



# Molecularly imprinted polymer as a synthetic antibody for the biorecognition of hazelnut Cor a 14-allergen

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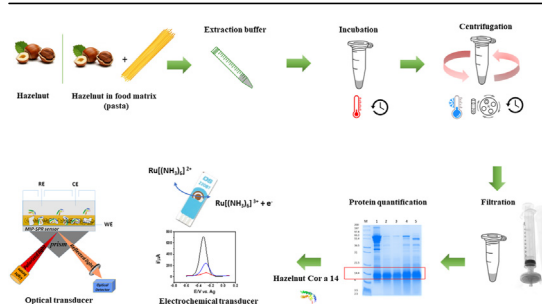
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## HIGHLIGHTS

- “Artificial antibody” targeting Cor a 14 allergen by molecularly imprinting strategy.
- MIP detects Cor a 14 down to 24.5 fg mL<sup>-1</sup> with high reproducibility/selectivity.
- Minor cross-reactivity for walnut/Brazil nut due to protein structural homology.
- Higher selectivity of Cor a 14-MIP than Cor a 14/anti-Cor a 14 IgG (raised in rabbit).
- Detection of 1 mg kg<sup>-1</sup> of hazelnut in wheat material (0.16 mg kg<sup>-1</sup> of protein).

## GRAPHICAL ABSTRACT



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## ABSTRACT

Artificial receptors that mimic their natural biological counterparts have several advantages, such as lower production costs and increased shelf-life stability/versatility, while overcoming the ethical issues related to raising antibodies in animals. In this work, the proposed tailor-made molecularly imprinted polymer (MIP)-allergen receptors aimed at substituting or even transcending the performance of biological antibodies. For this purpose, a MIP was proposed as an artificial antibody for the recognition of hazelnut Cor a 14-allergen. The target protein was grafted onto the conducting polypyrrole receptor film using gold screen-printed electrodes (Au-SPE). The electrochemical assessment presented a linear response for the dynamic range of 100 fg mL<sup>-1</sup>–1 µg mL<sup>-1</sup> and a LOD of 24.5 fg mL<sup>-1</sup>, as determined by square wave voltammetry from the calibration curves prepared with standards diluted in phosphate buffer. Surface plasmon resonance (SPR) was used as a secondary transducer to evaluate the performance of the Cor a 14-MIP sensor, enabling a linear dynamic range of 100 fg mL<sup>-1</sup>–0.1 µg mL<sup>-1</sup> and a LOD of 18.1 fg mL<sup>-1</sup>. The selectivity of the tailored-made Cor a 14-MIP was tested against potentially cross-reactive plant/animal species based on the rebinding affinity (Freundlich isotherm-K<sub>F</sub>) of homologues/similar proteins, being further compared with custom-made polyclonal anti-Cor a 14 IgG immunosensor. Results evidenced that the MIP mimics the biorecognition of biological antibodies, presenting higher selectivity (only minor cross-reactivity towards walnut and Brazil nut 2S albumins) than the Cor a 14/

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anti-Cor a 14 IgG immunosensor. The application of electrochemical Cor a 14-MIP sensor to model mixtures of hazelnut in pasta enabled quantifying hazelnut down to  $1 \text{ mg kg}^{-1}$  (corresponding to  $0.16 \text{ mg kg}^{-1}$  of hazelnut protein in the matrix). To the best of our knowledge, Cor a 14-MIP is the first sensor based on an artificial/synthetic biorecognition platform for the specific detection of hazelnut allergens, while presenting high-performance parameters with demonstrated application in food safety management.

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## 1. Introduction

Food allergies are considered a current problem of public health, affecting a significant fraction of the global population (up to 10%) [1–3]. Among the reported foods triggering relevant allergic reactions, tree nuts (e.g. hazelnut) are a very important group, encompassing a wide botanical diversity that is responsible for inducing severe/frequent clinical symptoms [4]. Since there is no cure for food-allergic diseases, their management by the allergic individuals is highly dependent on the adoption of preventive measures, such as the elimination of the offending food(s) and all the potentially cross-reactive ones from their daily diet [5]. Still, those individuals may be at risk of accidental exposure to food allergens, either by incorrect food labelling or inadvertent cross-contamination during food production/storage [6]. Protecting the health of the sensitised/allergic population is a top priority for food allergen management programmes and whose effectiveness is greatly reliant on the available analytical methodologies to verify food labelling compliance [7]. So far, the most common methods to trace allergens in foods rely on allergen-antibody interaction (enzyme-linked immunosorbent assays - ELISA, immunosensing), or peptide analysis by liquid chromatography coupled to mass spectrometry (LC-MS) [8]. In the specific case of hazelnut, several commercial ELISA kits can detect traces of hazelnut protein ( $0.5\text{--}2.5 \text{ mg kg}^{-1}$ ), as well as in-house developed ELISA and immunosensors that have been proposed for the specific detection/quantification of minute amounts ( $0.2\text{--}10 \text{ mg kg}^{-1}$ ) of hazelnut in processed foods [9–13].

Recently, molecular imprinting polymers (MIP) have become essential tools to overcome the shortfalls associated with immunoassays based on allergen-antibody interaction [14]. Biorecognition platforms based on MIP technology should take into consideration the effects of food processing and sample extraction procedures on the protein structure, or sera complexity in the case of clinical diagnostic purposes [15]. The detection of food allergens may be accomplished using an allergen as a template molecule imprinted in a polymer to produce an artificial antibody (synthetic molecular recognition) that mimics the receptor sites based on antibody-antigen interaction [16]. Concerns linked with monomer type, electrode potential, pH and template removal, as well as difficulties with low selectivity and with large-scale production of MIP, greatly restrains the potential commercialisation of biosensors based on MIP technology. The successful accomplishment of polymer networks with a targeted stable host-guest cavity structure and preserved shape has been well demonstrated [17], although its application to food allergens as target analytes is still at the early beginning [18]. So far, only a few studies have been advanced in the literature, namely, focused on the detection of Hev b 1 from rubber latex [19], Bos d 9/Bos d 10 ( $\alpha$ -casein) [20] and Bos d 5 ( $\beta$ -lactoglobulin) from cow's milk [21], presenting good sensitivities and specificities.

Considering that MIP technology can offer promising alternatives to animal-raised antibodies, this work proposes a simple and

effective procedure to construct a MIP-based mimetic bioreceptor to detect hazelnut Cor a 14 allergen as a case study. Cor a 14 is a storage protein belonging to the 2S albumin family of proteins, which is classified as an important hazelnut allergen due to its great stability towards extreme processing conditions and resistance to gastrointestinal digestion [22]. Mature Cor a 14 is a small acidic protein (14–16 kDa, 125 amino acids) with a monomeric globular structure that confers high stability towards heat treatments, returning to its native structure even after being submitted to temperatures above  $100^\circ\text{C}$  [23]. Besides its relevant physico-chemical properties, Cor a 14 is a molecular marker for hazelnut allergy that is frequently associated with severe clinical outcomes (e.g. anaphylaxis) [24]. For specificity/selectivity purposes, the Cor a 14-MIP was tested against other non-target homologues/similar proteins (2S albumins) extracted from 11 different sources (cross-reactivity assessment studies) sharing a high potential for structural homology (tree nuts, legumes, and cereals). Additionally, the binding affinity of Cor a 14-MIP was compared with a polyclonal anti-Cor a 14 IgG (raised in rabbit) to evaluate the suitability of Cor a 14-MIP as a viable alternative to Cor a 14/anti-Cor a 14 IgG immunoassay platform.

## 2. Material and methods

Electrochemical stability of the conductive polymer PPy, AFM setup, immunoassay construction and Zeta Potential/DLS characterisation are fully described in the supplementary material.

