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Food allergen control: Tropomyosin analysis through electrochemical immunosensing

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ABSTRACT

Regulations of the EU obliges the indication of the presence of allergens on food labels. This work reports the development of an electrochemical immunosensor to determine tropomyosin (TPM) – a major shellfish allergen – prevailing in the muscles of crustacean species. Two linear ranges between the signal and TPM concentration were obtained: between 2.5 and 20 ng mL^{-1} and between 30 and 200 ng mL^{-1}, with a lowest limit of detection of 0.47 ng mL^{-1}. The selectivity of the optimized immunoassay, tested with other food allergens (e.g., Cyp c 1, a fish allergen), assures the effective detection of TPM, enabling successful control of foodstuff labelling. Several (12) foods, containing high and low TPM concentrations and TPM-free samples, were analysed using the sensor. A conventional ELISA kit and recovery assays were used to evaluate the accuracy of the results.

1. Introduction

Food allergy is a public health problem with a rising incidence over the past few years, posing a threat for consumers and a challenge for the food industry. According to legislation, 14 allergenic foods were identified and associated with adverse food-related allergic reactions (Patel et al., 2021; Sicherer & Sampson, 2018). Food allergen intake, skin contact, or occupational exposure can cause clinically diagnosable symptoms from mild urticaria to life-threatening anaphylactic shock (Abrams & Sicherer, 2016; van Ree, 2021). To ensure food safety for allergen-sensitive individuals, the approval of the Food Allergen Labeling and Consumer Protection Act (FALCPA) in 2004 (FDA, 2004) and the Labelling Guide established by the European Parliament and of the Council in 2011 (EC Regulation (EU) No 1169/2011) advertised manufacturers to properly label foodstuff that contain or may contain food allergens (Hosu et al., 2018; Ross et al., 2018).

The consumption of seafood - defined as marine and freshwater fish and shellfish - increased between 2014 and 2018 from 19.9 to 20.5 kg per capita (FAO, 2020; Melissa Shahbandeh, 2020). Despite being recognized as a healthy food choice due to its low-fat protein content, it may contain undesirable substances for the human body (e.g., heavy metals, biogenic amines, allergens) (Torre et al., 2020). Tropomyosin (TPM), an allergenic protein present in shellfish muscles, is resistant to peptic acidic digestion and is more stable to thermal denaturation than non-allergic substances, thus leading to a higher likelihood of sensitization (Kamath et al., 2013). Among shellfish (shrimp, crab, lobster, bivalves, etc.), shrimp is associated to 80% of allergic reactions (Cheng et al., 2022; De Marchi et al., 2021; Kumar et al., 2020). So, the development of cost-effective technologies for the detection of traces of TPM can effectively aid consumers and manufacturers, refining food production considering hazard analysis of critical control points, risk assessment and good manufacturing practices.

Currently, multiple approaches have been developed to identify the presence of TPM in food, including polymerase chain reaction (PCR) (Kim et al., 2019; Suh et al., 2020), microarray technology and qualitative/semi-quantitative lateral flow assays (Koizumi et al., 2014; Liang et al., 2020). The disadvantages of the use of lateral flow assays are the fact that these generally only provide qualitative or semi-quantitative results. The main drawbacks of the analysis of allergens by the PCR method, although general sample extraction procedures can be applied, include significant matrix effects and the detection of target sequences resulting from cross-contamination during the analysis, leading to false positive results. Immunoanalytical methods (e.g. ELISA) are the most used, offering a quantitative/qualitative analysis (Khedri et al., 2018; Wammes et al., 2015) due to its high sensitivity, selectivity and reproducibility with practical analytical performance. Still,

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conventional ELISA presents some disadvantages, namely centralized analysis at specific laboratories with expensive microtiter plate spectrophotometers and laborious and long-time assay procedures that lead to high costs (Fuller et al., 2006; Lin et al., 2018; Zhang et al., 2014; Zhao et al., 2022). In contrast, electrochemical immunoassays are a prominent alternative for TPM analysis since they combine specific and high affinity antibodies, that can bind to different sites of a specific epitope and recognize distinct non-overlapping regions of the allergen, with the potential of the miniaturization with improved electrochemical detection (Angulo-Ibáñez et al., 2019; Jiang et al., 2013; Mohamad et al., 2020). Furthermore, when the assay is applied on screen-printed carbon electrodes (SPCEs) the final platform can be applied *in situ*, which simplifies TPM analysis, and increases speed, sensitivity, precision, and reduces the sample and reagent consumption (Sena-Torralba et al., 2020; Smart et al., 2020; Torre et al., 2020).

The sensor developed in this work is based on a sandwich-type assay using SPCE transducers. The analytical signal was obtained through the employment of an alkaline phosphatase-labelled secondary antibody and 3-indoxyl phosphate / silver nitrate (enzymatic substrate). The detection of the enzymatically deposited silver was performed by linear sweep voltammetry. Several foods (TPM-containing, with low TMP concentrations, and TPM-free samples were analysed using the sensor, and a conventional ELISA kit and recovery assays were used to evaluate the accuracy of the results.

2. Materials and methods

2.1. Equipment

Voltammetric analyses were carried out with a PGSTAT204 potentiostat/galvanostat from Methrohm Autolab (Utrecht, The Netherlands). Screen-printed carbon electrodes (SPCE, with a 4-mm carbon working electrode (WE), a carbon counter electrode, and a silver pseudoreference electrode) from Methrohm Dropsens (Oviedo, Spain) were used.

Sample preparation was performed with a block thermostat (Tembloc, Selecta, Barcelona, Spain) and a centrifuge (Heraues Megafuge 16R, Thermo Fisher Scientific, Osterode am Harz, Germany). An ELISA kit was used for immunosensor validation; data were acquired by a multi-mode microplate reader (Synergy HT W/TRF) and treated using Gen5 software (Version 2.0) from BioTek Instruments (Winooski, VT, USA).

Scanning Electron Microscopy (SEM) images were obtained by a FEI Quanta 400 FEG ESEM/EDAX Genesis X4M equipment, and the energy dispersive spectroscopy (EDS) was subsequently carried out to assess the elemental analysis. This study was conducted at the Centro de Materiais da Universidade do Porto (CEMUP, Porto, Portugal).

2.2. Reagents and solutions

Mouse IgG_1 monoclonal antibody MA-1A6 (capture antibody, C-Ab), purified natural shrimp Tropomyosin standard ST-STM (antigen, TPM), rabbit polyclonal antiserum shrimp tropomyosin PA-SHM (detection antibody, D-Ab) and a shrimp Tropomyosin ELISA 2.0 kit (product ID: EPC-TPM-1) were obtained from Indoor Biotechnologies (Cardiff, UK). Goat Anti-Rabbit IgG Fc - Alkaline Phosphatase conjugate (AP-Ab) was purchased from Invitrogen–Thermo Fisher Scientific (Oslo, Norway). 3-indoxyl phosphate (3-IP), bovine serum albumin (BSA), casein sodium salt from bovine milk, magnesium nitrate hexahydrate, reduced graphene oxide (rGO), single-walled carbon nanotubes (SWCNTs), N,*N*-dimethylformamide (DMF), hydrochloric acid, nanodiamonds (NDs), tetrachloroauric(III) acid, silver nitrate, tris(hydroxymethyl)aminomethane (Tris) and nitric acid were acquired from Merck (Algés, Portugal).

