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Development of sandwich ELISA and lateral flow immunoassay to detect almond in processed food

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ABSTRACT

Almond (*Prunus dulcis*) represents a potential allergenic hazard that should be included in Allergen Control Plans. In this study, sandwich ELISA and lateral flow immunoassay (LFIA), using amandin (Pru du 6) as the target protein, were developed to detect almond in processed food and validated according to international guides. ELISA could detect 2 ng/mL and LFIA 30 ng/mL of pure amandin. No cross-reactivity was found on a panel of 50 food commodities with the exception of Pecan nut, Brazil nut and chestnut for which the cross-reactivity was lower than 0.02%. Furthermore, ELISA and LFIA were able to detect 0.12 and 0.70 ppm of almond protein in foods spiked with almond extract whereas 0.20 and 2.0 ppm could be detected in baked cookies incurred with almond, respectively. Both techniques could be applied for food manufacturers and control agencies for monitoring the presence of almond traces in food and working surfaces.

1. Introduction

Almond (*Prunus dulcis*) is the main tree nut produced worldwide, about 3.5 million metric tons in 2019, United States being the first producer followed by Spain and Iran (FAO, 2020). Almonds are consumed raw or processed in snacks, as well as an ingredient in a variety of products like bakery, pastry, chocolate and confectionary products. The consumption of almond in developing countries has increased in the last years due to its health benefits (Kamil & Chen, 2012).

However, almond proteins are recognized as a source of dietary allergens for humans. Data about the prevalence of tree nut allergy are quite limited, but several reports indicate that it is increasing in recent years, especially in Western countries. A systematic review and *meta*-analysis on the prevalence of tree nut allergy showed a rate of about 2% for oral food challenge-confirmed allergy and between 0.05% and 4.9% for probable allergy (including reported IgE-mediated reactions or doctor's diagnosis) (McWilliam et al., 2015).

Almond allergy also deserves special attention because very low doses can induce severe allergic reactions, often an anaphylactic shock, and because this allergy usually persists throughout life (Costa, Mafra, Carrapatoso & Oliveira, 2012).

Until now, eight proteins have been identified as allergens in almond but only six of them, Pru du 3, Pru du 4, Pru du 5, Pru du 6, Pru du 8 and Pru du 10 are recognized and included in the repertoire of allergens by the World Health Organization and International Union of Immunological Societies (WHO/IUIS, 2021).). Pru du 6 also known as amandin or Almond Major Protein (AMP) is considered a major allergen of almond. It is also a major storage protein that accounts for 65% of total almond protein (Roux, Teuber, Robothan & Sathe., 2001). Amandin belongs to the 11S globulin family and it has a hexamer structure with molecular weight 360 kDa. Each subunit is composed by acidic and basic polypeptides of 34–42 kDa and 20-22 kDa, respectively, linked by a disulfide bond, which confers a high resistance to thermal treatments and pepsin degradation (Costa et al., 2012).

It is well established that the most effective way to prevent food allergic reactions is simply elimination diet. Therefore, most regulations have focused on providing information about the presence of food allergens through label declarations to protect allergic consumers. Tree nuts are included among the allergenic foods that must be labelled when they are used as ingredient in most countries including those of the EU and USA, Australia, New Zealand, Canada, China and Mexico (Gendel,

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2012). However, despite these regulations, hidden food allergens can still be present in a product due to cross contamination. As no specific legislative framework in UE is available for the labelling of cross-contaminated food products, producers could use the precautionary allergen labelling (PAL) "may contain". However, the abuse of PAL induces confusion in allergic consumers and restricts the food available for them. In 2005, the Allergen Bureau established an initiative, called Voluntary Incidental Trace Allergen Labelling (VITAL) Program, to provide a risk based methodology for food manufacturers to be used in assessing the impact of cross contact allergens, and providing appropriate PAL, thus avoiding its indiscriminate use (Holzhauser et al., 2020).

In spite of the effort made by the food industry to include allergens in the Hazard Analysis and Critical Control Points (HACCP) plans, 16% of notifications reported in 2018 by The Rapid Alert System for Food and Feed (RASFF) were due to the presence of undeclared allergens on food labels, being about 30% more than the previous year (RASFF, 2019).

As the almond is often used as ingredient in food industry, the unintentional presence of this allergen exists. Therefore, the development of reliable techniques would help food producers to control the risk of cross-contaminations and verify the presence of this ingredient in raw materials and final products. Several methods for almond detection have been developed mainly relying on immunochemical (Masiri et al., 2016; Su et al., 2013), mass spectrometry (Heick, Fischer & Pöpping, 2011) and DNA-based (Prieto et al., 2014) techniques. Currently, immunochemical techniques are the most widely used in the food industry to detect food allergens as they show a high specificity and sensitivity. Additionally, they have some advantages like technical simplicity, the possibility to test a large number of samples and the use of relatively inexpensive equipment (Cucu, Jacxsens & Meulenaer, 2013).

Enzyme-linked immunosorbent assay (ELISA) could be employed to check the presence of target allergens in ingredients and final products and to validate the cleaning process, obtaining quantitative results. Lateral flow immunoassay (LFIA) could be of choice to verify routine cleaning processes and to test ingredients, intermediary or finished products on site due to its simplicity and quick response (5–10 min), which allows taking corrective actions in a short time.

