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Competitive immunosensor based on gliadin immobilization on disposable carbon-nanogold screen-printed electrodes for rapid determination of celiotoxic prolamins

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Abstract

The first competitive disposable amperometric immunosensor based on gliadin-functionalized carbon/nanogold screen-printed electrodes was developed for rapid determination of celiotoxic prolamins. To date, no competitive spectrophotometric or electrochemical immunoassays have yet been successfully applied to gluten detection in processed food samples, which require the use of complex prolamin extraction solutions containing additives with denaturing, reducing and disaggregating functions. Thus, in this work great effort was put into the optimization and performance evaluation of the immunosensor in terms of suitability as a screening tool for analysis of cereal-based food samples. For this purpose aqueous ethanol or complex extracting mixtures, as the patented Cocktail Solution[®], were proved effective in the extraction of gliadin. Good sensitivity was achieved after optimization of the immunocompetitive assay, giving limit of detection and limit of quantitation of 8 and 22 ng/ml of gliadin, respectively, for ethanol extracts. The immunosensor was proved to be suitable also for samples extracted with Cocktail Solution[®] after a proper dilution. Analysis of real samples of different flours proved the suitability of the immunosensing device as a powerful tool for safety assessment of raw materials used for the formulation of dietary products for celiac disease patients. Findings evidence as the immunosensor combines good analytical performances with very simplified set-up protocol and suitability for rapid screening analysis performed with inexpensive and portable instrumentation.

Keywords: *Celiac Disease, Food Safety, Competitive Immunosensor, Gluten, Cocktail Solution.*

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1. Introduction

Strict lifelong observance of a gluten-free diet is currently the only effective treatment of celiac disease (CD), defined as a chronic immune-mediated enteropathy triggered by exposure to dietary gluten in genetically predisposed individuals. Recent epidemiologic data suggest a prevalence of approximately 1% in most Western countries [1]. CD patients have to avoid all gluten-containing foods produced from wheat, rye, barley, and certain varieties of oats.

According to the Codex Alimentarius Standard 118–1979 revised in 2008, “gluten-free” foods must not contain more than 20 mg/kg of gluten, whereas “very low gluten” products are allowed to contain gluten levels up to 100 mg/kg [2]. In addition, gluten is defined as “a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/l NaCl” [3]. This protein fraction comprises hundreds of different components and corresponds to the storage proteins exclusively present in the starchy endosperm of the grains. Following the classical definition of Osborne, cereal storage proteins soluble in aqueous alcohols without reduction of disulphide bonds are designated as “prolamins” (gliadins for wheat) and the insoluble proteins as “glutelins” [4]. Although Shewry and coworkers used the term “prolamins” for all storage proteins (including glutelins) [5], in this paper we will refer to the classical definition of prolamin, according to Osborne.

The prolamin/gluten conversion factor is usually set to 2 [6], but such a conventional value is not fully reliable, since gluten composition can vary according to its botanical origin (e.g., cereal species, varieties), agricultural conditions in which the plants are produced (e.g., climate, soil, fertilization), and food processing procedures (e.g., heating, enzymatic degradation, mechanical and chemical processing).

Detection and accurate quantitation of gluten in foods is essential not only for CD patients but also for food safety control in the industry producing gluten-free dietary products, usually formulated with nontoxic cereals (e.g. corn, rice, sorghum, millet) and pseudocereals (e.g. amaranth, buckwheat, quinoa). As very recently reviewed by Scherf and Poms [7], the scientific community devoted considerable efforts in research aimed at investigating different approaches for accurate gluten determination but without reaching a widely accepted solution.

Minimum requirements for reliable methods should include sufficient sensitivity, selectivity, and a certified reference material for calibration, currently not yet available. In addition, they should be applicable not only to raw materials, but also to processed foods (i.e. heat-treated, fermented, acid-treated foods). The key stages involved in the procedure for accurate analysis of gluten in foods are *i)* complete extraction of gluten proteins/peptides from the matrix, *ii)* application of a representative

protein/peptide reference for calibration and *iii*) accurate quantitation of extracted gluten proteins/peptides. Undoubtedly, the real bottlenecks of the whole analytical process are the exhaustive extraction of gluten proteins, especially from processed foods, and the lack of an univocally standardized material for the assessment of their concentration [6]. In addition, knowledge about the toxic epitopes within the proteins in gluten is still incomplete [8].

As for sample treatment, the extraction of prolamins with 40-70 % (v/v) aqueous ethanol can be applied only for raw materials, as cereal flours. Conversely, processed food requires the use of more complex extraction solutions containing denaturing and reducing agents, because the chemical properties and gluten solubility are modified by thermal, chemical, mechanical or enzymatic processes. The extraction Cocktail Solution (CS), developed and patented by D.E. Mendez [9], was endorsed by the Association of Official Agricultural Chemists (AOAC) of the U.S. Department of Agriculture. CS was proved suitable for food and raw materials, containing mercaptoethanol (ME) and guanidine as denaturing and disaggregating agents, respectively.

To date, immunological methods, in particular Enzyme-linked Immunosorbent Assay (ELISA), are the most used screening techniques for rapid assessment of gluten content in dietary products specifically formulated for CD patients.

On the other hand, non-immunological techniques such as Polymerase Chain Reaction [10,11] and liquid chromatography-mass spectrometry (LC-MS) [12-14] are equally important as independent control methods; in particular, MS-based approach permits to provide more targeted analytical information on marker peptides related to prolamins specifically occurring in different cereals [15,16]. Long analysis time and high cost are the main drawbacks of non-immunological methods, whereas immunological approaches are more rapid and less expensive, not requiring complex instrumentation and/or specifically trained analysts. However, immunological methods involve significant limitations in terms of possible cross-reactivity of antibodies among CD-toxic or harmful proteins.

