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# Electrochemical immunosensing of walnut and hazelnut allergenic proteins in processed foods

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ABSTRACT

Nuts are a well-known cause of food allergy and, once this has been diagnosed, due to the likelihood of cross-sensitization to multiple tree nut allergens, their strict avoidance from the diet is advisable. In this context, we present electrochemical bioplatforms to detect traces of hazelnut and walnut in processed foods through the determination of their respective allergenic proteins Cor a 9 and Jug r 1 in a fast and sensitive assay. First, the evaluation of the single determination of both proteins was performed by building sandwich immunoconjugates on the surface of magnetic microbeads relying on specific antibodies unmodified or conjugated to horseradish peroxidase. Amperometric transduction was made upon trapping the magnetic bioconjugates on the surface of disposable carbon electrodes, using the hydroquinone/hydrogen peroxide system. The great analytical performance achieved with the individual platforms (detection limits of 0.12 and 0.56 ng $mL^{-1}$ for Jug r 1 and Cor a 9, respectively), led us to the individual and dual quantification of both proteins in raw dough and baked cookies incurred with ground nuts. The developed method allowed detecting baked cookies incurred with 0.0025% ground walnut and 0.00002% ground hazelnut with results comparable to those provided by ELISA techniques. The feasibility of performing the dual determination of both allergens in a single run was demonstrated.

## 1. Introduction

Tree nut consumption has increased lately due to both their incorporation in many healthy eating guidelines and media coverage of evidence linking their consumption to a host of health benefits [1,2]. Although most people appreciate the intake of nuts, it may pose a health risk due to the possibility of inducing hypersensitivity in sensitized/allergic patients, for whom the best 'treatment' is the complete exclusion of the offending food from the diet. This makes necessary to include precise information on allergenic ingredients in the labeling of processed foods. Although the EU regulation [3] requires that all major allergenic food groups, including tree nuts, are declared on labels, it does not regulate the presence of these allergens due to cross-contamination during food processing. It should be noted that the voluntary precautionary allergen labeling (PAL) that can be used in this case, consisting of including the statement "may contain" [4], can cause confusion among allergic consumers.

Among tree nuts, hazelnut and walnut are by far the most popular worldwide [5]. Walnut is one of the most consumed tree nuts and is the most allergenic nut in North America, while hazelnut is the most common tree nut allergy in Europe [6]. Cor a 9, also known as corylin, is a major kernel storage protein in hazelnut, belonging to the 11S legumin-like protein family [7]. However, it is also one of the major allergenic proteins of hazelnut and is recognized for its high diagnostic accuracy

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for hazelnut allergy in children [8]. Furthermore, Cor a 9 is stable at roasting [9] and it has been proven that at least two peptides remain stable after high-temperature treatments [10]. On the other hand, Jug r 1 and Jug n 1, included in the prolamin superfamily of proteins, have been identified as allergens with respect to the species *Juglans regia* (English walnut) and *Juglans nigra* (black walnut), respectively, involved in walnut allergy. Jug r 1 is considered a major allergen in walnut since more than 50% of sera from allergic patients to walnut are reactive to it [11,12]. Furthermore, Jug r 1 is of great clinical importance because it has been associated with severe allergic symptoms in walnut-allergic subjects [13]. Besides, the high stability of the protein intrinsic structure allows it to retain its allergenicity during food processing [14] and to cause sensitization directly through the gastrointestinal tract [11].

The severity of tree nut allergy makes crucial the precise determination of their residues in food, not only as food components but also for monitoring cross-contamination of materials during production lines, therefore minimizing the serious consequences of possible errors and/or omissions in food labeling [15]. However, the lack of available reference materials and the absence of official methods for allergen detection and quantification, complicate this task. Additionally, since even small amounts of these nuts can cause severe reactions, strict monitoring is required to minimize the potential harm. This worrying context demands reliable and sensitive methods to guarantee both compliance with food labeling and consumer protection. The most widely used methods for allergenic protein detection are immunoassays, such as enzyme-linked immunosorbent assay (ELISA), and more recently, mass spectrometry (MS). MS provides higher sensitivity, specificity, and reproducibility, but its application is restricted as it requires expensive instrumentation and specialized expertise [16]. Furthermore, ELISA tests can provide quantitative information, although the involved methods are relatively laborious, time-consuming, and require non-portable instrumentation [17]. Moreover, available ELISA kits generally target a single nut [12,18], while multiple targeting would be preferred to avoid false-negative results due to either disruption or disappearance of some protein ingredients during food processing and/or cross-contamination issues [19].

In this framework, the innovative transformation of electrochemical biosensors, driven by their unique features of high sensitivity, ease of operation, low manufacturing cost, possibility of miniaturization and simultaneous multi-target detection, as well as the versatility to provide profiling on complex and poorly treated samples at the point of need, requiring shorter assay times and smaller sample quantities [20,21], currently positions electrochemical biosensors as interesting tools, alternative to conventional methods, for the determination of food protein allergens [22–29]. Within the wide variety of strategies for the development of electrochemical bioplatforms, magnetic microparticles (MBs) stand out for their easy integration into these devices and the enhancement of sensitivity through efficient capturing and preconcentration of the target analyte. Moreover, they allow easy magnetic-based washing and isolation protocols, thus helping eliminating sample matrix effects and reducing the likelihood of non-specific binding [30]. Although some bio-electrochemical approaches involving the use of MBs and disposable screen-printed electrodes (SPEs) have been reported for the single determination of allergenic proteins [22–27], the multiplexed approach, so necessary to offer more reliable results with less costs, shorter assay times and using a smaller amount of sample, is still scarce. In this context, few electrochemical platforms have been reported to detect several allergens at the same time [28,29]. It is important to highlight that, so far, no biosensors have been reported for the determination of walnut allergens. Moreover, considering the important issue of cross-contamination in their determination, the possibility of determining more than onein the same assay becomes particularly relevant.

To meet this need, this manuscript reports the first electrochemical immunosensing platforms for the single and dual determination of the main walnut and hazelnut allergenic proteins, Jug r 1 and Cor a 9, respectively. The implemented sandwich-type methodologies entailed the use of carboxylic-groups functionalized magnetic beads (HOOC-MBs), specific pair sets of antibodies for sandwiching each target protein, and amperometric transduction at screen-printed carbon electrodes (SPCEs) making use of the hydroquinone (HQ)/horseradish peroxidase (HRP)/$H_2O_2$ system. Once optimized, characterized, and confirmed the successful application of both bioplatforms for the single analysis of each target protein in raw dough and baked cookie samples, they were integrated into a dual platform for the dual protein determination, which was also characterized and applied with very promising results.

## 2. Experimental

### 2.1. Apparatus and electrodes

A CHI1140A and a CHI1030B multichannel potentiostats (CH Instruments, Inc.) were used to perform single and multiple amperometric measurements, respectively. Disposable screen-printed carbon electrodes with one (DRP-110, SPCE) or two (—DRP-C1110, SPdCE) sensing surfaces, along with the corresponding connector cables (DRP-CAC and DRP-BICAC, respectively), were provided by Metrohm-Dropsens S.L. A pH-meter (Basic 20+, Crison), a Vortex (Velp Scientifica), an MPW-65R centrifuge (MPW Med. Instruments), a Digiterm 100 water bath (P-Selecta), a thermomixer MT100 incubator shaker (Universal Labortechnik), a magnetic stirrer (Inbea S.L.) and a DynaMag™-2 magnet (Invitrogen-ThermoFisher Scientific) were also employed. Efficient trapping of the modified magnetic beads (MBs) onto the working electrodes (WEs) of SPCEs and SPdCEs was carried out by using lab-made polymethylmethacrylate (PMMA) casings embedding one or two neodymium magnets (AIMAN GZ). Spectrophotometric measurements were carried out with a SpectroStar-Nano spectrophotometer (BMG Labtech). An ÄKTA Start automated system was used for protein purification. Characterization of chromatographic fractions was performed by SDS-PAGE with the Mini-PROTEAN Tetra Cell (Bio-Rad) at 180 V. The purity degree of isolated proteins was determined by densitometry (EPSON EU-88 Image Scanner III, Long Beach, California). A Kenwood Titanium Chef KM010 (Kenwood, Woking, United Kingdom) was employed for cookies preparation.

