



Sensitive Gluten Determination in Gluten Free Foods by an Electrochemical Aptamer-based Assay

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3 **Sensitive Gluten Determination in Gluten Free Foods by an Electrochemical**
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5 **Aptamer-based Assay**
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Abstract

Enzyme-immune assays are currently the methods of choice for gluten control in foods labelled as “gluten free”, providing a mechanism for assessing food safety for consumption by coeliac and other allergic patients. However, their limitations, many of them associated to the reactivity of the different antibodies used and their degree of specificity, have prevented the establishment of a standardized method of analysis. We explore new methods for quantitatively determine gluten content in foods based on the use of two recently described aptamers, raised against a 33-mer peptide recognised as the immunodominant fragment from $\alpha 2$ -gliadin. The assays use the target peptide immobilized onto streptavidin-coated magnetic beads in combination with a limited amount of biotin-aptamer in a competitive format, followed by streptavidin-peroxidase labelling of the aptamer that remains bound to the magnetic beads. The enzyme activity onto the beads, measured by chronoamperometry in disposable screen-printed electrodes, is inversely related to the target concentration in the test solution. We find that while the assay using the aptamer with the highest affinity towards the target (Gli 4) achieves low detection limits (~ 0.5 ppm) and excellent analytical performance when challenged in samples containing the intact protein, gliadin, it fails in detecting the peptide in solution. This problem is circumvented by employing another aptamer (Gli 1), the most abundant one in the SELEX pool, as a receptor. The proposed assays allow the convenient detection of the allergen in different kind of food samples, including heat-treated and hydrolysed ones. The obtained results correlate with those of commercially available antibody-based assays, providing an alternative for ensuring the safety and quality of nominally gluten-free foods.

Keywords: Aptamer, Coeliac disease, Electrochemical detection, Gliadin analysis, Gluten, 33-mer peptide.

1. Introduction

Gluten detection is an outstanding problem because of its relation with coeliac disease (CD) and gluten-related allergies or intolerances. CD is one of the most prevalent autoimmune diseases affecting 1% of population and is related to genetic factors and triggered by the ingestion of gluten [1]. The only treatment for CD is the strict following of a lifelong gluten free diet. Because of the seriousness of the CD and its prevalence, EU legislation enforces the labelling of food that may contain gluten-containing cereals, and allows to label as “gluten free” products those containing less than 20 ppm of gluten [2]. However, there is not universal agreement on what the term “gluten free” means. For example, Spanish Federation of CD patients Associations (FACE) establishes a “Gluten Free Certification”, which assures that the product contains less than 10 ppm of gluten. But there are especially sensitive patients who can develop symptomatology after the ingestion of minimal amounts of gluten that makes those limits insufficient. The lowering of the existent limits is being frustrated by the need for more sensitive and reliable gluten detection methods to be applied in the food industry.

The development of gluten detection methods has to confront some challenges because of the nature of the analytical problem. First, the lack of a clearly defined chemical target, as gluten is the popular name given to major storage proteins of certain dietary grains. Gliadins, the alcohol soluble fraction of wheat gluten, and homologous proteins in barley, rye and possibly oats, globally termed as prolamins, are the main target in gluten analysis. Additionally, during food processing the proteins composing gluten may suffer alterations induced by thermal or enzymatic treatments. Gluten proteins have a relatively large amount of –SH containing amino acids. These residues form intrachain S-S bonds that suffer different changes depending on the thermal food

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3 processing. Breaking of the S-S intrachain bonds and formation of new ones between
4 different chains originates a polymeric structure with low solubility, avoiding
5 quantitative extraction of the proteins and affecting its further analysis [3]. The most
6 common sample treatment involves extraction of gluten prolamins using a 60 % ethanol
7 solution. This extraction is not capable of fully dissolving gluten in heat processed
8 foods [4]. In order to overcome this problem different extraction solutions have been
9 developed, incorporating a reducing reagent, for breaking interchain S-S bonds, and
10 chaotropic agents, to solubilize gluten proteins in the extraction solution [4,5]. The most
11 used extraction mixture, called “cocktail” is composed of 2-mercaptoethanol and
12 guanidine hydrochloride [5]. Another important alteration of the proteins occurs when
13 food processing involves treatments in which gluten can be hydrolysed. In the
14 production of starch and syrup and in brewery enzymatic processing breaks down the
15 proteins into small peptides that are also toxic for CD patients [6-8]. These
16 immunotoxic small peptides must also be detected to estimate the toxicity of these
17 products for celiac patients.

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19 Enzyme-linked immunosorbent assays (ELISA) are the currently accepted methods for
20 gluten determination in foods. Several antibodies targeting different fragments of gluten
21 proteins are employed [9-12]. Despite their many positive characteristics, the traditional
22 sandwich ELISA is not suitable for quantifying gluten in hydrolysed foods because it
23 requires the presence of at least two epitopes in the target molecule, which is unlikely in
24 the fragments obtained after hydrolysis. To overcome this drawback, competitive
25 immunoassays have been developed based on the R5 antibody and using as calibration
26 standard gliadin [4] or a mixture of whole protein and hydrolysed fragments [13] or on
27 the G12 antibody and tracing the calibration curve using whole gliadin [11]. However,
28 neither the sandwich assays, nor the competitive ones are fully compatible with the
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cocktail extraction solution because their components may denature the protein receptor [14]. A non-protein receptor for gliadin would thus contribute to the improvement of gluten detection methods.

