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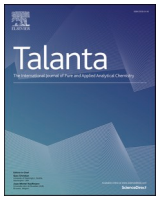

Electrochemical tracking of gluten in marketed foods by using a recombinant antibody fragment based-platform

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ABSTRACT

The only treatment to effectively manage celiac disease is the avoidance of gluten containing foods. Therefore, and given its high prevalence, it is of utmost importance to have reliable and efficient methods for the detection of gluten to ensure the well-being and quality of life of celiacs. This work presents the development of an electrochemical immunoplatform exhibiting many practical advantages including simplicity, reduced cost and high sensitivity for the screening of gluten-containing products. The methodology exploited the unique features offered by a recombinant antibody fragment with high affinity towards gliadin together with the use of magnetic microcarriers (MμCs) as scaffolds for the implementation of an indirect competitive immunoassay. Using amperometric transduction on disposable electrodes and the horseradish peroxidase/hydrogen peroxide/hydroquinone system, a dynamic range between 7.3 and 1982 ng mL^{-1} was obtained for gliadin standards, with a limit of detection of 1.4 ng mL^{-1}. The developed immunoplatform was successfully employed for the analysis of a variety of processed foodstuffs, demonstrating the ability to discriminate between gluten-free and gluten-containing foods according to the legislated threshold (20 mg kg^{-1} of gluten). The agreement with the results provided by the R5-based ELISA and qPCR methods confirmed the suitability of the bioplatform as a competitive tool in terms of assay time (results in just 60 min after gliadin extraction) sensitivity and applicability, even at the point of need.

1. Introduction

Gluten is the general name used to describe a group of storage proteins found in wheat (gliadins and glutenins), barley (hordeins), rye (secalins), oats and related products. Gluten, which provides elasticity to dough, helping it rise and hold its shape during baking, is commonly found in many staple foods, including bread, pasta, and cereals. Celiac disease, a major public health problem with a prevalence of 1 %, is an autoimmune disorder characterized by an intolerance to gluten. When people with the disease ingest gluten, their immune system reacts by attacking the small intestine, causing inflammation and damage to the intestine lining [1,2]. The only treatment for celiac disease is a strict lifelong gluten-free diet. Considering the impact of gluten on various diseases and health, several countries have established gluten labelling legislation, defining foods containing less than 20 mg kg^{-1} of gluten (10 mg kg^{-1} of gliadin) as gluten-free and those with gluten concentrations between 20 mg kg^{-1} and 100 mg kg^{-1} (10 and 50 mg kg^{-1} of gliadin, respectively) as low-gluten [1–3].

Gliadin, the main alcohol-soluble prolamin of wheat gluten (as opposed to hordein and secalin, which are the prolamins of barley and rye, respectively), cannot be completely digested by intestinal enzymes. In fact, the remaining 33-mer gliadin peptide is currently considered the main trigger of celiac disease. This is why many of the analytical methods for gluten detection in foods interrogate gliadin or its immunogenic peptides [4]. Several analytical methods have been developed to detect and quantify gluten in foodstuffs, including PCR [5],

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chromatography and mass spectrometry [6] and immunological techniques, including lateral flow immunoassays (LFIAs) [7] and the most widely used enzyme-linked immunosorbent assays (ELISAs) [8]. However, it is important to highlight that the reliability of the results obtained by the existing immunoanalytical methods depends on different factors, e.g. complexity of the food matrix, effects of food processing on the physical-chemical properties of the target proteins, the extraction method, sensitivities and specificities of the antibodies used (dependent on both the source of (wheat, rye, or barley) and the fraction (prolamins or glutelins)), and the materials against which the methods were calibrated [1,9–13]. Although currently there is no reference method, the ELISA using the R5 gliadin antibody is the one recommended by the Codex Alimentarius Commission and accepted by AOAC International as the "first official action" method [1]. However, this method based on a sandwich-type assay, can only be used for the detection of non-hydrolyzed gluten, as it requires the presence of at least two epitopes in a protein or peptide. This aspect is particularly relevant for analyzing processed samples, with smaller gliadin fragments which contain only one epitope, and for which quantification is no longer accurate [14]. It is important to mention also that the R5 gliadin antibody shows cross-reactivity with certain oat cultivars, attributed to the high similarity of certain peptides found in avenins (gluten-like proteins in oats) with gluten peptides [15]. In addition, although R5 antibody was raised against rye prolamins, due to its cross-reactivity, it reacts with wheat, barley and rye gluten, which may result in an inaccurate gluten estimation in some samples [10,16,17]. It is worth mentioning here also that there are other ELISAs based on monoclonal antibodies, such as the G12 and A1, which offer greater specificity towards gluten immunogenic peptides [18–20].

Nevertheless, the observed increase in the prevalence of gluten-related disorders together with the demands of the society to employ simple, affordable and point-of-care (POC) technologies has prompted the development of novel technologies for sensitive and selective gluten detection in foodstuffs that should ideally combine the use of tools capable of meeting these characteristics with that of new sustainable receptors obtained by molecular evolution avoiding animal immunization, such as phage display. Notable among them are recombinant antibodies which stand out as versatile and customizable receptors that offer improved performance over classical monoclonal and polyclonal antibodies. They are produced using in *vitro* techniques, thus avoiding the need for animal immunization and the presence of unwanted light chains generated by the hybridoma cells. Moreover, they can be produced in large amounts with reduced batch-to-batch variability, therefore increasing the reproducibility and reliability of the detection [15,21]. Between recombinant antibodies, single-domain (sdAbs) and Fab antibody fragments have showed benefits for the detection of food allergens [15,21–26] due to their characteristics, such as small size and high stability, which give them privileged access to different antigenic regions in proteins compared to conventional antibodies.

In this context, electrochemical affinity bioplatforms are versatile biotechnologies suitable for the determination of allergens of different nature. Over the last years they have gathered significant attention for the reliable determination of allergenic proteins [27,28] both in processed and unprocessed incurred samples and in commercial products, genetic targets in different organelles (nuclei [29], chloroplasts [30]) as well as oligosaccharide-type allergens, such as galactose-α-1,3-galactose (α-Gal) [31] involved in allergy to red meat. The state of the art also includes some electrochemical immunoplatforms for the determination of gluten employing commercial conventional antibodies in sandwich [32,33] or direct [34] formats that offer attractive analytical characteristics but require the use of nanomaterials as electrode modifiers and long preparation times of the biosurfaces [32–34].

With all this in mind, this work reports the development of the first electrochemical immunoplatform for the determination of gluten in foodstuffs employing a directed evolved antibody fragment obtained from the transformation of a recombinant sdAb into a Fab format by phage display (we will name it in this work as $rFab_{Gliadin}$) and a competitive format. The developed method combined in a pioneering way the unique characteristics of such designed antibody fragment with high affinity towards gliadin and sustainably produced without using laborious *in vivo* procedures and animal immunization, of magnetic driven μ-carriers (MμCs) for the implementation of an indirect competitive immunoassay, and, of amperometric transduction using the H_2O_2/hydroquinone (HQ) system on disposable electrodes, which facilitate interfacing with existing acquisition platforms such as smart potentiostats and personal glucose meters. The method stands out for its sensitivity, reduced assay time and satisfactory applicability for the simple analysis of a good representation of foodstuffs, with very variable matrices and processing. Indeed, it provides competitive results compared to the R5-based ELISA and qPCR methodologies in terms of assay time and applicability at the POC and has been applied to the analysis of a variety of foodstuffs.