### 2.1. Reagents

The reagents included ethanol (96%, AGA, Portugal); potassium hexacyanoferrate (II) ( $\geq 99.5\%$ , Honeywell International Inc, Charlotte, NC, USA); petroleum ether 40–60  $^\circ\text{C}$  (Carlo Erba Reagents SAS, Barcelona, Spain) and sulfuric acid (95–97%, Merck, Darmstadt, Germany). Hexaammineruthenium (II) chloride ( $[\text{Ru}(\text{NH}_3)_6\text{Cl}_2]$  98%), potassium hexacyanoferrate(II), potassium hexacyanoferrate(III) (99%), sodium dodecyl sulphate ( $\geq 99.9\%$ ), pyrrole (98%), sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ), acetic acid (99.7%), sodium acetate anhydrous ( $\geq 99\%$ ) acquired at Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained passing deionized water through a Milli-Q purification systems (water resistivity  $\geq 18 \text{ M}\Omega \text{ cm}$ ).

### 2.2. Selection of plant species and defatting process

For this study, different plants containing 2S albumin fractions were selected and submitted to a defatting process to concentrate the amount of proteins per species. The selection was based on the criteria of genetic proximity, meaning that different plant species may express similar 2S albumins, both considering the primary and conformational structures. Therefore, the selection included tree nuts (hazelnut, almond, walnut, pecan nut, cashew nut, macadamia nut, Brazil nut and chestnut), as well as different legumes (peanut

and lupine) and cereals (wheat and rye). Additionally, milk protein concentrate (a technological ingredient used by the food industry that consists of a mixture of multiple milk proteins) was also used for specificity assays (as examples of proteins from animal sources), which were already in their defatted state. The defatting process was performed by Soxhlet for 24 h, using 5 g of each milled species and petroleum ether, as the solvent to remove the fat content. In the case of plants with high content of fat (e.g. walnut, peanut), the defatting process was repeated twice. At the end of this process, the defatted portion of all species was dried in an oven (50 °C) to remove the excess of solvent, giving rise to a fine and loose powder, free of lipids.

### 2.3. Extraction and purification of 2S albumins

The selective extraction of Cor a 14 was fully optimised in a previous study [25]. The 2S albumin fraction has a high affinity for acidic buffers, thus different extraction buffers had to be tested to isolate the Cor a 14 allergen from hazelnut. Briefly, four extraction buffers were used (glycine-HCl 0.1 mol L<sup>-1</sup> pH 2.5; acetate 0.1 mol L<sup>-1</sup> pH 3.6, citrate 0.1 mol L<sup>-1</sup> pH 4.0 and citrate-HCl pH 4.0) to selectively extract the Cor a 14 from 150 mg of defatted hazelnut, following incubation in a thermoblock (Thermomixer comfort, Eppendorf AG, Hamburg, Germany) for 2 h at 60 °C and with constant stirring (950 rpm). During the incubation process, the tubes were vortexed every 20 min to increase the yield of extracted protein. Then, the tubes were centrifuged at room temperature at 9000×g for 30 min. All the supernatant phases were transferred into new 2.0 mL reaction tubes and submitted to new centrifugation in the same conditions. The supernatant was further filtered (sterile syringe filter 0.45 µm polypropylene, VWR International, LLC, Radnor, Pennsylvania, USA) to remove any suspended particles and transferred to a new 2.0 mL reaction tube. Protein yield and purity were determined by UV spectrophotometry on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take3 micro-volume plate accessory and with the protein280 protocol in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA). The quality and selectivity of the acidic buffer for Cor a 14 was also evaluated by SDS-PAGE in a discontinuous system (5–12%) and run at 150 V in non-denaturing conditions, using a Mini-PROTEAN® Tetra System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 1 × Tris/Glycine/SDS buffer. The buffer allowing the best selective extraction of Cor a 14, thus evidencing the highest yield, purity and quality was the acetate buffer 0.1 mol L<sup>-1</sup> pH 3.6. The results for this analysis are presented in previous work [25].

Each defatted plant material (tree nuts, legumes, and cereals) (150 mg) was also extracted with 1.5 mL of acetate buffer 0.1 mol L<sup>-1</sup> pH 3.6, following the extraction procedure described above. Cor a 14 and the other 2S albumins were lyophilised before being used for polymer imprinting or for cross-reactivity studies. The lyophilised Cor a 14 was also sent to a specialised company (Davids Biotechnologie GmbH, Regensburg, Germany) to produce antibodies. The immunoassay Cor a 14/anti-Cor a 14 IgG used for the cross-reactivity assessment was prepared considering a sandwich immunoassay format, via EDC/NHS coupling to the target allergen-antibody onto Au screen-printed disposable electrodes linked to an Au anchored thiol (mercaptop succinic acid) [25].

### 2.4. Cor a 14-MIP/NIP sensor construction

Before electropolymerization, Au-SPE were cleaned with ethanol, acetone and rinsed with ultrapure water followed by electrochemical cleaning with 0.5 mol L<sup>-1</sup> sulfuric acid by scanning the potential between -0.2 and 1.2 V, for 15 cycles, at a scan rate of

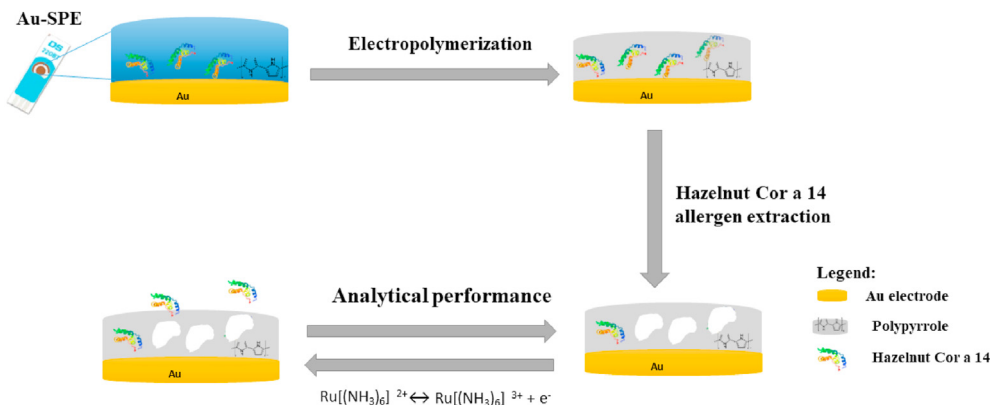
100 mV s<sup>-1</sup>. The MIP films were produced by covering the working area of the Au-SPE with Cor a 14 solution, 4.0 mg mL<sup>-1</sup> and 0.01 mol L<sup>-1</sup> pyrrole prepared in 0.01 mol L<sup>-1</sup> phosphate buffer (PB, pH 7.4). The electropolymerization step was also carried out in the absence of the target Cor a 14 (NIP), to allow a correct understanding of whether the existing change in the MIP depends on the interaction of allergen with its specific cavities rather than to other unspecific interactions. For the removal of Cor a 14 from the polymer matrix, MIP was incubated for 12 h in the absence of light at 4 °C with a solution of 25 mmol L<sup>-1</sup> SDS with 10% methanol. This step ensured the cleavage of the protein structure, thus allowing its removal from the constructed polymer network while preserving the specific cavities for Cor a 14 recognition. To correct any eventual changes in film structure, the NIP was also subjected to a similar treatment. After the incubation period, the sensor was washed with ultrapure water. Then, electrochemical cleaning (-0.6 to +0.1 V) was carried out with 0.01 mol L<sup>-1</sup> PB buffer (pH 7.4) to remove residues of SDS and protein that may persist in the polymeric network. In Scheme 1 is resumed the step-by-step construction of the Cor a 14-MIP sensor.

