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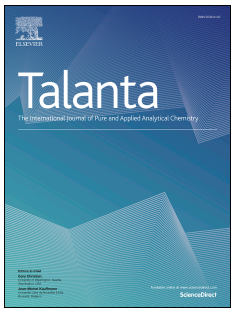

First electrochemical immunosensor for the rapid detection of mustard seeds in plant food extracts

M. Gamella, C. Bueno-Díaz, V. Ruiz-Valdepeñas Montiel, E. Povedano, A.J. Reviejo, M. Villalba, S. Campuzano, J.M. Pingarrón

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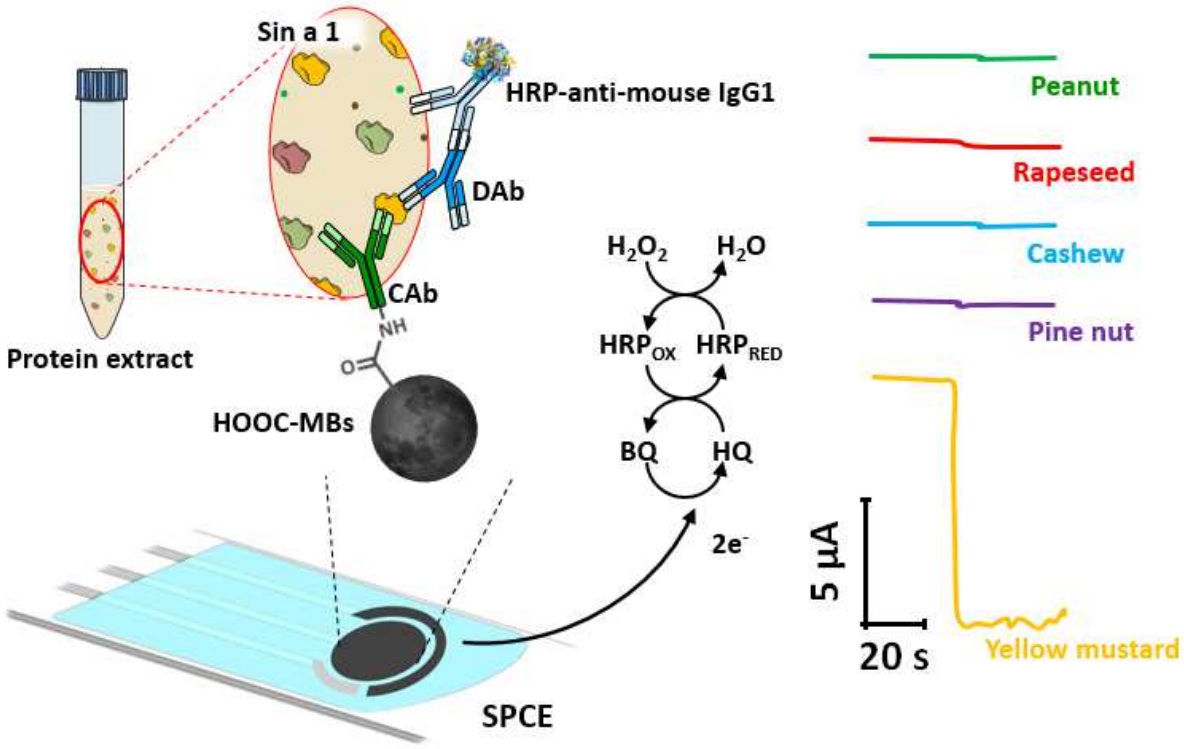
Graphical Abstract



First electrochemical bioplatform of mustard seeds through sensitive and selective amperometric determination of Sin a 1.

FIRST ELECTROCHEMICAL IMMUNOSENSOR FOR THE RAPID DETECTION OF MUSTARD SEEDS IN PLANT FOOD EXTRACTS

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Abstract

This paper describes the first biosensor reported to date for the determination of mustard seed traces. The biosensor consists of an amperometric immunosensing platform able to sensitively and selectively determine Sin a 1 content, the major allergen of yellow mustard and the most abundant protein of these seeds. The immunosensing platform exploits the coupling of magnetic microbeads (MBs) modified with sandwich-type immune complexes, comprising polyclonal and monoclonal antibodies, selective to the target protein for its capturing and detection, respectively. In addition, a HRP-conjugated secondary antibody was used for enzymatic labelling of the monoclonal antibody, and amperometric transduction was made at screen-printed carbon electrodes (SPCEs) using the hydroquinone (HQ)/H_2O_2 system. The electrochemical immunosensor allows the simple and fast detection (a single 1-h incubation step) of Sin a 1 with a limit of detection of 0.82 ng mL^{-1} (20.5 pg of protein in 25 µL of sample) with high selectivity against structurally similar non-target allergenic proteins (such as Pin p 1 from pine nut). The developed immunoplatform was successfully used for the analysis of peanut, rapeseed, cashew, pine nut and yellow mustard extracts, giving only positive response for the yellow mustard extract with a Sin a 1 content, in full agreement with that provided by conventional ELISA methodology.

Keywords: electrochemical immunosensor; mustard seed; Sin a 1; plant food extracts; magnetic beads; screen-printed carbon electrodes.

1. Introduction

Food allergies are a topic of increasing importance in public health. Since there is not effective treatment for them, the strict avoidance of the allergy-causing food is the only way to get rid of adverse reactions [1]. Many spices lead hypersensitivity reactions after their ingestion and among them mustard has been reported as a frequent cause of food allergy being one of the most reactive when tested *in vivo* [2]. Mustard is consumed worldwide as a food ingredient in various forms to enhance flavor and nutritional values. Common foods containing mustard seeds include pickled products, processed meats, seasoning mixes, salad dressings, sauces, spice mixtures and instant foods and mustard oil, which is also widely used as an edible oil and as flavoring agent [3-5]. Therefore, mustard traces can be inadvertently masked in many manufactured and processed products for flavoring, which makes its avoidance difficult and increases the risk for sensitized individuals of suffering unexpected allergic reactions [6].

The mustard plant belongs to the *Cruciferae* (*Brassicaceae)* family, which includes other vegetables such as radish, rutabaga, cabbage, broccoli, turnip, watercress, horseradish, castor oil plant, and rapeseed [7]. Mustard is mainly originated from three species: *Sinapis alba* (yellow mustard), *Brassica nigra* (black mustard) and *Brassica juncea* (oriental mustard) [1, 8] being the protein fraction, particularly the seed storage proteins, responsible of severe allergic reactions, as with other major allergens identified in seeds, nuts and legumes [9]. In fact, the proteins Sin a 1 and Bra j 1 from yellow and oriental mustard seeds, respectively, belonging to the 2S albumin class, have been described to trigger severe hypersensitivity reactions after mustard consumption [10]. It is estimated that mustard seed allergy accounts 1-7% of all food allergies in European regions, being considered the fourth most important food allergen for children population in France [8, 11]. Among the four allergenic proteins identified to date from yellow mustard seeds (Sin a 1-4), major allergen 2S albumin Sin a 1 is a napin (NAP) characterized as a basic, polymorphic, low-molecular-mass protein (14.1 kDa) [12], highly resistant to proteolysis and remains stable under heat treatments [6]. It is also considered a diagnostic marker for sensitization to mustard [2, 13].