The biomolecule solutions (BSA, antibodies and antigen) were prepared daily using Buffer 1 (B1 – 0.1 M Tris-HNO_3 pH 7.4), and the mixed enzymatic substrate solution (3-IP (1.0×10^{-3} M) + $AgNO_3$ (4.0×10^{-4} M)) was prepared with buffer 2 (B2 – 0.1 M Tris-HNO_3 pH 9.8, containing 2.0×10^{-2} M $Mg(NO_3)_2$). An extraction buffer (0.1 M Tris-HNO_3 pH 8.2) was used for sample preparation. Buffers were prepared using ultra-pure water (Simplicity 185, Millipore, Molsheim, France).

2.3. Immunoassay & electrochemical measurements

A representative scheme of the optimized immunosensor's construction and detection strategy is presented in Fig. 1. The WE of the SPCE was (1) coated with C-Ab (10 μL, 20 μg mL^{-1}, overnight) through physical adsorption and left to incubate at 4 °C in a humidified chamber. After rinsing the biosensor with B1 to remove unbound C-Ab, (2) the surface was blocked (40 μL, BSA (2% (m/V)), 30 min). An additional washing step was performed using B1, and (3) a 40-μL aliquot of a previously prepared mixed solution (10 min before use) containing the antigen (target analyte or food sample) and the D-Ab (2000×, in B1-BSA (1% (m/V)) was incubated during 60 min. After rinsing with B1, (4) an AP-Ab solution (40 μL, 40,000 × in B1-BSA (1% (m/V)), 60 min) was placed on the electrode surface, followed by a final washing step with B2. Once the immunological interactions were completed, (5) the enzymatic reaction took place (20 min) by adding 40 μL of a mixed solution containing 3-IP (enzymatic substrate, 1.0×10^{-3} M) and $AgNO_3$ (4.0×10^{-4} M). Briefly, the hydrolyzation of 3-IP by AP generates indigo blue while silver ions are reduced to metallic silver. (6) The silver particles, co-deposited with indigo blue, were re-oxidized by applying linear sweep voltammetry (LSV) (potential range: −0.03 V to + 0.4 V, scan rate: 50 mV/s). The peak potential was near + 0.2 V and the deposited metallic silver is proportional to the TPM concentration present in the sample. The LSV analyses were performed in triplicate and carried out at 25 °C.

2.4. Sample preparation

Fresh shrimp (*Litopenaeus vannamei*) and a set of commercial food products and ingredients that contain, may contain, or do not contain crustaceans were purchased at local supermarkets and used to evaluate and validate the immunosensor's applicability to food analysis. The food products/ingredients were ground with a mincer (Moulinex) and were prepared as follows: (a) 1 g of each sample was mixed with 10 mL of extraction buffer and the solution was incubated at 60 °C (water bath, 15 min); (b) the obtained suspension was centrifuged (2500 rpm, 20 min) and (c) the supernatant was collected and stored at −20 °C until use (Indoor Biotechnologies, 2012). Prior to analysis, the food extracts were adequately diluted (using B1-BSA 1% (m/V)) according to the amount of crustacean (indicated on the label): shrimp-containing foods (shrimp sauce, Asian gourmet chips, prawn crackers, crab paste, shrimp noodles, seafood sticks) – 1000×; extracts that may contain traces of crustaceans or contain unknown amounts (mussel) – 100 ×. The allergen- and crustacean-free products (hake, anchovies, meat and chicken paste) were diluted 1 × and the fresh shrimp extract (which contains a higher amount of TPM) was diluted 2000 ×.

3. Results

3.1. Immunoassay optimization

A simple methodology was applied for TPM analysis using a portable and small-sized electrochemical cell to develop the immunosensor. In the sandwich-type assay TPM is captured and recognized through matched anti-TPM antibodies (C-Ab immobilized on the WE and D-Ab mixed with the sample) both able to recognize the allergen at specific epitopes. A highly specific goat anti-rabbit IgG Fc coated enzyme tag (AP-Ab) efficiently binds to the previously formed immunocomplex. Optimization studies were carried out and the selected values were chosen based on the signal-to-blank ratio (S/B) in the presence (signal (S), 10 ng mL^{-1}) and absence (blank (B), 0 ng mL^{-1}) of TPM (reference

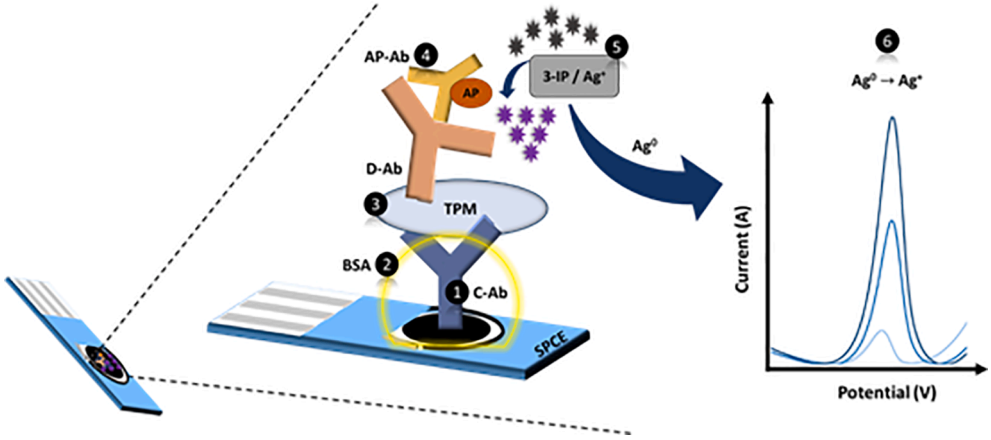

Fig. 1. Schematic representation of the optimized construction of the immunosensor's sensing phase, immunoassay configuration, enzymatic reaction, and electrochemical detection. (1) C-Ab immobilization, (2) blocking step with BSA, (3) TPM + D-Ab incubation, (4) AP-Ab addition, (5) enzymatic reaction (3-IP and silver ion addition) and (6) voltammetric analysis.

concentration according to manufacturer's recommendation). Initially, to verify the adequate performance of the reagents the following tests were carried out: complete immunoassays in the presence and absence of target analyte (TPM) (control assays), and assays without C-Ab, D-Ab, AP-Ab, 3-IP and $AgNO_3$. As expected, the obtained results, presented in Fig. 2**A,** confirmed that the presence of all immunoreagents (control assays) was crucial for the effective detection of the allergen and demonstrated the correct performance and the suitability of the present immunosensor. Representative cyclic voltammograms (CV) of the re-oxidation of silver in the presence and absence of the target analyte are displayed in Fig. 2**B**.