The electrochemical analytical performance of the Cor a 14-MIP sensor was assessed by square wave voltammetry (SWV) at a frequency of 10 Hz, a step potential of 0.0010 V and amplitude of 0.050 V using  $5.0 \times 10^{-3}$  mol L<sup>-1</sup> [Ru(NH<sub>3</sub>)<sub>6</sub>]<sup>3+</sup> (in PB, pH 7.4) as an electrochemical probe. A similar procedure was followed for the [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> redox pair. After 30 min of incubation with increasing concentration of Cor 14 standard solutions, the electrode surface was washed with ultrapure water and placed in the DRP-DSC interface, following the addition of the electrochemical redox probe. Cyclic voltammetry (CV) and SWV were performed in this sequence after each addition. The repeatability of the electrochemical/optical MIP was evaluated in triplicate. Three distinct Au-SPE and Au-SPR MIP were constructed, and the analytical performance was evaluated independently in successive days or within a short interval of time. The storage stability impact on the Cor a 14-MIP response was studied given that Cor a 14 aliquots used were frozen, and others were kept at 4 °C, and the response was not affected. Cor a 14-MIP sensor was stored at 4 °C and with an average relative standard deviation of 8% it can be reused twice. When it was reused for the third time, it was not possible to obtain linearity with an acceptable R<sup>2</sup> (<96%).

### 2.5. Apparatus for electrochemical and optical analysis

A potentiostat/galvanostat PGSTAT302 N (Metrohm Autolab, Utrecht, Netherlands) was used to perform the electrochemical measurements. This apparatus is equipped with an impedance module, controlled by NOVA software version 2.1.3 (Metrohm Autolab, Utrecht, The Netherlands). The biorecognition element was constructed on gold screen-printed electrodes (Au-SPE, 4 mm diameter, DRP-220AT, Metrohm, DropSens, Asturias, Spain) and the electrodes were placed in a DRP-DSC, which acts as an interface with the PGSTAT302 N.

A double-channel ESPRIT SPR equipment (Kinetic Evaluation Instruments B.V., Utrecht, The Netherlands) coupled to data acquisition software version 4.3.1 (KEI bv, Leusden, The Netherlands) was used to monitor the optical properties of the MIP constructed on Au-SPR sensors (Kinetic Evaluation Instruments B.V., Leusden, The Netherlands). The SPR was attached to the potentiostat/galvanostat PGSTAT302 N to produce in situ the MIP/NIP on the Au-SPR sensor and the temperature was controlled using a Julabo F32-HE water bath (Julabo GmbH, Seelbach, Germany). Before the SPR measurements, the stabilisation of the electropolymerised films was accomplished by continuously flowing (12.5 µL s<sup>-1</sup>) 0.01 mol L<sup>-1</sup> of phosphate buffer (PB) pH 7.4 on the substrate until a



**Scheme 1.** Schematic representation showing the different steps involved in the MIP assembly and electrochemical detection of hazelnut Cor a 14 allergen.

stable SPR response was achieved. The representation of the angle variation as a function of the logarithmic value of Cor a 14 concentration allowed the evaluation of the allergen rebinding (concentration ranging from  $1 \text{ fg mL}^{-1}$  to  $0.1 \text{ mg mL}^{-1}$ ) to the MIP cavities using changes in the film optical properties.

### 2.6. Preparation of model mixtures of hazelnut in pasta (incurred food matrices)

For the application of the developed electrochemical Cor a 14-MIP sensor to real foods, model mixtures of pasta containing known amounts of hazelnut (12 concentration levels,  $1\text{--}500,000 \text{ mg kg}^{-1}$ ) were prepared. Pasta was selected for the preparation of these model mixtures, since pasta is a complex food matrix, being composed of *Triticum aestivum* (soft wheat), *T. durum* (durum wheat), dehydrated eggs and water, and that suffer several processes (kneading, extrusion, drying, among others) during production. Hazelnut kernels were previously retrieved from shell and ground with a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) using decontaminated containers and material. For the first model mixture ( $500,000 \text{ mg kg}^{-1}$ ), 100 g of hazelnut were added to 100 g of pasta and vigorously mixed to ensure a good homogenisation of the mixture. The following model mixtures were serially diluted by successive additions with pasta until the level of  $1 \text{ mg kg}^{-1}$  in the equivalent proportion [26]. Total protein was extracted from 150 mg of each model mixture using 1.5 mL of Tris-HCl  $0.1 \text{ mol L}^{-1}$ , following the extraction procedure described in section 2.3.

## 3. Results and discussion

### 3.1. Electrosynthesis of Cor a 14-MIP grafted in overoxidized polypyrrole

Molecular imprinting is proposed as an effective and less expensive method to prepare “plastic or artificial” antibodies as biorecognition elements targeting hazelnut Cor a 14 allergen. For this purpose, a conductive polymer with  $\pi$ -conjugated electronic system polypyrrole (PPy) was selected, which has been extensively studied for several purposes [27–31]. PPy presents high electrical conductivity, good stability, environmental and biocompatibility and a low toxicological impact [32]. Cyclic voltammetry was considered for the preparation of the polymeric film on the surface of the electrode. The effect of Cor a 14 (template) concentration (Fig. 1A) and the number of scans considered for the CV (Fig. 1B) were assessed in the Cor a 14-MIP construction.

The pyrrole monomer electrochemical oxidation occurs at the

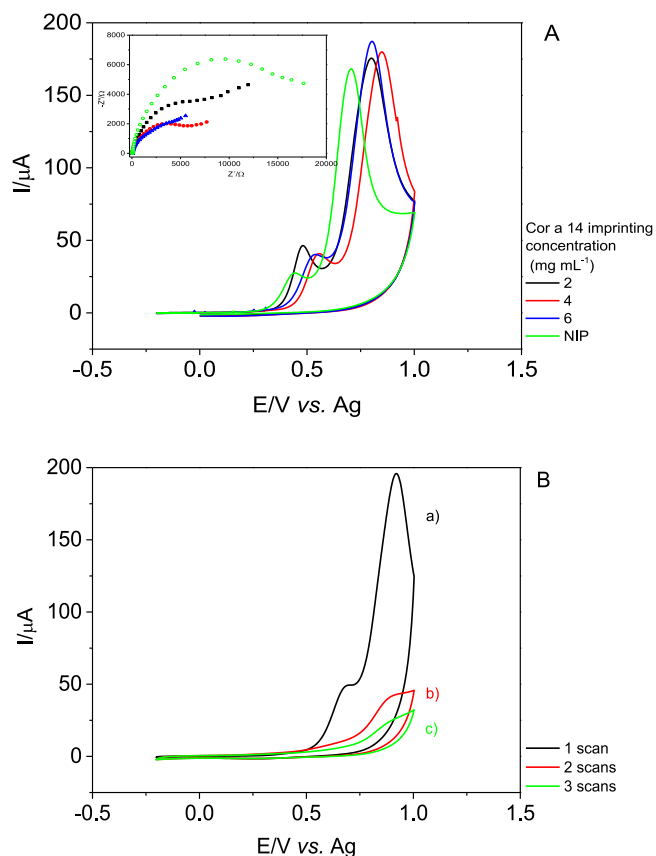


**Fig. 1.** (A) Cor a 14-concentration effect on the cyclic voltammograms obtained during electropolymerization of MIP (inset: Nyquist plots), (B) the number of scans a) 1, b) 2 and c) 3 scans during the electropolymerization stage of  $4 \text{ mg mL}^{-1}$  Cor a 14 and scan rate of  $100 \text{ mV s}^{-1}$ .

electrode surface under controlled potential conditions, thus resulting in the polymer film growth onto the surface of Au-SPE [33]. The Cor a 14 concentration was optimised to determine the best imprinting concentration into the PPy matrix. The CV for the NIP control (Fig. 1A) shows two oxidation peaks that occur at distinct potentials and with different current intensities ( $27 \mu\text{A}$  (+0.45 V vs. Ag/AgCl) and  $168 \mu\text{A}$  (+0.73 V vs. Ag/AgCl)). Comparing the CV profiles of MIP and NIP, no additional peaks were observed

within the accessible electrochemical window. This fact indicates that the allergen presence does not seem to have an impact on the electrochemical oxidation mechanism of the monomer, except on the potential oxidation, in which a shift towards more negative potentials is observed for NIP (Fig. 1A). The difference reported in the intensity of oxidation peaks may result from the number of cavities formed on the MIP produced by the incorporation of the allergen into the polymeric network, a trend that was also observed in other studies [29].