With the aim of solving this problem, we have recently obtained a panel of aptamers for specific binding to gliadin [15]. These non-protein receptors, obtained against one of the main immunogenic gluten peptides known as 33-mer [7], are able to bind not only the peptide used as target for selection but also the whole protein and homologous ones in rye, barley and oats, suggesting they might serve as viable receptors for the detection of gluten in foods at concentrations below the 20 ppm threshold proposed by the Codex Alimentarius Commission. Here, we expand this approach developing and validating two electrochemical competitive assays on magnetic particles for the determination of gluten. These assays rely on the use of two of the selected aptamers i.e. Gli 4, the ligand with the highest affinity towards gliadin, and Gli 1 the most abundant in the selection pool, whose binding to the peptide may be kinetically favoured. Their analytical performance is comparatively evaluated and validated against gliadin-ELISA kits. Both assays are complementary, proving to be reliable tools for sensitive detection of gluten in a wide variety of foods, including heat-treated and hydrolysed ones.

2. Materials and methods

Reagents

DNA aptamers (5'→3'), both labelled in 5' with biotin,

Gli 4: (CCAGTCTCCCGTTTACCGCGCCTACACATGTCTGAATGCC)

and Gli 1: (CTAGGCGAAATATAGCTACAACGTCTGAAGGCACCCAAT), which

are tagged with biotin at its 5'-terminus, were obtained from Integrated DNA

Technologies (IDT, Leuven, Belgium).

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3 Unmodified (LQLQFPQPQLPYPQPQLPYPQPQLPYPQPQP; 33-mer) and
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5 biotinylated (LQLQFPQPQLPYPQPQLPYPQPQLPYPQPQPFFFFFFF-lysine-
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7 Biotin; Biotin-33-mer) peptides and QQFP were purchased from Biomedal (Sevilla,
8
9 Spain).

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11 Streptavidin modified magnetic beads with a binding capacity of 4 nmol mL⁻¹ of free
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13 biotin (Dynabeads® MyOne™ streptavidin C1, 1 µm Φ, 10 mg mL⁻¹) were provided by
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15 Life Technologies (Madrid, Spain). Gliadin standard solutions were prepared using the
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17 gliadin standard provided by the Working Group on Prolamin Analysis (PWG) and
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19 acquired to R-Biopharm. Streptavidin-Peroxidase conjugate (Str-HRP₂, 176 units mg⁻¹
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21 peroxidase activity) was obtained from Thermo Fisher (Madrid, Spain). Reagents for
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23 buffer preparation, guanidinium hydrochloride, mercaptoethanol, polyvinylpyrrolidone,
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25 fish gelatine and enzymatic substrate 3,3',5,5'-tetramethylbenzidine (TMB) liquid
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27 substrate system for ELISA were purchased from Sigma-Aldrich. Solutions were
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29 prepared using MilliQ purified water.
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33 34 *Procedures*

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36 Modification of streptavidin coated magnetic beads: An aliquot of 50 µL of the
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38 streptavidin-magnetic beads stock solution was washed twice with BLmod (1 mL PBS
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40 + 0.01 % Tween-20) for 2 min and then resuspended in 1 mL of 2 µM biotinylated 33-
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42 mer in BLmod. The tube was incubated for 30 min at 30 °C in a Thermomixer
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44 (Eppendorf Ibérica, Spain) under continuous shaking. After that, the beads were washed
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46 twice in BLmod and blocked with 500 µM biotin in BLmod for 30 min. After two new
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48 washing steps, they were reconstituted in 500 µL BS (50 mM TRIS pH 7.4+0.25 M
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50 NaCl+5 mM MgCl₂) and used within the same day.
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54 Competitive assay: For Gli 1 assay, 30 µL of modified magnetic beads were incubated
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56 in 500 µL of BS with 250 nM of biotinylated aptamer and selected amounts of
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3 calibration standard (or 10 μL of extracted sample) for 30 min at 30 $^{\circ}\text{C}$ in Thermomixer
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5 under shaking. After that, the beads were washed twice with BSL (50 mM TRIS pH
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7 7.4+0.25 M NaCl+5 mM MgCl_2 + 0.01 % Tween-20), and incubated with an excess of
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9 enzymatic conjugate ($2.5 \mu\text{g mL}^{-1}$ Str-HRP₂) in 500 μL of BSL for 30 min at 30 $^{\circ}\text{C}$
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11 under shaking. Finally, the beads were washed twice with BSL, once with BS and
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13 resuspended in 30 μL of BS.
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17 For Gli 4 assay, 10 μL of modified magnetic beads were incubated in 1 mL of BS with
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19 250 nM of biotinylated aptamer and selected amounts of calibration standard (or 20 μL
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21 of extracted sample) for 30 min at 30 $^{\circ}\text{C}$ in Thermomixer under shaking. After that, the
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23 beads were washed twice with BSL, and incubated with an excess of enzymatic
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25 conjugate ($2.5 \mu\text{g mL}^{-1}$ Str-HRP₂) in 1 mL of BSL for 30 min at 30 $^{\circ}\text{C}$ under shaking.
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27 Finally, the beads were washed twice with BSL, once with BS and resuspended in 10
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29 μL of BS.
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33 Amperometric measurement: The resuspended beads (10 μL) were captured on screen
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35 printed carbon electrodes (Dropsens, Oviedo, Spain) placing a magnet under the
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37 working electrode, and the electrochemical cell was covered with 40 μL of 3,3',5,5'-
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39 tetramethylbenzidine + H_2O_2 substrate. The enzymatic reaction was developed for 30 s
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41 and the enzymatically obtained product measured by chronoamperometry at 0 V using a
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43 μ -AutoLab type II potentiostat (Ecochemie, The Netherlands).
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47 Extractions: For the extraction of flours and no heat-processed foods, 0.8 g of flour or
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49 homogenised food sample was placed in a 15 mL Falcon tube and washed with an
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51 aqueous solution of 2 M NaCl. After 20 minutes of shaking at RT, the tube was
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53 centrifuged in a Heraeus Multifuge 1L-R (ThermoScientific) for 15 min at 7500 g and
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55 the supernatant was discarded. Prolamins were extracted twice with ethanol 60 %
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57 solution. For this purpose 4 mL of ethanol solution were added to the tube and the
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3 mixture was incubated at RT during 20 min and then centrifuged for 15 min at 7500 g,
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5 and both supernatants were combined.
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8 Some food samples were also extracted as follows: 0.25 g of ground sample was mixed
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10 with 2 mL of cocktail solution (250 mM mercaptoethanol, 2 mM guanidine in 0.1×
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12 PBS). The suspension was incubated in an oven at 50 °C for 40 min. Once at room
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14 temperature, 3 mL of ethanol were added and extraction in a shaker was extended for 1
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16 hour. Finally the samples were centrifuged for 30 min at 2500 g, and the supernatant
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18 was used in the analysis. The sample containing chocolate was extracted in the presence
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20 of 0.25 g of gelatin and 0.1 g of polyvinylpyrrolidone, which were added to 0.25 g of
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22 ground sample before the extraction.
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27 **3. Results and discussion**