Some immunochemical techniques, which use antibodies raised against almond total soluble proteins, were developed to detect almond. These antibodies have been used in ELISA (Ben Rejeb et al., 2005) and LFIA (Masiri et al., 2016). Likewise, an indirect competitive ELISA using polyclonal anti-amandin antibodies was developed (Acosta, Roux, Teuber & Sathe, 1999). Using this ELISA, further studies showed that amandin maintains a considerable immunoreactivity after blanching, roasting or autoclaving of almonds or flour (Roux et al., 2001). However, the assay showed considerable cross-reactivity with certain food matrices that could interfere with specific detection of almond traces in foods (Tiwari et al., 2010).

Su et al. (2013) developed an indirect sandwich ELISA using rabbit polyclonal antibodies to almond soluble proteins as capture antibodies and a murine monoclonal antibody (4C10) that recognizes a conformational epitope of amandin as detector antibodies. This ELISA showed high specificity when assayed using a wide variety of foods commodities and good recovery in foods spiked with an almond protein extract. However, authors observed a considerable reduction in amandin immunoreactivity when they assayed samples of roasted almonds or heat treated model foods incurred with 0.5–5% almond flour such as cookies, sponge cakes or almond bars. This reduction was attributed to the epitope denaturation or to the loss of amandin solubility due to aggregation (Chhabra et al., 2017).

In this study, sandwich ELISA and LFIA using polyclonal antibodies raised to purified amandin were developed. This is the first paper published on the development of LFIA to detect amandin. Results obtained by visual interpretation and an electronic reader were compared. The performance of both assays were evaluated using cookies incurred with minute amounts of almond proteins as ingredient and several commercial complex matrices spiked with almond protein extract. An *in house* validation of both techniques was performed following standardized guidelines established by the AOAC (2016).

2. Materials and methods

2.1. Materials

Nonpareil shelled almonds, food commodities and processed foods with almond not included in the ingredient list were purchased from local supermarkets.

2.2. Isolation of amandin

The shell of almonds were removed and almonds seeds were ground, defatted three times using *n*-hexane at a ratio 1/5 (w/v) and residual hexane was evaporated overnight at room temperature (RT). Defatted almond flour was extracted with 0.02 M Tris-HCl buffer, pH 8.0 (ratio 1/10, w/v) for 1 h at RT and the mixture centrifuged at 9,000 × g for 30 min. Afterwards, the supernatant was loaded onto a HiTrap DEAE Cellulose column (5 mL) (GE Healthcare, Piscataway, NJ). The column was washed and bound proteins were released by elution with a linear gradient of NaCl (0–0.4 M). Fractions containing amandin were pooled, concentrated and loaded onto a Sepharose 6B-CL column (85 × 1 cm) (GE Healthcare, Piscataway, NJ) equilibrated with 0.1 M NaCl, 0.02 M Tris-HCl buffer, pH 8. Protein profiles of chromatographic fractions were analyzed by SDS-PAGE.

2.3. SDS-PAGE

SDS-PAGE under reducing conditions was performed as described by Laemmli (1970). Proteins were separated in 4–20% polyacrylamide gradient gels on a Mini-PROTEAN Tetra Cell (Bio-Rad) at 180 V, and stained with Coomasie Brilliant Blue R.

2.4. Antibody preparation and conjugation

Rabbits were immunized with purified amandin to obtain antisera according to Wehbi et al. (2005). Briefly, a volume of 0.5 mL of pure amandin solution (2 mg/mL) was homogenized with 0.5 mL of complete Freud's adjuvant and injected subcutaneously in the back. After three weeks, the animals were boosted following the same protocol but using incomplete Freund's adjuvant. Fifteen days later, the rabbits were bled from the ear vein. Thereafter, rabbits were immunized and bled with an interval of two weeks between each one. The titer of antisera, determined by a non-competitive indirect ELISA using wells coated with Pru du 6, ranged between dilutions 10^{-5} and 10^{-6} depending of animal and extraction.

All procedures were carried out using guidelines approved by the Ethic Committee of Zaragoza University for Animal Experiments (Project Licence 30/19). The care of animals was performed on regarding Spanish Policy for Animal Protection RD 53/2013 which meets the EU Directive 2010/63 on the animal's protection used for scientific purposes.

Purification of specific antibodies was performed by affinity chromatography using a HiTrap NHS-activated HP column (GE Healthcare) that was previously insolubilized with the corresponding pure target protein according to manufacturer instructions. A volume of 15 mL of antiserum was applied to the column. The gel was washed with 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl and 140 mM NaCl, pH 7.4 (PBS) until the absorbance at 280 nm of the eluate was lower than 0.02. The antibodies retained in the gel were eluted with 0.1 M HCl-glycine, 0.5 M NaCl solution (pH 2.8) and neutralized immediately with 0.5 M Tris solution (pH 8.0), and afterwards dialyzed against 15 mM sodium phosphate, 150 mM NaCl solution (pH 7.4) and concentrated.

For ELISA test, anti-amandin antibodies were conjugated with

horseradish peroxidase (HRP) using the Lightning-link HRP conjugation Kit (Innova Biosciences, Cambridge, UK).

For LFIA test, red and blue carboxyl-modified dyed latex particles (Estapor, Merck, Darmstadt, Germany), were coupled to anti-amandin or anti-internalin A antibodies, respectively, following manufacturer instructions (EMD Millipore Corporation, 2015). An antibody final concentration of 0.2 mg/mL was incubated with particles during 2.5 h at RT with gentle agitation at 12 rpm and 90° angle. Afterwards, 30 μL/mL of ethanolamine was added to stop the reaction and after 30 min incubation at room temperature, the mixture was centrifuged at 17,000 × g for 15 min, discarding the supernatant. Beads were blocked using 1% (w/v) BSA during 2 h at room temperature with gentle shaking. Conjugate beads were analyzed by dynamic light scattering (Zetasizer Nano Range, Malvern Instruments). Finally, beads conjugated with anti-amandin (test line) or anti-internalin A (control line) antibodies were mixed (ratio 1:1) and dispensed over the conjugate pad of glass fiber membrane (GE Healthcare) using a ZX 1010 Dispenser (Bio-Dot, Irvine, USA).

