

# A multiplex competitive ELISA for the detection and characterization of gluten in fermented-hydrolyzed foods

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**Abstract** A novel competitive ELISA was developed utilizing the G12, R5, 2D4, M10BS, and Skerritt antibody-HRP conjugates employed in nine commercial ELISA test kits that are routinely used for gluten detection. This novel multiplex competitive ELISA simultaneously measures gliadin-, deamidated gliadin-, and glutenin-specific epitopes. The assay was used to evaluate 20 wheat beers, 20 barley beers, 6 barley beers processed to reduce gluten, 15 soy sauces, 6 teriyaki sauces, 6 Worcestershire sauces, 6 vinegars, and 8 sourdough breads. For wheat beers, the apparent gluten concentration values obtained by the G12 and Skerritt antibodies were typically higher than those obtained using the R5 antibodies. The sourdough bread samples resulted in higher apparent gluten concentration values with the Skerritt antibody, while the values generated by the G12 and R5 antibodies were comparable. Although the soy-based sauces showed non-specific inhibition with the multiple R5 and G12 antibodies, their overall profile was distinguishable from the other categories of fermented foods. Cluster analysis of the apparent gluten concentration values obtained by the multiplex competitive ELISA, as well as the relative response of the nine gluten-

specific antibodies used in the assay to different gluten proteins/peptides, distinguishes among the different categories of fermented-hydrolyzed foods by recognizing the differences in the protein/peptide profiles characteristic of each product. This novel gluten-based multiplex competitive ELISA provides insight into the extent of proteolysis resulting from various fermentation processes, which is essential for accurate gluten quantification in fermented-hydrolyzed foods.

**Keywords** Gluten · Detection and quantification · Fermentation-hydrolysis · Competitive ELISA

## Introduction

Approximately 1 in 141 people in the USA have celiac disease (CD), an immune-mediated disorder triggered by the interaction of gluten (from wheat, rye, barley, and possibly oat) with the intestinal mucosa of sensitive individuals [1]. Wheat and related grains are commonly used in fermented-hydrolyzed foods such as beers, soy sauces, vinegars, and sourdough breads, where concurrent enzymatic hydrolysis/proteolysis occurs during the fermentation process. Enzymatic action during the fermentation process results in hydrolysis of gluten into peptides which may retain immunopathogenic activity [2–4]. As a result of the complexity of the hydrolytic process, even slight changes in the fermentation can lead to wide variation in the peptide profile. In 2013, the US Food and Drug Administration (FDA) issued the gluten-free final rule, which addressed the uncertainty in interpreting the results of current gluten test methods for fermented-hydrolyzed foods in terms of intact gluten. Further, in 2015, a rule regarding the use of gluten-free label for fermented, hydrolyzed, and distilled foods was proposed [5, 6]. Attempts to accurately quantify hydrolyzed gluten derived from 20 ppm (µg/mL) intact gluten

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in foods subjected to fermentation using routine analytical methods have failed [7, 8]. Further complicating the quantitative analysis of hydrolyzed gluten is the lack of appropriate standards that reflect the protein/peptide profiles characteristic of the various forms of fermentation. Any analytical method that is used to analyze multiple fermentation products must be able to distinguish between the different protein/peptide profiles to ensure accurate quantification.

Several studies have evaluated the effects of fermentation on the resulting peptides and on the detection and characterization of gluten proteins and peptides in both wheat and barley beers [9–17]. However, there are no reports that evaluate similar parameters for other categories of fermented-hydrolyzed foods such as sourdough breads and vinegars. A recent study indicated the inability of different commercial sandwich, competitive ELISAs and lateral flow devices to detect gluten in soy sauces [8]. Studies on sourdough breads have shown that certain strains of lactic acid bacteria used in sourdough fermentation can hydrolyze the gliadin fraction of wheat gluten under specific processing conditions [18–20]. However, there still is a gap in the literature regarding how enzymatic hydrolysis that occurs during the fermentation process affects the quantification of gluten and its immunopathogenicity.

Celiac disease, unlike food allergies, requires a single epitope for immunopathogenesis [21]. As such, sandwich ELISAs which require two epitopes cannot detect short peptides that do not have two antibody binding sites but are sufficiently long to contain a single immunopathogenic element [7, 15]. Competitive ELISAs based on the G12 and R5 antibodies are marketed for the detection of hydrolyzed gluten. However, these competitive ELISAs cannot distinguish between different fermented foods and while they target gliadins, they do not accurately detect glutenins, which also contain immunopathogenic sequences [7, 13, 16, 22–26]. Further, the pepsin/trypsin digest used as the calibrant in the R5 competitive ELISA does not represent the peptide profiles generated during fermentation processes [7].

A majority of the commercial gluten detection assays employ monoclonal antibodies such as Skerritt, R5, and G12, with the Morinaga Institutes of Biological Sciences, Inc. (MioBS) an exception. The Skerritt antibody was raised against wheat gliadin and has been shown to specifically recognize the high molecular weight (HMW) glutenins [7, 27, 28]. The R5 antibody was raised against rye secalin and strongly binds to the QQPFP, QQQFP, LQPFP, and QLPFP epitopes in  $\alpha$ -/ $\beta$ -,  $\omega$ -, and  $\gamma$ -gliadins [29, 30]. The G12 antibody was produced against the synthetic 33-mer of toxic  $\alpha$ 2-gliadin and recognizes the QPQLPY epitope [22, 23]. Both the R5 and G12 antibodies recognize gliadin [22, 23, 30] with the G12 recognizing more immunogenic peptides compared to the R5 [31].

In recent years, a transamidation reaction catalyzed by microbial transglutaminase has been utilized to improve wheat

gluten functionality for various applications [32, 33]. This enzyme has also been applied in an attempt to reduce the immunopathogenicity of gluten in fermented beverages produced with gluten-containing grains, such as beer [12, 34, 35]. Transglutaminase can also catalyze a deamidation reaction. Further, deamidation can occur non-enzymatically during the fermentation process. Deamidation of glutamine can generate T cell stimulatory gluten peptides, favoring the binding of gluten peptides to HLA-DQ2 and HLA DQ8 [36]. Deamidation has also been shown to drastically reduce the affinity of the Skerritt, G12, and the R5 antibodies towards gluten peptides and proteins [37]. Therefore, gluten detection methods employing these antibodies may underestimate the concentration of deamidated gluten that could be present in fermented foods. Recently a novel monoclonal antibody, which recognizes deamidated gliadin, has been generated by Pi Bioscientific Inc. [38, 39].

Utilizing multiple antibodies, which recognize different gluten epitopes (gliadin, deamidated gliadin, and glutenin) from established commercial gluten ELISA kits, we developed a novel multiplex competitive ELISA assay. The aim was to develop a method that can recognize the proteolytic pattern resulting from differences in the fermentation processes applied during the manufacture of different categories of fermented-hydrolyzed foods. The apparent gluten concentration profiles were generated for several different fermented-hydrolyzed foods. Only foods where concurrent enzymatic proteolysis/hydrolysis occurs during the fermentation process were analyzed. Cluster analysis distinguished among the different types of fermentation processes. This assay can be used to help identify appropriate calibration standards of comparable digestion and similar peptide composition, which is essential for accurate gluten quantification in fermented-hydrolyzed foods.

## Materials and methods

### Reagents

Wheat gluten (G5004), 10 mM PBS (phosphate buffered saline), Tween-20®, and other chemical reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). HRP-conjugated gluten-specific detector antibodies from commercial ELISA test kits, as listed in Table 1, were used in the competitive ELISA (Table 1). Fermented-hydrolyzed food samples were purchased from local grocery stores or online.

### ELISA standard and sample preparation

Intact wheat gluten was used as a standard in the multiplex competitive ELISA as previously described [7, 15].

**Table 1** HRP-conjugated detector antibodies from commercial ELISA kits used in the multiplex competitive ELISA

| Antibody                   | Type <sup>b</sup> | Target                    | ELISA kits <sup>d</sup>             | Manufacturer                                    | Dilution <sup>e</sup> |
|----------------------------|-------------------|---------------------------|-------------------------------------|-------------------------------------------------|-----------------------|
| G12                        | MAB               | QPQLPY                    | GlutenTox ELISA Competitive G12     | Biomedal Diagnostics                            | No dilution           |
| G12                        | MAB               |                           | AgraQuant Gluten G12                | Romer Labs                                      | 1 to 3                |
| R5                         | MAB               | QQQFP, QQQFP, LQFP, QLPFP | RIDASCREEN Gliadin                  | R-BioPharm, AG                                  | 1 to 33               |
| R5                         | MAB               |                           | RIDASCREEN Gliadin Competitive      | R-BioPharm, AG                                  | 1 to 33               |
| R5                         | MAB               |                           | Veratox for Gliadin R5 (Cat # 8510) | Neogen Corp.                                    | 1 to 25               |
| USDA Antibody <sup>a</sup> | MAB               | Gluten                    | Veratox for Gliadin (Cat # 8480)    | Neogen Corp.                                    | 1 to 20               |
| MIoBS                      | PAb               | Gliadin                   | Wheat/Gluten (Gliadin) MIoBS        | Morinaga Institute of Biological Sciences, Inc. | 1 to 2                |
| Skerritt                   | MAB               | HMW <sup>c</sup> glutenin | AllerTek Gluten                     | ELISA Technologies Inc.                         | 1 to 2                |
| 2D4                        | MAB               | Deamidated gliadin        | Microbiologique Gluten Sandwich     | Pi Bioscientific Inc.                           | 1 to 40               |

<sup>a</sup> Gluten-specific monoclonal antibody developed and licensed by the US Department of Agriculture (USDA)<sup>b</sup> MAB monoclonal antibody, PAb polyclonal antibody<sup>c</sup> High molecular weight<sup>d</sup> Name of the ELISA test kits<sup>e</sup> Dilution was done on a volume/volume basis using ultrapure water

Specifically, gluten at 10,000 µg/mL was mixed in PBST (PBS with 0.1% Tween-20) for 2 h at room temperature. The resulting solution was diluted into UD buffer (105 mM sodium phosphate, 75 mM NaCl, 2.5% Difco skim milk powder, 0.05% Tween 20, pH 7.4) to generate 100, 33, 11, 3.7, 1.2, 0.41, and 0.13 µg/mL gluten standards.