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29 We chose a competitive assay format, well suited to detect both intact protein and small
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31 protein fragments, using two previously selected aptamers targeting the immunotoxic
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33 α 2-gliadin fragment, 33-mer [15]. Magnetic microparticles are employed as support for
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35 the sensing phase to take advantage of the efficient magnetic separation offered by this
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37 platform, which facilitates subsequent exhaustive washing steps thus minimizing major
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39 problems associated with non-specific interactions. The preparation of the sensing phase
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41 is very simple. A commercially available 33-mer, modified at its carboxy-terminus with
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43 a biotin, is immobilized onto the surface of streptavidin-modified magnetic particles and
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45 then the unoccupied sites of streptavidin are blocked with biotin. The analytical assay
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47 we developed using this platform comprises the following steps (**Figure 1**): first, the
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49 sample and the biotin-labelled aptamer are incubated with a fixed amount of the
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51 modified magnetic beads. In this step, the analyte from the test solution competes with
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53 the immobilized peptide for a limited amount of aptamer. As a result, part of the biotin-
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3 aptamer is specifically captured and separated from the sample after applying an
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5 external magnetic field. In order to detect the bound aptamer, particles are incubated in
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7 a second step with a streptavidin-peroxidase conjugate containing two molecules of
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9 enzyme per streptavidin as reporter molecule. Finally, the enzyme activity on the
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11 surface is electrochemically quantified by magnetically entrapping the modified beads
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13 onto a screen-printed carbon electrode. After the addition of tetramethylbenzidine and
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15 hydrogen peroxide we measured by chronomaperometry the reduction of the product
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17 enzymatically produced. The higher the concentration of gliadin in the sample is the
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19 lower the reduction current we obtain.
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22 **Figure 1.**

23 *3.1. Assay Optimization*

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25 To obtain optimal conditions for the competitive assay, the concentration of biotin-
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27 aptamer was first selected, fixing the amount of magnetic particles modified with the
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29 peptide in 0.06 mg mL^{-1} . In this study, different concentrations of the aptamers in the
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31 range 0.5 nM - $2 \mu\text{M}$ were tested without protein or peptide in solution. This analysis
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33 produces typical binding curves for both aptamers (**Figure 2**). 250 nM of aptamer, the
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35 concentration that yield around 50% of the maximum obtained signal, was selected for
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37 competition. In the absence of the aptamers, we observe only a small reduction current
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39 ($0.4 \pm 0.1 \mu\text{A}$), which confirmed that the contribution of the nonspecific interaction of
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41 Str-HRP₂ conjugate is not significant. The assay also gives negligible responses when it
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43 is performed using a randomized biotinylated DNA library at a 250 nM concentration
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45 level. Conversely, we registered a maximum signal value of $4.7 \pm 0.1 \mu\text{A}$ and 3.6 ± 0.1
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47 μA for Gli 4 and Gli 1, respectively at this aptamer concentration. These results are
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49 consistent with the specific interaction of both aptamers with the immobilized peptide
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51 and a higher affinity for the aptamer Gli 4 [15].
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Figure 2

The second variable we took into consideration is the composition of the competition buffer. To evaluate the effect of ethanol in the aptamer/peptide interaction, we performed the competitive assay in buffer containing different concentrations of this alcohol. The presence of 1.2% of ethanol, which will correspond to a 1:50 dilution of samples extracted using 60 % ethanol, increases the maximum signal obtained while the blank is not affected. The improved signal amplitude at 1.2% ethanol can be explained given the apolar nature of the peptide modifying the surface of the beads, which may cause their slight aggregation. A small amount of ethanol may act as disaggregating agent, favouring the aptamer/peptide interaction. Consistent with this, the competitive assay achieves improved signal decrease with this amount of ethanol in the buffer. At 3% of ethanol the binding of Gli 1 to the peptide decreases to about 35% of the maximum while Gli 4 interaction is less affected, which again is in agreement with the lower affinity constant for the peptide-Gli 1 reaction.