2.5. Sandwich ELISA

Microtiter wells (Nunc, Roskilde, Denmark) were coated with 120 μL/well of specific antibodies to amandin (1 μg/mL) in 0.05 M carbonate buffer, pH 9.6 and kept overnight at 4 °C. Then, wells were blocked with 300 μL/well of 3% (w/v) ovalbumin solution in PBS during 2 h at RT. Before using, wells were washed three times with 300 μL/well of PBS containing 0.5% Tween 20 (PBST), and incubated with 100 μL of samples or standards for 30 min at RT. After washing three times with PBST, wells were incubated for 30 min at RT with 100 μL of a solution of peroxidase labelled anti-amandin antibodies (1.4 mg/mL) diluted 1/300,000 in PBS. Then, after washing again, wells were incubated with 100 μL/well of 3,3́,5,5́-tetramethylbenzidine (TMB) substrate. The enzymatic reaction was stopped by adding 50 μL/well of 2 M H_2SO_4 and the absorbance was read at 450 nm in an ELISA plate reader (Labsystem, Helsinki, Finland).

2.6. Lateral flow immunoassay

The anti-amandin and anti-internalin A antibodies for test and control lines, respectively were sprayed onto a nitrocellulose membrane at 0.5 mg/mL using a ZX 1010 dispenser (Bio-Dot, Irvine, USA). The nitrocellulose membrane, conjugate and adsorbent pads were assembled on an adhesive baking card with an overlapping among the components of 2 mm. Finally, strips of 4 mm width were cut using a CM4000 guillotine cutter (Bio-Dot, Irvine, USA) and store with desiccant in closed tubes at room temperature. The test procedure was performed by dipping the strip into 150 μL of food extract an incubated for 10 min.

Results were gathered with naked eye interpretation but also with an electronic strip reader (IRIS, ZEULAB, Zaragoza, Spain) following manufacture instructions. The internal software calculated the value of the signal of each line.

2.7. Preparation of amandin standards

A stock solution of purified amandin (1 mg/mL) was prepared assuming an extinction coefficient value at 280 nm of 0.7. Amandin standards (0, 5, 10, 25, 50, 75 and 100 ng/mL) for ELISA assays were prepared by diluting the stock solution with PBS containing 0.25% bovine serum albumin. Amandin standards for LFIA were prepared by diluting the stock solution in extraction buffer.

2.8. Preparation of spiked and incurred foods

Whole and defatted ground almond contained 20.0% and 35.2% protein as determined by the Kjeldahl method, respectively. In this study results are expressed as almond protein content (ppm or μg/g), which was estimated considering those percentages.

Spiked foods were prepared by adding different proportions of almond protein extract to blank matrices. Model cookies incurred with whole almond flour were elaborated at the Pilot Plant of Food Science and Technology of Zaragoza University following Method 10-50D of the American Association of Cereals Chemists (AACC, 2000). Ingredients (128 g butter, 263.7 g sugar, 4.2 g salt and 5 g sodium bicarbonate and 86.3 g water) were mixed using a Kenwood kitchen mixer. Raw ground almond was added to obtain a final concentration of almond protein of 40 ppm. Round cookies (20 g, 7 cm diameter) were prepared and baked at 205 °C for 10 min (internal temperature reached was 135 °C). Lower percentages of incurred almond protein in cookies were prepared by mixing adequate proportions of baked incurred almond cookies of 0 and 40 ppm.

Finally, ground model cookies, spiked foods, blank matrixes or food commodities (1 g or 1 mL) were mixed with 10 and 9 mL of extraction buffer, respectively in a filter-plastic bag (Seward Stomacher®, Worthing, UK). After bags were manually blended for 5 min, the filtered extract was collected. For all samples, at least two sample extractions were analyzed in at least three assays.

2.9. In-house validation

Validation of ELISA and LFIA was performed according to the standardized procedures established by the Association of Official Analytical Chemists (AOAC) (Abbott et al., 2010; AOAC, 2016). Cross-reactivity in both assays was tested using a wide variety of food commodities including different types of tree nuts and ubiquitous basic ingredients of vegetal and animal origin following recommendations of AOAC guidelines (Abbott et al., 2010).

2.9.1. Validation of ELISA test

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated as the average concentration of ten replicates of the zero standard plus three or ten times the standard deviation, respectively.

Recovery study to know the effect of food matrix was performed at different levels of almond protein in spiked food and it was calculated as the ratio of amandin concentration between determined and predicted values. The predicted value of amandin was calculated using the following conversion factor (1 ppm of almond protein correspond to 0.45 ppm of amandin) as it is explained in section 3.8 of Results and Discussion. Recovery study to know the effect of thermal processing was calculated as the ratio of amandin concentration obtained in model cookies before and after the baking process.

Precision parameters were determined in cookies and orange juice at different levels of almond protein. Repeatability was determined using ten replicates of the same extract in one assay. Intra-assay reproducibility was evaluated by analyzing ten extracts of the same sample in one assay. Inter-assay reproducibility was determined analyzing three extracts of the same sample in three independent assays. Robustness was estimated by introducing deliberate changes in the established procedure in the same experiment according to a Youden matrix design that included seven variables related with the volume, time and temperature of the assay (Table S1). The standard deviation of the differences (SDi) was estimated as indicated by Karageorgou & Samanidou (2014).

