



Electrochemical tropomyosin allergen immunosensor for complex food matrix analysis

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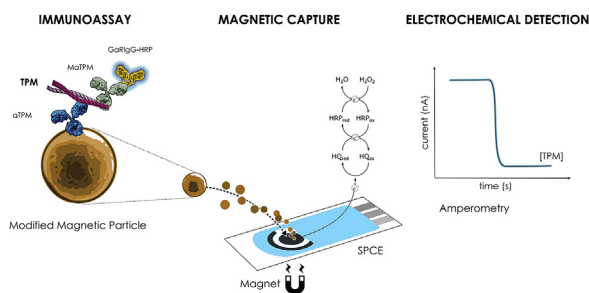
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HIGHLIGHTS

- An amperometric immunosensor has been developed for the accurate detection of shrimp tropomyosin.
- A limit of detection of 47 pg mL^{-1} has been obtained in under 3 h.
- Crustacean and mollusk tropomyosin has been clearly differentiated, a relevant issue for the food processing industry.
- The applicability of the sensor for the determination in both raw and cooked foods has been assessed.

GRAPHICAL ABSTRACT



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ABSTRACT

A shrimp tropomyosin (TPM) immunosensor has been developed and optimized to detect trace amounts of shrimp (in the ppm range), based on a combination of an amperometric transduction, magnetic particles and disposable screen-printed electrodes. The approach is based on the implementation of a sandwich immunoassay format on the surface of magnetic beads and their coupling onto disposable screen-printed electrodes to finally register the amperometric response at -200 mV vs. Ag pseudo-reference electrode, using H_2O_2 as enzymatic substrate and hydroquinone as redox mediator. The use of carboxyl-functionalized magnetic microbeads (MBs) and in-house made magnetic nanoparticles (MNPs) as solid supports have been evaluated and compared. Our experimental results confirm that the use of MBs, in addition to simplifying the test protocol, improves the resulting sensitivity, so they were selected for the implementation of the immunosensor. In the optimized experimental conditions, the developed immunosensor offered a LOD of 47 pg mL^{-1} for amperometric determination of shrimp TPM standards and great selectivity against TPM from other sources, thus allowing differentiation between crustaceans (shrimp) and mollusks (squid). Applicability studies demonstrated successful determination both in crude and cooked samples using very simple protocols. Additionally, processed foods based on fish and mollusks that could potentially include crustaceans in their composition have been analyzed using the sensor and compared to the declared ingredients.

The sensitivity and specificity showed by the sensor in the analysis of heterogeneous food samples without a previous purification or enrichment stage, also outperforms existing solutions in terms of time and cost effectiveness and permits its direct and smooth implementation in the food industry for routine allergen analyses.

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1. Introduction

Food allergies constitute an issue of increasing concern to public health [1], affecting around 5% of adults and 8% of children [2]. Although important age and geographic variations exist, incidence of allergic reactions to shellfish is high and it is catalogued as one of the 8 key food allergens, which account for over 90% of all food allergies [3–5]. It is also one of the most dangerous food allergens, with severe manifestations and a major cause of anaphylaxis and the leading cause of emergency visits to hospital. Additionally, in contrast to other allergens, allergic reactions to shellfish do not tend to diminish with age and avoidance is still the principal recommended measure to manage it.

Shellfish can be separated into two families: mollusks (that include scallops, oysters, clams, mussels...) and crustaceans (shrimp, lobster, crab...). These two groups are taxonomically unrelated, which is translated into a low cross-reactivity between them. Circumscribing to crustacean allergies, tropomyosin (TPM) has been identified as the major shellfish allergen. TPM is a muscle protein that, together with the troponin complex, plays a very important role in the activation process that leads to myofilament sliding and force generation [6]. This highly conserved protein, cross-reactive among many arthropods, is also the main shrimp allergen.

Food allergens are proteins that usually trigger Immunoglobulin E (IgE) -mediated immune reactions in sensitive individuals. IgEs are found in the tissue of the gastrointestinal tract, bound to the surface of the mast cells, basophils or eosinophils and hypersensitive subjects develop them as a result of the allergens penetrating through skin, gut or respiratory lining [3]. These IgEs bind to the active sites (epitopes) of the allergen, triggering the allergic immune response through the release of different mediators (histamine, leukotrienes and prostaglandins) from the mast cell, inducing various inflammatory alterations and causing the common hypersensitivity symptoms, that could eventually lead to anaphylaxis [7]. These manifestations usually appear within the first minutes after the exposure and last several hours. Although thresholds to initiate the allergic response vary, usually very small amounts of substance are sufficient to trigger the reaction in hypersensitive individuals.

At present, there is no cure for food allergies, merely symptomatic treatments through anti-histamines and corticosteroids complemented with epinephrine to reverse the symptoms of anaphylaxis at the most. Accordingly, avoidance of the allergenic foods is still the main measure for hypersensitive individuals. Therefore, an accurate and truthful control of the ingredients of processed foods, together with an exact food labelling is highly advocated. Moreover, there is still room to improve the understanding and management of food allergies through advances in the diagnosis, treatment and prevention strategies.