Fermented-hydrolyzed food samples included 20 wheat beers, 20 barley beers, and 6 barley beers that were processed to reduce their gluten content (referred to as the gluten-reduced beers), 15 soy sauces, 6 teriyaki sauces, 6 Worcestershire sauces, 6 vinegars, and 8 sourdough breads. Two gluten-free beers and two gluten-free sourdough bread samples were also included in the analysis. Liquid samples were diluted to at least two different concentrations (1:2, 1:50, or 1:500) using UD buffer prior to analysis. The sourdough bread samples were ground using an IKA tube mill to a fine powder and extracted using UD buffer (1:20 w/v) at room temperature for 2 h. The sourdough extracts were then centrifuged at 3000 g for 20 min and subsequently diluted with UD buffer (1:50 and 1:500) for analysis using the multiplex competitive ELISA.

### Multiplex competitive ELISA

Microtiter plates (Nunc-Immuno™ MicroWell™ Maxisorp™ 96-well polystyrene plates, Sigma # 442404) were coated with intact gluten diluted in 0.2 M carbonate-bicarbonate buffer, pH 9.4 (Fisher). A coating antigen concentration of 10 µg/mL gluten was found to be optimum after evaluating different concentrations and was used to coat the plates for all antibodies. For the Skerritt antibody, a 25 µg/mL coating antigen concentration was also used. After coating, the plates were incubated

overnight at 4 °C. The plates were then washed four times using a wash buffer (PBS containing 0.5% Tween 20). The wells were then blocked with 300 µL/well of 5% Difco skim milk powder in PBST, at 37 °C for 1 h. The plates were then washed four times with wash buffer before adding 50 µL of UD buffer diluted gluten standards or UD buffer diluted hydrolyzed samples. Afterwards 50 µL of each of the diluted nine antibody–HRP conjugates (Table 1) were added to the standards and samples, mixed and incubated at 37 °C. Antibodies were diluted using ultrapure water as indicated in Table 1. The GlutenTox G12 antibody was used as supplied. Dilution using PBS in place of water had negligible effect on the RIDASCREEN Gliadin R5 and the Microbiologique gluten antibodies. No effect was observed on the other diluted antibody–HRP conjugates in the ELISA. Wells with only UD buffer and each of the nine antibodies were included as controls to assess the direct binding signal of the antibodies to the absorbed gluten. The plates were then washed four times with the wash buffer before adding 100 µL of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate to each well. The plates were then incubated for 30 min at room temperature in the dark and the reaction was stopped by adding 100 µL of 0.16 M sulfuric acid to each well. The optical density (absorbance) of each well was measured using a Tecan M200 microplate reader at 450 nm.

### Western blot analysis

Proteins of six different soy sauces samples were separated by SDS-PAGE and western blot analysis was performed according to a previously described protocol [13]. HRP-conjugated

detector antibodies from the MIOBS Gliadin, ELISA Systems Gluten, Veratox for Gliadin R5 8510, and Veratox for Gliadin 8480 ELISA kits were used in the western blot. The MIOBS antibody was diluted 1:10 v/v and the other three antibodies were diluted 1:100 v/v with the blocking buffer for analysis. A Syngene G: Box Chemi XX9 imager (Syngene, Frederick, MD) was used to measure the chemiluminescence. Gluten standards were prepared by mixing extensively finely ground gluten in PBST at 10,000  $\mu\text{g/mL}$  and allowing it to equilibrate for 2 h at room temperature. The resulting solution was diluted with PBST to obtain 100 and 2.5  $\mu\text{g/mL}$  gluten standards, which were used in the western blots alongside the soy sauce samples. The gluten standards were extensively mixed immediately prior to transfer using wide bore pipette tips.

### Statistical analysis

Competitive ELISA standard curves were obtained for each individual antibody by plotting the measured absorbance values against the gluten concentration ( $\mu\text{g/mL}$ ) and fitting a four-parameter logistic model using the GraphPad Prism software. The normalized absorbance signal was also plotted against the gluten concentration. The upper and lower limits of the dynamic range for each standard curve were determined by calculating the onset of a saturation response, as evidenced by a 25% deviation from an exponential correlation between the gluten concentration and the absorbance response. The slopes of the standard curves were determined from the upper to the lower limits of the dynamic ranges of the standard curves. Assay sensitivity was determined by calculating the concentration of the analyte producing a 50% inhibition of maximum absorbance response (IC<sub>50</sub>). The apparent gluten concentration values ( $\mu\text{g/mL}$ ) of the hydrolyzed samples were estimated from the developed standard curves using the Graph Pad Prism software. Duplicate ELISA plates were analyzed each day. For some hydrolyzed samples, a single estimate was obtained with some of the antibodies as the ELISA absorbance values were out of the range of the standard curves.

Cluster analysis was performed on the apparent gluten concentration values of each sample obtained by the multiplex ELISA. Further, analysis was also conducted on the relative response of the nine antibodies used in the assay to different gluten proteins/peptides by comparing the responses obtained by each antibody to that of either the RIDASCREEN Gliadin R5 or the GlutenTox G12 antibody. Hierarchical clustering was performed using a Pearson correlation coefficient as a distance measure and the complete clustering method. All calculations were done in “R”. The R package pvclust was used to assess the uncertainty in hierarchical cluster analysis. In complete linkage, hierarchical clustering, the distance between two clusters is defined as the longest distance between

two points in each cluster. This method helps to prevent linking clusters together simply because one of their points is within close proximity to a point from a separate cluster. In complete linkage, outlying cases prevent close clusters from merging together because the measure of the furthest neighbor exacerbates the effects of outlying data. For assessing the uncertainty in the analysis, for each cluster in hierarchical clustering, quantities called *p* values are calculated via multiscale bootstrap resampling. The *p* value of a cluster is a value between 0 and 1, which indicates how strong the cluster is supported by data. The R package pvclust provides two types of *p* values: AU (approximately unbiased) *p* value and BP (bootstrap probability) *p* value. The AU *p* value, which is computed by multiscale bootstrap resampling, is a better approximation to unbiased *p* value than BP value computed by normal bootstrap resampling. For a cluster with AU *p* value greater than 0.95, the hypothesis that “the cluster does not exist” is rejected with significance level 0.05. This indicates that “the cluster does not exist” finding is caused by sampling error, but may be stably observed if we increase the number of observations. The calculated *p* values and the complete clustering method reduce the chances of concluding that clusters exist when in fact they do not [40].

## Results

### Competitive ELISA

Figure 1a shows the calibration/standard curves of the developed multiplex competitive ELISA for each of the nine antibodies plotted as normalized absorbance signal intensity versus gluten concentration. The standard curves of the three different R5 antibodies (from the RIDASCREEN Gliadin, RIDASCREEN Gliadin competitive, and the Veratox 8510 ELISA kits) were plotted separately for better comparison (Fig. 1b). Similarly, the two different G12 antibodies (from the AgraQuant Gluten and the GlutenTox Gluten ELISA kits) were plotted together (Fig. 1c). Results for the Skerritt antibody (from the AllerTek Gluten ELISA kit) at two different coating concentrations (10 and 25  $\mu\text{g/mL}$ ) were also plotted together (Fig. 1d). The plots of the gluten concentration versus absorbance values have been provided in the Electronic Supplementary Material (ESM) Fig. S1A–D. The standard curves had negative slopes typical of the competitive binding ELISAs (higher antigen concentration in the standard yield lower absorbance). Table 2 summarizes the dynamic ranges of the developed standard curves for each antibody, their respective slopes, and IC<sub>50</sub> values. The lower limit of quantification (LOQ) was in the range of 2.6–2.9  $\mu\text{g/mL}$  for all the antibodies except for the Skerritt (0.85–0.95  $\mu\text{g/mL}$ ) and the MIOBS (1.0  $\mu\text{g/mL}$ ), which were more sensitive. The standard curves of the two G12 antibodies had very different slopes at