To obtain an optimum immunoassay, several experimental parameters were optimized. First, the influence of two different blocking agents was studied: β-casein (2% (m/V)) and BSA (2% (m/V)) (using a fixed C-Ab concentration of 10 μg mL^{-1}, D-Ab dilution of 250×, and an AP-Ab dilution of 20,000 ×). In the presence of BSA higher i_p and S/B values were noticeable (**Fig. S1A**). Thus, after establishing BSA as blocking agent, the influence of the C-Ab concentration was tested using 2.5, 5.0, 10 and 20 μg mL^{-1} solutions (using a fixed D-Ab dilution of 250 × and an AP-Ab dilution of 20,000 ×). The obtained results, present in **Fig. S1B,** shows that the i_p increased with the C-Ab concentration and levelled off after 10 μg mL^{-1}. Nevertheless, the S/B ratio increased up to 20 μg mL^{-1}. Thus, this concentration was selected to proceed the studies since the S/B ratio is analytically preferable. Subsequently, using the optimized C-Ab concentration, seven serial D-Ab dilutions, from 250x to 12,000x, were tested (using a fixed C-Ab concentration of 20 μg mL^{-1}, and an AP-Ab dilution of 20,000x) (**Fig. S1C**). The 250×, 500 × and 1,000 × dilutions revealed the highest i_p values for both the signal and the blank; however, the blank values were significantly reduced for the 2,000×; 4,000×, 8,000 × and 12,000 × dilutions. As a compromise between the obtained values, a 2,000 × dilution was selected. To reduce the steps and/or the total analysis time, the following assay formats were evaluated: (I) (1) blocker, (2) TPM, (3) D-Ab, (4) AP-Ab, (5) 3-IP/Ag^+; (II) (1) blocker, (2) **TPM + D-Ab**, (3) AP-Ab, (4) 3-IP/Ag^+; (III) (1) blocker, (2) TPM, (3) **D-Ab + AP-Ab**, (4) 3-IP/Ag^+; (IV) (1) blocker, (2) **TPM + D-Ab + AP-Ab**, (3) 3-IP/Ag^+. Detailed information regarding this optimization is indicated in **Table S1**. The obtained results shown in **Fig. S1D** reveal that assay Format II (previous mixing of TPM and D-Ab) provided the highest i_p in the presence of TPM and the best S/B ratio. Although Format I (step-by-step assay) provided similar results, Format II considerably reduces the analysis time and was chosen for the subsequent studies. To conclude the immunoassay optimization, the AP-Ab dilution (10,000×, 20,000 × and 40,000 ×) and incubation time (15, 30 and 60 min) were studied. Based on the obtained results (**Fig. S1E**) a 40,000 × dilution was desirable over the lower dilutions since a lower

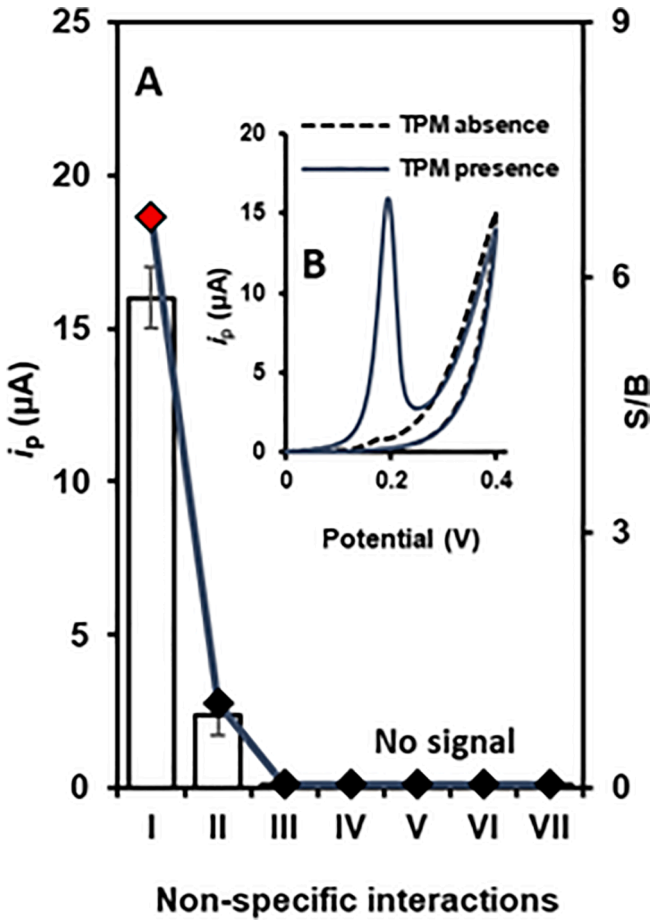

Fig. 2. (A) Peak current intensities (i_p) obtained for complete assays in the presence (10 ng mL^{-1}) (I) and absence (II) of TPM (control assays), and assays with TPM (10 ng mL^{-1}) without C-Ab (III), D-Ab (IV), AP-Ab (V), 3-IP (VI) and $AgNO_3$ (VII). **(B)** Evaluation of the adequacy of the electrochemical detection mechanism based on 3-IP/Ag^+ substrate: voltammograms obtained in the absence (dashed line) and presence (solid line) of TPM (10 ng mL^{-1}). Experimental conditions: C-Ab: 20 μg mL^{-1}, D-Ab: 2000×, AP-Ab dilution: 40,000×. Other conditions: BSA: 2% (m/V), 3-IP: 1.0 × 10^{-3} M, $AgNO_3$: 4.0 × 10^{-4} M. Results are presented as average ± standard deviation (n = 3). The red point indicates the best S/B value.

blank signal and a higher S/B ratio were achieved and, moreover,

involves lower reagent consumption. Regarding the incubation time (Fig. S1**F**), 60 min was selected since the highest S/B ratio was obtained. A summary of the optimized experimental variables, the tested ranges and the optimum results is indicated in Table S2. Additionally, the influence of two temperatures was tested (20 and 25 °C) (Fig. S2) and the best results were obtained at 25 °C, at which the biomolecule interactions are favoured. No higher temperatures were tested because of energy consumption issues.