Symptomatology of mustard seed allergy range from mild skin or gastrointestinal manifestations, to severe systemic reactions, such as anaphylactic shock in hypersensitive patients [5, 14]. Small amounts of this spice are capable of trigger these reactions, constituting a high risk for sensitized individuals due to potential inadvertent exposures (so-called “hidden” food allergens) when using contaminated cooking utensils, an inadequate cleaning of sharing processing equipment, or because of the consumption of food products

contaminated with ‘foreign’ food traces during shipping, storage, processing. The inadvertent presence of this spice is aggravated by the high thermal resistance of some of its allergens, whose structures are not altered during food processing [6, 15]. All these reasons led mustard to be included in the most recent European Directive concerning food safety (No. 1169/2011) which lists 14 main allergenic food ingredients forced to be declared by producers and manufacturers on food labels [16]. Therefore, analytical methods with the required sensitivity and selectivity to verify the presence of mustard traces in a wide variety of foodstuffs are highly demanded to ensure correct labeling and to guarantee the safety of the sensitized population.

Several methods have been reported to detect mustard traces in food samples including real-time or quantitative polymerase chain reaction (qPCR) [17-21], enzyme-linked immunosorbent assay (ELISA) [3, 9, 11, 22], flow cytometry [23] or mass spectrometry (MS) [7]. Although these methods have advanced significantly in terms of sensitivity and selectivity, they are time-consuming, labor-intensive, and need expensive equipment (such as a micro-plate reader or thermocycler). Moreover, they are poorly compatible with point-of-care and multiplexed analyses. Therefore, the development of analytical methods that can be applied in a fast and easy to-use manner to check food products for the presence of allergenic ingredients, offering manufacturers or consumers a tool for on-site testing during food production or consumption to avoid cross-contamination and health problems, is highly demanded.

In this sense, electrochemical immunosensors have emerged as powerful alternatives to the above-mentioned techniques for the detection and quantification of allergenic proteins. However, despite the advantages of electrochemical immunosensors regarding compatibility with miniaturization and multiplexing, simplicity, low cost and possible use in decentralized settings [24] to the best of our knowledge, no electrochemical immunosensor has been reported so far for the determination of mustard traces. We describe here the first electrochemical immunosensor for the determination of mustard residues through the determination of the Sin a 1 protein. The method involves the use of a capture antibody (CAb) and a detector antibody (DAb) labeled with a secondary antibody conjugated with horseradish peroxidase (HRP) to implement a sandwich format. Magnetic microbeads (MBs), providing improvements in assay time, sensitivity and minimization of sample matrix effects [25, 26], were employed to prepare CAb-functionalized MBs used for the efficient and selective capture of the target protein. After sandwiching the protein captured onto the CAb-MBs with the DAb and the HRP-labeled secondary antibody and magnetic capturing the final modified

MBs on the disposable screen-printed carbon electrodes (SPCEs), amperometric detection using hydroquinone (HQ) as electron transfer mediator and H_2O_2 as HRP substrate was performed. The analytical performance of the immunoplatform was evaluated by using the natural allergen purified from mustard seed and the immunosensor was successfully used to determine the endogenous concentration of Sin a 1 protein in plant extracts.

2. Materials and methods

2.1. Materials

A CHI812B potentiostat (CH Instruments) controlled by CHI812B software was used to perform the amperometric measurements. Screen-printed carbon electrodes (SPCEs, DRP-110), consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode and the specific cable connector (DRP-CAC) were acquired from DropSens, S.L. All measurements were carried out at room temperature. A Magellan V 7.1 (TECAN) ELISA plate reader was also used.

A Thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik) and a Bunsen AGT-9 Vortex were used. Magnetic separation for MBs incubation/washing processes was made using a Dynal MPC-S magnetic particle concentrator (product No. 120.20, Dynal Biotech ASA). Capture of the modified-MBs onto the working electrode (WE) surface of the SPCE was controlled by using a home-made polymethylmethacrylate (PMMA) casing with an encapsulated neodymium magnet (AIMAN GZ) embedded.

All the reagents used were of the highest available grade. Sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, Tris-HCl, NaCl and KCl were purchased from Scharlab. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS) were from Fluorochem. Ethanolamine, hydroquinone (HQ) and hydrogen peroxide (30%, w/v) were acquired from Sigma-Aldrich and 2-(N-Morpholino)-ethanesulfonic acid (MES) was purchased from Gerbu. A commercial casein blocking solution (a ready-to-use, PBS solution of 1 % w/v purified casein) was from Thermo Scientific. Carboxylic acid-modified MBs (HOOC-MBs, 2.7 µm Ø, 10 mg mL^{-1}, Dynabeads® M-270 Carboxylic Acid, Cat. No: 14305D) were purchased from Dynal Biotech ASA. IgG1 mouse-monoclonal (mAb) and rabbit-polyclonal (pAb) Sin a 1 specific antibodies were obtained in collaboration with the Hospital Clínico de Madrid (Spain). The mAb and pAb solutions were diluted and used as indicated in the text. HRP-labeled goat anti-mouse IgG Fc or IgG1 (Abcam, ab97265 and ab97240, respectively) and HRP-anti-rabbit IgG (BioRad, 170-6515) were tested as secondary antibodies. Natural Sin a 1 was purified from mustard seeds following the protocol described

by Menéndez-Arias et al. [27] and was used as standard to establish the analytical performance of the developed immunosensing platform.

All buffer solutions were prepared with Millipore Milli-Q purification water system (18.2 MΩ cm): phosphate-buffered saline (PBS) consisting of 0.01 M phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, pH 7.5; 0.05 M phosphate buffer, pH 6.0; 0.1 M phosphate buffer, pH 8.0; 0.025 M MES buffer, pH 5.0 and 0.1 M Tris-HCl buffer, pH 7.2. Activation and blocking steps of the HOOC-MBs were carried out using an EDC/sulfo-NHS mixture solution (50 mg mL^{-1} each in MES buffer, pH 5.0) and a 1 M ethanolamine solution (prepared in 0.1 M phosphate buffer solution, pH 8.0), respectively. Substrate (3,3′,5,5′-Tetramethylbenzidine, TMB/H_2O_2) and stop (H_2SO_4 2 N) solutions provided in the commercial ELISA kits were employed to apply this method.