Nyquist spectra were measured by considering solutions containing 2, 4 and 6 mg mL<sup>-1</sup> of Cor a 14-template (Fig. 1A (inset)). Generally, the diameter of the Nyquist semicircle is directly proportional to the resistance (or impedance) of the electron flow at the interface (charge transfer resistance). A larger diameter was recorded in the Nyquist plot of the NIP control, representing a higher resistance to the electron flow in the cell. The radius of the semicircle of MIP is narrower than with NIP, thus revealing a lower resistance (more permeable film) due to the presence of the Cor a 14 tangled in the imprinted polymer. A concentration of 4 mg mL<sup>-1</sup> of Cor a 14 was considered as optimal for the imprinting stage.

The increase in the number of scans leads to a decrease in the intensity of the oxidation peak current after the first cycle, which may indicate the formation of an insulating thicker film deposited on the electrode surface (Fig. 1B). This optimization parameter influences the electrochemical oxidation pathway required for the pyrrole monomer oxidation on the Au electrode [34], thus establishing a compromise between thickness-tuned surface imprinted MIP film properties with imprinting performance and template extraction [35]. A correlation was established between the number of scans and the electropolymerization scan rate considered during MIP construction vs. the electrochemical performance/rebinding of the Cor a 14 (Table 1). Freundlich's isotherm was used to determine the binding affinity of the polymeric film of the MIP, by fitting to the experimental data with equation (1).

$$\frac{I_0 - I}{I_0} = K_F \cdot C_e^{1/n_F} \quad (1)$$

$K_F$  is the Freundlich constant,  $n_F$  is the Freundlich exponent,  $\frac{I_0 - I}{I_0}$  is the amount of adsorbed protein on equilibrium and  $C_e$  (mg mL<sup>-1</sup>) the equilibrium concentration. The quality of the fitting of Cor a 14 rebinding was judged based on the  $\chi^2$ , which were invariably lower than 10<sup>-3</sup>.

### 3.2. Optimization of the electrochemical parameters for MIP construction

An extensive correlation was established between the analytical performance and binding affinity ( $K_F$ ) of the Cor a 14-MIP biorecognition element vs. the electrochemical parameters adopted within the electropolymerization process (number of scans and the scan rate) (Table 1). The thickness of the imprinted PPy film is expected to influence the amount of Cor a 14 intercalated in the polymer matrix, which necessarily impacts the sensitivity of the biorecognition element. The analytical performance included the study of three scanning rates, 20, 50 and 100 mV s<sup>-1</sup> and for each scan rate, 1, 2 and 3 scans were also considered. The electropolymerization parameters significantly influence the analytical performance of the MIP, including its dynamic range response and sensitivity (Table 1). In general, faster scan rates and fewer scans led to higher sensitivity and larger dynamic range intervals. The best imprinting protocol allows the construction of a MIP with an optimal thickness vs. analytical performance relationship. Highly thick polymer films present poor accessibility to recognition sites and low binding capability ( $K_F$ ) of Cor a 14-MIP, due to possible high mass-transfer resistance [36]. The thickness of polymer film was adjusted by controlling the number of scans and the scan rate during cyclic voltammetric electropolymerization. Accordingly, the electropolymerization of Cor a 14 when carried with a specific number of scans and a specific scan rate, seems to enhance the polymer recognition ability, thus providing a 3D architecture containing effective imprinted sites for the allergen. The conductive film must be built without compromising both allergen template extraction and site accessibility during the rebinding stage. The MIP construction protocol that led to a larger dynamic range, higher sensitivity and higher  $K_F$  included 1 scan for the electropolymerization process at 100 mV s<sup>-1</sup>.

### 3.3. Evaluation of the electrochemical performance of the biorecognition platform

After the removal of the template, the rebinding of Cor a 14 was carried out to evaluate the performance of the recognition sites. SWV was considered to follow the Cor a 14-MIP rebinding performance (Fig. 2A). Freundlich's binding isotherms of synthetic Cor a 14-MIP and NIP receptor films are represented in Fig. 2B.

A linear response is observed for the MIP as a function of log  $C_{Cor}$

**Table 1**  
Correlation between the optimization of electropolymerization parameters with the analytical performance of the MIP.

| Scan rate (mV s <sup>-1</sup> ) | Number of scans | Dynamic range concentration (mg mL <sup>-1</sup> )                  | Calibration curve equation          | Freundlich isotherm |                |           |
|---------------------------------|-----------------|---------------------------------------------------------------------|-------------------------------------|---------------------|----------------|-----------|
|                                 |                 |                                                                     |                                     | k                   | n              | $\chi^2$  |
| 20                              | 1               | $1 \times 10^{-11} - 0.01$                                          | $y = 8.04x + 410$<br>$R^2 = 0.97$   | $0.42 \pm 0.02$     | $22.9 \pm 2.4$ | $8.17E-3$ |
|                                 | 2               | $1 \times 10^{-12} - 1 \times 10^{-6}$                              | $y = 5.14x + 340$<br>$R^2 = 0.99$   | $0.16 \pm 0.02$     | $14.8 \pm 3.3$ | $5.75E-3$ |
|                                 | 3               | $1 \times 10^{-12} - 1 \times 10^{-6}$                              | $y = 5.85x + 320$<br>$R^2 = 0.97$   | $0.21 \pm 0.02$     | $21.8 \pm 4.2$ | $5.02E-3$ |
| 50                              | 1               | $1 \times 10^{-12} - 1 \times 10^{-7}$                              | $Y = 1.40x + 450$<br>$R^2 = 0.98$   | $0.58 \pm 0.05$     | $18.5 \pm 2.6$ | $3.17E-2$ |
|                                 | 2               | $1 \times 10^{-6} - 0.1$<br>$1 \times 10^{-12} - 1 \times 10^{-9}$  | $Y = 1.40x + 360$<br>$R^2 = 0.98$   | $1.06 \pm 0.05$     | $21.1 \pm 1.9$ | $4.08E-2$ |
|                                 | 3               | $1 \times 10^{-12} - 1 \times 10^{-8}$<br>$1 \times 10^{-7} - 0.01$ | $Y = 6.50x + 270$<br>$R^2 = 0.98$   | $0.43 \pm 0.04$     | $21.3 \pm 4$   | $2.86E-2$ |
| 100                             | 1               | $1 \times 10^{-11} - 1 \times 10^{-3}$                              | $Y = 12.24x + 330$<br>$R^2 = 0.99$  | $1.49 \pm 0.07$     | $22.9 \pm 2.1$ | $7.78E-2$ |
|                                 | 2               | $1 \times 10^{-12} - 1 \times 10^{-8}$                              | $Y = 14.00x + 340$<br>$R^2 = 0.99$  | $0.85 \pm 0.07$     | $27.9 \pm 4.9$ | $7.93E-2$ |
|                                 | 3               | $1 \times 10^{-12} - 1 \times 10^{-5}$                              | $Y = 10.70x + 0.32$<br>$R^2 = 0.97$ | $0.86 \pm 0.06$     | $17.2 \pm 2.0$ | $4.97E-2$ |

