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Article

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LABEL-FREE DETECTION OF GLIADIN IN FOOD BY QUARTZ CRYSTAL MICROBALANCE-BASED IMMUNOSENSOR

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ABSTRACT: Gluten is a protein composite found in wheat and related grains, including barley, rye, oat, and all their species and hybrids. Gluten matrix is a bio-molecular network of gliadins and glutenins contributing to the texture of pastries, breads and pasta. Gliadins are the main responsible for the coeliac disease, one of the most widespread food-related pathologies in Western world. In view of the importance of gliadin proteins, by combining the quartz crystal microbalance technology - a cheap and robust piezoelectric transducer - with the so-called Photonic Immobilization Technique - an effective surface functionalization method providing spatially oriented antibodies on gold substrates- we realized a sensitive and reliable biosensor for quantifying these analytes extracted from real samples in a very short time. The resulting immunosensor has a limit of detection of about 4 ppm and, more remarkably, shows excellent sensitivity in the range 7.5-15 ppm. This feature makes our device reliable and effective for practical applications since it is able to keep low the influence of false positives.

KEYWORDS: Gliadin, immunosensor, quartz-crystal microbalance, Photonic Immobilization Technique, antibodies, surface functionalization.

INTRODUCTION

Gliadins are the main responsible for the coeliac disease, a genetically determined autoimmune pathology characterized by stimulation of helper T-cells resulting in a chronic inflammation of the mucosal tissue of the small intestine.[1,2] This immunological disease is the most common food intolerance in the Western population showing an incidence of about 1%.[3] In view of the lack of effective medical treatments, coeliac patients have to strictly follow a gluten-free diet to avoid intestinal mucosal inflammation and other complications. Such a requirement motivates the quest for fast and sensitive procedures apt to detect gliadins in complex matrices. In this respect, as an alternative to conventional analytical techniques (e.g. high-performance liquid chromatography, HPLC) or immunological assays (e.g. flow cytometry enzyme-linked immunosorbent assay, ELISA), an appealing perspective is to use biosensor-based approaches, which are expected to provide devices with high specificity, appropriate sensitivity and easiness-of-use.[4,5]

Gliadins and glutenins are two classes of plant storage proteins with high proline content. They are the main constituents of gluten, a protein composite found in wheat and related grains.[6,7] Gliadins are monomeric alcohol soluble proteins having a molecular weight ranging from 30 to 80 kDa.[8] They are classified into four groups, named α-, β- (these two have similar structural characteristics), γ- and ω-gliadins. On the other side, glutenins are polymeric proteins connected through intermolecular disulfide bonds and are divided into high molecular weight (HMW), from 100 to 140 kDa, and low molecular weight (LMW), from 30 to 55 kDa, subunits.[9] The present regulation by the international Codex Alimentarius defines as gluten-free a food with gluten content <20 mg/kg (ppm) (CODEX STAN 118 – 1979). In addition, food products labelled as "very low gluten" can have a gluten level ranging between 20 and 100 ppm. These limitations are consistent with those set by other countries and international bodies like the European Union and the American Food and Drug Administration (FDA). According to Codex Alimentarius, the prolamin fraction (i.e. gliadin

content) is generally taken as 50% of gluten so that the amount of gluten is estimated by doubling prolamin concentration.

Conventional detection procedures used to quantify gliadins in real samples involve indirect techniques like gluten-specific polymerase chain reaction (PCR)[10] and direct approaches like ELISA,[11] HPLC[12], LC-MS-MS[13] and cytometry.[14] The standard method for gluten determination (according to the Codex Alimentarius) is an ELISA based on the monoclonal antibody R5.[15] This approach is exploited in several commercial immunoassays (RIDASCREEN®), which provide a limit of detection (LOD) of about 1.5 ppm (3 ppm of gluten). However, all of the above procedures are time-consuming, expensive and require extensively trained operators to be performed.

As an attempt to develop an approach for easy detection of gliadins, Chu *et al.*[16] developed an effective assay combining a purification procedure carried out by using immunomagnetic beads with a detection step involving immunoliposomal nanovescicles containing fluorescent dyes. This method proved to be suitable for quantifying gliadins in real samples with a LOD of 0.6 μg/mL. An additional approach has been proposed by Cimaglia *et al.*[17] who developed a protein microarray functionalized by using a recombinant glutamine-binding protein (GlnBP) and 4F3 monoclonal antibodies. Their device showed a good response up to 5 ppm of gliadin although its application as a biosensor requires additional improvements.

In the context of biosensing approach, De Stefano *et al*. exploited a GlnBP from *Escherichia coli* which recognizes an amino acid sequence typical of prolamins.[18] This bio-receptor was immobilized onto a nanostructured porous silicon (PSi) surface thus achieving a linear response between 2.0 and 8.0 μM of gliadins. The main limitation of this procedure is that samples require several complex and time consuming manipulation steps to make available the amino acid sequence detectable by GlnBP. Nassef *et al.*[19] realized amperometric and impedimetric immunosensors for the detection of gliadins using antigen-binding fragments (Fab). These immunoglobulin fragments were immobilized onto the gold electrodes using a self-assembled monolayer (SAM) approach thus

achieving a LOD of 3.29 ng/mL and 0.42 μg/mL for amperometric and impedimetric devices, respectively. However, these immunosensors were not tested against a real sample such as a wheat extract, which in principle can contain species affecting gliadin detection. Other electrochemical approaches have been developed by Laube *et al.*[20] who achieved a LOD lower than 1 μg/L by using a novel competitive electrochemical magneto immunosensor as well as by Chiriacò *et al.*[21] who showed a limit of detection lower than 1 ppm with an immunochip; however both these methods were not validated with the certificated ELISA assay.

