



Western blot analysis of fermented-hydrolyzed foods utilizing gluten-specific antibodies employed in a novel multiplex competitive ELISA

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Received: 21 February 2019 / Revised: 25 April 2019 / Accepted: 3 May 2019

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Abstract

Horseradish peroxidase (HRP) conjugated gluten-specific antibodies (G12, R5, 2D4, M10BS, and Skerritt), from nine commercial gluten ELISA test kits, previously utilized in the development of a multiplex competitive ELISA for the detection of fermented-hydrolyzed gluten, were utilized in western blot analyses of 59 fermented-hydrolyzed foods from four food groups (beer, soy-based sauces, vinegar, and sourdough bread). The protein/peptide profiles generated by the nine gluten-specific antibodies varied in size distribution and intensity dependent on the type of food, with minor differences between related products. Cluster analysis of the estimated gluten concentration values (based on western blot band intensities relative to intact gluten standards at 2.5 µg/mL and 100 µg/mL) and that of the relative response of the nine gluten-specific antibodies to different gluten proteins/peptides, distinguished among the different categories of fermented-hydrolyzed foods; comparable to what was observed in the multiplex competitive ELISA. Further, unlike the competitive ELISA, the western blot analyses distinguished between the presence of antigenic proteinaceous materials and false positives due to the presence of binding inhibitors (as observed with four soy-based sauces and one vinegar). Limitations of western blot analysis often include lower sensitivity than the comparable competitive ELISA and problems quantitating gluten-derived peptides and proteins. As a result, western blot analysis provides an orthogonal approach that can be used to both confirm the multiplex competitive ELISA while also providing additional insight into the protein/peptide profile of fermented-hydrolyzed foods.

Keywords Gluten · Fermentation · Western blot · Detection

Introduction

Celiac disease (CD) is an immune-mediated food intolerance with a prevalence of 1 in 141 individuals in the USA. The prevalence is approximately 1% in regions populated by individuals of European origin [1, 2]. The inflammatory responses in CD are typically triggered by the interaction of gluten proteins from wheat, rye, and barley with the intestinal mucosa,

leading to a reduction of the intestinal villi, ultimately leading to total atrophy [3, 4]. Gluten is a collective term for the alcohol soluble prolamins (gliadin from wheat, secalin from rye, and hordein from barley) and the alcohol insoluble glutelins (glutenins from wheat) [5]. A common feature of gluten proteins and peptides is the presence of multiple proline and glutamine residues that makes them resistant to the intestinal proteases and a preferred substrate of tissue transglutaminase [4, 6]. Although the gliadin fraction of wheat gluten is mainly responsible for exacerbating CD, glutenin proteins have also been shown to stimulate celiac small intestinal T cells and can induce a toxic response in CD patients [7, 8].

At present, the only effective treatment for CD is lifelong abstinence from products that are made from gluten-containing grains. To assist the sensitive consumer in reliably identifying products safe for consumption, the 2013 Gluten-free Regulation, which defines gluten free as less than 20 ppm (mg/kg) gluten, was enacted by the USA [9, 10]. Further, according to the Codex and the European commission (EC)

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00216-019-01893-0>) contains supplementary material, which is available to authorized users.

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Regulation No. 41/2009, only foods not exceeding a level of 20 mg gluten/kg may bear a gluten-free label [11]. Several commercial ELISA test kits have been validated and are suitable for the detection and quantitation of intact wheat gluten in foods [12–20]. However, detection of gluten in fermented-hydrolyzed foods poses a challenge due to highly variable proteolytic peptide patterns and the lack of suitable hydrolytic standards. This is further complicated by the lack of clinical information correlating peptide content with biological activity [20–23]. Recognizing the unique problems associated with the accurate detection and quantitation of gluten in fermented foods, a regulation targeting fermented-hydrolyzed and distilled foods was proposed in 2015 in the USA [10, 24].

