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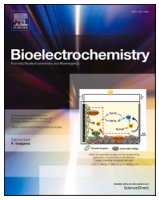

First PCR-free electrochemical bioplatform for the detection of mustard Sin a 1 protein as a potential "hidden" food allergen

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Keywords:
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ABSTRACT

A disposable electrochemical PCR-free biosensor for the selective detection of a fragment encoding the protein Sin a 1, a 2S albumin considered a diagnostic marker for sensitization to mustard, is reported. The methodology is based on the formation of DNA/RNA heterohybrids by sandwich hybridization of a specific fragment of the Sin a 1 allergen coding sequence with appropriately designed RNA probes. Labeling with commercial antibodies specific to the heteroduplexes and secondary antibodies conjugated with horseradish peroxidase (HRP) was carried out onto the surface of magnetic beads (MBs). Amperometric transduction was undertaken on screen-printed electrodes using H_2O_2 as enzyme substrate and hydroquinone (HQ) a redox mediator. The electrochemical biosensor allows the simple and fast detection (75 min) of Sin a 1 reaching a limit of detection of 3 pM. The bioplatform was successfully applied to the analysis of the targeted *Sin a 1* gene specific region using just 50 ng of non-fragmented denatured genomic DNA extracted from yellow mustard seeds.

1. Introduction

Mustard can trigger allergic reactions in sensitized people; indeed, severe incidents related to mustard allergy are growing across the world. Mustard can be present in a great variety of food stuffs such as sauces, meat, spice mixtures and instant foods. Among members of the mustard's plant family, black, brown, oriental, and yellow mustards are probably the most frequently used in the food industry, the latter two being responsible for most allergic reactions [1]. Four major allergenic proteins from yellow mustard have been identified: Sin a 1, Sin a 2, Sin a 3, and Sin a 4. The protein Sin a 1, a 14 kDa 2S albumin, has been found to be the most potent allergen in yellow mustard. The 2S albumins are the major allergens from nuts and seeds, affecting particularly to childhood, and they are considered as the most suitable diagnostic markers to analyze their prevalence and sensitization [2].

Currently, there is no cure for mustard allergy and, therefore, patients must exclude it from their diet to avoid an allergenic reaction, which can be severe and occasionally life threatening [3]. On the other hand, allergen successful avoidance depends on its identification; therefore, some countries require mustard to be labeled as a major allergenic source on food packaging. In Europe, the regulation (EU) 1169/2011 imposes including mustard among the 14 allergenic ingredients that must be listed on food labels [4]. However, this regulation fails to consider the accidental introduction of allergens during production, transportation or storage, as even small amounts of allergen can trigger severe reactions [5,6].

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In this context, the development of reliable and specific tools to detect traces of food allergens is fundamental to improve the quality of life of sensitized individuals [7]. The main allergen detection methods can be divided into two groups: immunological assays and DNA-based assays [8,9]. Immunoassays such as enzyme-linked immunosorbent assay (ELISA) [10] and lateral flow [8], and mass spectrometry (MS) [11] are the most common analytical techniques used to detect small amounts of allergenic proteins. These methods are quantitative or semi-quantitative, require specific equipment to perform the measurements, and can be time-consuming and expensive, particularly if a small number of samples is analyzed [12]. Besides, the impact of food processing on proteins should also be considered as, sometimes, production processes involve heat treatment and/or high-pressure industrial practices which can modify proteins, and thus alter their detection [13,14]. In the case of Sin a 1 specific quantification, liquid chromatography (LC) coupled to MS/MS was used by Posada-Ayala *et al.* achieving a detection limit of 0.25 ppm [15]. However, the complex sample treatment (protein extraction, reduction, and enzymatic digestion into peptides) along with the expensive and non-portable instrumentation makes Sin a 1 interrogation by LC-MS/MS not suitable for on-site applications.

DNA-based methods involve the extraction of specific allergen encoding-DNA fragments followed by polymerase chain reaction (PCR) amplification. Although food processing may also truncate DNA [14], it maintains its integrity better than proteins during food processing [16]. Therefore, the DNA determination of specific food proteins has been proposed as robust and reliable alternative for food allergen determination [17]. Methods for detection of allergenic food based on PCR include PCR-ELISA, real-time PCR, primer multiplex-PCR or loop-mediated isothermal amplification [12,16]. These methods are sensitive but are time-consuming and need expensive reagents and instrumentation. Therefore, it is necessary to develop easy-to-use and fast devices able to effectively replace the classical methodologies and suitable to be applied at the point of care.

Although biosensors are still not very much used for routine analysis, they appear as alternative technology to overcome the problems associated with current analysis systems [18]. Among them, electrochemical biosensors have been widely used for specific gene detection due to their unique properties such as low cost, simplicity, rapidity, good selectivity, and sensitivity [19]. In addition, they are easy to be automated and/or miniaturized, and can be used with simple, portable, and low-cost peripheral instruments with low power consumption and meeting important market demands. All these features are pushing the ongoing development of nucleic acids electroanalytical bio-devices linked to food sensing by means of specific DNA fragments of coding sequences of allergens such as hazelnut [20] or tomato seeds [21]. However, and despite the importance of mustard as a relevant allergen, no electrochemical DNA sensor for mustard has been reported so far. To fulfill this need, this work describes the first PCR-free electrochemical biosensing platform for the detection of mustard by targeting a 60-mer fragment of the genomic Sin a 1 allergen coding-sequence. The proposed assay is based on a sandwich hybridization format of the target genomic *Sin a 1* DNA fragment with a specific RNA biotinylated capture probe and an RNA detector probe at the surface of neutravidin-functionalized magnetic microcarriers (Neu-MBs). The captured DNA/RNA heteroduplexes were recognized with a specific antibody and subsequent labeling with a secondary antibody conjugated with HRP was accomplished. Amperometric detection at screen-printed carbon electrodes (SPCEs) involving the H_2O_2/HQ system was carried out after magnetic capturing the modified MBs onto the working electrode surface.

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were performed with an 812B potentiostat (CH Instruments) controlled by CHI812B software. SPCEs (DRP-110) consisting of a 4 mm diameter carbon working electrode, a carbon counter electrode, and an Ag pseudo-reference electrode, and a specific cable connector (DRP-CAC) were purchased from Metrohm Dropsens.

An incubator shaker Optic Ivymen® System (Comecta S.A, Sharlab), a Bunsen AGT-9 Vortex, a Raypa steam sterilizer, a biological safety cabinet Telstar Biostar, a thermocycler (SensoQuest LabCycler, Progen Scientific Ltd.) and a magnetic particles' concentrator DynaMag™-2 (123.21D, Invitrogen Dynal AS) were employed. The quantity and quality of the extracted DNA were evaluated by UV using a Shimadzu UV-1800 spectrophotometer and by Gel-Red staining (Biotium) after 0.7 % agarose gel electrophoresis, visualized using UV transilluminator (BioRad), respectively. A neodymium magnet (AIMAN GZ) embedded in a homemade poly(methyl methacrylate) (PMMA) casing was used to magnetically capture the modified-MBs on the surface of the SPCEs.

2.2. Reagents and solutions

Magnetic neutravidin coated microparticles (Neu-MBs, Ø = 1.0 μm, 10 mg mL^{-1}) were purchased from SpeedBeads™ GE Healthcare and streptavidin-modified magnetic beads (Strep-MBs, Ø = 2.8 μm, 10 mg mL^{-1}, Dynabeads M−280 Streptavidin, 11206D) were provided by Invitrogen-ThermoFisher™.