2.9.2. Validation of LFIA test

The Probability of Detection (POD) was estimated by analyzing between 20 and 80 independent samples of purified amandin or almond protein at different levels of protein concentration. POD was estimated as the ratio between positive results and total number of samples analyzed at each level (AOAC, 2014). The POD analysis was performed in different days, by 3 different analysts. Lower and upper control limits (LCL and UCL) were calculated according to Wehling, LaBudde, Brunelle, & Nelson (2011). To confirm the limit of detection, spiked foods with almond protein and incurred cookies were also assayed.

The robustness of the method was evaluated by introducing deliberate changes in the assay procedure and evaluating the effects on the results. These changes included deviations in the extraction procedure (sample portion and extraction buffer volume) and in the analysis procedure (volume, time and temperature of the assay).

2.10. Detection of almond residues in working surfaces

Analysis of working surfaces was also performed. Stainless steel (AISI-304) was chosen because is one of the most common surfaces in food industry. Different quantities of almond protein were prepared in a volume of 50 μL. Diluted almond was spread over a surface of 100 cm^2 according to FoodDrinkEurope (2013) recommendations and dried at room temperature for at last three hours. Samples were obtained by dipping the polystyrene swab in 0.5 mL of extraction buffer and then, rubbing the swab in all possible directions of the delimited surface. Swab was placed again in the extraction buffer. After shaking few seconds, the swab was discarded and the same buffer was analyzed simultaneously using LFIA and ELISA. Recovery was calculated as the ratio of amandin concentration in extraction buffer after rubbing and shaking respect to the initial concentration.

3. Results and discussion

3.1. Purification of amandin

The electrophoretic profile of almond extract and pure amandin is shown in Fig. S1. Purified amandin shows the presence of two major types of polypeptides with molecular weight of 38–45 and 20–22 and kDa, which correspond to acidic and basic subunits of the protein. The purity of amandin was determined by densitometry and found to be higher than 95%.

3.2. Development and optimization of sandwich ELISA for amandin

Sandwich ELISA was optimized for protein concentration and buffer composition of coating and detection antibodies, washing buffer composition, as well as incubation temperature and time of the different steps of the assay.

Calibration curves using pure amandin as standards were obtained using the relationship between the absorbance and their concentration values (Fig. 1). Each data point represents the mean ± SD of ten measurements of the absorbance values. The best fit was obtained with a 2nd degree polynomial curve within the range of concentrations from 5 to 100 ng/mL, giving all assays regression coefficients (R^2) higher than 0.98. The estimated LOD and LOQ were 2 and 6 ng/mL which corresponds to 0.02 and 0.06 ppm. These values were calculated by dividing the amandin concentration, in ng/mL, from the calibration curve by 100, considering extraction buffer dilution (1/10). In each assay, amandin concentration in food samples was estimated by interpolation of the absorbance values in the corresponding calibration curve.

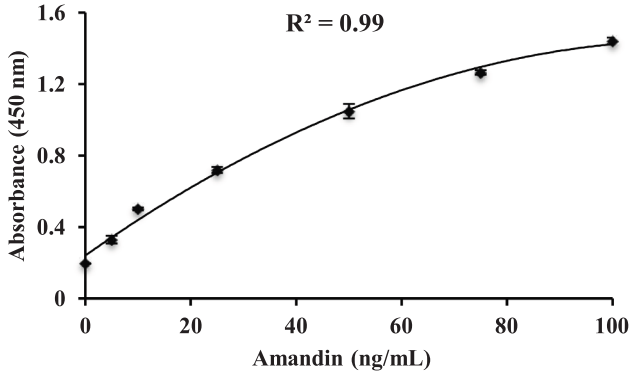

Fig 1. Calibration curve obtained for the determination of amandin by sandwich ELISA. Standards consisted of purified amandin.

The sensitivity of developed sandwich ELISA was similar to that previously estimated by Su et al. (2013) using an indirect sandwich ELISA with a monoclonal antibody to amandin as detector, which was 2.44 ng/mL. However, the LOD obtained in our study (2 ng/mL) is better than that indicated using an indirect competitive ELISA with polyclonal anti-amandin antibodies, which was of 20 ng/mL (Acosta et al., 1999). The higher sensitivity obtained when using a sandwich format instead a competitive format has been also reported for other allergenic proteins such as β-conglycinin and Ara h 1 (Montserrat et al., 2015, Segura-Gil et al., 2019).

3.3. Development of the strip test and POD concentration study

The concentration of the antibodies immovilized in test and control lines and the antibodies coupled to latex particles were optimized to obtain a suitable balance between the maximal signal in the test line with positive samples but avoiding background or unspecific signal when negative samples are analyzed. A negative result is considered when only the blue control line appears and a positive result when both test and control lines are visualized. The test is considered as invalid when the control line is not displayed.

When using the strip reader, a threshold value was established to determine when a sample is considered positive. Cut-off was estimated as the mean of the obtained signals from the tested negative food commodities plus 3 times the SD. A cut-off value of 3.8 was obtained (Abbott et al., 2010).

When an extract of almond protein was tested at different dilutions, the lowest level that showed a POD of 1 was 0.70 ppm, both with naked eye and strip reader (Table 1). In the case of isolated amandin dissolved in extraction buffer, a POD value of 1 was obtained at 0.30 ppm of amandin with naked eye and 0.60 ppm with strip reader (Table S2).

