Food Control 183 (2026) 111930

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

# ELISA and lateral flow immunoassay validation for a sensitive and rapid detection of crustaceans in thermally processed food and working surfaces

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ARTICLE INFO

*Keywords:*
Tropomyosin
Crustacean
Allergen
Immunoassays
Incurred food
Working surfaces

ABSTRACT

Crustaceans are one of the major triggers of food allergies and can be found as hidden allergens. A sandwich ELISA and lateral flow immunoassay (LFIA) were developed and validated to detect crustaceans by identifying prawn tropomyosin. Both methods could detect crustacean protein in complex food matrices spiked with prawn extract at levels of 1.25 and 5 μg/g, respectively, with high recoveries (59 %–98 %). Crustacean protein was also identified in model thermally processed foods (sausages, croquettes, and broth) containing prawn at levels of 1.25 and 10 μg/g using ELISA and LFIA. Among 63 basic ingredients analyzed, cross-reactivity was observed with mollusks, enabling their detection. ELISA and LFIA could detect 0.1 and 1.0 μg of crustacean protein on contact surfaces, respectively. Both tests proved to be robust. Laboratory and on-site analysis using these immunoassays can support allergen management strategies, helping to prevent unnecessary precautionary labelling.

## 1. Introduction

Crustaceans are one of the major food sources capable of inducing allergic reactions, affecting around 0.5–2.5 % of the general population, being higher in adults than in children (Fu et al., 2019). Shrimp allergy has been indicated to be the main cause of crustacean allergy. In Europe, a multi-center survey reported that 4.8 % of adults have IgE sensitization to shrimp, reaching to 10.2 % in Italy (Burney et al., 2010; Qu et al., 2025).

The symptoms of IgE-mediated allergy to crustaceans include urticaria, diarrhoea and asthma, among others (Cheng et al., 2022). In addition, crustaceans (especially shrimp and crab) can often trigger anaphylactic shock, which can be life-threatening (Ruethers et al., 2018).

The best way to prevent food allergies in sensitized individuals is to avoid the intake of the offending food. Therefore, the regulations of numerous countries have established the obligation to label allergenic foods when they are used as ingredients, including crustaceans.

Global shellfish consumption (crustaceans and mollusks) has increased markedly, being about 42 million tons in 2021 (FAO, 2024). Crustaceans such as prawn, shrimp, crab and lobster are widely used in diverse food categories due to their organoleptic and nutritional properties. They constitute a good source of proteins, essential amino acids, long-chain polyunsaturated fatty acids, vitamins and minerals (Venugopal & Gopakumar, 2017). Consequently, crustaceans can also be present in food as hidden allergens due to cross-contamination from shared production equipment, improper cleaning, procedures and mislabelling (Somorin et al., 2021; Soon & Abdul Wahab, 2021), which implies a risk for allergic consumers. The voluntary precautionary allergen labelling (PAL) “may contain” is a useful tool to be implemented by food producers in these situations, but its indiscriminate use imposes a food restriction on the consumer and induces risky behavior. Therefore, PAL should only be used when, after having assessed the risk and taken all possible preventive measures, the absence of allergenic ingredients cannot be guaranteed (Holzhauser et al., 2020).

In this regard, the Allergen Bureau developed the VITAL® (Voluntary Incidental Trace Allergen Labelling) program to provide a risk-based methodology to be used by food manufacturers to assess the

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https://doi.org/10.1016/j.foodcont.2025.111930
Received 24 October 2025; Received in revised form 10 December 2025; Accepted 20 December 2025
Available online 23 December 2025

impact of allergen cross-contamination, to perform a suitable PAL (Allergen Bureau, 2024; Brooke-Taylor et al., 2018; Holzhauser et al., 2020). In addition, the recent FAO/WHO report "Risk Assessment of Food Allergens" has included the threshold levels for the main allergenic foods (FAO & WHO, 2022). These programs take into account the reference doses reported for the allergenic food and the common serving sizes to establish different action levels. The high prevalence and severity of crustacean allergy make it necessary to have sensitive and specific analytical techniques for its detection at the corresponding action levels, to allow self-monitoring by the food industry.

In crustaceans, the majority of allergic reactions are attributed to a family of proteins designated as tropomyosins (Cheng et al., 2022; WHO/IUIS Allergen Nomenclature, n.d.), as it has been reported that 72–98 % of shrimp-allergic patients have positive IgE binding to purified tropomyosin (Gámez et al., 2011).

Tropomyosin, together with actin and myosin, intervenes in muscle contraction (Behrmann et al., 2012). The structure of shrimp tropomyosin comprises two intertwined α-helix subunits, which have a molecular weight of 35–38 kDa. Tropomyosin consists of a heptamer pattern that is repeated about 40 times along the entire chain (James et al., 2018; Qu et al., 2025). The stability of tropomyosin results from the interaction of these sequences along its entire length (James et al., 2018), which makes it resistant to heat, high pressure and acid treatment, preserving its allergenicity (Kamath et al., 2013; Lasekan et al., 2017).

Immunochemical techniques are currently the most widely used for allergen detection in the food industry, due to their high sensitivity, specificity and operational practicality. Enzyme-Linked Immunosorbent Assay (ELISA) provides quantitative results for allergens in ingredients and finished products. It is also useful for validating cleaning procedures and identifying contamination hot spots in food manufacturing facilities. Lateral Flow ImmunoAssay (LFIA) could be used to check raw materials, intermediate or final products, and to verify routine cleaning processes on site due to its ease of use and rapid response (5–10 min), allowing immediate corrective actions (FoodDrinkEurope, 2022).

Several ELISA and LFIA techniques have been developed to detect crustaceans based on tropomyosin determination, using monoclonal (Jeoung et al., 1997; Shi et al., 2011; Zeng et al., 2019), polyclonal (Fuller et al., 2006; Li et al., 2022; Werner et al., 2007; Yu et al., 2019) or both types of antibodies (Koizumi et al., 2014; Seiki et al., 2007; Zhang et al., 2014). Besides, a sandwich ELISA test has been reported for the detection of other shellfish species belonging to the mollusk category, specifically clams. This test can detect 2.5 ppm of clam protein in food samples (Koppelman et al., 2021).

One of the most important aspects to consider in the development of analytical techniques to detect allergenic food is their validation using model food incurred with the allergenic ingredient and then processed, in a similar way to that applied in the food industry. In fact, some regulatory authorities do not consider approving validation studies that do not incorporate results obtained from incurred food (AOAC International, 2014; Taylor et al., 2009). However, most studies use spiked food with added crustacean extract or pure tropomyosin (Fuller et al., 2006; Jiao et al., 2024; Shi et al., 2011; Wang et al., 2019; Werner et al., 2007; Yu et al., 2019) or blended food with added crustacean as an ingredient where the effect of processing is not considered (Koizumi et al., 2014). Other studies use commercial food in which the amount of allergen present is unknown (Jiao et al., 2024; Lin et al., 2018; Wang et al., 2019; Werner et al., 2007; Yu et al., 2019; Zeng et al., 2019; Zhang et al., 2014). Only a few studies that developed ELISA (Seiki et al., 2007; Zhao et al., 2022) or LFIA tests (Koizumi et al., 2014; Li et al., 2022) used incurred model food in their validation. Seiki et al. (2007) and Koizumi et al. (2014) could detect 5 μg of prawn protein per g of model food, and Zhao et al. (2022) and Li et al. (2022) could detect between 0.01 and 1 % of shrimp powder in different model foods. To our knowledge, there is only one study in which ELISA and LFIA tests were developed and compared (Zeng et al., 2019), but the validation was performed using commercial food containing crustaceans.