The nanostructuration and/or functionalization of the transducer surface is frequently used to improve its retention capacity of

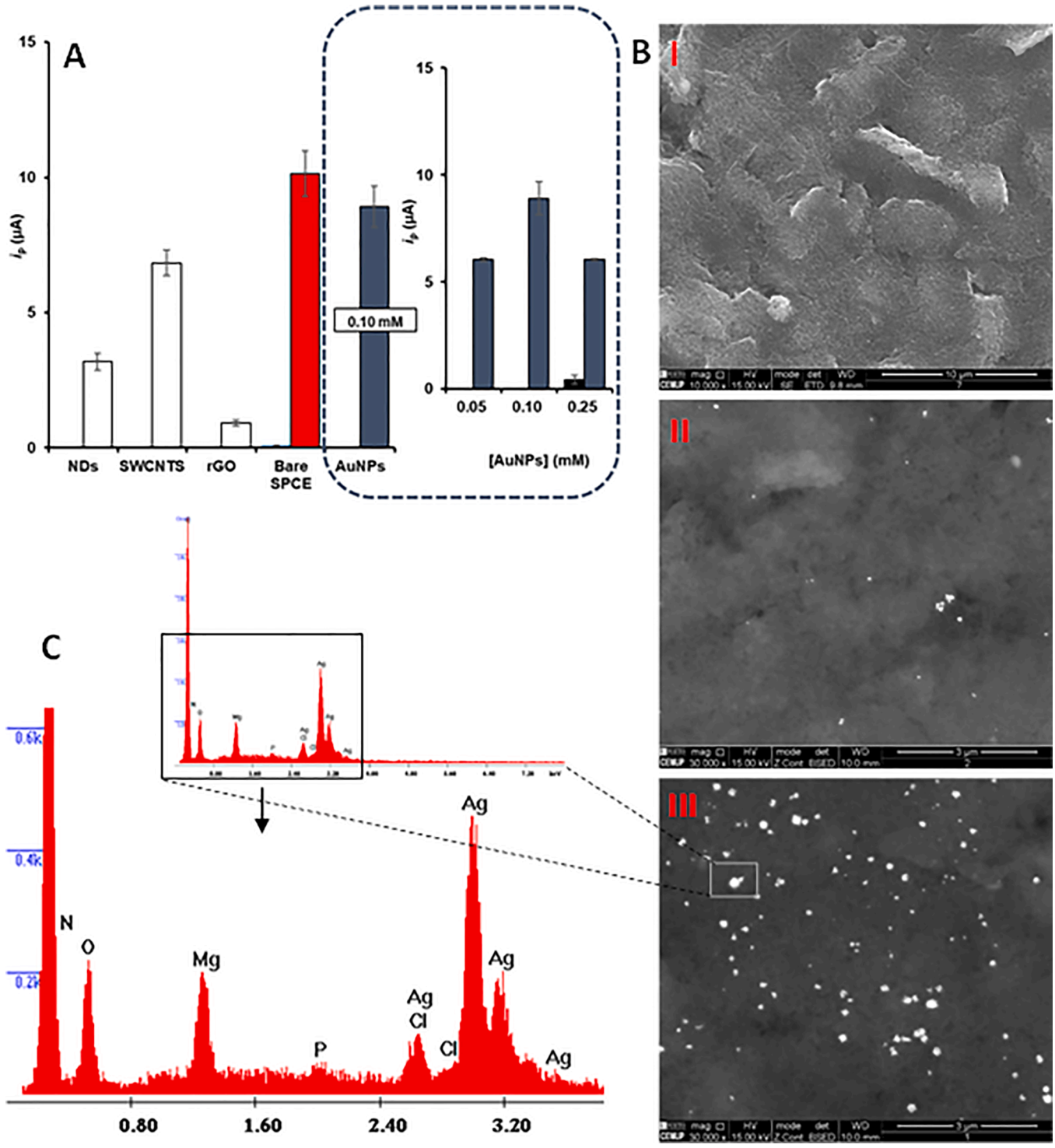

Fig. 3. (A) Peak current intensities (i_p) obtained using bare SPCE and SPCE modified with carbon-based nanomaterials: nanodiamonds (NDs, 0.10 mM), single-walled carbon nanotubes (SWCNTS, 0.10 mM), reduced graphene oxide (rGO, 0.10 mM), and gold nanoparticles (AuNPs, 0.05 mM, 0.10 mM, and 0.25 mM). Black bars: blank assay. White/red/blue bars: TPM (10 ng mL^{-1}). **(B)** SEM images of (I) bare SPCE, (II) optimized immunosensor in absence of TPM, and (III) optimized immunosensor in the presence of TPM 100 ng mL^{-1}. **(C)** EDS analysis of silver deposition acquired from SEM image (III) corresponding to the AP-Ab/D-Ab/TPM/C-Ab modified electrode. Experimental conditions: BSA: 2% (m/V), C-Ab: 20 μg mL^{-1}, D-Ab: 2000×, AP-Ab: 40,000×, 3-IP: 1.0×10^{-3} M, $AgNO_3$: 4.0×10^{-4} M. Results are presented as average ± standard deviation (n = 3).

biomolecules and to enhance the analytical signal (Mohamad et al., 2020). In this perspective, distinct carbon-based nanomaterials were tested to study the formation of an immunoaffinity layer through the antibody's adsorption on the working electrode. Nanodiamonds (NDs), single-walled carbon nanotubes (SWCNTs) and reduced graphene oxide (rGO) were tested (fixed concentrations of 0.10 mM, SPCE modification by drop casting). Additionally, a gold-based nanomaterial was tested (gold nanoparticles (AuNPs), produced by electrodeposition of a 0.10-mM tetrachloroauric(III) acid solution (Freitas et al., 2019)) to observe if chemisorption could be a better option. The results, depicted in Fig. 3**A**, revealed that the carbon-based nanomaterials provided a lower analytical signal compared to the bare SPCE, which can be explained by the high affinity between the capture antibody and the carbon sheets that make up the working electrode's surface and/or by the broad adsorption of dissimilar non-specific antibody-binding sites on the sensing phase related to the nanostructured electrode that minimize the voltammetric signal (Freitas, Neves, et al., 2021; Freitas, Nouws, et al., 2021). The gold nanoparticle coated SPCE showed similar results to the ones obtained for the bare SPCE. To explore this similarity other tetrachloroauric(III) acid concentrations were tested: 0.05 mM and 0.25 mM, but these did not improve the analytical signal (Alves et al., 2015). For this reason, a bare SPCE was chosen to develop this immunosensor, avoiding time- and reagent consuming transducer surface modification. Characterizations of the bare SPCE (Fig. 3**B I**) and the optimized sensing phase (Fig. 3**B II and III**) were carried out by scanning electron microscopy (SEM). The images of the AP-Ab/D-Ab/TPM/C-Ab modified-SPCE clearly indicate the presence of dense silver particles on the SPCE after the enzymatic reaction (Fig. 3**B II and III**). In addition, EDS analysis (Fig. 3**C**) confirmed the previous results through elemental mapping analysis.

