Microchemical Journal 195 (2023) 109403

Contents lists available at ScienceDirect

Microchemical Journal

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

First biosensing platform for detecting traces of almonds in processed foods by electrochemical determination of the allergenic protein Pru du 6

Alba Civera [a,b], Sofiia Tvorynska [c,d], Maria Gamella [a], Rebeca M. Torrente-Rodríguez [a], María Pedrero [a], Patricia Galán-Malo [e], Luis Mata [e], Lourdes Sánchez [b], Jiří Barek [d], José M. Pingarrón [a], Susana Campuzano [a,*], María D. Pérez [b,*]

[a] Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, 28040 Madrid, Spain
[b] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón (IA2) (Universidad de Zaragoza-CITA), Miguel Servet, 177, 50013 Zaragoza, Spain
[c] J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences, Dolejskova 3, 182 23 Prague 8, Czech Republic
[d] Charles University, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry, Hlavova 2030/8, 128 43 Prague 2, Czech Republic
[e] ZEULAB S.L., Polígono PLAZA, Bari, 25 Duplicado, 50197 Zaragoza, Spain

ARTICLE INFO

Keywords:
Electrochemical immunoplatform
Almond
Pru du 6
Incurred cookies
Biosensor

ABSTRACT

A novel electrochemical bioplatform for Pru du 6 determination, one of the most abundant and allergenic almond proteins, was developed to detect traces of almonds in processed foods. The bioplatform combines the application of a sandwich immunoassay on the surface of magnetic beads using specific antibodies unmodified or conjugated to horseradish peroxidase as capture and detection antibodies. The resulting magnetic bioconjugates are trapped on the surface of disposable carbon electrodes, and amperometry in the presence of hydrogen peroxide and hydroquinone is employed to measure the change in the cathodic current, which is proportional to Pru du 6 concentration. The developed bioplatform exhibited good selectivity and sensitivity, offering a detection limit of 0.12 ng mL^{-1} of Pru du 6. It could determine Pru du 6 in raw dough and baked cookies incurred with 0.2 μg g^{-1} of almond protein with acceptable precision, providing results comparable to those obtained using an ELISA technique.

1. Introduction

The prevalence of nut allergy has been reported to range from 0.05 to 4.9 % for probable allergy, which includes reported reactions mediated by IgE or a doctor's diagnosis, and less than 2 % for confirmed oral food challenges. In addition, nut allergy is characterized by its tendency to persist throughout life or to have low rates of resolution in childhood [1]. Currently, rigorous abstention of allergen ingestion is the most efficient way to avoid an allergic reaction.

Although the exact prevalence of almond allergy remains unknown, it has been estimated to affect approximately 0.3 % children [2] and 0.7 % adults, with severe reactions occurring in approximately 57.2 % of allergic individuals [3]. In addition, almond is considered a priority allergen worldwide, as it causes a high proportion of anaphylaxis, which can be fatal or near fatal, even if only traces are consumed [4].

Tree nuts are widely used in the manufacture of many food products such as bakery, pastry, chocolate and confectionary, among others, due to their nutritional properties, flavor characteristics and health benefits [5]. When added as ingredients, regulations in most countries, including the European Union (Regulation EU 1169/2011) and the United States (Food Allergen Labeling and Consumer Protection Act of 2004, FALCPA), it is required mandatory labelling of the tree nuts, including almonds, so that consumers can identify relevant allergens. However, the presence of hidden allergens due to accidental contamination during processing is a serious health hazard for allergic consumers that is not considered in this legislation.

To overcome this limitation, food producers can voluntarily apply Precautionary Allergen Labelling (PAL) in case of unintended presence of allergens to advise allergic consumers [6]. However, the deficiency of a regulatory framework for the management of hidden allergens and the absence of quantitative thresholds for many allergens have led the food industry to make an abusive use of PAL, reducing the range of food

* Corresponding authors.
E-mail addresses: susanacr@quim.ucm.es (S. Campuzano), dperez@unizar.es (M.D. Pérez).

https://doi.org/10.1016/j.microc.2023.109403
Received 30 July 2023; Received in revised form 29 August 2023; Accepted 20 September 2023
Available online 25 September 2023

choices and inducing risky behavior in allergic consumers [7]. Therefore, the issue of risk assessment and management of hidden allergens is a major challenge for food producers. The indiscriminate use of PAL is mainly because reference doses have not yet been established for many allergenic foods.

The Voluntary Incidental Trace Allergen Labelling (VITAL) program was developed in Australia and New Zealand to provide a risk-based methodology for the food industry to assess the impact of allergen cross-contamination and implement appropriate use of PAL. VITAL program recommends protein action levels considering the reference doses of the allergen to protect 99 % of allergic consumers (ED01) and the serving size. Although the updated VITAL 3.0 version does not include the reference dose for almond, a similar dose to that indicated for hazelnut (ED01 of 0.1 mg) could be assumed [8]. However, a higher reference dose of 3 mg and 1 mg of total protein from the allergenic source has been established for hazelnut and almond, respectively, in the Summary report of the FAO/WHO [9]. These estimates are useful as suggestive thresholds for food industry and laboratories to make decisions on whether to include PAL. As it can be deduced, the advancement of sensitive, specific and reliable analytical techniques to detect low concentrations of allergenic ingredients in food is necessary to support the risk assessment for management of food allergens.

Among the almond allergenic proteins, Pru du 6 or amandin or almond major protein (AMP) is the major storage protein, accounting for more than 50 % of the total almond protein. It belongs to the cupin superfamily and the 11S globulin family. Its hexametric structure of about 360 kDa is composed of basic (34–42 kDa) and acidic (20–22 kDa) subunits linked by a disulfide bond, which confers the protein a high thermal resistance [10]. Recently, Pru du 6 has been reported to be a good specific biomarker for almond allergy, as approximately 83 % of almond allergic patients have specific IgE against it [11].

Several analytical techniques have been used to detect traces of almonds, such as immunochemical tests, mass spectrometry [12] and DNA-based methodologies [13,14]. Immunochemical techniques such as enzyme-linked immunosorbent assays (ELISAs) and lateral flow immunoassays (LFIAs) are widely employed to detect allergens due to their high sensitivity, specificity, technical simplicity and quick response [15–20].

In this context, electrochemical bioplatforms have been successfully used to determine molecular markers of relevance in different fields including the food industry. They provide unique features such as simplicity, high sensitivity and selectivity, cost-effectiveness, miniaturization, customization, versatility and adaptability. These characteristics allow point-of-need multiplexed and/or multiomics biomarker profiling in complex and scarcely treated samples, as well as shorter assay times and the requirement of smaller sample quantities for allergen determination in comparison with other available methods. Indeed, electrochemical bioplatforms have been successfully applied in recent years for the investigation and determination of less explored allergens at both protein [21–24] and genetic [25] levels.

Knowing this state of the art, this work reports the first electrochemical bioplatform for the selective and sensitive quantification of almond traces in processed foods through the determination of the protein Pru du 6. This bioplatform also exploits the advantages offered by the implementation of sandwich immunoassay formats on the surface of magnetic beads (MBs) in terms of sensitivity, speed and minimization of the matrix effect and amperometric transduction on screen-printed carbon electrodes (SPCEs) as others previously described by our research group for the determination of other allergenic proteins [21–24]. However, the biotool proposed in this work is novel in the use of this type of devices for the target protein (Pru du 6), of polyclonal antibodies produced against it purified and prepared in the laboratory. Furthermore, it is novel in its application to the analysis of defatted almond flour and cookie incurred with different percentages of whole ground almond before and after the baking processing, also prepared in the laboratory. The implemented methodology involves the use of unmodified and horseradish peroxidase (HRP) labelled specific polyclonal antibodies against Pru du 6. The magnetic bioconjugates, bearing the HRP-labelled sandwich immunocomplexes, are captured on disposable electrode substrates to perform amperometric detection in the presence of H_2O_2 and hydroquinone (HQ) generating a cathodic current variation proportional to the concentration of Pru du 6 and, therefore, of almond in the analyzed sample.