The aim of this study has been the development of two immunoassays, an ELISA and a rapid test based on the detection of prawn tropomyosin. "In-house" validation to determine sensitivity, specificity, precision, recovery and robustness was carried out in accordance with international AOAC guidelines. Their performance was evaluated using complex food matrices spiked with a prawn extract, and three thermally processed model foods (sausages, croquettes and broth) incurred with minute amounts of prawn as an ingredient and subjected to industrial-like processing. In addition, the detection of crustacean protein on contact surfaces has also been analyzed by these two methods.

## 2. Materials and methods

### 2.1. Materials

Crustaceans [prawn (*Penaeus kerathurus*), shrimp (*Parapenaeus longirostris*), Norway lobster (*Nephrops norvegicus*), European lobster (*Homarus gammarus*) and river crayfish (*Austropotamobius pallipes*)], basic ingredients and commercial foods were obtained from local retailers. Sephadex G-75 gel and HiTrap NHS-activated HP column were obtained from Cytiva (Uppsala, Sweden). SDS-PAGE gels were acquired from Bio-Rad (Berkeley, California, USA). MaxiSorp loose Immunomodules were purchased from Nunc (Roskilde, Denmark). The Lightning-Link Horseradish Peroxidase conjugation kit was acquired from Abcam (Cambridge, UK). Red and blue carboxyl-modified latex microparticles were obtained from Estapor (Merck, Darmstadt, Germany) and Stomacher 80 Biomaster Strainer Closure Bags were purchased from Seward (Worthing, UK).

### 2.2. Purification and identification of prawn tropomyosin

Whole prawns (*Penaeus kerathurus*) were immersed in water and boiled for 20 min. After removing the head and exoskeleton, the muscle was homogenized in 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 3 mM KCl, 140 mM NaCl buffer, pH 7.4 (PBS) containing 10 mM EDTA at the ratio of 1:3 (w/v) using an ultraturrax, and the slurry was agitated for 2 h at 4 °C and centrifuged at 9000×*g* for 20 min. The supernatant was subjected to gel filtration on a Sephadex G-75 column (75 × 1.5 cm). Chromatographic fractions containing tropomyosin were collected and incubated with hexane at a ratio of 1:4 (v/v) for 90 min at room temperature (RT). Then, after centrifugation at 8000×*g* for 5 min, the aqueous fraction was collected.

The protein profile of the fractions obtained during the purification steps was analyzed by SDS-PAGE under reducing conditions with β-mercaptoethanol according to Laemmli (1970). Precast polyacrylamide gels (4–20 %) were run on a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, USA) and stained with Coomassie Brilliant Blue R-250. The gels were then scanned in an Image Scanner III (GE Healthcare, Chicago, Illinois, USA).

### 2.3. Preparation of prawn tropomyosin standards and food extracts

Prawn tropomyosin standards were prepared by dilution of a stock solution of the purified protein in extraction buffer (0.2 M Tris-HCl, pH 8.0, containing 0.5 M NaCl, 0.1 % (w/v) fish gelatine, 0.1 % (v/v) Tween-20) whose concentration was determined by spectrophotometry, considering an extinction coefficient ($A^{0.1\ \%}_{280\ nm}$) of 0.15 (DeWitt et al., 2004).

Prawn protein standards were prepared from freeze-dried flesh whose protein concentration was previously determined by the Kjeldahl method (AOAC International, 2002), using a nitrogen conversion factor of 6.25 (Marcó et al., 2002), obtaining a protein concentration of 24.3 %.

For protein extraction from food, solid samples were ground using a mixer. Afterwards, 1.00 ± 0.01 g or 1.00 ± 0.01 ml for liquid samples was added to a Stomacher bag and 10 or 9 ml of extraction buffer were

added, respectively, performing manual blending for 1–2 min. The extracts were analyzed immediately after extraction.

### 2.4. Procedure to prepare spiked and incurred samples

Commercial spiked foods and incurred foods were prepared considering the amount of protein determined in prawn flesh by Kjeldahl (24.3 %).

To prepare spiked foods, the prawn extract was added to various commercial liquid or ground solid food matrices at concentrations of 1.25, 2.5, 5.0 and 10 μg of prawn protein per g or ml of food.

Thermally processed incurred foods (Frankfurt sausages, croquettes and chicken broth) were prepared by adding ground freeze-dried prawn as an ingredient at a concentration of 400 μg of prawn protein per g of food. Then, the food matrices were subjected to a processing similar to that used in the food industry. Lower concentration levels (1.25, 2.5, 5.0, 10, 20 and 50 μg/g) were obtained by mixing the food samples containing 400 μg/g of prawn protein with the respective blank model food prepared without prawn.

Frankfurt sausages were prepared with 3 kg of pork leg meat, 3 kg of pork belly, 200 g of salt, 100 g of spices, 50 g of phosphates, 5 g of sodium ascorbate, 1 g of nitrites and 3 kg of ice using a cutter (CM-41, MAINCA, Barcelona, Spain). The ground freeze-dried prawn was added and the mixture was kneaded using a homogenizer. Then, the mixture was stuffed into cellulose casings (2.8 cm diameter) and cooked in a thermostatic bath for 40 min at 75 °C. Finally, the sausages were vacuum-packed and pasteurized in a thermostatic bath for 25 min at 95 °C.

Chicken and ham croquettes were elaborated with 400 g of milk, 100 g of cooked skinless chicken breast, 85 g of wheat flour, 50 g of butter, one hard-boiled egg, 30 g of diced cured ham, 25 g of olive oil and 15 g of onion using a Thermomix TM31 (Vorwerk, Wuppertal, Germany). The mixture was prepared according to the instructions, and then, the ground freeze-dried prawn was added, homogenized and the mixture was cooked for 8 min at 100 °C.

Chicken broth was prepared from a commercial product. The ground freeze-dried prawn was added, homogenized and the mixture sterilized in a pressure cooker for 20 min.

These model foods were also elaborated without the addition of prawn and considered as blank samples.

### 2.5. Production and labelling of antibodies against prawn tropomyosin

Antisera against prawn tropomyosin were raised in rabbits by immunization with the purified protein as previously described by Wehbi et al. (2005). All procedures performed with animals were approved by the Ethic Committee for Animal Experimentation from the University of Zaragoza (Project Licence PI 47/24). The care of animals was carried out in agreement with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63.