The reliability and cost-effectiveness provided by quartz crystal microbalance (QCM) technology make QCM devices valuable tools for a wide range of applications, including material science and biosensing.[22,23] Moreover, by adopting an appropriate fluidic setup it is possible to integrate QCM-based detection tools in industrial pipeline for monitoring the presence of contaminants and other harmful species. Recently, Chu et al.[24] developed a QCM-based immunosensor incorporating gold nanoparticles to increase the effective surface area of the electrode. In that case, LOD of 8 ppb was achieved, but the gold sensor surface was functionalized by non-commercial antibodies and the enhancement of the QCM response was reached by a complex surface modification procedure involving functionalized gold nanoparticles. Furthermore, the sensitivity exhibited by the sensor was relatively poor in the relevant range of 1-10 ppm thereby limiting practical applications. In fact, most of the gluten-free food actually contains few ppm of gliadins,[25] possibly as a consequence of the widespread presence of these proteins in food production processes; therefore, it becomes crucial the availability of biosensors with very high sensitivity able to reduce the occurrence of false positives. Essentially, the detection of gliadins in food demands high sensitivity in the range 1-10 ppm rather than very low limit of detection.

In this paper we propose a simple QCM-based immunosensor for quantifying gliadins, which exploits an unconventional functionalization method named Photonic Immobilization Technique (PIT),[26] whose effectiveness has been proven in detecting two harmful species like parathion (a

pesticide)[27] and patulin (a mycotoxin).[28] This functionalization method exploits the selective reduction of disulphide bridges in proteins produced by UV irradiation of near aromatic amino acid, which causes the upright immobilization of antibodies onto gold surfaces, i.e. with at least one binding site well exposed to the solution.[29] Since gliadin is a heavy protein, its detection does not require any ballasting procedure if LODs in the range of ppm are required. In this manuscript we prove that the PIT-functionalized QCM-based immunosensor has high sensitivity in the range 5-15 ppm while being able to detect gliadins in real food with a LOD as low as 4 ppm, a value smaller than the law limit required to label a food as gluten-free.

MATERIALS AND METHODS

Materials and instrumentation

Tris(2-carboxyethyl)phosphine (CA706) (TCEP), N-Lauroylsarcosine (61739), purified anti-gliadin polyclonal antibody from rabbit (G9144), gliadin from wheat (G3375) and bovine serum albumin (BSA) (A2153) were from Sigma Aldrich, while FluoSpheres Amine-Modified Microspheres (F8765; diameter 1μm; absorption and emission maxima were at 505 and 515 nm, respectively) were from ThermoFisher. A set of processed gliadin assay controls (R7012) was purchased from RBiopharm AG. Before analyzing these samples by QCM-based immunosensor, the amount of denaturing agents in the extracts was reduced by means of Amicon Ultra-15 3K Centrifugal Filter Devices (UFC900308). This step was necessary to preserve the functional characteristics of immobilized antibodies and prevent their denaturation once in contact with sample solution. Basically, 10 mL of extract were loaded in the tube and after spinning for 60 min at 5000 *g*, protein pellet was re-suspended with 1× PBS to its original volume. By repeating this procedure 3 times, the amount of denaturants was significantly reduced while gliadin concentration was the same of the original extract.

Other materials were 1× PBS buffer solution pH 7.4, MilliQ water, sulphuric acid 98%, hydrogen peroxide 30% and ethanol. The QCM device was a μLibra by Technobiochip (Italy) while the

piezoelectric sensors were AT-CUT quartz with a fundamental frequency of 10 MHz from Industria Elettronica Varese (Italy). The quartz crystals were cleaned using the so-called Piranha solution (3:1 ratio between concentrated sulphuric acid and 30% hydrogen peroxide) in the fume hood. The QCM microfluidic apparatus consisted of a HNP Mikrosysteme continuous pump, Tygon tubes (internal diameter 1.02 mm) and the cell containing the crystal. The QCM electrode was placed on the electronic console for the measurement of frequency oscillation, which was monitored using a proprietary software. The volume of the cell was about 30 μL while the whole circuit was about 300 μL. QCM experiments were performed with a constant flow rate of 2.2 μL/s.

RP-HPLC analysis of gliadins

Gliadin fractions were extracted from a rice flour with the procedure described by Mejias et al.[30] The Sigma Aldrich gliadin from wheat (G3375) was dissolved in 250 μL of 60% (v/v) ethanol, incubated with gentle shaking at 200 rpm for 20 min at RT, and centrifuged for 20 min at 10,000 *g*. The supernatant was recovered and the protein concentration was estimated by the Bradford method (1976) using an UV-VIS Spectrophotometer CARY 1 (Varian Medical Systems, Inc. 3100 Hansen Way, Palo Alto, CA).

RP-HPLC was performed through the E-Alliance HPLC system (Waters Corp., Milford, Massachusetts). Samples (an aliquot of 20 μL) were injected on a Zorbax Eclipse xdB C8 (150 × 3.0 mm, 2.5 μm particle size) column equipped with a Zorbax Eclipse xdB guard column (12.5 × 2.1 mm) (Agilent Technologies, Palo Alto, CA, USA). Separation was carried out using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% (v/v) formic acid, with a flow rate of 0.8 mL min^{-1}. The initial solvent composition consisted of 80% (v/v) of A and 20% (v/v) of B, then a linear gradient was applied firstly increasing it to 40% A and 60% B in 50 min and retaining it for 1 min, next increasing it to 10% of A and 90% of B in 2 min and retaining it for 4 min. Finally, it was returned to 80% of A and 20% B in 2 min. The column was equilibrated to 80% A and 20% B for 7 min before next injection. The analysis lasted 66 min setting column

139 temperature to 60 °C. Absorbance was monitored at a detection wavelength of 210 nm. Resulting
140 data were analyzed using the Waters Empower software.

141 **Gliadin extraction and quantification by ELISA**

142 Gliadins were extracted from commercial rice flour (not labeled as gluten-free), gluten-free corn
143 flour (Maizena) and from a set of three commercial standards (named as A, B and C) with a known
144 amount of gliadins (RIDASCREEN cod. R7012, RBiopharm AG Darmstadt, Germany) using two
145 buffers: the Cocktail extraction solution RIDASCREEN (cod. R7006/R7016, RBiopharm AG)
146 (patent WO 02/092633 A1)[31] and the UPEX buffer (patent WO 2011/07039 A2)[32] and following the
147 RIDASCREEN manual instructions.