Often concurrent with the fermentation process is the proteolysis of proteins. The peptide profile generated is highly dependent on numerous parameters (e.g., ingredients, time, temperature, type of fermenting organisms, or enzymes used). Further, due to the high redundancy of some amino acids and the presence of multiple immunopathogenic sequences, some redundant, it has been difficult to identify unique peptides as markers for accurately measuring the concentration of immunopathogenic gluten [25–28]. With an aim to quantify gluten in fermented-hydrolyzed foods and identify suitable reference materials whose peptide profile was sufficiently similar to the food, a multiplex competitive ELISA was recently developed [25]. The multiplex ELISA utilized multiple gluten-specific antibodies that recognized different gluten epitopes. The ELISA successfully recognized peptide profile differences in the fermented-hydrolyzed foods and was capable of classifying foods based on the type and the degree of fermentation [25]. However, false positive responses were observed with soy-based sauces. As with any competitive ELISA, the presence of anything that inhibited specific antigen-antibody binding of gluten proteins with the gluten-specific antibodies resulted in false positives. Typically, such non-specific background (propensity for false positives) is handled by increasing the stringency of the binding conditions or increasing the analytical threshold, the latter decreasing sensitivity and the dynamic range of the assay. An alternative approach involves the use of western blot analysis. Western blot analysis is routinely used for confirmatory analysis for the presence of egg and milk allergens in regulatory samples by the government of Japan [29]. Like typical competitive ELISAs and unlike sandwich ELISAs, western blot analysis requires only a single epitope or antigenic determinant for antibody binding, although the epitope structure/sequence can determine the specificity and the affinity of antibody binding (e.g., R5 antibody targeting the QQPFP epitope versus the G12 antibody targeting the QLPYPQP epitope [20]). This makes western blot ideally suited for peptide analysis while providing orthogonal information (e.g., peptide size) and distinguishing between antigenic proteinaceous material and inhibitors of specific antigen-antibody binding that may cause false positives in competitive ELISAs.

The possibilities of false positive reactions still exist in western blot analysis due to lectins or anything resulting from the fermentation process that might result in sticking of the HRP conjugated antibodies or peroxidase activity and such can be detected using appropriate controls and other confirmatory tests (e.g., mass spectrometry). Western blot analyses were performed with 59 different fermented-hydrolyzed foods utilizing all nine gluten-specific HRP-antibody conjugates from the multiplex competitive ELISA [25]. The aim was to determine if western blot analysis could confirm the protein/peptide profile differences indicated by the previously developed multiplex competitive ELISA and to possibly gain insight into the antigenic targets being detected by separating various proteins/peptides by size. Another aim was to extend the evaluation of soy-based sauces that generated non-specific responses (false positives) in the multiplex competitive ELISA.

Materials and methods

Reagents, antibodies, and food samples

Intact wheat gluten (Sigma-Aldrich cat # G5004) was used as a reference standard. Due to the insolubility of gluten, the finely ground gluten was made fresh daily by mixing 10 mg/mL in PBST (0.01 M phosphate-buffered saline (NaCl 0.138 M; KCl 0.0027 M); pH 7.4, with 0.1% Tween-20) for 2 h at room temperature. The working solution was then diluted to 100 µg/mL and 2.5 µg/mL intact gluten to serve as reference standards in the western blots.

The HRP conjugated, gluten-specific, detector antibodies from nine commercial ELISA test kits (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) were used for the western blot analyses. The target epitopes of these antibodies and their manufacturers are summarized in Table 1.

A subset of the fermented-hydrolyzed food samples analyzed by the multiplex competitive ELISA were used in the western blot analyses. The samples were randomly chosen based on ready availability. To facilitate comparison between the results of this western blot study and the prior multiplex competitive ELISA, the same identifying codes (letters) were used to identify the various products. Included in this study are 11 barley beers (seven lager (E, J, B, H, I, G, D), four ale (P, A, K, N)), one gluten-free beer FA (made from millet, buckwheat, corn and rice), 12 wheat beers (three ale (WI, WF, and WP), four witibiers (WS, WG, WB, WN), and five hefeweizens (WK, WH, WJ, WC, WL)), 12 soy sauces (six regular (SD, SE, SI, SA SB, ST), three gluten-free (SC, SX, SF), and three low sodium (SJ, SS, SV)), six teriyaki sauces (three regular

Table 1 HRP conjugated detector antibodies from commercial ELISA kits used in the western blot analysis