The Mendez group developed a sandwich ELISA based on a monoclonal antibody (R5) directed against epitopes occurring in CD-toxic sequences of prolamins [9]. R5 ELISA proved capable of recognizing prolamins from wheat, rye, and barley to the same degree. The R5-based sandwich ELISA kit is commercialized by R-Biopharm under the trade name of RIDASCREEN® and has been also endorsed by AOAC as official analysis method for quality assessment of gluten-free food. In 2012, a competitive ELISA using R5 monoclonal antibody was developed [17]. The competitive system is cheaper and faster than the sandwich system, since only one antibody is used, and sample and antibody incubation is performed in one step operation within the assay. This test needs only one epitope for binding and also detects small peptides derived from partially hydrolysed

prolamins, such as those present in fermented cereal foodstuffs. Since the competitive ELISA kit is not compatible with CS, a new extracting solution called UPEX[®] (Universal Prolamin and glutelin Extractant Solution), based on reducing Tris (2-carboxyethyl)-phosphine (TCEP) and anionic surfactant N-lauroylsarcosine (patent WO 2011/07039 A2) has been developed, resulting compatible with all gluten analysis procedures [18].

An ELISA competitive assay involving the monoclonal antibody G12 was developed by Amaya-González and co-workers in 2011 [19]. In this study the immunodominant peptide 33-mer from alpha2-gliadin was proposed as standard for calibration of the assay, exploiting amperometric flow-injection analysis as detection system.

Concerning non-immunochemical approaches, the same research group recently reported a successful selection of unmodified DNA aptamers bound to magnetic beads for amperometric detection of celiac disease-triggering proteins, showing improved performance with respect to the reference immunoassay for the same target [20].

Amperometric immunosensors are very versatile tools for rapid and user-friendly screening analysis [21], thanks to their compatibility with portable and compact devices, exploiting disposable screen printed electrodes as sensing substrates [22,23].

Until now, there are a limited number of publications showing electrochemical immunosensors being used for the determination of gluten: a sandwich immunosensor developed by Nassef et al. [24] was based on the combination of a capture antibody, anchored to the electrodic surface through dithiol compounds, and a reading antibody directed against the immunodominant CD epitope α 56–75, allowing to perform reliable and sensitive detection of gliadin in a short time with minimal requirement of operator manipulation. Laube et al. [25] reported a competitive magneto immunosensor for the determination of gliadin in natural or pre-treated food samples extracted with aqueous ethanol.

Herein we propose the use of a novel disposable competitive amperometric immunosensor for safety assessment of gluten-free products. The immunosensor was based on carbon/nanogold screen-printed electrodes directly functionalized with gliadin, exploiting an immunocompetition with a polyclonal rabbit anti-gliadin antibody. To date, competitive electrochemical immunosensors have not yet been successfully applied to the analysis of processed food samples, which require CS or UPEX for prolamin extraction. Thus, in this work a great effort was put to the optimization and evaluation of the immunosensor performance in terms of suitability for screening analysis of food samples.

2. Experimental

2.1. Reagents and standards

Gliadin from wheat (powder, G3375 product number), ethanol (EtOH), Trizma® base, Tween-20, sodium chloride, potassium chloride, Tris(2-carboxyethyl)phosphine hydrochloride, N-laurylsarcosine sodium salt, α -casein from bovine milk, polyclonal anti-gliadin (wheat) fractionated antiserum from rabbit (Ab anti-Gli) and Alkaline Phosphatase conjugated Anti-Rabbit IgG (Ab-AP) were purchased from Sigma–Aldrich (Milan, Italy). Sodium phosphate bibasic, Sodium phosphate monobasic and magnesium chloride were purchased from Carlo Erba (Milan, Italy). Hydroquinone Diphosphate (HQDP) was purchased from Metrohm Italiana (Origgio-VA, Italy). Deionized water was obtained from an in-house Milli-Q water purification system Alpha Q-Water (Millipore, Billerica, MA, USA). RIDASCREEN® Gliadin ELISA kit and patented Cocktail extracting Solution (CS) were purchased from R-Biopharm AG (Darmstadt, Germany).

Phosphate Buffered Saline (PBS 10 x) was prepared according to the following composition: 1.37 M NaCl, 0.027 M KCl, 0.015 M KH_2PO_4 , 0.08 M Na_2HPO_4 (pH 7.4). Diluted Phosphate Buffered Saline (PBS 1 x) was prepared by dilution of PBS 10 x in water. The washing buffer PBS-T consisted of PBS containing 0.05 % (v/v) of the surfactant Tween-20. TRIS buffer was prepared according to the following composition: 0.1 M Trizma® base, 0.02 M MgCl_2 (pH 7.4). The washing buffer TRIS-T consisted of TRIS containing 0.05 % (v/v) of the surfactant Tween-20. “Reading buffer” (RB) has the same composition of TRIS buffer but pH 9.8.

2.2 Fortified sample preparation and extraction

Rice, corn, barley, rye, buckwheat, oats, mile, chestnut, chickpeas, quinoa and potato flours as well as durum wheat pasta, breadcrumb, crackers and biscuits were obtained from local supermarkets.

Immunosensor calibration and validation was performed using rice flour as sample blank: for this purpose, after spiking with wheat gliadin powder, purchased from Sigma, homogenization and extraction of mixtures was carried out. Prolamin content of whole gliadin was assessed by RIDASCREEN® Gliadin ELISA kit and using the NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, Germany), as described in our previous work dealing with a LC-MS/MS method [16].