Specific antibodies were collected by immunoaffinity using a HiTrap NHS-activated HP column (1 ml) previously coupled with prawn tropomyosin. A volume of 15–20 ml of antiserum was applied onto the column and washed with 10 vol of PBS. Anti-tropomyosin antibodies were eluted using 0.1 M glycine, 0.5 M NaCl buffer, pH 2.8 and instantly neutralized with 0.5 M Tris buffer, pH 8.0. The purified antibodies were dialyzed against PBS, concentrated and used as capture antibodies in the immunoassays. In addition, a part of the antibodies was used to prepare detection antibodies, by conjugation with horseradish peroxidase using the Lightning-Link HRP conjugation kit following manufacturer's instructions for the ELISA, and by linking to red carboxyl-modified latex microparticles for the rapid test as previously described (Civera et al., 2022).

### 2.6. Sandwich ELISA

Microtiter wells were coated as previously described (Civera et al., 2022). Briefly, plates were coated with 100 μl of specific anti-prawn tropomyosin antibodies at 5 μg/ml. The blocking step was performed with ovalbumin at 3 % (w/v) in PBS. To carry out the assay, 100 μl of standards (6.25–100 ng/ml) or food samples were added and the wells were incubated for 30 min. After washing three times with 300 μl of PBS containing 0.1 % (v/v) Tween-20 (PBST), the wells were incubated for 30 min with 100 μl of a solution of peroxidase-conjugated anti-tropomyosin antibodies. The wells were then washed again three times and 100 μl of TMB (3,3′,5,5′-Tetramethylbenzidine) peroxidase substrate were added. After 30 min, the enzymatic reaction was stopped by adding 50 μl of 2 M $H_2SO_4$. The absorbance of the wells was measured at 450 nm on a Multiskan MS reader (Labsystem, Helsinki, Finland). All the procedure was performed at RT.

### 2.7. Lateral flow immunoassay

The rapid test was produced as previously described (Civera et al., 2022). Briefly, anti-tropomyosin antibodies (test line) and the control line were dispensed as independent lines on a nitrocellulose membrane at 0.5 mg/ml. Antibodies labelled with latex microparticles were applied over a pad of glass fiber membrane at a ratio of 1:1. The nitrocellulose membrane, conjugate pad and adsorbent pads were mounted on an adhesive backing card with an overlap between components of 2 mm. Strips of 4 mm width were cut.

The assays were performed by dipping the end of the strip in 150 μl of the standards or samples and incubating for 10 min. The results were read both visually and with an optical strip reader (IRIS, ZEULAB, Zaragoza, Spain) that allows an objective interpretation by translating the intensity of the control and test lines into a digital signal. The assay was performed at RT.

### 2.8. Single-laboratory validation

The developed ELISA was validated following the standardized guidelines described by AOAC (AOAC International, 2023). The parameters of sensitivity, specificity, precision, recovery and robustness were determined as follows.

Sensitivity is defined by the limit of detection (LOD) and the limit of quantification (LOQ), which were calculated from the mean concentrations obtained in 10 extracts by duplicate of the blank incurred foods (sausages, croquettes and broth) plus 3 and 10 times their standard deviation, respectively (Abbott et al., 2010; Cantwell, 2025).

Specificity or cross-reactivity was determined in extracts of 63 basic ingredients, including vegetal (tree nuts, legumes, seeds, fruit and vegetables, cereals and spices) and animal foods (meat, fish, mollusks, arthropods and milk) (Table 1). For all ingredients, two independent extractions were analyzed by duplicate.

Precision was determined using the thermally processed model foods. Three independent extractions (blank and three levels of added prawn protein) were analyzed by duplicate. Repeatability was determined as the variation coefficient of the results obtained in a single assay and intermediate precision as the variation coefficient of the results obtained in two assays conducted on different days.

Recovery was determined in spiked foods to assess the matrix effect. Prawn extract was added to several commercial liquid or ground solid food matrices (corn snack, croquette, chicken broth, soy sauce, wine vinegar, and red wine) at concentrations of 1.25, 2.5 and 5.0 μg of prawn protein per g or ml of food. Then, protein extraction was performed as previously described in Section 2.3. The expected concentration was assumed, considering the content of prawn protein according to the result obtained by Kjeldahl method. This parameter was calculated as the ratio between the prawn protein experimental concentration obtained by ELISA and the expected concentration, expressed in

**Table 1**
Food commodities (63) used in the cross-reactivity study tested by ELISA and LFIA.

| Tree nuts | ELISA | LFIA | Legumes | ELISA | LFIA | Seeds | ELISA | LFIA | Fruit and vegetables | ELISA | LFIA | Animal food | ELISA | LFIA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Almond | < LOD | N | Chickpea | < LOD | N | Pumpkin | < LOD | N | Apple | < LOD | N | Anchovy | < LOD | N |
| Brazil nut | < LOD | N | Lentil | < LOD | N | Quinoa | < LOD | N | Carrot | < LOD | N | Beef | < LOD | N |
| Cashew | < LOD | N | Lupin | < LOD | N | Sesame | < LOD | N | Cocoa | < LOD | N | Chicken | < LOD | N |
| Hazelnut | < LOD | N | Peanut | < LOD | N | Sunflower | < LOD | N | Coconut | < LOD | N | Cod | < LOD | N |
| Pecan nut | < LOD | N | White bean | < LOD | N | | | | Kiwi | < LOD | N | Cow milk | < LOD | N |
| Pine nut | < LOD | N | | | | | | | Orange juice | < LOD | N | Pork | < LOD | N |
| Pistachio | < LOD | N | | | | | | | | | | Salmon | < LOD | N |
| Walnut | < LOD | N | | | | | | | | | | Surimi | < LOD | N |
| | | | | | | | | | | | | Trout | < LOD | N |
| | | | | | | | | | | | | Tuna | < LOD | N |
| | | | | | | | | | | | | Whiting | < LOD | N |

| Cereals | ELISA | LFIA | Spices | ELISA | LFIA | Others | ELISA | LFIA | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Corn | < LOD | N | Black pepper | < LOD | N | Potato flakes | < LOD | N | | | | Clam | >LOD | P |
| Oats | < LOD | N | Cinnamon | < LOD | N | Red wine | < LOD | N | | | | Cricket | >LOD | P |
| Rice | < LOD | N | Curry | < LOD | N | Salt | < LOD | N | | | | Cuttlefish | >LOD | P |
| Rye | < LOD | N | Garlic | < LOD | N | Shrimp bread | < LOD | N | | | | Mussel | >LOD | P |
| Wheat | < LOD | N | Green anise | < LOD | N | Soy sauce | < LOD | N | | | | Octopus | >LOD | P |
| | | | Nutmeg | < LOD | N | Sugar | < LOD | N | | | | Scallop | >LOD | P |
| | | | Oregano | < LOD | N | White vinegar | < LOD | N | | | | | | |
| | | | Rosemary | < LOD | N | | | | | | | | | |
| | | | Thyme | < LOD | N | | | | | | | | | |
| | | | Turmeric | < LOD | N | | | | | | | | | |
| | | | White pepper | < LOD | N | | | | | | | | | |

LOD: limit of detection. N: negative. P: positive.

percentage.