2. Experimental section

2.1. Apparatus and electrodes

Amperometric recordings were conducted at room temperature using a CHI812B potentiostat (CH Instruments, Inc., USA) using the CHI812B software. Screen-printed carbon electrodes (SPCEs, DRP110, working electrode: 4-mm Ø, active area 12.6 mm^2) and the electrode connector (DRP-CAC) were acquired from Metrohm-DropSens (distributed by Metrohm Hispania, Spain). A polymethylmethacrylate (PMMA) platform was built and designed to magnetically capture the prepared immunoconjugates. The platform contained an embedded neodymium magnet (AIMAN GZ S.L., Spain) positioned just under the SPCE working electrode allowing the efficient magnetic capture of the immunoconjugates.

The modification of the magnetic microcarriers (MμCs) implied the use of a Dynamag-2 Magnet magnetic separator (Invitrogen-ThermoFisher Scientific). A vortex (VELP Scientifica, Italy, distributed by Scharlab, Spain), a Basic 20+ (Crison Instruments S.L., Spain) pH-meter and a thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik GmHb, Germany, distributed by Scharlab, Spain) were used. The sample extraction protocol required a flask shaker (Vibromatic, PSelecta, distributed by Scharlab, Spain), and a centrifuge (Centrifuge 5804 R, Eppendorf Ibérica S.L.U., Spain).

2.2. Reagents and solutions

All the reagents employed were of the highest available analytical grade. Carboxylic acid-functionalized MμCs (HOOC-MμCs, 2.8 μm Ø, 2 × 10^9 beads mL^{-1}, Dynabeads™ M − 270 carboxylic acid, 14305D, Invitrogen, USA) were purchased in ThermoFisher™ (USA, distributed by ThermoFisher, Spain) 2-(N-morpholino)ethanesulfonic acid (MES) and Tris-hydroxymethyl-aminomethane-HCl (Tris-HCl), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were acquired from Scharlab (Spain). N-hydroxysulfosuccinimide (Sulfo-NHS), N-(3-dimethyl-aminopropyl)-N′-ethyl-carbodiimide (EDC), ethanolamine (ETA), hydrogen peroxide (H_2O_2, 30 % (w/v)) and hydroquinone (HQ) were acquired from Sigma-Aldrich (Spain). Commercial Blocker™ Casein in PBS (blocking buffer, BB) was acquired from Thermo Scientific (USA, distributed by ThermoFisher, Spain).

The gliadin reference material of the Prolamin Working Group (PWG) (Hans-Dieter-Belitz-Institute, Freising, Germany), obtained from 28 European wheat cultivars, was used to prepare the calibration with standards. PWG-gliadin stock solution (1 mg mL^{-1}) in 60 % ethanol was prepared in glass vials and stored at room temperature no longer than one month. A recombinant antibody fragment specific to gliadin ($rFab_{Gliadin}$) obtained from the supernatant of induced *E. coli* K12 RV308 by IMAC (Immobilized Metal Affinity Chromatography) [15] and a

commercial rabbit polyclonal antibody anti-human IgG (heavy and light chains) conjugated with horseradish peroxidase (HRP-RAH, reference ab6759, Abcam, United Kingdom) were used as recognition and tagging antibodies, respectively. The $rFab_{Gliadin}$, isolated by phage display from the transformation of sdAb against gluten into a Fab format by combining the heavy variable sequence of the sdAb with immunoglobulin light sequences derived from celiac patients, was previously fully characterized for the specific detection of the gluten-containing cereals [21].

The preparation of the used buffer solutions (0.025 mol L^{-1} MES buffer pH 5.0; 0.1 mol L^{-1} phosphate buffer (PB) solution pH 8.0; 0.1 mol L^{-1} Tris-HCl pH 7.2; 0.01 mol L^{-1} and 0.05 mol L^{-1} PB solution pH 6.0) was carried out in deionized water type I from a Millipore Milli-Q purification system (18.2 MΩ cm). The modification of the HOOC-MμCs required an EDC/Sulfo-NHS mixture solution (50 mg mL^{-1} each, prepared in MES buffer pH 5.0) and a 1.0 M ethanolamine (ETA) solution prepared in 100 mM PB buffer pH 8.0. To perform the amperometric measurements, 0.1 M HQ and 0.1 M H_2O_2 solutions were freshly prepared in 0.05 M PB buffer pH 6.0.

2.3. Preparation of the gliadin-magnetic driven μ-carriers (Gliadin-MμCs)

The modification of HOOC-MμCs was made in 1.5 mL microcentrifuge tubes while shaking (950 rpm) at constant temperature (25 °C) using incubation volumes of 25 μL. On the other hand, 50 μL of the appropriate buffer solution were used to perform the washing steps, which consisted of placing the microcentrifuge tubes containing the MμCs suspension in a magnetic concentrator for 3 min to remove the supernatant with minimal MμCs loss.

Each assay was carried out by placing a 2 μL-aliquot of the HOOC-MμCs suspension (4×10^6 beads) into a microcentrifuge tube and washing twice for 10 min with 50 μL MES buffer. The covalent linkage of gliadin was performed using the EDC/Sulfo-NHS chemistry, involving the activation of the HOOC-MμCs suspension with a freshly prepared EDC/Sulfo-NHS solution for 35 min. After washing twice with MES buffer, the activated HOOC-MμCs were incubated in a 1.5 μg mL^{-1} gliadin solution (made in MES buffer) for 30 min. The as-prepared Gliadin-MμCs were washed twice with MES buffer and incubated in 1.0 M ETA solution for 60 min to deactivate the residual groups. After washing once with Tris-HCl buffer and twice with BB buffer, the Gliadin-MμCs were kept in PBS buffer until used. It is important to note that, although the storage stability of Gliadin-MμCs was not evaluated in this work, regarding our previous works, the bioconjugates prepared by immobilizing other proteins using the same supports and the same EDC/Sulfo-NHS chemistry were stable for at least 1 month [35].

2.4. Implementation of the indirect immunoassay and electrochemical detection

The determination of gliadin was performed using an indirect competitive immunoassay between the Gliadin-MμCs immunoconjugates and gliadin free in solution for binding the limited sites of a fixed $rFab_{Gliadin}$ concentration. For this purpose, a mixture solution containing the Gliadin-MμCs immunoconjugates, gliadin standard (or the sample to be analyzed) and $rFab_{Gliadin}$ (1/200 dilution) prepared in BB was incubated for 45 min. Thereafter, the HRP labelling of $rFab_{Gliadin}$ antibodies was performed by incubating the resulting MμCs immunoconjugates in a 1000-fold diluted HRP-RAH solution prepared in BB for 10 min.

The resulting modified MμCs immunoconjugates were washed twice with BB solution and re-suspended in 50 μL of 0.05 M PB, pH 6.0, to perform the amperometric measurements, which implied capturing the as-prepared MμCs immunoconjugates on the corresponding working electrode surface by inserting the SPCE into the lab made PMMA casing with an embedded Nd magnet. The SPCE/magnet holding block was immersed into an electrochemical cell containing 10 mL of 0.05 M PB (pH 6.0) and 1 mM HQ to measure the cathodic current, under continuous stirring, at a constant potential of −0.2 V (*vs.* Ag *pseudo*-reference electrode) upon the addition of 50 μL of 0.1 M H_2O_2 solution until reaching the steady state. The cathodic current values provided in the manuscript correspond to the difference between the steady state and background current of three replicates, estimating the error bars as the standard deviation of the replicates.

Cyclic voltammetry was performed between −500 and 800 mV (*vs.* Ag *pseudo*-reference electrode) at a scan rate of 50 mV s^{-1} in 0.05 M PB (pH 6. 0) in the presence of 1 mM HQ and 5 mM H_2O_2.