Fortified samples were extracted with both aqueous ethanol and the cocktail solution. Briefly, 200 mg of sample were suspended in 2 ml of 60% (v/v) ethanol and incubated for 1 hour at room temperature under agitation. After centrifugation at 5800 rpm for 10 minutes at room temperature, the supernatant was transferred to a clean tube. The pellet was further treated following the

previously described procedure [16] and resulting supernatants were merged in a clean tube. The extraction with the CS was carried out according to the experimental procedure published by Valdes et al. [9] and suggested by RIDASCREEN[®] ELISA Kit.

2.3 Immunosensor setup

Immunosensors were assembled on DropSens[®] disposable Gold Nanoparticles-modified Screen-Printed Carbon Electrodes (GNP-SPCEs) purchased from Metrohm Italiana (Origgio-VA, Italy). GNP-SPCEs were made in a ceramic substrate (L 33 x W 10 x H 0.5 mm) and based of 4 mm-diameter carbon-GNP working electrode, carbon counter electrode and silver pseudo-reference electrode.

GNP-SPCEs were incubated with a 0.5 mg/l gliadin solution obtained by dilution of the ethanolic extract of Sigma gliadin powder in RIDASCREEN[®] sample diluent. For this purpose, working electrodes were drop-casted with 30 μ l of gliadin solution, allowing to immobilize gliadin on gold nanoparticles at +4°C overnight. After removal of unreacted gliadin by careful washing with PBS-T, a blocking treatment aimed at preventing nonspecific responses during sample incubation was carried out by casting 30 μ l of 20 mg/ml α -casein solution in PBS-T on each electrode at room temperature for 1 hour, followed by careful washing with PBS-T and PBS buffer. All washing steps were repeated three times. Thereafter, the gliadin-modified GNP-SPCEs were used to carry out the competitive electrochemical immunoassay. Standard solutions and/or fortified sample extracts (30 μ l) were mixed with 3 μ l of a 1:25 diluted solution of Ab anti-Gli in PBS (1 x) to reach a 1:250 final dilution of the antibody. The mixture was transferred to the working electrode of the immunosensor and the competition reaction was allowed to take place for 1 hour at room temperature. Because of the ability of gliadin to inhibit antibody binding to the immobilized gliadin, increasing analyte concentration will reduce the amount of Ab anti-Gli bound to the modified immunosensor surface. After immunocompetition, the sensors were carefully washed with PBS-T and TRIS to remove non-specifically bound material. In order to detect the Ab anti-Gli immobilized on the electrodic surface, each immunosensor was incubated at room temperature for 1 hour with 30 μ l of a solution of the enzyme-conjugated secondary reading antibody Ab-AP, diluted in TRIS buffer by a proper factor from the original stock solution. Finally, careful washings with TRIS-T and RB to remove unreacted enzyme were performed. Figure 1 shows a schematic representation of the protocol developed for the realization of the immunosensors. Competitive immunoassays were performed using Differential Pulse Voltammetry (DPV) as a method of electrochemical detection, scanning the potential between -0.5 V and +0.3 V, with a pulse amplitude of 0.05 V, a step potential of 0.005 V and a pulse time of 100 ms. Non-electroactive HQDP was used as enzyme substrate,

since Alkaline Phosphatase (AP)-promoted dephosphorylation yields electroactive HydroQuinone (HQ) that is in turn oxidized to Quinone (Q) during the DPV scan, giving the gliadin-related analytical signal. A 1 mg/ml solution of HQDP dissolved in RB was used as reading solution. After drop-casting of the reading solution on the sensor, an equilibration time of 90 s and a preconditioning stage of 30 s at -0.5 V were applied prior to run DPV in order to reach an exhaustive enzymatic treatment and to preconcentrate HQ in its reduced form.

All electrochemical measurements were performed with a μ Autolab III electrochemical workstation (EcoChemie, Utrecht, NL) equipped with GPES 4.0 version customized software.

Response curves were obtained normalizing the current values observed for each concentration (S) as a function of the signal from zero level (S_0) obtained without gliadin in competition. The normalized signals, expressed as percentage values ($S/S_0 \times 100$), were plotted *versus* the logarithm of the gliadin concentration and fitted using a four-parameter logistic function (1), as conventional for competitive immunoassays [26,27]:5

$$S/S_0 = S_{min} + \frac{(S_{max} - S_{min})}{1 + ([C]/I_{50})^B} \quad (1)$$

where S_{min} and S_{max} are the asymptotic minimum and maximum, respectively (S_{max} is recorded in the absence of analyte) and B is the curve slope at the inflection point I_{50} , corresponding to the gliadin concentration $[C]$ giving 50% of signal inhibition. Data fitting was performed using the software product Microcalc OriginPro 8.5

2.4 Immunosensor optimization and validation

Two-factors and 3-levels experimental design was performed in order to optimize the concentrations of the solution used for the immobilization of gliadin on GNP-SPCEs and Ab anti-Gli in competition, as discussed in section 3.1. Data were processed by 2-way Analysis of Variance (ANOVA) with interactions. ANOVA was carried out using the Statgraphics Centurion XV statistical software.

Validation of the sensing device was performed on fortified rice flour according to the Eurachem guidelines [28]. The absence of gluten in rice flour, used as blank matrix, was previously verified by RIDASCREEN[®] Gliadin ELISA. The detection (LOD) and quantitation (LOQ) limits were assessed as the concentration of analyte giving a signal that is $2ts_b$ and $10s_b$ above the mean blank signal, respectively, where s_b is the standard deviation of the blank signal obtained from ten independent blank measurements and t is the constant of the *t-Student* distribution (one-tailed) at 95 % confidence level. At least three replicate measurements (independent immunoassays carried out with different gliadin-modified GNP-SPCEs on the same extract) were carried out for all standards

and samples. Trueness was calculated in terms of percent recovery as a ratio of the concentration determined in fortified rice flour, compared to the true concentration of the pure authentic standard at two concentration levels (i.e. LOQ and intermediate calibration level).