In this sense, great efforts are being made from the industry and regulatory organisms to offer more accurate and reliable information to the consumers. These initiatives, that try to improve manufacturing and labelling practices, require the support of new sensitive and reliable analytical tools, to lower the detection limits towards relevant values and substitute inconvenient techniques that hinder their implementation. At present, lateral flow and dipstick tests are the main methods for allergen testing, either in ingredients or finished products, as well as to check the cleanliness of different surfaces and avoid any cross-contamination between batches. These immunochemical methods allow the direct determination of the allergen, but are usually qualitative (semi-quantitative with the use of a reader) and offer modest detection limits. As an alternative, Enzyme Linked Immunosorbent Assays (ELISAs) allow quantitative determinations with an improved sensitivity,

but require relatively expensive equipment and trained personnel. Additionally, the influence of the food matrix is very significant, and the technique is highly dependent on the sampling and protein extraction protocol. On the other hand, DNA-based tests, such as Polymerase Chain Reaction (PCR), are becoming more ubiquitous as the required instrumentation is becoming more common and affordable. However, these tests target specific DNA sequences indicative of allergenic species, not the allergen itself; therefore, the absence of DNA does not univocally imply the absence of the protein. Although highly specific and high-throughput, the allergen detection is indirect and qualitative, the analysis and sample preparation require specialized personnel and due to the amplification process, the risk of cross-contamination is high. Alternatively, mass spectroscopy allows the detection of specific amino acid sequences of the protein, unequivocally identifying the allergen, but the high cost of the equipment and the materials, cause this labor-intensive technique to be unsuitable for routine analyses.

Focusing on the determination of the target allergen, fluorescent and ELISA assays, immunochromatographic strips and biosensors have been proposed. An attractive label-free aptamer-based fluorescent assay has been developed using functionalized magnetic nanoparticles (MNPs) as separation carrier and commercially available OliGreen dye as signal probe [8]. With the purpose of studying the interspecies TPM similarity, Jeoung et al. [9] analyzed brown shrimp TPM cross-reactivity and incorporated the generated specific antibodies in an ELISA test. Fuller et al. [10] also employed a sandwich-ELISA scheme, obtaining very low LOD (1 ng mL^{-1}) in under 2 h, but without getting around the fundamental shortcomings of the technique. Interestingly, Kamath et al. [11] generated and purified highly specific anti-TPM antibodies and integrated them in an ELISA assay targeting aerosolized TPM, with significant sensitivity and selectivity values. Other sensitive ELISA assay (LOD of 0.030 ng mL^{-1}) with feasibility to perform the determination in processed shrimps was proposed by Lin et al. [12]. Zeng et al. developed also a sandwich ELISA and a immunochromatographic strip for the determination of the target allergen [13]. Several biosensors have also been described for shrimp TPM detection based on different approaches and types of detection.

Tabrizi et al. [14] proposed a visible-light-driven photoelectrochemical (PEC) aptasensor with attractive detection limits, but involving a cumbersome surface preparation. Using a different approach, Xiulan et al. [15] employed a quartz crystal microbalance-based system to build a label-free immunosensor that, despite its rapid analysis time, relies on a technology not suitable for on-site testing. Wang et al. [16] developed a biosensor based on high spatial resolution imaging ellipsometry for the rapid detection of TPM. This method allows a fast and a label-free analysis that shows the binding kinetics of different ligands, but at the same time requires a setup and equipment not quite appropriate for routine analyses. An AuNPs trimer-based biosensor using circular dichroism (CD) detection with a LOD of 21 pg mL^{-1} and applied to identify adulteration in shellfish products has been recently proposed by Wang et al. [17]. From a different perspective, Jiang et al. [18] developed a novel cell-based impedimetric sensor to quantify shrimp TPM, obtaining a reasonable LOD ($0.15 \text{ } \mu\text{g mL}^{-1}$) but through a technique not apt for its use in a real industrial environment. Conversely, the electrochemical immunosensor developed in this work combines excellent analytical properties with simplicity and cost-effectiveness, especially suitable for point-of-care and routine applications where a portable and robust sensing method is required.

In short, we present the first electrochemical immunosensor reported so far for shrimp TPM determination, based on a sandwich immunoassay, involving a horseradish peroxidase (HPR)-labeled

secondary antibody, constructed onto magnetic particles and coupled to an electrochemical transduction system, requiring inexpensive and portable instrumentation that perfectly suits the needs of the food industry. The use of magnetic particles yields an improved efficiency in the capture of the TPM in an unpurified and unfiltered medium, minimizing possible matrix effects that could lead to false results. At the same time, the sample preparation process is simplified, allowing the use of the sensor directly on the field [19,20]. The final amperometric detection to monitor the affinity reaction and determine the TPM concentration is carried out using the system $\text{H}_2\text{O}_2/\text{HQ}$ [21] at screen-printed disposable electrodes, a very cost-effective platform that prevents cross-contaminations. The applicability of the immunosensor was exhaustively evaluated by analyzing different food samples which include pork, beef, chicken, shrimp and squid, these last two both cooked and uncooked.

2. Experimental

2.1. Apparatus

All the electrochemical measurements were performed using a DRP-STAT400 potentiostat from Metrohm-DropSens. The DRP-C110 Screen Printed Carbon Electrodes (SPCEs), consisting of a circular carbon Working Electrode (4-mm diameter, WE), a carbon Counter Electrode (CE) and a silver pseudo-Reference Electrode (RE) were also acquired from Metrohm-DropSens. The specific cable connector (DRP-CAC), used as interface between the SPCEs and the potentiostat, were also from Metrohm-DropSens. A DynaMag-2 magnet for microcentrifuge tubes from Invitrogen and a MixMate vortex mixer from Eppendorf were employed for the magnetic separation and incubation of the magnetic particles. A homemade poly(methyl methacrylate) (PMMA) dock with an encapsulated neodymium permanent magnet from AimanGZ was employed to capture the magnetic particles onto the WE surface.