Tris–HCl, NaCl, KCl, NaH_2PO_4 and Na_2HPO_4 were purchased from Scharlab, HQ, and H_2O_2 (30 %, w/v) were acquired from Sigma-Aldrich and ethylenediaminetetraacetic acid (EDTA) from Merck. Mouse RNA–DNA hybrid antibody (S9.6, $Ab_{DNA/RNA}$) from Kerafast and HRP-anti-mouse IgG (anti-mIgG-HRP) from Abcam were used. A commercial blocker casein solution (PBS solution of 1 % w/v purified casein, BB) was purchased from Thermo Scientific. DNA extraction was carried out using Nucleospin Plant II kit (740770, Macherey-Nagel). All the used oligonucleotides, whose sequences are described in Table 1, were purchased from Sigma-Aldrich. Upon receipt, they were reconstituted in nuclease-free water to a final concentration of 100 μM, divided into small aliquots, and stored at − 80 °C.

All buffer solutions were prepared in deionized water from a Millipore Milli-Q purification system (18.2 MΩ cm): phosphate-buffered saline (PBS) consisting of 0.01 M phosphate buffer solution containing 0.137 M NaCl and 0.0027 M KCl, pH 7.5; 0.05 M phosphate buffer, pH 6.0 (PB), and Binding and Washing buffer (B&W) consisting of 10 mM Tris–HCl solution containing 1 mM EDTA and 2 M NaCl, pH 7.5.

2.3. Samples

Genomic DNA was extracted from yellow mustard seeds (*Sinapis alba*). Radish seeds (*Raphanus sativus*) and hazelnut (*Corylus avellana L.*) were used as DNA for selectivity control. Seeds or hazelnuts were homogenized in a potter in the presence of liquid nitrogen, and delipidated with cold acetone (10 mL/g) for 2 h at room temperature. The samples were centrifuged for 10 min at 5000 g and let dry for 18 h, and the powder stored at − 20 °C.

2.4. Genomic DNA extraction

20 mg of dry powder were used for DNA extraction according to NucleoSpin Plant II protocol with minor variations. Optimal lysis time was established at 30 min and elution was performed using DNAse-free water instead of the recommended buffer to avoid interferences with the measurements.

Just before the analysis with the developed biosensing platform, the extracted double-stranded genomic DNA was thermally denatured at 95 °C for 7 min (in the thermocycler). DNA strand re-annealing was retarded by keeping the sample in ice until using.

2.5. Preparation of the magnetic immunoconjugates

The whole protocol was carried out in 1.5 mL microcentrifuge tubes.

Table 1
Oligonucleotides used for the development of the electrochemical DNA bioplatform for the determination of a specific region of Sin a 1 allergen coding sequence.

Role	Short name	Sequence ($\acute{5} \rightarrow \acute{3}$)
Biotinylated capture probe	bRNA-Cp*	biotin-UAGCAGUCUGGUAGAUACGG CUAAUUAC
Biotinylated detector probe	bRNA-Dp*	UUGCCUGAUGUUGCAAACUUUAGG UAAGUG-biotin
Detector probe	RNA-Dp*	UUGCCUGAUGUUGCAAACUUUAGG UAAGUG
Sin a 1 synthetic target	*Sin a 1* target DNA**	GTAATTAGCCGTATCTACCAGACTGC TACACACTTACCTAAAGTTTGCAACATCAGGCAA
Sola l 7 synthetic target	*Sola l 7* target DNA	CTTGGCACCCTGTGTCCCGTTCCTGACACAGGGTGGCGAGCCCGGTGCAGCATGCTGTAG
Cor a 9 synthetic target	*Cor a 9* target DNA	GAATGGGAGCGACAGGAGAGACAAGAGA GGGAGAGTGAGCAAGAGCGGGA
Peanut synthetic target	Peanut target DNA	AGGAGCAATAGAAACTGCGTTGATATTGCTCCTTTACTTTCAAAAACTCGT ATCCCTTAAGACAAAAA

* Cp and Dp (both 30 mer) were designed to hybridize contiguously with the selected 60 *mer*-Sin a 1 target DNA to achieve maximum sensitivity in the sandwich hybridization format [20,21].
** Sin a 1 contains two immunodominant regions which are allergenically relevant. The epitope recognized by the monoclonal antibody 2B3 was specific to mustard allergens (Sin a 1 and Bra j 1) since this monoclonal antibody did not recognize the 2S albumins from other species [22]. According to the authors, this specific region, which is in the "hypervariable region" of the 2S albumins included in the heavy chain of the molecule [23], contained an important allergenic determinant of the protein [24]. Therefore, a DNA fragment from genomic Sin a 1 DNA encoding the "hypervariable region" epitope was selected for the development of the platform due to the specificity of the sequence for yellow mustard.

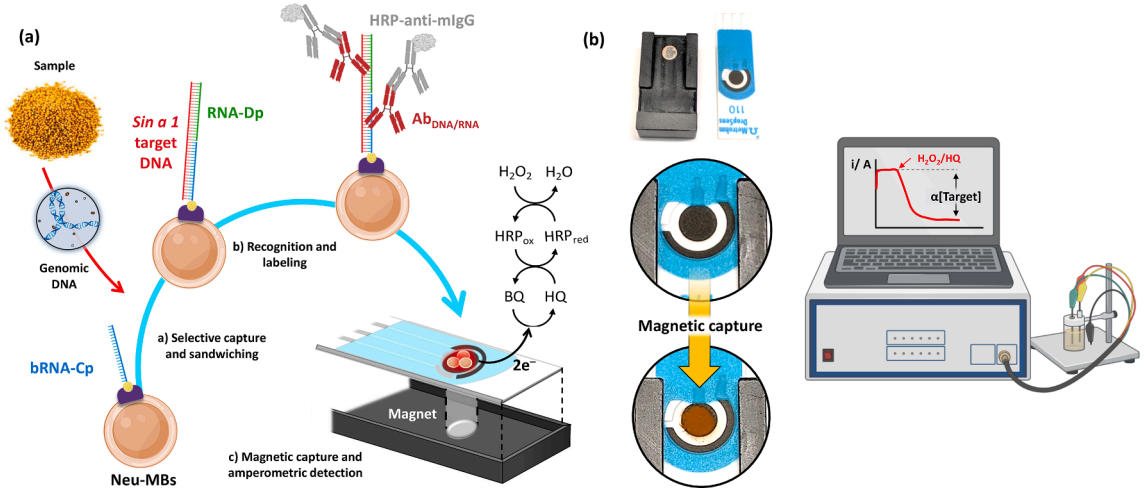
Unless otherwise indicated, all incubation steps were made with 25 μL of the corresponding solution at 37 °C under continuous shaking (950 rpm). All washings were performed with volumes of 50 μL, and the tubes with the MBs suspension were placed in the magnetic concentrator for 2 min to remove the corresponding supernatant without loss of MBs. The preparation of the bioconjugates required for each determination involved the use of a 4 μL aliquot of Neu-MBs suspension that was washed twice with B&W buffer solution. Thereafter, the Neu-MBs were incubated with a 0.25 μM bRNA-Cp solution in B&W for 30 min and subsequently washed twice with PBS solution. The sandwich assay was implemented by incubating for 60 min the bRNA-Cp-Neu-MB bioconjugates with a mixture solution containing the target DNA (synthetic *Sin a 1* target DNA or extracted plant-derived genomic *Sin a 1* DNA) and the RNA-Dp solution prepared in PBS. After washing twice with BB solution, the modified RNA-Dp-target DNA-bRNA-Cp-Neu-MBs were incubated for 15 min in a mixture solution containing $Ab_{DNA/RNA}$ (1.0 μg mL^{-1}) and HRP-anti-mIgG (1/2500 dilution) prepared in BB solution. Finally, the modified MBs were washed twice with BB solution and resuspended in 50 μL of 0.05 M sodium phosphate buffer solution (pH 6.0) until performing the amperometric detection. All the MBs manipulations prior the amperometric measurements were carried out in a laminar flow cabinet to avoid RNase contamination and prevent RNA degradation.