3. Results and discussion

3.1 Optimization of the immunocompetition

The experimental conditions related to the performance of the fabricated immunosensor were thoroughly evaluated and optimized in order to reach a dynamic response range useful for safety assessment of gluten-free products. The experimental design was aimed at finding the optimal conditions leading to a 50 % signal inhibition for a gliadin concentration of 50 ng/ml, corresponding to 10 mg/kg gliadin in the raw standard material (i.e. rice flour spiked with gliadin), extracted with aqueous ethanol. For this purpose, the concentration of the solution used for the immobilization of gliadin on GNP-SPCEs was explored over the 0.5-50 mg/l range, whereas the Ab anti-Gli dilution factor (with respect to the commercial stock solution with 6.5 mg/ml concentration) was varied in the 1:1000 to 1:10 range. Two-way ANOVA results show that both factors (Gli and Ab anti-Gli) and their interaction have a significant effect ($p\text{-value} < 0.05$) on the immunosensor response (Figure 2). According to the ANOVA results, gliadin concentration for the functionalization of GNP-SPCEs was fixed at 0.5 mg/l and optimal dilution factor of Ab anti-Gli was 1:250.

The absence of nonspecific responses, as well as the maintenance of the immunoreactivity of the gliadin, after its chemisorption on the nanogold substrate was verified by “blank” experiments carried out on gliadin-modified GNP-SPCEs, blocked with casein and incubated only with Ab-AP. A signal not significantly different from zero was recorded in the absence of Ab anti-Gli. Analogous experiments carried out with unmodified GNP-SPCEs, blocked with casein and incubated with both Ab anti-Gli and Ab-AP gave signals not significantly different from zero.

3.2 Immunosensor performance

The analytical performance of the immunosensor was assessed under the optimized experimental conditions carrying out competitive assays with standard solutions extracted with aqueous ethanol from the commercial gliadin. Figure 3 shows DPV scans recorded over the 0.25-250 ng/ml gliadin concentration range. The inhibition curve obtained interpolating the dataset with the four-parameter logistic function (1), is shown in Figure 4. Under optimal conditions, the developed immunosensor showed good analytical performance in terms of sensitivity, precision and trueness. LOD and LOQ

values of 8 and 22 ng/ml, respectively, were calculated. A comparison with the performance obtained with other analytical approaches is reported in Table 1. Concerning precision, relative standard deviation (RSD) always lower than 10% (n = 3) were observed over the explored concentration range. Trueness was assessed analysing standard solutions not included in the calibration dataset, giving recovery rates ranging from 89 to 104 %. As for shelf-life of the developed immunosensors, we verified as the blocking treatment with casein, as well as preventing the occurrence of non-specific responses, also guarantees the maintenance of the reactivity of the gliadin-modified GNP-SPCEs electrodic substrate. Focused experiments carried out over one month (at least) did not show significant changes in the responses, under dark storage at 4°C.

3.3 Matrix effect assessment

Our developed immunosensor did not show significant nonspecific response ascribable to matrix effects on the binding properties of the polyclonal anti-gliadin antibody. Cross-reactivity of the electrochemical immunoassay was evaluated in terms of signal inhibition rates observed analysing solutions obtained by extracting corn, barley, rye, buckwheat and oats flours with aqueous ethanol. The cross-responses obtained towards the corresponding prolamins, i.e. zeins, hordeins, secalins and avenins, agreed with the cross-reactivity data declared for the Ab anti-Gli, so indicating as the developed immunosensor is not affected by nonspecific signal inhibition. The cross-reactivity rates observed with the different cereals under investigation, calculated using the response to rice flour as zero-reference, are reported in Table 2.

3.4. Compatibility with patented Cocktail Solution

Applicability of the developed immunosensor for safety assessment of both raw materials and processed foods specifically formulated for CD patients represents a crucial challenge of the present study. An important bottleneck in the immunosensing determination of gliadin at trace levels lies in the sample treatment and extraction. In fact, as discussed in the Introduction, aqueous ethanol has been demonstrated suitable only for the extraction of raw materials, whereas processed food requires the use of more complex solutions containing reducing and/or denaturing agents.

Concerning the use of simpler and less time-consuming competitive assays, the kit launched by the same manufacturer in 2012 (RIDASCREEN® Gliadin competitive) is declared not compatible with the use of CS; this fact is also confirmed by the findings of Laube et al. [25] dealing with a competitive magneto immunosensor developed on tosyl-activated magnetic beads and only tested

for analysis of milk and beer spiked with gliadin and extracted with aqueous ethanol. To date, the system developed by Laube and coworkers is the only example of competitive immunosensor for gliadin reported in literature. Conversely, our competitive immunosensor combines assay set-up simplicity with implementation on disposable SPEs. Taking into account the good immunosensor performance, we investigated its suitability for complex extraction solutions to be used for treatment of processed food samples.

Since Preliminary experiments proved that UPEX[®] solution is not compatible with our competitive electrochemical immunoassay. In more detail, a strong interference in terms of signal inhibition occurred, also in the absence of gliadin standard (false positive), when the extracting solution contained the sodium salt of N-laurylsarcosine, whereas other components did not affect the response. On the basis of these findings, showing that such a surfactant strongly interferes with the immunocompetition, we focused the study on the compatibility of CS with the developed immunodevice.

In a first stage, the solutions obtained extracting rice flour (blank) with CS were subjected to different further dilutions, i.e. 12.5- or 62.5-fold, as suggested in the protocol of the RIDASCREEN[®] Sandwich-type ELISA kit for gluten free or low-gluten samples, respectively. The diluted extracts were subsequently spiked with different amounts of standard gliadin, in order to evaluate the response range in terms of signal inhibition associated with gliadin concentration.