Robustness was determined by introducing slight variations in the experimental conditions of the test (incubation temperature, incubation time, assay volume, number of washing steps and assay reading time) using a Youden matrix design (Karageorgou & Samanidou, 2014). This method implements a fractional factorial design combining seven variables (Supplementary Table 1). Two levels of added prawn protein (1.25 and 2.5 μg/g) were tested using the model chicken broth and the standard deviations of the differences (SDi) between the values obtained in the established and modified conditions of the ELISA were calculated.

The developed LFIA was validated following AOAC guidelines (AOAC International, 2023). The parameters of sensitivity, specificity and robustness were determined as follows.

Sensitivity was determined by the probability of detection (POD) method. Several dilutions of prawn tropomyosin were assayed, as well as prawn extracts at different protein concentrations. A number of 40 independent samples at concentrations close to the limit of detection were tested to calculate that limit. The POD value was calculated as the ratio between the number of samples with a positive result and the total number of samples analyzed. A POD value greater than 0.95 ensures a positive sample with at least 95 % confidence (AOAC International, 2014; Wehling et al., 2011).

In order to confirm the lowest level of detection, spiked foods were also tested. Furthermore, incurred foods containing different levels of added prawn protein were assayed to evaluate the effect of processing on the detection limit.

Specificity was determined by analyzing the undiluted extracts of the 63 basic ingredients by duplicate.

Robustness was determined using the model chicken broth at two levels of added prawn protein (10 and 20 μg/g). The effect of deliberate changes in sample portion, extraction buffer volume and assay volume, as well as in incubation time and temperature were studied (Supplementary Table 1).

### 2.9. Monitoring of crustacean protein on surfaces

Stainless steel and melamine surfaces were selected due to their common use in the food industry. Prawn protein solutions were prepared at different concentrations and 50 μl were evenly spread over a 10 cm side square surface and then left to air dry overnight, following FoodDrinkEurope (2022) guidelines. A polystyrene swab was soaked in 0.5 ml of extraction buffer and used to rub the square surface. The swab was then dipped again in the extraction buffer and discarded, and the same buffer analyzed using both immunoassay methods. The prawn protein recovery from the surface was measured by ELISA, considering the amount of prawn protein in the extraction buffer after rubbing and shaking in relation to the initial spread amount applied.

## 3. Results and discussion

### 3.1. Characterization of prawn tropomyosin

The analysis of purified prawn tropomyosin by SDS-PAGE under reducing conditions showed a single band of 35–38 kDa (Supplementary Fig. 1), similar to that reported by other authors (Lv et al., 2025; Wang et al., 2023). The purity of the protein, as determined by densitometry, was over 95 %.

### 3.2. Quantitative immunoassay to determine tropomyosin

The concentrations of anti-tropomyosin antibodies used for capture and detection in ELISA were optimized. The most effective results were achieved with a coating concentration of 5 μg/ml of antibodies in the wells and with conjugated antibodies (1.0 mg/ml) diluted 1/20000.

Fig. 1 shows the calibration curve obtained with prawn extract and Supplementary Fig. 2 the calibration curve obtained with purified prawn tropomyosin. The concentration of prawn protein of the standards is expressed as μg/g, considering the concentration of protein in prawn determined by Kjeldahl and the 10-fold dilution that is applied to the food samples for the extraction. The calibration curves were

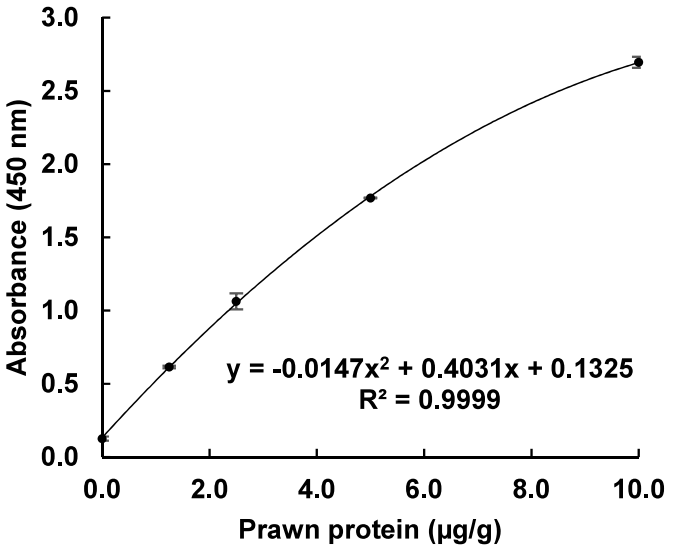


**Fig. 1.** Calibration curve obtained with prawn protein extract by ELISA. Error bars indicate the standard deviation of the absorbance values.

obtained by representing the absorbance values versus the concentration of prawn protein (µg/g) or tropomyosin (ng/ml), which were adjusted to a polynomial curve with regression coefficients higher than 0.99. The working ranges of both calibration curves were between 1.25 and 10 µg/g for prawn protein and between 6.25 and 100 ng/ml for prawn tropomyosin. The concentration of prawn protein or tropomyosin in the samples was calculated by interpolation of the absorbance values into the calibration curve obtained for each assay.

The LOD and LOQ of the method were 0.13 and 0.41 µg/g using the prawn protein standard curve and 2.4 and 7.4 ng/ml using the prawn tropomyosin standard curve, respectively. These values are slightly higher than those determined by other authors, which reported tropomyosin LOD values of 0.75 ng/ml (Fuller et al., 2006), 0.71 ng/ml (Seiki et al., 2007) and 0.008 µg/g (Zhao et al., 2022). Comparing the LOD and LOQ obtained in the developed method with the statements of commercial kits (Supplementary Table 5), the results are within the expected range.

To determine the relationship between tropomyosin and prawn protein, prawn protein standards (quantified by Kjeldahl) and pure tropomyosin standards (quantified by absorbance at 280 nm) were analyzed in the same ELISA assay, both in µg/g. The absorbance values at 450 nm of prawn protein standards were interpolated into the calibration curve of pure tropomyosin. Then, the calculated concentration of pure tropomyosin in prawn protein standards (X axis) was plotted vs. prawn protein concentration (Y axis), giving a linear relationship ($r^2 >$ 0.99). From the slope of the equation obtained (5.6), the equivalence between tropomyosin and prawn protein was 1 µg/g tropomyosin = 5.6 µg/g prawn protein (18 %), under the extraction and assay conditions applied.

### 3.3. Rapid on-site immunoassay to determine tropomyosin

The results of the LFIA test are interpreted as follows: when both blue (control) and red (test) lines appear, the result is considered positive and when only the blue line appears, the result is considered negative. If the blue line, which indicates that the test has been performed correctly, does not appear, the result is considered invalid.

In order to enhance the signal in positive samples and reduce the background signal in negative samples, the concentration of capture antibodies dosed on the membrane and the proportion of dyed latex microparticles to antibodies were previously optimized.