2. Experimental

2.1. Materials

Food commodities and raw tree nuts were acquired from local markets. Carboxylic acid-modified magnetic beads (HOOC-MBs, 2.7 μm Ø, Dynabeads M-270 carboxylic acid, Cat. No. 14305D) were purchased from Invitrogen™ (Waltham, Ma, EEUU). HiTrap DEAE Cellulose column, Sepharose 6B-CL gel and HiTrap NHS-activated HP column were acquired from GE Healthcare (Piscataway, NJ) and the Lightning-link Horseradish Peroxidase conjugation Kit from Innova Biosciences (Cambridge, UK). ELISA wells maxisorp flat bottom were purchased from Nunc (Roskilde, Denmark). Blocker™ Casein (BB solution) and Blocker™ BSA (10X) were purchased from Thermo Scientific (Waltham. EEUU) and the ELISA substrate containing 3,3́,5,5́-tetramethylbenzidine (TMB) from ZEULAB S.L., (Zaragoza, Spain). N-(3-dimethy-aminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), ethanolamine, hydroquinone (HQ), hydrogen peroxide (H_2O_2, 30 % w/v) and morpholino ethanesulfonic acid (MES) buffer were provided by Sigma. All the reagents used were of analytical grade and solutions containing them were prepared in purified deionized water provided by a Millipore Milli-Q system.

2.2. Apparatus and electrodes

A multi potentiostat (model 1030B, CH Instruments, Austin, TX, USA) driven by the CHI1030B software, screen-printed carbon electrodes (SPCEs, DRP-110 consisting of a single carbon working electrode, ϕ 4 mm), a carbon auxiliary electrode and a silver pseudo-reference electrode, and the specific cable connector (DRP-CAC) (Metrohm-DropSens S.L., Oviedo, Asturias, Spain) and a homemade polymethylmethacrylate (PMMA) casing that has an embedded neodymium magnet (AIMAN GZ) were used for amperometric measurements. A thermomixer MT100 incubator shaker (Universal Labortechnik) and a Dynamag-2 Magnet magnetic separator (Invitrogen Dynal AS) were used for MBs modification.

Spectrophotometric measurements were carried out with a Magellan V 7.1 (TECAN) ELISA plate reader. An ÄKTA Start automated system was used for purification of the target protein and anti-Pru du 6 antibodies. A Mini-PROTEAN Tetra Cell (Bio-Rad) was employed to perform electrophoresis. A Kenwood Titanium Chef KM010 (Woking, United Kingdom) was used for cookies elaboration.

2.3. Purification of Pru du 6

Pru du 6 was isolated as previously described [20]. Briefly, ground almonds were defatted with hexane and proteins were extracted with 0.02 M Tris-HCl buffer, pH 8.0 (1/10 w/v) for 1 h at room temperature. The mixture was centrifuged at 9-000 × g for 30 min and the supernatant was applied onto a HiTrap DEAE Sepharose (5 mL) anion exchange column (dynamic binding capacity 110 mg human serum albumin/mL medium). After washing, retained proteins were eluted using a linear gradient of NaCl (0.0–0.4 M). Fractions containing Pru du 6 were pooled, concentrated, and applied to a Sepharose 6B-CL (85 × 1 cm) column equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 0.1 M NaCl. Chromatographic fractions were analyzed by SDS-PAGE according to Laemmli et al. [26]. The electrophoretic profile of purified Pru du 6 showed the presence of acidic (34–45 kDa) and basic (20–22

kDa) subunits [20]. The purity degree of isolated Pru du 6 determined by densitometry was higher than 95 %.

2.4. Antibody purification and conjugation

Antisera to Pru du 6 were raised in rabbits by immunization with the purified protein as previously described [20]. All the procedures were carried out according to the Ethic Committee Guidelines of the University of Zaragoza for Animal Protection RD 53/2013 following EU Directive 2010/63 used for scientific purposes (Project License 30/19). Purification of polyclonal anti-Pru du 6 antibodies was performed by immunoaffinity using a HiTrap NHS-activated HP column (1 mL) previously coupled with Pru du 6, and conjugation of purified anti-Pru du 6 antibodies with horseradish peroxidase using a commercial conjugation kit as described by [20]. Specific unmodified antibodies were used as capture (CAb) and conjugated antibodies (HRP-DAb) as detection receptors for amperometric and spectrophotometric ELISA determinations.

2.5. Preparation of the magnetic bioconjugates

The determination of Pru du 6 required the generation of sandwich immunocomplexes on HOOC-MBs. Unless stated otherwise, all the incubation steps to prepare the MBs were carried out in microcentrifuge tubes using 25 μL of the corresponding solutions under constant stirring (950 rpm) at room temperature and were followed by several washing steps with 50 μL of the corresponding buffer solution. A magnetic separator was employed to remove the supernatant avoiding the loss of MBs.

The modification procedure of the magnetic particles involved 3-μL aliquots of the HOOC-MBs suspension. After washing twice with 0.025 M MES buffer, pH 5.0 for 10 min, the activation of the MBs HOOC groups was performed by incubation with a freshly prepared EDC/Sulfo-NHS mixture solution containing 50 mg mL^{-1} each in MES buffer for 35 min. After two washing steps, the activated HOOC-MBs were incubated with 25 μL of CAb solution (2.5 μg mL^{-1} in MES buffer) for 30 min. After two washing steps, the residual active groups were blocked with 1 M ethanolamine in 0.1 M phosphate buffer, pH 8.0 for 1 h. The prepared CAb-MBs were washed once using 0.1 M Tris-HCl buffer, pH 7.2, and twice using BB solution, and then filtered and stored at 4 °C until further use.

The immunoassay procedure started with the incubation of the CAb-MBs with 25 μL of standard solutions of Pru du 6 or food extracts for 15 min. After two washings with commercial BB solution, the MBs were incubated with 25 μL of an appropriate dilution of DAb-HRP in BB solution for 15 min. After washing twice with BB solution, the MBs bearing the sandwich immunocomplexes were suspended in 50 μL of 0.05 M phosphate buffer, pH 6.0, to carry out the amperometric determination.

2.6. Amperometric measurements

The magnetic immunoconjugate suspension was pipetted onto the surface of the SPCE working electrodes pre-placed in the PMMA homemade magnetic holder. The SPCE-PMMA holding block was connected to the potentiostat and immersed into an electrochemical cell containing 10 mL of 0.05 M phosphate buffer, pH 6.0, supplemented with 1.0 mM freshly prepared HQ. The measurements were performed under continuous stirring at room temperature, using a detection potential of − 0.20 V vs. the Ag pseudo-reference electrode. After adding 50 μL of 0.1 M H_2O_2 solution in 0.05 M phosphate buffer, pH 6.0, the variation of the cathodic current was monitored until the steady state was reached. The given amperometric signals correspond to the average value of the difference between the steady state of standards or samples and the corresponding background current measured for three replicates. Error bars represent the standard deviation of each set of replicates.