2.6. Amperometric measurements

Amperometric readout was performed using commercial disposable SPCEs. The modified MBs suspension was deposited and magnetically captured on the corresponding working SPCE surface which was previously placed on a PMMA homemade casing containing an embedded neodymium magnet. The ensemble SPCE-casing was immersed in the electrochemical cell containing 10 mL of a 1 mM solution of HQ prepared in 50 mM PB (pH 6.0). The amperometric measurements were carried out under stirring by applying − 0.20 V *vs.* the Ag pseudo-reference electrode after adding 50 μL of fresh 100 mM H_2O_2 solution (also prepared in 50 mM PB, pH 6.0) until the steady state was reached. The analytical responses given through the text correspond to the difference between the steady state and the background currents and were the mean values of three replicates. The displayed error bars were estimated as three times the standard deviation of each set of replicates ($\alpha = 0.05$).

3. Results and discussion

The developed bioplatform is based on a sandwich hybridization format for targeting a genomic *Sin a 1* DNA fragment. The fabrication procedure is displayed in Scheme 1. Briefly, the bRNA-Cp-Neu-MBs were used to selectively capture the target DNA which in turn hybridizes to a specific RNA detector probe (RNA-Dp). For this purpose, the bRNA-Cp-Neu-MBs were incubated in a mixture solution containing the synthetic target DNA (or the extracted denatured gDNA) and the RNA-Dp. The DNA-RNA heteroduplexes of the MBs were recognized using an $Ab_{DNA/RNA}$ further labeled with an anti-mIgG conjugated with HRP. The resulting magnetic bioconjugates, bearing the sandwich RNA/DNA



Scheme 1. Development of the MBs-assisted amperometric biosensing platform for targeting a specific fragment of the Sin a 1 allergen coding sequence (a). Capture of the modified magnetic bioconjugates to perform the amperometric detection (b). (Created using biorender.com).

heterohybrids labeled with multiple HRP molecules, were captured on the surface of the SPCE working electrode previously placed on a custom-fabricated magnetic holding block for amperometric detection at − 0.20 V (against the Ag pseudo-reference electrode) in stirred solutions using the HQ/H_2O_2 system. This detection potential was previously optimized to carry out amperometric detection at SPCEs using the HRP/HQ/H_2O_2 system [25]. According to the bioassay format employed, the measured cathodic current variation was directly proportional to the concentration of the target DNA.

3.1. Optimization of experimental variables

To achieve the best analytical performance of the proposed methodology, the key experimental parameters involved in the fabrication and operation of the bioplatform were carefully optimized without compromising the pursued objectives of simplicity, affordability, and time-saving. The selection criterion was the largest ratio between the amperometric signals measured with the bioplatforms at − 0.20 V (*vs.* the Ag pseudo-reference electrode) for 1.0 nM of synthetic *Sin a 1* target DNA (signal, S) and for 0.0 nM (blank, B), signal-to-blank, S/B, ratio. All the checked variables, as well as the selected values, are summarized in Table 2.

Firstly, a comparison study was performed using either streptavidin- (Strep-MBs) or neutravidin- (Neu-MBs) functionalized MBs as solid supports. The calculated S/B ratios were 2.5 and 4.5, for Strep-MBs and Neu-MBs, respectively (Fig. 1**a**). These results agree with those previously reported and are attributed to the higher affinity of neutravidin towards biotin [26]. Accordingly, Neu-MBs were selected for developing the proposed methodology. Moreover, a comparison between the results obtained by performing sandwich assays by immobilizing the bRNA-Cp on the surface of the Neu-MBs and the bRNA-Dp as detector probe (bRNA-Cp/bRNA-Dp) or by immobilizing the bRNA-Dp on the surface of the Neu-MBs and using the bRNA-Cp as detector probe (bRNA-Dp/bRNA-Cp (Fig. 1**b**) was carried out. A better S/B ratio was achieved using the probes for what they were initially designed (bRNA-Cp/bRNA-Dp), thus, this protocol was selected for the development of the methodology. Moreover, further work was made using a non-biotinylated RNA-Dp.

The optimization experiments regarding the Neu-MBs suspension volume (Fig. 2**a**) shows as the S/B ratio increased with Neu-MBs suspension volume up to 4.0 μL and decreased for larger volumes, which can be attributed to the increase in the electron transfer resistance occurring for large MBs loadings on the SPCE [27]. The dependence of the bRNA-Cp loading onto Neu-MBs is displayed in Fig. 2**b**. The specific amperometric current (grey bars) increased with the amount of the bRNA-Cp over the 0.05–0.5 μM range and decreased for larger concentrations, probably due to impaired hybridization to the target DNA when too many bRNA-Cp molecules are immobilized on the Neu-MBs [21]. Therefore, 0.5 μM was selected for further work. As it can be observed in Fig. 2**c, 30** min was enough to allow an efficient immobilization of the bRNA-Cp with the target DNA. To develop a simpler and shorter assay protocol, the number of steps involved in the bioplatform preparation was optimized by comparing the amperometric responses provided by the bioplatforms prepared using the following protocols (all of them involving 30 min incubation steps and starting from the bRNACp-MBs):

- Protocol 1: two sequential incubation steps with solutions containing (1) the synthetic target DNA and RNA-Dp and (2) $Ab_{DNA/RNA}$ and HRP-anti-mIgG.
- Protocol 2: three sequential incubation steps with (1) a mixture solution containing the synthetic target DNA and RNA-Dp, (2) a $Ab_{DNA/RNA}$ solution and (3) a HRP-anti-mIgG solution.
- Protocol 3: three sequential incubation steps with the (1) synthetic target DNA, (2) RNA-Dp and (3) a mixture solution containing $Ab_{DNA/RNA}$ and HRP-anti-mIgG.
- Protocol 4: four sequential incubation steps with the (1) synthetic target DNA, (2) RNA-Dp, (3) $Ab_{DNA/RNA}$ and (4) HRP-anti-mIgG.

Fig. 2**d** shows as the protocol 1 provided the best S/B ratio. This can be attributed to the improved efficiency of the hybridization and labeling reactions in homogeneous solutions. Since this protocol also allowed developing a simpler and shorter assay methodology, it was selected for the bioplatform preparation. The effect of the RNA-Dp concentration on the bioplatform response was evaluated over the 0.05 to 0.5 μM concentration range (Fig. 2**e**). Larger S/B ratio was found for a 0.25 μM RNA-Dp concentration. Fig. 2**f** shows as the specific currents measured in the presence of the synthetic target DNA increased with the mixture RNA-Dp/synthetic target DNA incubation time. However, the maximal S/B ratio was reached for 60 min, which was selected for further work. A larger S/B ratio was found for a 1.0 μg mL^{-1} $Ab_{DNA/RNA}$ concentration with slightly higher non-specific signals for larger antibody concentrations (Fig. 2**g** white bars). Fig. 2**h** allowed selecting an optimal HRP-anti-mIgG dilution of 1/2500 and, regarding the incubation time for the $Ab_{DNA/RNA}$ and HRP-anti-mIgG mixture (Fig. 2**i**), 15 min were sufficient to achieve larger S/B ratios, decreasing for longer incubation times due to an increase in the non-specific adsorptions.