The hook effect that appeared at very high levels of the target protein was also evaluated. This effect, which is inherent to the sandwich LFIA, results in the decrease in color signal, even giving false negative results, due to saturation on the binding sites of the antibodies. Results showed that the test line was not displayed at concentrations higher than 20,000 ppm of almond protein in food samples (data not shown). As this test is designed to detect low amounts of almond that could come from cross contamination, it is expected that foods to be assayed will not contain amandin concentrations that produce an overloading effect.

The LOD of the developed LFIA is lower than that reported by Masiri et al. (2016) using antibodies to an almond protein extract, which detected 1 ppm of almond protein in foods.

The LOD for amandin by LFIA is about one order of magnitude higher than that obtained by ELISA (0.30 and 0.02 ppm, respectively). This difference is in the order of that observed for the detection of other allergenic proteins, like β-lactoglobulin, using both immunoassays (Galan-Malo et al., 2019; de Luis et al., 2009) and could be attributed to the limitation of reagent and sample volume availability and/or the short incubation times of LFIA assays.

3.4. Cross-reactivity study

Sandwich ELISA and LFIA were tested for cross-reactivity by assaying more than 50 food commodities, which included other nuts and ubiquitous food ingredients such as egg, milk, meat and fruits, among others, following recommendations of the AOAC to validate immunoassays (Abbott et al., 2010).

As can be seen in Table 2, no cross-reactivity was found for the ingredients analyzed with the exception of Pecan nut which showed reactivity below 0.01% when tested by LFIA and Brazil nut, Pecan nut and chestnut which showed cross-reactivity below 0.002% when tested by ELISA.

Table 1
Limit of detection of almond protein determined in almond extracts by LFIA using naked eye and electronic strip reader. N: number of replicates analysed, x: number of positives POD: probability of detection (POD), UCL: upper control limit, LCL: lower control limit, CI: confidence level, SD: standard deviation of electronic reader signal.

	Almond protein (ppm)	Mean	SD	N	x	POD	LCL (95% CI)	UCL (95% CI)
Naked eye	0.30			20	6	0.30	0.15	0.52
	0.40			40	36	0.90	0.77	0.96
	0.50			80	71	0.89	0.80	0.94
	0.70			60	60	1.00	0.94	1.00
	1.10			60	60	1.00	0.94	1.00
	2.20			20	20	1.00	0.84	1.00
	7.00			20	20	1.00	0.84	1.00
Strip reader	0.30			20	0	0.00	0.00	0.16
	0.40			40	18	0.45	0.31	0.60
	0.50	4.5	1.7	80	67	0.84	0.74	0.90
	0.70	9.9	3.2	60	60	1.00	0.94	1.00
	1.10	9.9	3.1	60	60	1.00	0.94	1.00
	2.20	13.4	2.3	20	20	1.00	0.84	1.00
	7.00	27.1	3.5	20	20	1.00	0.84	1.00

Su et al. (2013) using a sandwich ELISA with a monoclonal antibody (4C10) to amandin as detector, did not observed cross-reactivity with a wide range of food commodities including tree nuts, legumes, cereals and animal-based foods. However, Acosta et al (1999) using an indirect competitive ELISA with rabbit polyclonal antiserum to amandin obtained cross-reactivity with three of the 10 ingredients tested, specifically with cashew, tepary and Great Northern bean phaseolins (Acosta et al., 1999). Further testing revealed that when that competitive ELISA was assayed using a wide variety of foods spiked with almond flour, food matrices from dairy, nuts, and vegetable products, and from some legumes and cereals resulted in over-estimation of amandin, with some values being up to three times greater than the corresponding amount of spiked almond. These findings suggest that competitive ELISA was susceptible to considerable interferences by most of tested food matrices. These interferences could be attributed to non-specific reaction of other food components with antibodies (Tiwari et al., 2010).

It has been shown that the configuration of an immunoassay could influence the interferences observed in ELISA assays due to matrix effect. Thus, it has been observed that the sandwich format developed for β-conglycinin showed less cross-reactivity with food commodities than the competitive format resulting in an assay of higher specificity (Segura-Gil et al., 2019). This fact could be due to the number of epitopes required to interact with antibodies, being two for the sandwich and just one for the competitive format. Besides, antiserum is usually employed in the competitive format whereas sandwich format requires previous immunoaffinity purification of antibodies, which could result in the collection of more specific antibodies.

3.5. Analysis of incurred model cookies

It has been indicated that a proper validation process of any analytical method should involve the use of model incurred foods in which the allergenic ingredients are incorporated into the food formulation, and then food is processed in a manner that mimics industrial food processing (Taylor, Nordlee, Niemann & Lambrecht., 2009). Although incurred samples are laborious and costly to prepare, some regulatory organizations may be reluctant to approve validation results if they have not been obtained using model foods incurred with the ingredient containing the allergen being targeted (AOAC International, 2016). In this study, baked cookies were selected as the matrix to evaluate the developed methods as cookies are foods in which almond proteins are often included as ingredients.

As can be seen in Fig. 2, the blank cookies (without almond flour) gave an amandin level below the LOQ of the ELISA. This finding indicates that no false positives were found, and thus, that none of the ingredients used in the elaboration of cookies produced interferences in the assay. Furthermore, all model cookies incurred with almond protein gave concentration levels of amandin above the LOQ of the assay, increasing the concentration of amandin with the increase of almond protein present in samples. These results indicate that the ELISA can detect at least 0.2 ppm of almond protein in model baked cookies.