2.2. Reagents and solutions

Carboxylic acid-modified magnetic microbeads (HOOC-MBs) from Invitrogen (Dynabeads M-270 Carboxylic Acid, $2.8\ \mu\text{m}$, $30\ \text{mg mL}^{-1}$) were used as received. Shrimp TPM standard, polyclonal Rabbit anti Shrimp TPM antibody (RaTPM) and monoclonal Mouse Immunoglobulin G antibody (aTPM) were purchased as an ELISA Kit (ref. EL-TPM, Indoor Biotechnologies Inc). Horseradish peroxidase (HRP) conjugated Goat anti-Rabbit Immunoglobulin G (GaRlgG-HRP) and Bovine Serum Albumin (BSA), were from Jackson ImmunoResearch (ref. 111-036-046 and 001-000-161, respectively).

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine hydrochloride (ETA), hydroquinone (HQ), hydrogen peroxide 30% (w/v) in H_2O (H_2O_2) and Tween 20 were from Sigma-Aldrich. Sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, 2-(N-morpholino) ethanesulfonic acid (MES) and sodium chloride (NaCl) were purchased from Scharlau. The rest of the used chemicals were of analytical grade and ultrapure water (Millipore Milli-Q) was used throughout.

The following buffers and solutions were prepared daily: 10 mM phosphate buffered saline at pH 7.5 (PBS), 50 mM MES buffer at pH 5.0, 50 mM phosphate buffer at pH 6.0 (PB), PB containing 1 mM HQ, PBS containing 0.05% (w/v) Tween 20 (PBST), PBST containing 1% BSA and 100 mM H_2O_2 .

2.3. Procedures

2.3.1. Biofunctionalization of the magnetic particles and sandwich immunoassay

Both sets of magnetic particles, Dynabeads M270 MBs and custom-made MNPs, were biofunctionalized in the same manner, according to the protocol described below. All incubation steps were carried out under continuous stirring at 950 rpm and $25\ ^\circ\text{C}$ and in volumes of $37.5\ \mu\text{L}$; washing operations were performed similarly but using $75\ \mu\text{L}$ of the wash buffer. To transition between the different steps of the procedure, the microtube containing the magnetic particle suspension was placed on the magnetic separator for 2 min before removing the supernatant.

$180\ \mu\text{g}$ (dry weight) of magnetic particles were pipetted into a 1.5 mL microtube and washed twice with MES buffer. The carboxyl groups on the surface of the particles were activated in a freshly prepared mixture of 100 mM EDC and 100 mM NHS in MES for 30 min. Two washing steps in MES buffer were carried out before resuspending the particles in a $2\ \mu\text{g mL}^{-1}$ aTPM solution prepared in MES for 1 h. After two washing steps in MES, the magnetic particles were resuspended in a 1 M ETA solution for 1 h to block the remaining unreacted groups on the particle surface. For the allergen capture, after two washing steps in PBST buffer, the anti-TPM biofunctionalized particles were resuspended in the different sample or standard solutions (prepared in PSBT + 1% BSA) and left to incubate for 1 h. To complete the sandwich immunoassay, the particles with the captured allergen were washed twice in PBST, and then incubated in a RaTPM solution prepared in PBST + 1% BSA for 1 h. After two washing steps in PBST, the magnetic particles were resuspended in a GaRlgG-HRP solution (prepared in PBST containing 1% BSA) and incubated for 1 h. Finally, the modified particles were washed twice in PBST and once in PB before resuspending them in $150\ \mu\text{L}$ of PB, leaving them ready for the amperometric measurement. The final volume of the modified particle suspension ($150\ \mu\text{L}$) was used to perform three replicates, so just $60\ \mu\text{g}$ of the initial magnetic particle stock was actually employed for each determination.

2.3.2. Amperometric detection

The amperometric measurements were performed by magnetically trapping the modified particles onto the WE of the SPCE which was previously fixed to the PMMA dock. A $50\ \mu\text{L}$ drop of the final magnetic particle suspension was gently pipetted over the WE, capturing the magnetic particles in a stable and reproducible way onto the SPCE by the neodymium magnet exactly placed under the WE.

The ensemble PMMA block/SPCE with the captured magnetic particles was connected through the cable to the potentiostat and introduced into a gently stirred 8 mL PB and HQ solution. The amperometric signal was monitored by applying a $-0.20\ \text{V}$ potential vs. Ag pseudo-reference electrode. After a 60 s stabilization period to obtain a steady baseline, $40\ \mu\text{L}$ of a 100 mM H_2O_2 solution were added, triggering a variation in cathodic current due to the HRP reduction of H_2O_2 mediated by HQ (see scheme reaction and a model amperometric response in Fig. 1), readily reaching a steady state. At least three replicates were measured for each standard/sample.

2.3.3. Application to the analysis of foods

The MBs/MNPs-based immunosensor was employed for the determination of TPM in raw and processed foods. For the protein extraction, the following procedure was used: 1 g of product and 4 mL of a specific protein extraction buffer (2 M NaCl, 50 mM Tris-HCl, pH 8.4) were mixed in a plastic bag to prevent cross-contaminations and the mixture was gently homogenized in a

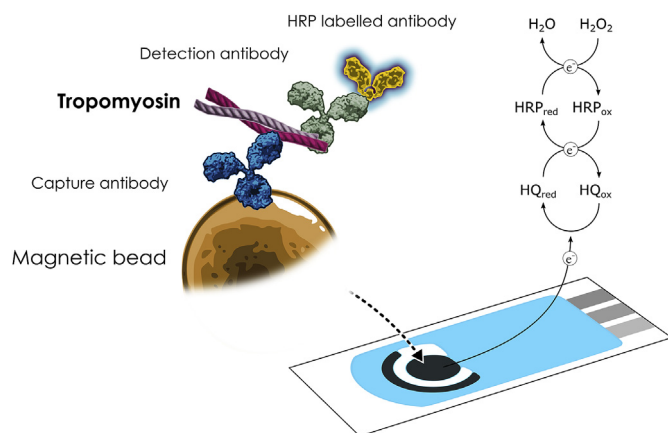


Fig. 1. Scheme of the sandwich immunosensing platform developed for shrimp TPM determination and the reactions involved in the amperometric detection.

mortar by hand. This blend was let sit for 30 min to extract the proteins from the matrix. In the case of the cooked foods, the raw samples were introduced in water at 95 °C for 30 min before the protein extraction procedure. In both cases, the homogenized solution was centrifuged for 4 min at 13,000 rpm and the supernatant recovered. Samples were finally diluted by a $1/10^6$ factor in PBST and used as described in Section 2.3.2.