148 The RIDASCREEN Gliadin kit (RBiopharm AG, cod. R 7001) was used to quantify gluten content
149 in samples according to the official R5-Mendez procedure approved by the AOAC (Association of
150 Official Agricultural Chemists). Each sample was tested in duplicate. The Multiskan FC (VWR
151 International s.r.l., Milan, Italy) plate reader was used to acquire data which were analyzed using
152 the RIDAsoftWIN.NET software (RBiopharm AG).

153 **SDS-PAGE and Western blot**

154 The trichloroacetic acid (TCA) precipitation method was used to prepare protein samples for SDS-
155 PAGE and Western blotting. Briefly, a volume of 100% TCA was added to 4 volumes of protein
156 samples. Then, samples were incubated for 10 min at 4°C and centrifuged at 14k rpm for 5 min.
157 The supernatant was removed and the pellet washed twice with cold acetone and centrifuged at 14k
158 rpm for 5 min. After drying the pellet in 95°C heat block for 10 min, precipitated proteins were
159 dissolved in 2× Laemmli sample buffer (Bio-Rad, Milan, Italy) with β-ME and boiled for 10 min in
160 95°C heat block.

Proteins were analyzed by SDS-PAGE on a Mini-Protean II mini-gel apparatus (Bio-Rad, Milan, Italy), using 6% (w/v) stacking polyacrylamide gel and 12% (w/v) separation gel.[33] SDS-PAGE gel was stained with Coomassie Blue.

Western blot was carried out by transferring proteins onto polyvinylidene difluoride (PVDF) membrane by electroblotting with Mini Trans-Blot Cell (Bio-Rad, Milan, Italy). The blot was probed with the anti-gliadin polyclonal antibodies (Sigma Aldrich), as a primary antibody (dilution 1:500) and Goat anti-rabbit IgG conjugated with Horseradish peroxidase (Sigma Aldrich) as a secondary antibody (dilution 1:2000).[34] Protein patterns were detected by chemiluminescence using the Clarity™ Western Blotting Substrate (Bio-Rad, Milan, Italy).

Microfluidic system characterization using polystyrene μbeads

In order to optimize the performances of the QCM-based immunosensor, a systematic study on the effects of the flow rate on surface coverage was carried out. Fluorescent beads were used as model system since they are safe and easy-to-handle probes which can be involved in studying flowing and deposition phenomena. Samples containing 80 μg/mL of fluorescent microspheres were conveyed onto gold surface of a QCM at different flow rates (2.2, 6.6 and 9.2 μL/s). Then, after air-drying, the electrode surface was analyzed by a Leica DM 2700M Fluorescence Microscope equipped with a L5 Filter Cube and a Mercurial Arc Lamp EL6000. By using a proprietary software it was possible to visualize the complete surface of the electrode thus obtaining the images reported in Figure 1. It is evident that a flow rate of 2.2 μL/s leads to a homogeneous coverage of the gold surface while higher flows result into a more irregular deposition. The value of 2.2 μL/s was the lowest reliable flow rate achievable with our setup and was used throughout the QCM analyses.

UV-activation of antibodies for sensor functionalization

Photonic Immobilization Technique (PIT) is a valuable functionalization method providing an effective anchoring of immunoglobulins onto thiol reactive surfaces (like QCM gold electrodes),

which greatly improves the detection efficiency.[26] This approach relies upon the selective photoreduction of disulphide bridges in antibodies caused by the UV activation of the trp/cys-cys triad, a typical structural feature of immunoglobulins[35]. As previously demonstrated, the thiol groups produced in such a way are able to immobilize the antibodies onto the gold sensor surface without affecting their recognition properties.[27,28] Moreover, the recently reported single molecule characterization of UV-activated antibodies by means of atomic force microscopy shows that PIT is able to steer the immobilized immunoglobulins in an upright orientation, i.e. with the sensitive portion of the biomolecule well exposed to the environment.[29]

In the present work, purified anti-gliadin polyclonal antibodies were used to functionalize the QCM gold sensor surfaces. Protein samples of 1 mL, containing 25 μg of immunoglobulins, were activated in a quartz cuvette placed close to a HERAEUS amalgam type lamp (mod. NNI 40/20) emitting in the UVC spectral range (wavelength ~254 nm, power 40 W). The samples were irradiated for 10 min, a time which guarantees the maximum efficiency in the photoreduction mechanism as verified through the so-called Ellman's assay.[36] After the activation, immunoglobulin samples were conveyed in the fluidic chamber containing the electrode for sensor functionalization.

RESULTS AND DISCUSSION

Reversed-phase HPLC gliadin profile

The spectrophotometric measurement of protein content extracted from the Sigma Aldrich gliadin (G3375, used as reference) and commercial rice flour provided 448 μg/mL and 176 μg/mL, respectively. As reported in literature[30], the elution profiles of gliadin fractions of the two samples could be appreciated at three different time intervals: 9–21 min for ω-gliadins, 21–30 min for α/β-gliadins and 30–35 min for γ-gliadins (Fig. 2A and B).

Gliadin quantification by R5 ELISA method

The RIDASCREEN Gliadin kit combined with the R5 Mendez ELISA method was used for gluten quantification in different samples. As described in the materials and methods section, gliadin fractions were recovered using both the cocktail extraction solution (R7006, R5-Mendez method) and the UPEX buffer. As confirmed by the ELISA output, these two procedures provided the same extraction yield (see Table I).