In previous studies in which an ELISA was developed to determine amandin, this technique was applied to quantify the concentration of the protein in commercial products or foods spiked with almond (Acosta et al., 1999; Tiwari et al., 2010; Chhabra et al., 2017). From our knowledge, only the study of Chhabra et al. (2017) included incurred foods prepared with almond flour at levels ranging between 0.5% and 5%. In this study, the presence of amandin could be detected in cookies, sponge cakes, almond bars and cornflakes at the levels assayed. However, it cannot be assumed if that ELISA could detect minute amounts of almond that could imitate cross-contaminated products, as we used in our study, 0.2–4 ppm protein that correspond to 0.0001%-0.002% of whole almond in cookies.

When model cookies were tested by LFIA test, positive results were found at almond protein levels of at least 2 ppm (data not shown).

3.6. Effect of food matrix

Immunoassay techniques can be susceptible to matrix effects. Matrices of particular interest are those most likely to be contaminated with a particular allergen and the ones containing compounds that commonly cause interferences like polyphenols, tannins, ethanol or acids since these components could hamper the detection of the target allergenic proteins (Abbott et al., 2010). In our study, a representative group of food matrices declared as almond-free was also assayed. Some of these matrices represent foods that could be in contact with almonds (in various forms) during food processing or elaboration like coffee liqueur or other vegetables drinks. Other matrices were selected as they possess characteristics that could challenge the test due to its acidity like fruit juice or salad-dressing, or due to its high tannin content like chocolate.

To determine the recovery, a factor conversion for amandin was estimated. To do that, different amounts of the almond powder were prepared in the extraction buffer, and analyzed by ELISA, obtaining an amandin percentage of 45% in almond protein. This value, which is lower to that previously reported of 65% (Roux et al., 2001) was used to calculate the predicted concentration of amandin. The lower level of amandin detected could be attributed to the incomplete extraction of proteins from the almond flour and /or to the lower content of amandin in the almond variety used.

Results showed that all matrixes gave a negative result when assayed by ELISA and LFIA. Furthermore, when samples spiked with 0.35 and 0.70 ppm of almond protein were analyzed by ELISA, the percentages of recovery ranged from 70% to 119% unless for salad dressing at 0.70 ppm

Table 2
Study of cross-reactivity in food commodities using ELISA and LFIA. Two sample extractions in at least three assays were analysed. LOQ: limit of quantification of ELISA assay, N: negative. Values are expressed as the concentration ratio respect to the almond extract (100%).

		ELISA	LFIA
Tree nuts	Hazelnut	<LOQ	N
	Walnut (nogal)	<LOQ	N
	Macadamia nut	<LOQ	N
	Pecan nut	<0.002%	<0.01%
	Brazil nut	<0.002%	N
	Pistachio	<LOQ	N
	Cashew	<LOQ	N
	Pinions (Pine nut)	<LOQ	N
	Chestnut	<0.002%	N
Legumes	Soy	<LOQ	N
	Soy lecithin	<LOQ	N
	Chickpeas	<LOQ	N
	Peanut	<LOQ	N
	Red beans	<LOQ	N
	Beans	<LOQ	N
	White beans	<LOQ	N
	Lentils	<LOQ	N
	Pea	<LOQ	N
Seeds	Sunflower seeds	<LOQ	N
	Pumpkin seeds	<LOQ	N
	Sesame	<LOQ	N
	Poppy seeds	<LOQ	N
Cereal	Legumes	<LOQ	N
	Rye	<LOQ	N
	Barley	<LOQ	N
	Wheat	<LOQ	N
	Buckwheat	<LOQ	N
	Rice	<LOQ	N
	Oats	<LOQ	N
	Corn	<LOQ	N
	Spelt Flour	<LOQ	N
Animal foods	Milk	<LOQ	N
	Egg	<LOQ	N
	Ham	<LOQ	N
	Meat chicken	<LOQ	N
	Beef	<LOQ	N
	Veal	<LOQ	N
	Pork	<LOQ	N
	Tuna	<LOQ	N
	Prawn	<LOQ	N
	Hake	<LOQ	N
	Trout	<LOQ	N
Fruits and Vegetable	Coconut	<LOQ	N
	Kiwi	<LOQ	N
	Pineapple	<LOQ	N
	Watermelon	<LOQ	N
	Medlar	<LOQ	N
	Apple	<LOQ	N
	Banana	<LOQ	N
	Orange	<LOQ	N
	Peach	<LOQ	N
	Apricot	<LOQ	N
	Carrot	<LOQ	N
	Cocoa	<LOQ	N

that was of 64% (Table 3). Values of recovery obtained are within the range that is considered acceptable for ELISA methods to quantify food allergens (Abbott et al., 2010).When the same matrixes spiked with 0.70 ppm of almond protein were assayed by LFIA, the percentage of positive samples was in all cases 100%.

3.7. Determination of precision and robustness of ELISA

Precision was studied using model cookies at two levels of incurred almond protein, (0.4 and 1.9 ppm) and at two levels of spiked almond protein in orange juice (0.2 and 0.7 ppm). As shown in Table 4 repeatability gave variation coefficients between 10.5 % and 14.6%, intra-assay-reproducibility between 4.2% and 35.1% and inter-assay reproducibility between 8.4% and 21.6%. These values are within the acceptance levels established by the AOAC for validation of quantitative food allergen by ELISA methods (AOAC, 2016). The higher CV was found in the reproducibility intra-assay for the cookies. This CV was even bigger than that found for this matrix in the reproducibility inter-assay. This result suggests that extraction step in complex food matrixes is the main variability factor, more than the ELISA method.