LFIA test results are qualitative as they are read visually, but interpretation might be subjective when the intensity of the lines is low. In our study, we have incorporated an objective interpretation of the results by means of an optical strip reader, which translates the intensity of the test and control lines into a digital signal that is expressed in arbitrary units (a.u.). A threshold value over which a sample is considered positive was calculated from the mean signal values of the negative food commodities analyzed in the study of cross-reactivity plus 3 times the SD (Abbott et al., 2010; Cantwell, 2025). The threshold value obtained was 3.4 a.u.

The sensitivity of the rapid test was determined by the probability of detection (POD) method. As can be seen in Table 3, when purified tropomyosin was tested at different concentrations, the detection limit value was set at 125 ng/ml (POD = 1). This value is higher than those obtained by Koizumi et al. (2014) and Li et al. (2022), who reported visual values of 25 ng/ml of shrimp protein and 50 ng/ml of shrimp tropomyosin, respectively. However, it is lower than the value obtained by Wang et al. (2019) of 500 ng/ml for shrimp tropomyosin. When a prawn extract, whose protein concentration was determined by Kjeldahl method, was analyzed, a low level of 5 µg/g of prawn protein was detected when the results were read with both naked eye and strip reader (Table 3).

The comparison of the LOD values obtained for the quantitative method (2.4 ng/ml tropomyosin or 0.13 µg/g prawn protein) and the qualitative assay (125 ng/ml of tropomyosin or 5 µg/g prawn protein) in the present study indicates that the ELISA is around 4–5 times more sensitive than the LFIA, as it has also been observed for other allergenic proteins determined using both tests (Civera et al., 2024).

Moreover, higher concentrations of purified tropomyosin or prawn protein were analyzed by the rapid test in order to evaluate the hook effect, which produces a decrease in the intensity of the test line due to the saturation of the binding sites with tropomyosin. This could lead to false negative results (Galan-Malo et al., 2019). The developed assay showed no hook effect for concentrations up to 10000 ng/ml of pure tropomyosin (138.0 a.u.) and 100000 µg/g of prawn protein (94.6 a.u.), although the test line showed a very small increase of intensity at levels of protein higher than 5000 ng/ml for tropomyosin, and even a small decrease at levels of 100000 µg/g for crustacean protein.

### 3.4. Specificity

Cross-reactivity is a relevant parameter to be determined in immunoassays to evaluate the potential presence of false positive results. Undiluted extracts of 63 food commodities selected according to AOAC guidelines, including nuts, cereals, legumes, spices, fruits and animal-based food were analyzed. All the basic ingredients analyzed by ELISA gave concentrations below the LOD, except mollusks (squid, octopus, scallop, clam and mussel) and arthropods (cricket) (Table 1). Likely, it has been indicated that an ELISA test developed to detect clam showed certain cross-reactivity with crustaceans (Koppelman et al., 2021). These cross-reactivities could be attributed to the high sequence homology between the tropomyosin present in those groups of animals and in crustaceans (Cheng et al., 2022; Palmer et al., 2020). Mollusks are also included among the allergens requiring mandatory labelling according to Regulation (EU) No. 1169/2011. Furthermore, studies have shown that individuals with a crustacean protein allergy may also react to mollusk and arthropod proteins (Scala et al., 2022). Therefore, detecting mollusks and arthropods provides a higher level of protection for sensitive individuals.

In our study, the lowest level of these mollusk proteins that could be detected by the immunoassay methods was analyzed (Table 2). Mollusk protein from octopus, mussel, scallop and clam was detected at levels ranging from 27 to 100 µg/g, both by the quantitative immunoassay and rapid test, respectively. Furthermore, arthropod protein from cricket was identified at 0.7 µg/g and 20 µg/g, respectively. It is also important to acknowledge the practical challenges of avoiding cross-contamination between crustaceans and mollusks, given that they

**Table 2**
Level of detection towards tropomyosin from crustacean, mollusk and arthropod species. Extracts from each species were analyzed by ELISA and LFIA, using naked eye and strip reader. Results are expressed in μg/g of crustacean or mollusk protein considering the percentage of protein from the Spanish Food Composition Database (BEDCA, n.d.). Two independent test portions were analyzed at least by duplicate.

| | | ELISA (μg/g) | LFIA naked eye (μg/g) | LFIA strip reader (μg/g) |
|---|---|---|---|---|
| **Crustaceans** | Shrimp (*Parapenaeus longirostris*) | 0.4 | 5 | 5 |
| | Prawn (*Penaeus kerathurus*) | 0.3 | 5 | 5 |
| | Norway lobster (*Nephrops norvegicus*) | 0.7 | 5 | 5 |
| | European lobster (*Homarus gammarus*) | 0.8 | 5 | 10 |
| | River crayfish (*Austropotamobius pallipes*) | 0.5 | 5 | 10 |
| **Mollusks** | Octopus (*Octopus vulgaris*) | 100 | 100 | 100 |
| | Mussel (*Mytilus galloprovincialis*) | 100 | 100 | 100 |
| | Scallop (*Pecten maximus*) | 100 | 100 | 100 |
| | Clam (*Ruditapes philippinarum*) | 100 | 100 | 100 |
| | Cuttlefish (*Sepia officinalis*) | 27 | 27 | 27 |
| **Arthropods** | Cricket (*Acheta domesticus*) | 0.7 | 20 | 20 |

a.u.: arbitrary units. SD: standard deviation of electronic reader signal.

frequently share facilities and utensils throughout the logistics chain. This cross-reactivity has also been observed by other authors using immunoassays to detect crustaceans, for example with clam, squid, octopus and abalone (Shi et al., 2011) and with squid, cockle and cockroach (Werner et al., 2007).

When extracts from other crustacean species (shrimp, prawn, Norway lobster, European lobster and river crayfish) were analyzed by ELISA, all of them gave positive results with protein concentrations ranging from 0.3 μg/g (prawn) to 0.8 μg/g (European lobster) (Table 2). The LFIA test could also detect crustacean species at protein concentrations ranging from 5 μg/g (shrimp, prawn and Norway lobster) to 10 μg/g (European lobster and river crayfish). The high cross-reactivity observed among the tested crustacean species is a very positive aspect as it indicates that the test is able to detect the most consumed crustaceans. Other authors also analyzed different crustacean species by ELISA and LFIA and found a high cross-reactivity to shrimp or prawn tropomyosin (Seiki et al., 2007; Shi et al., 2011; Zhao et al., 2022).

The reactivity of the antisera used in the developed ELISA and LFIA is unlikely to match perfectly the reactivity of human IgE in individuals with a shellfish allergy. Nevertheless, these immunoassays are probably as good as can be achieved using any immunological detection method, and this limitation will apply to all published crustacean immunoassays.