The ELISA confirmed that the corn flour labeled as gluten-free did not contain gluten as the amount of gliadins resulted to be lower than 2.5 ppm, whereas the rice flour had a gliadin content of about 492 ppm. This high value may be ascribed to the use of equipments previously involved in gluten-containing food processing or to the intentional addition of gluten for improving the rice flour texture properties. Gluten content estimated in the commercial standards (A, B and C) was in agreement with the values provided by the manufacturer (RBiopharm AG). Finally, gliadin quantification performed using the certificated ELISA method has been found in fair agreement with the values estimated by using the QCM-based approach proposed in this paper (see the last column in Table I). The characteristics of the immunosensor and the comparison between QCM outcomes and ELISA results are described in the QCM procedure section.

SDS-PAGE and Western blot

The same protein samples analyzed by the R5 Mendez ELISA were also characterized by SDS-PAGE and Western blotting, the latter performed using polyclonal anti-gliadin antibodies (Sigma Aldrich). The profiles of the standard Sigma Aldrich gliadin, rice and corn flour are shown in Fig. 3. As expected, SDS-PAGE (Fig. 3A) revealed the presence of gliadins together with other proteins having lower molecular weight, which are not recognized by anti-gliadin antibodies in the Western blot (Fig. 3B). This shows comparable protein patterns for the Sigma Aldrich standard gliadin and rice flour, although in the latter the band intensity is lower, while the corn flour (labeled as gluten-free) has no hybridization signals in the immunoblotting.

Detection of gliadin by QCM-based immunosensor

The response of the QCM-based immunosensor was calibrated using gliadins extracted from a commercial rice flour using the UPEX buffer. This sample was quantified by using the R5 Mendez ELISA method, thus showing an amount of gliadins of about 500 ppm. Since prolamins recovered by using the UPEX buffer are not stable for more the 24h,[32] it is important analysing the extract immediately after the recovering procedure. For this reason, a protocol allowing the sequential analysis of several gliadin samples at increasing concentrations was developed. Samples were treated by diluting the extraction stock solution with 1x PBS thus covering a gliadin concentration ranging between 2.5 and 35 ppm, and significantly reducing the amount of denaturing species (i.e. ethanol, TCEP and N-Lauroylsarcosine) in the solution flowing onto the functionalized electrode. In Fig. 4A we report a sensorgram corresponding to the analysis of three samples at different gliadin concentration ([G_I]=5 ppm, [G_{II}]=10 ppm and [G_{III}]=25 ppm), while in panel B of the same figure the different steps of the measurement are sketched. Each concentration was tested twice providing the data shown in Fig. 5 where the calibration of the QCM-based immunosensor is reported.

Firstly, the fluidic cell containing the electrode was washed with 1× PBS until basal frequency stabilization was reached [Fig. 4A, step (a)]. Then, the solution of UV-activated anti-gliadin antibodies (25 μg/mL) was conveyed onto the gold sensitive surface resulting in the first drop in frequency at about 500 s, which is due to the immunoglobulin immobilization onto the sensor surface [step (b)]. When the electrode reached the stabilization, the cell was washed with PBS [step (c)]. The next step was the blocking of all remaining available sites on the sensor surface with a BSA solution (100 μg/mL), thereby avoiding non-specific binding and saturating the gold surface [step (d)], and then the circuit was purged again with PBS [step (e)]. The whole functionalization procedure is highlighted in Fig. 4A in green, while the analysis on gliadin samples and the following washing phases are reported in red and blue, respectively. Once the QCM achieved the new stabilization, the sensor was ready for the analysis of the samples. Gliadin samples were prepared by diluting the extract in 1× PBS. When the first sample (5 ppm of gliadin) reached the

electrode at about 3200 s, there was an evident drop in frequency and some fluctuations due to the change of the solvent [step (f)]. Since the quartz crystal microbalance is extremely sensitive to changes in density and viscosity of the liquid in contact with the electrode, when the UPEX/PBS mixture flowed on the gold surface, the new solution induced a frequency perturbation. Indeed, when we purged the circuit with PBS, the frequency rose and stabilized at a higher value [step (g)]. Because of this effect, the frequency shift due to gliadin molecules recognized by immobilized antibodies was measured at the end of the washing phase. The difference in frequency between the values before the flowing of the first gliadin sample and after the washing with PBS was due to the binding of the antigens by the immobilized antibodies onto the sensor surface and corresponds to Δf_1 in Fig.4A. The same procedure was used for the remaining two samples (second and third), which brought about the frequency shifts Δf_2 and Δf_3, respectively [steps (h)-(k)]. When the second sample (10 ppm of gliadin) reached the electrode [step (h)], the immobilized antibodies were partly bound to the antigens of the first sample and hence, the new equilibrium condition was reached by a smaller frequency shift. Thus, the contribution due to the presence of analytes in the second sample in our measurement is $\Delta f[G_{II}])=\Delta f_1 + \Delta f_2$, whereas for the third sample we have $\Delta f[G_{III}]) = \Delta f_1 + \Delta f_2 + \Delta f_3$. This procedure allowed us to consecutively test more samples with increasingly gliadin concentrations, which eventually led to the dose-response curve reported in Fig. 5 (black square points). It is worth noticing that these points were collected in different days by extracting gliadins from the same rice flour thus proving the reproducibility and the reliability of both extraction and sensing procedures.

The experimental data were fitted using the following Hill type equation, a mathematical model quite similar to Michaelis-Menten law, which describes cooperative binding events:[37]

$$\Delta f([G]) = \frac{\Delta f_{sat}[G]^h}{K_H^h + [G]^h} \quad (1)$$

where [G] is gliadin concentration, $(\Delta f)_{sat}$ is the maximum frequency shift achieved by the sensor at saturating antigen concentration, K_H is an average dissociation constant depending on all the binding events and h is the so-called Hill coefficient. The latter parameter provides an estimation of the cooperative effect in protein-protein and protein-ligand interactions. For $h>1$, a cooperative event is described, whereas $h<1$ suggests an anti-cooperative binding; on the opposite, when $h=1$, the Hill equation reduces to the non-cooperative Michaelis-Menten law. The best fit of the experimental data provides $(\Delta f)_{sat}$=178±10 Hz, K_H=11±1 ppm and h=3.5±0.5, the latter value being consistent with a cooperative process, which can be ascribed to the aggregation tendency of the gliadins.[38,39] In fact, these proteins, which are classified into four subcategories, α-, β-, γ-, and ω-gliadins (being α, γ, and ω-types responsible for the coeliac disease), are naturally assembled with glutenins to form the gluten (the bases of these connections being intermolecular disulphide bridges, hydrophobic and hydrogen bonding interactions).