3.2. Analytical characteristics and method validation

With the optimized conditions the immunosensor's response towards increasing TPM concentrations was evaluated between 2.5 and 200 ng mL^{-1}. Two distinct linear ranges between i_p and TPM concentration were obtained: (1) 2.5–20 ng mL^{-1}, i_p (μA) = 0.456 [TPM] (ng mL^{-1}) + 4.74 (r = 0.993; n = 5), and (2) 30–200 ng mL^{-1}, i_p (μA) = 0.0384 [TPM] (ng mL^{-1}) + 14.9 (r = 0.995; n = 4). The calibration straights are presented in Fig. 4**A** and representative voltammograms of linear range (1) are shown in Fig. 4**B**. Tracking TPM in food products is essential to prevent severe symptoms caused by the presence of allergens that can be attributed to hidden and undeclared allergenic substances, or even food adulteration, cross-contamination, and fraud. Thus, screening lower concentrations allows the detection of trace amounts; however, the usefulness of both ranges is practical since the quantification of TPM in food products can be effectively assessed over a wide range. The limits of detection (LOD = $3S_b/m$) and quantification (LOQ = $10S_b/m$) were calculated based on the calibration data, where S_b is the standard deviation of the blank and m is the slope. The lowest LOD and LOQ obtained (for linear range (1)) were 0.47 ng mL^{-1} and 1.6 ng mL^{-1}, respectively. The precision of the method was adequate since a coefficient of variation (V_{x0}) below 10% was obtained. Additional figures of merit are indicated in **Table S3**.

The repeatability of the results was evaluated by performing three successive inter-electrodic measurements using separate immunosensors and the reproducibility was checked in a 3 inter-day evaluation. Average coefficients of variation (CV) of 7.3 % and 4.3%, respectively, were achieved, indicating that the immunosensor provides precise results.

Moreover, to access the stability of the sensing surface, sensors were

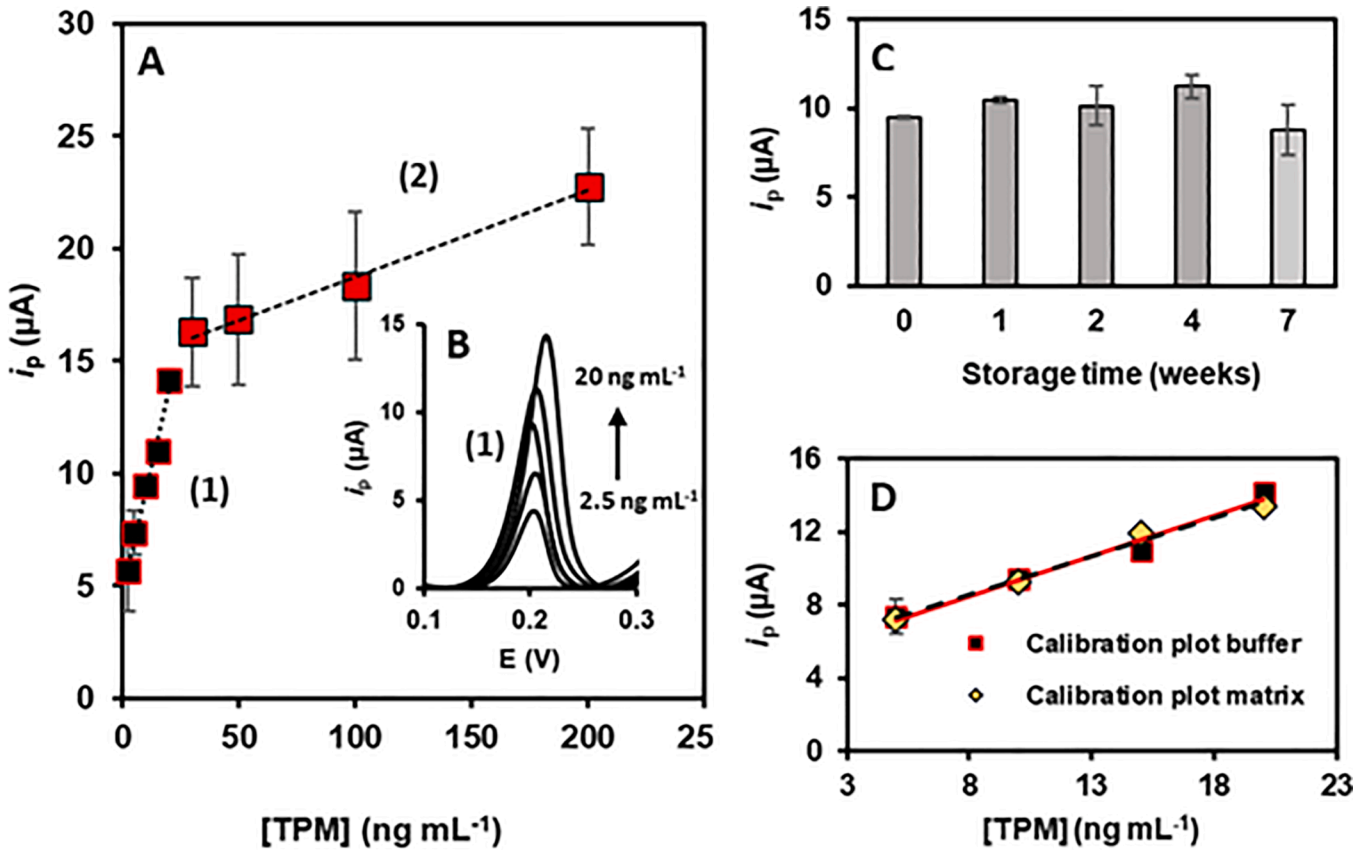

Fig. 4. **(A)** Calibration straights for the analysis of TPM: (1) 2.5–20 ng mL^{-1} and (2) 30–200 ng mL^{-1}. **(B)** Examples of voltammograms in linear range (1): 2.5, 5.0, 10, 15 and 20 ng mL^{-1}. **(C)** i_p values obtained on the day of preparation and after 1, 2, 4 and 7 weeks (TPM 10 ng mL^{-1}). **(D)** Comparison between the analysis of TPM solutions (2.5, 5.0, 10, 15 and 20 ng mL^{-1}) prepared in buffer and in hake extract. Experimental conditions: C-Ab: 20 μg mL^{-1}; BSA: 2% (m/V); D-Ab: 2000×, AP-Ab dilution: 40,000×; 3-IP: 1.0 × 10^{-3} M, $AgNO_3$: 4.0 × 10^{-4} M. Results are presented as average ± standard deviation (n = 3).

modified with C-Ab, stored at 4 °C, and used to analyse a TPM solution (10 ng mL^{-1}) on the day of its preparation (control) and after 1, 2, 4 and 7 weeks. The results of the LSV measurements revealed that the developed sensing phase was stable for up to 4 weeks, without significant changes of the i_p values (Fig. 4**C**). After 7 weeks a slight decrease (7.2%) of the initial i_p value was observed. So, the prepared platform can be used within a 30-day period, when stored in humid and refrigerated conditions, demonstrating suitable storage stability when compared to a reported immunosensor with a stability of 6 days (Mohamad et al., 2020). This proves that the adsorption of the C-Ab antibody on the electrode surface enables a stable, simple, and inexpensive immobilization, without the need for additional steps. The stability of the immunosensor is limited to a few weeks even if appropriate conditions are guaranteed because of the possible loss of functionality of the antibodies after this period (Freitas, Nouws, et al., 2021).