3. Results and discussion

The basic configuration of the proposed immunosensing platform is described in Fig. 1. All the steps involved in the specific capture and labelling of the TPM are performed onto MBs (or MNPs), through a sandwich format, as detailed in Section 2.3.2.: HOOC-modified magnetic particles are activated through EDC/NHS chemistry to covalently bind RaTPM capture antibodies; the unbound active sites are blocked using ETA, to avoid any succeeding non-specific binding events; these functionalized MBs (or MNPs) are then used to specifically catch shrimp TPM amidst all the constituents of the food extracts or standard solutions; finally, bound TPM is sandwiched through a detector aTPM antibody and a secondary labeled antibody (GaRlgG-HRP).

The MBs (or MNPs) with the fully formed sandwich immunocomplexes are brought to the surface of the WE of the sensing electrodes to carry out the final amperometric detection upon addition of the H₂O₂ enzymatic substrate.

3.1. MNP synthesis

In order to study the influence of the magnetic particle size and surface chemistry in the sensitivity of the determination, custom made MNPs were manufactured. For this purpose, the best method for magnetite nanoparticle synthesis was sought, that offers both a high carboxylic group density and the appropriate stability in a water solution. A metallic salt co-precipitation synthesis protocol further coupled with a polyacrylic acid (PAA) functionalization was selected to obtain the required carboxylic groups for the covalent binding of the capture antibodies through amine coupling. This protocol results in a very high colloidal stability of the MNPs so it is necessary to finely tune the optimal PAA concentration to maintain the capability of concentrating the particles via a neodymium magnet.

The fabricated MNP size and distribution was estimated by Dynamic Light Scattering (DLS), resulting in an average 22 ± 4 nm particle size. The full particle size distribution is shown in Fig. 2, and

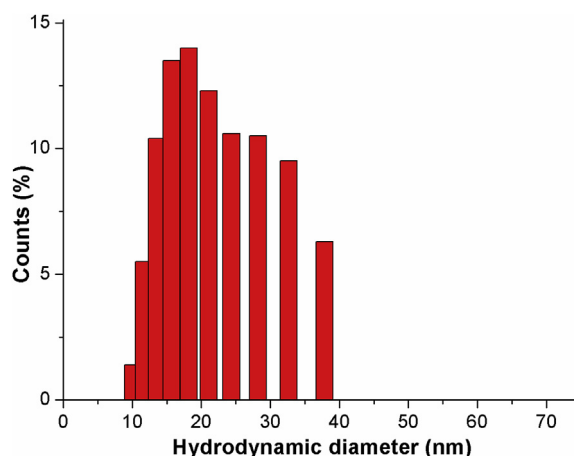


Fig. 2. Calculation of the hydrodynamic diameter of the synthesized MNPs by DLS.

reflects a good particle size homogeneity. The surface electrical charge was characterized by means of Zeta potential measurements, yielding values of -4 ± 4 mV and -35 ± 6 mV for the unmodified and PAA functionalized MNPs, respectively. These values show on the one hand the satisfactory modification of the MNPs with -COOH groups (that produce a more negative charged particle) and on the other that this strategy leads to stable particle dispersion, minimizing aggregations in further functionalization steps. These characterization results were complemented by those achieved by infrared spectroscopy and thermogravimetry. The infrared spectrum (Fig. 3a) clearly shows the characteristic peaks of the carboxylic groups: two O-H groups between 3,000 and 3,500 cm⁻¹, one due to environmental humidity and the other related to the O-H vibration and another double peak between 1,500 and 1,700 cm⁻¹, corresponding to the symmetric and asymmetric coordination of the carboxylic group. On the other hand, the thermogravimetry analysis (Fig. 3b), shows a first dissociation at low temperatures (200–400 °C) corresponding to the PAA chains bound electrostatically and by hydrogen bonds and a second at higher temperatures, related to the coordinated carboxylic groups of the MNP surface. This analysis permits the quantification of the PAA (27% of the total mass), confirming the preparation of highly colloidal stable MNPs with high density of carboxylic groups in their surface.

3.2. Optimization of the experimental variables

The immunosensor is based on a sandwich structure composed of a capture antibody, a detection antibody and an HRP-labeled secondary antibody. The concentration of each one of these elements, together with other experimental variables, were selected for optimal analysis conditions.

All the variables involved in the MBs/MNPs-based immunosensor were optimized by taking as selection criterion the ratio between the current values measured at -0.20 V (vs. Ag pseudo-reference electrode) in the presence (S) and absence (N) of 10 ng mL^{-1} TPM standard, using as starting values the reference concentrations recommended by the commercial ELISA kit. The detection potential value and other trans-assay experimental variables were previously optimized for a similar electrochemical detection system [22,23] and the HOOC-modified magnetic particle activation procedure, the successive washing steps and the unreacted carboxylic group blocking were performed according to the protocol given by the supplier.