The error in the measurements was due to instrumental limitations of the QCM device as well as to random fluctuations in some steps of sample preparation, thus the uncertainty in the measurement appears to be larger in the linear region of the calibration plot (7.5-15 ppm), where steeper dependence is observed, than in the regions where gentler dependence on the concentration is measured (e.g. initial and saturation regime). In particular, at very low concentrations, where the QCM starts to detect the gliadins, an error σ_d=2 Hz can be estimated; thus, by assuming a safe 99% confidence interval (i.e. signal-to-noise ratio >3 σ_d) a frequency shift ≥6 Hz is required to consider the measurement as significantly different from zero. As a result, the LOD can be worked out by the dose-response curve reported in Fig. 5 where the frequency shift of 6 Hz occurs at a gliadin concentration of 4 ppm. Such a value fulfills the requirements dictated by current regulation (10 ppm) and the change of the steep of the dose-response curve occurring around 4-5 ppm allows us to assess that any slightly higher concentration can be safely measured. This is very important in practical applications for gliadins detection in real samples since most of the gluten free foods

actually contain gliadins in the range 1-10 ppm.[25] In these conditions, it may be difficult to fix a threshold for gliadin content, which rejects 99% of the contaminated food while keeping very low the fraction of the false positives (i.e. safe food not detected as such). We can easily show that our device fulfills such a requirement at a high degree; in fact, even by considering an uncertainty on the single measurement in a range around 10 ppm as σ=10 Hz, the threshold frequency warranting 99% confidence level is

$$\Delta f_{th} = \Delta f(10\,\text{ppm}) - 3\sigma = 75\,\text{Hz} - 30\,\text{Hz} = 45\,\text{Hz} = \Delta f(8\,\text{ppm}). \quad (2)$$

Such a high value (8 ppm) is very close to the law limit of 10 ppm and is a consequence of the high sensitivity (≈15 Hz/ppm) our device shows in a range around 10 ppm. In the context of QCM-based biosensors, it is worth to compare our threshold with that resulting from a dose-response curve with a much smoother dependence of the frequency shift on the gliadin concentration. Although extending on a very broad dynamical range, the dose-response curve reported by Chu *et al.*[24] only shows a sensitivity of approximately 3 Hz/ppm in the range 1-10 ppm. Thus, even by considering an optimistic value of the standard deviation on the single measurement of only 4 Hz, by applying the same criteria leading to Eq. (2), we retrieve $\Delta f_{th} = \Delta f(2\,\text{ppm})$. This value is significantly lower than ours and would lead to consider as contaminated most of the gluten free food[25] with obvious undesirable consequences.

In order to verify the robustness and the reliability of the QCM-based immunosensor, the response of this device was measured against real samples like commercial standards with known gliadin content and Maizena, a corn flour being intrinsically gluten-free which is considered safe for people affected by the coeliac disease. One gram of this flour was treated using the same extraction procedure as rice samples and gliadin content was quantified using first the certificated ELISA and then the QCM-based method. In both cases, a negligible response was measured, such a result consistent with a gluten-free matrix (for QCM measurements see the red circles in Fig. 5).

To test a positive response, three processed snacks (A, B and C) from RBiopharm AG were analyzed by the standard ELISA method, which provided 3.8 ppm (A), 21 ppm (B) and 38 ppm (C), respectively. The sensorgram of the same samples produced by QCM is reported in Fig. 6, where the frequency jumps occurring at the injection of the samples are due to the solvent change (diluted UPEX against PBS). We point out that prior their injection in the QCM fluidic samples A, B and C were treated using the Amicon Ultra-15 Centrifugal Filter devices (see Materials and Methods) to reduce the amount of denaturing agents (ethanol, TCEP and N-Lauroylsarcosine) thereby reaching a dilution level comparable to that used for the calibration curve. In fact, the frequency jumps occurring in Fig. 6 are comparable to those in Fig. 4 measured at high dilution levels, thus guaranteeing a non-denaturing environment for the immobilized antibodies.

QCM measurements provide an estimation of the gliadin content in the standards A, B and C (see blue triangles in Fig. 5 and Table I), which results to be 4±2 ppm, 20±1 ppm and 25±3, respectively. The values for samples A and B are in excellent agreement with the data provided by the ELISA method, whereas a significant difference is observed between the two methods for the sample C. We can safely ascribe such a discrepancy to the lack of sensitivity of our method for the sample C, whose gliadin concentration lies in the saturation region of the dose-response curve. In fact, such a curve shows an abrupt change of slope just at concentration of approximately 23-24 ppm, which represent the upper limit of our dynamical range.

In conclusion, the risks associated with the presence of allergens, contaminants and harmful pathogens in food motivates the quest for fast and sensitive procedures apt to detect these species in complex matrices and fresh fruit and vegetables (e.g. apple, celery and tomato). The spread of the coeliac disease makes the reliable detection of gliadins in the food supply chains an attracting perspective in view of the inappropriateness of conventional analytical techniques (e.g. HPLC) or immunological assays (e.g. ELISA). Because of their extremely high specificity and easy-of-use, biosensor-based approaches can effectively address the issue. We showed that an immunosensor

based on a quartz-crystal microbalance, conveniently functionalized by PIT (an optical technique able to orient antibodies upright on a gold surface), is able to measure gliadin content in food with a LOD of 4 ppm, which is lower than the law limit to label food as gluten-free. Even more important, our biosensor can be used in practical situations by fixing a safe threshold limit for contaminated food at 8 ppm, thereby keeping the fraction of false positives at very low level. The extraction procedure we adopted is very effective and involves the so-called UPEX buffer, a PBS-based recovering method, which provides mostly the same extraction yield of the recommended Cocktail buffer. The amount of gliadins estimated by using the immunosensor herewith described is in fair agreement with the values obtained using the certificated R5 Mendez ELISA method for gluten quantification. The intrinsic high specificity provided by immobilized antibodies combined with the robustness and portability of QCM devices make this immunosensor a valuable tool for *in situ* analysis without the need of trained personnel and complex and expensive facilities.