### 2.2. Reagents and solutions

Carboxylic acid-modified magnetic beads (HOOC-MBs, 2.7 μm Ø, Dynabeads M 270 carboxylic acid, Cat. No. 14305D) were purchased from Invitrogen™. Sepharose CL-6B was acquired by GE Healthcare (Piscataway, NJ), Concanavalin A-Sepharose from Merck, (Darmstadt, Alemania) and HiTrap NHS-activated HP, HiTrap Phenyl HP and HiTrap SP HP from Cytiva (Uppsala, Sweden). The labeling of antibodies was performed using the Lightning-link Horseradish Peroxidase conjugation Kit (Innova Biosciences).

Sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, sodium chloride, potassium chloride, tris(hydroxymethyl)aminomethane (Tris)-HCl, and sodium hydroxide were acquired from Scharlab. Tween®20, N-(3-dimethy-aminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), ethanolamine, hydroquinone (HQ), hydrogen peroxide ($H_2O_2$, 30% *w/v*) and bovine serum albumin (BSA) were purchased from Sigma. 2-Morpholinoethanesulfonic acid (MES) was acquired from Gerbu. BSA Blocker™ (10×) in PBS and Blocker™ Casein in PBS (Blocking buffer solution containing 1% casein, BB solution) was from Thermo Scientific.

Phosphate-buffered saline (PBS, consisting of 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 0.137 M NaCl and 0.3 mM KCl, pH 7.5); phosphate-buffered saline with Tween (PBST, consisting of PBS with 0.05% Tween®20); 0.1 M phosphate buffer (PB), pH 8.0; 0.05 M PB, pH 6.0; 0.025 M MES buffer pH 5.0; and 0.1 M Tris-HCl, pH 7.2 were prepared in type I water from a Millipore Milli-Q purification system (18.2 MΩ cm). A freshly prepared EDC/sulfo-NHS mixture solution (50 mg $mL^{-1}$ each, made in 0.025 M

MES buffer, pH 5.0) and a 1 M ethanolamine solution in 0.1 M PB, pH 8.0 were used for activation and blocking of the MBs surface, respectively. 0.1 M HQ and 0.1 M $H_2O_2$ solutions in 0.05 M PB (pH 6.0) were prepared just before the amperometric readings. Other solutions are specified in the corresponding section. Food commodities and raw tree nuts were acquired in local markets.

### 2.3. Purification of Cor a 9 and Jug r 1

Cor a 9 was purified as reported previously [31]. Briefly, hazelnut seeds were ground and defatted with n-hexane. Proteins from defatted flour were extracted with 50 mM Tris-HCl, 200 mM NaCl, pH 7.0. After centrifugation, the supernatant was concentrated and subjected to gel filtration on a Sepharose CL-6B column. Fractions containing Cor a 9 were dialyzed and loaded onto a Concanavalin A-Sepharose column, and retained proteins eluted with 400 mM methyl α-D mannopyranoside.

Jug r 1 was purified as reported previously [32]. Briefly, walnut seeds were ground and proteins were extracted with 25 mM Tris-HCl buffer, pH 8.2 containing 1 M NaCl, 0.1% PVPP, 1 mM EDTA and 0.02% sodium azide. After centrifugation, the supernatant was added with ammonium sulphate to reach 90% saturation and centrifuged. Proteins obtained in the pellet were dialyzed and applied on a HiTrap Phenyl Sepharose HP column. Retained proteins were eluted with 0.05 M sodium phosphate buffer, pH 7.0, dialyzed and loaded on a HiTrap SP Sepharose HP column. Retained proteins were eluted with a gradient of sodium chloride (0-0.4 M) in 0.05 M sodium phosphate, pH 8.0.

Chromatographic fractions of 1.5 mL were collected and analyzed by SDS-PAGE under reducing and non-reducing conditions.

### 2.4. Preparation of immunochemical reagents

Antisera were raised in rabbits by inoculating purified Cor a 9 and Jug r 1 as reported previously [31]. The correct use and care of the involved animals have been assured by applying the EU Directive 2010/63 on protecting animals used for scientific purposes (Spanish policy RD53/2013). The Ethical Animal Experiment Committee of the University of Zaragoza approved all procedures framed in this work (License Project 30/19). Specific antibodies were isolated using immunoadsorbents prepared by insolubilization of Cor a 9 or Jug r 1 in a HiTrap NHS-activated HP column as described in Civera et al. [33]. Purified antibodies were conjugated with horseradish peroxidase using a commercial conjugation kit. Specific antibodies were used as capture ($cAb_{Jug\ r\ 1}$ or $cAb_{Cor\ a\ 9}$) and conjugated antibodies (HRP-$dAb_{Jug\ r\ 1}$ or HRP-$dAb_{Cor\ a\ 9}$) as detection receptors for amperometric and colorimetric determinations.

### 2.5. Characterization of purified proteins and antibodies by SDS-PAGE and Western blotting

SDS-PAGE under reduced and nonreduced conditions was carried out using 4–20% precast polyacrylamide gels and a Mini-PROTEAN Tetra Cell equipment (Bio-Rad Laboratories, Hercules, CA). Gels were stained using Coomassie Brilliant Blue R and the image was captured using an Image Scanner III (GE Healthcare, Chicago, IL). Western blotting was performed as described previously [31] using anti-Cor a 9 or anti-Jug r 1 antisera and goat anti-rabbit IgG labeled with horseradish peroxidase as secondary antibody.

Purified Cor a 9 and Jug r 1 were characterized by SDS-PAGE (Fig. S1 in the Supporting Information). The electrophoretic profile in reduced conditions of Cor a 9 mainly contains intensive bands of 20–25 kDa and 35–38 kDa which correspond to basic and acidic subunits of the protein, and some minor bands lower than 20 kDa, which probably represent products of partial hydrolysis of Cor a 9 as previously mentioned by Trashin et al. [10]. The electrophoretic profile of Jug r 1 in non-reduced conditions showed a band of about 14 kDa, similar to that obtained by Doi et al. [18]. The analysis by densitometry showed that the purity degree of Cor a 9 and Jug r 1 was larger than 90%. Furthermore, the analysis by Western blotting showed that the corresponding antibodies recognized both acidic and basic subunits of Cor a 9 and Jug r 1.

### 2.6. Formation of the sandwich-type immunocomplexes for the single and dual determination of Jug r 1 and Cor a 9

The single determination of Jug r 1 and Cor a 9 allergens relied on sandwich-type assays and MBs involving successive incubation and washing steps with 25 and 50 μL, respectively, of the corresponding solutions. Regardless of the target allergen, 3 μL-aliquots from the HOOC-MBs commercial suspension were transferred into 1.5 mL centrifuge tubes and washed twice with 50 μL of 0.025 M MES buffer, pH 5.0 for 10 min with continuous stirring at controlled temperature (950 rpm, 25 °C). Thereafter, between each incubation step required to form the corresponding immunocomplexes, the MBs were washed by placing them in a magnetic holder for 2 min to easily remove the supernatant through magnetization. The preparation of Jug r 1- and Cor a 9-sandwich-type bioconjugates onto HOOC-MBs involved several successive steps:

I. Activation of the carboxylic groups on the MBs surface using a freshly prepared EDC/sulfo-NHS mixture solution (50 mg $mL^{-1}$ each, in 0.025 M MES buffer, pH 5.0) for 35 min, followed by two washings of the activated MBs with 50 μL of the same buffer solution.
II. Covalent immobilization of $cAb_{Jug\ r\ 1}$ or $cAb_{Cor\ a\ 9}$ by incubating the activated MBs with a 5.0 $cAb_{Jug\ r\ 1}$ or 25 $cAb_{Cor\ a\ 9}$ μg $mL^{-1}$ solution (both in 0.025 M MES buffer, pH 5.0) for 10 and 30 min, respectively. Thereafter, the resulting $cAb_{Jug\ r\ 1}$-MBs or $cAb_{Cor\ a\ 9}$-MBs were washed twice with 0.025 M MES buffer, pH 5.0.
III. Blocking off the residual unreacted activated–HOOC groups on the MBs with ethanolamine (1 M in 0.1 M PB, pH 8.0) for 60 min. Thereafter, $cAb_{Jug\ r\ 1}$-MBs or $cAb_{Cor\ a\ 9}$-MBs conjugates were washed once with 0.1 M Tris-HCl buffer, pH 7.2, and twice with the commercial blocker casein solution (BB); then the corresponding cAb-MBs conjugates were re-suspended in 50 μL PBS buffer, pH 7.5, until used.
IV. Immunoassays for the single determination of Jug r 1 and Cor a 9: the $cAb_{Jug\ r\ 1}$-MBs or $cAb_{Cor\ a\ 9}$-MBs conjugates were re-suspended in the correspondent Jug r 1 or Cor a 9 standard solution or the sample (prepared in BB) and incubated for 10 or 15 min, respectively. Then, the modified MBs were washed twice with BB and incubated in an HRP-$dAb_{Jug\ r\ 1}$ or HRP-$dAb_{Cor\ a\ 9}$ solution diluted 1/1000 with BB for 30 or 15 min, respectively. Two washing steps with BB solution were performed and the resultant HRP-$dAb_{Jug\ r\ 1}$/Jug r 1/$cAb_{Jug\ r\ 1}$-MBs or HRP-$dAb_{Cor\ a\ 9}$/Cor a 9/$cAb_{Cor\ a\ 9}$-MBs conjugates were kept in PB, pH 6.0 to perform the amperometric detection.

Once the magnetic immunoconjugates for the determination of Jug r 1 or Cor a 9 were independently prepared using the described protocols, the individual or dual determination of both proteins was carried out using electrochemical transducers with either one or two sensing surfaces. That is, the magnetic bioconjugates were prepared in the same way (optimized protocol for each target) regardless of whether the individual or dual determination of both proteins was carried out.

### 2.7. Amperometric measurements

Either SPCEs (for single determinations) or SPdCEs (for dual determinations) were inserted into the lab-made PMMA blocks with either one (for single determinations) or two (for dual determinations) neodymium magnets right beneath the corresponding working carbon electrode(s). The MBs-immunocomplexes, prepared as detailed in the previous section, were re-suspended either in 50 or 5 μL of 50 mM PB,

pH 6.0 and quantitatively placed onto the surface of the corresponding WE(s) of a SPCE or SPdCE, respectively. Then, the magnet holding block/SPCE (or /SPdCE) assembly was immersed into an electrochemical cell containing 10 mL of freshly prepared 1.0 mM HQ in 0.05 M PB, pH 6.0 solution. Amperometric measurements were performed by applying a constant potential of −0.20 V (*vs.* Ag pseudoreference electrode) in stirred solutions and recording the change in the cathodic current after the addition of 50 μL of a freshly made 0.1 M $H_2O_2$ solution in the same buffered media. The amperometric signals given in this manuscript correspond to the difference between the steady-state (after $H_2O_2$ addition) and background (before $H_2O_2$ addition) currents, and error bars were estimated as the standard deviation (SD) for a set of three replicates ($n$ = 3). New SPCEs/SPdCEs were used for each measurement.

### 2.8. Preparation and analysis of food extracts

The protein content on ground nut and defatted nut flour was determined by Kjeldahl method obtaining 12.0 and 31.4% protein for hazelnut and 14.9 and 30.7% protein for walnut, respectively. Model cookies were prepared at the Pilot Plant of Zaragoza University following the Method 10-50D of the American Association of Cereals Chemists [34]. 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 mixed using a Kenwood kitchen mixer. Once mixed, different amounts of ground nut (0, 0.2 and 2% nut w/w) were added and the dough was mixed again. Rounded cookies (20 g, 7 cm diameter) were prepared and baked at 205 °C for 10 min. Lower percentages of nut proteins were obtained by mixing adequate proportions of cookie samples without nut with those containing 0.2% nut (either walnut or hazelnut). Both dough and cookies were stored at −20 °C until use.

Protein extraction from food samples involved the addition of 10 mL of PBS into a plastic tube containing (1.00 ± 0.01) g of sample and shaking the suspension with a vortex for 1 min. The mixture was then heated at 60 °C in a water bath for 15 min and centrifuged at 3000 *g* for 15 min. The supernatant was collected and directly analyzed. As matrix effect was observed only for hazelnut determination, to unify protocols, the quantification of both allergens was performed by interpolating the sample response into calibration plots built in the supernatant extracted from nut-free raw dough or baked cookie.

### 2.9. ELISA methodology

The food protein extracts obtained according to the above section were also analyzed by applying independent ELISA methods using the same immunoreagents as those employed for the construction of the amperometric sandwich-type immunoplatforms [27]. Briefly, microtiter wells were coated with 120 μL/well of the correspondent cAb solution (3.0 μg $mL^{-1}$ $cAb_{Jug\ r\ 1}$ or 5.0 μg $mL^{-1}$ $cAb_{Cor\ a\ 9}$ in 0.05 M carbonate buffer, pH 9.6), and kept overnight at 4 °C. After three washing steps with 300 μL/well with type I water, an incubation step with 300 μL/well of blocking solution (containing 1% w/v BSA in PBS) was carried out at room temperature (RT) for 2 h. Subsequently, the wells were washed thrice with PBST and incubated either with 100 μL of the correspondent standards of the target proteins or the sample extracts for 30 min (RT). Once the wells were washed five times with PBST, 100 μL of the correspondent HRP-labeled dAb solution (HRP-$dAb_{Jug\ r\ 1}$ or HRP-$dAb_{Cor\ a\ 9}$, respectively) 1/100000 diluted with PBS, were incubated for 30 min at RT and five washing steps were performed with PBST. Finally, 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) commercial substrate was added, stopping the enzymatic reaction after 15 min by adding 50 μL/well of 2 M $H_2SO_4$. The absorbance of the wells was measured at 450 nm.

## 3. Results and discussion

The fundamentals of the sandwich-type bioplatforms for the determination of walnut and hazelnut traces through the quantification of the allergenic proteins Jug r 1 and Cor a 9, respectively, as well as the reactions involved in the amperometric detection, are schematized in Fig. 1. Briefly, magnetic microcarriers (MBs) were modified with specific capture antibodies against Jug r 1 ($cAb_{Jug\ r\ 1}$) or Cor a 9 ($cAb_{Cor\ a\ 9}$) which were used to selectively bind the corresponding target protein. This was further sandwiched with the appropriate detector antibody conjugated to HRP (HRP-$dAb_{Jug\ r\ 1}$ for Jug r 1 and HRP-$dAb_{Cor\ a\ 9}$ for Cor a 9). The resultant sandwich-immunocomplexes modified-MBs were magnetically deposited on the WE surface of SPCEs or SPdCEs to perform single or dual determination of both allergenic proteins using amperometric transduction by monitoring the variation in the measured cathodic current using the $H_2O_2$/HQ system ($E_{app}$ = − 0.20 V *vs.* the Ag pseudoreference electrode).

### 3.1. Single determination of Jug r 1 and Cor a 9 proteins

#### 3.1.1. Optimization of experimental variables

The key variables involved in the performance of the sandwich immunoassays were carefully optimized (Fig. S2 in the Supporting Information). The parameters, the checked ranges and the selected values, according to the signal (S)/blank (B) ratios measured in the absence (B) and in the presence (S) of 10 and 50 ng $mL^{-1}$ Jug r 1 and Cor a 9, respectively, are summarized in Table 1. The experimental variables regarding amperometric detection and the volume of HOOC-MBs used per assay were optimized previously [35,36].