2.5. Samples preparation

Whole grain kernels of wheat, barley and rye were provided by the Spanish National Centre for Plant Genetic Resources (CRF-INIA, Madrid, Spain). These gluten-containing grains were used as reference samples and included seeds from ten different cultivars of *Triticum aestivum vulgare* (common wheat), *T. aestivum spelta* (spelt wheat), *T. turgidum turgidum* (rivet wheat), *T. turgidum durum* (durum wheat), *Hordeum vulgare* (barley) and *Secale cereale* (rye).

All cereal kernels were grounded individually employing an IKA A11 analytical mill (IKA®, Staufen). With the aim of obtaining a wheat-barley-rye mixture (WBRm) containing one third of each target cereal, cultivars belonging to the same cereal type were homogenized and proportionally combined to get the so-called WBRm. A total of 100 g of the WBRm placed on disposable baking trays was subjected to two different heat treatments at 160 °C for 13 min and 200 °C for 25 min in a conventional laboratory oven (Heraeus, Thermo Scientific).

Subsequently, laboratory-scale binary mixtures of the raw and heat-treated WBRm were prepared using a matrix of maize flour because of the absence of background signal when testing this flour with the used detection system. These binary mixtures were prepared to include representative gluten spiked levels, assuming as overall content of gluten proteins a 10 % of the cereal weight [21,36] since the protein content of the grains ranged between 8 and 15 % with 80–90 % representing the gluten proteins. Thereby the analyzed binary mixtures with 1000, 200 and 100 mg of WBRm per kg could be equated to samples containing theoretical gluten levels of approximately 100, 20 and 10 mg kg^{-1}. To guarantee the homogeneity of the incurred samples, a first mixture containing 100000 mg of WBRm per kg (10 %) was obtained by thoroughly mixing 5 g WBRm with 45 g of maize flour for 5 min in a food grinder. This sample was subsequently diluted in maize flour to obtain the desired concentration levels of 1000, 200 and 100 mg kg^{-1}.

A total of 37 commercial food products and three fermented beverages were acquired in retail markets of Madrid (Spain) to include a wide representation of products with different labelling declarations regarding cereals.

2.6. Gluten extraction from food and fermented beverages samples

50 g of each sample were ground ensuring thorough cleaning between samples and stored at −20 °C. The gluten-like proteins were extracted from 250 mg of finely ground samples (either experimental binary mixtures or food products) using 2.5 mL of the SENSISpec Gluten extraction solution (Gold Standard Diagnostics, Madrid, Spain) and 7.5 mL of ethanol 80 % (v/v). The samples were incubated for 1 h at room temperature in a vertical rotator and centrifuged at 2000×*g* for 10 min. The supernatants containing the gluten-like proteins were transferred to a glass vial and stored in darkness until analysis.

For validation purposes, the protein extracts from food products were analyzed in parallel with the R5 monoclonal antibody-based sandwich ELISA (SENSISpec INgezim Gluten kit with a limit of detection of 3 mg kg^{-1} of gluten, Gold Standard Diagnostics) according to the manufacturer's protocol. Additionally, the samples were analyzed by two different previously lab-developed ELISA methods using the $rFab_{Gliadin}$. These assays were performed following the methodology described by Garcia-Calvo et al. [15] for the indirect ELISA and [37] for

the sandwich ELISA. Finally, an alternative non-immunological method based on a previously reported qPCR assay was also performed following the methodology described by García-García et al. [5].

3. Results and discussion

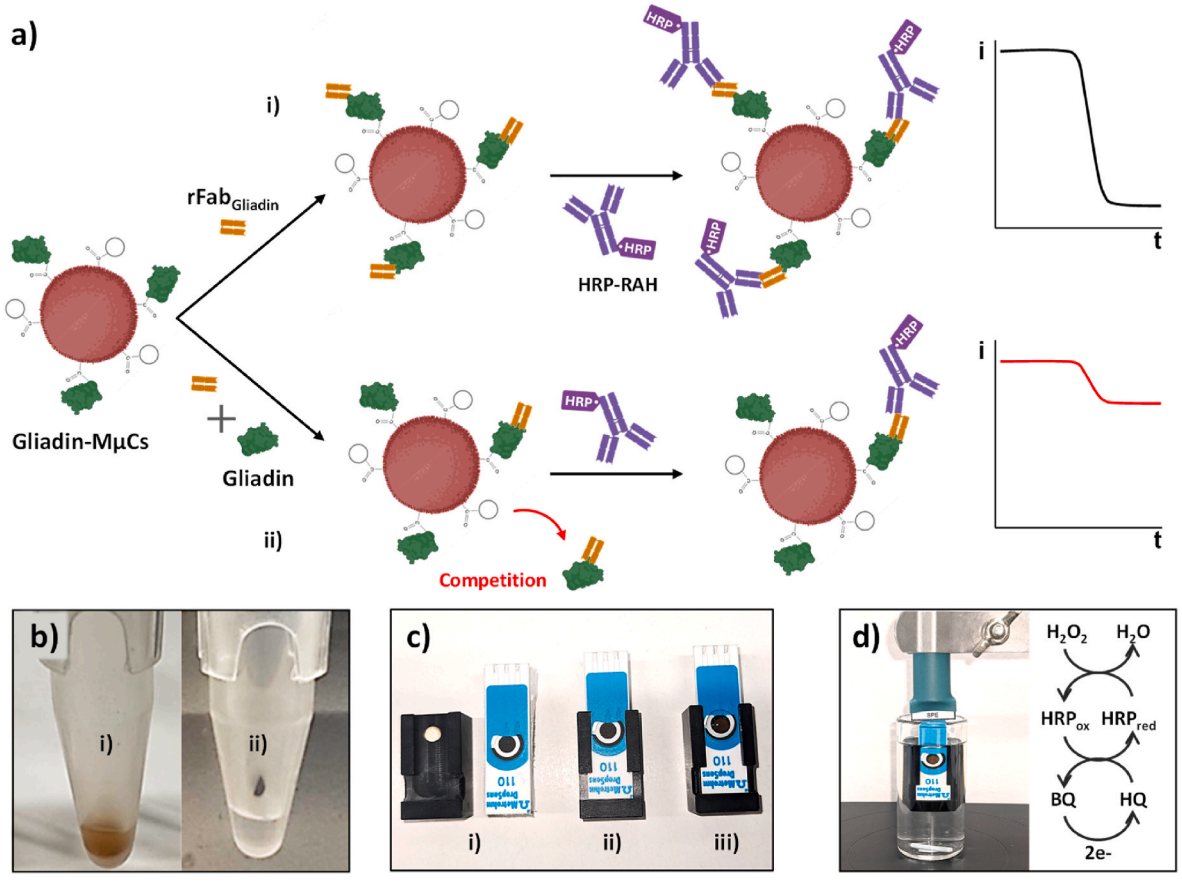
The overall workflow involved in the preparation of the amperometric immunoplatform for the determination of gluten in foods is depicted in Scheme 1. An indirect competitive immunoassay format was implemented to determine the major gluten protein, gliadin. The proposed immunoassay utilizes a recombinant antibody fragment (rFab$_{Gliadin}$) specific to gliadin from wheat, rye, and barley, which does not cross-react with other heterologous species [21]. In addition, gliadin-covalently linked magnetic driven μ-carriers (Gliadin-MμCs) were employed as competitors and polyclonal IgG antibodies specific to human antibodies, conjugated to horseradish peroxidase (HRP-RAH), were employed for tagging and electrochemical tracking of the resulting bioconjugates. The immunorecognition events were monitored by recording the cathodic current generated at −200 mV (*vs.* the Ag *pseudo*-reference electrode) in stirred solutions using the HRP/H_2O_2/HQ system [38,39]. For this, the bioconjugates were previously trapped on the SPCEs by positioning, under the working electrode surface, a Nd magnet integrated in a PMMA casing designed specifically for proper SPCE-magnet assembly.