### 3.5. Spiked food analysis

Specific food matrices with a complex composition that could hinder the interaction of antibodies with the target protein, thus affecting its detection, were tested. The selected matrices included acidic food and food containing polyphenols and tannins, such as vinegar, red wine and soy sauce. Furthermore, other matrices susceptible to cross-contamination due to the possibility of sharing processing lines in the industry with products containing crustaceans were also analyzed, including a corn snack, chicken broth and croquettes. None of the selected products showed cross-reactivity before the addition of the prawn protein extract when tested by both immunoassay methods (Table 4).

For the quantitative method, concentrations of 1.25, 2.5 and 5.0 μg/g of added prawn protein were assayed for each food matrix (Table 4). Results showed recovery values from 58.7 % to 98.3 % with variation coefficients from 0.3 % to 12.1 %, depending on the food matrix. The recoveries of around 60 % obtained in croquettes can be attributed to the difficulty of extraction due to the viscosity and stickiness of the matrix. Although ideal recovery rates should range from 80 % to 120 %, it must be taken into account that recovery levels are affected by both the efficiency of the extraction step and the procedure. Therefore, the AOAC guidelines for the quantification of food allergens by immunoassays consider acceptable recoveries between 50 % and 150 % (Abbott et al., 2010; AOAC International, 2023).

Regarding the rapid test, all spiked food showed positive results at concentrations of added prawn protein of 5.0 and 10 μg/g with both naked eye and strip reader. These results confirm the detection limit value of 5 μg/g of crustacean protein previously determined for this method with prawn extracts (Table 4).

Several authors have conducted recovery studies during the development of ELISA (Seiki et al., 2007; Yu et al., 2019; Zeng et al., 2019; Zhao et al., 2022) and LFIA tests (Jiao et al., 2024; Li et al., 2022; Shi et al., 2011; Wang et al., 2019) for the detection of crustaceans.

**Table 3**
Limit of detection of crustacean protein determined in prawn extracts and pure tropomyosin solutions by LFIA using naked eye and strip reader.

| | units | N | Naked eye | Strip reader | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | x | x | Signal (a.u.) | SD | POD | LCL | ULC |
| **Crustacean protein (μg/g)** | 1.25 | 20 | 0 | 0 | 0.8 | 0.4 | 0.00 | 0.00 | 0.16 |
| | 2.5 | 41 | 37 | 13 | 3.3 | 1.0 | 0.32 | 0.20 | 0.47 |
| | 5.0 | 41 | 41 | 41 | 9.2 | 1.7 | 1.00 | 0.91 | 1.00 |
| | 10.0 | 41 | 41 | 41 | 27.8 | 5.2 | 1.00 | 0.91 | 1.00 |
| | 50.0 | 7 | 7 | 7 | 91.4 | 6.9 | 1.00 | 0.65 | 1.00 |
| | 100 | 7 | 7 | 7 | 116.9 | 4.7 | 1.00 | 0.65 | 1.00 |
| | 1000 | 7 | 7 | 7 | 104.3 | 4.6 | 1.00 | 0.65 | 1.00 |
| | 10000 | 7 | 7 | 7 | 127.2 | 6.7 | 1.00 | 0.65 | 1.00 |
| **Tropomyosin (ng/ml)** | | | | | | | | | |
| | 62.5 | 21 | 16 | 1 | 2.4 | 0.3 | 0.05 | 0.01 | 0.23 |
| | 125 | 41 | 41 | 41 | 6.8 | 1.2 | 1.00 | 0.91 | 1.00 |
| | 250 | 41 | 41 | 41 | 14.9 | 1.9 | 1.00 | 0.91 | 1.00 |
| | 500 | 41 | 41 | 41 | 39.0 | 4.4 | 1.00 | 0.91 | 1.00 |
| | 1000 | 7 | 7 | 7 | 74.8 | 7.7 | 1.00 | 0.65 | 1.00 |
| | 5000 | 7 | 7 | 7 | 134.8 | 2.8 | 1.00 | 0.65 | 1.00 |
| | 10000 | 7 | 7 | 7 | 138.0 | 5.1 | 1.00 | 0.65 | 1.00 |

N: number of replicates assayed. X: number of positive results. POD: probability of detection. CI: confidence interval. LCL: lower control limit (95 % CI). UCL: upper control limit (95 % CI). a.u.: arbitrary units. SD: standard deviation of electronic reader signal.

**Table 4**
Level of detection and recovery of crustacean protein in food matrices spiked with prawn protein extract analyzed by ELISA and LFIA. Two independent extracts were analyzed by duplicate.

| Food matrix | Spiked level (μg/g) | LFIA | | | | ELISA | |
|---|---|---|---|---|---|---|---|
| | | Naked eye | Strip reader | | | | |
| | | P (%) | P (%) | Signal (a.u.) | SD | Recovery (%) | CV (%) |
| **Corn snack** | **Blank** | 0 | 0 | 0.4 | 0.4 | nd | |
| | **1.25** | na | na | – | – | 94.9 | 1.7 |
| | **2.5** | 100 | 100 | 7.4 | 1.0 | 92.5 | 5.2 |
| | **5.0** | 100 | 100 | 17.9 | 3.9 | 89.6 | 6.9 |
| | **10** | 100 | 100 | 44.4 | 3.0 | na | na |
| **Croquettes** | **Blank** | 0 | 0 | 0.7 | 0.4 | nd | |
| | **1.25** | na | na | – | – | 58.7 | 4.5 |
| | **2.5** | 100 | 50 | 3.6 | 1.2 | 66.5 | 10.0 |
| | **5.0** | 100 | 100 | 6.1 | 1.2 | 61.0 | 9.4 |
| | **10** | 100 | 100 | 18.6 | 6.9 | na | na |
| **Chicken broth** | **Blank** | 0 | 0 | 0.7 | 0.4 | nd | |
| | **1.25** | na | na | – | – | 98.3 | 7.7 |
| | **2.5** | 100 | 0 | 2.9 | 0.3 | 87.2 | 4.6 |
| | **5.0** | 100 | 100 | 7.9 | 2.3 | 82.2 | 0.3 |
| | **10** | 100 | 100 | 19.7 | 1.9 | na | na |
| **Soy sauce** | **Blank** | 0 | 0 | 0.2 | 0.1 | nd | |
| | **1.25** | na | na | – | – | 89.6 | 3.6 |
| | **2.5** | 50 | 0 | 1.7 | 0.5 | 92.6 | 12.1 |
| | **5.0** | 100 | 75 | 3.0 | 0.7 | 81.5 | 7.9 |
| | **10** | 100 | 100 | 7.2 | 2.4 | na | na |
| **Vinegar** | **Blank** | 0 | 0 | 0.5 | 0.3 | nd | |
| | **1.25** | na | na | – | – | 90.0 | 3.0 |
| | **2.5** | 100 | 0 | 2.0 | 0.9 | 86.2 | 0.8 |
| | **5.0** | 100 | 100 | 6.9 | 1.6 | 84.5 | 6.9 |
| | **10** | 100 | 100 | 14.1 | 4.2 | na | na |
| **Red wine** | **Blank** | 0 | 0 | 0.7 | 0.6 | nd | |
| | **1.25** | na | na | – | – | 93.5 | 4.2 |
| | **2.5** | 100 | 0 | 2.4 | 0.7 | 84.9 | 7.3 |
| | **5.0** | 100 | 100 | 5.5 | 1.0 | 91.9 | 0.4 |
| | **10** | 100 | 100 | 13.4 | 2.2 | na | na |

P: percentage of positive results. nd: not detected. na: not analyzed. CV: coefficient of variation. a.u.: arbitrary units. SD: standard deviation of electronic reader signal.