2.2. Sandwich immunoassay onto the MBs

A 3-µL aliquot of the HOOC-MBs suspension was placed into a 1.5 mL microcentrifuge tube and washed twice with 50 µL MES buffer solution for 10 min under continuous stirring (950 rpm, 25 °C). The washing steps were performed by placing the microcentrifuge tubes for 4 min in the magnetic concentrator, then discarding the supernatant. Next, the carboxylic groups on the MBs surface were activated by incubation in 25 µL of the EDC/sulfo-NHS mixture solution for 35 min (950 rpm, 25 °C). The activated MBs were washed twice with 50 µL of MES buffer solution, and re-suspended in 25 µL of a 1/1,000 diluted CAb solution in MES buffer to perform the covalent immobilization of CAb (25 °C under continuous stirring at 950 rpm for 30 min). Subsequently, the CAb-modified MBs were washed twice with 50 µL of MES buffer solution. The unreacted activated groups on the MBs were blocked by adding 25 µL of the 1 M ethanolamine solution and incubating the suspension under continuous stirring (950 rpm) for 60 min at 25 °C. The modified MBs were washed with 50 µL of 0.1 M Tris–buffer (pH 7.2) and twice with 50 µL of the commercial casein blocking solution. The resulting modified MBs were kept at 4 °C in PBS buffer solution (sterilized in an autoclave) until use.

The immunoassay protocol consisted of incubating the CAb-coated MBs in 25 µL of a mixture solution, prepared in commercial casein blocking solution, containing the purified Sin a 1 (or the diluted sample extract), 100-times diluted DAb and HRP-anti-mouse IgG1 (0.2 µg mL^{-1}) for 60 min (950 rpm, 25 °C). After two washings with 50 µL of casein blocking solution, the MBs bearing the sandwich immunocomplexes were re-suspended in 50 µL of 0.05 M sodium phosphate buffer solution (pH 6.0) to perform the amperometric detection.

2.3. Amperometric measurements

After inserting the SPCE in the PMMA casing, the 50 µL modified-MBs suspension was magnetically captured on the WE surface of the SPCE in an easy, reproducible and stable way. The ensemble SPCE/magnet holding block was immersed into an electrochemical cell containing 10 mL of 0.05 M phosphate buffer of pH 6.0 and 1.0 mM HQ solution (freshly prepared) [28]. Amperometric measurements in stirred solutions were performed by applying a detection potential of −0.20 V *vs.* Ag pseudoreference electrode. This detection potential was previously optimized for the HQ/H_2O_2 system [29]. Upon addition of 50 µL of a 0.1 M H_2O_2 solution the current was recorded until the steady-state was reached. The amperometric signals given through the manuscript correspond to the difference between the steady-state and the background currents. Unless otherwise stated, the given values are the average ones of three replicates and error bars were estimated as triple of the standard deviation of the replicates (confidence intervals calculated for $\alpha = 0.05$).

2.4. Food extracts analysis

Food extracts were prepared following the protocol described by Bueno-Díaz et al. [30]. The extracts were adequately diluted in casein blocking solution and the protocol detailed in section 2.2 was followed. For comparative purposes, the same extracts were analyzed using the same immunoreagents by the ELISA method. Briefly, each well was coated with 100 µL of a 1/1,000 diluted in PBS CAb solution and incubated overnight at 4 °C. After washing each well with 350 µL PBST, a blocking step was carried out by incubating at room temperature for 1 h with 350 µL of PBS buffer containing 1% BSA. After a subsequent washing, each well was incubated for 90 min at 25 °C with a 100 µL-aliquot of a mixture solution, prepared in commercial casein blocking solution, containing a variable concentration of the Sin a 1 standard (or diluted sample extract), 1/100 diluted DAb and HRP-anti-mouse-IgG1 (0.2 µg mL^{-1}). After another washing step, color reaction was generated by adding 100 µL of Substrate Solution to each well and incubating for 10 minutes at room temperature (the well microplate was coated with aluminum foil to avoid direct light). The end of color reaction was accomplished by adding 50 µL of Stop Solution to each well, and the absorbance was measured immediately using the microplate reader set at 450 nm.

Due to the absence of matrix effect in the diluted extracts, the Sin a 1 concentration was determined by simple interpolation of the amperometric or absorbance signals measured in the diluted extracts into the calibration plots prepared with Sin a 1 standards.

3. Results and discussion

The fundamentals of the proposed sandwich immunosensing configuration are depicted in Fig. 1. Briefly, magnetic microcarriers were modified with specific Sin a 1 capture antibodies (CAb) and used to selectively isolate the target protein which was sandwiched in solution with the detector antibody (DAb) further conjugated with an HRP-labeled secondary antibody (HRP-anti-mouse IgG). The resulting modified MBs with the immunocomplexes immobilized on their surface were magnetically captured on the SPCE WE surface, and the amperometric transduction was performed by monitoring the variation in the measured cathodic current using the H_2O_2/HQ system (E_{app} = –0.20 V *vs.* the Ag pseudoreference electrode).