Therefore, the best performance in terms of sensitivity is obtained employing 0.06 mg mL⁻¹ of the modified beads and 250 nM aptamer, in the presence of 1.2% ethanol. Upon the addition of 10 µg L⁻¹ of PWG standard to the competition buffer in the presence of Gli 1, a 13% of signal decrease is observed with a coefficient of variation of 6%. Under the same conditions, Gli 4 gives rise to higher sensitivity with about 20% of decrease at the same concentration of PWG. We find, however, that this aptamer leads to worse reproducibility, more than 15% of RSD at 10 µg L⁻¹, probably related to the fact that a higher tendency of the 33-mer-modified magnetic particles to aggregate is observed with this aptamer. To overcome this difficulty we performed the competitive assay with Gli 4 using a higher dilution of the magnetic beads during the competition i.e 0.01 mg mL⁻¹, while maintaining the amount of beads on the electrode surface for measurement

(10 µg). In this way sensitivity of the assay was not affected and precision was improved, with a RSD of 6% for 10 µg L⁻¹ of PWG.

3.2. Analytical performance

The performance of both aptamers in competitive assays under the optimum conditions was compared using PWG gliadin or 33-mer peptide as standards. The selection of the standard in order to obtain a calibration to which refer the measurements of the samples have to be carefully done to avoid deviations in the value obtained. Most used calibration and validation standard is PWG gliadin, a material obtained after processing of 28 different cultivars of wheat [16]. We used this material as a gliadin standard to obtain the calibration curve.

In **Figure 3A**, the current intensities obtained after challenging the modified beads with increasing concentrations of PWG using both aptamers is depicted. As expected, aptamer Gli 4 gives rise to higher currents than aptamer Gli 1 as a result of the difference in affinity.

Data were fitted to a four parameters logistic (equation 1)

$$y = A + \frac{(B-A)}{1 + \left(\frac{x}{EC_{50}}\right)^p} \quad (1)$$

where A is the response at an infinite concentration of analyte, B is the response at a zero concentration of analyte, EC₅₀ is the concentration of analyte that corresponds to the 50 % of the maximum signal and p is the Hill slope that represents the slope at the inflexion point of the sigmoidal curve. Due to the low solubility of gliadin in aqueous solution concentrations higher than 1 µg L⁻¹ were not assayed, which precluded the observation of the complete sigmoidal curve. The best fitting were obtained fixing A parameter to zero and the equations are the following:

$$I(\mu\text{A}) = (3.60)/(1 + ([PWG (\mu\text{g L}^{-1})]/721)^{0.5}); r = 0.980 \text{ for Gli 1 and}$$

$$I(\mu\text{A}) = (5.17)/(1 + ([PWG (\mu\text{g L}^{-1})]/273)^{0.6}); r = 0.997 \text{ for Gli 4.}$$

The limit of detection of each assay was calculated as the concentration that gives the 95 % of the maximum signal and corresponds to 4.9 $\mu\text{g L}^{-1}$ of gliadin when using Gli 1 as recognition element and 0.5 $\mu\text{g L}^{-1}$ of gliadin when using Gli 4. Taking into account the sample extraction, the dilution of the extract and the gliadin to gluten ratio, the limit of detection in real samples can be calculated in order to verify whether the methods meet the requirements to evaluate the accomplishment of the EU legislation. The overall sample dilution is 1:500 and the content of gliadin in gluten is considered as 50 %, [17] though values ranging from 32% to 67 % have been found [18], so the limit of detection expressed in gluten for each assay is 4.9 ppm and 0.5 ppm for Gli 1 and Gli 4, respectively. Those values are below the limits needed to check the legislation accomplishment and are similar or slightly lower than competitive immunoassays even those currently marketed [4,11,19-21].

Figure 3

The repeatability of the assays calculated as the coefficient of variation at 1 and 10 $\mu\text{g L}^{-1}$ of gliadin concentration were 7 and 8%, respectively for Gli 1 aptamer and 9 and 6 %, respectively for Gli 4. The inter assay reproducibility, calculated with the signal obtained for 10 $\mu\text{g L}^{-1}$ in three different days is 9.5 % for Gli 1 assay and 6% for Gli 4 assay.

Competitive assays are specifically designed for hydrolysed samples where the proteins are digested to small peptides containing a single epitope for the antibody. The competitive format of R5 antibody uses a peptide fragment (QQPFP) as a standard even though the equivalence between peptide fragments and the intact protein is lacking [13].