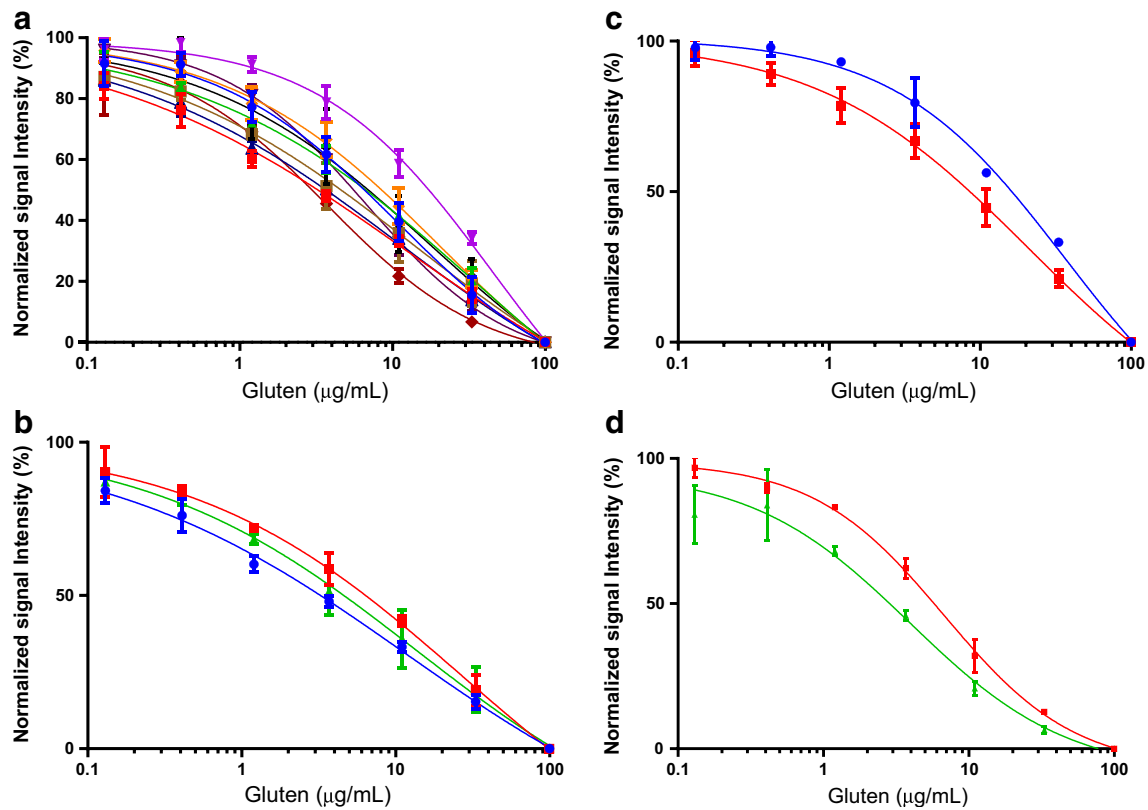


**Fig. 1** Competitive ELISA standard curves developed using the detector antibodies: **a** use of the Morinaga Institute of Biological Sciences, Inc. (MIoBS) Gliadin (●), Microbiologique Gluten (▲), RIDASCREEN Gliadin (■), RIDASCREEN Gliadin Competitive (▼), Neogen Veratox for Gliadin R5 (■), Veratox for Gliadin, cat # 8480 (●), GlutenTox ELISA Competitive G12 (◆), AgraQuant Gluten G12 (▼), and AllerTek Gluten (▼) and (◆) (10 μg/mL and 25 μg/mL, respectively coating antigen concentration). **b** Comparison of the standard curves of the detector antibodies of the RIDASCREEN Gliadin (●), RIDASCREEN Gliadin Competitive

(■), and the Veratox for Gliadin R5(▼) ELISA kits. **c** Comparison of the standard curves of the detector antibodies of the AgraQuant Gluten G12 (●) and the GlutenTox ELISA Competitive G12 (■) ELISA kits. **d** Comparison of the standard curves of the detector antibody of the AllerTek Gluten ELISA kit at 10 μg/mL (▼) and 25 μg/mL (■) coating concentrations. Data represents gluten concentration (μg/mL) plotted against the normalized signal intensities. Each data point represents the average of triplicate analysis conducted on three different days. Error bars indicate ± one standard deviation

their respective dynamic ranges. Out of the three R5 antibodies, the standard curve at the dynamic range for the RIDASCREEN Gliadin competitive antibody had a different slope than that of the other two R5 antibodies (RIDASCREEN Gliadin and the Veratox 8510). The slopes of the standard curves at the dynamic ranges for the Skerritt antibodies at two different coating concentrations were similar. The Skerritt antibody, which preferentially binds to HMW glutenins, displayed a slope comparable to the MIoBS polyclonal, which recognizes HMW glutenins in addition to gliadins ([13], Table 2). The differences in the slopes of the standard curves among the antibodies of the same classes can be attributed to the differences in the level of conjugation and the concentrations of antibodies that were provided in the ELISA kits. For the purpose of this assay, the apparent gluten concentration values were calculated from the standard curves of the different antibodies utilizing an intact gluten standard and

were compared, to distinguish among the different fermented-hydrolyzed foods.

### Analysis of beers

Both wheat (WA–WT) and barley beers (A–T) were analyzed by the multiplex competitive ELISA. In addition, six gluten-reduced barley beers (RA–RF) and two gluten-free beers (FA and FB) were included in the analysis (Table 3). The wheat beers included the Belgian style *witbiers* (WA, WB, WD, WG, WM, WN WR, and WS), the German-style *hefeweizens* (WC, WE, WH, WJ, WK, WL, and WT), and the ale-style beers (WF, WI, WO, WP, and WQ). The ABV (alcohol by volume) of the wheat beers ranged from 4.2 to 6%. The barley beers included both the lager (B–J and Q) and the ale type of beer (A, K–P and R–T) with ABV values ranging from 4.2 to 9%.

**Table 2** Analytical parameters of the multiplex competitive ELISA standard curves

| Antibody code | Antibody sources               | Antibodies                  | Dynamic range       |                     | Slope <sup>c</sup> | IC50 (µg/mL) <sup>d</sup> |
|---------------|--------------------------------|-----------------------------|---------------------|---------------------|--------------------|---------------------------|
|               |                                |                             | Lower limit (µg/mL) | Upper limit (µg/mL) |                    |                           |
| Ab 1          | AgraQuant Gluten               | G12                         | 2.9 ± 0.3           | 45 ± 2 <sup>b</sup> | 20.3 ± 3.5         | 45 ± 20                   |
| Ab 2          | GlutenTox Gluten               | G12                         | 2.8 ± 0.3           | ≥ 100               | 3.4 ± 0.1          | 21 ± 8.0                  |
| Ab 3          | RIDASCREEN Gliadin             | R5                          | 2.9 ± 0.3           | ≥ 100               | 7.4 ± 0.7          | 12 ± 5.0                  |
| Ab 4          | RIDASCREEN Gliadin Competitive | R5                          | 2.9 ± 0.4           | ≥ 100               | 2.6 ± 0.3          | 36 ± 19                   |
| Ab 5          | Veratoxfor Gliadin 8510        | R5                          | 2.8 ± 0.2           | ≥ 100               | 6.6 ± 0.6          | 14 ± 8.0                  |
| Ab 6          | Veratoxfor Gliadin 8480        | USDA Antibody <sup>a</sup>  | 2.6 ± 0.1           | ≥ 100               | 7.0 ± 0.5          | 18 ± 13                   |
| Ab 7          | Microbiologique Gluten         | 2D4                         | 2.8 ± 0.2           | ≥ 100               | 5.9 ± 0.3          | 11 ± 3.6                  |
| Ab 8          | MloBS Gliadin                  | Polyclonal                  | 1.0 ± 0.1           | ≥ 100               | 2.2 ± 0.2          | 11 ± 3.0                  |
| Ab 9          | AllerTek Gluten                | Skerritt (10 µg/mL coating) | 0.85 ± 0.04         | 38 ± 2              | 2.1 ± 0.1          | 3.8 ± 0.7                 |
| Ab 10         | AllerTek Gluten                | Skerritt (25 µg/mL coating) | 0.95 ± 0.06         | 43 ± 5              | 1.6 ± 0.1          | 6.7 ± 0.5                 |

Data presented as the average ± standard deviation

<sup>a</sup> Gluten-specific monoclonal antibody developed and licensed by the US Department of Agriculture (USDA)

<sup>b</sup> Upper limit of the dynamic range was only calculated for the standard curves where the upper limit was less than 100 µg/mL, which is the highest gluten concentration used to develop the standard curves

<sup>c</sup> The standard curve slopes were negative typical of competitive binding ELISAs. Slopes were calculated from the upper limit of quantification (LOQ) to the lower LOQ of the standard curves

<sup>d</sup> Concentration of analyte producing a 50% inhibition of maximum signal

The gluten-reduced barley beers were from both the lager (RE and RF) and ale type (RA–RD) with ABV ranging from 4.6 to 7.7%. One of the two gluten-free beers (FB) was made from sorghum with the other (FA) made from cereals such as millet, buckwheat, corn, and rice.

Table 3 shows the apparent gluten concentration values (µg/mL) of the wheat, barley, gluten-reduced barley, and the gluten-free beers obtained with the nine gluten-specific antibodies used in the multiplex ELISA. As expected, for individual beer samples, the apparent gluten concentration values with both the G12 antibodies (Ab 1 and 2) were similar to each other with only a few exceptions. Similarity was also observed between the two RIDASCREEN R5 antibodies (Ab 3 and 4) and also between the two different coating concentrations of the Skerritt antibody (Ab 9 and 10). The overall apparent gluten concentration values of the wheat beers (30–65,090 µg/mL) were higher compared to the barley beers (4–7520 µg/mL). In the case of wheat beers, the apparent gluten concentration values obtained by both the G12 antibodies (Ab 1 and 2) and the Skerritt antibody (Ab 9 and 10) were higher compared to those obtained by the other gliadin detectors (Ab 3–7) and MloBS (Ab 8). Exceptions were found, where four *hefeweizens* (WE, WH, WK, and WL) also showed higher values with some of the other gliadin detectors. Similarly, two ale beers (WF and WI)

showed comparatively lower apparent gluten concentration values with the Skerritt antibody. In contrast, the barley beers showed lower apparent gluten concentration values with the G12 antibodies (Ab 1 and 2) compared to some of the other gliadin detectors (Ab 3–7), MloBS (Ab 8), and the Skerritt antibody (Ab 9 and 10). Exceptions were found in three ale beers (M, O, and S), which showed comparatively higher apparent gluten concentration values with the G12 antibodies.