Abbreviations Used

PIT, photonic immobilization technique; QCM, quartz crystal microbalance; BSA, bovine serum albumin; UPEX, universal prolamin and glutelin extractant solution; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography.

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481 **Figure captions**

Figure 1. Fluorescence images of QCM electrodes functionalized using fluorescent microspheres at different flow rates: 2.2 (a), 6.6 (b) and 9.2 μL/s (c). The brighter spots in the bottom left portion of the electrode correspond to the input channel of the fluidic cell containing the QCM sensor.

Figure 2. Elution profiles of Sigma Aldrich G3375 gliadin fractions from wheat A) and from rice flour B). Time intervals of gliadin fractions (ω, α/β and γ) are also indicated.

Figure 3. (A) Electrophoretic separation of gliadin fractions from flours on SDS-PAGE and (B) Western blotting with polyclonal anti-gliadin antibodies. Lanes: 1, Molecular Weight Markers; 2**,** commercial Gliadin (Sigma Aldrich); 3, rice flour gliadin extracted with UPEX buffer; 4, corn flour gliadin extracted with UPEX buffer.

Figure 4. (A) QCM output of the experimental procedure used for detecting gliadins in real sample extracts and (B) sketches of the different phases of the experiment. The vertical dashed lines show the steps described in the text while the functionalization phases, the gliadin sample analysis and the following washing phases are highlighted in green, red and blue, respectively. (a) Basal frequency stabilization; (b) UV-activated antibody immobilization onto the QCM gold surface. The immunoglobulin concentration is 25 μg/mL; (c) washing with PBS; (d) blocking step with BSA (100 μg/mL); (e) washing with PBS; (f) analysis of the first gliadin sample (5 ppm); (g) washing with PBS; (h) analysis of the second gliadin sample (10 ppm); (i) washing with PBS; (j) analysis of the last gliadin sample (25 ppm); (k) final washing with PBS. When the first gliadin sample ($[G_I]$=5 ppm) reaches the electrode (f) there is a first drop in frequency due to the solution properties because the sample contains a fraction of the original extraction solution, which differs from PBS and causes this huge change in frequency. The purge of the circuit with PBS leads to an increase of

frequency. This behavior is more evident for the second [[G_{II}]=10 ppm, (h)] and third [[G_{III}]=25 ppm, (j)] gliadin samples where the contribution of extraction buffer is larger. The frequency shifts are shown on the right as Δf_1, Δf_2 and Δf_3, respectively (horizontal dashed lines).

Figure 5. Response of the QCM versus gliadin concentration. The experimental points obtained using rice extracts (black squares) are fitted by a Hill-type equation (black solid line) while three negative controls performed by analyzing a corn flour (i.e. Maizena) sample are the red circles in the low concentration region. The commercial standards having a known gliadin content (i.e. A, B and C) led to the blue triangles in the graph while the vertical dashed lines indicates the corresponding gliadin content. The range of concentration where the response is approximately linear is highlighted in grey.

Figure 6. QCM output for gliadin from samples A, B and C (sensor functionalization not shown). The frequency shifts and the washing with 1x PBS (W) are reported in red and blue, respectively. The big frequency jump at the beginning of the injection is caused by the change of the solvent.

Table captions

Table I. Gliadin content estimated in flour samples using the R5 Mendez ELISA. Standard A, B and C (RIDASCREEN cod. 7010, RBiopharm AG Darmstadt, Germany) were commercial samples with known gliadin content. ELISA data were compared with the values obtained using the QCM-based immunosensor. *Cocktail is the RBiopharm AG product (cod. R7006/R7016).

528 **Tables**

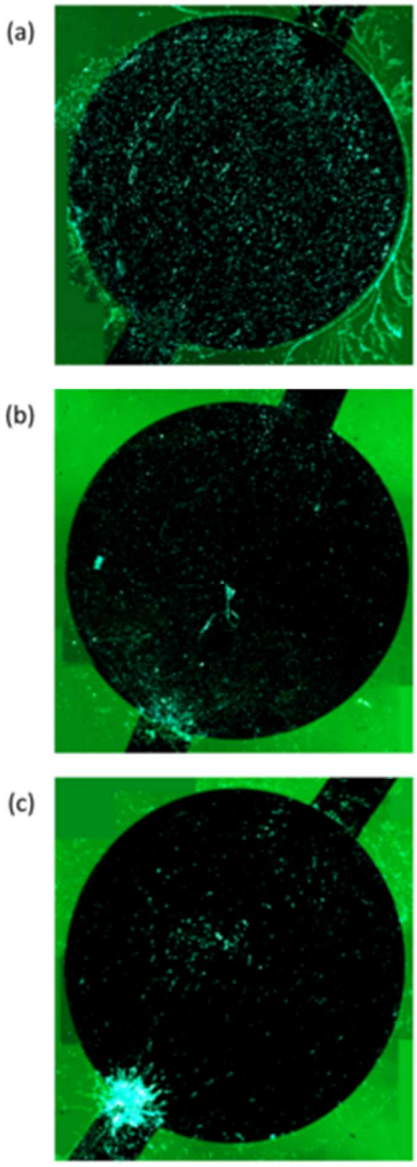
529 TABLE 1

Technique	ELISA		QCM
Extraction buffer	*Cocktail	UPEX	UPEX
Sample	Gliadin (ppm)		Gliadin (ppm)
Standard A	3.8 ± 0.1	3.8 ± 0.1	4 ± 2
Standard B	21 ± 1	21 ± 1	20 ± 1
Standard C	38 ± 1	38 ± 1	25 ± 3 (saturation)
Rice	493 ± 1	491 ± 1	Reference sample
Corn	<2.5	<2.5	<<4