According to the rationale of the proposed assay, in the absence of gliadin, the total rFab$_{Gliadin}$ were immobilized on Gliadin-MμCs and subsequently tagged with the HRP-RAH, resulting in a large cathodic response (Scheme 1a). However, in the presence of free target gliadin, which competes with the immobilized gliadin for the available binding sites of the specific antibody fragment, a lower amount of rFab$_{Gliadin}$ was immobilized on the MμCs, and thus, a decrease in the cathodic response was observed (Scheme 1b). Consequently, the recorded amperometric responses were inversely proportional to the concentration of free gliadin in the sample.

3.1. Optimization of critical experimental variables for effective immunoassay implementation

The primary considerations for preparing the immunoplatform were to develop a simple and rapid methodology with potential for on-the-spot application, while ensuring the specificity and sensitivity demanded for the accurate detection in gluten-containing foods. To achieve these goals, a systematic evaluation of all key variables involved in the methodology's implementation was made. Given the challenges to optimize the conditions of competitive immunoassays, it is especially relevant to control the amount of both rFab$_{Gliadin}$ and competitor involved in the assay, in addition to maximizing the electrochemical signal window in the absence of the target analyte, while ensuring adequate competition in the lowest concentration range. In this regard, a higher competition ratio −defined as the relationship between the amperometric signals measured in the absence and in the presence of 50 ng mL^{-1} of gliadin− was used as criterion for selecting the values of experimental variables. The overall set of the tested conditions and the corresponding selected values to implement the immunoplatform are summarized in Table 1 and displayed in Fig. 1, respectively.

Competitor optimization entailed checking the amount of MμCs and



Scheme 1. a) Schematic diagram of the indirect competitive immunoassay developed for the gliadin detection involving the use of rFab$_{Gliadin}$, HRP-RAH and Gliadin-MμCs. Workflow both in the absence (i) and presence of free gliadin target (ii). (Created in part with Biorender, https://www.biorender.com). Real images of the MμCs-assisted amperometric immunosensing platform for gliadin determination: b) MμCs manipulation, showing suspension (i) and magnetic capture (ii); (c) SPCE and magnet holding block, depicted separately (i), as an assembly (ii), and with bioconjugates captured onto the assembly (iii); and d) amperometric readout process in stirred solutions using the HRP/HQ/H_2O_2 system.

Table 1
Experimental variables, evaluated ranges, and values selected for the implementation of the gliadin determination immunoplatform.

Variable	Range studied	Selected value
MμCs × 10^6	2.0–10.0	4.0
[Gliadin], μg mL^{-1}	0.0–2.5	1.5
Conjugation time, min	15–60	30
Steps	1–2	2
$rFab_{Gliadin}$ dilution (concentration in μg mL^{-1})	1/300–1/100 (1.3 – 3.8)	1/200 (2.0)
Competition time, min	30–90	45
HRP-RAH dilution	1/5000–1/500	1/1000
Tagging time, min	5–30	10

immobilized gliadin, as well as the conjugation time for the covalent assembly of gliadin onto the MμCs (Fig. 1a)). An increase of the MμCs quantity led to increased cathodic responses in all cases, attributed to the more efficient trapping of more detection antibodies using larger amounts of Gliadin-MμCs. However, this also resulted in a smaller competition ratio above 4 × 10^6 beads, achieving a higher value for this amount of MμCs. The optimization of the concentration of gliadin immobilized on MμCs revealed that the responses, both in the absence and in the presence of free gliadin in solution, increased with the amount of immobilized gliadin up to 2 μg mL^{-1}. Beyond this concentration, the response in the absence of free-gliadin decreased, most likely due to steric hindrance effects impairing the recognition of the detection antibodies [40]. Additionally, large concentrations of immobilized gliadin may also impede competition. Therefore, a larger competition ratio was observed for a 1.5 μg mL^{-1} gliadin concentration. It should be remarked that responses below 100 nA were measured in the absence of immobilized gliadin (Fig. 1a), bars 0), demonstrating the appropriateness of the methodology based solely on gliadin-$rFab_{Gliadin}$ recognition and confirming the absence of nonspecific adsorptions onto the MμCs. The conjugation time was evaluated over a 15–60 min range, selecting 30 min for further work as it provided a larger competition ratio.

Although immunoassays are often streamlined to reduce the number of steps, an optimization of this aspect is crucial to exploit the efficiency of the recognition reactions. Therefore, two approaches were tested: a single 30-min step, combining competition and tagging through the simultaneous incubation of the target, Gliadin-MμCs, $rFab_{Gliadin}$, and HRP-RAH; and 2 steps (30 min each), starting by the competition among the target, Gliadin-MμCs, and $rFab_{Gliadin}$, and followed by tagging the immunocomplexes with the HRP-RAH. Fig. 1b) shows as the 2-step protocol provided larger amperometric responses and competition ratio, with very low responses when the 1-step immunoassay was performed. Even though the use of antibody fragments is advantageous due to their small size, enabling their access to different antigenic sites on proteins more effectively than conventional antibodies [26], combining competition and tagging in a single step can quickly lead to the formation of $rFab_{Gliadin}$/HRP-RAH conjugates, thus hindering the successful recognition of the immobilized gliadin on the MμCs by steric hindrance. Therefore, competition and tagging in separate steps were chosen for further work.

After optimizing the competitor, the other variables involved in the competition, including the amount of $rFab_{Gliadin}$ and competition time, were also evaluated. Since the number of antibody-specific binding sites is critical for tuning the competition, its concentration was optimized by