As expected, the gluten-reduced barley beers (RA–RF) showed lower apparent gluten concentration values compared to the regular barley beers (≤ 850 µg/mL). Very low values were observed with the G12 (Ab 1 and 2), MloBS (Ab 8), and the RIDASCREEN R5 antibodies (Ab 3 and 4). A moderate value was observed with the deamidated gliadin-specific antibody (Ab 7). The apparent gluten concentration values for beers RA, RC, RD, and RE, obtained with both the Veratox antibodies (Ab 5 and 6), were higher compared to the other two gluten-reduced barley beers (RB and RF). The gluten-reduced barley beers also showed high apparent gluten concentration values with the Skerritt antibody (Ab 9 and 10). The two gluten-free beers resulted in very low apparent gluten concentration values with all antibodies (≤ 13 µg/mL), except for the two Veratox antibodies (Ab 5 and 6) and the deamidated gliadin-specific antibody (Ab 7) where moderate values were observed (20–80 µg/mL). Nevertheless, the



values obtained with these three antibodies, for the gluten-free beers, were lower than the values of all the other beers analyzed.

### Analysis of soy-based sauces, vinegars, and sourdough breads by ELISA

In addition to the beer samples, other fermented-hydrolyzed foods such as soy sauces (SA–SX), teriyaki sauces (TA–TH), Worcestershire sauces (WoA–WoF), vinegars (VA–VF), and sourdough breads (BA–BH) were also analyzed by the multiplex competitive ELISA (Table 4). The soy sauces included regular (SA, SB, SD, SE, SG, SI, ST), gluten-free (SC, SF, SX), and low-sodium (SH, SJ, SS, SU, SV, SX) varieties. Out of the six teriyaki sauces, two were gluten-free (TC and TH) and one had less sodium in it (TD). Four of the Worcestershire sauces (WoC, WoD, WoE, WoF) did not have wheat listed as an ingredient in them, whereas the other two (WoA and WoB) had wheat in the ingredient lists. Vinegars VA–VE had malt, barley, or grain listed as ingredients. Vinegar VF was gluten-free. The sourdough breads had wheat flour (BE, BH), wheat flour and malted barley flour (BA, BB, BC, BD), wheat flour and rye flour (BF), or only rye flour (BG) in their ingredient lists. Two gluten-free sourdough breads (BI and BJ), made from gluten-free cereals such as rice, sorghum, and millets, were also included in the analysis.

Similar to the beer samples, the apparent gluten concentration profiles for individual soy-based sauces, vinegars, and sourdough breads, as shown in Table 4, were similar between the two G12 antibodies (Ab 1 and 2), between the two RIDASCREEN R5 antibodies (Ab 3 and 4), and also between the two different coating concentrations of the Skerritt antibody (Ab 9 and 10); exceptions were found only in a few samples. The soy sauces (20–100,010  $\mu\text{g/mL}$ ), teriyaki sauces (13–9920  $\mu\text{g/mL}$ ), Worcestershire sauces (20–1170  $\mu\text{g/mL}$ ), and vinegars (10–1070  $\mu\text{g/mL}$ ) resulted in a wide range of apparent gluten concentrations. Very high values were observed with both of the Veratox antibodies (Ab 5 and 6) and the deamidated gliadin-specific antibodies (Ab 7), particularly in case of the soy sauces and the teriyaki sauces. With both these sauces, the apparent gluten concentration values obtained with the Skerritt (Ab 9 and 10) and the MIOBS (Ab 8) antibodies were typically lower than that of the gliadin detectors (Ab 1–7). No differences in the profiles were observed among the gluten-free, regular, or the reduced sodium categories of sauces. One (WoA) of the two Worcestershire sauces, which has wheat in the ingredient list, resulted in higher apparent gluten concentration values with both the Veratox antibodies (Ab 5 and 6) and the deamidated gliadin-specific antibody (Ab 7)

compared to the other antibodies. Worcestershire sauce WoB resulted in higher apparent gluten concentration values with the Veratox 8510 (Ab 5) and the deamidated gliadin (Ab 7) antibodies and comparatively lower values with all other antibodies. The Worcestershire sauces without wheat in their ingredient lists (WoC, WoD, WoE, and WoF) showed lower apparent gluten concentration values with all antibodies compared to the Worcestershire sauces that had wheat in their ingredient list (WoA and WoB). The gluten concentration values obtained with the five grain-containing vinegars were similar to that of the gluten-free vinegar.

The soy sauces showed high apparent gluten concentration values with both the Veratox antibodies (Ab 5 and 6) and the deamidated gliadin-specific antibody (Ab 7). In order to investigate whether these antibodies can recognize any antigenic protein in the soy sauces, western blot was performed (ESM Fig. S2B–D). Samples included the regular (SD, SE), gluten-free (SC, SF, SX) as well as the reduced sodium (SS, SX) categories of the soy sauces. MIOBS antibody, which showed comparatively lower apparent gluten concentration values with all the soy sauces, was also included in the western blot analysis (ESM Fig. S2A). Gluten solutions at 100 and 2.5  $\mu\text{g/mL}$  were included along with the samples in the western blot as reference standards. As observed by Cao et al. [8], preferential binding to protein bands corresponding to the gliadin fraction of gluten in the reference lanes (ESM Fig. S2A, lanes 1 and 3) was observed with the MIOBS antibody. The two Veratox and the deamidated gliadin-specific antibodies also resulted in binding to protein bands corresponding to the gliadin fraction of gluten (ESM Fig. S2B–D, lanes 1 and 3). No binding was observed with any of the soy sauce samples that were run alongside the gluten reference standards in the western blot.

The apparent gluten concentration values of the sourdough breads (BA–BH) were in the range of 160–10,780  $\mu\text{g/mL}$  and the profile was vastly different from the soy-based sauces and beers (Table 4). In the case of four sourdough breads (BA, BB, BF, and BH), the values obtained were nearly identical for all the gliadin detectors (Ab 1–7) and the MIOBS (Ab 8) antibody, with only slight variations. Two other bread samples (BC, BD) resulted in lower values with the MIOBS (Ab 8), the two Veratox (Ab 5 and 6), and the deamidated gliadin detector (Ab 7) antibodies compared to the other gliadin detectors (Ab 1–4). Sourdough breads BE and BG showed lower apparent gluten concentration values with the MIOBS (Ab 8) antibody compared to all other gliadin detectors (Ab 1–7). All the sourdough breads resulted in comparatively higher apparent gluten concentration values with the Skerritt antibody (Ab 9 and 10). The apparent gluten concentration values obtained with the two gluten-free sourdough breads (BI and BJ) were

**Table 4** Apparent gluten concentration ( $\mu\text{g/mL}$ ) profiles of the soy-based sauces (A) and sourdough breads (B) as analyzed by the multiplex competitive ELISA