As it can be seen in "bars 0" of Fig. S2a, neither Jug r 1 or Cor a 9 protein nor HRP-$dAb_{Jug\ r\ 1}$ or HRP-$dAb_{Cor\ a\ 9}$ were adsorbed on MBs in the absence of the corresponding cAb ($cAb_{Jug\ r\ 1}$ or $cAb_{Cor\ a\ 9}$), thus confirming the feasibility of the proposed sandwich-type configurations. Better S/B ratios were found when 5.0 μg $mL^{-1}$ of $cAb_{Jug\ r\ 1}$ or 25 μg $mL^{-1}$ or $cAb_{Cor\ a\ 9}$ were immobilized on the HOOC-MBs (Fig. S2a). Larger $cAb_{Jug\ r\ 1}$ concentrations provoked a decrease in the S/B ratio due to a slight increase in the "B" signals and a significant decrease in the "S" signals, which can be attributed to a poorer protein recognition due to steric hindrance when many cAb molecules are immobilized on the MBs [37]. However, a plateau was observed in the case of $cAb_{Cor\ a\ 9}$ (Fig. S2a). On the other hand, the cAbs immobilization efficiency did not improve for incubation times longer than 10 min for Jug r 1 and 30 min for Cor a 9 (Fig. S2b), due to a more hindered antigen-antibody recognition, as well as to a significant increase in the "B" signals, particularly in the case of Cor a 9, attributed to a certain non-specific interaction between cAb and HRP-dAb. The number of steps involved in the sandwich-type assays was checked by comparing the results obtained using two different procedures: (i) a one-step-protocol, where the $cAb_{Jug\ r\ 1}$- or $cAb_{Cor\ a\ 9}$-MBs were incubated for 30 min in a mixture solution containing Jug r 1 and HRP-$dAb_{Jug\ r\ 1}$ or Cor a 9 and HRP-$dAb_{Cor\ a\ 9}$, respectively, and (ii) a two-step-protocol consisting of a 30-min incubation of the $cAb_{Jug\ r\ 1}$- or $cAb_{Cor\ a\ 9}$-MBs in the Jug r 1 or Cor a 9 solution followed by a 15-min incubation step in the HRP-$dAb_{Jug\ r\ 1}$ or HRP-$dAb_{Cor\ a\ 9}$ solution, respectively.

Fig. S2c shows as, in both cases, discrimination between the absence and the presence of the correspondent target allergen was achieved only when the two-step protocol was employed, probably due, otherwise, to steric hindrance effects and to a possible competition between both antibodies for the target protein [27]. Noteworthy, Jug r 1 capturing was performed in just 10 min while a 30-min incubation time for Cor a 9 was selected (Fig. S2d). The resultant S/B decreased for longer incubation times, trends which could be attributed to less efficient labeling of the immobilized protein by sterical hindrance when too much protein is captured on the MBs. Regarding the optimization of HRP-$dAb_{Jug\ r\ 1}$ and HRP-$dAb_{Cor\ a\ 9}$ concentration, although a larger S/B ratio was observed for a 1/500 HRP-$dAb_{Jug\ r\ 1}$ dilution, a 1/1000 dilution was selected for

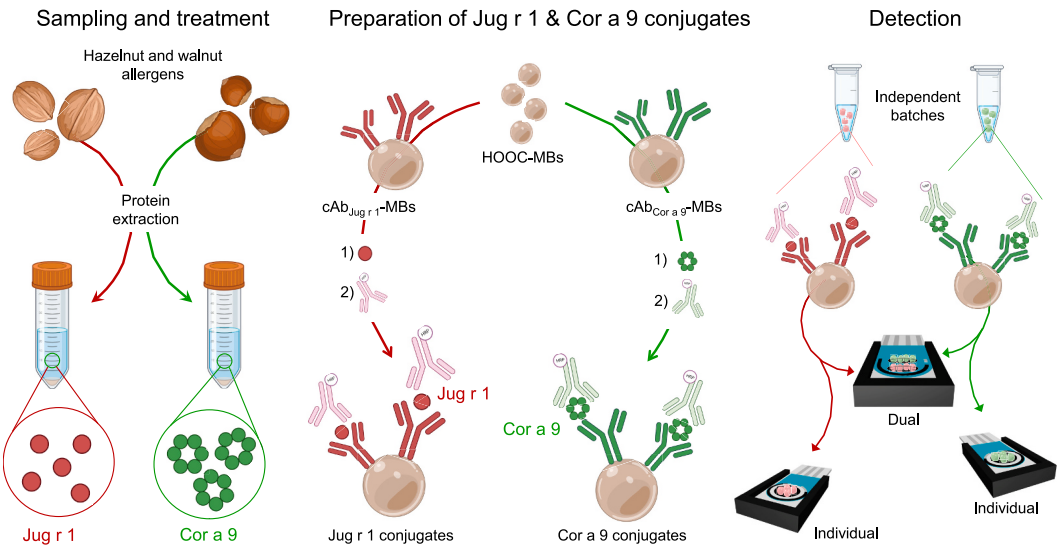


**Fig. 1.** Schematic display of the sandwich immunosensing configurations using MBs developed for the individual or dual amperometric determination of Jug r 1 and Cor a 9 allergenic proteins.

**Table 1**
Optimization of the experimental parameters and selected values affecting the performance of the bioplatforms for the single determination of Jug r 1 and Cor a 9.

| Parameter | Jug r 1 | | Cor a 9 | |
|---|---|---|---|---|
| | Checked range | Selected value | Checked range | Selected value |
| [cAb], μg mL$^{-1}$ | 1.0–50.0 | 5.0 | 2.5–50.0 | 25.0 |
| $t_{cAb}$, min | 0.5–60 | 10 | 15–90 | 30 |
| Number of steps | 1 or 2 | 2 | 1 or 2 | 2 |
| $t_{target}$, min | 5–60 | 10 | 5–60 | 30 |
| HRP-dAb dilution | 1/5000–1/500 | 1/1000 | 1/5000–1/500 | 1/1000 |
| $t_{HRP\text{-}dAb}$, min | 10–60 | 30 | 10–60 | 15 |
| Time for the assay*, min | 40 | | 45 | |

* Starting from cAb-MBs.

further work in both assays (Fig. S2e), as a compromise between sensitivity and cost of the analysis. Incubation times for HRP-dAb$_{Jug\ r\ 1}$ and HRP-dAb$_{Cor\ a\ 9}$ longer than 30 and 15 min, respectively, led to a decrease in the S/B ratios due to a relatively more significant increase for the "B" signals than for the "S" responses (Fig. S2f).

### 3.1.2. Analytical and operational characteristics

Under the optimized conditions, calibration graphs were constructed for Jug r 1 and Cor a 9 standards (Figs. 2a and c, respectively) whose analytical characteristics are summarized in Table 2.

To our knowledge, no biosensors have been reported so far for the determination of walnut allergens. On the other hand, several bioplatforms have been proposed for hazelnut detection. Hellenäs's Group reported a surface plasmon resonance (SPR) method for detecting several allergenic proteins and claiming a LOD for hazelnut of 5 μg g$^{-1}$ in chocolate. However, this direct immunoassay showed extensive problems in food extracts due to nonspecific interactions with other food components [38]. Monoclonal antibodies were used for the quantitative SPR analysis of hazelnut allergenic proteins in oil, reaching a LOD of 0.08 μg g$^{-1}$ [39]. Despite the short assay time, high degree of automation, and possibility of label-free detection, some factors such as the cost of equipment, specialized personnel requirement and cost of analysis must be considered when carrying out the determination of food allergens. To our knowledge, only Trashin et al. [10] reported a sandwich-

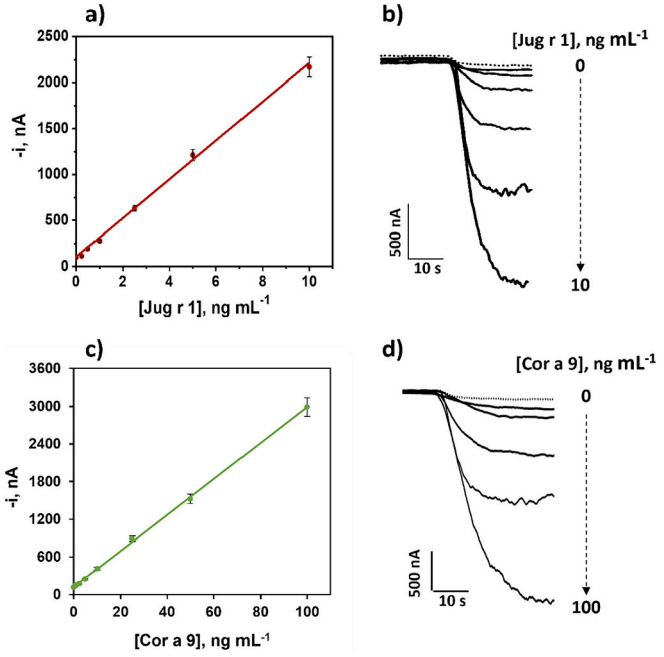


**Fig. 2.** Calibration plots constructed with the developed bioplatforms for the amperometric determination of Jug r 1 (a) and Cor a 9 (c). Amperometric traces recorded for 0, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng mL$^{-1}$ Jug r 1 standards (b) and for 0, 5, 10, 25, 50 and 100 ng mL$^{-1}$ Cor a 9 standards (d).