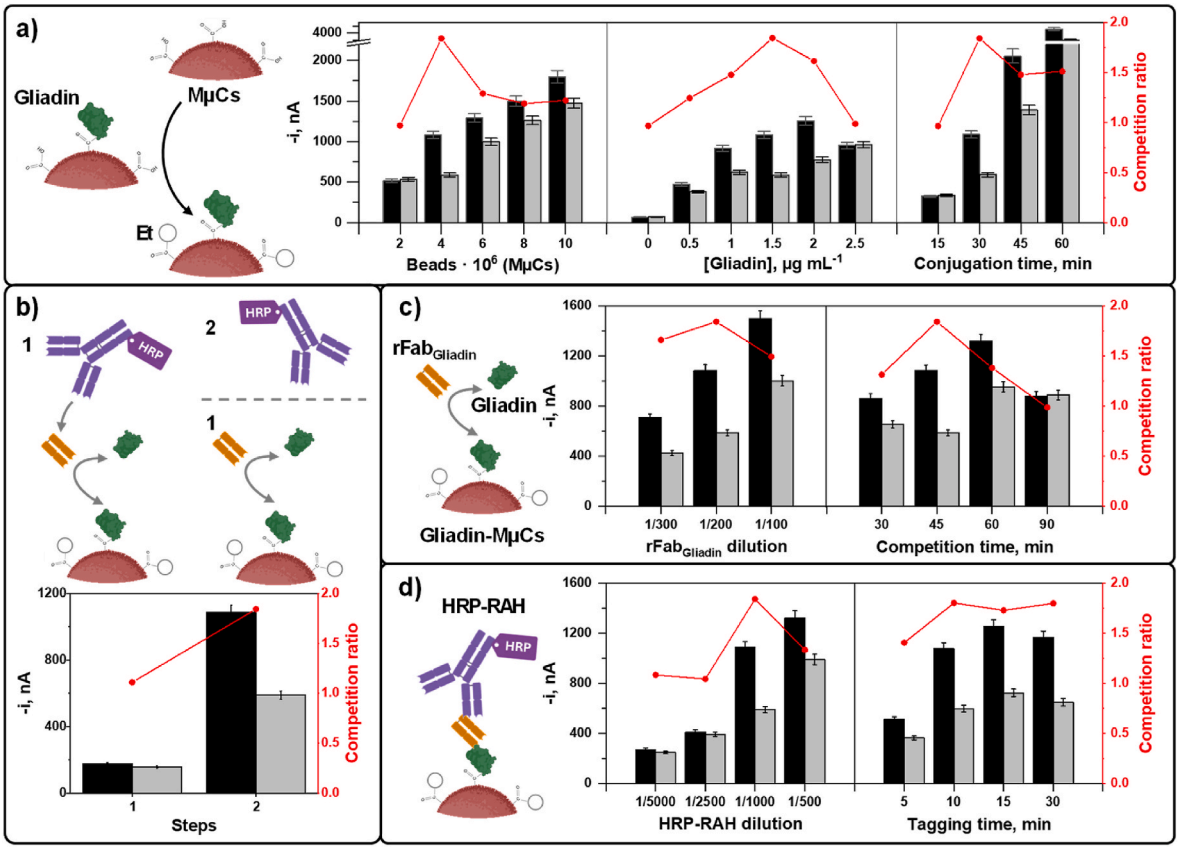


Fig. 1. Dependence of the amperometric responses provided by the developed immunosensing platform in the absence (black bars) and in the presence of 50 ng mL^{-1} gliadin (grey bars), and the resulting competition ratio values (red circles connected by lines), with the MμCs and gliadin loadings and gliadin-MμCs conjugation time a), number of steps involved in the immunoassay b), $rFab_{Gliadin}$ dilution and competition time c) and HRP-RAH dilution and incubation time for tagging d). Schematic diagrams of each set of optimized variables inserted along with the figures. (Created in part with Biorender, https://www.biorender.com). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

testing three different antibody fragments working dilutions. Fig. 1c) shows that, regardless of the increase in the cathodic responses with the quantity of $rFab_{Gliadin}$, both in the absence and presence of target gliadin, the competition ratio decreased for the lower $rFab_{Gliadin}$ dilution tested (1/100), because an excess of antibody does not promote effective competition [31]. Consequently, a 1/200 dilution was selected for subsequent experiments. The study of the incubation time for the competition mixture containing Gliadin-MμCs, free-gliadin, and $rFab_{Gliadin}$ (Fig. 1c)) showed that a higher competition ratio was achieved for 45 min.

Finally, the amount of secondary antibody conjugated to the enzymatic tracer, as well as the time for tagging were optimized (Fig. 1d)). A decrease in the HRP-RAH dilution provoked an increase in the cathodic responses in all cases. In fact, large dilutions (1/5000−1/2500) provided low responses. Dilutions lower than 1/1000 allowed implementation of the competitive immunoassay. Specifically, 1/1000 dilution of HRP-RAH resulted in a better competition ratio and involved a lower enzyme tracer concentration ensuring efficient immunocomplex tagging and minimizing nonspecific adsorptions onto the MμCs. Regarding the time for tagging, both the amperometric responses and competition ratio increased up to 10 min, and a plateau for the competition ratio was observed for longer times. Therefore, 10 min was selected as the incubation time. This rapid detection time supports our earlier discussion on the number of involved steps. We suggested that the inability to combine the competition and tagging into a single step might be due to the swift formation of the $rFab_{Gliadin}$/HRP-RAH conjugates.

According to the optimized variables summarized in Table 1, the used protocol allowed gliadin analysis within ng mL^{-1} range in an overall time of 55 min (starting from Gliadin-MμCs), accomplished in just two steps. Regarding the applied potential, as it can be observed in Fig. S1 (in the Supporting Information), a potential of −200 mV (*vs.* the Ag *pseudo*-reference electrode) provided the best amperometric signals, thus it was selected for the amperometric measurements using the HRP/H_2O_2/HQ system. Other variables involved in the amperometric transduction such as the concentrations of the enzyme substrate (H_2O_2) and redox mediator (HQ), were optimized in previous works [41,42].

3.2. Analytical and operational parameters for gliadin determination

During the optimization of the immunoplatform preparation, the strategy demonstrated adequate sensitivity for gliadin detection in the low ng mL^{-1} range. However, a comprehensive characterization of the methodology was necessary to ensure a robust correlation between the analytical signal and the gliadin concentration. Therefore, various gliadin standard solutions, over the 1−5000 ng mL^{-1} concentration range, were tested under the optimized working conditions, resulting in the amperometric responses and calibration curve shown in Fig. 2a). As expected, the results were fitted to a sigmoidal curve (r^2 = 0.991), consistent with the nature of competitive immunoassays, through the equation:

$$y = i_{min} + \frac{(i_{max} - i_{min})}{1 + \left(\frac{x}{EC_{50}}\right)^{-p}}$$

In addition to the analytical signal (y, nA) and gliadin concentration (x, ng mL^{-1}), the parameters included in the equation are: the lowest cathodic signal for a hypothetical infinite concentration of gliadin (i_{min}), the highest cathodic response in the absence of gliadin (i_{max}), the concentration that reduces i_{max} by half (EC_{50}), and the Hill slope of the calibration curve (p) (parameter values summarized in Table 2). Based on the fitting, the limit of detection (LOD), defined as the concentration at which i_{max} is reduced by 10 %, was calculated to be 1.4 ng mL^{-1} (35 pg in 25 μL). Additionally, the dynamic range was set between 7.3 and 1982 ng mL^{-1}. A linear dependence (r^2 = 0.989) between the cathodic responses and the logarithm of gliadin concentration, with slope and intercept values of (−311 ± 10) nA and (−1178 ± 21) nA, respectively, was found (Fig. 2b)). Although this fitting does not enhance the assay's sensitivity, it allows extending the dynamic range up to 5000 ng mL^{-1}. Additionally, the application of linear regressions may facilitate more convenient and accurate quantification of real samples.

The reproducibility of the developed methodology was evaluated by comparing the amperometric responses for 50 ng mL^{-1} gliadin with eight immunoplatforms constructed on different working days. A relative standard deviation (RSD) value of 4.2 % demonstrated the excellent reproducibility of all the steps involved in the fabrication of the magnetic driven competitors, immunoassay, and electrochemical readout.

The widespread incidence of celiac disease has led to the proliferation of reported papers and commercial methodologies for the detection of gluten in food, hindering a complete and comprehensive comparison.

Table 2
Fit-related parameters corresponding to the sigmoidal curve shown in Fig. 2a) obtained with the developed immunoplatform for the determination of gliadin.