This precludes the conversion of the results into gluten content as required in the

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3 legislation [17]. On the contrary, the competitive G12 assay uses the intact protein [11].
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5 We have previously found a correlation between Sigma gliadin and 33-mer using G12
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7 antibody in an electrochemical competitive immunoassay [20]. Therefore, the feasibility
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9 of using 33-mer peptide as a standard for the competitive apta-assay was studied. In
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11 **Figure 3B**, the response of Gli 1-based method to increasing concentrations of 33-mer
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13 is shown and compared with the calibration plot using PWG both expressed in nM. It is
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15 apparent that the aptamer recognizes the peptide but at much larger concentrations than
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17 the intact protein, which indicates that PWG contains several binding sites in its
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19 structure. However, the number of those “aptatopes” would be very high, about 200,
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21 which seems unrealistic. In our previous work two binding sites for the antibody G12
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23 were found in each gliadin standard, which is reasonable. Recently, we have found that
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25 Gli 1 exhibits positive cooperativity when binding to PWG, which can explain the
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27 apparent higher affinity towards the intact protein even taking into consideration the
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29 presence of several binding sites [22].
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34 On the contrary, proper calibration plots using Gli 4 aptamer as recognition element and
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36 33-mer peptide as standard were not obtained. This fact is attributed to microscopic
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38 aggregation of the beads in presence of this aptamer. Therefore, Gli1 aptamer was
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40 selected as the receptor for subsequent testing of hydrolysed samples.
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43 The response of Gli 1 aptamer to the recognition epitope of the official R5 method, that
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45 is, the pentapeptide QQPFP [23] was also tested. This specific sequence is not present
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47 in the immunodominant 33-mer peptide used for aptamer selection but it is present in
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49 wheat, rye and barley prolamins. The aptamer was able to recognize this peptide but at
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51 much higher concentrations than 33-mer (above 1 μ M), which suggests a much lower
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53 affinity accordingly to its absence in the selection target.
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3.3. Cross-reactivity against other cereals and grains

Not only wheat proteins are toxic for coeliac disease patients, prolamins from barley and rye have almost the same toxicity. However, the toxicity of oat for coeliac patients is still controversial [24]([25] and citation therein), so oat prolamins also have to be evaluated towards the quantification of the toxicity in food products. For this reason it is necessary to evaluate the response of the competitive assays using Gli 1 and Gli 4 aptamers against prolamins from other toxic cereals and the selectivity against not-CD-triggering cereals and grains. Because of the unavailability of prolamins standards or reference materials from other grains, the ethanol-soluble fraction was extracted from each grain flour. After the extraction, the total protein present in solution was quantified by the Bradford method using PWG as standard, obtaining a content in prolamins for each extract as follows: wheat 5.4 mg mL⁻¹, rye 4.6 mg mL⁻¹, barley 5.2 mg mL⁻¹ and oat 4.7 mg mL⁻¹. All the extracts were diluted at different levels to obtain the desired protein concentration in the competition buffer. As it can be seen in **Figure 4** both assays with Gli 1 and Gli 4, recognise gliadins (wheat), hordeins (barley) and secalins (rye) in a similar extension within the experimental error. However, the response to avenins (oat) is different. Gli 1 aptamer is not able to bind oat proteins while Gli 4 aptamer recognises avenins but with less affinity than the rest of prolamins from CD toxic cereals. This result is interesting because the antibodies G12 and A1, also raised against the 33-mer peptide, weakly binds to avenins although 33-mer is not present in their sequence [26]. The sensitivity order to prolamins was, however, different: hordenins \approx gliadins > secalins \gg avenin for G12 and hordeins \approx rye > gliadin \gg avenins for the aptamers. In our case, differences between hordeins, gliadins and secalins are less pronounced in both cases. The official R5 method does not respond to the controversial oat prolamins [10].

Figure 4

It is also important to ensure the non-response of the developed method against proteins from non-toxic flours such as soya, maize and rice often used as substitutes of toxic cereals to elaborate CD-safe food. To evaluate the cross-reactivity, the response to increasing concentrations of proteins extracted from soya, maize and rice flours were assayed. In all concentration range, the analytical responses expressed as current percentage of the maximum current obtained in the absence of protein were around 100%. Specifically, percentages obtained with Gli 1 assay were: soya 112 ± 11 % maize 135 ± 26 % and rice 112 ± 9 %. For the Gli 4 assay were: soya 125 ± 37 %, maize 121 ± 36 % and rice 113 ± 25 %. According to this, aptamers do not cross react with non-toxic proteins.

3.4. Application of the competitive assays to food samples

The competitive methods developed are sensitive and selective enough to be applied to gluten determination in gluten free labelled foods in order to evaluate the compliance with the law and to provide more information to the consumer. To do this, we tested samples provided by two external laboratories. All of them had unknown gluten content. The samples provided by Laboratory I were divided, according to the information given by the company, in two groups: non-hydrolysed (Table 1) to be quantified by the Gli 4 assay that is more sensitive, and hydrolysed samples (Table 2) to be quantified by Gli 1 assay, the peptide- responsive one. Liquid samples were just diluted with 60% ethanol and solid samples were extracted with the conventional extraction procedure using 60% ethanol, which is the method recommended by the commercial kit based on G12, the antibody used by the company for comparison. Samples are considered as positive when the gluten content is above the limit of quantification of the method used in each case, even in cases where samples meet gluten free specifications (less than 20 ppm gluten).