530

Figure graphics

FIGURE 1



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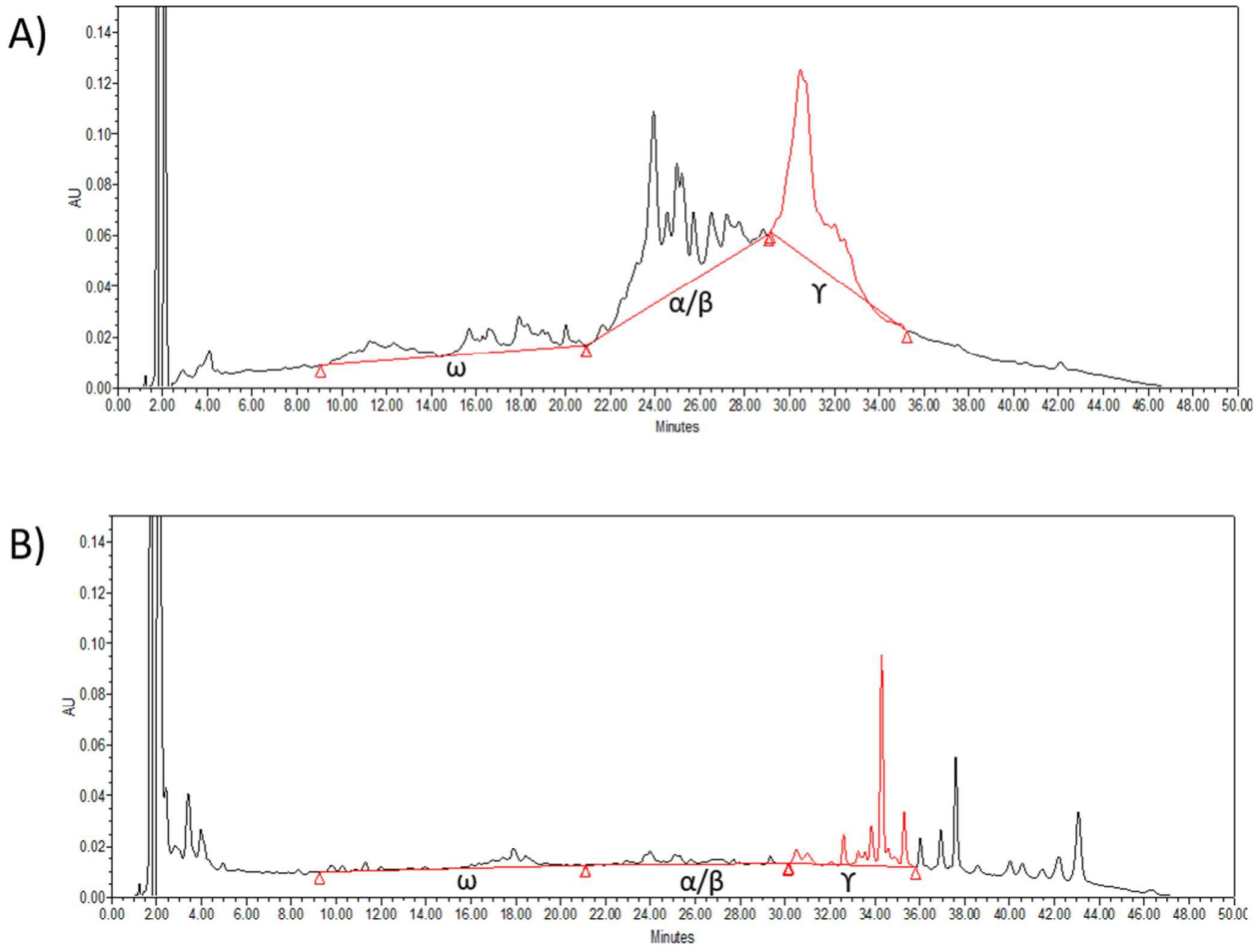

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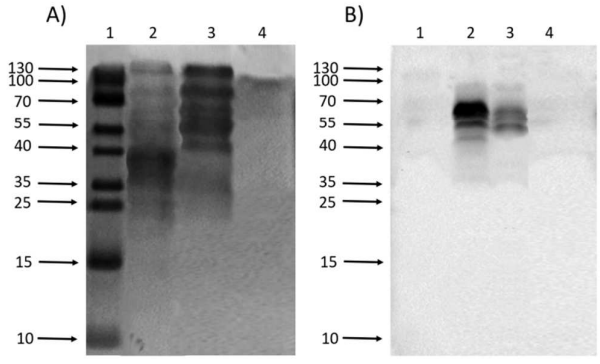