It is essential to emphasize at this point that, working under the optimized experimental conditions, the labeling of the sandwich

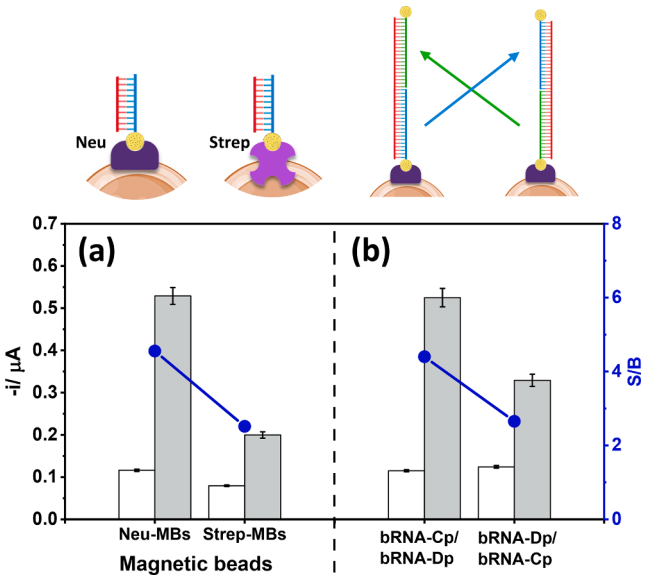


Fig. 1. Comparison of the amperometric responses provided by the bioplatforms for 0 (blank, B, white bars) and 1 nM of synthetic *Sin a 1* target DNA (signal, S, grey bars) corresponding to a specific fragment of the Sin a 1 allergen coding sequence, and the obtained S/B ratio values (blue line and circles) using Strep-MBs or Neu-MBs (a) and different sandwich hybridization configurations (b). (Created using biorender.com).

Table 2
Assayed and selected experimental variables involved in the performance of the developed bioplatform for Sin a 1 determination.

Parameter	Tested interval	Selected value
MBs	Neu or Strep	Neu
Neu-MBs, μL	1 – 7	4
[bRNA-Cp], μM	0.05 – 2.5	0.5
$t_{bRNA\text{-}Cp}$, min	15 – 120	30
Steps	2 – 4	2
$t_{synthetic\ Sin\ a\ 1\ target\ DNA\ +\ RNA\text{-}Dp}$, min	0 – 120	60
[RNA-Dp], μM	0 – 0.5	0.1
$[Ab_{DNA/RNA}]$, μg mL^{-1}	0 – 4.0	1.0
[HRP-anti-mIgG], dilution	1/500 – 1/10000	1/2500
$t_{AbDNA/RNA\ +\ HRP\text{-}anti\text{-}mIgG}$, min	0 – 90	15

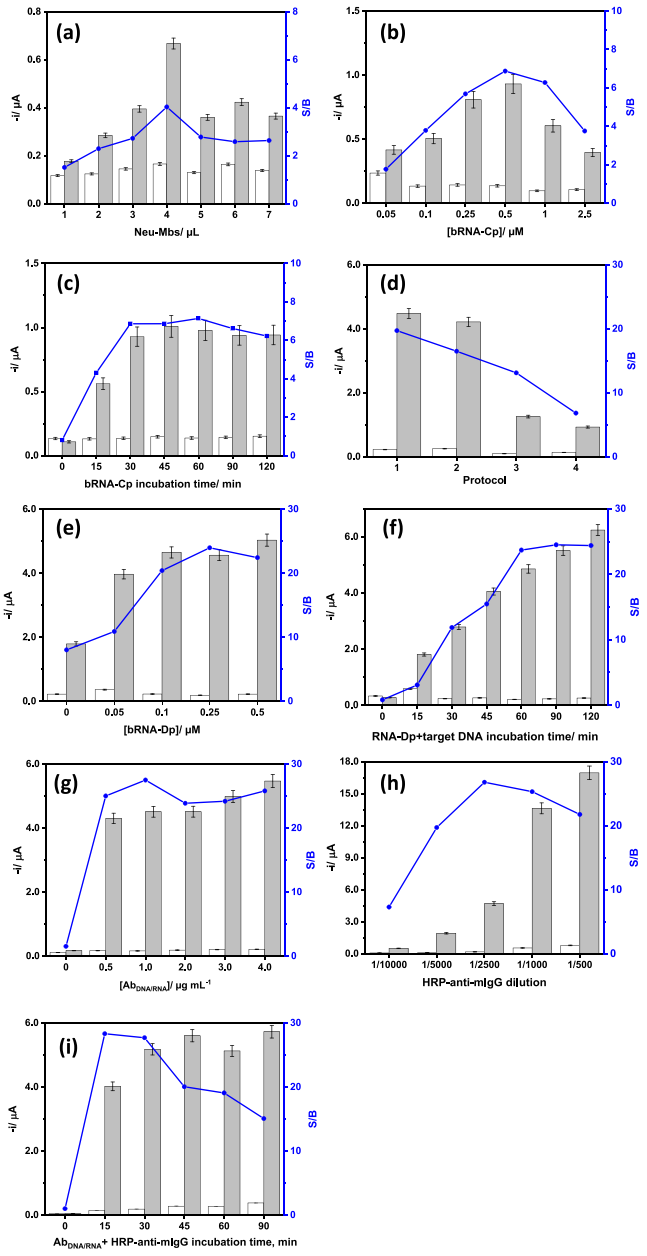


Fig. 2. Optimization of the experimental variables involved in the Sin a 1 bioplatform performance. Dependence of the amperometric responses measured with the bioplatforms for 0 (blank, B, white bars) and 1 nM synthetic *Sin a 1* target DNA (signal, S, grey bars) corresponding to a specific fragment of the Sin a 1 allergen coding sequence, and the obtained S/B ratio values (blue line and circles) with the volume of Neu-MBs (a), bRNA-Cp concentration (b), incubation time of the Neu-MBs in the bRNA-Cp solution (c), number of steps involved in the protocol (d), RNA-Dp concentration (e), incubation time in the mixture solution containing the synthetic target DNA and RNA-Dp (f), concentration of Ab$_{DNA/RNA}$ (g), HRP-anti-mIgG dilution (h), and Ab$_{DNA/RNA}$ and HRP-anti-mIgG mixture solution incubation time (i).

heterohybrid with Ab$_{DNA/RNA}$ and HRP-anti-mIgG provided the necessary response amplification for detecting low concentrations of the synthetic target DNA. As it can be seen in Fig. 3, the responses obtained using conventional labeling (Strep-HRP) of the biotinylated sandwich hybrid formed using bRNA-Dp did not allow detecting the presence of 1 nM synthetic target DNA ("Conventional" bars in Fig. 3, S/B = 1). However, a clear discrimination between the absence and presence of this synthetic target DNA concentration was possible when the sandwich heterohybrid was labeled with Ab$_{DNA/RNA}$ and HRP-anti-mIgG ("60 bp"