**Table 2**
Analytical characteristics provided by the developed immunoplatforms for the single determination of Jug r 1 and Cor a 9.

| Parameter | Jug r 1 | Cor a 9 |
|---|---|---|
| Linear range, ng mL$^{-1}$ | 0.4–10.0 | 1.5–100 |
| Slope, nA mL ng$^{-1}$ | 211 ± 4 | 28.7 ± 0.6 |
| Intercept, nA | 89 ± 19 | 120 ± 20 |
| R | 0.997 | 0.999 |
| LOD*, ng mL$^{-1}$ | 0.12 | 0.56 |

* Calculated as 3$s_b$/slope ($s_b$: standard deviation of ten "B" signals).

ELISA assay using chicken egg yolk antibodies conjugated or not with HRP using both electrochemical and optical modes for Cor a 9 determination. In the optical mode, the method had a LOD in phosphate buffer of 4 ng $mL^{-1}$ and of 0.1 μg $g^{-1}$ in cookies. In the electrochemical approach, involving an amperometric sandwich immunosensor on graphite electrodes modified with carboxylic groups, which allowed the covalent immobilization of the capture antibody, a LOD of 0.1 μg $mL^{-1}$ was achieved. However, these methods exhibited longer assay times (4 h and 1 h *vs.* 45 min) and higher LODs in comparison with those achieved with our developed immunoplatform (0.56 ng $mL^{-1}$). In addition, the amperometric immunosensor reported by Trashin et al. was not applied to the analysis of real samples.

An indirect ELISA test using polyclonal antibodies against soluble walnut protein was developed and it was able to detect 10 ng $mL^{-1}$ walnut proteins; however, precise quantification in food extracts was not possible [40]. As an alternative to animal antibodies, a biotinylated soluble fragment-single chain and multimeric antibody allowed the detection of walnut in food samples with a LOD of 1616 mg $kg^{-1}$ [41]. Besides, sandwich formats were developed to detect walnut proteins [19,42], as well as the 2S albumin fraction, down to 0.39 ng $mL^{-1}$ (0.156 μg $g^{-1}$ in food) [18] or 0.25 ng $mL^{-1}$ (0.04 μg $g^{-1}$ in food) [32]. Specifically, Jug r 1 was targeted in an indirect competitive assay reaching LOD values of 2.2 ng $mL^{-1}$, equivalent to 0.44 μg $g^{-1}$ of food sample [12]. Moreover, immunoelectrophoresis [43,44], lateral flow immunoassay (LFIA) and ELISA methods have been used for detecting hazelnut proteins in food products. Although some competitive ELISAs either for hazelnut total soluble proteins [45,46] or Cor a 9 [47] reaching LODs from 0.45 to 10 ng $mL^{-1}$ were reported, sandwich assays are more common, with LODs ranging from 0.7 to 30 ng $mL^{-1}$ [48–53] for hazelnut proteins and Cor a 9 standard solutions [10,31], and able to detect from 0.2 to 1 ppm of hazelnut proteins in food extracts. Additionally, commercial ELISA kits with nominal LODs of 0.03 or 2.5 mg $kg^{-1}$ hazelnut protein in various foods are available [54]. In terms of comparison, the LOD values obtained with the developed immunoplatforms are similar or even lower than those reported by ELISA methods for both allergens. Noteworthy, the LOD achieved for Cor a 9 was 4 times lower than that claimed with the ELISA assay that used the same immunoreagents (0.56 *vs.* 2.1 ng $mL^{-1}$) [31]. In addition, ELISAs are relatively labor-intensive and time-consuming, and require non-portable instrumentation and qualified personnel. LFIA methods have been developed due to their simplicity and portability which promote their on-site application by non-specialized users. Nevertheless, they provide just semi-quantitative detection of hazelnut and walnut residues reaching visual LODs of 0.5 μg $g^{-1}$ [31,32,55].

Other methods such as peptide analysis by liquid chromatography coupled to MS for hazelnut [53,56–59] and walnut allergens [60], polymerase chain reaction (PCR) [61–63] or PCR-ELISA [64] for the detection and quantification of hazelnut in food matrices (LODs from 0.2 to 10 μg $g^{-1}$), as well as PCR [65], and real-time PCR [66–70] for walnut assessment have been reported. However, despite these methods allowed multiplexed analysis, showed high precision and reproducibility, and achieved a sensitivity similar to that of the developed immunoplatforms, they are more complex and require non-portable equipment that limits their applicability for *on site* detection. In this sense, the developed bioplatforms stand up as good candidates to carry out this type of analysis due to the inherent features of electrochemical devices such as high sensitivity, ease of operation, low manufacturing cost, multi-target ability, and miniaturization.

The amperometric measurements for 1.0 and 25 ng $mL^{-1}$ Jug r 1 or Cor a 9 standards, obtained with five immunoplatforms prepared in the same manner and in the same day provided relative standard deviation (RSD) values of 4.5 and 5.5%, respectively. RSD values of 4.2 and 8.9% for Jug r 1 and Cor a 9, respectively, were obtained when the immunoplatforms were prepared in different days. These data suggest a good reproducibility of the measurements and the robustness and reliability of the assay protocols.

The storage stability of the $cAb_{Jug\ r\ 1}$- and $cAb_{Cor\ a\ 9}$-MBs conjugates was evaluated. After they were prepared, they were re-suspended in filtered PBS, and kept at 4 °C until used. The amperometric responses obtained with immunoplatforms prepared with the stored conjugates in the absence and in the presence of 5.0 and 25.0 ng $mL^{-1}$ Jug r 1 and Cor a 9 standards, showed no significant differences in the calculated S/B ratios for at least 50 and 32 days (longer periods were not evaluated), respectively (data not showed). This excellent storage stability of the $cAb_{Jug\ r\ 1}$- and $cAb_{Cor\ a\ 9}$-MBs conjugates greatly facilitates and shortens the preparation of sandwich immunocomplexes for the determination of both allergenic proteins using the developed bioplatforms.

### 3.1.3. Evaluation of the selectivity

Even though cross-reactivity seems more common among nuts from the same taxonomic family, it has been shown that they can be categorized into two general groups of cross-reactive nuts: (i) walnut, pecan and hazelnut, and (ii) hazelnut, cashew, Brazil nut, pistachio, and almond [71]. In this sense, cross-reactivity has been reported between walnut (*Juglans regia*) and hazelnut (*Corylus avellana*) allergens, at the level of both 2S albumins and 11S globulins. In fact, the alignment of amino acid sequences of 2S albumins and legumins from walnut and hazelnut shows an identity ranging between 61.8 and 71.7% [5].

Thus, the selectivity of the developed immunoplatforms was independently evaluated using purified proteins from hazelnut (Cor a 9, 11S globulin), walnut (Jug r 1, 2S albumin) and from almond (Pru du 6, 11S). Fig. 3a shows that no noticeable changes were observed in the S/B ratio calculated for the determination of Jug r 1 in the presence of Cor a 9 or Pru du 6 standards. Therefore, these proteins did not interfere in the determination of Jug r 1 if they are present at the same concentration level. On the other hand, the results shown in Fig. 3c demonstrated that, despite the amino acid sequences showed 50 and 41% identity and homology, respectively, between Pru du 6 and hazelnut Cor a 9 [72], Pru du 6 did not produce a significant interference when both proteins were at the same concentration level (10 ng $mL^{-1}$), nor did Jug r 1, despite the reported cross-reactivity between walnut and hazelnut allergens [5].