Parameter	Value
r^2	0.991
Dynamic range, ng mL^{-1}	7.3–1982
EC_{50}, μg mL^{-1}	121 ± 33
i_{min}, nA	−99 ± 58
i_{max}, nA	1146 ± 76
p	0.50 ± 0.08
LOD, ng mL^{-1}	1.4

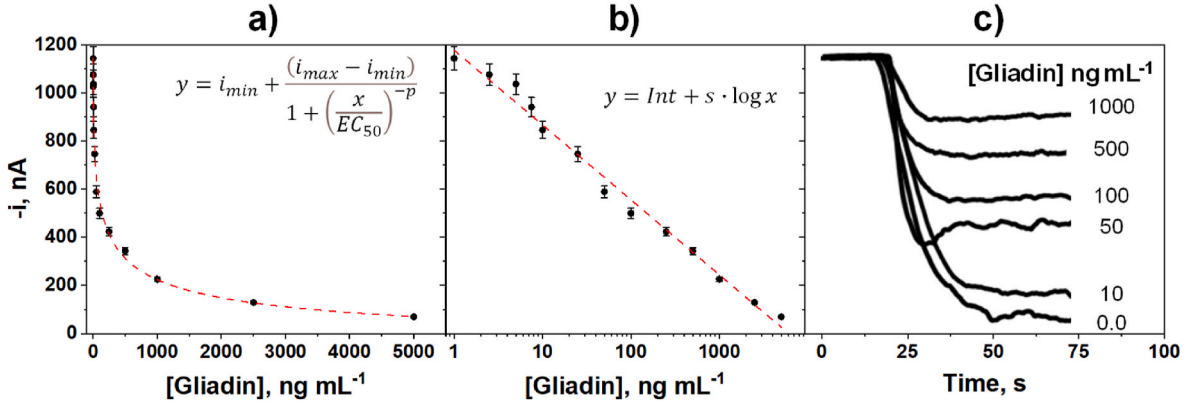


Fig. 2. Sigmoidal a) and linear b) fitting curves for the cathodic responses versus gliadin concentration or logarithm of the gliadin concentration, respectively, obtained with the gliadin immunosensing platform. Amperograms recorded for different gliadin concentrations c). In b) Int: intercept; s: slope; and x: [Gliadin].

Consequently, we will focus exclusively on comparing the results achieved with the developed immunoplatform with those of electrochemical affinity biosensors reported in the past five years as well as with the gold standard ELISA method based on the R5 gliadin antibody. The LOD achieved was significantly lower [2,33,34,43–45] or similar [32,46] to those reported for most of the electrochemical immuno- or aptasensors (see Table 3). Nevertheless, Tertis et *al.* reported an ultrasensitive electrochemical aptasensor (LOD of 0.003 ng mL^{-1}) utilizing a lab-made graphite screen-printed electrode decorated with gold nanoparticles. In this work, the aptamer was deposited using a multipulse method consisting of 3000 cycles over 3 h, alternating +0.5 V for 30 ms and −0.2 V for 30 ms [47]. In contrast to the complex architecture and electrochemical fabrication methodology used by Tertis et *al.*, our methodology is not only sensitive enough to detect gluten at levels set by legislation [3] but also implies a simpler fabrication process. Furthermore, our method matches the sensitivity claimed for gliadin detection by ELISA using the R5 gliadin antibody (e.g., the INgezim Gluten Quick commercial kit commercialized by Gold Standard Diagnostics). Our glucometer-inspired approach provides adequate sensitivity while enabling simple and timely analysis without the need for sophisticated and expensive benchtop equipment. Additionally, it benefits from the use of highly specific recombinant antibody fragments, synthetically obtained by phage display, adhering to non-animal and sustainable biofabrication standards.

3.3. Application to the analysis of real samples

To assess the usefulness of the developed methodology for gluten analysis in various food samples, the performance of the immunosensor platform was initially evaluated using samples incurred with known concentrations of gluten-containing flour. A crucial factor to consider is the amount of sample required for each analysis. In this sense, it is important to find the appropriate amount/dilution of sample used to maximize sensitivity while minimizing matrix effects. Besides, ethanol, used as gluten extracting agent, may interfere with antigen binding of antibodies [22], thus, requiring careful attention to its possible effects on the immunoassay performance.

For this purpose, we prepared maize-based binary mixtures both gluten-free (Fig. 3, dark blue bars) and spiked with 100 mg kg^{-1} of gluten-containing flours (Fig. 3, light blue bars). Once the gluten was extracted, the analysis was performed using different dilutions of the corresponding extracts, to select the dilution allowing the highest discrimination between the absence and the presence of gluten. The results displayed in Fig. 3 show that the developed method was able to discriminate the presence of gluten upon dilution of the extracts from between 50 and 200 times. A 1/100 dilution was selected due to the higher discrimination observed. The smaller amperometric responses, along with an almost null discrimination of gluten was observed for lower dilutions, which may be attributed to the detrimental effects associated with large ethanol amounts that may compromise the integrity of the antibodies, denature the enzymatic mark and/or interfere with antigen-antibody recognition [22].

According to the legislation, 20 mg kg^{-1} is the established threshold value to differentiate between gluten-free samples from those containing gluten [1,3,4], regardless of the food processing applied. Food

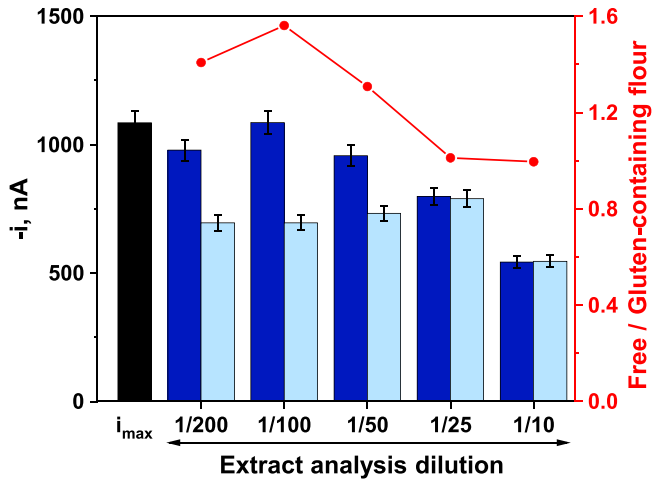


Fig. 3. Amperometric responses provided by the developed immunosensor platform in the analysis of maize gluten-free flour (dark blue bars) or incurred (light blue bars) with 100 mg kg^{-1} of a wheat, barley and rye flours (WBRm) mixture as well as the resulting signals ratio (red circles connected by lines) at different dilutions of extracts; i_{max} (black bar) are the amperometric responses measured in the absence of target gliadin in buffered solutions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Main characteristics of the electrochemical affinity sensors/assays reported in the last 5 years for the determination of gliadin.

Fundamentals	Electrode/Electrochemical detection	LOD (gliadin)	Time needed for support preparation/Determination	Stability	Application/Number of samples tested	Reference
Sandwich type aptasensor	SPCE/Chronoamperometry	1 ng mL^{-1} (LOQ)	~12 h/70 min	–	Dessert powders/2	[46]
Label-free AuNPs-based aptasensor	SPCE/EIS	50 ng mL^{-1}	~12 h/90 min	5 days	Beer and sauce samples/3	[44]
Paper-based aptamer-antibody biosensor	Paper modified SPCE/ Chronoamperometry	200 ng mL^{-1} (Gluten)	~14 h/30 min	–	Food samples/2	[43]
Label-free AuNPs-based aptasensor	SPCE/DPV	0.003 ng mL^{-1}	~10 min/30 min	7 days	Food samples/4	[47]
Label-free Parylene double-layer coated SPCE aptasensor	SPCE/Capacitante	7 μg mL^{-1}	~16 h/50 min	7 days	Wheat flour samples/2	[2]
Paper-based sandwich immunoassay and CNFs modified SPCE	SPCE/Amperometry	5 ng g^{-1}	3 h/~22 min	3 months	Flour and control food samples/11	[32]
Zein-CNT-based direct immunosensor	GCE/CV	0.5 ppm	~18 h/~30 min	1 month	–	[34]
Graphene oxide/AgNPs-based sandwich immunosensor	GCE/DPV	23.6 ng mL^{-1}	~13 h/~45 min	–	Spiked food samples/1	[33]
$rFab_{Gliadin}$ based immunoassay onto MμCs	SPCE/Amperometry	1.4 ng mL^{-1}	~2 h (Gliadin-MμCs)/55 min (starting from Gliadin-MμCs)	–	Food and beer samples/ 40	This work

CNF: carbon nanofiber; CNT: carbon nanotubes; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; GCE: glassy carbon electrode; Gliadin-MμCs: gliadin-magnetic μ-carriers; LOD: limit of detection; LOQ: limit of quantification; NPs: nanoparticles; SPCE: screen-printed carbon electrode; ZIF: zeolite imidazole frame.

processing can affect the detection of gluten in foods because heat treatment may provoke certain protein changes and chemical modifications, which may have a significant impact on the antibody-binding activity and protein solubility [1]. Thus, this possible effect should be addressed when developing alternative gluten detection methods. For this purpose, samples with representative gluten levels in terms of the regulatory requirement (0, 10, 20 and 100 mg kg^{-1} of gluten), were subjected to different thermal treatments (raw, 160 °C for 13 min and 200 °C for 25 min) and analyzed with the immunoplatform. Fig. 4 shows the obtained results and the Student's t-test, at a 95 % confidence level, was applied to evaluate whether there was any significant difference between the responses obtained in the absence and in the presence of 20 mg kg^{-1} gluten after each treatment. As seen in Table S1 (in the Supporting Information), significant differences were found in all cases, indicating that the developed immunoplatform was able to detect as low as 20 mg kg^{-1} of gluten independently of the treatment applied to the samples.