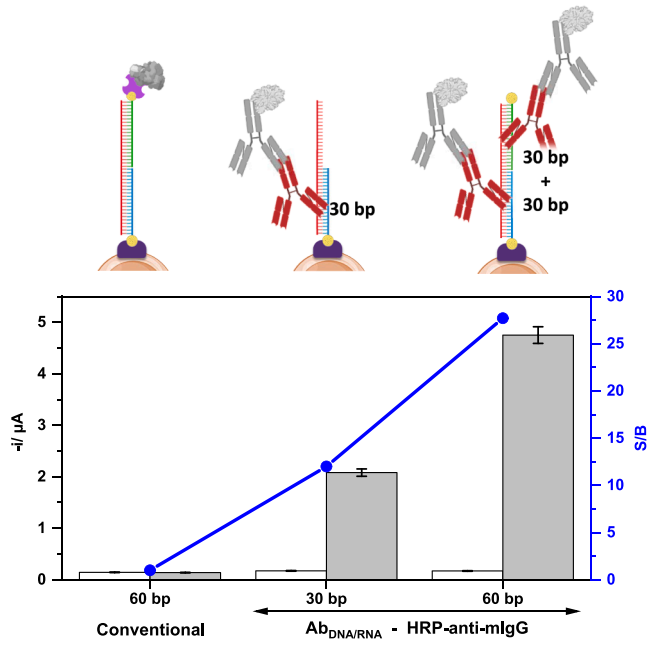


Fig. 3. Comparison of the amperometric responses provided by the developed bioplatform for 0 (blank, B, white bars) and 1 nM synthetic *Sin a 1* target DNA (signal, S, grey bars) corresponding to a specific fragment of the Sin a 1 allergen coding sequence, and the obtained S/B ratio values (blue line and circles) using direct ("30 bp" bars) or sandwich ("60 bp" bars) hybridization formats and Strep-HRP ("Conventional" bars) or Ab$_{DNA/RNA}$ and HRP-anti-mIgG "Ab$_{DNA/RNA}$ and HRP-anti-mIgG" bars) for labeling the sandwich heterohybrids. (Created using biorender.com).

bars in Fig. 3, S/B = 27.7). This amplification factor was provided by the use of Ab$_{DNA/RNA}$ that allows binding several HRP molecules per heterohybrid (ideally 10 considering the length of the heterohybrid and the 6-bp of the antibody epitope [28,29]). This effect was confirmed by comparing the responses obtained using direct hybridization of the 60 *mer*-selected synthetic *Sin a 1* target DNA with the 30 *mer*-biotinylated capture probe (bRNA-Cp) ("30 bp" bars in Fig. 3) or with those obtained using the sandwich-type hybridization configuration involving, apart from the 30 *mer*-bRNA-Cp, a 30 *mer*-detector probe (RNA-Dp) ("60 bp" bars in Fig. 3). The sandwich-type hybridization format provided a 2.3 times larger S/B ratio compared to the direct hybridization strategy (27.7 *vs.* 12.0), as expected considering that the double length of the formed heterohybrid. It is important to note that the use of a sandwich hybridization format instead of a direct one implies two independent hybridization reactions, which also leads to improved selectivity.

3.2. *Analytical performance of the MBs-assisted biosensing platform*

A 60-mer synthetic target DNA whose sequence is complementary to a partial region of the *Sin a 1* gene (synthetic *Sin a 1* target DNA) was used to evaluate the analytical performance of the developed bioplatform. Under the optimized working variables, the calibration curve depicted in Fig. 4 was obtained. A linear dependence (r = 0.998) between the amperometric signal and the concentration of the synthetic *Sin a 1* target DNA was found between 0.01 and 2.0 nM, with slope and intercept values of (3.9 ± 0.2) µA nM^{-1} and (0.2 ± 0.1) µA, respectively. The limit of detection (LOD) was calculated according to the $3s_b/m$ criterion where s_b is the standard deviation of 10 measurements recorded in the absence of synthetic *Sin a 1* target DNA and m the slope value of the calibration plot. A LOD value as low as 3 pM was achieved.

The reproducibility of the amperometric measurements for 0.5 nM synthetic *Sin a 1* target DNA was checked by testing 10 different bioplatforms prepared under the same experimental conditions and in the

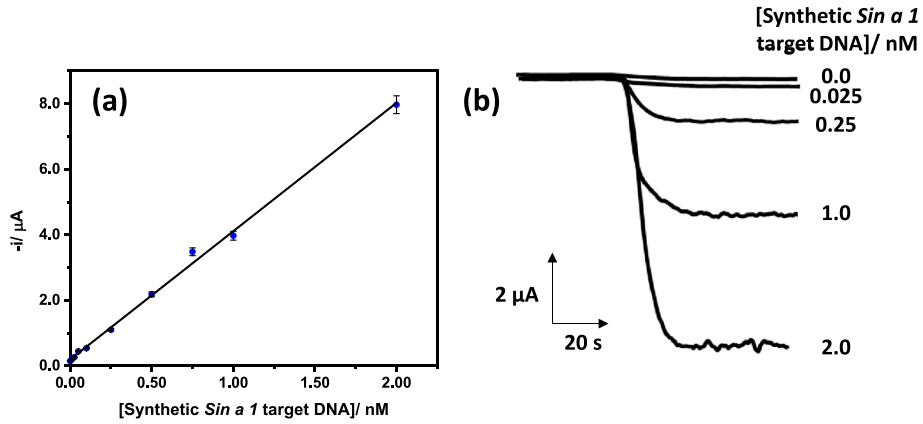


Fig. 4. Calibration plot (a), and amperometric traces (b) obtained with the developed bioplatform for the determination of synthetic *Sin a 1* target DNA.

same day. The calculated relative standard deviation (RSD) value was 3.8 %, thus verifying a good reproducibility for both the bioplatform fabrication and the amperometric transduction.

In addition, bRNACp-MBs conjugates were prepared and stored at 4 °C in microcentrifuge tubes containing 50 µL of filtered B&W solution. No significant differences (results not shown) in the measured S/B ratio for 0.0 and 1.0 nM synthetic *Sin a 1* target DNA were apparent for a period of 14 days (no longer times were assayed). The good storage stability of the bioconjugates allows the preparation of bRNACp-MBs batches and their storage at 4 °C, for the further performing of the determination, when necessary, in just 75 min. This assay time is similar or slightly shorter than that required by the previously reported strategies for detecting adulteration with horsemeat [30] or the Sola l 7 allergenic protein [21], 75 and 90 min, respectively.