Additionally, cross-reactivity towards nut protein extracts from defatted almond, hazelnut and walnut flour was tested for each sandwich-type immunoplatform. The results shown in Fig. 3b evidenced the correct extraction of the target protein Jug r 1 and further confirmed the selectivity of the methodology, since only the 10-fold diluted walnut extract provided a substantial amperometric response. Neither hazelnut nor almond 10-fold diluted flour protein extracts provided any noticeable amperometric signal, thus proving that it was possible to determine Jug r 1 in the presence of other hazelnut or almond proteins. In addition, regarding the single determination of Cor a 9, Fig. 3d shows that no significant response was found upon a 1000 times dilution for almond flour (S/B = 1.8) or walnut flour (S/B = 0.9) compared to that obtained for similarly diluted hazelnut flour (S/B = 46.8), indicating that no apparent cross-reactivity was observed with the proteins from the selected nuts. It is important to note that different dilutions were tested for the extracts of both proteins due to the Hook effect [73]. Such effect was observed for extracts diluted less than 1/1000 for the determination of Cor a 9. All these results agree with the previously reported cross-reactivity among nuts [71] and with those reported by Civera et al. who found cross-reactivity by ELISA with almond and walnut below 0.04% using the same anti-Cor a 9 antibodies [31]. Therefore, the excellent specificity of the employed antibodies to recognize Jug r 1 and Cor a 9 was demonstrated.

### 3.1.4. Analysis of raw dough and baked cookies incurred with hazelnut and walnut

The use of incurred samples is highly recommended in international guidelines because the practice of adding the allergenic food at the ingredient state before performing the heat processing is much more realistic than adding an extract of the allergenic food to the final manufactured product. In this sense, the use of incurred foods can reveal

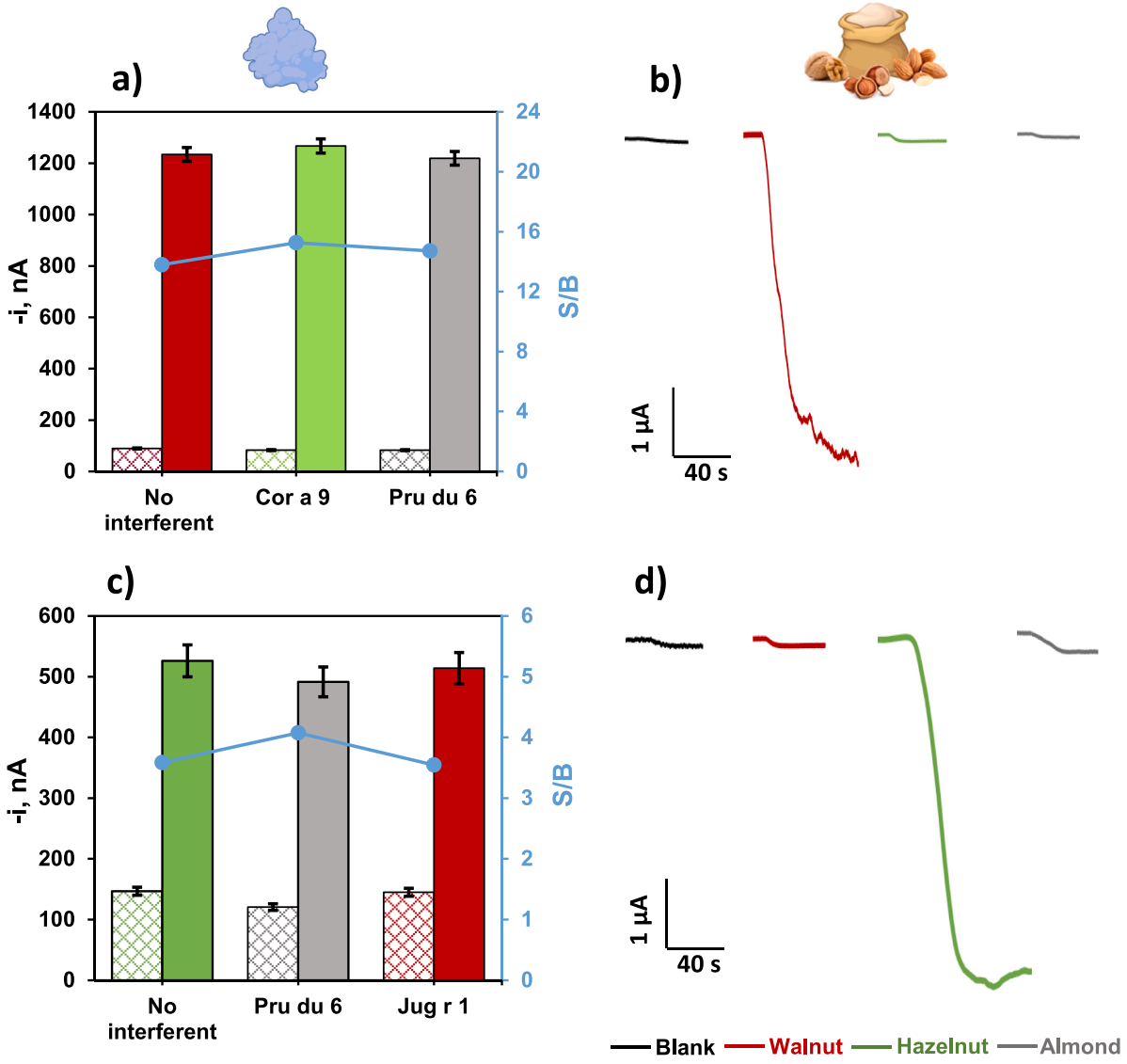


**Fig. 3.** Assessment of the selectivity provided by the developed immunoplatforms for the single determination of Jug r 1 (a, b) and Cor a 9 (c, d) against different allergenic proteins. The amperometric responses were tested for 0.0 (striped bars) and 5.0 (filled bars) ng $mL^{-1}$ Jug r 1 and Cor a 9 prepared in the absence ("No interferent" bars) and in the presence of 10 ng $mL^{-1}$ pure Cor a 9 and Pru du 6 proteins (a), or 10 ng $mL^{-1}$ pure Pru du 6 and Jug r 1 (c). Real amperometric traces measured in the absence (Blank) and in the presence of 10-fold (b) and 1000-fold (d) diluted protein extracts from hazelnut, almond and walnut flours.

some of the limitations of immunoassays as the target protein may be affected during processing [74]. In this work, cookies were selected as model food as they are bakery products in which nuts are frequently added. Thus, cookie doughs made with hazelnut or walnut as ingredients were prepared and baked at the Pilot Plant of Zaragoza University in a similar way to that applied in the food industry.

In this context, the analysis of protein extracts from raw dough and baked cookies prepared without or with different amounts of ground hazelnut and walnut was performed using the Cor a 9 or Jug r 1 developed bioplatforms. Raw and baked blank samples gave signals below the calculated LOQs for both allergens and, therefore, no false positives were found. The analysis of walnut incurred raw dough and baked cookie samples showed that the minimum amounts of added walnut that provided detectable signals were 0.0001% (1 µg of walnut/g of raw dough) and 0.0025% (25 µg of walnut/g of cookie), respectively. Although it has been reported that Jug r 1 exhibits good resistance to heat denaturation after thermal treatment at 90 °C [75,76], we observed some modifications induced by the baking processing at 205 °C affecting the protein immunoreactivity. Considering that walnut contains 14.9% of protein, the developed immunosensor can detect 3.7 µg of walnut protein/g in baked cookies and 0.15 µg $g^{-1}$ in raw cookie dough. These results are better than those reported by Doi et al. that detected 10 µg of walnut protein/g in several model foods (porridge, meatballs, bread, cake, biscuits, jelly and orange juice) using an ELISA test for Jug r 1 as the target protein [18].