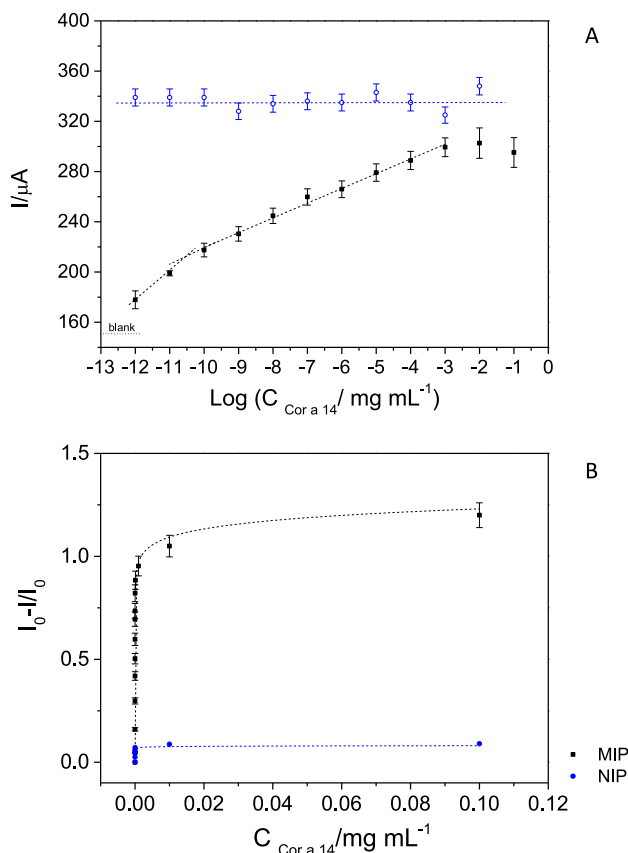


**Fig. 2.** (A) Calibration curves of Cor a 14 rebinding to MIP/NIP receptor films using electrochemical transduction in a concentration range of  $1.0 \text{ fg mL}^{-1}$ – $0.1 \text{ mg mL}^{-1}$ , in  $0.01 \text{ mol L}^{-1}$  PB pH 7.4 for 30 min using  $5 \text{ mmol L}^{-1}$   $[\text{Ru}(\text{NH}_3)_6]^{3+}$  redox probe. (B) Binding isotherm of synthetic MIP and NIP receptor films as a function of the concentration of Cor a 14. Dotted lines represent the fitting of Freundlich isotherms to the experimental data.

a 14 for the dynamic range of  $100.0 \text{ fg mL}^{-1}$ – $1.0 \text{ μg mL}^{-1}$  (Fig. 2A). The sensitivity was determined from the slope of the calibration curve ( $11.8 \pm 0.4 \text{ μA decade}^{-1}$ , correlation coefficient of 0.99152). The limit of detection (LOD) for the Cor a 14-MIP biorecognition element was  $24.5 \text{ fg mL}^{-1}$ , according to the International Union of Pure and Applied Chemistry (IUPAC) recommendations for ion-selective electrodes [37]. The analytical performance of NIP presented a random (non-linear) behaviour, with the increasing concentration of Cor a 14 (Fig. 2A), thus revealing a negligible contribution of nonspecific interactions. For a scan rate of  $100 \text{ mV s}^{-1}$  and one scan for the electropolymerization step,  $K_F$  was approximately  $1.49 \pm 0.07 \text{ mg}^{-1} \text{ mL}$ , which relates to favourable adsorption of Cor a 14 to the specific cavities. Under these conditions, the Freundlich exponent,  $n_F$ , corresponds to an estimated value of  $22.9 \pm 0.21$ , which allows concluding that the interaction between Cor a 14 and the polymeric film is favourable (Fig. 2B). The slope increases 1.36-fold from  $2 \text{ mg mL}^{-1}$  to  $4 \text{ mg mL}^{-1}$  of imprinted Cor a 14, and a slight decrease of 0.9-fold is observed for  $6 \text{ mg mL}^{-1}$ . Furthermore, the highest concentration of Cor a 14 considered during the imprinting stage presumably adds a challenge to achieve an efficient template extraction from the PPy. The surface coverage of the polymer, the electrochemical behaviour of the biorecognition platform in all stages of MIP construction with the best performance and the topographic analysis (AFM) are represented in Fig. S1 and S2 as supplementary material.

### 3.4. Redox probe – charge effect

The effect of the redox charge on the sensitivity of the hazelnut Cor a 14-MIP bioreceptor using  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox pair was also evaluated, evidencing that the protein charge plays a relevant role in the redox mechanism of an electrochemical probe. It is known that several factors can influence the performance and stability of the electrochemical biosensors, such as the case of appropriate protocols for cleaning the electrodes surface, electrolyte, incubation times and charge of the redox probe, among others. However, little is known about the adsorption behaviour of these probes onto the gold surface when routine electrochemical techniques (e.g. CV or pulse techniques) are used during the construction of the biorecognition and detection elements with potential application in biosensors.

In this part of the work, the redox probe  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was replaced by  $[\text{Fe}(\text{CN})_6]^{3-/4-}$ , which is often used to characterise the surface of electrodes and recognised to be sensitive to the surface modifications, requiring a specific surface interaction for the transfer of electrons. In Fig. 3, it is presented the analytical performance of hazelnut Cor a 14-MIP using a negative  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox pair probe.

The analytical performance curve presents a negative slope (Fig. 3), in contrast with the positive slope determined for the redox probe  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ . Contrarily to what was observed for the positively charged redox probe, the peak intensity decreases with the re-adsorption of increasing concentrations of Cor a 14. Regarding the sensitivity, the redox pair  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  present a slope of  $-1.73 \pm 0.06 \text{ μA}$  and a LOD of  $1 \text{ pg mL}^{-1}$ , which is lower than the value obtained with the  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  redox probe. The  $K_F$  and  $n_F$  determined is also lower  $-0.21 \pm 0.01 \text{ mL mg}^{-1}$  and  $7.67 \pm 0.66$ , respectively.

PPy is a positively charged conductive polymer in the oxidised form. The charge determined by the zeta potential for Cor a 14 is approximately  $-18.5 \pm 0.7 \text{ mV}$  [25]. Consequently, the charge on the PPy film can be counterbalanced after binding the oppositely charged Cor a 14, thus affecting the redox probe diffusion. The slope variation resulting from the variation in the peak current intensity can be attributed to the thickness of the film and to the electrostatic repulsions between the charge of the redox probe and the PPy thin film, which in the presence of the Cor a 14 may be dominant due to a possible heterogeneous distribution of the protein charge. Consequently, the accumulation of the negative charge can lead to constrained diffusion of the redox probe presenting the same

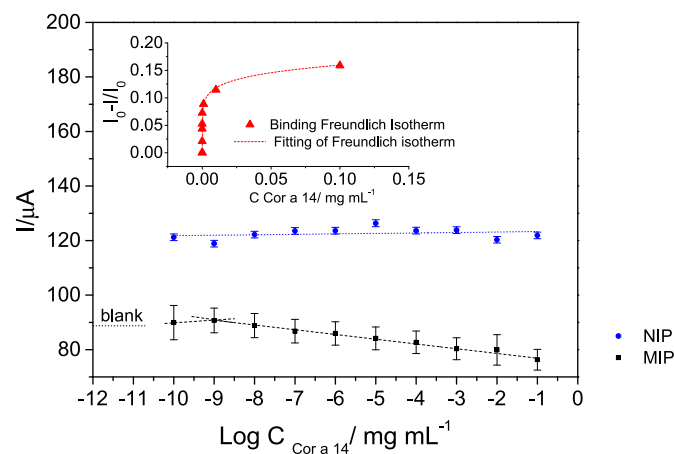


**Fig. 3.** Effect of the redox charge  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox probe. Analytical performance for hazelnut Cor a 14 rebinding to the PPy MIP.

charge [38].

In the literature, it is possible to find some basis for this double effect, given the potential attractive/repulsive electrostatic interactions of the protein with the redox probe, instead of a blocking effect of the electrode surface due to its size [39]. During the electropolymerization process, Cor a 14 was incorporated in an interleaved way in the polymeric network, which may favour the formation of a film with heterogeneous properties and asymmetric charge distribution, resulting in a decrease/increase in the electrostatic interactions established between the redox probe and the PPy film. This assumption is supported by the random response obtained at the NIP control electrode for both electrochemical probes. The charge of the redox probe selected to evaluate the analytical performance of the biorecognition element (MIP) should be carefully assessed, considering the film and the analyte properties. Depending on the interfacial properties, a positively charged redox probe like  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  could be more suitable instead of the most commonly used negatively charged  $[\text{Fe}(\text{CN})_6]^{4-}/[\text{Fe}(\text{CN})_6]^{3-}$  probe [38].

### 3.5. Surface plasmon resonance – step by step construction and sensing performance

Transducers are a key element in biosensor performance since the signal transduction process depends on their operating principle (signal generated from the bioreceptor-analyte interaction). Surface nature materials (conductivity), catalytic activity and optical properties (e.g. refractive index) will affect transducer performance (sensitivity of the analytical method, time analysis, reproducibility, detection). The possibility of combining electrochemical and optical transductions using the same biosensor (distinct dynamic range assessment) may lead to a great advance since a more accurate control over the sensing environment could be achieved (e.g. simultaneous determination of different allergens) [40].