The lack of reported biosensors, electrochemical or not, for the determination of mustard using the Sin a 1 allergen coding sequence avoids the comparison of the developed bioplatform analytical performance with other biosensing strategies. However, Sin a 1 was determined by LC-MS/MS [15], achieving a detection limit of 0.25 ppm. On the other hand, the development of rapid, portable, and inexpensive analytical systems is a trend in the development of analytical methods, as real time and on-site measurements are highly demanded [31]. In this regard, electrochemical sensors possess attractive properties compared to more traditional techniques like high performance liquid chromatography (HPLC), LC-MS/MS, capillary electrophoresis, colorimetry or fluorimetry [32], which require more expensive instrumentation and, in general, involve time consuming methods with multistage sample preparation and expert operators [31]. In this sense, electrochemical devices need much less cumbersome operation and less energy consumption, which is translated to greater affordability and user-friendliness [31–34]. Besides, some PCR-based methods have been reported for the determination of mustard targeting different allergens and using different allergen coding sequences. In this context, Musrtop *et al.*, developed a real-time PCR method for the detection of mustard which, due to the primers design, was not specific for mustard showing cross-reactivity with all *Brassica* species tested, including rapeseed and radish [35]. The real-time PCR method reported by Fuchs *et al.* enabled the specific detection of white mustard and did not show cross-reactivity with the tested species including 12 *Brassica* species [36]. Later, the same research group developed a real-time PCR assay for the simultaneous determination of black and brown mustard down to 0.1 pg, with minor cross-reactivity with white mustard and other species [37]. A duplex real-time PCR method was reported allowing the simultaneous detection of traces of white mustard and celery roots. The duplex assay did not show cross-reactivity with different members of the *Brassicaceae* family, and the achieved LOD was 1 ng mL^{-1} for white mustard [38]. Following the same concept, a duplex real-time PCR assay for the simultaneous detection of white, black, and brown mustard did not show cross-reactivity with other *Brassicaceae* species [1]. The achieved LODs for black and white mustard DNA were 0.2 ng mL^{-1} (1 pg DNA) and 2 ng mL^{-1} (10 pg), respectively. In addition, for mixtures of white and brown mustard DNA, LODs were 0.2 ng mL^{-1} (1 pg DNA) and 0.02 ng mL^{-1} (0.1 pg), respectively. Mustrop *et al.*, also reported a multiplex quantitative ligation dependent probe amplification (MLPA) assay to simultaneously determine mustard and seven allergenic foods. However, the assay also detected other members of the *Brassicaceae* family [39]. When the comparison is made with the results provided by the developed bioplatform, a similar LOD was achieved (3 pM; ~0.11 ng mL^{-1}; ~2.75 pg). However, unlike the PCR-based assays, this high sensitivity was achieved by replacing the amplification of the target nucleic acid by amplification of the signal through multienzymatic labeling employing commercial reagents and using affordable cost and low power requirement instrumentation for detection. These analytical characteristics make the developed bioplatform competitive in terms of assay time, affordability, handling by non-specialized personnel and applicability at the point of care, with other available technologies.

Importantly, LOD of few pM were obtained with a PCR-free biosensing methodology exploiting the formation of DNA/RNA heterohybrids and their tagging with multiple HRP molecules using $Ab_{DNA/RNA}$. A similar methodology was reported by our group for the determination of DNAs corresponding to specific fragments of the horse mitochondrial DNA D-loop region [30] or of the gene encoding the *Sol a 7* allergenic protein [21] enabling the detection of adulterations with horsemeat and tomato seeds, respectively.

3.3. Selectivity

The selectivity of the method was tested by comparing the amperometric responses obtained in the absence and in the presence of 0.5 nM synthetic *Sin a 1* target DNA prepared in the absence and in the presence of the same concentration of synthetic DNAs corresponding to specific regions of Sola l 7, Cor a 9 and peanut allergens (*Sola l 7*, *Cor a 9* and peanut target DNAs given in Table 1). The results in Fig. 5 show that the discrimination of synthetic *Sin a 1* target DNA was not significantly affected by the presence of synthetic DNAs from these other plant-relevant allergens although a slight decrease in the S/B ratio was observed in the presence of synthetic *Cor a 9* target DNA.

3.4. Application to the analysis of genomic DNA extracts

The developed electrochemical bioplatform was applied to the determination of the *Sin a l* allergen coding sequence in extracted genomic DNA from yellow mustard seeds. The method required only 50 ng of non-fragmented plant-derived genomic DNA (gDNA) extract. Since the method should allow the detection of mustard in food, the biosensing strategy should not show any cross-reactivity with other sample

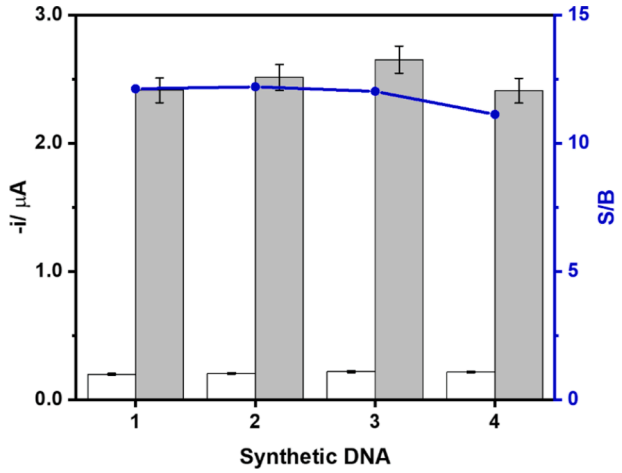


Fig. 5. Amperometric responses obtained with the developed bioplatform in the absence (blank, B, white bars) and in the presence (signal, S, grey bars) of 0.5 nM synthetic *Sin a 1* target DNA prepared without (1) and with synthetic DNA coding sequences from other plant-derived allergens: *Sola 1 7* (2), peanut (3) and *Cor a 9* (4).

components and/or other members of the *Brassicaceae* family. The close phylogenetic proximity between mustard and rapeseed and other members of the *Brassicaceae* family including turnip, radish, cabbage, cauliflower, and broccoli, implies that the characteristics of their 2S allergens are very similar (sequence identity between 86 and 92 %) and they can provoke cross-reactivity between species [22,40]. In this context, genomic DNA extracted from radish was selected to check the cross-reactivity of the bioplatform against the *Brassicaceae* family. On the other hand, a significant association between allergies to nuts and other spices, such as walnuts and sesame, have also been reported [41]. In fact, it has been claimed that more than 50 % of patients allergic to mustard exhibit hypersensitivity to other different vegetable foods, mainly nuts and legumes. Mustard and nuts are both seeds, thus they may have common allergens with similar functions. In fact, different seeds share 2S storage albumins or their precursors, which have been recognized as major allergens [42].

Accordingly, the applicability of the developed bioplatform for the selective and sensitive detection of Sin a 1 was tested by comparing the amperometric responses provided by the developed bioplatform for 50 ng of gDNA extracted from yellow radish, hazelnut, and yellow mustard seeds. Fig. 6 shows as amperometric responses significantly different from that of the blank were only obtained for gDNA extracted from yellow mustard, thus demonstrating the high selectivity of the developed methodology involving these challenging samples, and the ability to perform the determination with no prior fragmentation or amplification and just after a denaturation step. These results should be attributed, in terms of selectivity, to the use of a sandwich hybridization format and the specificity of the commercial antibodies for the type of generated heterohybrids. As already commented, the high sensitivity is due to the use of MBs as solid supports which improves the efficiency of the affinity reactions and to the amplification of the signal derived from the heterohybrid labeling with multiple HRP molecules [29,43].