Antibody	Target	ELISA kits ^c	Manufacturer	Dilution ^e
G12	QPQLPY	GlutenTox ELISA Competitive G12	Biomedal Diagnostics	1 to 10
G12		AgraQuant Gluten G12	Romer Labs	1 to 10
R5		RIDASCREEN Gliadin	R-BioPharm, AG	1 to 110
R5		RIDASCREEN Gliadin Competitive	R-BioPharm, AG	1 to 110
R5		Veratox for Gliadin R5 (Cat # 8510)	Neogen Corp.	1 to 100
USDA antibody ^a	Gluten	Veratox for Gliadin (Cat # 8480)	Neogen Corp.	1 to 100
MiBS	Gliadin	Wheat/Gluten (Gliadin) MiBS	MiBS ^d , Inc.	1 to 10
Skerritt	HMW ^b glutenin	AllerTek Gluten	ELISA Technologies Inc.	1 to 50
2D4	Diamidated gliadin	Microbiologique Gluten Sandwich	Pi Bioscientific Inc.	1 to 1000

^a Gluten-specific monoclonal antibody developed and licensed by the US Department of Agriculture^b HMW, high molecular weight^c Name of the ELISA test kits^d Morinaga Institute of Biological Sciences^e Dilution was done on a volume/volume basis using 3% BSA

(TA, TB, TE), two gluten-free (TC, TH), and one less sodium (TD)), six Worcestershire sauces (four without wheat as ingredient (WoC, WoD, WoE, and WoF) and two with wheat (WoA and WoB)), six vinegars (one gluten-free (VF) and five with malt, barley or grain as ingredients (VA, VB, VC, VD, VE)), and six sourdough breads (one gluten-free (BI), five made from gluten-containing flour (BE, BB, BG, BC, BF)). Liquid samples were analyzed without any further concentration or extraction. Sourdough bread samples were ground using an IKA tube mill to a fine powder and extracted using PBST (1:20 *w/v*) at room temperature for 2 h. The extracts were then centrifuged at 3000g for 20 min and subsequently mixed with the SDS-PAGE sample buffer and loaded onto the gel.

Western blot analysis

Western blot analyses were conducted of the SDS-PAGE, electrophoretically separated proteins as previously described [19]. This entailed fractionation using a Novex 4–12% Bis-Tris gels using the XCell SureLock™ Mini-Cell (Invitrogen Corp.), followed by transfer to a PVDF (polyvinylidene difluoride) membrane, and immunoblotting with the various antibodies. All fermented-hydrolyzed samples and the gluten standards (100 and 2.5 µg/mL) were separated by electrophoresis without any further dilution. Gels were loaded with the maximum amount (15 µL/lane) of the reduced-denatured samples, without artificially concentrating or diluting the samples, with the goal of visualizing the proteins/peptides that are present at both high and low concentrations and avoiding any selective pressure that might inadvertently alter the distribution of any proteins or peptides. Precision Plus marker protein standards (Bio-Rad Laboratory Inc., CA # 161-0374) were used as molecular weight markers. The marker proteins were pre-stained and did not contain IgG binding sites; therefore,

their presence was not obvious in the chemiluminescent images of the immunoblots. However, the marker proteins could be visualized under white light. Table 1 summarizes the dilution of each of the nine antibodies used in the western blot. The dilution of antibody was chosen based on the results of preliminary experiments to obtain a good signal to noise ratio.

A Syngene G: Box Chemi XX9 imager (Syngene, Frederick, MD) was used to measure the chemiluminescence. Different exposure levels were used during the chemiluminescent imaging to help visualize less intense protein bands and to distinguish between adjacent bands. Intact gluten standards ran alongside the food samples made it possible to directly compare the images obtained at different exposure levels and estimate the relative content of proteinaceous materials. By superimposing the white light images of the blots (where the marker proteins were visible) with the chemiluminescent images, the molecular weights of the peptides and proteins were estimated. The superimposed (white light image and chemiluminescent image of the same blots) western blot images of six barley beers (H, I, G, K, D, N) with nine gluten-specific antibodies are shown in Electronic Supplementary Material (ESM) Fig. S1, as examples to help display the positions of the non-chemiluminescent molecular weight markers. All western blot analyses were performed twice to confirm reproducibility. GeneTool software from Syngene (provided with the Syngene G:BOX imaging system) was used to estimate the gluten concentrations in the sample and those of specific proteins based on the relative intensities compared to the 2.5 µg/mL and 100 µg/mL intact gluten standards.