Thus, for a deeper understanding of its sensing performance, a sensorgramme was recorded by SPR during the construction of the Cor a 14-MIP (Fig. 4A), where it can be observed a clear instantaneous increase of the SPR angle at the start of the anodic scan (0 to +0.91 V) and a faint decrease at more positive potentials than the oxidation peak. The increase in the SPR angle resulted from the electropolymerization process reported for one scan varying in a discontinuous manner from  $-1286^\circ$  to  $831^\circ$ , caused by the variation of the refractive index of growing polymer film. This sequential oscillation is normally attributed to changes in the optical dielectric properties and the thickness of the electropolymerization of the conducting polymer film onto the Au substrate, as it shifts from oxidised to reduced state [41]. The reversal scan caused a slight decrease in the SPR angle, probably induced by the reduction of the PPy film thickness that resulted from counter-ions release into the bulk solution [42]. The Cor a 14-MIP optical sensor shows a reasonable sensing response, creating a shift in the SPR angle (refractive index) of up to  $\sim 38$  mdeg, with Cor a 14-rebinding producing a linear response in the range of  $100 \text{ fg mL}^{-1}$ – $0.1 \mu\text{g mL}^{-1}$  (Fig. 4B). The LOD was estimated to be  $18.1 \text{ fg mL}^{-1}$  with a sensitivity of  $5.41 \pm 0.16 \text{ mdeg mL mg}^{-1}$ . The  $K_F$  determined with SPR was approximately  $0.22 \pm 0.04 \text{ mdeg}^{-1} \text{ mL}$  and  $n_F$   $7.78 \pm 0.94$ , 6-fold lower compared to an electrochemical transducer. A random response in the SPR angle was obtained during the rebinding of Cor a 14 onto the PPy film, thus allowing to conclude that the contribution of nonspecific binding was also negligible (inset Fig. 4B).

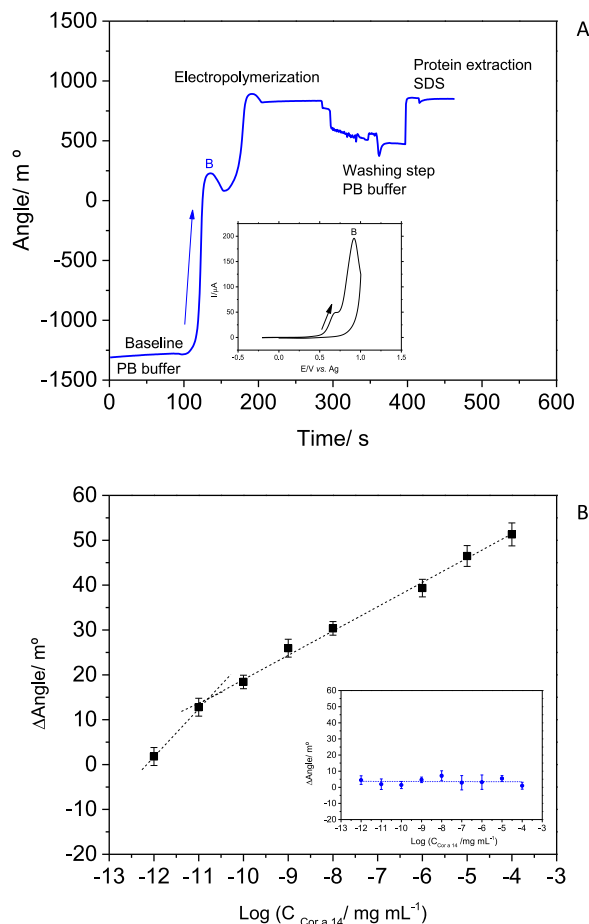


**Fig. 4.** (A) Sensorgramme of hazelnut Cor a 14-MIP construction process and (B) calibration curve of Cor a 14 rebinding to MIP using SPR as a transducer. Angle values at each concentration are the mean  $\pm$  SD (standard deviation) values of triplicate measurements. Inset: The plots of peak current as a function of Cor a 14 concentration on NIP receptor films.

### 3.6. Selectivity of Cor a 14-MIP versus Cor a 14/anti-Cor a 14 IgG

Ideally, a useful biorecognition element should respond to only one analyte. However, in practice, it is more common that recognition platforms may also respond to structurally analogue compound. For this reason, it is mandatory to evaluate the specificity/selectivity of a biorecognition receptor to verify its potential affinity to other non-target molecules (e.g. structurally homologue proteins). In this work, Cor a 14 was used as a template to imprint a polymer that would function as an “artificial” antibody for its biorecognition in real foods. Cor a 14 is a member of a specific family of proteins (2S albumins), which contains several other potent allergens from different plant species. Considering that the members of this family have high sequence and structural similarities [23], they are known to induce cross-reactive allergic reactions in sensitised/allergic individuals, especially among those with a tree nut allergy [43]. Cross-reactivity phenomena make testing and diagnosis of food allergies very challenging tasks since homologue proteins can trigger similar allergic responses [3]. When a person is clinically diagnosed with an allergy towards a specific nut (e.g. cashew nut), this often implicates the total avoidance of other nuts due to the high risk of cross-reactive allergic reactions or co-sensitisation/co-allergy (e.g. cashew nut vs. pistachio allergies) phenomena [44,45].

Among the tree nuts, some species have more genetic proximity than others, which means that their respective proteins are expressed with higher homology (high sequence similarity that is traduced in high protein structural homology) [23].

Therefore, Cor a 14-MIP was tested against 2S albumins from other plant sources, such as tree nuts (almond, Brazil nut, cashew nut, chestnut, macadamia, pecan nut and walnut), as well as from legumes (lupine, peanut) and cereals (wheat, rye). The selectivity of the Cor a 14-MIP was also evaluated towards proteins from animal origin, namely milk protein concentrate (mixture of multiple allergens) that includes the caseins (Bos d 8) and the whey (e.g. Bos d 4-Bos d 7) fraction, thus presenting numerous examples of different conformational structures (2D to 4D) [23,46]. The selectivity was evaluated considering the Freundlich constant estimated from the adjustment of its isotherms to the binding response of the 2S albumins against the Cor a 14-MIP, with each protein generating independent responses (Fig. 5A). A MIP is considered as a “plastic” or “artificial” antibody, therefore among all the other performance parameters, it is also of major importance to test its specificity and to compare it with a natural biological antibody (raised in animals). In this sense, the specificity of Cor a 14-MIP was compared with the specificity of a custom-made anti-Cor a 14 IgG polyclonal antibody (raised in rabbit) against several non-target proteins (from different plant/animal species. From previous work [25], the anti-Cor a 14 IgG demonstrated to present good parameters of quality, affinity and specificity towards the intended target Cor a 14, being further selected as the comparative biorecognition element for Cor a 14-MIP.

Cor a 14-MIP biorecognition element is highly specific to the intended target, despite showing some minor reactivity with 2S albumins from two species (Fig. 5A). The MIP  $K_F$  values for the other species follow the trend: walnut > Brazil nut > almond ~ lupine ~ wheat ~ milk protein > rye > peanut > pecan

nut > macadamia >> chestnut >> cashew nut. From the 11 different sources evaluated, the walnut and Brazil nut 2S albumins presented the highest reactivity, and the cashew nut 2S albumin exhibited the lower affinity for Cor a 14-MIP. This trend is supported by the close structural homology among the 2S albumins of different tree nuts [45]. As an example, the cross-reactivity reported for walnut (*Juglans regia*) and hazelnut (*Corylus avellana*) has been strongly correlated with their close botanical origin, as they are genetically related species belonging to the same Fagales order. In patients with severe walnut and/or hazelnut allergies, the sera from most individuals were IgE-reactive to Cor a 14 (hazelnut) and its counterpart in walnut (Jug r 1), thus reinforcing the high structural and epitopic homology among these two 2S albumins [47]. Likewise, the Ber e 1 (Brazil nut) presented a high affinity for the MIP cavities because of its high structural similarity to Cor a 14, as supported by the strong clinical cross-reactivity among both proteins along with walnut [48,49]. The selectivity of Cor a 14-MIP is higher for species with less homologue 2S albumins (more distant related species). Additionally, it is important to highlight that all 2S albumins were in their native state, suffering purification processes to minimise the presence of other proteins, but which may not be efficient to fully eliminate them. Some reactivity of Cor a 14-MIP might also result from the occupation of the cavities with proteins of small molecular size (<12 kDa), which might lead to some increment in the signal.