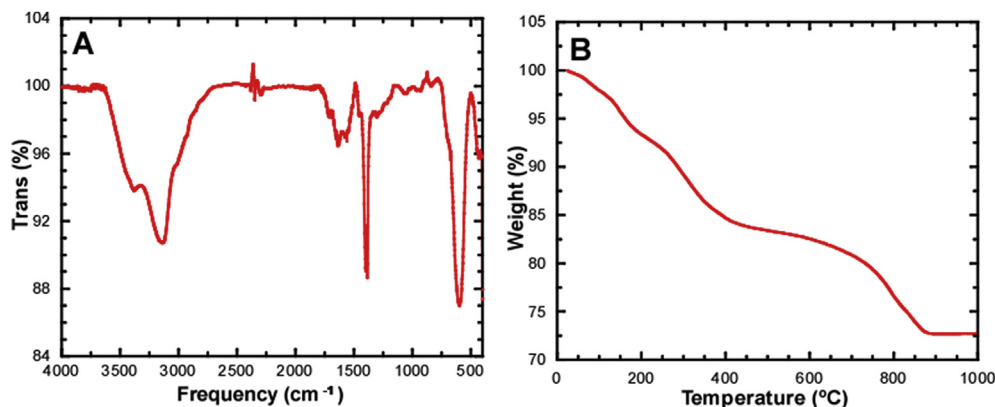


Fig. 3. Infrared spectrum (a) and thermogram (b) of the PAA-functionalized MNPs.

3.2.1. Magnetic particle size selection

Magnetic particles are a widely used tool for enhancing detection sensitivity through an increased active area, reduction of matrix effect and drop of assay time, qualities highly influenced by the properties of the particle, particularly size [24]. With the aim of improving the efficiency of the detection through the study of the influence of the particle size, two different sets of HOOC-modified magnetic particle were tested: standard commercial superparamagnetic polystyrene microbeads with a magnetite core (Dynabeads M270, 2.8 μm in diameter) (MBs) and home-made magnetite MNPs joined with a polyacrylic acid functionalization (MNPs synthesized in-house, 22 nm in diameter). The same amount in weight of both MBs were modified with carboxyl groups in their surface to bind the capture antibodies via amine coupling in the same manner, as described in Section 2.3.1.

Nanometric particles remarkably improve the total effective surface, which translates into a larger capture area. This reflects into larger currents, which are linked to more binding events, but not necessarily into an improvement of the specific vs. unspecific binding ratio. As shown in Fig. 4.a, this increment in the amperometric response is observed both in the presence and absence of TPM. The results shown in Fig. 4a demonstrate higher non-specific adsorptions (in agreement with the higher matrix effects reported using MNPs [25,26]) and a 30% lower S/N ratio using NPMs than MBs. Additionally, the smaller size of the MNPs translates into more stable suspensions, which makes the concentration step more arduous, prolonging the duration and worsening the reproducibility of the assay. Considering these outcomes, commercial micrometric sized magnetic particles (MBs Dynabeads M270) were selected as optimal and used throughout the research. Nevertheless, in other applications where the specificity of the used capture molecules is not the limiting factor, the improved ability of the smaller sized particles to capture a greater quantity of analyte could be a remarkable asset even if it means employing more powerful magnets or longer times for their magnetic isolation.

3.2.2. Immunoreagent concentration optimization

The GaRIgG-HRP concentration was optimized in the 200–1,600 ng mL^{-1} range. Only considering the amperometric response obtained in presence of TPM standard, a linear increase of the signal with the concentration of GaRIgG-HRP is observed, saturating at higher values. In the absence of TPM standard, the amperometric response also increases with the GaRIgG-HRP concentration, indicating a higher amount of non-specific binding events. Nevertheless, as shown in Fig. 4.b, the S/N ratio peaks at 200 ng mL^{-1} and therefore this has been selected as the optimal labelling antibody

concentration, at the equilibrium point between easily detectable amperometric signals and limited nonspecific bindings.

The RaTPM concentration was optimized in the 1:4,000–1:250 dilution range of the stock solution (the concentration is not specified by the supplier). As it can be observed, the amperometric responses obtained in the presence of TPM standard increase with the RaTPM concentration (lower dilution). However, for dilutions lower than 1/2,000, the non-specific responses increase more significantly than the specific ones and the S/N ratio decreases drastically (Fig. 4c). Therefore, a 1/2,000 dilution factor was selected as optimal detection antibody concentration.

Finally, the aTPM concentration was optimized in the 0.25–4.0 $\mu\text{g mL}^{-1}$ range. As displayed in Fig. 4.d, both in the presence and absence of TPM, the amperometric response increases linearly with the capture antibody concentration immobilized. Considering the value that maximizes the S/N ratio, a 2 $\mu\text{g mL}^{-1}$ concentration of capture antibody was selected as optimal. Additionally, the limited amount of used reagents remarkably improves the cost-effectiveness of the test.

Table 1 summarizes the selected values that maximize the analytical performance of the test.

3.3. Analytical characteristics

Under the optimized conditions, the analytical characteristics of the MBs/MNPs-based immunosensor were evaluated by constructing a calibration curve for TPM standard solutions prepared in PBST contacting 1%BSA in the 0–218.7 ng mL^{-1} concentration range (see Fig. 5). The sigmoidal shape of the calibration curve perfectly fits a 1:1 biomolecular binding model and a LOD of 46.9 pg mL^{-1} was calculated using the 3s criteria (expressed in concentration units, where s is the standard deviation of six amperometric determinations in the absence of TPM), 4 times lower than the stated for the ELISA kit employing the same immunoreagents (0.2 ng mL^{-1}).