Sandwich ELISA analysis

The soy-based sauce samples that bound any of the nine antibodies and resulted in western blot bands were analyzed using

both the MIOBS Gliadin and the RIDASCREEN R5 Gliadin sandwich ELISA test kits for intact gluten. The analyses were carried out according to the kit manufacturer's protocol. Samples were extracted in triplicate and analyzed by two different ELISA kits from the same lot. The absorbance values were measured using the Infinite® M200 (Tecan, Inc., Morrisville NC) plate reader. The apparent concentration of gluten in the samples was determined by interpolating the responses relative to third-order polynomial trendlines ($R^2 > 0.99$) based on the test kit standards.

Cluster analysis

Hierarchical Cluster Analysis using the Ward's Minimum method (JMP® 12.0.1, SAS Institute, Inc., Cary, NC) was performed on the western blot-derived gluten concentration estimates ($\mu\text{g/mL}$) for each sample. Further, analyses were performed on the responses of the nine antibodies relative to the responses generated by the Skeritt (AllerTek Gluten) antibody. The Skeritt antibody was chosen for the relative comparisons because it bound to proteins from most of the fermented-hydrolyzed samples (except for soy-based sauces and vinegars) in western blot. The clustering patterns were illustrated using Constellation plots.

Results and discussion

Western blot was used as an orthogonal approach to detect gluten proteins/peptides in a subset of the fermented-hydrolyzed food samples used in the development of a multiplex competitive ELISA [25]. By employing multiple antibodies, the goal was to characterize the proteins/peptides present by monitoring different antigenic epitopes. The inclusion of the same antibody (e.g., R5 and G12) from multiple sources served as a form of confirmatory analysis and to provide insight into antibody-specific and HRP conjugation effects on antigen recognition. The inclusion of several gluten-free samples as controls ruled out the likelihood of non-fermentation based non-specific false positive responses in the western blot. A gluten-free beer (FA) was used as a control for beers, gluten-free sourdough bread (BI) was used as a control for the sourdough breads, and gluten-free vinegar (VF) and several gluten-free soy sauces (SC, SX, SF, TC, TH) were used as controls for the vinegars and soy-based sauces respectively. The gluten-free samples, except for soy sauce SF and Worcestershire sauce WoE, did not show any bands with the gluten-specific antibodies indicating that the bands observed in the gluten-containing samples were gluten-derived.

Unlike the multiplex competitive ELISA, the western blot was not designed for quantitation, but instead for qualitative analysis and characterization of the sizes of antigenic peptides

and proteins. However, by comparing the intensity of specific bands to those associated with reference material at defined concentrations, it is possible to estimate protein content. When performing such analyses, the sources of variance, reducing the precision and accuracy, are numerous (e.g., variation in sample loading, sample transfer efficiency from the polyacrylamide gel to the PVDF membrane, rate of transfer between the samples in separate lanes and separate membranes, and differences in substrate availability); however, useful information can still be obtained. The two intact gluten standards ran alongside the samples in the western blots provided direct comparison controls for proteins present at 2.5 and 100 $\mu\text{g/mL}$ non-fermented, intact gluten. Assuming linearity between the intensities of the bands and gluten concentration, estimates of gluten content in the samples were made by using the two standards. Estimation was performed by interpolation and in some cases extrapolation, in some wheat beers and sourdough breads, where the gluten concentration was high. Due to the inherent error associated with the process (use of only two standards and extrapolation), the estimations were used primarily for comparison purposes in generating profiles and cluster analysis (comparing the various fermented foods and their recognition by the nine antibodies) and not for fine quantitation of gluten content.