To evaluate the immunosensoŕs selectivity towards TPM and the possible interference of other allergens, a fish allergen (Cyp c 1, 20 ng mL^{-1}) and a peanut allergen (Ara h 1, 250 ng mL^{-1}) were used. The concentrations of these allergens were selected based on the minimum quantities required to induce mild symptoms in sensitive individuals (Fernandes et al., 2015; Freitas, Neves, et al., 2021). The immunosensoŕs results, displayed in **Fig. S3**, showed similar values for the blank assays and the assays with the non-target allergens. Moreover, very similar responses for TPM and for solutions containing both TPM and the non-target allergens were obtained. These results allow to conclude that the presence of these other food allergens do not significantly affect the analysis of TPM, thus corroborate the high selectivity of the selected anti-TPM antibodies for the specific tracking of TPM in food products.

Certified reference materials are ideally employed for the evaluation of the accuracy of the results of a method. However, no such reference material was available, so recovery experiments using a TPM-free hake extract were conducted. The hake extract was spiked with increasing TPM concentrations (5.0, 10, 15 and 20 ng mL^{-1}) within linear range (1). The linear relationship between i_p and TPM concentration was described by the following equation: i_p (μA) = 0.422 [TPM] (ng mL^{-1}) + 5.19 (r = 0.989, n = 4). The calibration straight (Fig. 4**D**) confirmed that no significant matrix effect was observed because the obtained slope and the one for the calibration straight in buffer were very similar ($slope_{buffer}/slope_{matrix}$ = 1.08). The calculated recoveries for TPM concentrations of 5.0, 10, 15 and 20 ng mL^{-1} were 90.9% (CV = 8.5%), 100.4 % (CV = 5.8 %), 94.9 % (CV = 2.9 %), and 105.3 % (CV = 3.3 %), respectively. These values and the associated CV prove the accuracy of the results and the suitability of the sensor for tracking purposes in commercial food products.

To evaluate the applicability of the sensor to (sea)food analysis, a set of commercial products (Table 1) were analysed. These products were chosen based on the presence/absence of TPM, the product label, and the protein composition. An easy-to-use method for allergen extraction was employed (section 2.3.) and the resulting extracts were diluted and analysed. The results were compared with the results of a reference method (ELISA) (**Fig. S4A**). The main characteristics of the samples and the results are indicated in Table 1. The concentration of TPM in fresh shrimp (*Litopenaeus vannamei*) was found to be 808 ± 28 μg g^{-1}. However, no reference values were found to validate this result. The concentrations of TPM in shrimp-containing foods (prawn crackers, Asian gourmet chips and shrimp sauce) are in accordance with the product label, thus demonstrating the feasibility of the proposed sensor and the compliance of manufacturers with legislation. Low amounts of crustaceans were quantified in some samples (crab paste - crab (3%) and seafood sticks - crab flavour (2.9%), crab extract (1.4%)), and trace amounts of TPM were found in shrimp noodles (shrimp dust (0.7%)). Moreover, a mussel sample (*Mytilus spp.*, a bivalve mollusc) with a label that adequately advertised the consumers ("may contain traces of fish and crustaceans") was analysed and TPM was successfully quantified (8.51 ± 2.96 μg g^{-1}). These latter results are particularly relevant because of the low amount of TPM that can effectively be quantified (between 8.51 and 9.82 μg g^{-1}), demonstrating the ability of this biosensor to detect trace amounts of the target allergen. For TPM-free samples (meat, anchovies, chicken paste and hake) no measurable analytical signal was obtained, indicating the absence of the allergen.

Table 1
Analysis of TPM in foods by the proposed immunosensor and ELISA (average results ± standard deviation (n = 3) are indicated).

Food/ Ingredient	Specie/Type	Crustacean (%)	TPM (μg g^{-1})	
			Immunosensor	ELISA
Shrimp	*Litopenaeus vannamei*	Shrimp (100 %)	808 ± 28	834 ± 36
Shrimp sauce	–	Shrimp (6%) Crab extract (0.7%)	466 ± 3	487 ± 29
Asian gourmet chips	–	Prawns (21%) (may contain traces of fish)	294 ± 17	309 ± 10
Prawn crackers	–	Shrimp meat (15.4%) (may contain traces of fish)	223 ± 19	226 ± 14
Seafood sticks	–	Crab flavour (2.9%) Crab extract (1.4%)	21.0 ± 1	19.7 ± 3
Crab paste	–	Crab (3%)	25.0 ± 2	26.1 ± 12
Shrimp noodles	–	Shrimp dust (0.7%)	9.82 ± 1	8.19 ± 0.3
Mussel	*Mytilus* spp.	May contain traces of fish and crustaceans	8.51 ± 3	10.3 ± 2
Meat	Bacon	n.a.	n.d.	n.d.
Anchovies	*Engraulis encrasicolus*	n.a.	n.d.	n.d.
Chicken paste	Chicken	n.a.	n.d.	n.d.
Hake	*Merluccius capensis*	n.a.	n.d.	n.d.

n.a. – not applicable; n.d. – not detected.

A good correlation between the results obtained with the proposed immunosensor and the ELISA results was observed (**Fig. S4B**), thus confirming the accuracy of the results obtained with the proposed voltammetric immunosensor.

The threshold value (mg of protein/kg sample) for TPM has not yet been reported by the regulatory agencies (FDA, 2006), so a biosensor capable to detect trace amounts is of utmost importance.

The characteristics of the present electrochemical sensor and previously reported methods for TPM analysis are presented in Table 2. The main advantages of our sensor, when compared with the ELISA methods, are its lower cost, portability, and wider (linear) concentration range. When compared with the electrochemical immunoassays, its much simpler construction (avoiding exhaustive transducer surface modification) and stability can be highlighted. Besides this, the sensor was applied to a much wider variety of samples (except for one study), using a fast and simple sample preparation method, demonstrating its applicability. On the other hand, the general drawbacks of the developed sensor are its LOD, analysis time (2 h50 min), and single-use format.

Nevertheless, the advantages of the sensing phase preparation (cheap, non-modified transducer), its small size (low sample volumes (40 μL)), that allows portability and *in-situ* analysis, as well as the calibration ranges, efficiently fulfil the purpose required for food safety and control.

4. Conclusion

In this work, a simple immunosensor was developed for tropomyosin analysis, using an SPCE as transducer. The biosensor is able to determine TPM in 2h50 min, with good precision and accuracy, and a low limit of

Table 2
Summary of the characteristics of immunoassays for tropomyosin analysis.