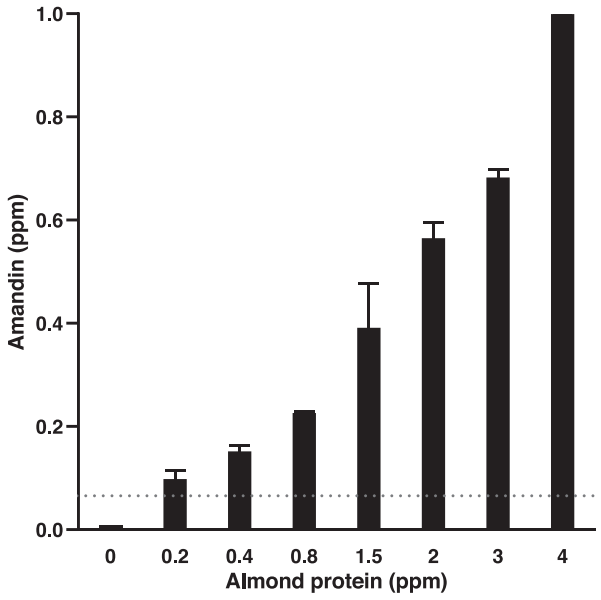

Fig 2. Concentration of immunoreactive amandin in model cookies incurred with different amounts of almond protein determined by sandwich ELISA. The line corresponds to the LOQ.

Table 3
Detection and recovery of amandin by LFIA and ELISA in spiked food matrixes. N: number of replicates analysed, P: percentage of positive replicates. nd: not detected. Recovery is the mean of at least two sample extractions analysed in at least two assays.

	LFIA			ELISA		
Food matrix	Almond protein (ppm)	N	P (%)	Almond protein (ppm)	Mean recovery[a] (%)	CV
Orange juice	0	2	0	0	nd	
				0.12	115.6	28.0
				0.35	111.0	12.0
	0.70	2	100	0.70	90.3	9.3
Coffee liquor	0	2	0	0	nd	
				0.12	86.0	27.8
				0.35	92.1	15.1
	0.70	2	100	0.70	90.9	9.3
Chocolate soy drink	0	2	0	0	nd	
				0.12	92.6	9.0
				0.35	91.0	23.8
	0.70	10	100	0.70	81.2	9.1
Salad dressing	0	2	0	0	nd	
				0.12	71.4	30.6
				0.35	82.9	2.7
	0.70	2	100	0.70	64.4	25.2
Rice ice cream	0	2	0	0	nd	
				0.12	96.3	31.8
				0.35	84.7	10.8
	0.70	2	100	0.70	70.5	26.3
Goat cheese	0	2	0	0	nd	
				0.12	98.6	28.8
				0.35	118.7	18.9
	0.70	2	100	0.70	91.7	27.1

[a] Recovery was calculated based on amandin accounting for the 45% of almond protein.

Table 4
Results of the precision study performed with the sandwich ELISA for amandin determination in model cookies incurred with almond protein and commercial orange juice spiked with almond protein. Values are expressed in ppm of amandin

	Cookies				Orange juice			
Almond protein (ppm)	0.40		1.90		0.20		0.70	
	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Repeatability	0.21	14.5	0.73	13.1	0.12	13.7	0.24	10.5
Intra-assay reproducibility	0.16	35.1	0.48	27.1	0.12	10.9	0.30	4.2
Inter-assay reproducibility	0.17	18.7	0.59	21.6	0.12	8.4	0.28	13.5
Day 1	0.21		0.73		0.13		0.30	
Day 2	0.14		0.54		0.13		0.24	
Day 3	0.17		0.49		0.11		0.28	

To determine robustness, orange juice samples spiked with 0.20 and 0.70 ppm of almond protein were assayed in experiments in which conditions were slightly changed. The value of the standard deviation of the differences (SDi) for each level was 0.0095 and 0.029 respectively (Table S3). These values are lower than the standard deviation of the values of the inter-assay reproducibility (0.010 and 0.038 respectively) which indicates that the sandwich ELISA is a robust test (Karageorgou & Samanidou, 2014).

3.8. Effect of food processing

In order to know the effect of processing on the determination of amandin, cookies incurred with 0.40 ppm of almond protein were analyzed before and after baking. Compared to the raw cookie dough, the concentration of amandin in baked cookie decreased to about 46 ± 6.1 %.

Several studies have been performed on the effect of thermal processing of whole almonds such as blanching, roasting and autoclaving on amandin immunoreactive concentration determined by ELISA (Roux et al., 2001; Su et al., 2017). Results of these studies showed that, with the exception of severe prolonged treatments, none of the processes caused significant decrease in amandin immunoreactivity. These findings suggest that amandin is considered quite stable towards heat processing methods and therefore, it is a useful marker protein for almond detection.

Few studies have investigated the effect of processing on allergenic proteins in complex food matrices (Taylor et al., 2009). Su et al. (2017) determined the immunoreactivity of amandin in roasted almond seeds or flour and observed that it significantly decreased in the presence of sucrose and syrup, probably due to the chemical modification induced by the Maillard reaction.

Chhabra et al. (2017) determined amandin reactivity in model foods incurred with almond flour using a sandwich ELISA. They found that, when using the monoclonal antibodies 4C10 that recognized a conformational epitope, the ratio of incurred food immunorreactivity to that of the reference samples was reduced between 3.8 and 6.10 times in matrices of cookies and sponge cakes that had been treated at 190 °C for 12 min and 163 °C for 30 min, respectively. The reduction in amandin reactivity was attributed to epitope masking or degradation, or to the loss of protein solubility due to thermal aggregation.