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539 FIGURE 4

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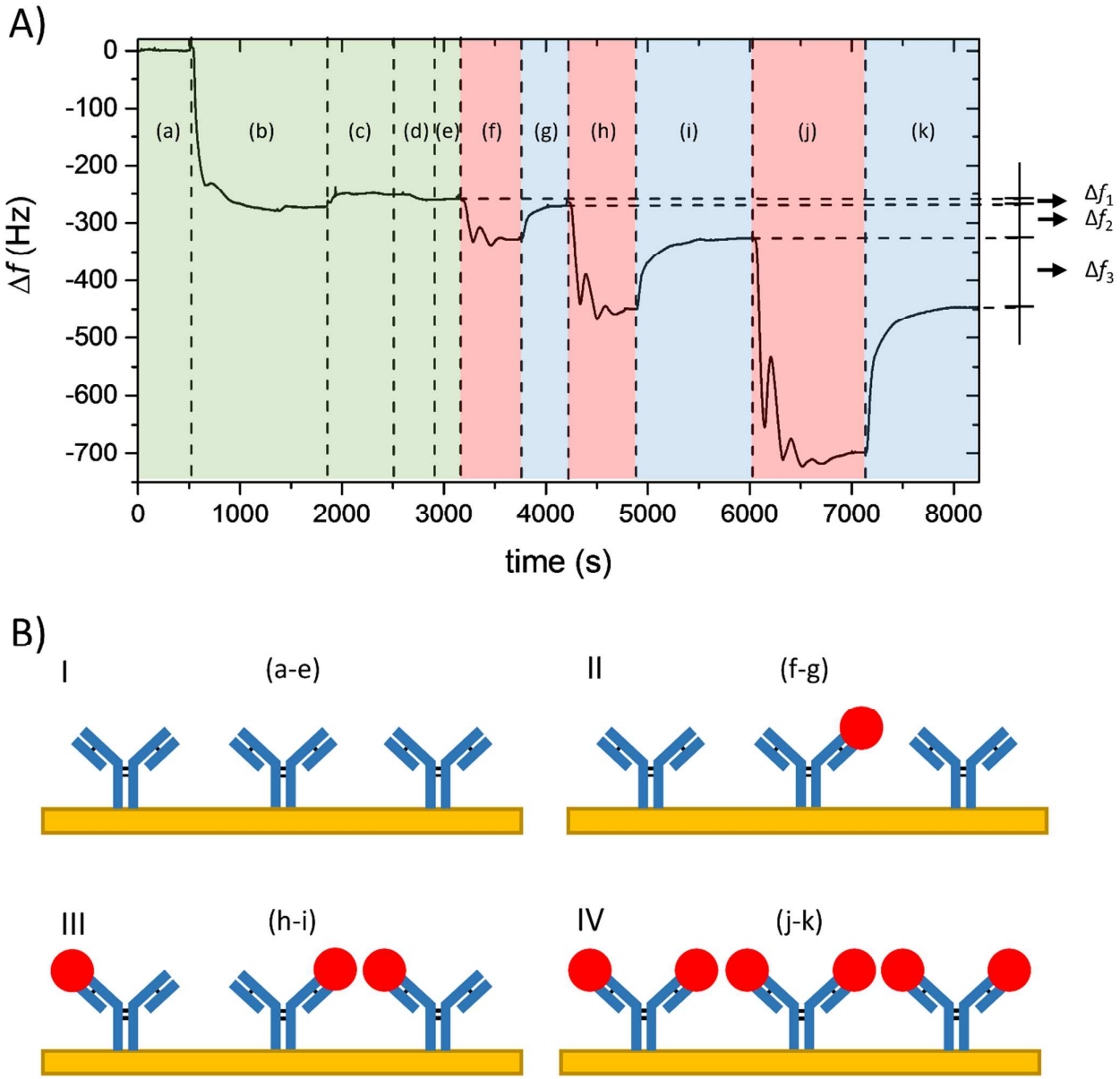

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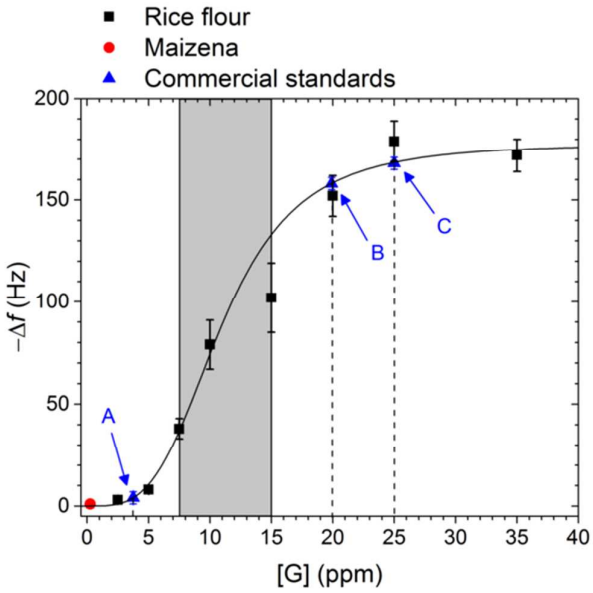
543 FIGURE 5



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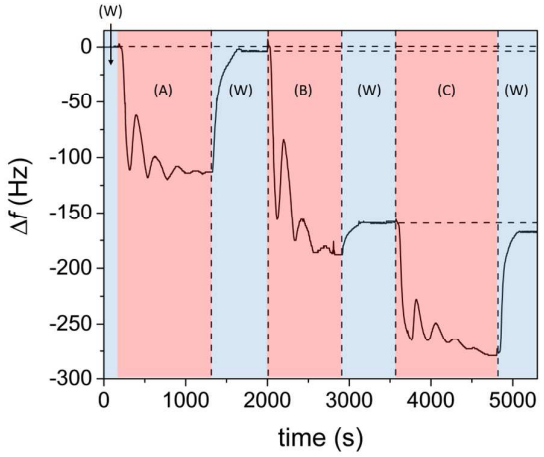
545 FIGURE 6



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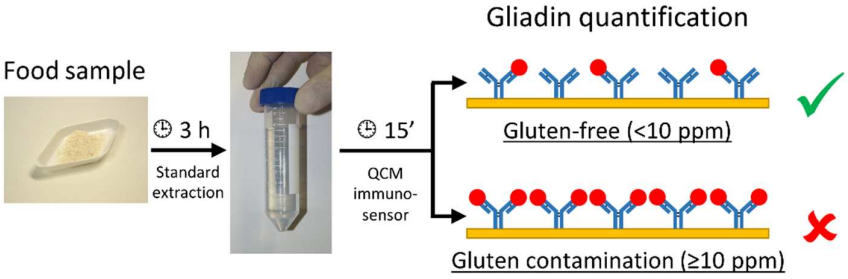
548 **Graphic for table of contents**



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