Results of these experiments evidenced that the 12.5-fold diluted extracts are not compatible with the competitive immunosensor, since no signal inhibition was observed upon spiking with gliadin over the 0.1-10000 ng/ml concentration range. Conversely, 62.5-fold diluted extracts resulted to be suitable for analysis with the immunosensor, showing a dynamic response over the same concentration range. The inhibition curve interpolated with equation (1) is reported in Figure 5. Under these conditions, LOD and LOQ values of 30 and 93 ng/ml, respectively, were calculated. High precision was also observed, RSD values being always lower than 5%.

On the basis of these findings, different samples of rice flour were spiked with solid gliadin standard, carefully homogenized and extracted with CS according to RIDASCREEN[®] assay protocol. The obtained extracts were 62.5-fold diluted and analysed with the immunosensor, giving the inhibition curve reported in Figure 6, where the gliadin concentration is referred to the original spiked amount of gliadin in rice flour (mg/kg). In this case, LOD and LOQ values of 162 and 498 mg/kg, respectively, were obtained. An excellent precision, with RSD values (three measurements with independent sensors on the same extract) always lower than 3%, was also observed.

It is important to underline that LOD and LOQ values referred to the original raw material (rice flour spiked with gliadin) are not ascribable to the intrinsic performance of the sensing device, that

would be suitable for safety assessment of gluten free products, but rather to the high dilution factor of the extracted solutions, made necessary to limit the interference of CS components on the immunocompetition process. Again, the inhibition cut-off value ($S_{min} = 43.7\%$) is explainable in terms of reaching the gluten solubility limit for spiked concentrations higher than 10000 mg/kg.

3.5 Analysis of real samples

Considering the findings of our studies about the extent of the interference of the CS to be used for the extraction of processed food samples, the immunosensor was tested with the analysis of different commercial flours, nominally gluten-free, but labelled with the warning of possible contamination from gluten at trace levels. Particularly, flours from mile, chestnut, chickpeas, quinoa and potato were extracted with aqueous ethanol and analysed both with the immunosensor and with RIDASCREEN® Sandwich-type ELISA kit, as reference method. The gluten content of all samples resulted less than LODs of both methods. As for trueness assessment, samples of durum wheat pasta, breadcrumb, crackers and biscuits were processed and extracted with CS, according to the experimental procedure reported in the commercial RIDASCREEN® Gliadin ELISA kit. Considering that the commercial assay kit is not intended for analysis of gluten-containing foods, the obtained extracts were 100.000-fold diluted with “sample diluent” included in the kit, before the execution of the assay. Another aliquot of each extract was analogously 10.000-fold diluted and analysed with our immunosensors. A good agreement between the results from ELISA reference assay and the responses of our immunosensor was obtained, as shown in Table 3.

3.6 Conclusions

The present study reports the first competitive disposable immunosensor based on screen-printed electrodes for rapid screening of gliadin in gluten-free food products: good analytical performance combined with very simplified set-up protocol performed with inexpensive and portable instrumentation are the main features of the device developed. Another aspect challenged for the first time was a systematic study aimed at investigating the compatibility of the competitive immunosensor with sample treatment protocols applicable to processed foods and involving the use of complex extracting solution as the patented CS. This easily fabricated immunosensor provides a new promising tool for safety assessment of raw materials used for the formulation of dietary products for CD patients. The method developed was characterized by inherent sensitivity, simplicity, speed, and cost benefits coupled to high selectivity.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Table and Figures captions

Table 1. Comparison of analytical performance of our immunosensor with respect to other methodological approaches.

Table 2. Cross-reactivity rates among prolamins in the cereals investigated.

Table 3. Gliadin content found with ELISA RIDASCREEN[®] kit and with our immunosensor in gluten-containing processed food samples. Means and standard deviations calculated from 3 replicated assays.

Figure 1. Schematic representation of the working principle and set-up of the competitive immunosensor implemented on disposable carbon/nanogold screen-printed electrodes functionalized with gliadin.

Figure 2. Interaction plot from ANOVA, evidencing the effects of Ab-anti-Gli and Gli concentrations on the signal inhibition extent.

Figure 3. DPV scans recorded analysing different gliadin standard solutions over the 0.25-250 ng/ml concentration range.

Figure 4. Inhibition curve obtained interpolating the dataset from analysis of matrix-free gliadin standard solution. Inset table: fitted curve parameters.

Figure 5. Inhibition curve obtained interpolating the dataset from analysis of 62.5-fold diluted cocktail solution, post-spiked with different amounts of gliadin. Inset table: fitted curve parameters.

Figure 6. Inhibition curve obtained interpolating the dataset from analysis of blank matrix (rice flour) samples spiked with different amounts of gliadin and extracted with the cocktail solution. Inset table: fitted curve parameters

Table 1

Analytical Approach	LOD (ng/ml)	LOQ (ng/ml)	Reference
Competitive amperometric immunosensor	8	22	Present study
Sandwich ELISA	1.5	N.D. ^a	[9]
Competitive ELISA	0.36	1.22	[18]
Competitive ELISA-Amperometric FIA	1.0 ^b	N.D. ^a	[19]
Amperometric immunosensor	5.5	N.D. ^a	[24]
Amperometric magneto-immunosensor	5.1	N.D. ^a	[25]
Magneto-ELISA	5.7	N.D. ^a	[25]

^a Not Declared

^b Not referred to gliadin, but to immunodominant 33-mer peptide

Table 2

Cereal	Related Prolamins	Cross-response rate ^a (%)
Corn	Zeins	No Inhibition
Barley	Hordeins	75 ± 3
Rye	Secalins	95 ± 1
Buckwheat	-	No Inhibition
Oats	Avenins	No Inhibition