2.7. ELISA measurements

Microtiter wells were coated with 120 μL of CAb against Pru du 6 at a concentration of 1 μg mL^{-1} in 0.05 M sodium carbonate buffer, pH 9.6, overnight at 4 °C. After washing the wells with 300 μL of distilled water, an incubation step with 300 μL of Bloker™ BSA 10-fold diluted in 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl and 140 mM NaCl buffer, pH 7.4 (PBS) was performed for 2 h at room temperature (RT). After three washing steps with PBS containing 0.5 % Tween 20 (PBST), wells were incubated with 100 μL of the standards or samples for 30 min at RT. The wells were then washed with PBST and incubated with an appropriate dilution of the HRP-DAb for 30 min at RT. Following a wash step with PBST, wells were incubated with 100 μL of TMB chromogen solution, and after 30 min of incubation at RT, the enzymatic reaction was stopped by the addition of 50 μL of 2 M H_2SO_4 per well. The absorbance was read at 450 nm using the ELISA plate reader.

2.8. Preparation and analysis of food samples

Whole ground almond, hazelnut and walnut were obtained using a blender and they were defatted with n-hexane (1/5 w/v). The protein content determined by the Kjeldahl method was 20.0 % for whole almond flour and 35.2, 31.4 and 30.8 % for defatted almond, hazelnut and walnut flour, respectively.

Model cookies were prepared at the Pilot Plant of Zaragoza University following the American Association of Cereals Chemists methodology (Method 10-50D) [27]. All the ingredients required to elaborate the cookies (128 g butter, 263.7 g sugar, 4.2 g salt, 5 g sodium bicarbonate and 86.3 g water) were blended with a Kenwood kitchen mixer. Then, percentages of 0.2 % and 2 % of whole ground almond (w/w), which correspond, to 400 and 4000 μg g^{-1} almond protein, respectively, were added, and the dough mixed again. Cookies (20 g, 7 cm diameter) were prepared and baked at 205 °C for 10 min. Lower percentages of almond protein were obtained by mixing adequate amounts of almond-free cookies with cookies containing 400 μg g^{-1} of almond.

For the extraction of proteins from almond, hazelnut and walnut flours and cookies, (1.00 ± 0.01) g was added with 10 mL of PBS and vortexed for 1 min. Then, the mixture was heated at 60 °C for 15 min in a water bath and centrifuged at 3000 × g for 15 min. The supernatant was collected and analyzed according to the protocols indicated above to determine amperometric and spectrophotometric measurements.

3. Results and discussion

Fig. 1 shows the basis of the developed sandwich immunoplatform. Briefly, specific capture antibodies (CAb) to Pru du 6 were immobilized on the surface of magnetic microcarriers (MBs) and used to selectively sandwich the target protein into the solution with the detector anti-Pru du 6 antibody tagged with horseradish peroxidase (HRP-DAb). The resulting MBs-immunocomplexes were captured on the SPCE working electrode surface with a magnet to perform amperometric reading by monitoring the variation in the measured cathodic current in the presence of the H_2O_2/HQ system (E_{app} = − 0.20 V vs. Ag pseudo-reference electrode).

3.1. Optimization of key experimental variables

The variables concerning the amperometric transduction such as the volume of MBs, pH, detection potential and composition of the supporting electrolyte were previously optimized [28,29]. Therefore, the variables regarding the formation of immunocomplexes on the surface of the MBs such as the concentration of CAb and incubation time with the activated HOOC-MBs, the incubation time of the target protein with the CAb-MBs, the number of steps of the protocol, and the dilution and incubation time of the HRP-DAb were optimized in this study. As the selection criterion for each assessed parameter, the amperometric

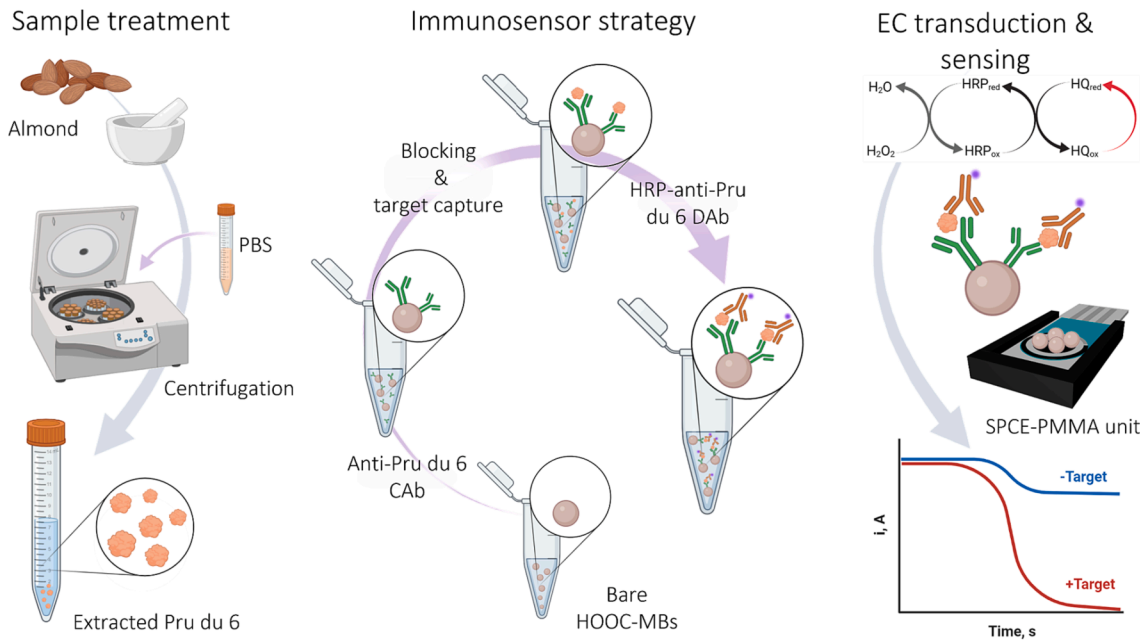

Fig. 1. Immunoplatform assisted by MBs for the determination of almond traces by determining the target protein Pru du 6. Schematic diagrams of the protocols used for the sample treatment, the preparation of the immunoconjugates and their amperometric transduction on screen-printed carbon electrodes (SPCE) and a polymethylmethacrylate (PMMA) unit using the HRP/H_2O_2/HQ system. Capture antibodies (CAb), detector antibodies (HRP-DAb), carboxylic acid-modified magnetic beads (HOOC-MBs), electrochemical (EC). Created with Biorender.

responses in the absence (B, blank) and in the presence of Pru du 6 at 10 ng mL^{-1} (S, signal) were compared and the larger signal-to-blank (S/B) ratio was chosen for further assays.