However, all these studies used pure tropomyosin to perform the spike and, therefore, their results are not comparable to those obtained in our study. To our knowledge, there is only one work in which a shrimp extract was added to different commercial food matrices for the recovery determination (Fuller et al., 2006). Recoveries of tropomyosin ranging between 74 % and 140 % were reported in that study, which were calculated by comparing the absorbance value obtained by ELISA with the spiked samples and the absorbance value of the equivalent dilution of prawn extract alone.

### 3.6. Incurred food analysis

In this study, the use of thermally processed incurred foods prepared in a pilot plant using prawn as an ingredient was intended to determine how industrial processing in a food matrix affects the detection of the target protein. Among the effects involved during processing are chemical alterations from the Maillard reaction, protein structural changes from homogenization, emulsion formation and pH variations, among others. It is worth noting that certain regulatory authorities might be reluctant to approve validation studies that do not incorporate results obtained from incurred samples (Abbott et al., 2010; AOAC International, 2014; Taylor et al., 2009).

Model Frankfurt sausages, croquettes and chicken broth containing different levels of prawn protein were analyzed by both methods. These matrices were selected because of the potential risk of cross-contamination with crustacean traces when sharing processing lines with other products.

Results showed that all the blank model foods gave a tropomyosin level below the LOD when tested by ELISA and LFIA, indicating the absence of interferences. The quantitative assay could detect the addition of all tested prawn protein concentrations (1.25, 2.5 and 5.0 μg/g) (Table 5), increasing the concentration of tropomyosin with the increase of prawn protein added to samples. The lower level of tropomyosin determined by ELISA in croquettes compared to sausages or broth could be attributed to the matrix effect produced by the high content of starch in flour, which causes an increase in viscosity and stickiness of the extract that could hinder the solubilization of the protein, despite being

**Table 5**
Results of the precision study obtained by ELISA in model foods incurred with prawn protein. Mean values correspond to the concentration of crustacean protein (μg/g).

| | Crustacean protein (μg/g) | Repeatability | | Intermediate precision | |
|---|---|---|---|---|---|
| | | Mean (μg/g) | CV (%) | Mean (μg/g) | CV (%) |
| **Sausages** | 1.25 | 0.33 | 3.3 | 0.31 | 11.4 |
| | 2.5 | 0.57 | 12.6 | 0.60 | 17.8 |
| | 5.0 | 1.13 | 5.7 | 1.11 | 4.7 |
| **Croquettes** | 1.25 | 0.17 | 26.0 | 0.18 | 41.1 |
| | 2.5 | 0.30 | 11.6 | 0.32 | 17.9 |
| | 5.0 | 0.63 | 5.4 | 0.62 | 7.3 |
| **Chicken broth** | 1.25 | 0.47 | 10.9 | 0.45 | 9.6 |
| | 2.5 | 0.95 | 6.7 | 0.97 | 7.5 |
| | 5.0 | 1.81 | 5.4 | 1.73 | 6.9 |

CV: coefficient of variation.

the food with the least intense heat treatment applied (Wood, 1991). The highest level of tropomyosin was obtained in the chicken broth, which may be due to the fact that it is a liquid matrix that allows a better extraction of the protein. When analyzed by the rapid test, it could detect the addition of 10 μg/g of prawn protein in all incurred matrices (Supplementary Fig. 3, Table 6). The lowest and highest intensity levels were found in croquettes and chicken broth, respectively, similar to what was found by the quantitative immunoassay. In this case, the texture of the extracts likely influences the fluidic of the test, being more viscous for croquettes and more fluid for broth.

Few studies based on ELISA (Seiki et al., 2007; Zhao et al., 2022) and LFIA (Koizumi et al., 2014; Li et al., 2022) to detect crustaceans have included model incurred food to validate the tests. Seiki et al. (2007) and Koizumi et al. (2014) reported detection levels of 5 μg/g of prawn protein in pasteurized sausages, which are lower than those found in our study.

On the contrary, the sensitivity reported in other studies is lower. Taking into account that the protein content of shrimp is around 24 %, Zhao et al. (2022) were able to detect 0.01, 0.05 and 1 % shrimp powder (about 24, 120 and 2400 μg/g of shrimp protein) in model cookie, sausage and sauce, respectively, and Li et al. (2022) could detect 0.01, 0.1 and 0.05 % shrimp powder (about 24, 240 and 100 μg/g of shrimp protein) in model roast fish fillet, boiled meatball and steamed meatball, respectively.

In order to know the effect of processing on the determination of tropomyosin, untreated model Frankfurt sausages, croquettes and broth, incurred with 5 μg/g of prawn protein, were also analyzed by ELISA and compared to the corresponding processed model products. Results showed that the concentration of tropomyosin in processed model Frankfurt sausages, croquettes and broth was reduced by 49.4 %, 60.2 % and 30.3 %, respectively, compared to the raw model food. These results confirm the high resistance of tropomyosin to heat processing.

According to the action levels recommended by VITAL® 4.0 (Allergen Bureau, 2024) and FAO & WHO (2022), based on reference doses (200 mg for crustacean protein) and calculated for predefined intake categories, the developed immunoassay tests are able to detect crustacean protein at levels much lower than that recommended (800 μg/g for a serving size of 250 g), providing safety margins of 6000 and 160 times greater sensitivity, respectively, taking into account the LOD of the developed ELISA and LFIA. Therefore, these techniques could be implemented in the risk management plan to ensure adequate precautionary allergen labelling by the food industry.

### 3.7. Precision of the ELISA test

The precision of the quantitative assay was determined by analyzing three independent extractions (blank and three levels of added prawn protein) in duplicate for each model incurred food. This parameter was determined in a single assay (repeatability) and in two assays conducted on different days (intermediate precision) (Table 5). For repeatability, the variation coefficients obtained ranged from 3.3 % to 26.0 % and for intermediate precision, from 4.7 % to 41.1 %. Both results are considered acceptable according to the AOAC guidance on food allergen immunoassay validation (AOAC International, 2023).

### 3.8. Robustness of the immunoassay methods

To determine the robustness of the quantitative test, two levels of added prawn protein (1.25 and 2.5 μg/g) were analyzed using the model chicken broth. The standard deviations of the differences (SDi) between the values obtained in the established and modified conditions of the assay were calculated for each concentration level (Supplementary Table 2). Considering that the SDi of the robustness (0.00050 and 0,00104 at 1.25 and 2.5 μg/g of added prawn protein) was lower than the standard deviation of the intermediate precision, the test proved to be robust (Commission Decision 2002/657/EC of 12 August 2002 Implementing Council; Karageorgou & Samanidou, 2014).