^a *relative to rice flour as zero-reference*

Table 3

Food	Found Gliadin Concentration (mg/kg)		Recovery Rate (%)
	ELISA commercial Kit	Our Immunosensor	
Breadcrumb	81983 ± 7962	67191 ± 3810	82
Durum Wheat Pasta	27070 ± 2626	22616 ± 2001	83
Crackers	60058 ± 6617	53633 ± 5120	89
Biscuit	66430 ± 7710	56698 ± 4689	85

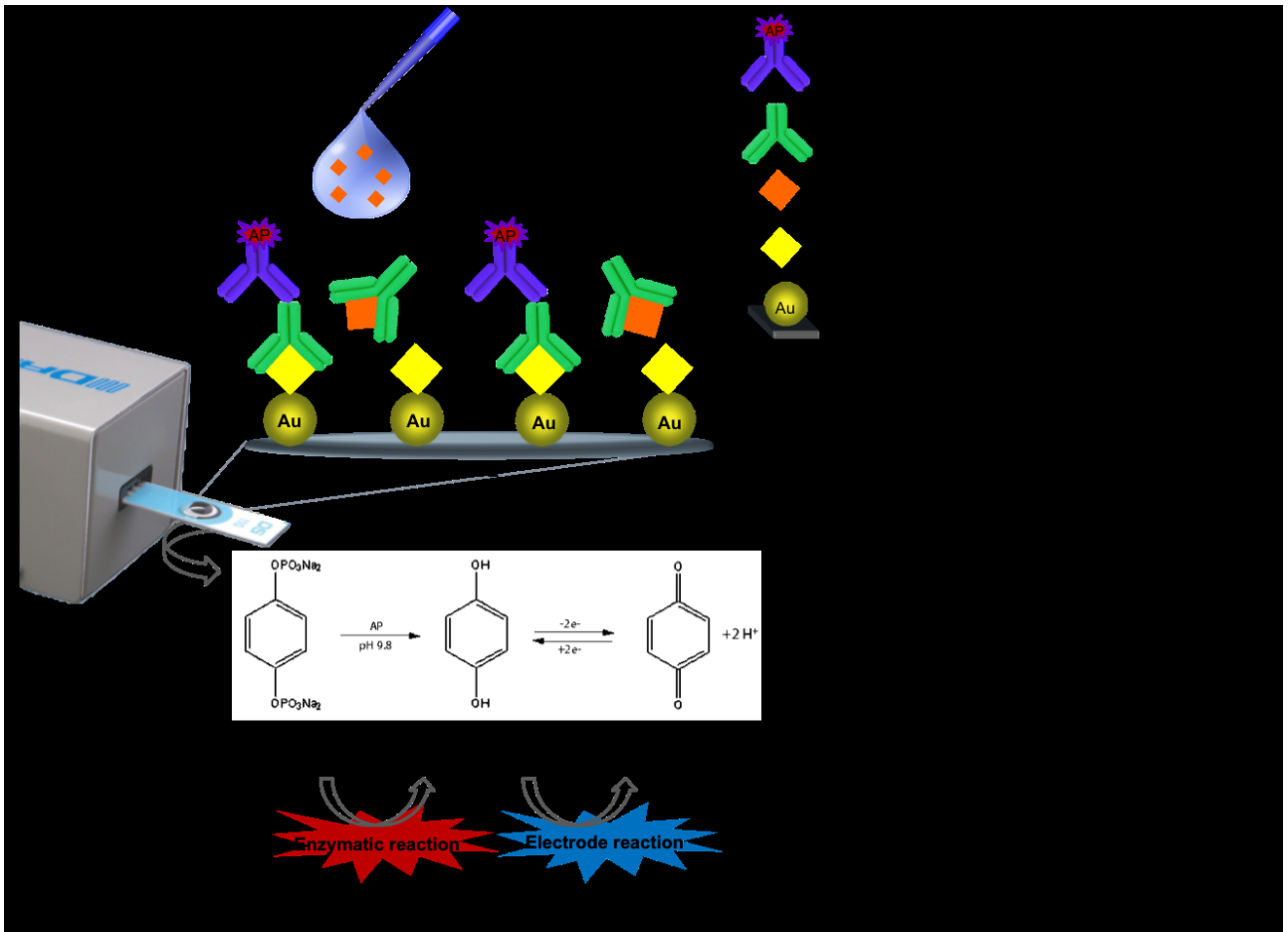


Figure 1

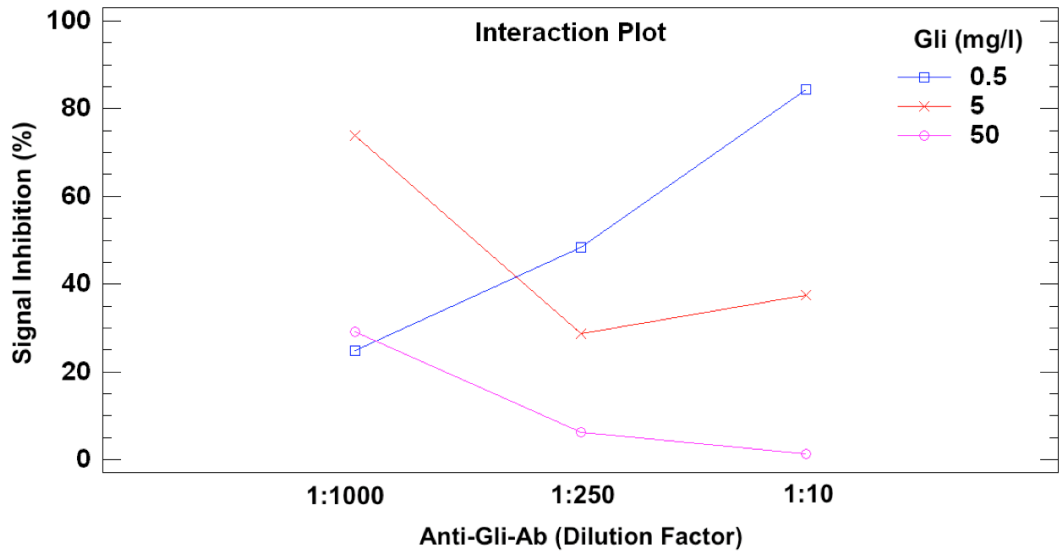


Figure 2

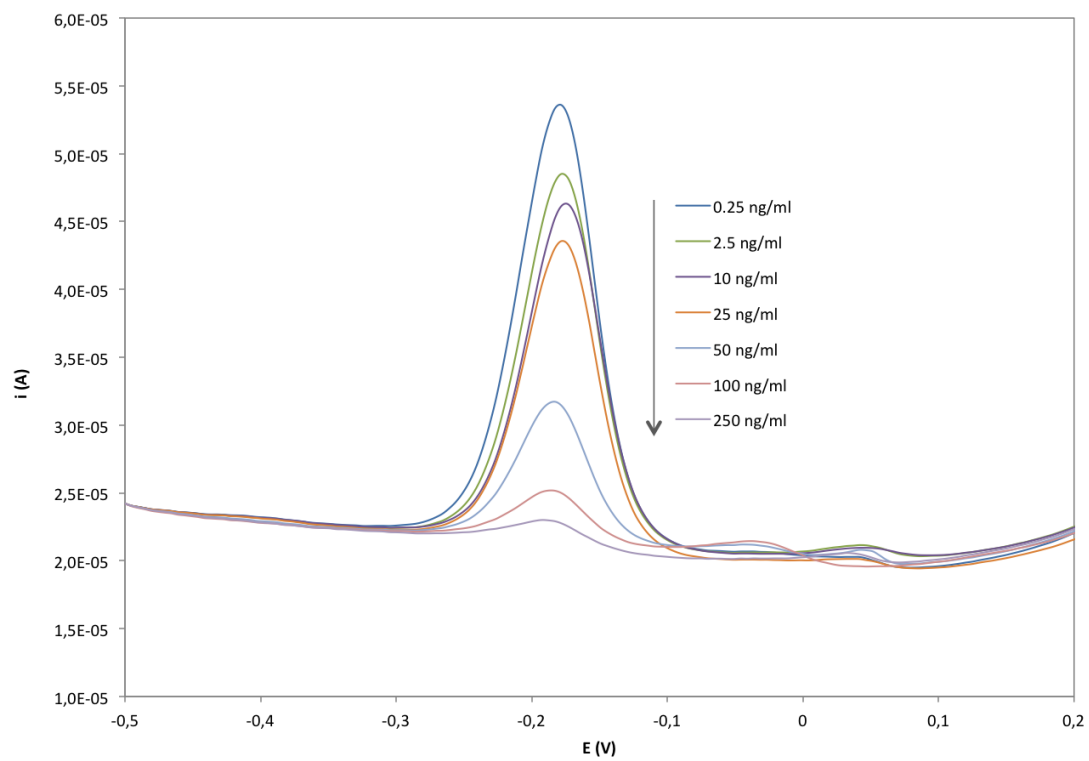