| Ab <sup>a</sup> |      |                 |      |       |      |       |      |       |      |       |       |       |      |       |      |       |      |       |       |       |       |    |
|-----------------|------|-----------------|------|-------|------|-------|------|-------|------|-------|-------|-------|------|-------|------|-------|------|-------|-------|-------|-------|----|
| A               | Ab 1 |                 | Ab 2 |       | Ab 3 |       | Ab 4 |       | Ab 5 |       | Ab 6  |       | Ab 7 |       | Ab 8 |       | Ab 9 |       | Ab 10 |       |       |    |
|                 | Mean | Rng/2           | Mean | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean  | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean  | Rng/2 |       |    |
|                 | SA   | 590             | 80   | 600   | 120  | 790   | 70   | 750   | 20   | 8300  | NE    | 4250  | 1500 | 4940  | 710  | 20    | 0.2  | 110   | 30    | 150   |       | 60 |
| SB              | 780  | 200             | 800  | 2     | 500  | 10    | 1520 | 120   | 5680 | NE    | 1370  | 180   | 3400 | 740   | 20   | 10    | 60   | 10    | 90    | 10    | 1100  |    |
| SC              | 1400 | 30              | 840  | 20    | 1350 | 140   | 300  | 140   | 3020 | 370   | 2970  | NE    | 2630 | NE    | 40   | 10    | 100  | 3     | 110   | 10    | 2200  |    |
| SD              | 1490 | 170             | 940  | 120   | 1580 | 280   | 640  | 130   | 2620 | 720   | 9090  | 1440  | 2100 | NE    | 30   | 10    | 80   | 7     | 100   | 0.4   | 3300  |    |
| SE              | 1900 | 310             | 1710 | 60    | 3240 | 200   | 1240 | 360   | 4640 | 1500  | 7100  | 60    | 3570 | 170   | 150  | 20    | 240  | NE    | 280   | NE    | 4400  |    |
| SF              | 1350 | 160             | 880  | 160   | 1540 | 300   | 770  | 20    | 1500 | 240   | 3320  | 260   | 1800 | NE    | 90   | 20    | 60   | NE    | 70    | 4     | 5500  |    |
| SG              | 900  | 50              | 320  | 160   | 1250 | NE    | 550  | 90    | 6450 | 320   | 2900  | 1270  | 3790 | 300   | 100  | 10    | 190  | 20    | 240   | 3     | 6600  |    |
| SH              | 750  | 240             | 180  | 20    | 340  | NE    | 250  | 80    | 3410 | 300   | 3590  | NE    | 1760 | 420   | 120  | 2     | 160  | 20    | 160   | 13    | 7700  |    |
| SI              | 1490 | 290             | 390  | 40    | 1510 | NE    | 690  | NE    | 1370 | 20    | 8310  | NE    | 1360 | 110   | 110  | 20    | 130  | 20    | 150   | 1     | 8800  |    |
| SJ              | 2050 | 120             | 930  | 30    | 3230 | NE    | 1420 | NE    | 3840 | 1250  | 8700  | NE    | 2200 | 40    | 150  | 5     | 240  | 2     | 230   | 8     | 9900  |    |
| SS              | 1600 | 1110            | 970  | 110   | 2290 | 190   | 860  | 110   | 2100 | 800   | 10010 | 1700  | 2970 | 590   | 110  | 20    | 60   | NE    | 70    | 4     | 11000 |    |
| ST              | 570  | NE <sup>d</sup> | 550  | 20    | 1380 | 40    | 400  | 30    | 2370 | 880   | 9820  | 500   | 1460 | 680   | 40   | 2     | 100  | 3     | 110   | 10    |       |    |
| SU              | 560  | NE              | 430  | 40    | 1020 | 90    | 350  | 40    | 1810 | 610   | 8750  | 1500  | 1360 | 640   | 30   | 2     | 80   | 7     | 100   | 0.4   |       |    |
| SV              | 500  | 280             | 460  | 20    | 1110 | 310   | 330  | 110   | 630  | 60    | 4190  | 1380  | 1490 | 180   | 60   | 2     | 250  | NE    | 280   | NE    |       |    |
| SX              | 930  | 70              | 420  | 70    | 760  | 130   | 270  | 20    | 1430 | 150   | 5200  | 3200  | 820  | 50    | 110  | 3     | 150  | 5     | 170   | 3     |       |    |
| TA              | 300  | 12              | 1100 | 140   | 620  | 100   | 400  | 50    | 1130 | 400   | 3070  | 2080  | 1320 | NE    | 60   | 3     | 40   | 20    | 40    | 0.6   |       |    |
| TB              | 500  | 70              | 300  | 3     | 1060 | 60    | 160  | 10    | 4780 | 1130  | 9920  | 10    | 3690 | 110   | 20   | 2     | 130  | 10    | 20    | NE    |       |    |
| TC              | 300  | 2               | 220  | 40    | 180  | 60    | 190  | 3     | 2570 | 200   | 5220  | 430   | 1120 | 150   | 60   | 10    | 120  | 20    | 100   | 4     |       |    |
| TD              | 200  | 50              | 220  | 40    | 370  | 40    | 250  | 19    | 5090 | 700   | 6650  | NE    | 1720 | 280   | 230  | NE    | 140  | 10    | 110   | 6     |       |    |
| TE              | 630  | 160             | 260  | 20    | 1940 | 750   | 580  | 50    | 6990 | 150   | 1710  | NE    | 4290 | 100   | 13   | NE    | 80   | 10    | 80    | NE    |       |    |
| TH              | 230  | 90              | 200  | 20    | 420  | 60    | 180  | 20    | 4000 | 240   | 1270  | NE    | 2800 | 220   | 90   | 5     | 70   | 7     | 80    | 30    |       |    |
| WoA             | 230  | 5               | 370  | 10    | 230  | 30    | 130  | 30    | 1280 | 220   | 4120  | 1010  | 640  | 50    | 130  | 40    | 120  | 20    | 100   | 40    |       |    |
| WoB             | 70   | 3               | 60   | 3     | 20   | 2     | 40   | 10    | 1170 | NE    | 260   | 40    | 390  | 40    | 60   | 4     | 80   | 10    | 50    | NE    |       |    |
| WoC             | 120  | 4               | 20   | 6     | 40   | 2     | 30   | 2     | 220  | 20    | 370   | 100   | 70   | 30    | 110  | 3     | 100  | 3     | 100   | 0.5   |       |    |
| WoD             | 100  | 20              | 40   | 0.4   | 50   | 3     | 30   | 7     | 230  | 40    | 90    | 10    | 120  | 3     | 100  | 20    | 70   | 7     | 70    | 5     |       |    |
| WoE             | 120  | 40              | 20   | 0.4   | 70   | 0.2   | 60   | 0.9   | 40   | NE    | 130   | 20    | 210  | 10    | 110  | 20    | 70   | 4     | 70    | 6     |       |    |
| WoF             | 50   | 5               | 40   | 4     | 40   | 3     | 30   | 6     | 100  | 5     | 360   | 20    | 70   | 9     | 200  | 30    | 70   | 2     | 60    | 3     |       |    |
| VA              | 30   | 10              | 150  | 40    | 190  | 30    | 60   | 30    | 570  | 70    | 10    | NE    | 240  | 70    | 20   | 0.5   | 50   | 6     | 50    | 8     |       |    |
| VB              | 65   | 20              | 200  | 40    | 1780 | 30    | 380  | 8     | 1070 | 110   | 250   | 140   | 280  | 20    | 20   | 4     | 150  | 6     | 120   | 10    |       |    |
| VC              | 20   | 8               | 70   | NE    | 280  | 20    | 250  | 5     | 170  | 20    | 60    | NE    | 90   | 8     | 20   | 1     | 60   | 3     | 60    | 6     |       |    |
| VD              | 50   | NE              | 70   | NE    | 50   | 7     | 40   | 20    | 230  | 7     | 30    | 10    | 80   | 0.2   | 50   | 5     | 140  | 6     | 130   | 8     |       |    |
| VE              | 30   | 6               | 210  | 20    | 450  | 10    | 210  | 40    | 560  | 7     | 60    | NE    | 160  | 7     | 30   | 4     | 120  | 10    | 100   | 10    |       |    |
| VF              | 40   | NE              | 110  | 7     | 150  | 9     | 70   | 7     | 210  | 10    | 570   | 80    | 110  | 20    | 35   | 0.8   | 70   | 15    | 670   | 2     |       |    |

| B  | Ab 1            |       | Ab 2 |       | Ab 3 |       | Ab 4 |       | Ab 5 |       | Ab 6 |       | Ab 7  |       | Ab 8 |       | Ab 9  |       | Ab 10 |       |      |
|----|-----------------|-------|------|-------|------|-------|------|-------|------|-------|------|-------|-------|-------|------|-------|-------|-------|-------|-------|------|
|    | Mean            | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean | Rng/2 | Mean  | Rng/2 | Mean | Rng/2 | Mean  | Rng/2 | Mean  | Rng/2 |      |
|    | BA              | 1600  | 280  | 1380  | 40   | 660   | 220  | 1300  | 80   | 1450  | 130  | 550   | 160   | 1290  | 460  | 1770  | 50    | 5380  | 900   | 5330  |      |
| BB | 2430            | 260   | 1510 | 140   | 1210 | 180   | 1640 | 100   | 2730 | 110   | 2070 | 980   | 2150  | 360   | 1730 | 20    | 3890  | 170   | 5450  | 390   | 1100 |
| BC | 1330            | 60    | 1160 | 250   | 1050 | 350   | 1550 | 130   | 840  | 70    | 500  | 170   | 1140  | 80    | 340  | 50    | 3400  | 90    | 2920  | 70    | 2200 |
| BD | 1420            | 160   | 1390 | 440   | 1200 | 370   | 1590 | 3     | 790  | 70    | 600  | 60    | 890   | 650   | 570  | 70    | 3460  | 250   | 2870  | 160   | 3300 |
| BE | 2130            | 780   | 2040 | 40    | 2190 | 750   | 2420 | 580   | 2940 | 30    | 2570 | 1200  | 2740  | 100   | 570  | 10    | 3460  | 210   | 3460  | 210   | 4400 |
| BF | 3340            | 100   | 2810 | 60    | 3100 | 920   | 2940 | 360   | 4270 | NE    | 3080 | NE    | 3680  | NE    | 1450 | 100   | 10700 | 960   | 10780 | 460   | 5500 |
| BG | 3270            | 1400  | 2320 | 1080  | 4460 | 80    | 4740 | 110   | 3270 | NE    | 5060 | NE    | 6400  | 1710  | 160  | NE    | 6230  | 510   | 6050  | 540   | 6600 |
| BH | 1450            | 260   | 1730 | 460   | 960  | 180   | 1760 | 260   | 1530 | 340   | 1410 | 490   | 1300  | 90    | 1480 | 90    | 6870  | 20    | 4820  | 30    | 7700 |
| BI | ND <sup>e</sup> |       | 0.1  | NE    | ND   |       | 0.02 | NE    | 3    | 1     | 1.6  | NE    | 0.002 | 0.001 | ND   |       | ND    |       | ND    |       | 8800 |
| BJ | ND              |       | ND   |       | ND   |       | ND   |       | 5    | 0.1   | 3    | NE    | 0.02  | 0.01  | ND   |       | 0.1   | 0.0   | ND    |       | 9900 |

Values shown are obtained by rounding the actual apparent gluten concentration values

<sup>a</sup> Ab 1 to Ab 10 as described in Table 2

<sup>b</sup> Average  $\mu\text{g/mL}$  gluten from duplicate analysis

<sup>c</sup> Range over two values from duplicate analysis

<sup>d</sup> Not estimated (mean represents  $\mu\text{g/mL}$  gluten from a single observation)

<sup>e</sup> Not detectable (absorbance values above the background)

less than  $2.5 \mu\text{g/mL}$  for all the antibodies, except for the two Veratox antibodies (Ab 5 and 6), which resulted in at most  $5 \mu\text{g/mL}$  gluten (Table 4).