It is worth to remark also that compared to the performance of other ELISA assays and biosensors reported so far for TPM determination (whose main features are summarized in Table 2), the immunosensor developed is fundamentally competitive in terms of sensitivity (offers one of the best LOD), simplicity of protocol and portability and affordable cost of the required instrumentation. It is important to highlight also the more than 3000 times lower LOD offered by the immunosensor developed in comparison with the only electrochemical biosensor described to date [18] which has also failed to demonstrate its applicability to the determination in real food samples.

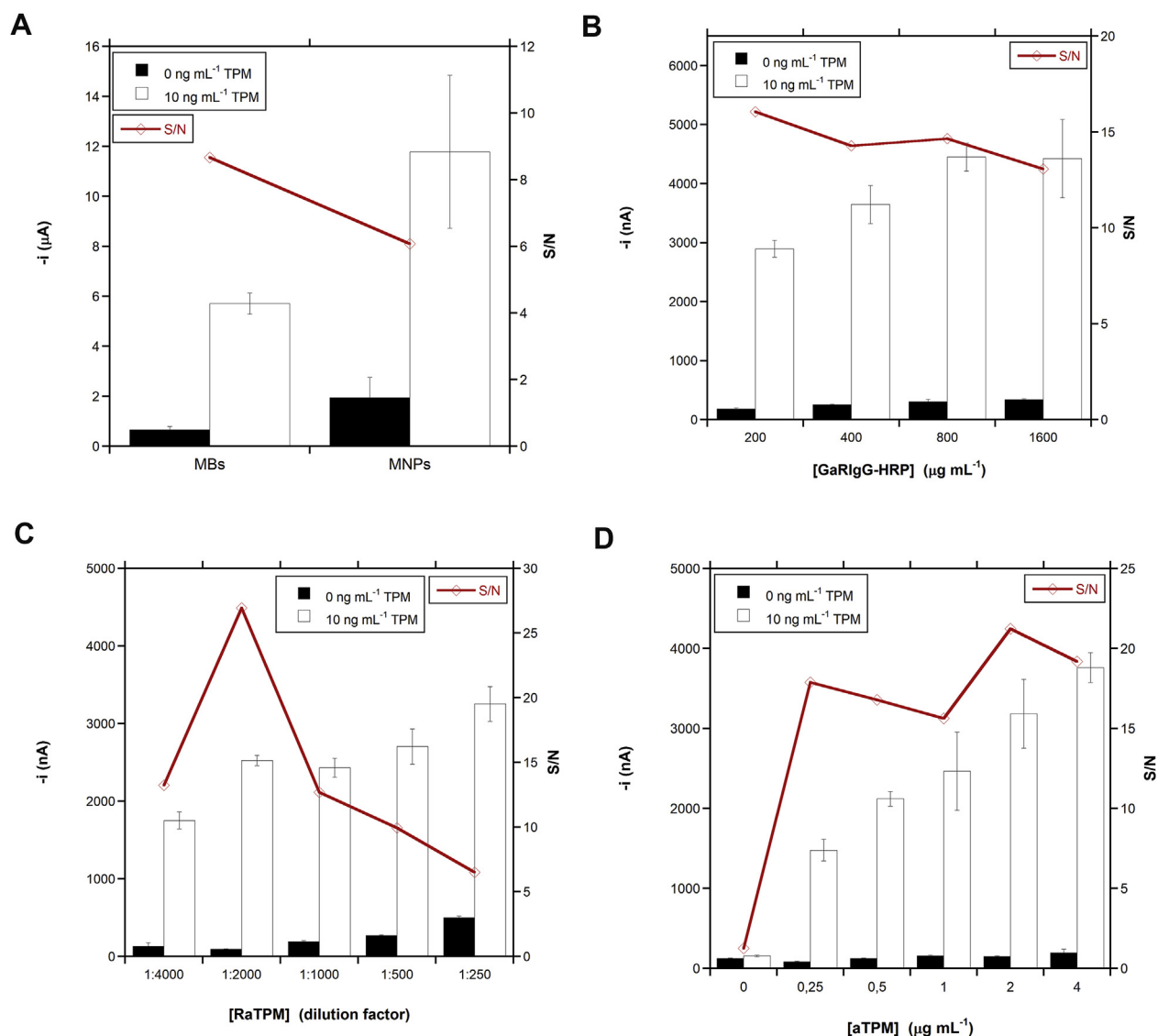


Fig. 4. Amperometric responses provided by the immunosensors in the absence (black bars) or in the presence (white bars) of 10 ng mL⁻¹ TPM standards as a function of: type of magnetic particles (a), HRP-labeled secondary antibody (GaRlgG-HRP) concentration (b), detection antibody (RaTPM) concentration (c) and capture antibody (aTPM) concentration (d). Error bars estimated as a triple of the standard deviation ($n = 3$).

Table 1
Optimization of the immunoreagents concentration involved in the preparation of the MBs/MNPs-based immunosensor for TPM.

Experimental variable	Tested ranges	Selected values
aTPM (capture antibody), μg mL ⁻¹	0.25–4	2
RaTPM (detector antibody), dilution factor	1/4,000–1/250	1/2,000
GaRlgG-HRP (labeled antibody), ng mL ⁻¹	200–1,600	200

3.4. Selectivity of the immunosensor

Eventually, the sensor is intended to be used to discriminate between safe foods and those that could potentially trigger an allergic response in the consumer. To evaluate the selectivity of the MBs/MNPs-based immunosensor, different animal source foods have been studied, together with different factors that could affect the affinity of the test, such as cooking or processing degree.

3.4.1. Identification of TPM from different sources

The TPM is a muscular protein present in all animals; the sensor, nevertheless, is intended to be specific to shrimp TPM. Accordingly, the affinity of the immunosensor against TPM from various sources was evaluated using different food samples: seafood, different meats and one processed food. At the same time, these experiments permit the evaluation of the efficiency of the sensor to analyze real samples, taking into account the complexity of the matrix, which contains a high amount of nonspecific proteins and other elements that could hinder the effective recognition of the analyte.

