels indicated by FAO/WHO for walnut, even for a serving size of 500 g. The combined use of ELISA and LFIA tests could be applied in the risk management plans of allergens to reduce the indiscriminate use of PAL in foods and thus to ensure protection for walnut allergic patients.

CRediT authorship contribution statement

Alba Civera: Investigation, Methodology, Validation, Writing – original draft. **Clara Esteban:** Investigation. **Luis Mata:** Conceptualization, Writing – review & editing. **Lourdes Sánchez:** Writing – review & editing. **Patricia Galan-Malo:** Conceptualization, Project administration, Supervision, Writing – review & editing. **María D. Pérez:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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 material

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