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3 Samples identified as positive with the reference methods are in general positive with our
4 method. There are two apparent exceptions, both liquid non-hydrolysed samples (liquids C
5 and H). These are gluten free samples that were spiked with gluten by the external
6 laboratory in a blind assay. The recovery values we found for these samples are close 100%
7 even in the case of sample C, spiked with a gluten concentration very near to the limit of
8 quantification of our method. So we can conclude that our method does not present false
9 negative results. Only one out of the 5 liquid non-hydrolysed samples analysed (liquid A)
10 was negative by the G12 immunoassay. In this sample our method detected gluten but with
11 a content below the 20 ppm threshold level. This difference may be due to the improved
12 sensitivity of our method with respect to G12. Gliadin levels measured in the solid non-
13 hydrolysed samples were comparable with levels detected by G12 assay in 50 % of samples
14 (3 out of 6). Both methods showed non-detectable gluten in a rice sample. In general, our
15 method detects higher gluten content than did the G12 kit. Interestingly, the three discordant
16 samples are negative by G12-assay, and positive by our method. These differences can be
17 explained by variations in the extracted target compounds detected by the two assays.

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Attending to the results for hydrolysed samples (Gli 1 assay, Table 2), there is a good
correlation between the results provided by the external analysis and those obtained using
the competitive assay developed in this work. This correlation demonstrates the
applicability of the competitive assay based on the aptamer Gli 1 to quantify gluten in
hydrolysed samples. Only a false positive result was obtained, corresponding to a sample of
a gluten free beer. It is accepted that samples where the gluten content differ most are the
hydrolysed ones because the value obtained strongly depends on the target sequence
detected [27]. In a previous study, hordein content in gluten was comparatively assessed
using ELISA and MS [28]. This study reveals significant differences between both
methods, showing that 10% of the tested beers give ELISA gluten contents below the limit

of quantification but near average hordein content by MS. Our assay, using an aptamer that detects hordeins [15], could be an alternative for this kind of samples.

Solid samples provided by Laboratory II (Table 3) were extracted alternatively with the conventional 60% aqueous ethanol extraction method and with cocktail solution to evaluate the influence of reducing and denaturing agents on our method. The results were compared with values obtained with the official method (R5) provided by the company after carrying out the analysis with the Gli 4 competitive method and with an independent analysis performed by a certified Spanish company for gluten analysis (Laboratory III). Most of the values obtained with R5 antibody were comparable with those obtained with our method after ethanol extraction except for cappuccino, where a false positive result is observed. Again, this is probably due to the increased sensitivity of our method. The influence of the cocktail solution on the aptamer-based competitive method was studied. In general higher results were obtained with the cocktail solution, probably because a larger yield of the extraction. The exception was the sample containing chocolate, even though PVP and fish gelatin were used to assist the extraction. It is remarkable the discrepancy between the analysis using the same procedure, which is an indication of the difficulty of such samples. Additional discrepancies are observed in corn flakes samples which would led to labelling as gluten free according to one of the laboratories but not to the other. Our results with ethanol extraction are in agreement with laboratory II and are closer to laboratory III after cocktail extraction for one of them. Therefore, it can be concluded that the alteration of the protein conformation during the extraction procedure does not significantly affect its recognition by the aptamer (no false negative detected) and the cocktail solution is compatible with our aptamer-based competitive method unlike competitive immunoassays.

4. Conclusions

The combination of magnetic beads and aptamers as receptors has proved to be an attractive method for the sensitive quantification of gluten, with a detection limit of 0.5 ppm in the case where the aptamer (Gli 4) with the highest binding affinity to the protein is employed, and 4.9 ppm when using Gli 1, the most abundant aptamer. The assays can quantify gluten in food samples below the legally established limits for the labelling of “gluten free” products. Gli 4-based method is compatible with cocktail extraction method, allowing the quantification of gluten in heated foods. The assay using Gli 1 aptamer can quantify gluten in hydrolysed samples. Both methods are complementary and can be valuable tools in the determination of gluten in foods intended for coeliac patients.

Acknowledgements

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TABLES

Table 1: Samples extracted with ethanol 60 % and analysed with Gli4 aptamer. They were compared with a G12 method carried out by the external laboratory

Sample	Gli 4 method (ppm gluten)	External Laboratory I (ppm gluten)
Millet flour	16 ± 2	15
Quinoa	76 ± 24	40
Maize flour	35 ± 20	Negative
Rice flour	< LOD	Negative
Chamtor sample	36 ± 25	Negative
Almond flour	116 ± 65	Negative
Liquid sample A	12 ± 1	Negative
Liquid sample B	30 ± 13	Positive
Liquid sample C	1 ± 0.6	Positive
Liquid sample G	35 ± 7	Positive
Liquid sample H	16 ± 5	Positive

Table 2: Analysis of liquid hydrolysed samples by the Gli 1 based competitive method and comparison with the values provided by the external laboratory.

Sample	Competitive Gli 1 (ppm gluten)	External Laboratory I
Wheat beer (Paulaner)	224 ± 5	Positive
Liquid sample D	46 ± 6	Dubious
Liquid sample E	92 ± 29	Positive
Spanish beer (Cruzcampo)	130 ± 27	Positive
Liquid sample F	130 ± 28	Positive
Daura Beer (gluten free)	113 ± 36	Negative

Table 3: Samples analysed with the assay using Gli 4 aptamer after two different extraction protocols and compared with R5 method carried out by two independent laboratories.