The decrease of reactivity of amandin in model foods reported by Chhabra et al. (2017) is higher than that we obtained by sandwich ELISA using rabbit specific polyclonal antibodies. This could be due in part to the binding capacity of monoclonal antibodies that can be markedly affected by small changes in the structure of the unique epitope as consequence of heat denaturation. However, because of polyclonal antibodies recognize a host of antigenic epitopes, the change of one or a small number of epitopes is less likely to be significant (Lipman, Jackson, Trudel & Weis-Gracia, 2005).

3.9. Determination of LFIA robustness

The robustness was determined by evaluating small variations in the standard procedure. All variations were assayed using orange juice at three different levels of almond protein addition (0.70, 0.90 or 1.20 ppm) depending on the condition tested (Table S4).

Respect to the deviation in the extraction procedure, results showed that only for samples spiked with 0.70 ppm of almond protein, a reduction of 20% in the sample portion, or an increase of 20% in the volume of the extraction buffer affects the detection limit of the assay. Considering the deviations in the analysis procedure, differences in the assay volume or temperature did not affect the sensitivity of the test. When differences in the assay time were evaluated, the test line was positive at 10 and 15 min at all levels of spiked almond protein. However, after 2 or 5 min, the test line was negative unless for sample spiked at 1.20 ppm of almond protein after 5 min. These results indicate that to obtain suitable results, it is crucial to incubate the strip for 10 min. Therefore, results derived from the robustness evaluation showed that critical factors to obtain the most suitable results are the sample portion, the volume of the extraction buffer and the assay time.

3.10. Environmental surface testing

The analysis of working surfaces is a useful tool to check the cleaning effectiveness in food industry and to conduct contamination mapping studies in food facilities. After performing the cleaning validation process, LFIA test could be used as the simplest, fast and cost-effective method for routine monitorization of residual allergen on working surfaces (Galan-Malo et al., 2017).

In this study, LFIA test was evaluated using stainless steel as it is a typical working surface in food industry. The lower quantity of almond protein detected with a POD of 1.0 was 0.9 μg. The analysis of this sample with the ELISA test showed a recovery of 4.3% at this level (Table S5). Despite this low recovery, the detection capacity of the test could to be enough to ensure a level of protection to sensitive people. VITAL 3.0 program provides a reference dose for hazelnut but not for almond (Holzhauser et al., 2020). Assuming this dose is similar for both tree nuts, 100 μg of almond protein might trigger an allergic reaction. In the present study, LFIA test has been able to detect 0.9 μg of almond proteins in a working surface of 100 cm^2, a hundred times lower than the level necessary to elicit an allergic reaction. Therefore, LFIA test can be a suitable tool to verify the correct cleaning of the working surfaces and hence to help manufacturers to reduce the abusive use of PAL.

Others studies have indicated that LFIA shows a good performance to evaluate egg and milk proteins from a stainless-steel surface with recoveries near 100% (Galan-Malo et al., 2017). The higher recovery observed in that study could be due to authors carried out a specific optimization by assaying different swab materials as well as extraction conditions, whereas in the present study optimal extraction conditions

for food products was directly applied to the surface analysis without further optimization.

4. Conclusions

In this study, a quantitative sandwich ELISA and a qualitative LFIA test to detect almond protein in food were developed. Each one has their strengths and weaknesses and the choice would depend on the purpose of use. Thus, ELISA is a quantitative method that allows determining the level of allergen in food ingredients and final products as well as identifying hot spots of contamination when performing a mapping of the food manufacturing facilities. LFIA tests are qualitative but they give a rapid response to make decisions and are very useful to rapid testing of raw materials and to perform routine checking of surfaces cleaning.

This is the first report of a LFIA based on amandin as the target protein to detect almond residues. Both assays showed a high specificity for almond, and only a very low cross-reactivity was detected with some nuts, whose presence must be also declared on the label due to their allergenic potential. ELISA test showed a linear range of amandin concentration between 5 and 100 ng/mL with a LOD of 2 ng/mL (0.02 ppm) and LFIA a LOD of 30 ng/mL (0.30 ppm). Furthermore, ELISA and LFIA could detect 0.12 and 0.70 ppm of almond protein respectively in complex food matrixes.

As expected, ELISA showed a higher sensitivity, than LFIA to detect amandin. This fact is due to the longer incubation times and the higher amount of sample and antibody used in the ELISA assay compared to LFIA. Nevertheless, ELISA and LFIA could detect very low levels of almond protein in baked incurred cookies. Likewise, both assays could also detect low levels of almond protein in spiked foods whose complex matrixes could hamper the detection of the allergenic target protein. According to VITAL guidelines, the sensitivity of developed LFIA or ELISA tests would allow to detect enough almond protein even for a serving size of 100 g. Furthermore, the in-house validation of the ELISA assay showed acceptable results of precision, recovery and robustness. The LFIA results of the robustness evaluation pointed out that the sample portion, the volume of the extraction buffer and the assay time are critical factors to obtain the most suitable results. From a practical point of view, initial validation to monitor the unintended presence of almond in food, ingredients and working surfaces should be performed by ELISA because of its higher sensitivity and quantitative response. However, LFIA could be applied as the checking technique during the manufacturing process because of its quickness and simplicity. On the other hand, the use of a calibrated electronic strip reader would allow obtaining an objective determination of the LFIA results, avoiding misinterpretation of the results. The combined use of both assays, ELISA and LFIA, could improve the plans for the allergen risk management in food industry and then, it would allow reducing the abusive use of precautionary labelling of foods.

CRediT authorship contribution statement

Alba Civera: Investigation, Writing – original draft, Validation. **Patricia Galan-Malo:** Methodology, Investigation, Writing – review & editing. **Isabel Segura-Gil:** Formal analysis. **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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131338.

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