Results obtained in these optimization studies are depicted in Fig. 2. All the tested variables and ranges as well as the chosen values are indicated in Table 1. A CAb concentration of 2.5 μg mL^{-1} to prepare the CAb-MBs provided a larger S/B ratio (Fig. 2**a**). Higher CAb concentrations did not increase the S/B ratio despite the noticeable signal increase

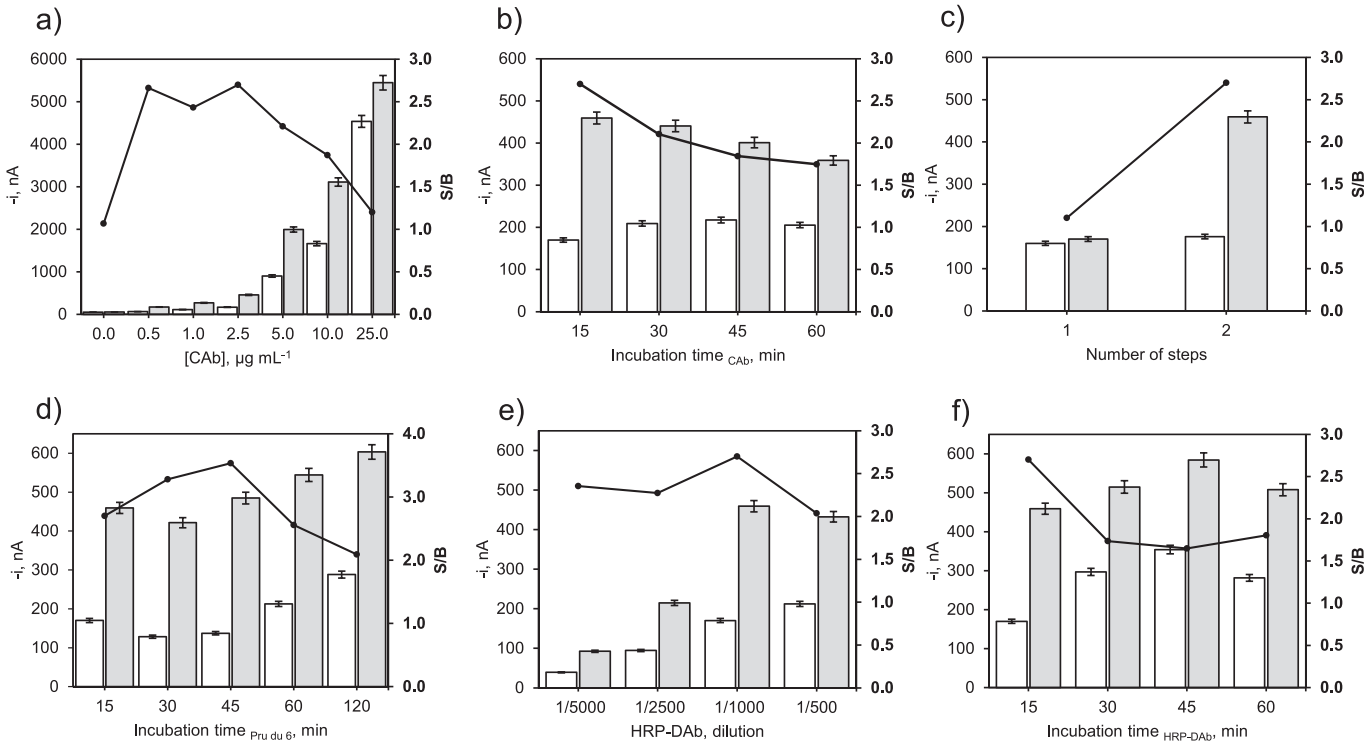

Fig. 2. Optimization studies for the key variables involved in the performance of the developed electrochemical sandwich bioplatform for the amperometric detection of Pru du 6 allergen. Dependence of the amperometric readings obtained with the developed bioplatforms in the absence (blank, B, white bars) and in the presence (signal, S, grey bars) of 10 ng mL^{-1} Pru du 6 standard with the concentration (a) and incubation time (b) of capture antibody (CAb), number of steps required for the assay (c), incubation time of Pru du 6 (d), and concentration (e) and incubation time (f) of detector antibodies (HRP-DAb). Ratio values between S and B (S/B) obtained for each tested variable are displayed as black line and dots.

Table 1
Optimized key variables, tested ranges and selected values for the amperometric determination of Pru du 6 protein with the developed bioplatform.

Variable	Tested range	Selected value
[CAb], μg mL^{-1}	0.0–25.0	2.5
Incubation time with CAb, min	15–60	15
Number of steps	1–2	2
Incubation time with Pru du 6, min	15–120	15
HRP-DAb dilution	1/5000–1/500	1/1000
Incubation time with HRP-DAb, min	15–60	15

that is due to a certain type of non-specific interaction between CAb and HRP-DAb when using high concentrations. These results also confirmed that it was not possible to discriminate the presence of Pru du 6 in the absence of immobilized CAb (0.0 bars), and that Pru du 6 was captured on the MBs through the CAb indicating that sandwich immunocomplexes were formed.

The incubation time selected for the binding of CAb at 2.5 μg mL^{-1} with the activated MBs was 15 min, as a decrease of the S/B ratio was observed for longer times (Fig. 2**b**). This fact was also observed with other electrochemical bioplatforms developed to detect soy allergenic proteins and was attributed to the poorer recognition of the target protein by steric hindrance when too many CAb molecules were immobilized in the MBs [24]. The number of steps employed in the immunoassay is a key parameter to simplify the whole procedure and thus, to reduce the time of the assay. In this study, two different protocols were tested for the formation of the sandwich immunocomplexes. A one-step assay involving the incubation of the CAb-MBs for 30 min with a mixture of Pru du 6 and HRP-DAb (Fig. 2**c**, bars 1), and a two-step assay involving two consecutive incubation steps of 30 min each, first with a solution of Pru du 6 and thereafter with a solution of HRP-DAb (Fig. 2**c**, bars 2). Results obtained showed that only the two-step assay allowed discriminating the presence of Pru du 6 (Fig. 2**c**), which is probably due to a steric hindrance effect or/and to a possible competition between CAb and HRP-DAb for the target protein to form the immunocomplexes.

Fig. 2**d** shows the effect of the incubation time of the CAb-MBs with Pru du 6. Although slightly larger S/B ratios were observed with the time increase until 45 min, a period of 15 min was selected as a remarkable reduction of the assay time could be achieved without substantial loss of response.

Regarding the concentration of HRP-DAb added to the Pru du 6-CAb-MBs to form sandwich immunocomplexes (Fig. 2**e**), results showed that the S/B ratios increased with the antibody concentration up to a 1/1000 dilution and lower S/B ratios were obtained for higher concentrations. Furthermore, the impact of the incubation time of the selected HRP-DAb concentration with Pru du 6-CAb-MBs for periods longer than 15 min resulted in a marked decrease of the S/B ratios (Fig. 2**f**). These facts could be attributed to an increase of the corresponding non-specific adsorption (larger B signals) under those conditions.

3.2. Analytical and operational characteristics

The relationship between the concentration of Pru du 6 and the cathodic current variation provided by the bioplatforms is shown in Fig. 3. The calibration plot showed a linear range between 0.36 and 50 ng mL^{-1} of Pru du 6 (r = 0.998), with a slope value of (26 ± 1) nA mL ng^{-1} and an ordinate intercept of (183 ± 20) nA. The limit of detection (LOD) and the limit of quantification (LOQ) values were determined according to the Ks_b/m criterion being s_b the standard deviation of ten measurements in the absence of Pru du 6, m the slope of the calibration plot and K a value of 3.3 or 10 times for LOD and LOQ, respectively. The obtained LOD and LOQ values were 0.12 and 0.36 ng mL^{-1}, respectively.