When the selectivity of the artificial antibody is compared with the antibody raised in a biological system targeting the same template (Cor a 14), it can be inferred that both approaches have a comparable performance for most species (Fig. 5A). Moreover, the selectivity of the MIP can be considered higher than the anti-Cor a 14 IgG because it might eliminate the recognition of specific epitopic regions (both conformational and linear epitopes), such as those from peanut, cashew nut, chestnut, and rye. The highest selectivity reported for MIP against peanut found some support in

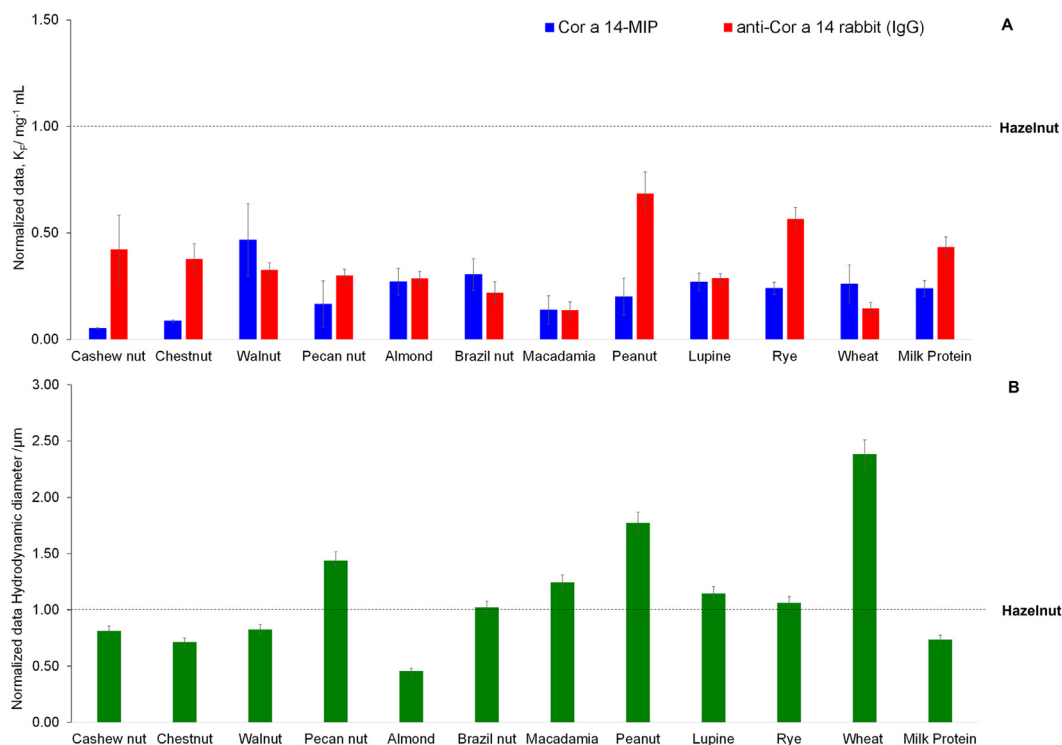


**Fig. 5.** (A) - Cross-reactivity assessment of the Cor a 14-MIP against plant 2S albumins and other animal proteins. Comparison of Cor a 14-MIP cross-reactivity with polyclonal anti-Cor a 14 IgG (rabbit). (B) - Hydrodynamic diameter obtained by dynamic light scattering and normalized for the Cor a 14 hydrodynamic diameter.

the normalized hydrodynamic diameter reported in Fig. 5B. The conformational structure, size, and shape of homologue proteins may play an important role in their binding mechanism to the cavities designed to recognise a specific target allergen. Cross-reactivity among 2S albumins is mainly associated with a high homology among their 3D structures rather than with their primary conformations [50]. Despite sharing high structural homology, 2S albumins from distinct plant species can present primary sequences with several variances in their residue composition, a factor that might contribute to some differences in their hydrodynamic diameters. For that reason, the dynamic light scattering (DLS) was performed for all species considered in the selectivity assessment of the Cor a 14-MIP sensor. Among the 7 tree nuts, the 2S albumins from pecan nut and Macadamia nut presented a higher hydrodynamic diameter compared to Cor a 14 (hazelnut), although both proteins exhibited a very limited affinity for the Cor a 14-MIP. Conversely, almond 2S albumin presented a lower diameter, but simultaneously a slightly higher affinity for Cor a 14-MIP. For the proteins with higher hydrodynamic diameter, such as the case of peanut, the selectivity of Cor a 14-MIP seemed to increase, thus presenting lower reactivity than to the anti-Cor a 14 IgG. Peanut Ara h 2/Ara h 6 and hazelnut Cor a 14 are 2S albumins with similar molecular weights (WHO/IUIS, <http://allergen.org/>), but this fact was not translated into similar hydrodynamic diameters (Fig. 5B), which could suggest some type of combined effects of Ara h 2/Ara h 6 allergens contributing to increase the hydrodynamic diameter. Consequently, the binding affinity ( $K_F$ ) and the DLS data seem to point out that the interaction of the potential cross-reactive 2S albumins with Cor 14-created cavities occurs due to a specific binding recognition rather than to protein shape/size. Lower hydrodynamic diameters were not correlated with higher cross-reactivity among 2S albumins, since the smaller proteins did not show a higher tendency to occupy the Cor a 14-MIP specific void (Fig. 5B).

Cor a 14-MIP exhibits antibody-like molecular recognition properties, which can be considered as a suitable and viable “artificial antibody” in alternative to the polyclonal anti-Cor a 14 IgG, thus offering higher selectivity and specificity compared to its biological counterpart.

### 3.7. Application of Cor a 14-MIP sensor in the context of real foods

Upon the optimization of the electrochemical Cor a 14-MIP sensor, it was important to test its applicability to real foods, which was performed using model mixtures of pasta containing known amounts of hazelnut (12 levels ranging from 1.0 to 500,000 mg kg<sup>-1</sup>) (Fig. 6A and B). The MIP-sensor presented appropriate performance parameters in a dynamic range of 1.0–1000 mg kg<sup>-1</sup> with a sensitivity of  $-10.02 \pm 0.89 \mu\text{A}$  and a correlation coefficient of 0.99127. At above 1000 mg kg<sup>-1</sup> of hazelnut in pasta, the Cor a 14-MIP seems to saturate, with no empty cavities being available for target occupation. However, the aim of most analytical methods for allergen analysis concerns the detection of trace levels, which means that the proposed Cor a 14-MIP sensor has excellent performance parameters over a higher dynamic range than most ELISA kits for hazelnut protein detection (e.g. dynamic range of 2.5–20 mg kg<sup>-1</sup> for Ridascreen® Fast Hazelnut, r-Biopharm AG, Darmstadt, Germany). The electrochemical MIP-sensor allowed detecting Cor a 14 in 1.0 mg kg<sup>-1</sup> of hazelnut incurred in pasta, which was the same LOD reported for the electrochemical immunosensor proposed for Cor a 14 quantification of real foods [25]. Likewise, the Cor a 14-MIP sensor allows the quantification of 0.16 mg kg<sup>-1</sup> of hazelnut protein in the matrix (using a conversion factor of 6.4 as reported by Wensing et al. [51]). The proposed method enables detecting hazelnut in a 500 g of serving size portion of food matrix (1.28 mg kg<sup>-1</sup>) [9], thus