Sample	ethanol extraction (ppm gluten)	cocktail extraction (ppm gluten)	Lab II (ppm gluten)	Lab III (ppm gluten)
Cake	>100	>100	78	>80
Fixamyl	8 ± 3	30	7	13.3 ± 1.1
Capuccino	23 ± 3	50	<5	<5
Rolled oats	63 ± 9	97 ± 9	77	>80
Corn flakes 2	34	58 ± 4	38	18.1 ± 1.3
Dessert Chocolate	11	<LD	13	<5
Fit snack	81 ± 8	90	77	>80
Vanilla cream	<6	<LD	<5	<5
Corn flakes 1	13	34 ± 7	17	30.8 ± 1.9
Panna Cotta	6 ± 1	< LD	<5	<5

CAPTION FOR THE FIGURES

Figure 1: Schematic representation of the competitive aptamer-based assay for gliadin determination.

Figure 2: Binding curves of aptamers Gli 4 (•, dashed line) and Gli 1 (□, solid line) to 33-mer-peptide immobilized onto magnetic beads through biotin-estreptavidin interaction.

Figure 3: Calibration curves for the competitive assay: (A) using Gli 4 (•, dashed line) and Gli 1 (□, solid line) aptamers with PWG gliadin as a standard (B) using Gli 1 aptamer with PWG gliadin (□, solid line) and 33-mer peptide (•, dashed line) as standards.

Figure 4: Response of the competitive assay expressed as percentage of the maximum signal obtained in the absence of protein to different toxic prolamins from wheat (◆, red line), rye (■, blue line), barley (•, green line), and oats (□, black line) using (A) Gli 1 and (B) Gli 4 aptamers.

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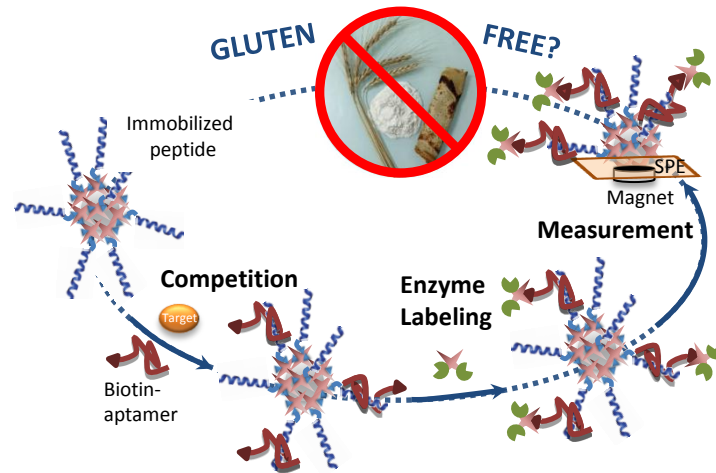
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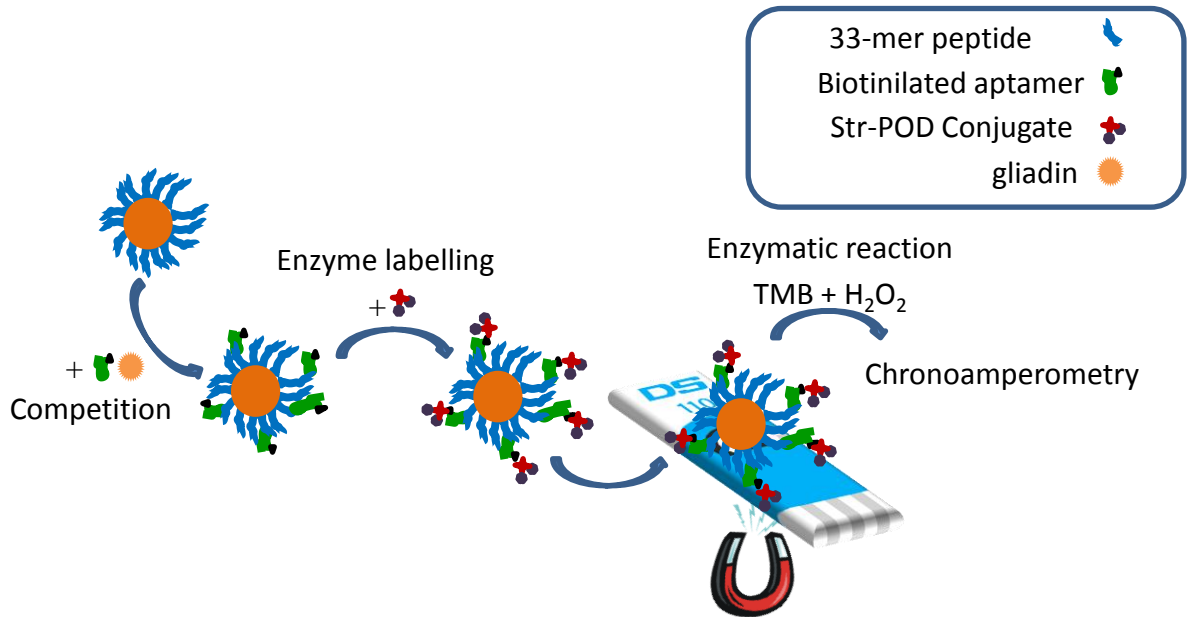