As this is the first reported electrochemical immunosensing platform for the determination of Pru du 6, results were compared with other

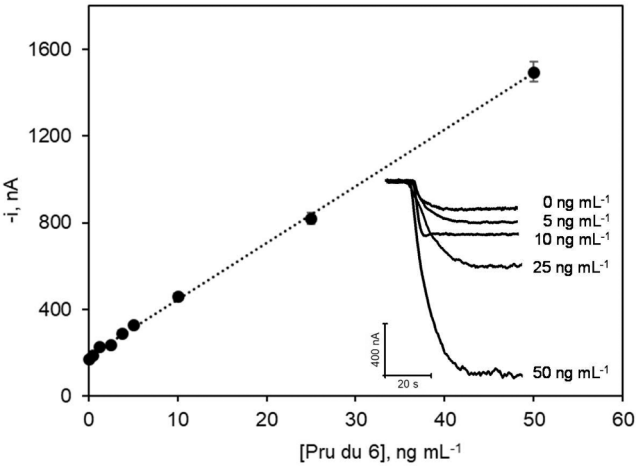

Fig. 3. Calibration plot constructed with the developed bioplatform for the amperometric determination of Pru du 6 standards. Inset: actual amperograms obtained for the indicated concentrations of Pru du 6.

techniques developed to detect almond traces in food. The multiplexed determination of six food allergens, including almond, using an opto-electrical microanalytical system performed by Morais' Group was accomplished with a standard digital compact disc functionalized with highly selective antibodies in a microarray format and an optical detector. This system enabled the simultaneous determination of the selected allergens, with a LOD for almond of 3.4 ng mL^{-1} [30]. When comparing with ELISA techniques based on the determination of Pru du 6, LOD values ranging between 3 ng mL^{-1} [17] and 20 ng mL^{-1} [15] have been reported. Furthermore, it is necessary to point out that the sandwich ELISA test using the same immunoreagents that those employed in this study, reported a LOD value of 2 ng mL^{-1} [20], which is about 15 times higher than that obtained with the electrochemical bioplatform. The better sensitivity of the amperometric immunoplatform compared to the ELISA test has been also reported for the determination of soy allergenic proteins glycinin and β-conglycinin [24]. It is also important to highlight here that, due to the instrumentation used in the detection step, the developed bioplatform is competitive with the ELISA methodology in terms of cost and applicability at the point-of-need.

Likewise, a LFIA using the same antibodies to Pru du 6 exhibited a 250-fold higher LOD (30 ng mL^{-1}) [20]. Despite the advantage of having a very rapid response (10 min), the LFIA test can give false negative results due to the hook effect associated with this technique and it does not provide quantitative results [31].

The amperometric measurements obtained using eight different bioplatforms prepared in the same way on three different days, for the analysis of 25 ng mL^{-1} Pru du 6 standard, gave a relative standard deviation (RSD) value of 3.1 %, indicating the great reproducibility of the analysis procedure. Furthermore, the CAb-MBs stored in filtered PBS at 4 °C were used for the preparation of the bioplatforms with no differences in the measured S/B ratios for 38 days. These results indicate a good stability of the prepared immunoconjugates, which simplifies and speeds up the complete analytical process, allowing the determinations to be performed in 30 min.

3.3. Selectivity

The selectivity study is intended to provide information on potential sources of cross-reactivity and interferences. In this study, the selectivity of the developed immunoplatform was determined by assaying protein extracts from defatted flours of hazelnut and walnut as they are the most widely nuts consumed and they could be found with almond in commercial food. In a previous study performed to develop ELISA and LFIA

tests, cross-reactivity was determined with the same anti-Pru 6 antibodies using 50 food commodities (other nuts and ubiquitous food ingredients such as egg, milk, meat and fruits). In that study, only Pecan nut, Brazil nut and chestnut showed certain reactivity, which was less than 0.01 % [20].

Likewise, reactivity with some of the most allergenic proteins from those sources, like Cor a 9 (hazelnut) and Jug r 1 (walnut) was also tested. As it can be seen in Fig. 4**a**, Cor a 9, with structural similarity to Pru du 6 (51 %) [32], did not cause any significant interference when the protein was assayed at the same concentration than Pru du 6. Likewise, Jug r 1 did not cause any interference in the assay. Similarly, the analysis of flour showed that undiluted hazelnut and walnut extracts gave a similar S/B ratio. Furthermore, when testing almond flour 100 times more diluted than hazelnut and walnut flours, the amperometric response gave S/B ratios of about 15 times higher for almond, suggesting that hazelnut and walnut proteins do not show any apparent cross reactivity in the assay with the developed platform (Fig. 4**b**).

These results are in good agreement with those reported by Civera et al. [20] as they did not find cross-reactivity with hazelnut and walnut when extracts were assayed by ELISA and LFIA tests using the same anti-Pru du 6 antibodies.

3.4. Determination of Pru du 6 in food samples

The developed bioplatform was applied to the analysis of defatted almond flour and of cookie incurred with different percentages of whole ground almond before and after the baking processing.

For defatted almond flour, the lowest level detected by the immunoplatform in extracts at different dilutions was 0.035 μg g^{-1} of protein (**Fig. S1**). Considering that Pru du 6 is approximately 50 % of almond protein [16], the bioplatform could detect 0.018 μg g^{-1} of the target protein.

International guidelines recommend that the analytical techniques developed to detect allergens must be tested using model foods in which the allergenic ingredient is incorporated before the food is processed [33]. Although the preparation of incurred food requires more time and effort, the food matrix is more realistic as it has been subjected to processing conditions like those applied in the food industry. In this study, cookies incurred with ground almond were selected as model food as nuts are ingredients commonly used in the elaboration of many bakery products.

The analysis of cookie dough and baked cookies added with different amounts of almond protein were performed to evaluate the performance of the developed biosensor. As shown in Fig. 5, the blank dough and cookies (without almond) gave a signal below the LOQ of the bioplatform, indicating that no false positives are found. Besides, all samples incurred with almond protein gave signals above the LOQ of the assay, which increased with the amount of almond protein added. Results obtained indicated that the developed bioplatform can detect at least 0.2 μg g^{-1} of almond protein in both, raw dough and baked cookies. These results agreed with those reported by Civera et al. [20] for the detection of amandin in baked cookies by a sandwich ELISA using the same immunoreagents, as they could also detect the addition of 0.2 μg g^{-1} of almond protein in baked cookies. Likewise, in the study of Chhabra et al. [19], Pru du 6 was detected by ELISA in cookies, sponge cakes, almond bars and cornflakes incurred with 0.5 % of almond, although they did not include the low percentages used in our study that could mimic cross-contaminated products, which correspond to 0.0001 % of whole almond (0.2 μg g^{-1} almond protein). On the other hand, results obtained with the developed bioplatform indicated that the signal level measured in baked cookies was about 11–35 % lower than that obtained in the corresponding raw dough. These results are in accordance with those described for incurred cookies analysed by sandwich ELISA using the same immunoreagents, where the Pru du 6 concentration decreased to about 46 % after the baking processing [20].

This could be due to the denaturation of the target protein, which induces changes in some conformational and/or lineal epitopes that interact with the antibodies, and to the aggregation of the target protein that reduces its solubility and hides the epitopes recognized by the antibodies [33,34].

However, our results contrast with those reported by Chhabra et al. [19] using a sandwich ELISA and monoclonal antibodies against Pru du 6. These authors observed a much higher reduction of immunoreactivity in model foods (between 3.8 and 6.1 times) than that obtained in this study. This difference is probably due to the fact that the treatments applied markedly affect the single epitope that is recognized by monoclonal antibodies, whereas these changes are less critical when polyclonal antibodies capable of recognizing different epitopes are used.