Regarding hazelnut, it was possible to detect it in samples incurred with 0.00002% hazelnut (0.2 µg hazelnut/g sample) in both raw dough and baked cookies (Fig. 4). As ground hazelnut contains about 12.0% of proteins, the immunoplatform was able to detect 0.024 µg hazelnut protein/g of sample. Therefore, the sensitivity achieved by the developed immunoplatform is much better than ELISA methods for the same protein that claimed a detectability of 0.10 µg protein/g [10] or 0.15 µg $g^{-1}$ [31] in incurred cookies. In addition, the results provided by the developed immunoplatform are also better than those reported for SPR methods for the detection of hazelnut added in olive oil (0.08 µg $g^{-1}$ of the final concentration of soluble proteins) [39] or chocolate (5 µg of

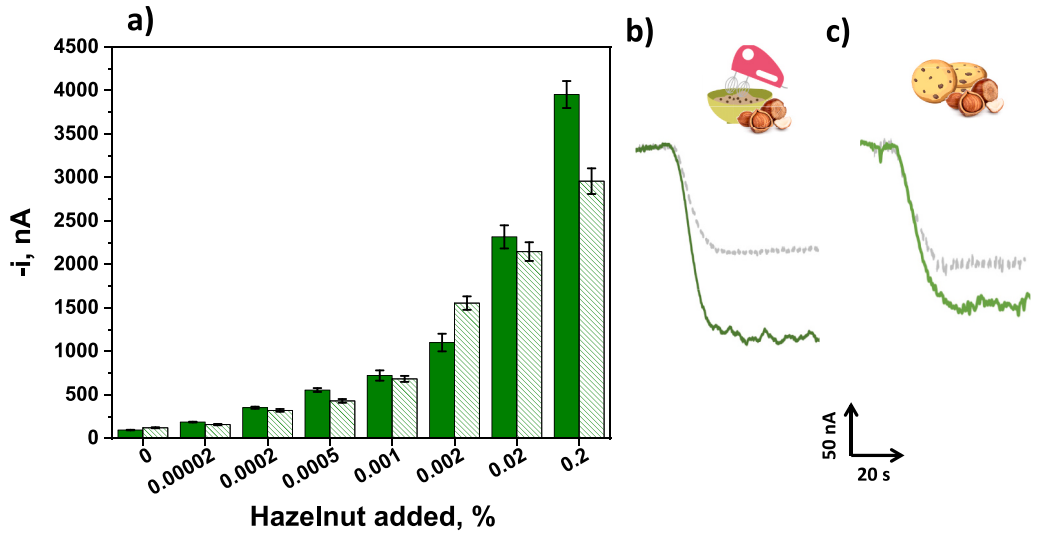


**Fig. 4.** Amperometric responses measured with developed bioplatform for the determination of Cor a 9 in dough (dark green) and baked cookies (pale green) incurred with different amounts of hazelnut (a); real amperometric traces obtained in raw cookie dough (b) and baked cookies (c) prepared without or with 0.00002% of hazelnut. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hazelnut/g) [38]. It has been reported that, in general, hazelnut immunogenicity is affected after roasting at 140 °C for 40 min [77,78]. However, specifically Cor a 9 was stable at 170 °C after 20 min of roasting [9]. In this sense, we have observed that, despite the minimum level of hazelnut detected is similar in dough and cookies, the current values obtained for the same incurred level are lower in baked cookies than in raw dough samples as it can be seen by comparing stripped *vs.* dark green bars in Fig. 4. This current decrease was probably due to denaturation or aggregation of Cor a 9 induced by the baking processing that decreased its solubility and/or decreased the reactivity with the employed antibodies. In fact, a decrease of Cor a 9 immunoreactivity of about 46–52% [31] and 23–44% [10] was reported in cookies after the baking process compared to the raw dough.

When comparing the detection of individual nuts, although the biosensor developed to determine Jug r 1 achieved a lower LOD value than that developed for Cor a 9 (see Table 2), the latter was able to detect lower levels of nut addition (0.00002% of hazelnut *vs.* 0.0025% of walnut in cookie samples). This is probably due to the lower thermal stability of Jug r 1 with respect to Cor a 9. In fact, the comparison of the slope values obtained with the bioplatforms for both proteins in baked cookie extracts with respect to the raw cookie dough shows that they decrease by 53% for Jug r 1 and only 10% for Cor a 9.

The developed bioplatforms were applied to quantify walnut and hazelnut in raw cookie dough and baked cookie samples through the determination of Jug r 1 and Cor a 9 proteins, respectively. Regarding quantification in raw cookie dough, calibration plots were constructed by spiking protein extracts obtained from free-nut raw cookie dough samples with different Jug r 1 (0.5, 1, 5, 10, 50 ng $mL^{-1}$) or Cor a 9 (1, 5, 10, 50, 100 ng $mL^{-1}$) concentrations. The slope values of these calibrations compared with those obtained for calibrations constructed with the corresponding standards of the purified proteins in buffered solutions, revealed that no significant differences were found in the case of walnut ($t_{exp} = 1.408 < t_{tab} = 2.262$; $\alpha = 0.05$), but they were important for hazelnut ($t_{exp} = 7.5 > t_{tab} = 2.776$; $\alpha = 0.05$) indicating the presence of a significant matrix effect. With the aim of unifying the protocols for the determination of both allergens, the analysis in raw cookie dough was performed by interpolating the obtained responses in the protein extract into the corresponding calibration graph constructed by spiking the extracts from raw cookie dough with protein standard solutions.

Table 3 summarizes the concentration of Jug r 1 and Cor a 9 determined in raw cookie dough containing 0.00025 and 0.0005% walnut and 0.0001 and 0.0003% hazelnut using the developed immunoplatforms. In addition, the results provided by ELISA methods using the same immunoreagents in cookie hazelnut dough are given [31]. Recoveries were calculated considering conversion values of allergenic nut protein in the nut content determined by ELISA. As can be seen, the results obtained with both methods were not statistically different ($t_{exp} < t_{tab}$ value, $\alpha = 0.05$), thus showing the good accuracy of the results provided by the developed immunoplatforms. Besides, both immunosensors showed acceptable RSD values (5–7%).

Once demonstrated the feasibility of the bioplatforms, the quantification of walnut and hazelnut in incurred baked cookie samples was also

**Table 3**
Determination of Jug r 1 and Cor a 9 (in ng $mL^{-1}$) in protein extracts from raw dough and baked cookie containing ground walnut and hazelnut using the developed immunosensors and the corresponding sandwich ELISA tests.

| Raw cookie dough (%, w/w) | | Immunoplatform | | ELISA | | | Baked cookie (%, w/w) | | Immunoplatform | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | [Jug r 1] [a] | RSD, % | [Jug r 1][a] | RSD, % | $t_{exp}$ [b] | | | [Jug r 1] [a] | RSD, % |
| Walnut | 0.00025 | 1.0 ± 0.1 | 6.1 | 1.05 ± 0.03 | 2.3 | 0.345 | Walnut | 0.005 | 1.5 ± 0.3 | 9.5 |
| | 0.0005 | 2.3 ± 0.1 | 6.1 | 2.6 ± 0.2 | 6.5 | 1.184 | | 0.01 | 6 ± 1 | 7.8 |
| | | [Cor a 9] [a] | RSD, % | [Cor a 9][a] | RSD, % | $t_{exp}$ [b] | | | [Cor a 9] [a] | RSD, % |
| Hazelnut | 0.0001 | 15 ± 3 | 6.7 | 15 ± 2 | 5.3 | 0.341 | Hazelnut | 0.0001 | 2.8 ± 0.6 | 8.0 |
| | 0.0003 | 35 ± 4 | 5.3 | 36 ± 3 | 3.7 | 0.879 | | 0.0003 | 6.9 ± 0.4 | 4.0 |

[a] Mean value ± t × s/√n (n = 3, α = 0.05).
[b] $t_{exp} < t_{tab}$ of 4.303 (n = 3, α = 0.05).

performed, following a similar protocol consisting of interpolating the sample signal in calibration plots built by spiking protein extracts obtained from free-nut baked cookie samples with different Jug r 1 or Cor a 9 proteins (Table 3). Incurred cookies containing 0.005 and 0.01% walnut and 0.0001 and 0.0003% hazelnut were analyzed by the developed immunoplatforms. A decrease in immunoreactivity of Cor a 9 and Jug r 1 was observed when comparing both raw and baked cookies.