### Cluster analysis

Cluster analysis was performed on the apparent gluten concentration values of 87 fermented-hydrolyzed foods. Figure 2 displays the dendrogram with the AU and BP  $p$  values and the edge number. Two significant clusters appeared at edge numbers 84 and 85. These clusters distinguished the soy-based

sauces from the wheat beers and the sourdough breads. However, barley beers appeared in both these clusters. The cluster that appeared at edge number 84 was further subdivided into seven different significant clusters (Fig. 3a). All of the 20 wheat beers generated significant clusters separately from the sourdough breads (edge numbers 73, 57, and 68). Some of the wheat beers were found to be more closely related to each other than the others. Three of the wheat beers generated significant cluster with one barley beer (O) at edge number 68. Seven of the eight sourdough breads generated significant clusters separately from the wheat beers and barley

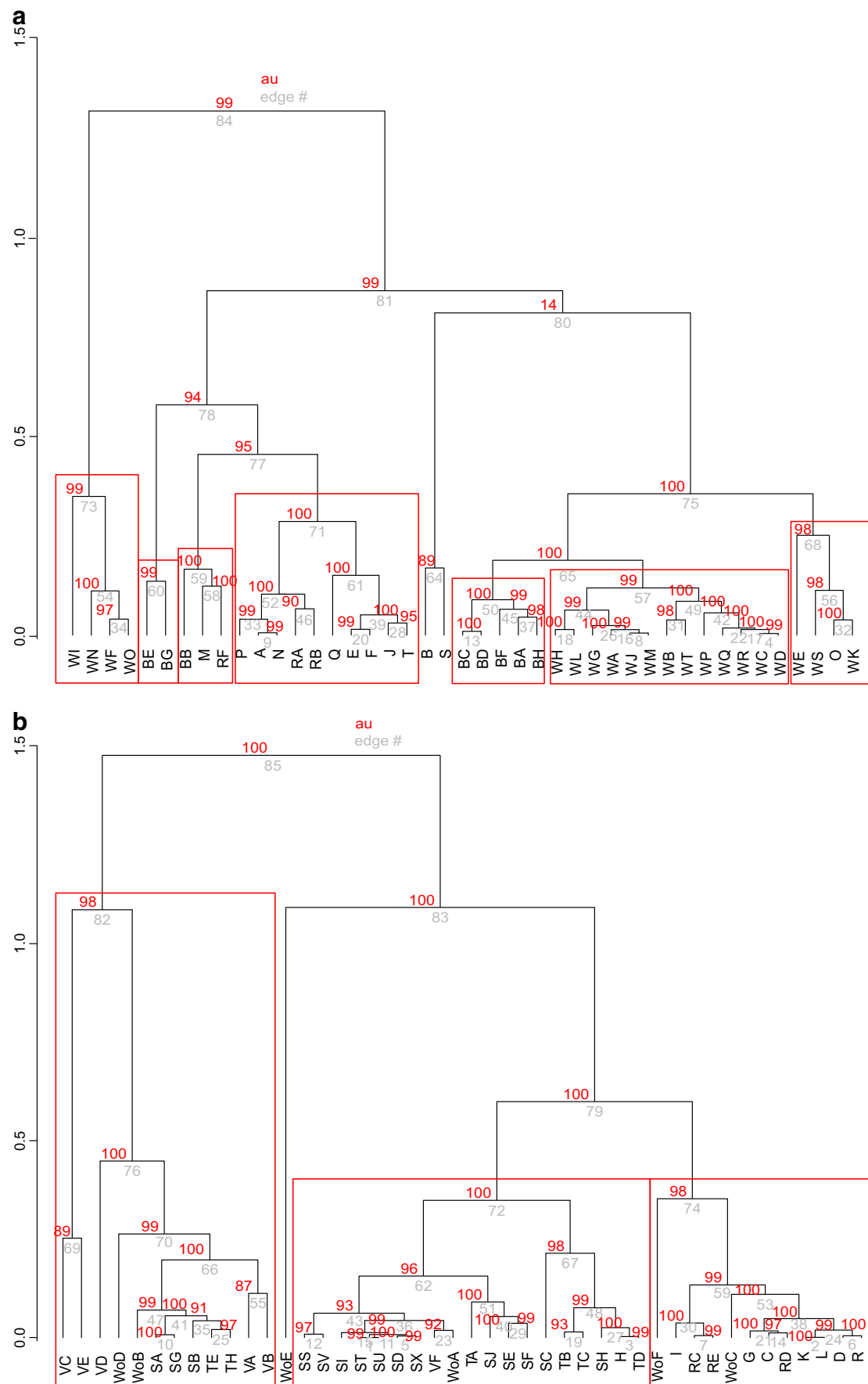
axis gives the distance (or dis-similarity measure) at which any two clusters are joined. Values on the edges of the clustering are  $p$  values (%). Red values are AU  $p$  values, green values are BP  $p$  values, and gray values are edge numbers. For a cluster with AU  $p$  value  $>0.95$ , the hypothesis that “the cluster does not exist” is rejected with significance level 0.05. Red boxes indicate significant clusters

other three gluten-reduced barley beers (RA, RB, and RF) brewed by three different companies (edge numbers 59 and 71, Fig. 3a). Similarly, two regular barley beers (C and D) brewed by the same company also clustered together (edge number 74, Fig. 3b).

Cluster analysis performed on the relative response of the nine gluten-specific antibodies used in the assay to different gluten proteins/peptides generated a clustering pattern that was identical to that observed in Fig. 2. This indicates that the different categories of the fermented-hydrolyzed foods are clustering on the basis not only of their apparent gluten concentration values obtained by the assay but also the relative response of different antibodies used in the assay (not shown).

## Discussion

A novel multiplex competitive ELISA was developed which simultaneously detects gliadin-, deamidated gliadin-, and glutenin-derived epitopes present in gluten proteins and peptides. Wheat gluten was used as a coating antigen as well as a calibrant in this study for consistency purposes as the material is commercially available, forms the regulatory basis for the analytical methods employed by the FDA (and several other governments) in assessing gluten content and potential health risks as well as keeping in mind the reported specificity of the



◀ **Fig. 3** Hierarchical cluster analysis dendrogram at edge number 84 (a) and edge number 85 (b) from Fig. 2. The vertical axis gives the distance (or dis-similarity measure) at which any two clusters are joined. Red values on the edges of clustering are AU *p* values (%) and gray values are edge numbers. For a cluster with AU *p* value >0.95, the hypothesis that “the cluster does not exist” is rejected with significance level 0.05. Red boxes indicate significant clusters

commercial gluten detection antibodies used in this assay. Although several research groups have studied hordein from barley and characterized the extracts, no barley hordein standard is available and variability between cultivars and methods of extraction/processing is significant making their utility somewhat problematic [41, 42]. Wheat gluten as a whole was used to avoid excluding any gluten protein fraction (gliadins or glutenins) from analysis. Further, specific antibodies were employed to detect different gluten fractions as well as any modified gliadin that may be present in a fermented food. Multiple gliadin-specific antibodies, such as both the G12 and R5 antibodies, were included in the multiplex ELISA to detect hydrolyzed peptides/proteins that are specifically recognized by these two classes of antibodies. Multiple R5 and G12 antibodies from different ELISA kit manufacturers were also included to confirm the profile pattern and to avoid any potential bias. The multiplex ELISA developed in this study was sensitive enough to detect gluten in various fermented-hydrolyzed foods, resulting in unique profiles of apparent gluten concentration. The profiles obtained highlight two important parameters measured by the assay. Firstly, the ratio of the different gluten peptides/proteins present in the different fermented foods, as illustrated by the relative response of the nine gluten-specific antibodies used in the assay and, secondly, the amount of those proteins/peptides present, measured as the apparent gluten concentration values.

The major difference between the wheat and barley beers was the high apparent gluten concentration values obtained by wheat beers with the G12 antibodies compared to the barley beers. High levels of 33-mer equivalent peptides, the peptides specifically recognized by the G12 antibodies, have been found in the wheat beers compared to the barley beers [12]. This may explain the high values of the wheat beers with G12 antibodies that we observed in our study. Since G12-reactive peptides in beer have been shown to be more immunogenic [31], our results indicate that the wheat beers may have higher immunogenic potential compared to the barley beers. Differences in the apparent gluten concentration profiles were identified in each category of beers. This is potentially attributable to different processing parameters applied during the manufacture of different beers. Examples of such parameters are the type of wheat (malting or unmalted) used for brewing a wheat beer, whether a filtration step was used during manufacture, as well as variations in the temperature and duration of fermentation. Further, different yeast strains used in brewing can lead to differences in the peptide profile of the beer and

thereby result in a difference in the apparent gluten concentration profile [43].