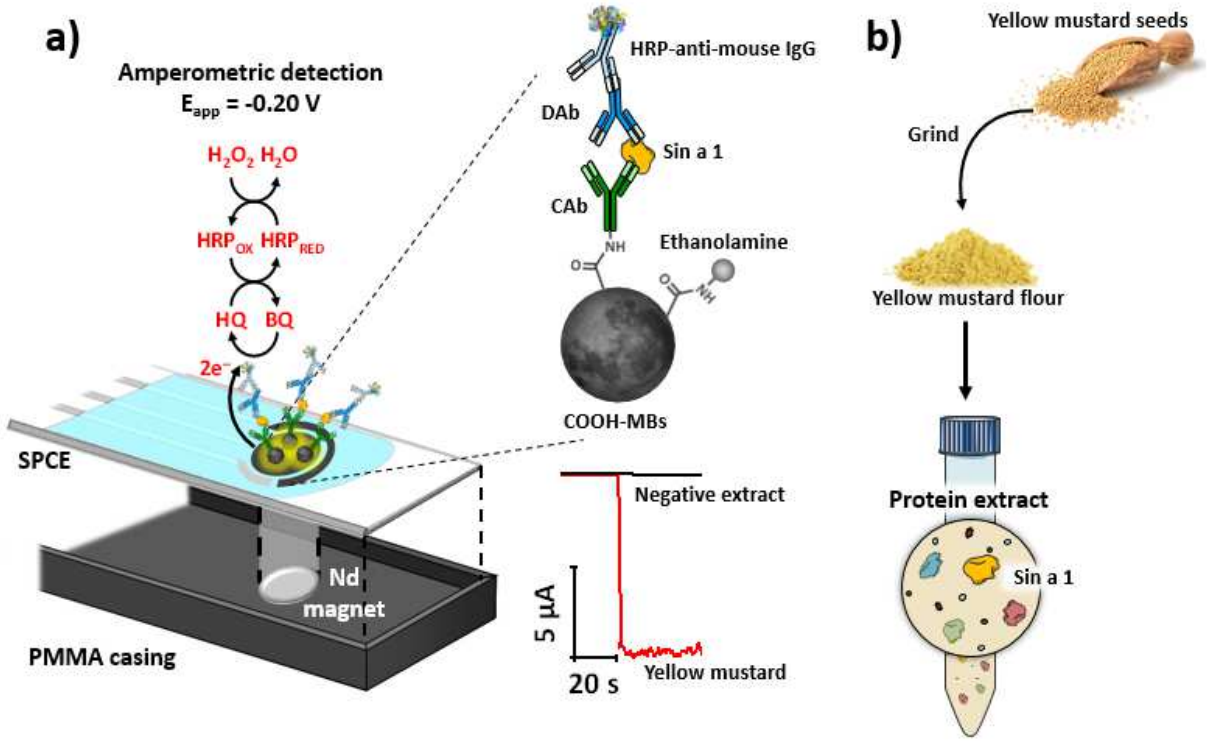

Fig. 1. Schematic display of the sandwich immunosensing configuration using MBs developed for the amperometric determination of Sin a 1 proteins (a) and plant extracts (b).

3.1. Optimization of experimental variables

The feasibility of the sandwich-type immunoassay was tested by comparing the amperometric responses obtained in the absence and presence of 50 or 2,000 ng mL^{-1} of Sin a 1 standards using unmodified MBs and MBs coated with the available Sin a 1 specific antibodies (an IgG1 mouse-monoclonal, mAb, Fig. 2a) and a rabbit-polyclonal, pAb (Figs. 2b and c), respectively. The obtained results showed that when the pAb and mAb were used as

capture (CAb) and detector (DAb) antibodies, respectively, (Fig. 2b) it was possible to detect a 40-times smaller Sin a 1 concentration (50 *vs.* 2,000 ng mL^{-1}) than using the mAb as CAb and the pAb as DAb (Fig. 2a). It is worth noting also the 106–times improvement in sensitivity allowed by this combination as indicated by the resulting slope values obtained with the antibodies coated-MBs (12.3 (Fig. 2b) *vs.* 0.116 (Fig. 2a) nA mL ng^{-1}, respectively). In view of these striking results, the pAb was selected as CAb for further assays.

The enzymatic labeling of the mAb used as DAb was evaluated using two different HRP-labeled secondary antibodies: HRP-anti-mouse IgG-Fc (Fig. 2b) or IgG1 (Fig. 2c). As it can be seen, the sensitivity jumped up to 31.4 nA mL ng^{-1} when the anti-mouse IgG1 antibody was used (Fig. 2c). These results are most likely due to the significant decrease of the non-specific interactions between the anti-mouse IgG-Fc and pAb used as capture antibody (white bars, Fig. 2b) as well as the enhanced affinity of anti-mouse IgG1 antibody for the DAb since mAb is a IgG1 isotype (Fig. 2c). Therefore, the HRP-anti-mouse IgG1 was selected as a secondary antibody for DAb enzymatic labeling. All these results show the critical role that both the disposition and type of antibodies play in the analytical performance of the immunosensor.

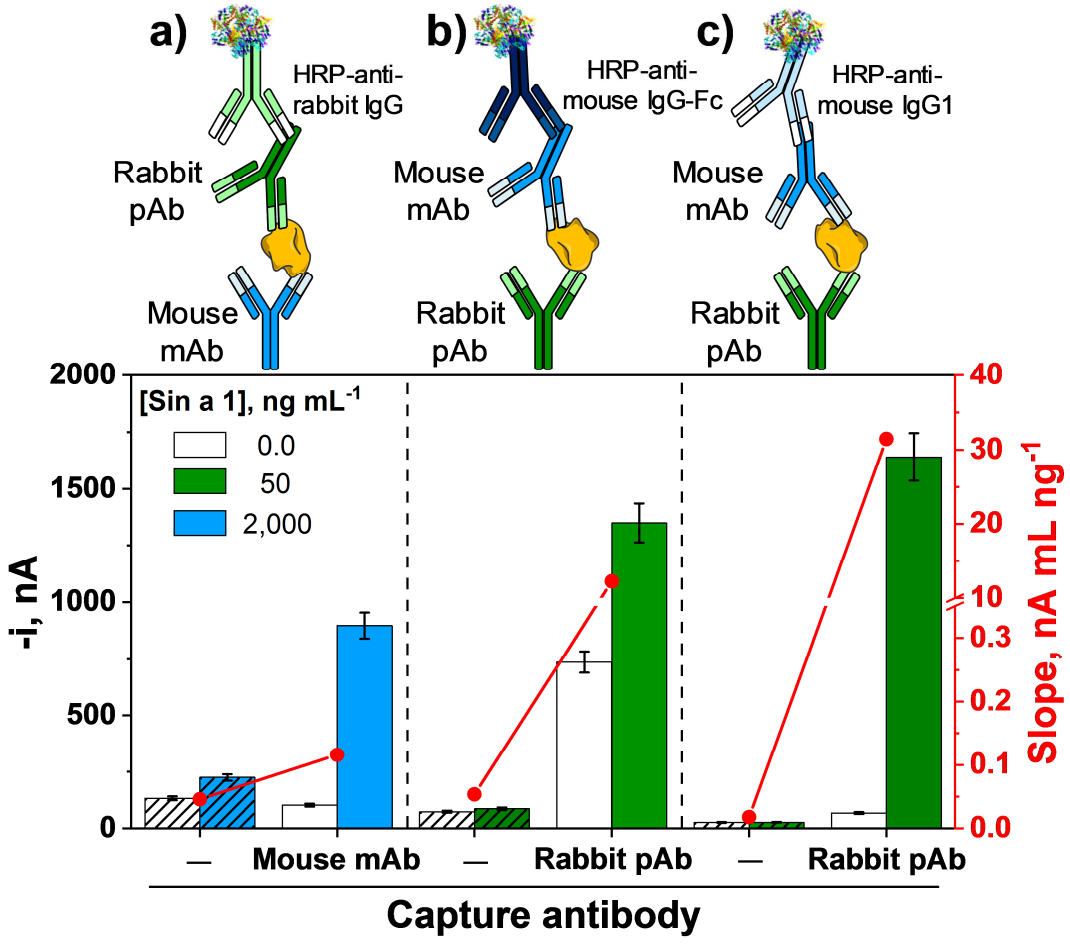

Fig. 2. Comparison between the amperometric responses obtained with unmodified-MBs (—, striped bars) or antibodies coated-MBs (non-striped bars) in the absence (white bars) or the presence of 50 (green bars) or 2,000 ng mL^{-1} (blue bars) of Sin a 1 standards and the resulting slope values calculated for each configuration (red circles and lines). Assayed configurations: (a) mouse mAb (CAb), rabbit pAb (DAb) and HRP-anti-rabbit IgG; (b) rabbit pAb (CAb), mouse mAb (DAb) and HRP-anti-mouse IgG-Fc; (c) rabbit pAb (CAb), mouse mAb (DAb) and HRP-anti-mouse IgG1.