The developed bioplatform was applied to determine the concentration of Pru du 6 in cookies incurred with almond protein. To perform this quantification, a calibration curve was constructed by spiking almond-free cookie extracts with increasing concentrations of Pru du 6 (2.5–25 ng mL^{-1}). The preparation of these standards was necessary as a matrix effect was observed for Pru du 6 standards prepared with the extract of the blank baked cookies compared to those prepared in buffer. Thus, the statistic study comparing both slopes showed that $t_{exp} = 4.22$

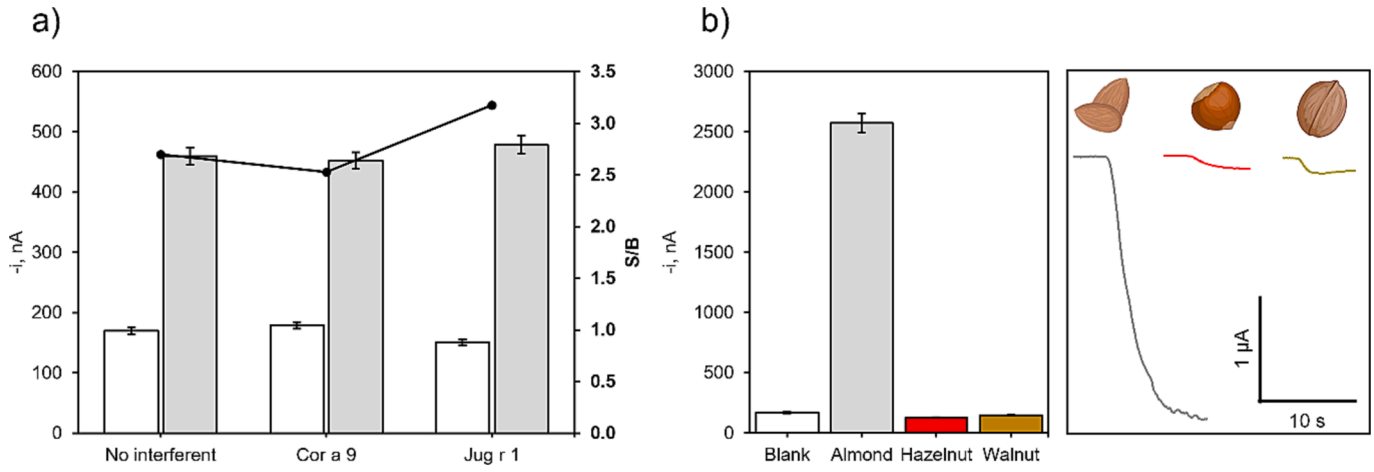

Fig. 4. Evaluation of the selectivity of the developed amperometric bioplatform using flour extracts and purified allergenic proteins from hazelnut and walnut. Amperometric responses obtained with the developed immunoplatform for blank signal (without Pru du 6) (white bars) and 10 ng mL^{-1} of Pru du 6 (grey bars) prepared in the absence and in the presence of 10 ng mL^{-1} of Cor a 9 and Jug r 1 (a). Amperometric responses obtained in the absence (blank) and in the presence of 100 times diluted almond flour extract and undiluted hazelnut and walnut flour extracts (b).

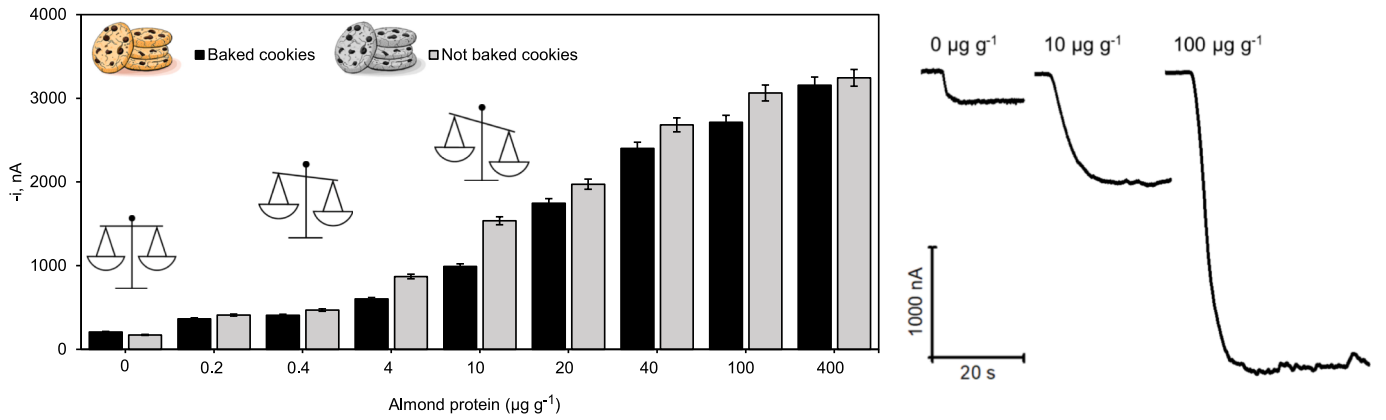

Fig. 5. Amperometric responses measured with the developed sandwich-based bioplatform for the determination of Pru du 6 in raw dough (grey bars) and baked cookies (black bars) incurred with different amounts of almond protein. Results are the mean value ± standard deviation of three cookie extracts. Representative amperometric traces obtained for incurred baked cookies with 0, 10 and 100 μg g^{-1} almond protein.

$> t_{tab} = 2.36$, considering a 95 % confidence level.

Table 2 shows the concentration values of Pru du 6 determined in cookies incurred with 0.4 and 0.6 μg g^{-1} of almond protein using the developed bioplatform. In addition, these results were compared with those obtained by a sandwich ELISA test previously developed using the same immunoreagents [20]. The good agreement among the results obtained with both methodologies ($t_{exp} < t_{tab}$ values) with the confidence intervals (at a significance level of $\alpha = 0.05$) indicates a good accuracy of the results provided by the developed immunoplatform. Although the statistical comparison confirms the good concordance between both methodologies, no correlation parameters are given due to the small number of compared results. In both cases, the concentration of Pru du 6 was lower than expected, probably due to denaturation and aggregation induced by the applied heat processing [33]. Furthermore, RSD values obtained for the determination of Pru du 6 in three different extracts of almond incurred cookies using both techniques yielded RSD values lower than 10 % indicating an acceptable reproducibility.

Results obtained show that the developed bioplatform can detect traces of almond in incurred baked cookies (0.2 μg g^{-1} almond protein). An allergen action level has not been established for almond in VITAL program in order to provide appropriate PAL. However, if the reference dose for hazelnut indicated in the VITAL program is considered (1 μg g^{-1} of hazelnut protein for a serving size of 100 g), the developed almond bioplatform could be applied by food producers as a suitable control tool for the allergen risk management.

4. Conclusions

This study reports the first electrochemical bioplatform for the sensitive and selective detection of almond through the determination of the allergenic protein Pru du 6. The bioplatform is based on the recognition of this allergenic target protein using unlabelled and HRP-labelled specific antibodies and on the amperometric monitoring of HQ-mediated enzymatic reduction of H_2O_2 after capturing the prepared magnetic bioconjugates on the surface of disposable electrodes.

This developed bioplatform allows determining Pru du 6 (LOD of 0.12 ng mL^{-1}) in only 30 min and has successfully coped with the analysis of almond flour and baked cookies incurred with almond, demonstrating in this latter case the ability to determine as little as 0.2 μg g^{-1} of almond protein. Noteworthy, the bioplatform is competitive with the ELISA methodology in terms of saving time, bioreagent consumption and compatibility of application at point-of-need.