The robustness of the rapid test was also determined using the model chicken broth, added with 10 and 20 μg/g of prawn protein (Supplementary Table 3). Results indicated that a 20 % variation in the sample portion weight, the extraction buffer volume or the assay volume has no impact on the sensitivity of the test. In addition, increasing the assay time to 15 min produced a slight increase in the signal of the test line, whereas reducing it to 5 min gave a negative result. These results indicate that the incubation time of the test is crucial for obtaining reliable results.

**Table 6**
Level of detection of crustacean protein in model foods incurred with different levels of prawn protein analyzed by LFIA.

| | Crustacean protein (μg/g) | N | Naked eye | Strip reader | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | x | x | Signal (a.u.) | SD | POD | LCL | UCL |
| **Sausages** | 0.0 | 20 | 0 | 0 | 0.4 | 0.0 | 0.00 | 0.00 | 0.16 |
| | 5.0 | 20 | 0 | 0 | 2.0 | 0.7 | 0.00 | 0.00 | 0.16 |
| | 10 | 40 | 40 | 39 | 7.0 | 2.7 | 0.98 | 0.81 | 0.98 |
| | 20 | 20 | 20 | 20 | 12.6 | 5.6 | 1.00 | 0.84 | 1.00 |
| | 50 | 6 | 6 | 6 | 24.8 | 8.9 | 1.00 | 0.61 | 1.00 |
| | 400 | 6 | 6 | 6 | 144.8 | 7.0 | 1.00 | 0.61 | 1.00 |
| **Croquettes** | 0.0 | 20 | 0 | 0 | 0.4 | 0.2 | 0.00 | 0.00 | 0.16 |
| | 5.0 | 40 | 0 | 0 | 1.2 | 0.7 | 0.00 | 0.00 | 0.09 |
| | 10 | 54 | 54 | 42 | 4.8 | 1.4 | 0.78 | 0.65 | 0.87 |
| | 20 | 20 | 20 | 19 | 7.8 | 3.6 | 0.95 | 0.76 | 0.99 |
| | 50 | 6 | 6 | 6 | 37.4 | 20.6 | 1.00 | 0.61 | 1.00 |
| | 400 | 6 | 6 | 6 | 85.4 | 11.3 | 1.00 | 0.61 | 1.00 |
| **Chicken broth** | 0.0 | 20 | 0 | 0 | 0.6 | 0.5 | 0.00 | 0.00 | 0.16 |
| | 5.0 | 20 | 20 | 1 | 2.5 | 0.7 | 0.05 | 0.01 | 0.24 |
| | 10 | 40 | 40 | 40 | 7.2 | 1.2 | 1.00 | 0.91 | 1.00 |
| | 20 | 20 | 20 | 20 | 16.2 | 3.2 | 1.00 | 0.91 | 1.00 |
| | 50 | 6 | 6 | 6 | 64.6 | 12.2 | 1.00 | 0.61 | 1.00 |
| | 400 | 6 | 6 | 6 | 152.5 | 3.8 | 1.00 | 0.61 | 1.00 |

N: number of replicates assayed. X: number of positive results. POD: probability of detection. CI: confidence interval. LCL: lower control limit (95 % CI). UCL: upper control limit (95 % CI). a.u.: arbitrary units. SD: standard deviation of electronic reader signal.

### 3.9. Crustacean protein detection on surfaces

Cross-contamination in the food industry can occur due to the use of shared processing lines and poor cleaning of contact surfaces. Therefore, the monitoring of these surfaces using adequate analytical techniques should be implemented to eliminate possible traces of food allergens. One of the most commonly used methods is environmental swabbing, as indicated in allergen management guidelines (FoodDrinkEurope, 2022). In the present study, stainless steel and melamine surfaces were tested by both methods due to their common use in the food industry. The lowest crustacean protein level detected in both surfaces by the quantitative test was 0.1 μg, with recoveries of 36 % in stainless steel and 40 % in melamine. When analyzed by the rapid test, the lowest crustacean protein level detected with a POD of 1 was 1.0 μg in stainless steel and 2.0 μg in melamine (Supplementary Table 4). To our knowledge, no studies have been conducted to detect crustacean traces on working surfaces. However, research on the detection of egg, milk and nut residues on surfaces has been previously performed. Courtney et al. (2016) could detect between 3 and 30 μg of non-fat dry milk (NFDM) in stainless steel surfaces using different commercial LFIAs. Galan-Malo et al. (2017) detected egg and milk powder by ELISA at levels of 0.04 and 0.2 μg, respectively, and by LFIA at levels of 0.07 and 0.6 μg, respectively, with recoveries between 30 % and 100 %. Civera et al. (2023) detected 0.6 and 0.3 μg of hazelnut protein by ELISA with recoveries of 13 % and 16 % in stainless steel and melamine, respectively, and 1.2 and 0.6 μg by LFIA in stainless steel and melamine, respectively. The recoveries obtained in the present study are similar to those obtained for egg and milk and higher than those obtained for hazelnut. These variations may be due to differences in protein size or to the efficiency of the recovery during swabbing and immersion in the extraction in buffer.

## 4. Conclusion

In this study, the development and validation of sandwich ELISA and LFIA tests for the detection of crustaceans based on the determination of tropomyosin has been performed. The "in-house" validation of both tests proved that they have a high sensitivity, a good specificity among more than 60 basic ingredients analyzed and an acceptable precision, recovery and robustness, according to international guidelines. To our knowledge, this is the first study that includes the development and validation of both types of tests and compares their effectiveness using thermally processed model foods and working surfaces. The importance of the use of model incurred foods in the validation procedure should be emphasized, as they offer a realistic approach to the detection of allergens in processed industrial products. Furthermore, complex food matrices that could interfere in the detection of the target protein spiked with prawn extract were tested with both techniques, showing good recoveries and verifying the sensitivity of the rapid test. The ELISA could be used to quantify the presence of crustacean protein in ingredients or final products and to identify areas of contamination in processing lines, when there is no time limitation. However, the simplicity and rapid response of LFIA makes it suitable to be used during processing when immediate on-site decisions are required. It can also be used to verify the proper cleaning of tools and contact surfaces between batches. Moreover, the integration of an optical strip reader enables the objective interpretation of the results, avoiding misinterpretation when faint lines appear. It is noteworthy that the developed ELISA and LFIA have enough sensitivity to protect 95 % of consumers with an allergy to crustaceans according to detection levels recommended by VITAL® 4.0 and FAO/WHO, and are suitable for being implemented in risk management plans in the food industry, thus avoiding the misuse of precautionary labelling.

## CRediT authorship contribution statement

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

## 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.

## Acknowledgments

Authors thank Aragon Regional Government (A20_23R) and the European Social Fund (ESF). Clara Esteban is grateful to the Government of Aragon for providing her a PhD grant. The authors acknowledge Ms. Alba Gutiérrez and Ms. Blanca Barranco for her technical assistance. Authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111930.

## Data availability

Data will be made available on request.

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