4. Conclusions

We report the first disposable electrochemical nucleic acid sensor for the detection of mustard through the selective and sensitive PCR-free detection of a specific fragment of the Sin a 1 allergen coding sequence. The developed bioplatform shows an excellent analytical performance without requiring nucleic acid amplification. It is based on a sandwich hybridization assay performed on the surface of magnetic microsupports that leads to the formation of RNA/DNA heteroduplexes

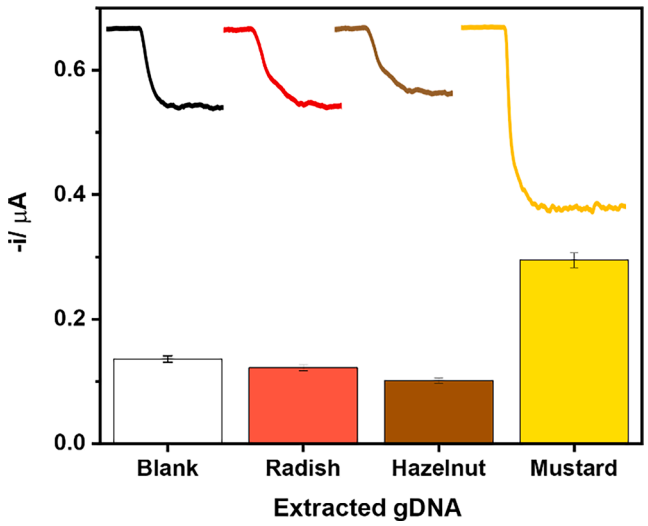


Fig. 6. Amperometric responses and real traces measured with the bioplatform constructed to determine the Sin a 1 allergen coding sequence in the absence of *Sin a 1* target DNA (blank) and for 50 ng of denatured gDNA extracted from radish, hazelnut, and mustard.

further labelled with a commercial antibody, which binds regions of only 6 bp in the RNA heterohybrids, and a secondary HRP-conjugated antibody, followed by the amperometric transduction at SPCEs. The developed MBs-assisted bioplatform exhibits a good sensitivity (LOD of 3.0 pM for the synthetic *Sin a 1* target DNA) and the analysis can be performed in just 75 min (starting from bRNACp-MBs) using only 50 ng of extracted raw gDNA without previous fragmentation and after a denaturing step.

The simple handling of the bioplatform, its versatility (it can be easily adapted to the determination of other targets just by changing the oligonucleotide sequence) and the relatively low cost make this biotool a good alternative against conventional methods used in routine analyses, not only in food safety control and consumer protection but also in diagnostics, and environmental monitoring.

CRediT authorship contribution statement

María Gamella: Methodology, Investigation, Supervision, Writing – review & editing. **Anabel Laza:** Methodology, Investigation. **Jorge Parrón-Ballesteros:** Methodology, Investigation. **Cristina Bueno:** Methodology, Investigation. **Víctor Ruiz-Valdepeñas Montiel:** Conceptualization, Supervision. **María Pedrero:** Supervision. **Franco A. Bertolino:** . **José M. Pingarrón:** Resources. **Mayte Villalba:** Conceptualization, Supervision, Resources, Funding acquisition. **Susana Campuzano:** Conceptualization, Supervision, Resources, Funding acquisition.

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

Data will be made available on request.

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References

[1] M. Palle-Reisch, M. Cichna-Markl, R. Hochegger, Development and validation of a duplex real-time PCR assay for the simultaneous detection of three mustard species (Sinapis alba, Brassica nigra and Brassica juncea) in food, Food Chem. 153 (2014) 66–73.

[2] A. Sharma, A.K. Verma, R.K. Gupta, N.D. Dwivedi, P. Dwivedi, Comprehensive review on mustard-induced allergy and implications for human health, Clinic. Rev, Allerg. Immunol. 57 (2019) 39–54.

[3] M. Planque, T. Arnould, N Gillard, Food allergen analysis: Detection, quantification and validation by mass spectrometry, in Allergen. London, United Kingdom. IntechOpen, 2017 [Online]. Available: https://www.intechopen.com/chapters/55777 doi: 10.5772/intechopen.69361.

[4] Regulation (Eu) No. 1169/2011 of the European parliament and of the council of 25 October 201. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1169&from=EN.

[5] M.Q.I. Spanjersberg, A.C. Knulst, A.G. Kruizinga, G. Van Duijn, G.F. Houben, Concentrations of undeclared allergens in food products can reach levels that are relevant for public health, Food Add. Contam. Part A 27 (2010) 169–174.

[6] B.C. Remington, J.L. Baumert, W.M. Blom, G.F. Houben, S.L. Taylor, A. G. Kruizinga, Unintended allergens in precautionary labelled and unlabelled products pose significant risks to UK allergic consumers, Allergy 70 (2015) 813–819.

[7] I. Pádua, A. Moreira, P. Moreira, F. Melo de Vasconcelos, R. Barros, Impact of the regulation (EU) 1169/2011: Allergen-related recalls in the rapid alert system for food and feed (RASFF) portal, Food Control 98 (2019) 389–398.

[8] L. Monaci, A. Visconti, Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives, Trends Food Sci. Technol. 21 (2010) 272–283.

[9] M. Prado, I. Ortea, S. Vial, J. Rivas, P. Calo-Mata, J. Barrox-Velázquez, Advanced DNA- and protein-based methods for the detection and investigation of food allergens, Crit. Rev. Food Sci. Nutr. 56 (2016) 2511–2542.

[10] M. Abbott, S. Hayward, W. Ross, S.B. Godefroy, F. Ulberth, A.J. Van Hengel, J. Roberts, H. Akiyama, B. Popping, J.M. Yeung, P. Wehling, S. Taylor, R.E. Poms, P. Delahaut, Validation procedures for quantitative food allergen ELISA methods: Community guidance and best practices, J. AOAC Int. 93 (2010) 442–450.

[11] I. Dominguez, A.G. Frenich, R. Romero-González, Mass spectrometry approaches to ensure food safety, Anal. Meth. 12 (2020) 1148–1162.

[12] R.C. Alves, M.F. Barroso, M.B. González-García, M.B.P.P. Oliveira, C. Delerue-Matos, New trends in food allergens detection: Toward biosensing strategies, Crit. Rev. Food Sci. Nutr. 56 (2016) 2304–2319.

[13] C.H. Parker, S.E. Khuda, M. Pereira, M.M. Ross, T.-J. Fu, X. Fan, Y. Wu, K. M. Williams, J. DeVries, B. Pulvermacher, B. Bedford, X. Zhang, L.S. Jackson, Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry, J. Agric. Food Chem. 63 (2015) 10669–10680.

[14] T. Holzhauser, Protein or no protein? Opportunities for DNA-based detection of allergenic foods, J. Agric. Food Chem. 66 (2018) 9889–9894.

[15] M. Posada-Ayala, G. Alvarez-Llamas, A.S. Maroto, X. Maes, E. Muñoz-Garcia, M. Villalba, R. Rodríguez, M. Perez-Gordo, F. Vivanco, C. Pastor-Vargas, J. Cuesta-Herranz, Novel liquid chromatography–mass spectrometry method for sensitive determination of the mustard allergen Sin a 1 in food, Food Chem. 183 (2015) 58–63.

[16] R. Linacero, A. Sanchiz, I. Ballesteros, C. Cuadrado, Application of real-time PCR for tree nut allergen detection in processed foods, Crit. Rev. Food Sci. Nut. 60 (2020) 1077–1093.

[17] S. Santiago-Felipe, L.A. Tortajada-Genaro, R. Puchades, A. Maquieira, Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis, Anal. Chim. Acta 811 (2014) 81–87.

[18] J.H. Shina, Y.V.M. Reddya, T.J. Park, J.P. Parka, Recent advances in analytical strategies and microsystems for food allergen detection, Food Chem. 371 (2022), 131120, https://doi.org/10.1016/j.foodchem.2021.131120.