The developed bioplatform, unlike other conventional methodologies, does meet the stringent requirements of the food industry, in terms of sensitivity, simplicity, rapidity and use of low-cost and simple instrumentation which is compatible with multiplexed and field determinations.

All these unique characteristics lead to consider it as a promising biotool to improve the allergen risk management by food producers and therefore, it could help to reduce the abusive use of PAL. Besides, the biotool could be used for further advancing in emerging allergenic targets research, and for assisting precision nutrition and medicine, the latter considering clinical problems arising from the ingestion of products to which certain individuals are particularly sensitive.

Table 2
Determination of Pru du 6 concentration (in ng mL^{-1}) in extracts of cookies incurred with almond protein using the developed bioplatform and the sandwich ELISA.

Incurred cookies (μg g^{-1})	Inmunoplatform		ELISA		t_{exp} [b]
	[Pru du 6] [a]	$RSD_{n=3}$, %	[Pru du 6] [a]	$RSD_{n=3}$, %	
0.4	3.0 ± 0.3	4.4	3.0 ± 0.7	9.9	0.243
0.6	5.7 ± 1.4	10.2	4.9 ± 0.6	5.1	2.313

[a] mean value ± $t \times s/\sqrt{n}$ ($n = 3$, $\alpha = 0.05$).
[b] $t_{exp} < t_{tab}$ of 4.303 ($n = 3$, $\alpha = 0.05$).

CRediT authorship contribution statement

Alba Civera: Methodology, Investigation, Writing – review & editing, Writing – original draft. **Sofiia Tvorynska:** Methodology, Investigation. **Maria Gamella:** Supervision, Methodology, Investigation, Writing – review & editing, Writing – original draft. **Rebeca M. Torrente-Rodríguez:** Supervision, Methodology, Investigation, Writing – review & editing, Writing – original draft. **María Pedrero:** Supervision, Writing – review & editing, Writing – original draft. **Patricia Galán-Malo:** Methodology, Resources, Writing – review & editing, Writing – original draft. **Luis Mata:** Methodology, Resources, Writing – review & editing, Writing – original draft. **Lourdes Sánchez:** Methodology, Resources, Writing – review & editing, Writing – original draft. **Jiří Barek:** Resources, Writing – review & editing, Writing – original draft, Funding acquisition. **José M. Pingarrón:** Methodology, Resources, Writing – review & editing, Writing – original draft. **Susana Campuzano:** Conceptualization, Supervision, Resources, Writing – review & editing, Writing – original draft, Funding acquisition. **María D. Pérez:** Conceptualization, Supervision, Resources, Writing – review & editing, Writing – original draft, 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.

Acknowledgements

The financial support of PID2019-103899RB-I00 (Spanish Ministerio de Ciencia e Innovación) Research Project, TRANSNANOAVANSENS-CM Program from the Comunidad de Madrid (Grant S2018/NMT-4349), Cátedra Agrobank (Ref 2019/0061), Aragon Government (Ref. A20_23R) and the European Social Found are gratefully acknowledged. Alba Civera is grateful to the Aragon Government for a predoctoral contract. Sofiia Tvorynska Erasmus+ Learning Agreement for Traineeship with Charles University. The authors would like to acknowledge the use of Servicio General de Apoyo a la Investigacion–SAI, Universidad de Zaragoza.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109403.

References

[1] S.H. Sicherer, H.A. Sampson, Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management, J. Allergy Clin. Immunol. 141 (1) (2018) 41–58, https://doi.org/10.1016/j.jaci.2017.11.003.

[2] V. McWillian, R. Peters, M.L.K. Tang, S. Dharmage, A.L. Ponsonby, L. Gurrin, K. Perrett, J. Koplin, K.J. Allen, T. Dwyer, A. Lowe, M. Wake, C. Robertson, Patterns of tree nut sensitization and allergy in the first 6 years of life in a population-based cohort, J. Allergy Clin. Immunol. 143 (2) (2019) 644–650.e5, https://doi.org/10.1016/j.jaci.2018.07.038.

[3] R.S. Gupta, C.M. Warren, B.M. Smith, J. Jiang, J.A. Blumenstock, M.M. Davis, R. P. Schleimer, K.C. Nadeau, Prevalence and Severity of Food Allergies Among US Adults, JAMA Netw. Open 2 (1) (2019) e185630.

[4] World Health Organization, Risk assessment of food allergens: part 2: review and establish threshold levels in foods for the priority allergens: meeting report. Retrieved from https://www.who.int/en/ (accessed 4 April 2023).

[5] D. Barreca, S.M. Nabavi, A. Sureda, M. Rasekhian, R. Raciti, A.S. Silva, G. Annunziata, A. Arnone, G.C. Tenore, I. Süntar & G. Mandalari. Almonds (Prunus dulcis Mill. D. A. webb): A source of nutrients and health-promoting compounds. Nutrients, *12*(3) (2020) 672. https://doi.org/10.3390/nu12030672.

[6] K.J. Allen, B.C. Remington, J.L. Baumert, R.W.R. Crevel, G.F. Houben, S. Brooke-Taylor, A.G. Kruizinga, S.L. Taylor, Allergen reference doses for precautionary labeling (VITAL 2.0): Clinical implications, J. Allergy Clin. Immunol. 133 (1) (2014) 156–164.

[7] S. La Vieille, J.O. Hourihane, J.L. Baumert, Precautionary Allergen Labeling: What Advice Is Available for Health Care Professionals, Allergists, and Allergic Consumers? J. Allergy Clin. Immunol. In Pract. 11 (4) (2023) 977–985, https://doi.org/10.1016/j.jaip.2022.12.042.

[8] T. Holzhauser, P. Johnson, J.P. Hindley, G. O'Connor, C.H. Chan, J. Costa, C. K. Fæste, B.J. Hirst, F. Lambertini, M. Miani, M.C. Robert, M. Röder, S. Ronsmans, Z. Bugyi, S. Tömösközi, S.D. Flanagan, Are current analytical methods suitable to verify VITAL® 2.0/3.0 allergen reference doses for EU allergens in foods? Food Chem. Toxicol. 145 (2020), 111709 https://doi.org/10.1016/j.fct.2020.111709.

[9] FAO/WHO (2021). Summary report of the Ad hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens. Part 2: Review and establish threshold levels in foods of the priority allergens Retrieved from https://www.who.int/news-room/events/detail/2021/03/15/default-calendar/ad-hoc-joint-fao-who-expert-consultation-on-risk-assessment-of-food-allergens-part2-review-and-establish-threshold-levels-in-foods-of-the-priority-allergens (accessed 4 April 2023).

[10] J. Costa, I. Mafra, M.B.P.P. Oliveira, High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods, Food Chem. 133 (3) (2012) 1062–1069, https://doi.org/10.1016/j.foodchem.2012.01.077.

[11] S. Kabasser, C. Hafner, S. Chinthrajah, S.B. Sindher, D. Kumar, L.E. Kost, A.J. Long, K.C. Nadeau, H. Breiteneder, M. Bublin, Identification of Pru du 6 as a potential marker allergen for almond allergy, Allergy: Eur. J. Allergy Clin. Immunol. 76 (5) (2021) 1463–1472, https://doi.org/10.1111/all.14613.