However, a slight increase in the amperometric response of the immunoplatform was observed for samples subjected to the more intense thermal treatment applied. This may be due either to the heat treatment affecting the immunorecognition of gliadin or to a change in the solubility of gliadin at 200 °C that causes its concentration in the extracts to be lower [1,26]. These results highlight the importance of including samples subjected to different treatments in the assay validation to evaluate their impact on target detection. Considering this, to apply our methodology to the analysis of various blind food matrices, which could be subjected to a wide variety of food processing methods, we established our experimental threshold limit value (TLV) according to the mean ± standard deviation (n = 3) of the amperometric responses obtained for the analysis of samples containing 20 mg kg^{-1} of gluten subjected to the different heat treatments shown in Fig. 4. Such value was used to discriminate among food samples with gluten contents above or below the legislated level (Fig. 5).

Currently, the most widely used methods for gluten detection in foods involve ELISA and lateral flow immunoassays, since they present advantages such as easy handling and availability of commercial kits. However, over the last years, several studies have shown large discrepancies in the quantification of gluten when comparing the results obtained with different kits. Multiple factors such as the type of antibody used, the protein extraction procedure, the biased detection of one family of gluten proteins over others or the selected reference standard give rise to highly discrepant gluten values, which raises doubts about the accuracy of the data obtained [12,17]. Taking these aspects into consideration, and to evaluate the applicability of the developed immunoplatform, we contrasted the results obtained for the analysis of

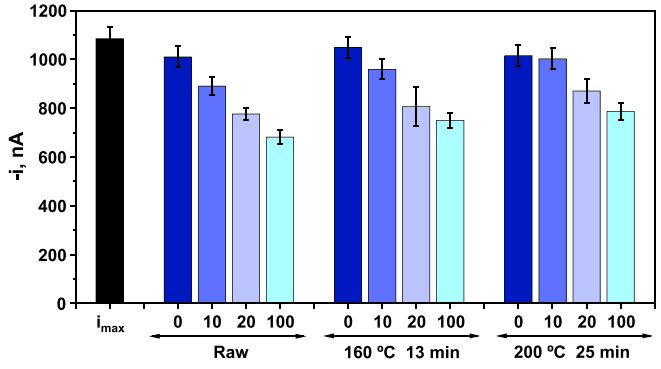


Fig. 4. Amperometric responses provided by the immunosensor platform in the analysis of raw and treated samples (160 °C 13 min or 200 °C 25 min) of maize gluten-free flour (0) or incurred with 100, 200 or 1000 mg kg^{-1} of a mixture of wheat, barley and rye (WBRm) flours that are equivalent to samples containing 10, 20 and 100 mg kg^{-1} of gluten; i_{max} (black bar) are the amperometric responses measured in the absence of target gliadin in buffered solutions.

thirty-seven commercial food samples with the $rFab_{Gliadin}$-based immunoplatform and with a commercially available sandwich ELISA using the R5 monoclonal antibody, as well as with two different lab-developed ELISA methods using the $rFab_{Gliadin}$, and with a non-immunological qPCR method. A qualitative comparison of all the results is provided in Table S2 (in the Supporting Information), although for simplicity only discussion of the most significant results and their comparison with the commercially available ELISA kit is included below.

Food samples were selected to include a wide variety of commercial food products, including processed (breads, cookies, drinks, breakfast cereals, baby foods, etc.) and raw products (seeds, flours, etc.). According to their labeling, samples were organized in four different categories: (A) products that declared to contain gluten (Fig. 5, samples 1 to 7, green bars); (B) corn-based products (Fig. 5, samples 8 to 14, orange bars); (C) oat-based products (Fig. 5, samples 15 to 22, blue bars); and (D) products with "gluten-free" labeling and/or certification (Fig. 5, samples 23 to 37, pink bars).

Most of the gluten-containing samples yielded amperometric responses under the TLV, confirming the capability of the immunoplatform to detect the presence of gluten above the established limit of 20 mg kg^{-1} (Fig. 5). Only sample number 2 showed values close to the limit for a gluten-containing sample, in agreement with the value of 30 mg kg^{-1} calculated by ELISA (Table 4) and with the elevated Cp value obtained by qPCR (Table S2 in the Supporting Information).

A broad representation of oat- and corn-based products was also included in the experiments since according to the evidence found by some authors the first ones may contain gluten [19], and both have been frequently found to be contaminated with other gluten-containing cereals [5].

Among the corn-based analyzed products, 6 out of 7 were identified by both the immunosensor and the ELISA with gluten contents under 20 mg kg^{-1}. Sample number 8, which showed an amperometric response in the limit to be considered a positive sample, gave absorbance values in the ELISA below the limit of detection of the assay. Regarding the 8 oat-based samples, only three of them (18, 20 and 22) were clearly discriminated as negative by both methodologies. Amperometric responses of four samples (15, 16, 17 and 19) were within the TLV range, in agreement with the low gluten values detected by the ELISA between 17 and 40 mg kg^{-1} (Table 4) and with the Cp values obtained by qPCR (Table S2 in the Supporting Information). These results confirmed also the high selectivity of the electrochemical immunoplatform using the $rFab_{Gliadin}$ in an indirect competitive format towards avenin peptides and therefore oats cultivars. It is worth highlighting that oat-based foods, which many celiac people must resort to, are among the products most frequently contaminated with other cereals containing gluten throughout the food production chain, so sensitive people should avoid consuming them unless they are certified as gluten-free [48].

Finally, among the 15 gluten-free products analyzed, 9 of them were classified by both methodologies as samples with a gluten content below 20 mg kg^{-1}. None of the samples analyzed in this category yielded amperometric responses below the TLV, although the results for the other six samples were within the TLV limits. The ELISA results for three of these samples (31, 32 and 36) showed values below the detection limit of the assay while the other samples were estimated to contain gluten levels lower than 20 mg kg^{-1} (13–16 mg kg^{-1}, Table 4) and were also detected by qPCR (Table S2).