Figure 2

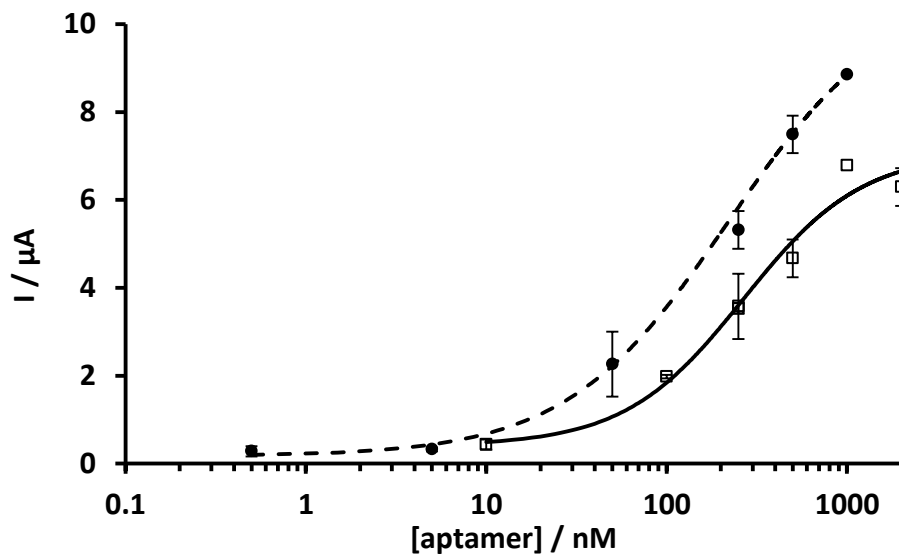


FIGURE 3

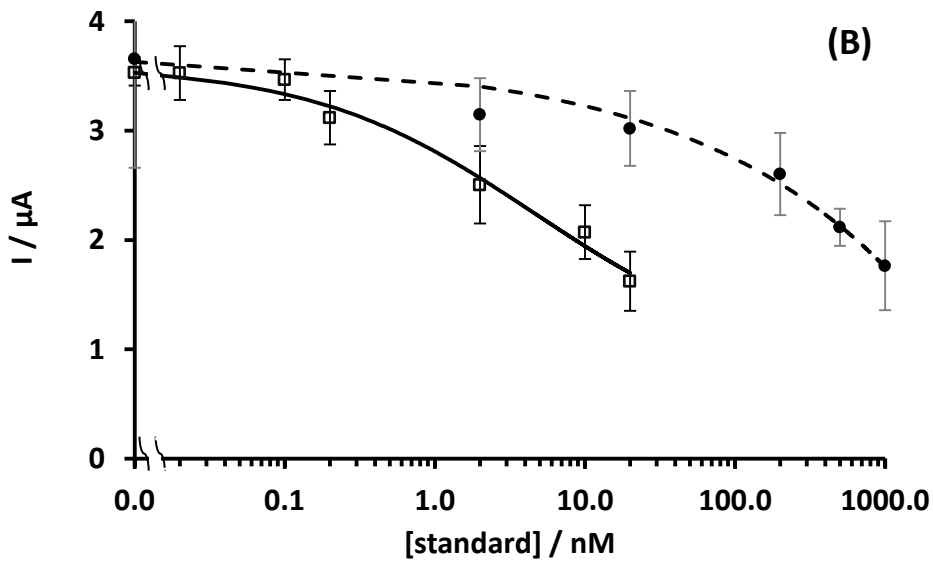
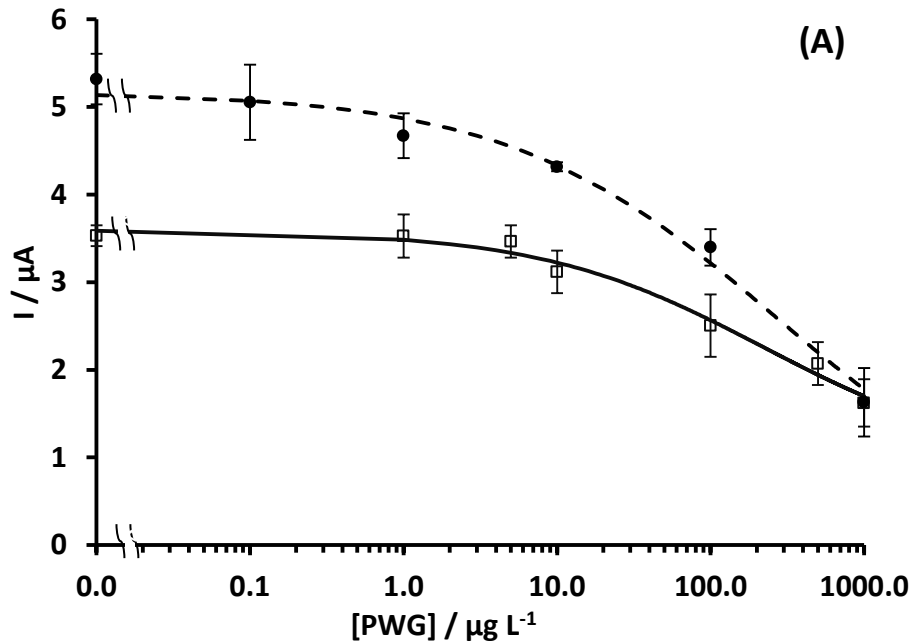


FIGURE 4