The overall apparent gluten concentration values of the two gluten-free beers were lower compared to all other beers analyzed. This was expected as these beers were made from non-gluten-containing grains such as sorghum, rice, corn, and millets. Higher apparent gluten concentration values were obtained for the gluten-reduced barley beers with the Skerritt antibody compared to the G12, M10BS, and the RIDASCREEN R5 antibodies. This is consistent with the western blot results from our previous study, where HMW glutenin, specifically recognized by the Skerritt antibody, was found to be more resistant to the effect of an enzyme used by some manufacturers to reduce the gluten level of beers [7]. Another mass spectrometry-based study also reported higher levels of peptides derived from D hordein (similar to HMW glutenin) in barley-based low-gluten beers, where the gluten level is reduced by proprietary processing steps during brewing [44]. The high apparent gluten concentration values obtained by some of the gluten-reduced barley beers with the Veratox antibodies and the moderate values obtained with the deamidated gliadin-specific antibodies by all gluten-reduced barley beers need further investigation. Specifically, since the gluten-free beers also resulted in moderate apparent gluten concentration values with these three antibodies, the specificity of these three antibodies to beer components other than gluten needs to be investigated.

In the case of the soy-based sauces and vinegars, the higher apparent gluten concentration values obtained by the multiplex ELISA can be attributed to non-specific inhibitory response. This is because the values from some of the gluten-free soy-based sauces and vinegars were indistinguishable from the gluten-containing counterparts. In addition, a recent study and our western blot results indicated the absence of specific interactions of antibodies employed in our ELISA with any antigenic elements that may be present in soy sauces [8]. Whether any antigenic protein survives the extensive hydrolysis process applied during manufacture of soy-based sauces is an important question that needs further research. However, that is beyond the scope of this manuscript. The non-specific responses by the soy-based sauces were observed even though UD buffer, a strong buffer, containing a blocking agent and a detergent, that has been shown to be useful in preventing false positive responses for problematic samples in antibody-based assays, was used for sample dilution [45, 46]. The non-specific inhibitory response of soy-based sauces in the multiplex ELISA can be attributed to the high salt concentrations usually found in soy-based sauces. However, the reduced sodium categories of soy-based sauces analyzed by the ELISA resulted in apparent gluten concentration values and overall profiles that were similar to the regular soy-based sauces. Therefore, it was concluded that in addition to high salt content, other soy sauce components such as sugar,

or other additives could be responsible for the non-specific responses observed. In competitive ELISA, any component in the analyzed product that prevents binding of the gluten-specific antibodies to the coated antigen results in a positive response. It is possible that high concentrations of salt, sugar, or some other additives present in soy-based sauces prevent binding of antibodies to the coated gluten, resulting in a false positive response. Further, it appears that the gliadin-specific antibodies are affected more by this non-specific inhibition than the others (MIoBS and Skerritt). Sourdough breads resulted in higher apparent gluten concentration values with the Skerritt antibody compared to the gliadin detectors. Studies have shown that the glutenin fraction of gluten is more resistant to hydrolysis by some strains of sourdough lactic acid bacteria compared to gliadin [19, 47]. Further, glutenin hydrolysis during sourdough fermentation has been shown to be dependent on pH. A pH of less than 4.00 during fermentation can activate the proteolytic cereal enzymes, thereby favoring digestion of HMW glutenin [48]. Therefore, depending on the strain of the lactic acid bacteria used and the pH during the fermentation of the analyzed sourdough breads, the HMW glutenin fractions may be degraded to a lesser extent compared to the gliadin during the fermentation of these bread samples. Variation in the apparent gluten concentration profiles obtained by the gliadin detectors among the different sourdough breads can be explained by the selection of flour type and other parameters such as the fermentation conditions, which affects the proteolytic activity of sourdoughs.

Cluster analysis performed on the apparent gluten concentration values of the samples obtained by the assay, as well as the relative responses of the different antibodies, distinguished among the different categories of fermented-hydrolyzed foods. The specificity of the different antibodies towards gliadins, glutenins, and deamidated-gliadin in this multiplex competitive ELISA means that the clusters reflect differences in antigenic peptide profiles. All of the wheat beers, sourdough breads, and the soy-based sauces resulted in significant clusters separately from each other. It is particularly noteworthy that the soy-based sauces clustered together separately from the sourdough breads and the wheat beers, in spite of showing non-specific interactions, thereby making the effects of these interactions negligible on the utility of the developed assay. Of the 26 barley beers analyzed, 25 clustered separately from wheat beers and 24 clustered separately from sourdough breads. Only one barley beer clustered with the majority of the soy-based sauces. Three gluten-reduced barley beers brewed by the same company clustered together separately from the other gluten-reduced barley beers. Similarly, two regular barley beers brewed by the same company also clustered together. This indicates that the developed assay also has the potential to differentiate the protein/peptide profile characteristics resulting from different processing methods applied by different beer manufacturers. Clustering of individual samples from

each category of fermented foods together in one group by having similar peptide/protein ratio, as well as the amount of those protein/peptides, indicates that the processes used by the manufacturers to produce those products are very much similar. Samples, particularly in case of barley beers, that clustered separately from others as a result of differences in both the peptide/protein ratio and the amount of those proteins/peptides, indicate differences in degree of digestion, which could be due to differences in the release of hordein from barley malt during mashing, the amount of hordein that enters fermentation, as well as other differences in fermentation processes (length of fermentation, temperature, pH, yeast strain used) applied by the manufacturers.

This novel multiplex assay is capable of classifying foods on the basis of type and degree of fermentation or gluten hydrolysis. In addition, by virtue of its clustering digested gluten-based on the protein/peptide profile, it can help in choosing appropriate hydrolyzed gluten standards of comparable digestion and similar peptide composition. The ability to choose appropriate standards, or at least to know how the use of a less than ideal standard may skew quantitation, should enable more accurate quantification of gluten content and aid in the identification of possible immunopathogenicity.

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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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**Analytical and Bioanalytical Chemistry**

**Electronic Supplementary Material**

**A multiplex competitive ELISA for the detection and characterization  
of gluten in fermented-hydrolyzed foods**

Rakhi Panda, Marc Boyer, Eric A.E. Garber

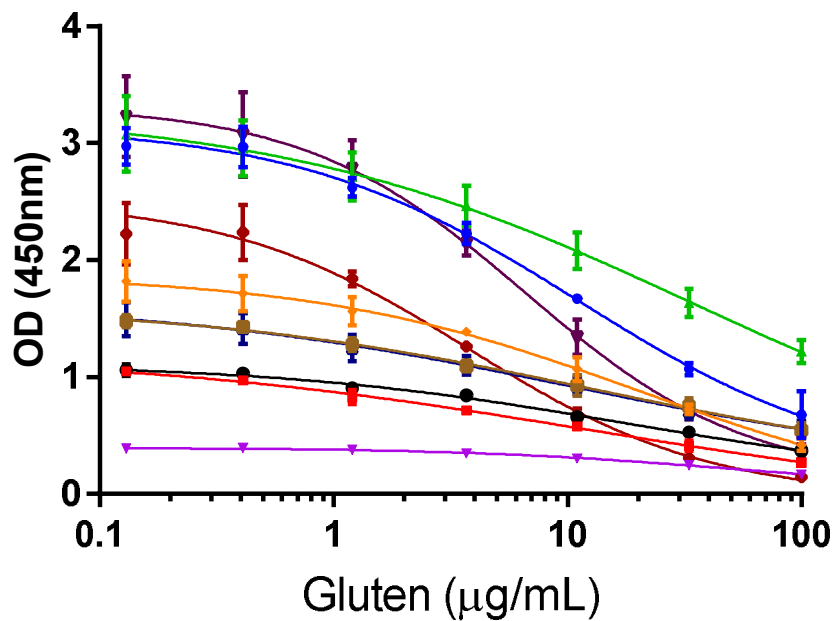


**Fig. S1A** Competitive ELISA standard curves developed using the detector antibodies of the Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (—●—), Microbiologique Gluten (—▲—), RIDASCREEN Gliadin (—■—), RIDASCREEN Gliadin Competitive (—▲—), Neogen Veratox for Gliadin R5 8510 (—■—), Varatox for Gliadin 8480 (—●—), GlutenTox ELISA Competitive G12 (—◆—), AgraQuant Gluten G12 (—▼—) and AllerTek Gluten (at 10 μg/mL (—▼—) and 25 μg/mL (—◆—) coating antigen concentration ELISA kits

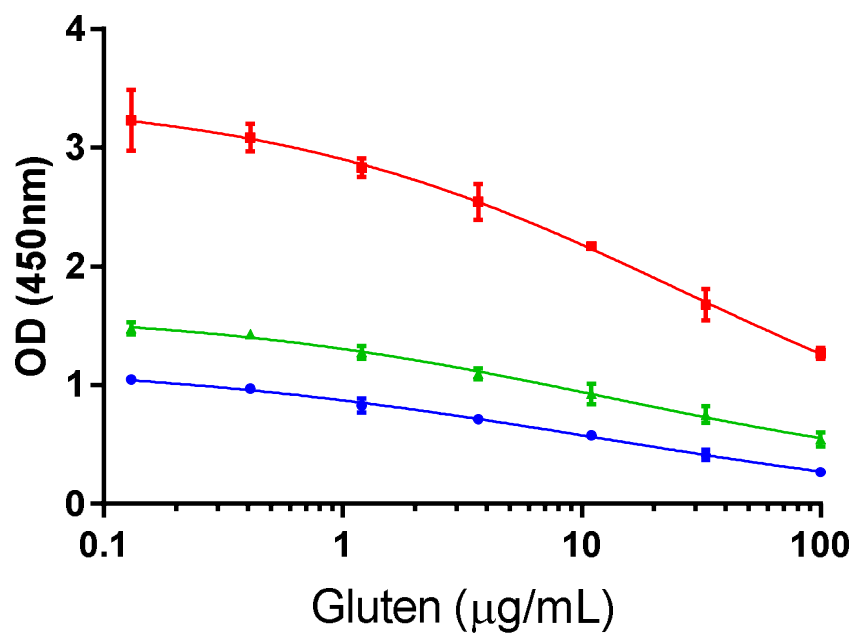


**Fig. S1B** Comparison of the standard curves of the detector antibodies of the RIDASCREEN Gliadin (—●—), RIDASCREEN Gliadin Competitive(—■—) and the Veratox for Gliadin R5 8510 (—▲—) ELISA kits