Methodology	Assay		Detection	Analytical features				Food Sample		Ref
Platform	Format	Time	Technique, Label	CV (%)	Stability	Conc. Range	LOD	Type	Recovery	
Immunosensor (Bare SPCE)	Sandwich	2 h50 min	LSV, 4.0×10^{-4} M silver	< 5%	30 days	2.5–20 ng mL^{-1} & 30–200 ng mL^{-1}	0.47 ng mL^{-1}	Shrimp, shrimp sauce, Asian gourmet chips, prawn crackers, crab paste, mussel, shrimp noodles, seafood sticks, meat, anchovies, chicken paste, hake	90–105 %	This work
Immunosensor (AuNPs/Cys/GE)	Label-free	30 min	EIS, 2.5 mM [$Fe(CN)_6]^{3-/4-}$	< 5%	–	0.5–0.25 μg mL^{-1}	0.15 μg mL^{-1}	Fresh brown shrimp	–	(Jiang et al., 2013)
Immunoassay (MBs/SPCE)	Sandwich	≥3h	Amperometry, 1 mM HQ	–	–	0.05–218.7 ng mL^{-1}	47 pg mL^{-1}	Shrimp, pork, chicken, beef, crab sticks	–	(Angulo-Ibáñez et al., 2019
Immunosensor (SPCM/AuMRs/PdNPs/PANI)	Label-free	20 min	DPV, 5 mM [$Fe(CN)_6]^{3-/4-}$	< 4%	6 days	0.01–100 pg mL^{-1}	0.01 pg mL^{-1}	Shrimp-free cream cracker	87–117 %	(Mohamad et al., 2020)
ELISA (Microtitre plate)	Sandwich	<2h	Spectrometry (vis) TMB	–	–	0.3–15 ng mL^{-1}	0.75 ng mL^{-1}	Soy sauce, tuna and sweet corn spread, pilau rice, vegetable balti, quiche, thai crackers, ocean pie, lemon and dill sauce	41–143 % (cooked) 74–140 % (raw)	(Fuller et al., 2006)
ELISA (Microtitre plate)	Sandwich	>3h	Spectrometry (vis) HRP	< 9%	–	0.125–2.0 ng mL^{-1}	0.030 ng mL^{-1}	Shrimp	–	(Lin et al., 2018)
ELISA (Microtitre plate)	Sandwich	>2h	Spectrometry (vis) TMB	< 6.1%	–	0.6–50 ng mL^{-1}	0.004 mg kg^{-1}	Sausage, cookies, fish sauce	68–87 %	(Zhao et al., 2022)

AuMRs – Gold-microrods; AuNPS – gold nanoparticles; CV – coefficient of variation; Cys-L-cysteine; DPV – differential pulse voltammetry; EIS – electrochemical impedance spectroscopy; ELISA, enzyme-linked immunosorbent assay; GE – gold electrode; LOD – Limit of detection; PANI – polyaniline; PdNPs – Palladium nanoparticles; SPCE – screen-printed carbon electrode; SPCM- screen-printed carbon micro-electrode; TPM – tropomyosin.

detection (0.47 ng mL^{-1}). Moreover, the immunosensor is highly selective to the target analyte and is stable for at least 30 days. The easy-to-use sample preparation method (~50 min) combined with the sensor's performance and the equipment's portability enables accurate and decentralized analysis, providing a reliable quantification of TPM.

CRediT authorship contribution statement

Ricarda Torre: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Maria Freitas:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Estefanía Costa-Rama:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Henri P.A. Nouws:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Cristina Delerue-Matos:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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References

Abrams, E. M., & Sicherer, S. H. (2016). Diagnosis and management of food allergy. *Canadian Medical Association Journal, 188*(15), 1087. https://doi.org/10.1503/cmaj.160124

Alves, R. C., Pimentel, F. B., Nouws, H. P. A., Marques, R. C. B., González-García, M. B., Oliveira, M. B. P. P., & Delerue-Matos, C. (2015). Detection of Ara h 1 (a major peanut allergen) in food using an electrochemical gold nanoparticle-coated screen-printed immunosensor. *Biosensors and Bioelectronics, 64*, 19–24. https://doi.org/10.1016/j.bios.2014.08.026

Angulo-Ibáñez, A., Eletxigerra, U., Lasheras, X., Campuzano, S., & Merino, S. (2019). Electrochemical tropomyosin allergen immunosensor for complex food matrix analysis. *Analytica Chimica Acta, 1079*, 94–102. https://doi.org/10.1016/j.aca.2019.06.030

Cheng, J.-H., Wang, H., & Sun, D.-W. (2022). An overview of tropomyosin as an important seafood allergen: Structure, cross-reactivity, epitopes, allergenicity, and processing modifications. *Comprehensive Reviews in Food Science and Food Safety, 21*(1), 127–147. https://doi.org/10.1111/1541-4337.12889

De Marchi, L., Mainente, F., Leonardi, M., Scheurer, S., Wangorsch, A., Mahler, V., … Zoccatelli, G. (2021). Allergenicity assessment of the edible cricket Acheta domesticus in terms of thermal and gastrointestinal processing and IgE cross-reactivity with shrimp. *Food Chemistry, 359*, Article 129878. https://doi.org/10.1016/j.foodchem.2021.129878

EC Regulation (EU) No 1169/2011. (2011). *on the Provision of Food Information to Consumers*. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32011R1169.

Fao. (2020). The State of World Fisheries and Aquaculture 2020. *FAO*. https://doi.org/10.4060/ca9229en

FDA. (2004). *Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA)*. FDA Silver Spring, MD, USA.

FDA. (2006). *Approaches to Establish Thresholds for Major Food Allergens and for Gluten in Food*. https://www.fda.gov/food/food-labeling-nutrition/approaches-establish-thresholds-major-food-allergens-and-gluten-food.

Fernandes, T. J. R., Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2015). An overview on fish and shellfish allergens and current methods of detection. *Food and Agricultural Immunology, 26*(6), 848–869. https://doi.org/10.1080/09540105.2015.1039497

Freitas, M., Neves, M. M. P. S., Nouws, H. P. A., & Delerue-Matos, C. (2021). Electrochemical Immunosensor for the Simultaneous Determination of Two Main Peanut Allergenic Proteins (Ara h 1 and Ara h 6) in Food Matrices. *Foods, 10*(8). https://doi.org/10.3390/foods10081718

Freitas, M., Nouws, H. P. A., & Delerue-Matos, C. (2019). Electrochemical Sensing Platforms for HER2-ECD Breast Cancer Biomarker Detection. *Electroanalysis, 31*(1), 121–128. https://doi.org/10.1002/elan.201800537

Freitas, M., Nouws, H. P. A., & Delerue-Matos, C. (2021). Voltammetric Immunosensor to Track a Major Peanut Allergen (Ara h 1) in Food Products Employing Quantum Dot Labels. *Biosensors, 11*(11). https://doi.org/10.3390/bios11110426

Fuller, H. R., Goodwin, P. R., & Morris, G. E. (2006). An enzyme-linked immunosorbent assay (ELISA) for the major crustacean allergen, tropomyosin, in food. *Food and Agricultural Immunology, 17*(1), 43–52. https://doi.org/10.1080/09540100600572651

Hosu, O., Selvolini, G., & Marrazza, G. (2018). Recent advances of immunosensors for detecting food allergens. *Current Opinion in Electrochemistry, 10*, 149–156. https://doi.org/10.1016/j.coelec.2018.05.022

Indoor Biotechnologies. (2012). *Multiplex Array for Food Allergens Kit 96 Well Plate Assay Magnetic MARIA ® for Foods*. https://inbio.com/maria-kits/magnetic-maria-food-allergens/mra-mf1-magnetic-food-maria-kit.