### 3.2. Dual determination of Jug r 1 and Cor a 9 allergenic proteins

Since walnut and hazelnut may be present in food products together and taking advantage of the multiplexed capacities offered by electrochemical biosensors, we have developed the first electrochemical platform for the dual determination of both target proteins by capturing the sandwich immunocomplexes for each target protein (prepared as for the single determinations) on the WEs of an SPdCE.

No significant cross-talking between the adjacent WEs was observed (Fig. 5a). Therefore, the dual immunoplatform, whose general ensemble is schematized in Fig. 1, was employed to carry out the dual determination of both allergenic proteins. The corresponding calibration graphs are displayed in Fig. 5b with the analytical characteristics summarized in Table S1 (in the Supporting Information). The analytical and operational characteristics of the dual immunoplatform were comparable to those obtained with the single bioplatforms, with the decrease observed in sensitivity attributable both to the smaller surface area of the SPdCEs compared to the SPCEs WEs (6.3 $mm^2$ *vs.* 12.6 $mm^2$, respectively) and to the fact of capturing the same amount of MBs on a smaller surface, which may hinder the electron transfer and/or imply a larger diffusion barrier on the WE surface [79]. Nevertheless, the achieved analytical characteristics are sufficient to allow the detection of both target proteins at very low levels in food extracts.

Therefore, the dual platform was applied to the determination of raw cookie dough and baked cookies with different percentages of ground walnut and hazelnut (Table 4) using the same protocol as for individual determinations. Results obtained in the dual platform were similar to those obtained with the single immunoplatforms for the individual determination of Jug r 1 and Cor a 9.

Several methods have been reported for the simultaneous detection of nut allergens. Among them, multianalyte profiling (xMAP®) technology has been used for qualitative evaluation of allergens (including hazelnut and walnut, LODs 5 ng $mL^{-1}$), though the analysis of 36 samples required 6 h [80]. Another approach for the detection of six food allergens consisted of a standard digital compact disc functionalized with capture antibodies in a microarray format, enabling the evaluation of 20 samples in 70 min with qualitative detection by naked eye, reaching a LOD for hazelnut of 171 ng $mL^{-1}$ (walnut was not included among the tested allergens) [81]. PCR-based methods were developed for the simultaneous determination of walnut and hazelnut, allowing their relative quantification using real-time PCR [82,83] or using an optical thin-film microarray of two multiplex PCR systems for simultaneous qualitative detection of eight food allergens [84]. Regarding simpler and portable technologies, a multiplexed LFIA was developed for the detection of casein, ovalbumin, and hazelnut allergenic proteins in commercial biscuits. However, the method only allowed qualitative detection with a visual LOD of 0.1 μg $mL^{-1}$ [85]. Most of these reported methods, apart from requiring relatively expensive instrumentation, provide in some cases higher LODs, or, in most cases, only semi/qualitative results. Moreover, as far as we know, there is not, until now, any electrochemical immunoplatform for the independent and dual determination of hazelnut and walnut allergenic proteins.

**Table 4**
Jug r 1 and Cor a 9 concentrations (in ng $mL^{-1}$) provided by the developed dual bioplatform in raw dough and baked cookies with ground walnut (W) and hazelnut (H) as indicated.

| Raw cookie dough | [Jug r 1]*/$RSD_{n=3}$, % | [Cor a 9]*/$RSD_{n=3}$, % |
|---|---|---|
| W (0.00025%) + H (0.0001%) | (1.2 ± 0.1)/3.8 | (17 ± 2)/4.7 |
| W (0.0005%) + H (0.0003%) | (2.1 ± 0.1)/3.4 | (35 ± 9)/6.8 |
| **Baked cookies** | **[Jug r 1]*/$RSD_{n=3}$, %** | **[Cor a 9]*/$RSD_{n=3}$, %** |
| W (0.005%) + H (0.0001%) | (1.6 ± 0.6)/9.8 | (3.0 ± 0.8)/9.3 |
| W (0.01%) + H (0.0003%) | (6.6 ± 0.6)/3.3 | (7 ± 1)/9.5 |

* Mean value ± $ts/\sqrt{n}$; n = 3; α = 0.05.

## 4. Conclusions

This work reports the first electrochemical bioplatforms developed for the single or dual determination of hazelnut and walnut through their major allergenic proteins, Cor a 9 and Jug r 1, respectively. The immunoplatforms were assisted using magnetic microsupports to implement sandwich immunoassays, using specific antibodies for each target protein raised in rabbits. Unmodified specific antibodies were used for protein capture, while antibodies labeled with the peroxidase enzyme were employed for their detection. Transduction was performed by amperometry by trapping the MBs carrying the HRP-labeled immunoconjugates on the surface of SPCEs or SPdCEs WEs for single or dual determination, respectively. Both single and dual platforms exhibit

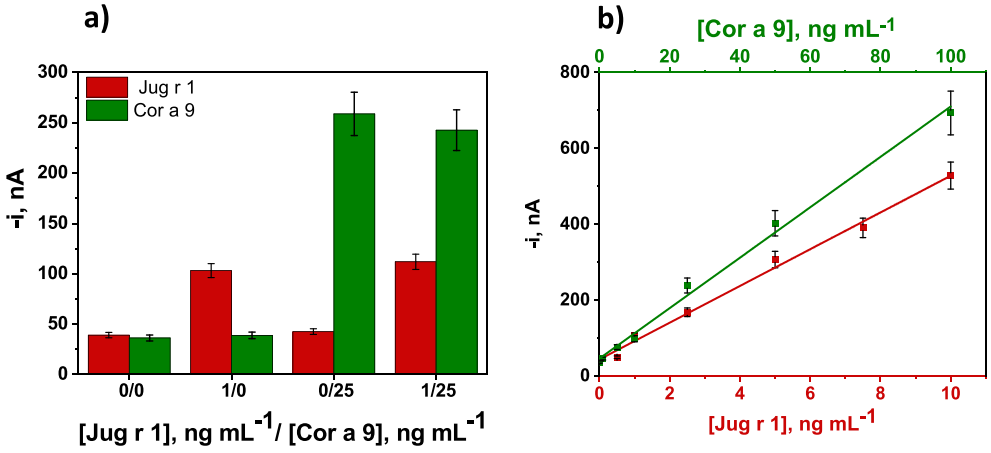


**Fig. 5.** Amperometric responses carried out with the dual immunoplatform (WE1: Jug r 1, red bars and WE2: Cor a 9, green bars) for Jug r 1/Cor a 9 standard mixtures containing: 0.0/0.0 ng $mL^{-1}$; 1.0/0.0 ng $mL^{-1}$; 0.0/25.0 ng $mL^{-1}$ and 1.0/25.0 ng $mL^{-1}$ (a) and calibration plots obtained for the amperometric determination with the dual immunosensing platform for Jug r 1 (red line and squares) and Cor a 9 (green line and squares) (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adequate analytical and operational characteristics for the sensitive and selective determination of the two allergenic proteins and have been successfully applied to the sensitive analysis of baked cookies incurred with ground walnut and hazelnut (allowing detecting as low as 0.0025% and 0.00002%, respectively). The unique advantages arising from combining MB-assisted immunodetection with amperometric transduction at SPEs make these bioplatforms particularly advantageous compared to other available methodologies, including ELISA, in terms of simplicity of handling, analysis time, multiplexing capacity and on site applicability, and as highly useful devices to guarantee precision nutrition and regain consumer confidence. Always prioritizing the accuracy and reliability of food processing, as well as the avoidance of food mislabeling, the implemented dual bioplatform denotes unquestionable features to be recognized as a very useful device to ensure general food safety and the trust of specific-allergic consumers. Furthermore, the widespread fear of developing food allergy episodes triggered by incorrect labeling and/or unintentional cross-contamination is on its way to be reduced, thanks to the development of multiplexed platforms for the reliable and simultaneous monitoring of panels of allergenic markers at different omics levels, something truly achievable, as demonstrated in this work, through electrochemical biosensing technologies.

## CRediT authorship contribution statement

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

## 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.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbsr.2024.100644.

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