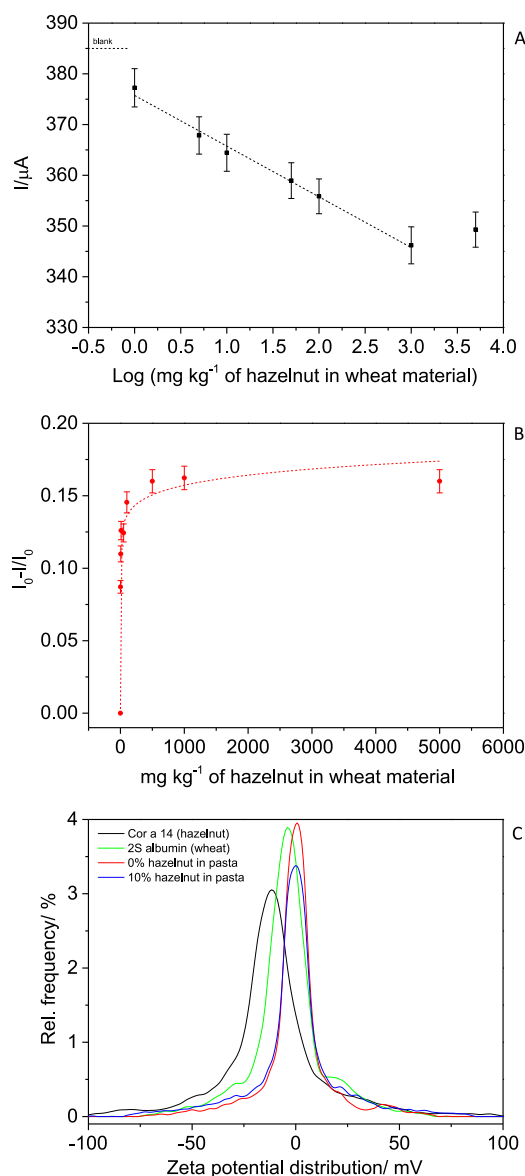


**Fig. 6.** (A) Calibration curve of Cor a 14-MIP sensor using incurred foods (1.0–5000 mg kg<sup>-1</sup> of hazelnut in pasta). (B) - Binding isotherm of Cor a 14-MIP at different percentages of hazelnut in incurred foods. Dotted lines representing the fitting of Freundlich isotherm to the experimental data. (C) - Zeta potential distribution of Cor a 14 (hazelnut), 2S albumin (wheat); 0% and 10% hazelnut in pasta in PB buffer pH 7.4.

highlighting their adequacy to quantify traces of hazelnut in foods. Moreover, the application of Cor a 14-MIP sensor for food safety assessment is expected to protect at least 95% of the hazelnut-allergic population from adverse reactions, considering the eliciting doses (ED) of 0.1 and 3.5 mg of hazelnut protein for 1% and 5% of hazelnut allergic population [52].

Biosensors for the detection/quantification of hazelnut are limited, but none of the reported is based on MIP technology or targets Cor a 14 allergen. There are few immunosensors and genosensors described in the literature for hazelnut, targeting protein markers/allergens or allergen encoding genes, respectively. Raz et al. [53] developed a SPR biosensor in a microarray format for the simultaneous detection of different allergenic foods, including

hazelnut. The authors used anti-hazelnut antibodies with no reference to target protein(s) and reported LOD of  $1.5 \text{ mg kg}^{-1}$  and  $4.3 \text{ mg kg}^{-1}$  of hazelnut in cookies and dark chocolate, respectively. Bremer et al. [54] also proposed an immunosensor targeting an antihazelnut MAb 50-5H9 monoclonal antibody to detect hazelnut in olive oil, describing a LOD of  $0.08 \text{ mg kg}^{-1}$  of hazelnut proteins in olive oil. Trashin et al. [12] developed an electrochemical immunosensor targeting Cor a 9 with polyclonal antibodies, reporting a LOD of  $10 \text{ mg kg}^{-1}$  of hazelnut in cookies. Bettazzi et al. [55] proposed two electrochemical genosensor targeting the genes encoding the Cor a 1.03 and Cor a 1.04 isoallergens, stating LOD of 0.3 and  $0.1 \text{ nmol L}^{-1}$ , respectively. The authors also declared the application of the genosensor to commercial foods, but with no reference to the relative proportion of the target in matrix. An electrochemical genosensor was also developed by Montiel et al. [56] targeting Cor a 9 encoding gene, reporting a LOD of  $0.72 \text{ } \mu\text{mol L}^{-1}$  for the synthetic target and the applicability to detect PCR products from 20 pg of hazelnut. In this regard, the proposed Cor a 14-MIP sensor presents similar analytical performances than the ones reported in the literature with the advantage of being of cheaper construction.

Compared to the Cor a 14-MIP-sensor optimization process, an inversion on the slope of the calibration curve was observed using incurred foods (Fig. 6A), despite presenting similar sensitivity. To deeper understand those unexpected results, the zeta potential was determined for Cor a 14 and wheat 2S albumin, as well as the extracts containing 0% and 10% of hazelnut in the incurred matrix (Fig. 6C). It was found a shift in the zeta potential towards less negative charged when comparing the target Cor a 14 with wheat 2S albumin. The zeta potential of the incurred matrix was estimated to be approx. 0 mV, which may be the basis for the reported slope inversion. This trend finds support in the redox probe charge effect assessment, which means that by changing the charge of the redox probe an inversion in the slope of calibration curve built-in PB buffer is also reported and discussed (Fig. 3). Accordingly, the overall net charge of the interface and the charge of the redox probe determine the positive or negative slope of the calibration curves, although presenting a negligible effect on the analytical performance of the MIP-sensor.

#### 4. Conclusion

This work describes for the first time a MIP sensor for the detection of hazelnut in foods, with target on the Cor a 14 allergen. The Cor a 14-MIP sensor was successfully developed as an alternative “artificial” antibody platform capable to mimic a specific molecular biorecognition. The Cor a 14-MIP sensor was developed with high sensitivity and specificity, presenting similar electrochemical or optical analytical performance, enabling a dynamic range of  $100.0 \text{ fg mL}^{-1}$  -  $1 \text{ } \mu\text{g mL}^{-1}$  or  $100.0 \text{ fg mL}^{-1}$  -  $0.1 \text{ } \mu\text{g mL}^{-1}$  with LOD of  $24.5 \text{ fg mL}^{-1}$  or  $18.1 \text{ fg mL}^{-1}$ , respectively.

The “artificial” antibody presented higher selectivity towards other non-target 2S albumins, exhibiting minor cross-reactivity with walnut and Brazil nut. Compared to anti-Cor a 14 IgG, Cor a 14-MIP can be considered as a promising and alternative “artificial” antibody for hazelnut Cor a 14 detection. The application of Cor a 14-MIP sensor to real foods was successfully achieved, enabling a LOD of  $1 \text{ mg kg}^{-1}$  ( $0.16 \text{ mg kg}^{-1}$  protein) of hazelnut in pasta with good performance parameters (dynamic range of 1–1000  $\text{mg kg}^{-1}$ ).

The proposed methodology should bring fundamental advances in resolving relevant shortcomings regarding the use of antibodies, namely limited shelf-lives, and instability to testing conditions. It is important to highlight that the use of proteins as templates in molecular imprinting poses some additional challenges owing to their conformational flexibility, function, molecular size, shape, and

solubility requirements. Additionally, the developed platform presents relevant advantages towards the use of biological-based recognition elements, such as higher stability, lower cost, and easier tailoring for allergen detection applications.

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#### CRediT authorship contribution statement

**Renata Costa:** Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Joana Costa:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Patrícia Moreira:** Conceptualization, Methodology, Formal analysis. **Ana T.S.C. Brandão:** Conceptualization, Methodology, Formal analysis. **Isabel Mafra:** Writing – review & editing, Project administration, Funding acquisition. **A. Fernando Silva:** Writing – review & editing, Resources. **Carlos M. Pereira:** Writing – review & editing, Resources, Funding acquisition.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2021.339310>.

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