Subsequently, all the experimental variables involved in the preparation of the immunosensing platform were optimized. Larger ratios between the amperometric responses obtained at –0.20 V (*vs.* Ag pseudo-reference electrode) for 25 and 0 ng mL^{-1} Sin a 1 standards (signal-to-blank ratio, S/B) were used as the selection criterion. The checked variables and the values selected are summarized in Table 1 and their dependence depicted in Figs. 3a-f.

Table 1

Experimental variables evaluated and values selected to perform the amperometric determination of Sin a 1 with the developed MBs-based immunosensor.

Variable	Tested range	Selected value
CAb dilution	1/10,000 – 1/50	1/1,000
Incubation time with CAb, min	15 – 60	30
Number of assay steps	1 – 3	1
DAb dilution	1/250 – 1/10	1/100
HRP-anti-mouse IgG1 concentration, µg mL^{-1}	0.04 – 1.0	0.2
Incubation time with Sin a 1 + DAb + HRP-anti-mouse IgG1 mixture, min	15 – 90	60

Figs. 3a and 3b show that larger S/B ratios were obtained by immobilizing a 1/1,000 diluted (in MES buffer solution) CAb solution (Fig. 3a) for 30 min (Fig. 3b), probably due to the steric hindrance effects that occur when larger CAb loadings are immobilized which led to a worse recognition of the target antigen. With the aim of simplifying as much as possible the whole protocol and reducing the assay time, the effect of the number of 30 min incubation steps involved in the immunoassay procedure was evaluated (Fig. 3c). The tested protocols were: a single step involving antigen capture and labeling by means of incubation in a mixture

solution containing Sin a 1, DAb and HRP-anti-mouse IgG1 (bars 1 in Fig. 3c); two sequential steps, (i) Sin a 1 + DAb mixture solution and (ii) HRP-anti-mouse IgG1 (bars 2A in Fig. 3c); two sequential incubation steps, (i) Sin a 1 solution and (ii) DAb + HRP-anti-mouse IgG1 mixture solution (bars 2B in Fig. 3c); three sequential incubation steps, (i) Sin a 1, (ii) DAb and (iii) HRP-anti-mouse IgG1 solutions (bars 3 in Fig. 3c). As it can be seen, a significantly larger S/B ratio was obtained when a single incubation step was carried out, which also simplifies and shortens the protocol considerably. This same behavior, previously observed in other sandwich immunoassays [31], is attributed to a higher efficiency of the immune and labeling reactions in homogeneous solution.

Fig. 3d shows that the S/B ratio increased with the DAb concentration up to a 1/100 dilution (in casein blocking solution) and decreased drastically for higher concentrations possibly due to agglutination phenomena in homogeneous solution that favored non-specific adsorptions and impair the specific recognition. Therefore, a 1/100 DAb dilution was selected for further work. The effect of HRP-anti-mouse IgG1 concentration (Fig. 3e) shows an increase of the S/B ratio up to 0.2 µg mL^{-1} and a dramatic decrease for larger HRP-anti-mouse IgG1 concentrations due to the significant increase of the secondary antibody non-specific binding. In addition, a better S/B ratio was found by incubating the CAb-MBs for 60 in the mixture solution containing Sin a 1 + DAb + HRP-anti-mouse IgG1 (Fig. 3f).