Figure 3

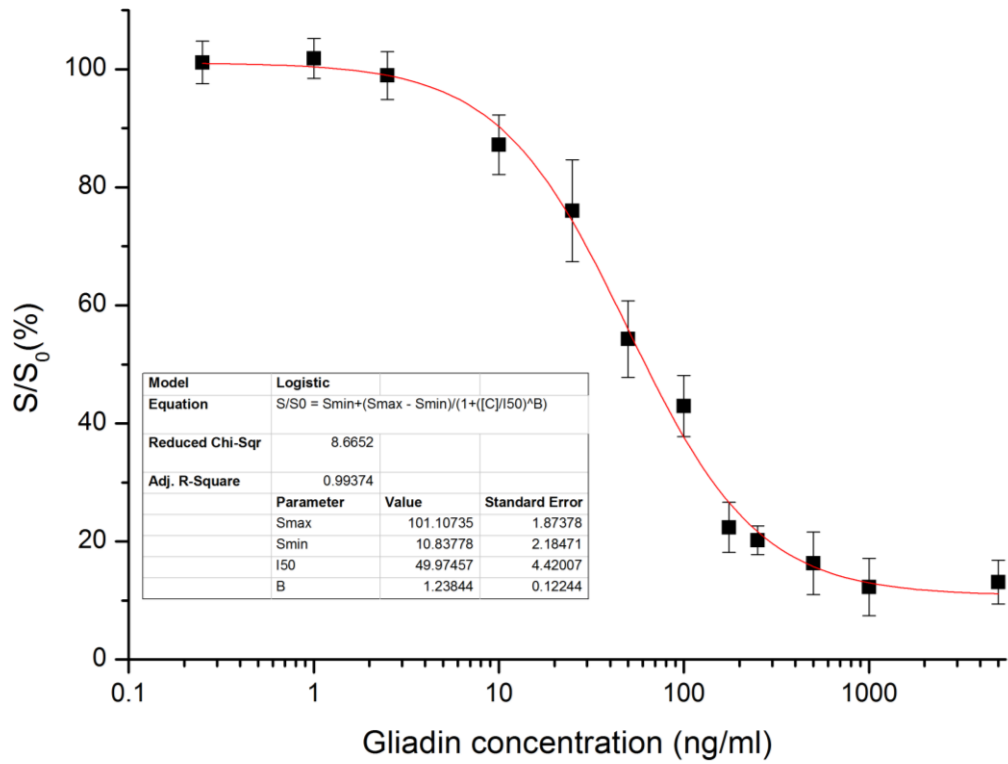


Figure 4

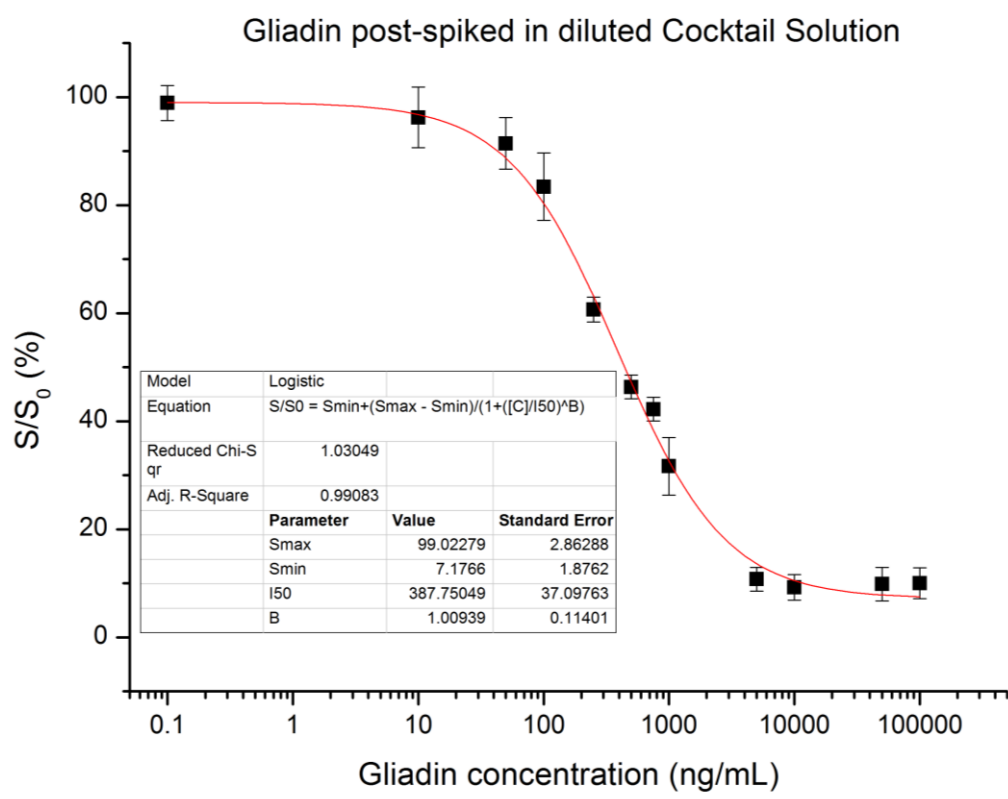


Figure 5

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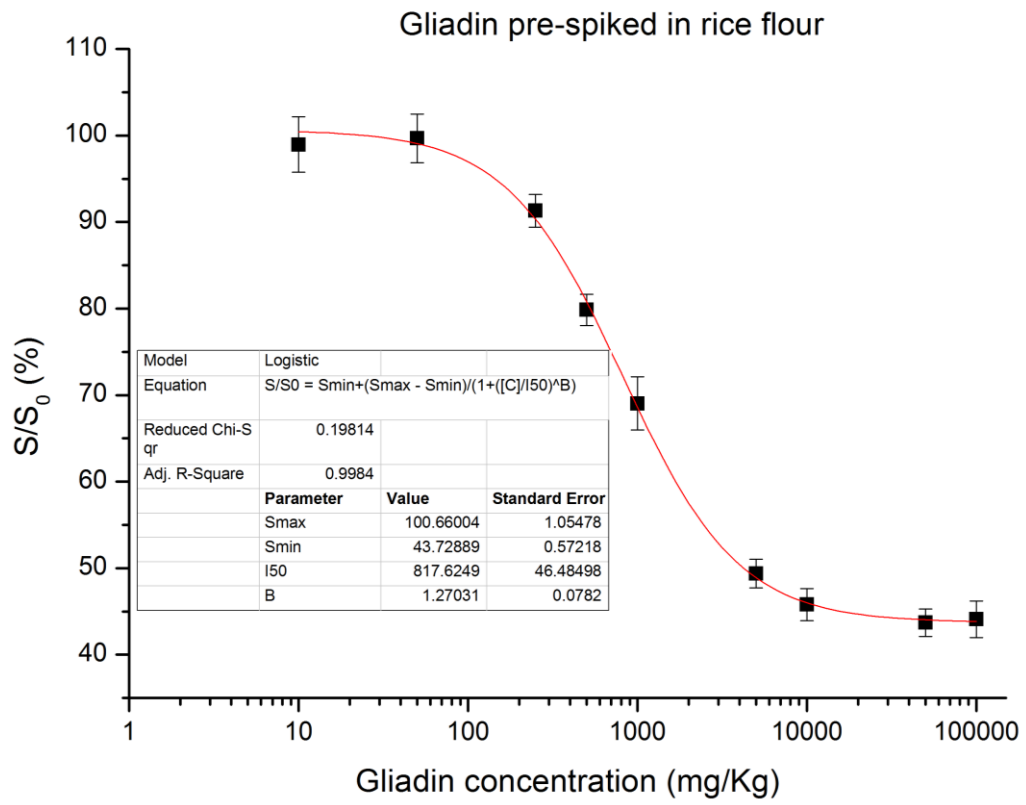


Figure 6