The appearance of diffused bands in western blots for some of the fermented-hydrolyzed samples (e.g., wheat beers and sourdough breads, where the gluten concentration was high) may be due to multiple variables and are not unique to this study [30–34]. It is a challenge to obtain well separated western blot bands when fermented-hydrolyzed samples are analyzed where the gluten protein/peptide profile is very diverse. Possible causes of the diffused appearance of the bands include high concentrations of proteins that caused the binding signal to surpass the saturation point during chemiluminescent imaging, overlap between adjacent protein bands due to insufficient separation during electrophoresis, and the complex composition of proteins in gluten and the associated peptides. Multiple approaches may be employed to improve resolution depending on the cause of the problem (e.g., varying sample volume, reducing sample concentration, electrophoretic conditions, sample processing, and fractionation). However, doing so may compromise detection of proteins/peptides inherently present in the sample at a lower concentration. Further, the most commonly used methods to improve band resolution can accidentally result in the loss of some proteinaceous components present in the sample and can potentially skew the results. It was therefore decided to analyze the whole samples with no further treatment other than reduction and denaturation. Fortunately, similar to the previously developed multiplex competitive ELISA, the western blot generated protein/peptide profile patterns characteristics of the fermentation processes. It is possible that improved resolution may provide further insight ultimately worth studying.

Barley beers

A diverse range of protein/peptide bands were recognized by the nine gluten-specific HRP-antibody conjugates used in the western blot with the 11 barley beers (Fig. 1). The gluten-free barley beer (FA) resulted in no bands with any of the nine antibodies (lane 15, Fig. 1A), whereas barley beer A (lane 7, Fig. 1A) resulted in moderate to high intensity bands with all the nine antibodies used in the western blot. The MIOBS polyclonal antibody (h) resulted in overall weak bands in the western blot for the remaining barley beers; no bands were observed with seven of the barley beers [P (lane 5, Fig. 1A), H (lane 5, Fig. 1B), I (lane 7, Fig. 1B), G (lane 9, Fig. 1B), K (lane 11, Fig. 1B), D (lane 13, Fig. 1B), and N (lane 15, Fig. 1B)] and very faint bands were observed above 50 kDa, with three barley beers [E (lane 9), J (lane 11), and B (lane 13), Fig. 1A].

The Microbiologique Gluten antibody (g) displayed similar band pattern as the R5 antibody from the RIDASCREEN competitive ELISA kit (d) for barley beers A (lane 7, Fig. 1A), E (lane 9, Fig. 1A), and J (lane 11, Fig. 1A). Bands were observed in the range of 15–100 kDa for A, 15–50 kDa for E, and 15–37 kDa for J. Barley beers E (lane 9) and J (lane 11) also displayed low intensity bands (approximately 25–30 kDa for E and 25–50 kDa for J) with the AgraQuant Gluten G12 antibody (a). Barley beer J (lane 11) further showed moderate intensity bands, ranging from 20 to 50 kDa, with the Neogen Veratox for Gliadin, cat # 8480 antibody (f) (Fig. 1A). A faint protein band at approximately 45 kDa was observed for barley beer B (lane 13, Fig. 1A), only with the Microbiologique Gluten antibody (g).

Barley beer H (lane 5, Fig. 1B) did not show any bands with any of the nine gluten-specific antibodies. For barley beer I (lane 7, Fig. 1B), very high intensity bands were observed in the MW range of 15–30 kDa with the Microbiologique Gluten antibody (g). The same beer also resulted in a moderate intensity band at approximately 17 kDa with the Neogen Veratox R5 antibody (e) and faint bands in the range of 20–50 kDa with the Neogen Veratox for Gliadin, cat # 8480 antibody (f). For barley beers G (lane 9, Fig. 1B), the Microbiologique Gluten antibody (g), the Neogen Veratox R5 antibody (e), and the Neogen Veratox for Gliadin, cat # 8480 antibody (f) resulted in bands at approximately 50 kDa. The band observed by the Neogen Veratox R5 antibody (e) was fainter and the band observed by the Neogen Veratox for Gliadin, cat # 8480 antibody (f) was of higher intensity compared to the Microbiologique Gluten antibody (g). Further, an additional band at approximately 37 kDa was observed only with the Neogen Veratox for Gliadin, cat # 8480 antibody (f). For barley beer K (lane 11, Fig. 1B), very faint bands at approximately 50 and 20 kDa were observed with the Microbiologique Gluten antibody (g), whereas the same beer showed a moderate intensity band at approximately 50 kDa with the Neogen Veratox for Gliadin, cat # 8480 antibody (f). For barley beer D