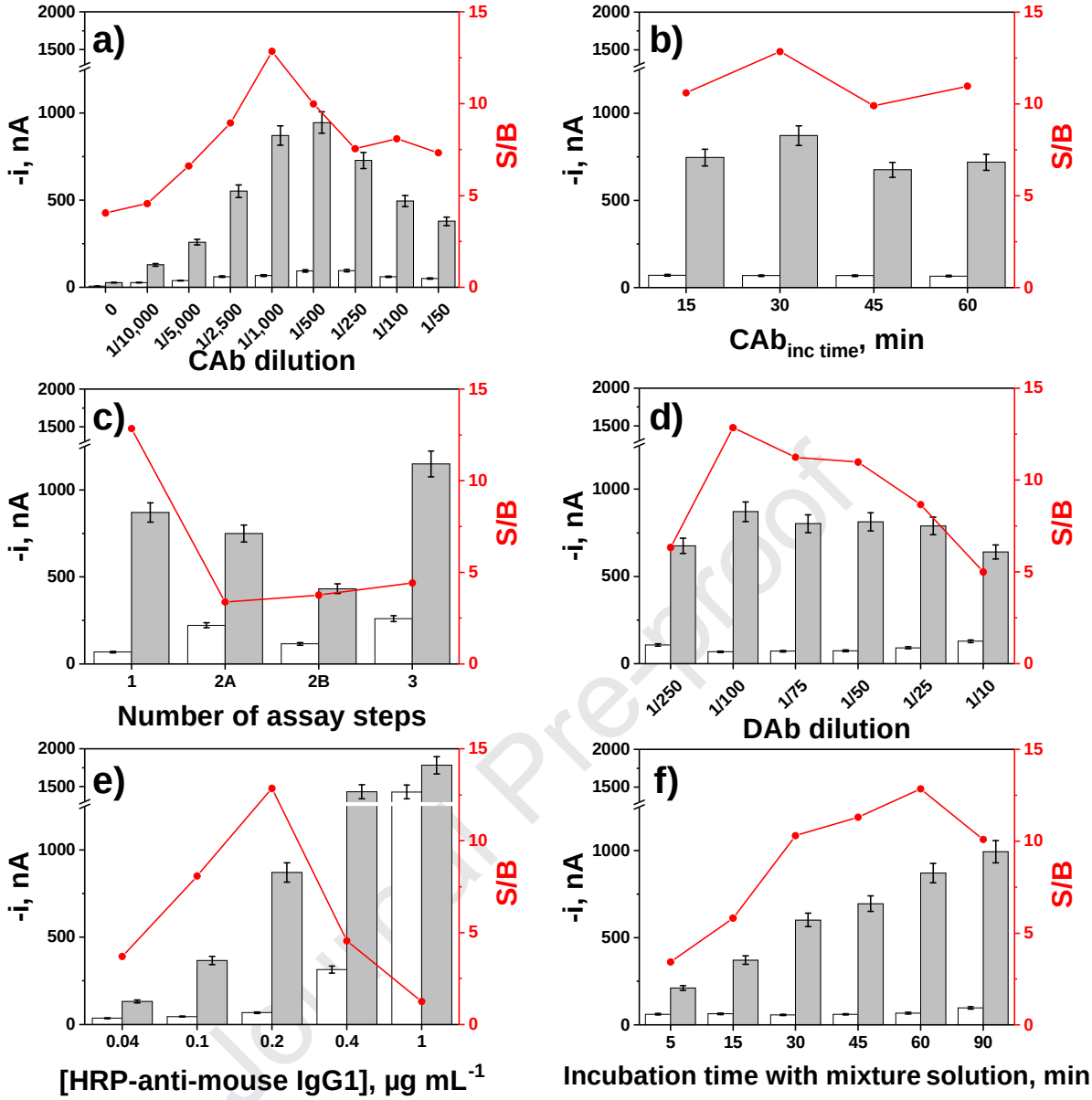

Fig. 3. Effect on the amperometric responses measured in the absence (white bars) or in the presence (grey bars) of 25.0 ng mL^{-1} Sin a 1 standards, and the resulting S/B ratio values (red circles and lines) with the CAb concentration (a) and incubation time (b), number of incubation steps used to perform the immunoassay (c), DAb dilution (d), HRP-anti-mouse IgG1 concentration (e), and incubation time with the Sin a 1 + DAb + HRP-anti-mouse IgG1 mixture solution (f).

3.2. Analytical characteristics

Fig. 4 shows the calibration curve, constructed under the optimized experimental conditions, for the amperometric determination of Sin a 1 standard solutions. The variation in the cathodic current increases linearly (r = 0.996) as a function of the Sin a 1 concentration

from 2.7 to 50 ng mL^{-1} showing a further saturation (not shown), with a slope value of (32 ± 2) nA mL ng^{-1} and an intercept of (25 ± 46) nA, respectively. The limit of detection (LOD) was calculated according to the $3 \times s_b / m$ criterion, where m is the slope of the linear calibration plot, and s_b was estimated as the standard deviation of ten amperometric signals obtained without target Sin a 1. The estimated LOD value was 0.82 ng mL^{-1} (0.82 ppb).

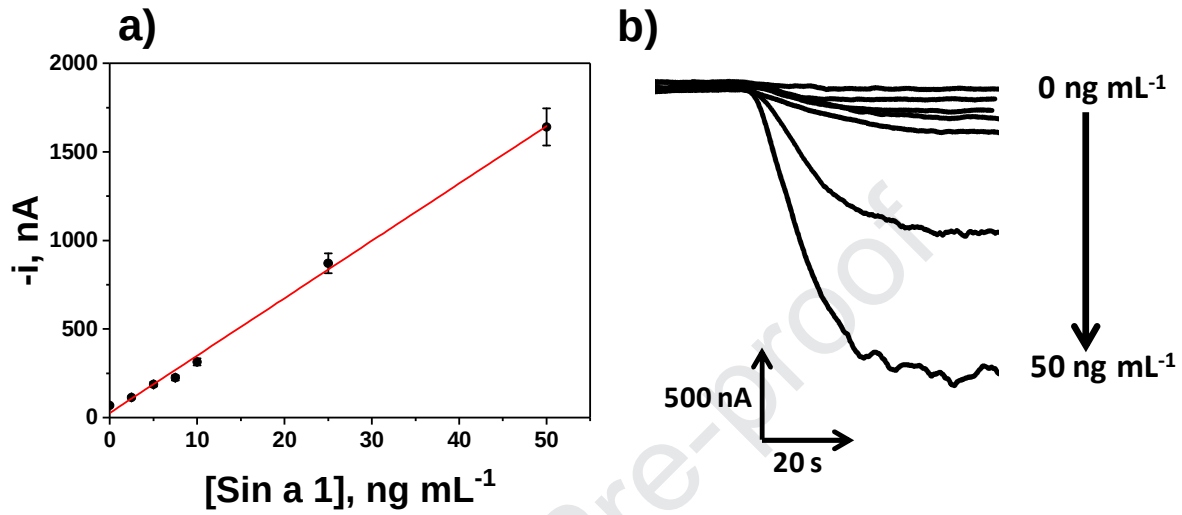

Fig. 4. Calibration plot constructed for the amperometric determination of Sin a 1 standards a) and amperometric traces recorded b) with the developed MBs-based sandwich immunoplatform.

The relative standard deviation (RSD) value calculated from the amperometric measurements for 25.0 ng mL^{-1} Sin a 1 provided by 8 different immunoplatforms prepared in the same manner and on the same day was 6.3 %, which confirmed a good reproducibility of the protocols involved in the immunosensor preparation and the amperometric detection.

The storage stability of the CAb-MBs conjugates, once the blocking step with ethanolamine was performed, was evaluated. The modified-MBs were kept at 4 °C in sterilized PBS. The stored CAb-MBs were used each working day to prepare the immunoplataforms, which were employed to measure the amperometric responses for 0 and 25.0 ng mL^{-1} Sin a 1 standards. No significant differences in the measured S/B ratio (results not shown) were observed for a period of at least 50 days (no longer times were assayed), indicating a great stability of the magnetic immunoconjugates. These results make it possible to prepare batches of CAb-MBs, storing them at 4 ºC and to carry out the determination when necessary in just 1 h.

The lack of other reported immunosensors neither electrochemical nor other, for the determination of Sin a 1 does not make it possible to compare the developed immunosensor analytical performance with that provided using other strategies. However, there is general agreement in the detection limits for different food products need to be somewhere between 1 and 100 ppm depending on the food and the sensitivity of the allergic individuals, with the minimal eliciting dose estimated for mustard proteins around 0.04 mg [8, 15, 32]. This value is achieved with the developed immunoplatform, because Sin a 1 makes up to 20% of the mustard seeds [27] and the reached LOD, 0.82 ppb, corresponds to the detection of 20.5 pg Sin a 1 in 25 µL of sample. It is important to note that this high sensitivity is particularly interesting for the detection of trace levels of this allergen, which are not detectable by conventional methodology and, therefore, do not appear on the food label or do not ensure the absence of hidden allergens due to the presence of cross-contamination phenomena.