[19] A. Curulli, Electrochemical biosensors in food safety: Challenges and perspectives, Macromolecules 26 (2021) 2940, https://doi.org/10.3390/molecules26102940.

[20] V. Ruiz-Valdepeñas Montiel, R.M. Torrente-Rodríguez, G. González de Rivera, A. J. Reviejo, C. Cuadrado, R. Linacero, F.J. Gallego, S. Campuzano, J.M. Pingarrón, Amperometric determination of hazelnut traces by means of Express PCR coupled to magnetic beads assembled on disposable DNA sensing scaffolds, Sens. Actuators B Chem. 245 (2017) 895–902.

[21] M.A. Pereira-Barros, M.F. Barroso, L. Martín-Pedraza, E. Vargas, S. Benedé, M. Villalba, J.M. Rocha, S. Campuzano, J.M. Pingarrón, Direct PCR-free electrochemical biosensing of plant-food derived nucleic acids in genomic DNA extracts. Application to the determination of the key allergen Sola l 7 in tomato seeds, Biosens. Bioelectron. 137 (2019) 171–177.

[22] R.I. Monsalve, M. Villalba, R. Rodríguez, Allergy to mustard seeds: The importance of 2S albumins as food allergens, Internet Symposium on Food Allergens 3 (2001) 57–69.

[23] R.I. Monsalve, C. López-Otín, M. Villalba, R. Rodríguez, A new distinct group of 2 S albumins from rapeseed. Amino acid sequence of two low molecular weight napins, FEBS Lett. 295 (1991) 207–210.

[24] R.I. Monsalve, M.A. González de la Peña, L. Menéndez-Arias, C. López-Otín, M. Villalba, R. Rodríguez, Characterization of a new oriental-mustard (Brassica juncea) allergen, Bra j IE: detection of an allergenic epitope, Biochem. J. 293 (1993) 625–1362.

[25] M. Gamella, S. Campuzano, F. Conzuelo, A.J. Reviejo, J.M. Pingarrón, Amperometric magnetoimmunosensors for direct determination of D-Dimer in Human Serum, Electroanalysis 24 (12) (2012) 2235–2243.

[26] T.T. Nguyen, L.K. Sly, J.C. Conboy, Comparison of the energetics of avidin, streptavidin, neutravidin, and anti-biotin antibody binding to biotinylated lipid bilayer examined by second-harmonic generation, Anal. Chem. 84 (2012) 201–208.

[27] O.A. Loaiza, S. Campuzano, M. Pedrero, M.I. Pividori, P. García, J.M. Pingarrón, Disposable magnetic DNA sensors for the determination at the attomolar level of a specific Enterobacteriaceae family gene, Anal. Chem. 80 (2008) 8239–8245.

[28] A.J. Qavi, J.T. Kindt, M.A. Gleeson, R.C. Bailey, Anti-DNA:RNA antibodies and silicon photonic microring resonators: Increased sensitivity for multiplexed microRNA detection, Anal. Chem. 83 (2011) 5949–5956.

[29] V. Ruiz-Valdepeñas Montiel, E. Povedano, E. Vargas, R.M. Torrente-Rodríguez, M. Pedrero, A.J. Reviejo, S. Campuzano, J.M. Pingarrón, Comparison of different strategies for the development of highly sensitive electrochemical nucleic acid biosensors using neither nanomaterials nor nucleic acid amplification, ACS Sens. 3 (2018) 211–221.

[30] V. Ruiz-Valdepeñas Montiel, M.L. Gutiérrez, R.M. Torrente-Rodríguez, E. Povedano, E. Vargas, A.J. Reviejo, R. Linacero, F.J. Gallego, S. Campuzano, J. M. Pingarrón, Disposable amperometric polymerase chain reaction-free biosensor for direct detection of adulteration with horsemeat in raw lysates targeting mitochondrial DNA, Anal. Chem. 89 (2017) 9474–9482.

[31] N. Jornet-Martínez, Y. Moliner-Martínez, C. Molins-Legua, P. Campíns-Falcó, in: Trends for the development of in situ analysis devices, in Encyclopedia of Analytical Chemistry, 2017, https://doi.org/10.1002/9780470027318.a9593.

[32] S. Saranya, J. Jency Feminus, B. Geetha, P.N. Deepa, Simultaneous detection of glutathione, threonine, and glycine at electrodeposited RuHCF/rGO–modified electrode, Ionics 25 (2019) 5537–5550.

[33] C. Pérez-Ràfols, Y. Liu, Q. Wang, M. Cuartero, G.A. Crespo, Why not glycine electrochemical biosensors? Sensors (Basel) 20(14) (2020) 4049, https://doi.org/10.3390/s20144049.

[34] D. Li, C. Wu, X. Tang, Y. Zhang, T. Wang, Electrochemical sensors applied for in vitro diagnosis, Chem. Res. Chin. Univ. 37 (4) (2021) 803–822.

[35] S. Mustorp, C. Engdahl-Axelsson, U. Svensson, A. Holck, Detection of celery (Apium graveolens), mustard (Sinapis alba, Brassica juncea, Brassica nigra) and sesame (Sesamum indicum) in food by real-time PCR, Eur. Food Res. Technol. 226 (2008) 771–778.

[36] M. Fuchs, M. Cichna-Markl, R. Hochegger, Development and validation of a real-time PCR method for the detection of white mustard (Sinapis alba) in foods, J. Agric. Food Chem. 58 (2010) 11193–11200.

[37] M. Palle-Reisch, M. Wolny, M. Cichna-Markl, R. Hochegger, Development and validation of a real-time PCR method for the simultaneous detection of black mustard (Brassica nigra) and brown mustard (Brassica juncea) in food, Food Chem. 138 (2013) 348–355.

[38] M. Fuchs, M. Cichna-Markl, R. Hochegger, Development and validation of a duplex real-time PCR method for the simultaneous detection of celery and white mustard in food, Food Chem. 141 (2013) 229–235.

[39] S.L. Mustorp, S.M. Drømtorp, A.L. Holck, Multiplex, quantitative, ligation-dependent probe amplification for determination of allergens in food, J. Agric. Food Chem. 59 (2011) 5231–5239.

[40] R.I. Monsalve, M.A. González de la Peña, C. López-Otín, A. Fiandor, C. Fernández, M. Villalba, R. Rodriguez, Detection, isolation and complete aminoacid sequence of an aeroallergenic protein from rapeseed flour, Clin. Exp. Allergy 27 (1997) 833–841.

[41] C. Bueno, L. Martín-Pedraza, J. Cuesta-Herranz, M. Villalba, Is the cross-reactivity of Sin a 1, 2S albumin from mustard seeds, exclusively restricted to Brassicaceae members? JSM Allergy Asthma 1 (2016) 1001.

[42] T. Caballero, M. Soledad San-Martín, M.A. Padial, J. Contreras, R. Cabañas, P. Barranco, M.C. López-Serrano, Clinical characteristics of patients with mustard hypersensitivity, Ann. Allergy Asthma Immunol. 89 (2002) 166–171.

[43] S. Campuzano, P. Yánez-Sedeño, J.M. Pingarrón, Electrochemical biosensing of microribonucleic acids using antibodies and viral proteins with affinity for ribonucleic acid duplexes, Electrochim. Acta 230 (2017) 271–278.