To carry out these experiments, different food products were selected: shrimp, pork, chicken, beef and crab sticks (see Fig. 6). 1 g of raw food was taken in each case, and the extracts were prepared following the protocol described in Section 2.3.4. As expected, the electrochemical signal obtained with shrimp extracts corresponds to a very high concentration of TPM, being 78% lower in the cases of chicken and beef and 92% for pork. These results demonstrate the lack of cross-reactivity between TPMs of different animal origin, highlighting the specificity of the test just for shrimp TPM.

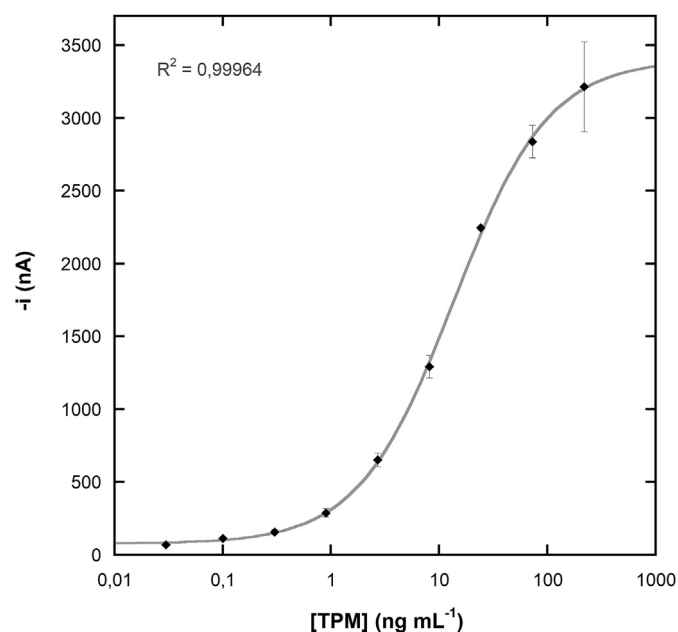


Fig. 5. Calibration curve constructed with the immunosensors for TPM standard solutions prepared in PBST containing 1% BSA. Supporting electrolyte, PB pH 6.0; $E_{app} = -0.20$ V vs. Ag pseudo-reference electrode. Fitting of the experimental results to a sigmoidal curve. Error bars estimated as a triple of the standard deviation ($n = 3$).

When analyzing the presence of TPM in the crab sticks, the obtained electrochemical response was a 93% lower than that obtained with the shrimp extracts. Therefore, despite being sold as a seafood product, the analyzed sticks are given a negative in the test. And, indeed, this specific product is made mainly of white fish (32% white fish, water, starch, egg white, vegetable oil, soy protein, flavorings and flavor enhancers) and does not contain any ingredient of crustacean origin. So, a product that is sold as “crab” stick and may apparently contain crustaceans in its composition is free of any shellfish among its ingredients, as the developed sensor indicates in this case. Therefore, this result confirms that the studied product does not pose any risk to people allergic to shrimp.

3.4.2. Differences between crustaceans and mollusks and effect of cooking

Shellfish can be divided into two groups: crustaceans and mollusks. Many crustacean-allergic people can eat mollusks safely, but in many fish/shellfish processing plants both are processed within the same installations, with the elevated risk of cross-reactivity between batches if the appropriate sanitary measures are not adopted. Additionally, several seafood substitutes and processed products include fish, crustacea and mollusks in their

composition. Therefore, the differentiation of mollusk and crustacean traces in processed foods is of utmost interest. This issue is hindered due to the greater structural similarity between invertebrate TPM compared to vertebrate TPM, increasing the possibility of cross-reactivity.

In order to adequately evaluate the specificity of the test, it is also important to take into account how the potential processing or cooking may affect the conformation of the protein: the structure of the epitopes may be modified, directly affecting the IgE antibody binding (altering the allergenic potential of the food) or the affinity of the antibodies used in the sensor [27].

To assess these issues, the developed sensor was used to determine the shrimp-specific TPM content of both raw and cooked samples of shrimp and California squid. The analyzed extracts were prepared as described in Section 2.3.4. and the measurements were carried out following the methodology detailed in Section 2.3.3. The results (Fig. 7) show the high selectivity of the sensor and its capability to differentiate between crustacean and mollusk TPM, regardless of the processing degree (raw and cooked). As shown in Fig. 7, the measured response of both raw and cooked shrimp is 87% and 92% higher than the squid.

These experiments demonstrate the capability of the immunosensor to distinguish between crustacean and mollusk TPM, regardless of their processing degree, which allows the direct implementation of the developed sensor in a real production environment to ensure maximum consumer satisfaction and protection.

4. Conclusions

Biosensors and specifically electrochemical biosensors represent the best option for an extremely sensitive quantitative sensor, of straightforward use and easily implantable in any production environment at a very low cost. Highly adaptable to the detection of any allergen, the use of functionalized MBs allows the selective determination of the analyte concentration directly in complex food matrixes, without requiring further sample purification steps.

Based on these findings, this work proposed a novel immunosensor involving a sandwich immunoassay onto MBs and amperometric detection at SPCEs. This approach provided a LOD of 47 pg mL⁻¹ for TPM standards determination in just 3 h. Moreover, to attest the usefulness of the sensor in a real environment, the developed sensor has been used to analyze food samples from different origins, to assess the selectivity of the sensor against TPM from other animals. The obtained results show a high affinity of the sensor for shrimp samples, and barely no cross-reactivity with TPM from other origins. Especially relevant features from the applicability studies include the selectivity of the sensing platform against mollusk TPM, the confirmation that crab sticks do not contain shrimp extract among its ingredients and the feasibility to perform

Table 2
Main features ELISA assays and biosensors reported so far for TPM determination.