Several ELISA methods have been published for the quantification of mustard proteins in mustard seed oil [9], yellow mustard seeds [11], meat [3, 4, 22] and salad dressings [3] or spiked meals [3, 4]. The achieved limit of detections ranges from 0.3 [11] to 3.08 ppm [4] significantly larger than that achieved with the developed immunoplatform. Although they satisfy the labeling requirements of the food industry, most methods are used to determine the total content of mustard proteins [3, 9, 22] and show cross-reactivity with other 2S albumin found in rapeseed [3, 11] or other proteins [9]. Only the methods reported by Shim et al. [11] and Marambe et al. [8] are specific for the determination of Sin a 1 protein, highly relevant even in processed foods for its high structural and immunological stability to heat and retaining immune reactivity after gastrointestinal digestion [4, 6, 8, 33].

Moreover, other less common methods to detect mustard allergens use test strips for qualitative analysis in only 10 min (LOD = 2 ppm). MS [7] and PCR-based [17-21] methods have been reported also for mustard allergens determination, with LODs of 0.25 ppm and from 0.1 to 10 ppm, for MS and PCR, respectively. However, despite their sensitivity and specificity, these methods are time-consuming, need expensive equipment and well-trained people, which vastly limits their use to centralized settings.

It is important to remark that the developed immunoplatform represents a substantial improvement in sensitivity compared to the commercial ELISA kits (providing a LOD about 3 orders of magnitude lower) in a rather similar assay time (from 40 to 90 min), once the plate or the MBs are coated with the CAb and the sample extract is ready. Moreover, the ELISA methods require larger sample/reagent volumes (100 *vs*. 25 µL) and relatively expensive instrumentation (ELISA plate readers) hardly portable and miniaturizable. Therefore, even

though ELISA is long adopted in centralized laboratories for protein antigens, it exhibits disadvantages to perform on site and/or multiplexed determinations, which are demanded to track and trace for allergen-free food production chains.

3.3. Selectivity

One of the most important aspects in bioassays is to evaluate the degree to which the method can distinguish the target molecule from other possible components of the sample. This aspect is particularly relevant in this work considering the reported cross-reactivity between mustard proteins and other proteins found in plant-derived foods, including nuts, spices, legumes, and rosaceae fruits [7, 34].

It is worth noting here as well that the 2S albumin class is an abundant group of seed storage proteins considered "universal allergens" and with structure related to Sin a 1 which have been isolated from other *Brassicaceae*, including rapeseed, radish and thale cress [33, 35]. For example, Bra j 1 is an allergenic 2S albumin isolated oriental mustard seed protein with very similar structural and immunological characteristics to Sin a 1 [36, 37]. It has been reported also cross-reactivity between the proteins of mustard and rape seeds, whose major allergen Bra n 1, has been identified as close homologue of Sin a 1, with 94 % of sequence similarity after their alignment [33, 38]. Other 2S albumins have also been described as major food allergens in tree nuts such as pine nut (Pin p 1) [39, 40], peanut (Ara h 2, Ara h 6) [41], walnut (Jug r 1) [42], cashew (Ana o 3) [43] or Brazil nut (Ber e 1) [44].

In this context, the selectivity of the developed immunoplatform was tested towards another purified protein from 2S albumin family such as Pin p 1 as well as towards plant extracts which can contain non-target 2S albumins from peanut, rapeseed, cashew and pine nut [12, 45]. Yellow mustard seeds extract was tested as a positive control. Fig. 5a shows that despite the structural similarity with Sin a 1, the non-target protein Pin p 1 does not provoke any significant interference when both proteins are at the same level (25 ng mL^{-1}). The results shown in Fig. 5b in the analysis of the raw plant protein extracts confirm the high selectivity of the developed immunoplatform. Only the extract from yellow mustard provided a noticeable amperometric response, thus indicating the extraction of the protein Sin a 1 from the seeds. It is important to mention that no significant amperometric response was found for smaller dilutions (from 1/10 to 1/10,000) of any tested plant extract different from yellow mustard. These results suggested that not apparent cross-reactivity was observed between the proteins of the selected plant extracts. It is remarkable the absence of cross-reactivity obtained for the rapeseed extract, whose cross-reactivity has been previously reported in the

development of Sin a 1 immunological tests [11]. Therefore, it can be pointed out the high selectivity of the antibodies used to implement the sandwich immunosensor and its competitiveness also in selectivity compared with commercial ELISA kits [3, 9, 11].

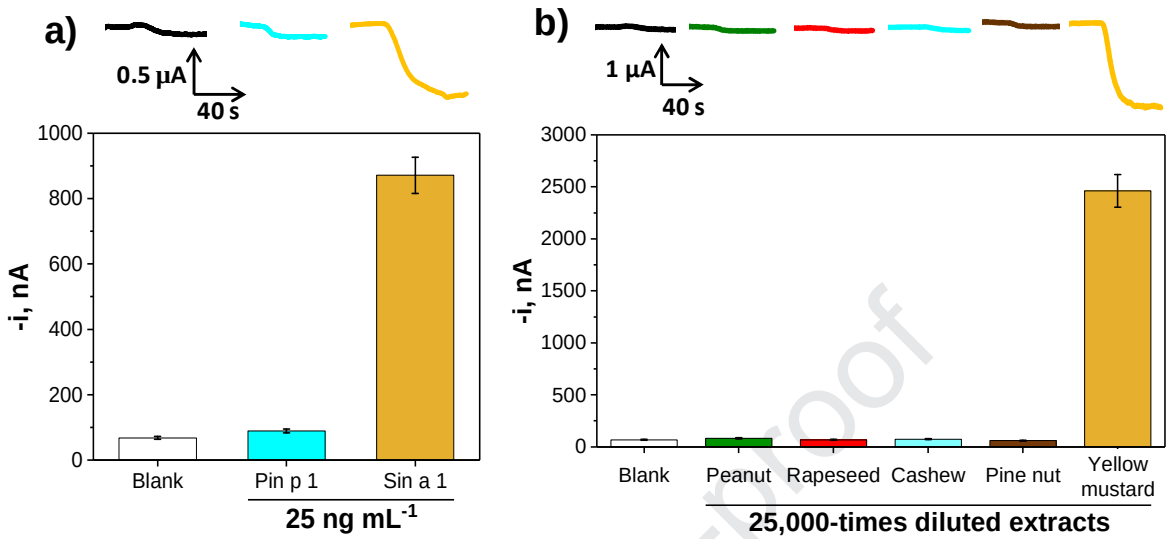

Fig. 5. Amperometric responses and real amperometric traces obtained with the developed immunoplatform at –0.20 V (*vs.* Ag pseudoreference) for: (a) 0 (white bar), 25 ng mL^{-1} Pin p 1 protein (blue bar) and Sin a 1 protein (yellow bar); (b) 0 (white bar) and 25,000-times diluted peanut (green bar), rapeseed (red bar), cashew (blue bar), pine nut (brown bar) and yellow mustard (yellow bar) extracts.