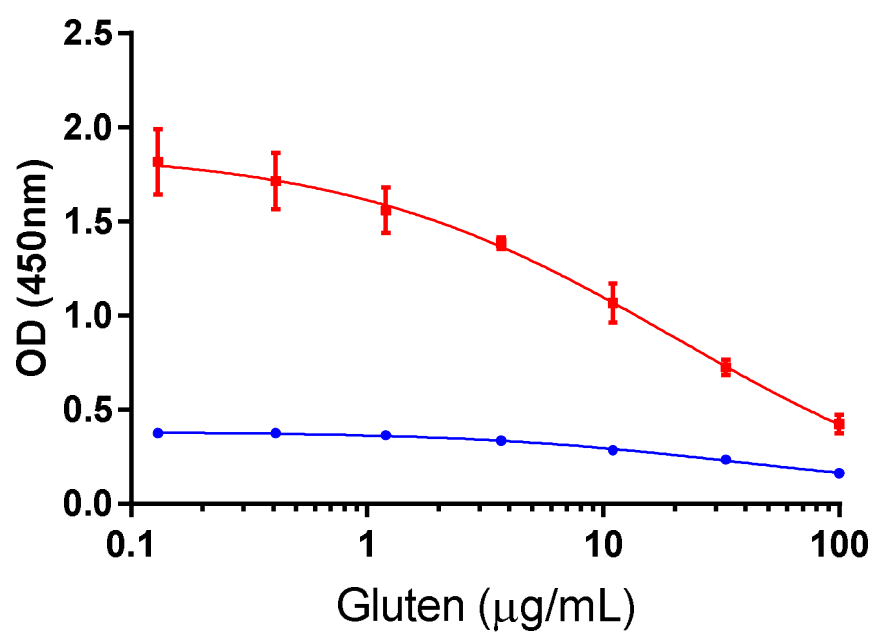


**Fig. S1C** Comparison of the standard curves of the detector antibodies of the AgraQuant Gluten G12 (●) and the GlutenTox ELISA Competitive G12 (■) ELISA kits

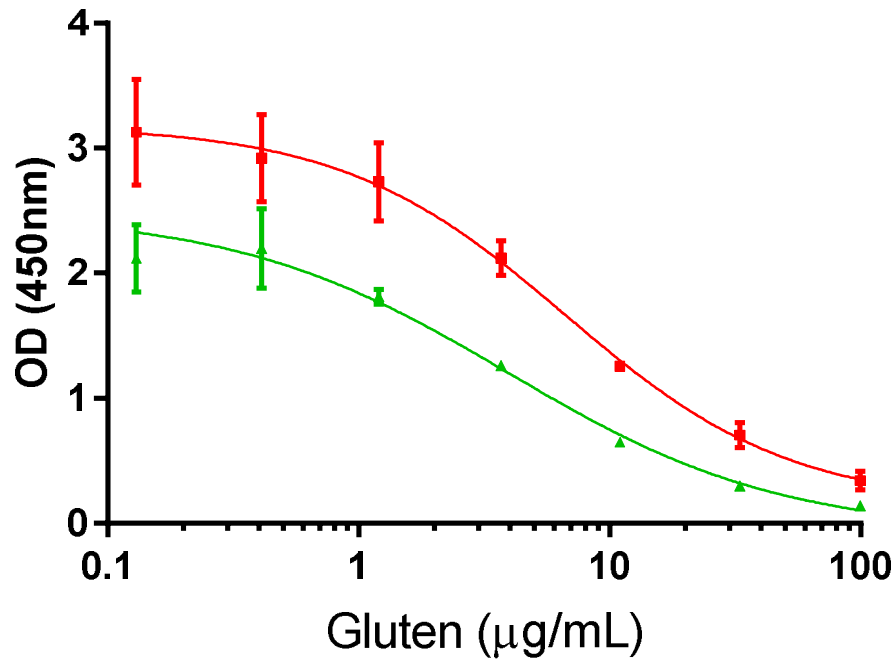


**Fig. S1D** Comparison of the standard curves of the detector antibody of the AllerTek Gluten ELISA kit at 10 µg/ml (—▲—) and 25 µg/ml (—■—) coating concentrations. Data represents gluten concentration (µg/mL) plotted against the absorbance (OD) values. Each data point represents the average of triplicate analysis conducted on three different days. Error bars indicate  $\pm$  one standard deviation

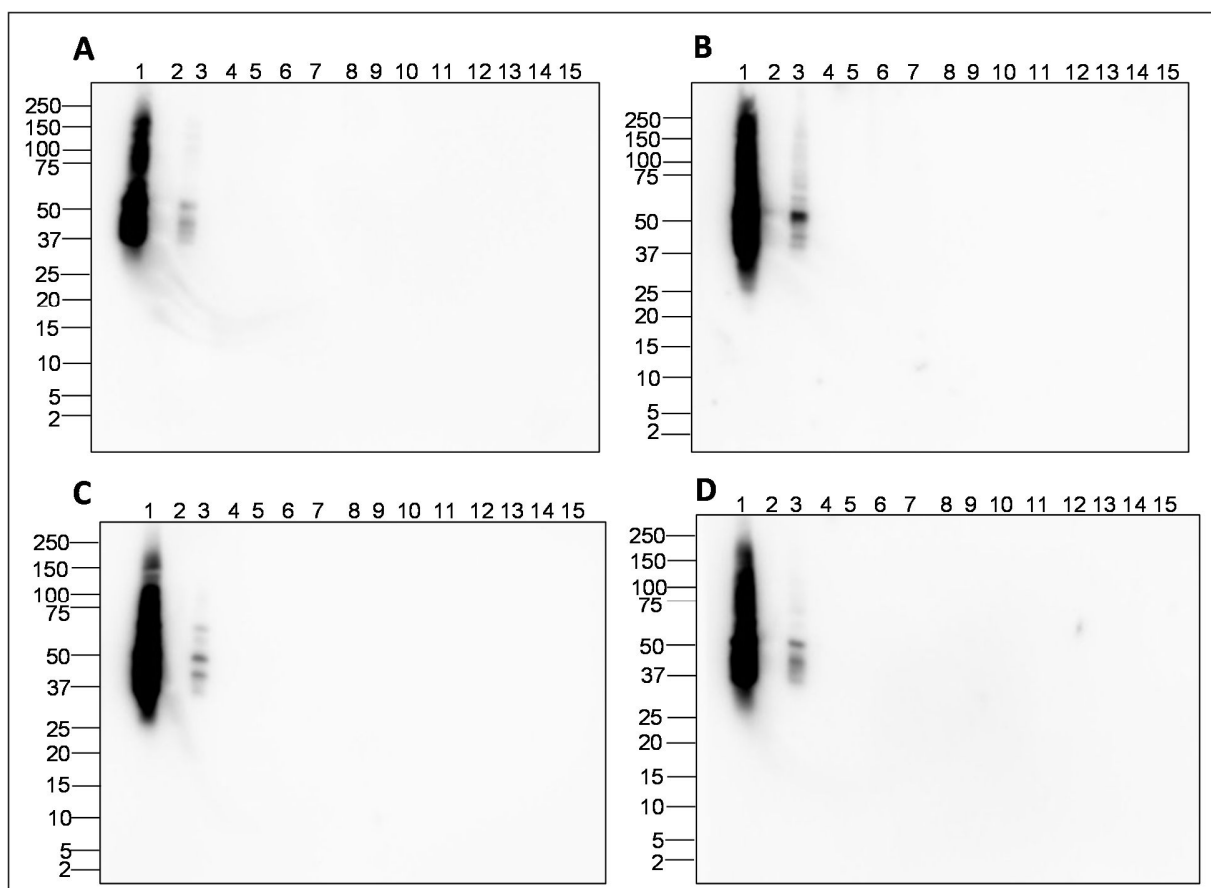


**Fig S2** Western blot of the soy sauce samples. The detector antibodies of the MloBS Gliadin (A), Veratox for Gliadin, cat # 8480 (B), Microbiologique Gluten (C) and Veratox for Gliadin R5 (D) ELISA test kits were used. Lane information; Lane 1- Intact gluten standard (100  $\mu$ g/mL), Lane 2- Molecular weight marker (250,000 – 2000 D) , Lane 3- Intact gluten standard (2.5  $\mu$ g/mL), Lane 4- Empty, Lane 5- Kikkoman Gluten free soy sauce (SC), Lane 6- Empty, Lane 7- Kikkoman organic soy sauce (SD), Lane 8- Empty, Lane 9-Kikkoman Tamari sauce (SE), Lane 10- Empty, Lane 11- San-J tamari gluten free soy sauce (SF), Lane 12- empty, Lane 13- San-J Gluten free less sodium soy sauce (SX), Lane 14- Empty, Lane 15- Kikkoman less sodium soy sauce (SS)