(lane 13, Fig. 1B), both the Microbiologique Gluten antibody (g) and the Neogen Veratox for Gliadin, cat # 8480 antibody (f) bound to a protein band at approximately 50 kDa. Barley beer N (lane 15, Fig. 1B) did not show any bands with eight gluten-specific antibodies (a, b, c, d, e, f, g, and h). The visualization of six marker proteins (250, 150, 100, 75, 50, and 37 kDa) in the chemiluminescent western blot images, with the Microbiologique Gluten antibody (g) in Fig. 1B, is possibly due to low level of cross-reaction of the Microbiologique Gluten antibody with those marker proteins, which became visible when images were captured at a high exposure level.

The binding pattern of the Skerritt (AllerTek Gluten) antibody (i) was different than the other antibodies. Skerritt antibody resulted in high intensity bands (ranging from as low as 20 kDa to as high as 250 kDa) with all the barley beers except for barley beers P (lane 5, Fig. 1A), where faint bands were observed around 37 kDa, and for beer H (lane 5, Fig. 1B), where no bands were observed. The high intensity binding by the Skerritt antibody to protein bands in most of the barley beers indicates that the high molecular weight (HMW) glutenins (known as D hordein in case of barley) preferred by the Skerritt antibody tend to remain in the finished barley beers after fermentation as HMW peptides. This finding is consistent with several previous studies [27, 28, 35].

Although the same antibodies from different sources were used in the western blot (e.g., R5 and G12), the band profiles were different for some barley beers. For example, bands at 25–30 kDa for barley beer E (lane 9, Fig. 1A) and at 25–50 kDa for barley beer J (lane 11, Fig. 1A) were fainter with the G12 antibody derived from the GlutenTox ELISA Competitive G12 test kit (b) than from the AgraQuant Gluten G12 test kit (a). Very faint bands below 10 kDa were observed for barley beer E (lane 9, Fig. 1A) and B (lane 13, Fig. 1A) with only the G12 antibody from the GlutenTox ELISA Competitive G12 test kit (b) and protein bands at approximately 20 kDa were observed for barley beers I (lane 7, Fig. 1B) and barley beer D (lane 13, Fig. 1B) with only the G12 antibody from the AgraQuant Gluten G12 test kit (a). A similar pattern was observed with the R5 antibodies. The R5 detection antibody derived from the RIDASCREEN competitive R5 ELISA kit (d) was more effective at visualizing protein bands than the R5 detection antibodies from either the RIDASCREEN (c) or the Neogen R5 (e) sandwich ELISA test kits (e.g., bands at 20–37 kDa for barley beers E (lane 9, Fig. 1A) and J (lane 11, Fig. 1A), observed only with d). Comparison between the two R5 detection antibodies from the sandwich ELISA test kits (c and e) indicated a slightly enhanced sensitivity of the R5 antibody from the Neogen Veratox for Gliadin R5 (e) test kit (e.g., visualization of a band at approximately 17 kDa for barley beer I (lane 7, Fig. 1B), only with the Neogen Veratox for Gliadin R5 antibody (e)). The greater sensitivity using the antibodies from some kits is characteristics of either a higher level of HRP conjugation or a

higher level of biological activity. Nonetheless, these results provide another perspective of the data and thereby support the use of the antibodies from multiple sources.

The western blot band intensity profile of the different barley beers, as recognized by the nine gluten-specific HRP-antibody conjugates are presented in the ESM Fig. S2. As illustrated by ESM Fig. S2 and Fig. 1, protein/peptide bands of varying size ranges and the intensities were observed by the nine gluten-specific antibodies with multiple barley beers. The low MW protein bands indicates the presence of gluten-derived peptides in the beers. Interestingly, for some of the beers, while one antibody was only recognizing the high MW protein bands

(intact gluten), another antibody was able to recognize both the high and low MW bands (intact proteins and peptides). The observation of differential responses with the antibodies is the reason why the use of a single antibody may inadvertently miss the detection of peptides in some samples.