Methodology	LOD	Assay time	Reference
mAb-based sandwich-ELISA	1,000 pg mL ⁻¹	(overnight + 4 h 45 min) 3 h 45 min	Jeoung et al. [9]
Sandwich-ELISA	1,000 pg mL ⁻¹	2 h	Fuller et al. [10]
Sandwich-ELISA	60 pg mL ⁻¹	(overnight + 4 h 30 min) 4 h 30 min	Kamath et al. [11]
Sandwich-ELISA	30 pg mL ⁻¹	—	Lin et al. [12]
mAb-based sandwich-ELISA	450 pg mL ⁻¹	~16 h	Zeng et al. [13]
Piezoelectric immunosensor	0.333 µg mL ⁻¹	(6 h) 10 min	Xiulan et al. [15]
High sensitive visible light-driven PEC aptasensor	230 pg mL ⁻¹	(5 h 10 min + 7 h 50 min) 80 min	Tabrizi et al. [14]
High spatial resolution imaging ellipsometry biosensor	1 mg mL ⁻¹	30 min (detection)	Wang et al. [16]
AuNPs trimer-based biosensor using CD detection	21 pg mL ⁻¹	≥2 h	Wang et al. [17]
Electrochemical immunosensor	0.15 µg mL ⁻¹	(6 h 45 min) 1 h 5 min	Jiang et al. [28]
Amperometric immunosensor	46.9 pg mL ⁻¹	3 h	This work

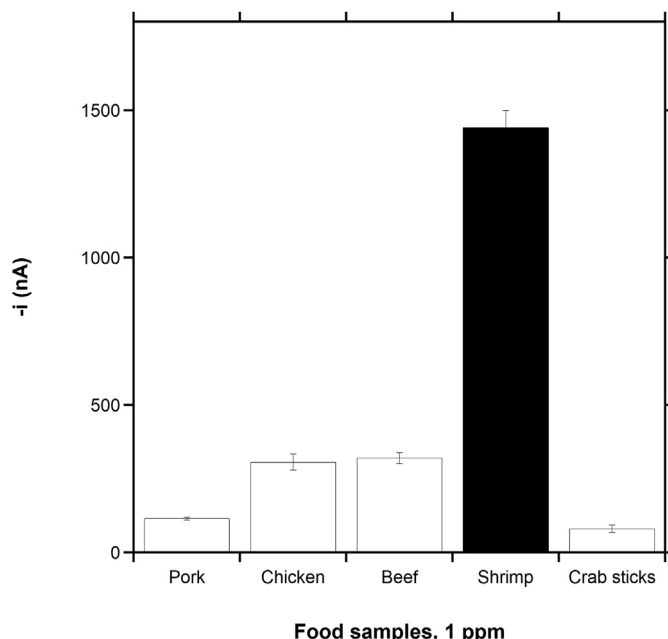


Fig. 6. Amperometric responses provided by the immunosensors in the analysis of different food extracts, diluted to a 1 ppm concentration, prepared in PBST containing 1% BSA. Supporting electrolyte, PB pH 6.0; $E_{app} = -0.20$ V vs. Ag pseudo-reference electrode. Error bars estimated as a triple of the standard deviation ($n = 3$).

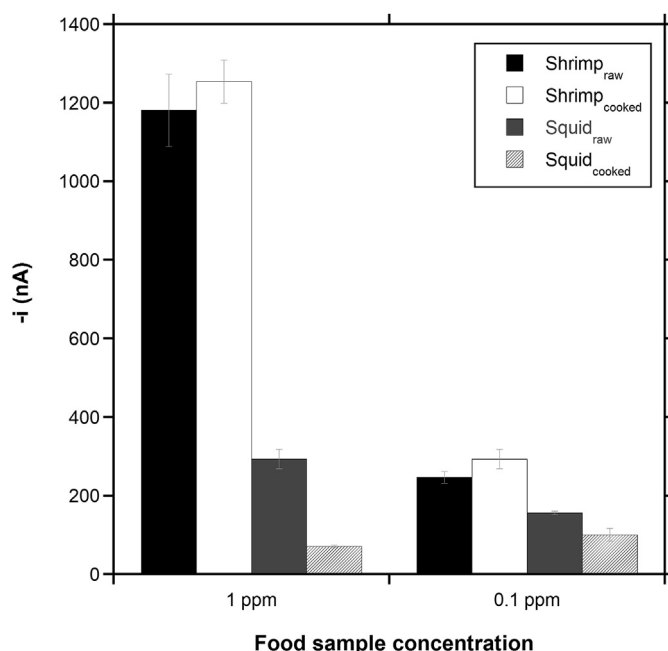


Fig. 7. Amperometric responses provided by the immunosensors in the analysis of shrimp and squid extracts, diluted at two different concentrations (0.1 ppm and 1 ppm, prepared in PBST containing 1% BSA), and two degrees of processing (raw and boiled). Supporting electrolyte, PB pH 6.0; $E_{app} = -0.20$ V vs. Ag pseudo-reference electrode. Error bars estimated as a triple of the standard deviation ($n = 3$).

the determination even in cooked samples. These capabilities are particularly interesting for the fish and seafood processing industry, where fish and seafood (both crustaceans and mollusks) are often manipulated in the same plant and eventually used in the preparation of diverse products. Furthermore, through a fine antibody selection, the method is readily adaptable to a wide variety of

analytes, leading to a multiplexed detection platform for the simultaneous analysis of several allergens of interest in a certain environment or application.

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