[12] L. Monaci, E. De Angelis, N. Montemurro, R. Pilolli, Comprehensive overview and recent advances in proteomics MS based methods for food allergens analysis, TrAC - Trends Anal. Chem. 106 (2018) 21–36, https://doi.org/10.1016/j.trac.2018.06.016.

[13] I.M. López-Calleja, S. de la Cruz, N. Pegels, I. González, R. Martín, T. García, Sensitive and specific detection of almond (Prunus dulcis) in commercial food products by real-time PCR, LWT 56 (1) (2014) 31–39, https://doi.org/10.1016/j.lwt.2013.10.039.

[14] N. Prieto, E. Iniesto, C. Burbano, B. Cabanillas, M.M. Pedrosa, M. Rovira, J. Rodríguez, M. Muzquiz, J.F. Crespo, C. Cuadrado, R. Linacero, Detection of almond allergen coding sequences in processed foods by real time PCR, J. Agric. Food Chem. 62 (24) (2014) 5617–5624, https://doi.org/10.1021/jf405121f.

[15] M.R. Acosta, K.H. Roux, S.S. Teuber, S.K. Sathe, Production and Characterization of Rabbit Polyclonal Antibodies to Almond (Prunus dulcis L.) Major Storage Protein, J. Agric. Food Chem. 47 (10) (1999) 4053–4059, https://doi.org/10.1021/jf980231d.

[16] K.H. Roux, S.S. Teuber, J.M. Robotham, S.K. Sathe, Detection and stability of the major almond allergen in foods, J. Agric. Food Chem. 49 (5) (2001) 2131–2136, https://doi.org/10.1021/jf001307k.

[17] M. Su, M. Venkatachalam, C. Liu, Y. Zhang, K.H. Roux, S.K. Sathe, A murine monoclonal antibody based enzyme-linked immunosorbent assay for almond (Prunus dulcis L.) detection, J. Agric. Food Chem. 61 (45) (2013) 10823–10833, https://doi.org/10.1021/jf402851k.

[18] J. Masiri, L. Benoit, M. Meshgi, J. Day, C. Nadala, M. Samadpour, A Novel Immunoassay Test System for Detection of Modified Allergen Residues Present in Almond-, Cashew-, Coconut-, Hazelnut-, and Soy-Based Nondairy Beverages, J. Food Prot. 79 (9) (2016) 1572–1582, https://doi.org/10.4315/0362-028X.JFP-15- 493.

[19] G.S. Chhabra, C. Liu, M. Su, M. Venkatachalam, K.H. Roux, S.K. Sathe, Effects of the Maillard Reaction on the Immunoreactivity of Amandin in Food Matrices, J. Food Sci. 82 (10) (2017) 2495–2503, https://doi.org/10.1111/1750-3841.13839.

[20] A. Civera, P. Galan-Malo, I. Segura-Gil, L. Mata, A.P. Tobajas, L. Sánchez, M. D. Pérez, Development of sandwich ELISA and lateral flow immunoassay to detect almond in processed food, Food Chem. 371 (2022), 131338, https://doi.org/10.1016/j.foodchem.2021.131338.

[21] S. Benedé, V. Ruiz-Valdepeñas Montiel, E. Povedano, M. Villalba, L. Mata, P. Galán-Malo, R.M. Torrente-Rodríguez, E. Vargas, A.J. Reviejo, S. Campuzano, J. M. Pingarrón, Fast amperometric immunoplatform for ovomucoid traces determination in fresh and baked foods, Sens. Actuators B 265 (2018) 421–428, https://doi.org/10.1016/j.snb.2018.03.075.

[22] V. Ruiz-Valdepeñas Montiel, E. Povedano, S. Benedé, L. Mata, P. Galán-Malo, M. Gamella, A.J. Reviejo, S. Campuzano, J.M. Pingarrón, Disposable Amperometric Immunosensor for the Detection of Adulteration in Milk through Single or Multiplexed Determination of Bovine, Ovine, or Caprine Immunoglobulins G, Anal. Chem. 91 (17) (2019) 11266–11274, https://doi.org/10.1021/acs.analchem.9b02336.

[23] M. Gamella, C. Bueno-Díaz, V. Ruiz-Valdepeñas Montiel, E. Povedano, A.J. Reviejo, M. Villalba, S. Campuzano, J.M. Pingarrón, First electrochemical immunosensor for the rapid detection of mustard seeds in plant food extracts, Talanta 219 (2020), 121247, https://doi.org/10.1016/j.talanta.2020.121247.

[24] M. Blázquez-García, B. Arévalo, V. Serafín, S. Benedé, L. Mata, P. Galán-Malo, I. Segura-Gil, M.D. Pérez, J.M. Pingarrón, S. Campuzano, Ultrasensitive detection of soy traces by immunosensing of glycinin and β-conglycinin at disposable electrochemical platforms, Talanta 241 (2022), 123226, https://doi.org/10.1016/j.talanta.2022.123226.

[25] M. Gamella, A. Laza, J. Parrón-Ballesteros, C. Bueno, V. Ruiz-Valdepeñas Montiel, M. Pedrero, F.A. Bertolino, J.M. Pingarrón, M. Villalba, S. Campuzano, First PCR-free electrochemical bioplatform for the detection of mustard Sin a 1 protein as a potential "hidden" food allergen, Bioelectrochemistry 150 (2023), 108357, https://doi.org/10.1016/j.bioelechem.2022.108357.

[26] U.K. Laemmli, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, Nature 227 (5259) (1970) 680–685, https://doi.org/10.1038/227680a0.

[27] AACC, Method 10–50D, Baking quality of cookie flour, Approved methods of the American Association of Cereal Chemists, American Association of Cereal Chemists, 2000.

[28] M. Pele, M. Brohée, E. Anklam, A.J. van Hengel, Peanut and hazelnut traces in cookies and chocolates: Relationship between analytical results and declaration of food allergens on product labels, Food Addit. Contam. 24 (12) (2007) 1334–1344, https://doi.org/10.1080/02652030701458113.

[29] 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, https://doi.org/10.1002/elan.201200503.

[30] A. Sena-Torralba, N.G.E. Smits, D. Blázquez, C. Albero-Pérez, Y. Pallás-Tamarit, Á. Maquieira, S. Morais, Simultaneous quantification of six major allergens in commercial foods for children using a multiplex array on a digital versatile disc, Food Chem. 404 (2023), 134570, https://doi.org/10.1016/j.foodchem.2022.134570.

[31] G. Mandalari, A.R. Mackie, Almond allergy: An overview on prevalence, thresholds, regulations and allergen detection, Nutrients 10 (11) (2018) 1706, https://doi.org/10.3390/nu10111706.

[32] J.M. Smeekens, K. Bagley, M. Kulis, Tree nut allergies: Allergen homology, cross-reactivity, and implications for therapy, Clin. Exp. Allergy 48 (7) (2018) 762–772, https://doi.org/10.1111/cea.13163.

[33] S.L. Taylor, J.A. Nordlee, L.M. Niemann, D.M. Lambrecht, Allergen immunoassays-considerations for use of naturally incurred standards, Anal. Bioanal. Chem. 395 (1) (2009) 83–92, https://doi.org/10.1007/s00216-009-2944-0.

[34] E. De Angelis, S.L. Bavaro, G. Forte, R. Pilolli, L. Monaci, Heat and pressure treatments on almond protein stability and change in immunoreactivity after

simulated human digestion, Nutrients 10 (11) (2018) 1679, https://doi.org/10.3390/nu10111679.