Even though the detection of gluten in foods remains a major challenge in food science because of the complexity of the analysis, the results obtained with the proposed methodology demonstrate the great potential of the developed immunoplatform to rapidly discriminate between gluten-free or gluten-containing products. In addition to the importance of the development of analytical tests for accurate gluten detection, which is crucial for expanding research and discussion on appropriate cut-off values, sample contamination, and cross-reactivity of methodologies, the integration of biosensors on MμCs with

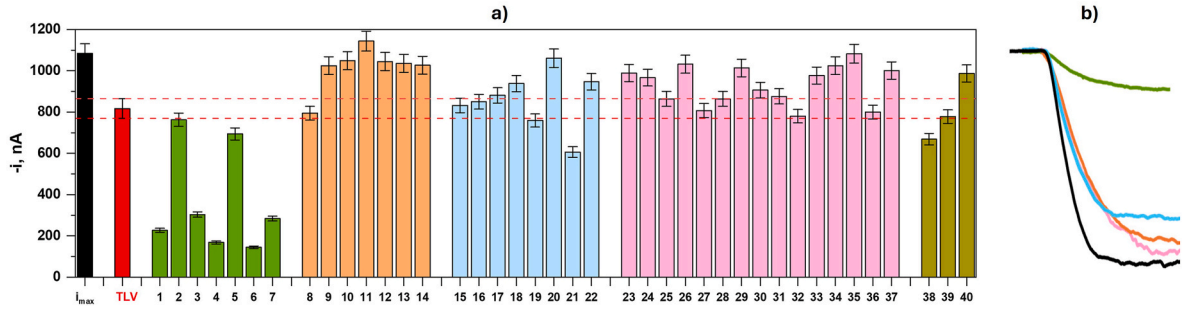


Fig. 5. Amperometric responses provided by the developed immunosensing platform for the analysis of 100-fold diluted extracts of food samples containing gluten (green bars), based on corn (orange bars) or oats (blue bars), labeled as gluten free (pink bars), and fermented beverages (mustard bars); i_{max} (black bar) are the amperometric responses measured in the absence of target gliadin in buffered solutions. TLV (20 mg kg^{-1} threshold limit value, red bar) and red dotted control lines are estimated as mean and standard deviation, respectively, of raw and treated corn flour samples incurred with 20 mg kg^{1} of a mixture of wheat, barley and rye (WBRm) flours a). Representative real amperometric signals provided by the immunosensor platform for each set of food samples b). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Results obtained for the quantitation of gluten using the R5-based sandwich ELISA for the commercial food products identified by the developed immunoplatform with amperometric signals within the threshold limit value (TLV).

Identification number	Food sample	Gluten concentration determined by ELISA (mg kg^{-1})
2	Vegetable soup	30
8	Corn flour	< LOD
15	Oats flakes	40
16	Oats bran	40
17	Oats-based drink	17
19	Oats bran	25
25	Rice flour	< LOD
27	Snack	< LOD
28	Pasta	< LOD
31	Cereal bar	13
32	Cereal bar	16
36	Rice cake	13

LOD: limit of detection of the R5 sandwich ELISA kit of 3 mg kg^{-1} of gluten.

electrochemical readout endows our strategy with the key attributes for set-up low-cost and user-friendly pocket meters or smartphone-coupled systems, similar to glucometers, enabling off-lab gluten screening without the need for sophisticated bench-top technology, promoting sustainable food production and reducing inequalities to access to protective technologies. In this line, and as a future perspective, the design of magnetic driven μ-carriers charged on their surface with multiple gliadins, used as competitors in the developed immunoplatform, could be easily applied to design strategies for monitoring the immune response to gluten consumption in minimally invasive diagnostic biofluids, where these methodologies have already proven potential [49], paving the way for the development of multipurpose strategies that will enable both allergen detection and organism response surveillance, closing the loop between food industry, nutrition and precision health. Additionally, further implementation of the immunoplatform would be of interest for the analysis of samples containing hydrolyzed gluten, such as beer or syrups. In this sense, and as a proof of concept, fermented hydrolyzed beverages (mustard bars 38–40 in Fig. 5) have been analyzed. Among the three samples tested (38—40), significant competition was only observed in the analysis of cereal beverages (38 and 39). The regular beer sample showed a gluten content above 20 mg kg^{-1}, with an amperometric response clearly below the TLV (38), whereas the hydrolyzed beer sample labeled as gluten-free showed gluten levels within the TLV range, which is consistent with previous reports [44,50,51]. It is also important to note here that other electrochemical affinity sensors/assays reported in the last 5 years for the determination of gliadin, compared in Table 3, have demonstrated their applicability in the determination of a much smaller number of samples: mostly in 2–4 samples [2,33,43,44,46,47] and only one in 11 samples [32], still far from the 40 tested in this work.

The platform's demonstrated ability to detect gluten in a wide range of commercial samples (including processed food and beer samples) together with results displayed in Fig. S2 (in the Supporting Information), proving that the sdAb successfully recognizes both the 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) and the consensus peptide (CPFPQQQPFPQQPFPQQQPFQQQPFQQPFQQQ PQQQP) used for antibody isolation via phage display further highlight the potential of this technology for the accurate and reliable analysis of real food samples.

4. Conclusions

This paper presents the design, optimization, and application of an electrochemical immunoplatform for detecting gluten in various foodstuffs by targeting gliadin, the alcohol-soluble protein responsible for triggering celiac disease. To develop a simple, fast, and user-friendly approach, while avoiding complex architectures, we explored for the first time to date the use of magnetically driven carriers for the implementation of an indirect competitive immunoassay, along with disposable electrodes for amperometric transduction. The developed immunoplatform also innovatively leverages evolution engineering and phage display technologies to produce a recombinant antigen-binding antibody fragment to ensure maximum sensitivity and specificity, together with reproducibility and sustainability in its production. The developed immunoplatform showed attractive analytical and operational characteristics, with a limit of detection of 1.4 ng mL^{-1} of gliadin, for the successful detection of gluten at the stablished legislative threshold of 20 mg kg^{-1} and, it was effectively applied to the target allergen screening in nearly 40 processed and unprocessed samples from various sources. The obtained results were consistent with those provided by different ELISAs and qPCR methodologies. In this line, the developed methodology displayed robust performance in detecting the gluten presence regardless of the food matrix or processing, which expands its application scope.

All the results presented, along with the inherent advantages of using immunosensors involving MμCs and disposable electrodes for electrochemical readout —such as ease of operation, portability, and affordability— demonstrate the significant potential of the developed methodology for implementing portable prevention biotools, to facilitate on-the-spot self-testing of suspicious foods, ensuring optimal protection for patients with celiac disease. Furthermore, the versatility of this strategy could be leveraged to develop multiplexed and multipurpose devices for the detection of other nutrition targets of interest and for self-assessing the autoimmune response, effectively bridging the gap

between health and foodomics in a precise and personalized way.

It is important to note, however, that, being aware the high variability and the wide range of samples where the presence of gluten must be determined and the multitude of factors already mentioned that can affect the accuracy of the results obtained in immunoassays, the proposed biotool still has a long way to go to leave the research laboratory to the industry and/or markets. This transition would involve, among many other things, facing the determination of different forms of gliadin (hydrolyzed or denatured) in a larger number of samples and gluten immunogenic peptides and selecting the most appropriate standards to provide quantitative results. Future efforts should also focus on simplifying its manufacture even further, avoiding, if possible, the need for an incubator, which complicates its applicability at the point of need to improve its robustness and integrate it into POC-type electrochemical devices. In addition, ensuring the reliability of the results, particularly when analyzing processed foods, must involve contrasting the results the biotool provides in the analysis of many more samples (variable in nature and processing) employed by different users and in different environments.

CRediT authorship contribution statement

Víctor Ruiz-Valdepeñas Montiel: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Conceptualization. **Eduardo Garcia-Calvo:** Methodology, Investigation, Conceptualization. **Maria Gamella:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Aina García-García:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Santiago Rodríguez:** Methodology, Investigation, Conceptualization. **Teresa García:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **José M. Pingarrón:** Writing – review & editing, Writing – original draft, Resources. **Rosario Martín:** Writing – review & editing, Writing – original draft, Supervision, Resources. **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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.127747.

Data availability

Data will be made available on request.

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