3.4. Determination of Sin a 1 in plant extracts

The developed immunosensor was used for the determination of Sin a 1 in protein extracts from peanut, rapeseed, cashew, pine nut and yellow mustard seeds. The content of Sin a 1 in extracts other that from yellow mustard is undetectable although they are tested 20,000 times less diluted.

Regarding the determination of endogenous Sin a 1 in yellow mustard seeds extract, the possible existence of matrix effect was tested by constructing a calibration plot in these extracts adequately diluted with blocker casein solution and spiked with growing amounts of the Sin a 1 protein up to 10 ng mL^{-1}. The slope values of the respective calibration plots constructed in extracts diluted 200,000 or more times were statistically similar to that obtained with Sin a 1 standard solutions (slope values of 33 ± 10 and 32 ± 2 nA mL ng^{-1}, respectively). Therefore, it was concluded that no significant matrix effect was apparent in such diluted extracts, and the endogenous Sin a 1 concentration was determined by simple interpolation of the amperometric signals obtained from the diluted extracts into the

calibration plot constructed with the Sin a 1 standards (Fig. 4). Table 2 summarizes the results obtained for Sin a 1 quantification with the developed electrochemical immunosensor and with an ELISA spectrophotometric method involving the same immunoreagents and according to the protocol detailed in section 2.4.

Table 2

Determination of the Sin a 1 protein endogenous content (in mg g^{-1}) in plant extracts using the developed amperometric magnetoimmunosensor and an ELISA method involving the same immunoreagents.

Extract	**Dilution**	**Electrochemical immunosensor**	**ELISA**
Peanut	1/10	ND	ND
Rapeseed	1/10	ND	ND
Cashew	1/10	ND	ND
Pine nut	1/10	ND	ND
Yellow mustard	1/200,000	$(29 \pm 9)^{*}$	$(31 \pm 4)^{*}$

ND: non-detectable; *Mean value ± ts/√n (n = 3; α = 0.05)

As it can be seen, the Sin a 1 content provided by the immunosensor is in good agreement with that obtained using the ELISA methodology (t_{exp} = 1.528, t_{tab} = 2.776) and within the ranges reported for Sin to 1 in the seeds of different yellow mustard varieties (Sin a 1 was 2.6–5.9% of yellow mustard seed weight) [8].

In addition, the sensitivity of the developed methodology for the analysis of different amounts of Sin a 1 in the yellow mustard seeds extract was assessed. Fig. 6 shows the linear dependence (r^2=0.9914) between the measured cathodic current and the logarithm of the total protein content in the yellow mustard seeds extract, in the range from 1.15 to 23.0 ng of protein extract (1/500,000–1/25,000 protein extract dilution), with a slope value (1,522 ± 82) nA and an intercept of (394 ± 71) nA, respectively. These results confirm the high sensitivity of the developed immunoplatform that is able to detect the presence of Sin a 1 in just 1.15 ng of total protein from the yellow mustard seeds extract.

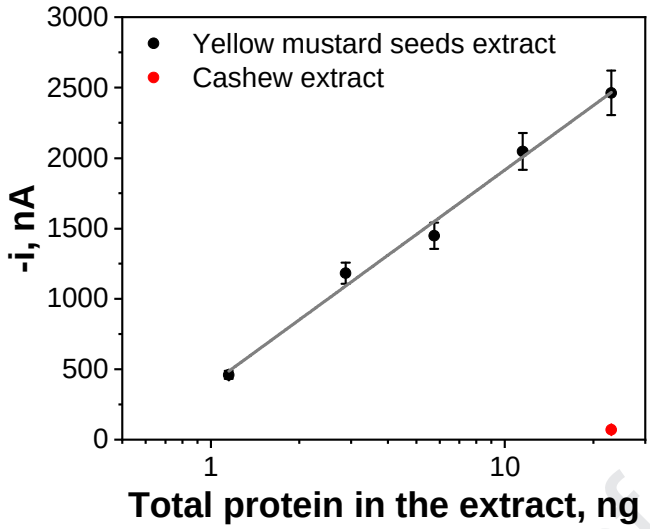

Fig. 6. Amperometric responses obtained with the developed immunosensing platforms in the analysis of 1.15 to 23.0 ng total extracted protein (1/500,000–1/25,000-diluted extract) from the yellow mustard seeds (black circles) and in 23.0 ng of the cashew extract (red circle) used as negative control.

4. Conclusions

The first immunosensor for the determination of mustard traces and products thereof is presented in this work by targeting the Sin a 1 protein, the major allergen found in yellow mustard. The platform implies the magnetic capture of immunocomplexes formed on commercial MBs by sandwiching the target protein with a pair of selective polyclonal and monoclonal antibodies, and enzymatically tagging the monoclonal antibody with a secondary antibody conjugated with HRP, on the surface of SPCEs to perform amperometric transduction in the presence of the HQ/H_2O_2 system. The resulting immunosensor exhibits a high sensitivity (LOD value of 0.82 ng mL^{-1}) and selectivity for the single-step and 1 h determination of Sin a 1 against other closely related allergenic proteins of the plant kingdom. The quantification of Sin a 1 in yellow mustard seed extracts were in agreement with that obtained using an ELISA method. The developed immunosensor is highly competitive in terms of sensitivity (with a detection limit three orders of magnitude lower than that obtained with ELISA), cost and compatibility with multiplexing and decentralized determinations. These characteristics make the developed immunoplatform a very attractive and affordable biotool for on-site testing to minimize consumer-safety concerns and comply with increasingly enforcing labelling regulations and quality-assurance procedures.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Maria Gamella: Conceptualization, Investigation, Writing, Review & Editing - original draft. **Cristina Bueno-Díaz:** Investigation, Resources, Review & Editing - original draft. **Víctor Ruiz-Valdepeñas Montiel:** Conceptualization, Investigation, Writing, Review & Editing - original draft. **Eloy Povedano:** Conceptualization, Investigation, Writing, Review & Editing - original draft. **A. Julio Reviejo:** Conceptualization. **Mayte Villalba:** Supervision, Resources, Review & Editing - original draft and Funding acquisition. **Susana Campuzano:** Conceptualization, Supervision, Writing, Review & Editing - original draft and Funding acquisition. **José M. Pingarrón:** Review & Editing - original draft and Funding acquisition.