Jiang, D., Ji, J., an, lu, Sun, X., Zhang, Y., Zhang, G., & Tang, L. (2013). Mast cell-based electrochemical biosensor for quantification of the major shrimp allergen Pen a 1 (tropomyosin). *Biosensors and Bioelectronics*, *50*, 150–156. 10.1016/j.bios.2013.06.032.

Kamath, S. D., Abdel Rahman, A. M., Komoda, T., & Lopata, A. L. (2013). Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chemistry, 141*(4), 4031–4039. https://doi.org/10.1016/j.foodchem.2013.06.105

Khedri, M., Ramezani, M., Rafatpanah, H., & Abnous, K. (2018). Detection of food-born allergens with aptamer-based biosensors. *TrAC Trends in Analytical Chemistry, 103*, 126–136. https://doi.org/10.1016/j.trac.2018.04.001

Kim, M.-J., Kim, H.-I., Kim, J.-H., Suh, S.-M., & Kim, H.-Y. (2019). Rapid on-site detection of shrimp allergen tropomyosin using a novel ultrafast PCR system. *Food Science and Biotechnology, 28*(2), 591–597. https://doi.org/10.1007/s10068-018-0479-x

Koizumi, D., Shirota, K., Akita, R., Oda, H., & Akiyama, H. (2014). Development and validation of a lateral flow assay for the detection of crustacean protein in processed foods. *Food Chemistry, 150*, 348–352. https://doi.org/10.1016/j.foodchem.2013.10.130

Kumar, V., Sinha, A. K., Uka, A., Antonacci, A., Scognamiglio, V., Mazzaracchio, V., … Arduini, F. (2020). Multi-potential biomarkers for seafood quality assessment: Global wide implication for human health monitoring. *TrAC Trends in Analytical Chemistry, 132*, Article 116056. https://doi.org/10.1016/j.trac.2020.116056

Liang, Z.-Y., Deng, Y.-Q., & Tao, Z.-Z. (2020). A quantum dot-based lateral flow immunoassay for the rapid, quantitative, and sensitive detection of specific IgE for mite allergens in sera from patients with allergic rhinitis. *Analytical and Bioanalytical Chemistry, 412*(8), 1785–1794. https://doi.org/10.1007/s00216-020-02422-0

Lin, S.-Y., Lee, C.-H., Huang, E. S., Sheu, S.-C., & Yu, H.-S. (2018). Quantification of Crustacean Tropomyosin, a Major Food Allergen, in Eight Species of Taiwanese Shrimp Based on Immunoassay. *Food Analytical Methods, 11*(9), 2607–2613. https://doi.org/10.1007/s12161-018-1242-x

Melissa Shahbandeh. (2020). *Average annual per capita consumption of seafood worldwide from 2014 to 2019*. Statista. https://www.statista.com/statistics/820953/per-capita-consumption-of-seafood-worldwide.

Mohamad, A., Rizwan, M., Keasberry, N. A., Nguyen, A. S., Lam, T. D., & Ahmed, M. U. (2020). Gold-microrods/Pd-nanoparticles/polyaniline-nanocomposite-interface as a peroxidase-mimic for sensitive detection of tropomyosin. *Biosensors and Bioelectronics, 155*, Article 112108. https://doi.org/10.1016/j.bios.2020.112108

Patel, N., Adelman, D. C., Anagnostou, K., Baumert, J. L., Blom, W. M., Campbell, D. E., … Turner, P. J. (2021). Using data from food challenges to inform management of consumers with food allergy: A systematic review with individual participant data meta-analysis. *Journal of Allergy and Clinical Immunology, 147*(6), 2249–2262.e7. https://doi.org/10.1016/j.jaci.2021.01.025

Ross, G. M. S., Bremer, M. G. E. G., & Nielen, M. W. F. (2018). Consumer-friendly food allergen detection: Moving towards smartphone-based immunoassays. *Analytical and Bioanalytical Chemistry, 410*(22), 5353–5371. https://doi.org/10.1007/s00216-018-0989-7

Sena-Torralba, A., Pallás-Tamarit, Y., Morais, S., & Maquieira, Á. (2020). Recent advances and challenges in food-borne allergen detection. *TrAC Trends in Analytical Chemistry, 132*, Article 116050. https://doi.org/10.1016/j.trac.2020.116050

Sicherer, S. H., & Sampson, H. A. (2018). Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *Journal of Allergy and Clinical Immunology, 141*(1), 41–58. https://doi.org/10.1016/j.jaci.2017.11.003

Smart, A., Crew, A., Pemberton, R., Hughes, G., Doran, O., & Hart, J. P. (2020). Screen-printed carbon based biosensors and their applications in agri-food safety. *TrAC Trends in Analytical Chemistry, 127*. https://doi.org/10.1016/j.trac.2020.115898

Suh, S.-M., Kim, M.-J., Kim, H.-I., Kim, H.-J., & Kim, H.-Y. (2020). A multiplex PCR assay combined with capillary electrophoresis for the simultaneous detection of tropomyosin allergens from oyster, mussel, abalone, and clam mollusk species. *Food Chemistry, 317*, Article 126451. https://doi.org/10.1016/j.foodchem.2020.126451

Torre, R., Costa-Rama, E., Nouws, H. P. A., & Delerue-Matos, C. (2020). Screen-Printed Electrode-Based Sensors for Food Spoilage Control: Bacteria and Biogenic Amines Detection. *Biosensors, 10*(10). https://doi.org/10.3390/bios10100139

van Ree, R. (2021). Grand Challenges in Food Allergy. *Frontiers in Allergy, 2*. https://doi.org/10.3389/falgy.2021.668479

Wammes, A. E. M., van Delft, F. L., van Hest, J. C. M., & Löwik, D. W. P. M. (2015). Surface plasmon resonance (SPR) sensors for detecting allergens in food. In S. Flanagan (Ed.), *Handbook of Food Allergen Detection and Control, 12* pp. 229–244). Woodhead Publishing. https://doi.org/10.1533/9781782420217.2.229.

Zhang, H., Lu, Y., Ushio, H., & Shiomi, K. (2014). Development of sandwich ELISA for detection and quantification of invertebrate major allergen tropomyosin by a monoclonal antibody. *Food Chemistry, 150*, 151–157. https://doi.org/10.1016/j.foodchem.2013.10.154

Zhao, J., Li, Y., Li, R., Timira, V., Dasanayaka, B. P., Zhang, Z., … Li, Z. (2022). Evaluation of poly- and monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (ELISA) for their performance to detect crustacean residues in processed foods. *Food Control, 138*, Article 108983. https://doi.org/10.1016/j.foodcont.2022.108983