Wheat beers

Figure 2A shows the western blot images of six wheat beers (WI WF, WS, WK, WH, WG) as recognized by the nine gluten-specific HRP conjugates. The western blot images of the other six wheat beers (WJ, WB, WP, WC, WN, WL) are

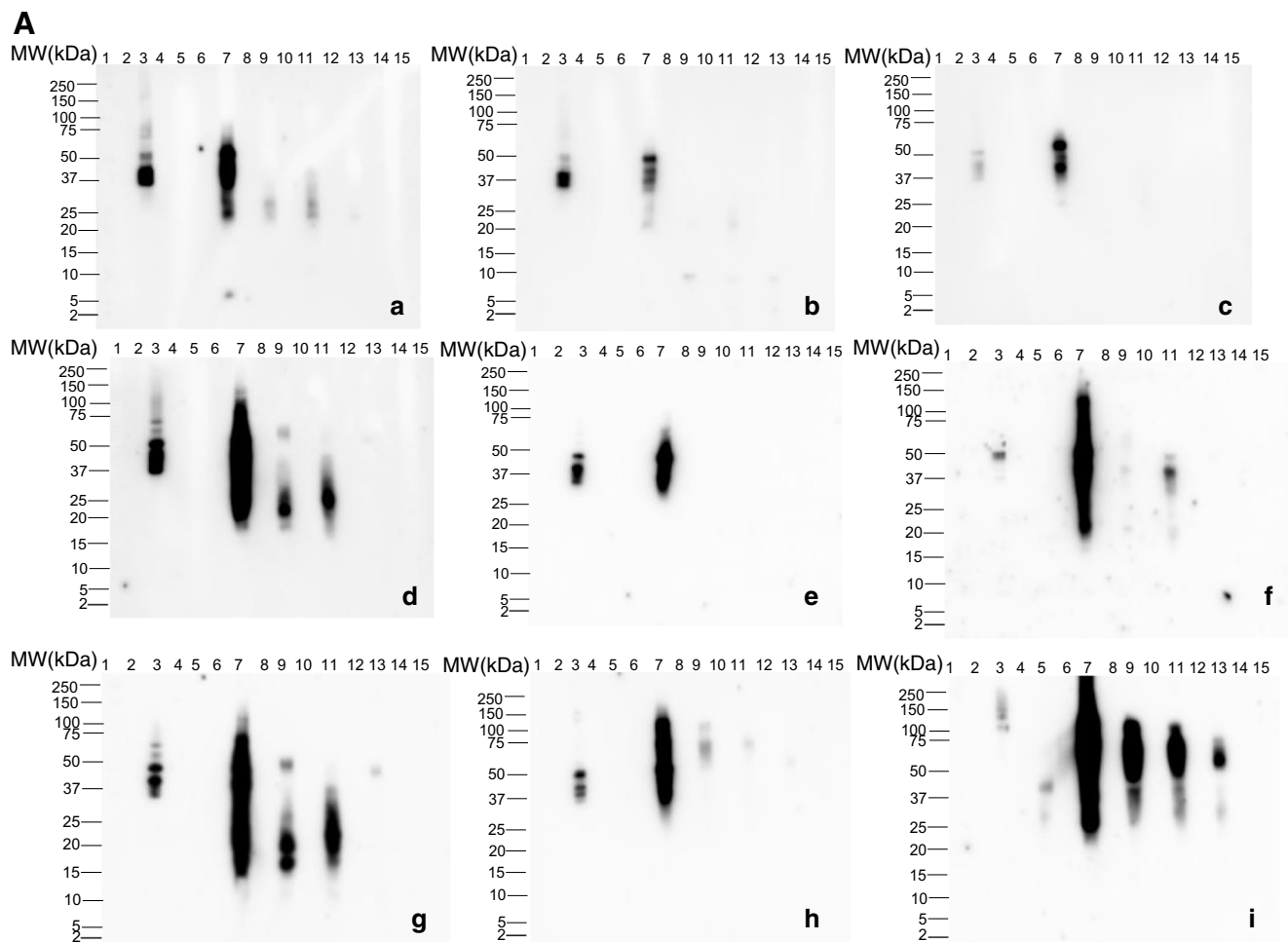
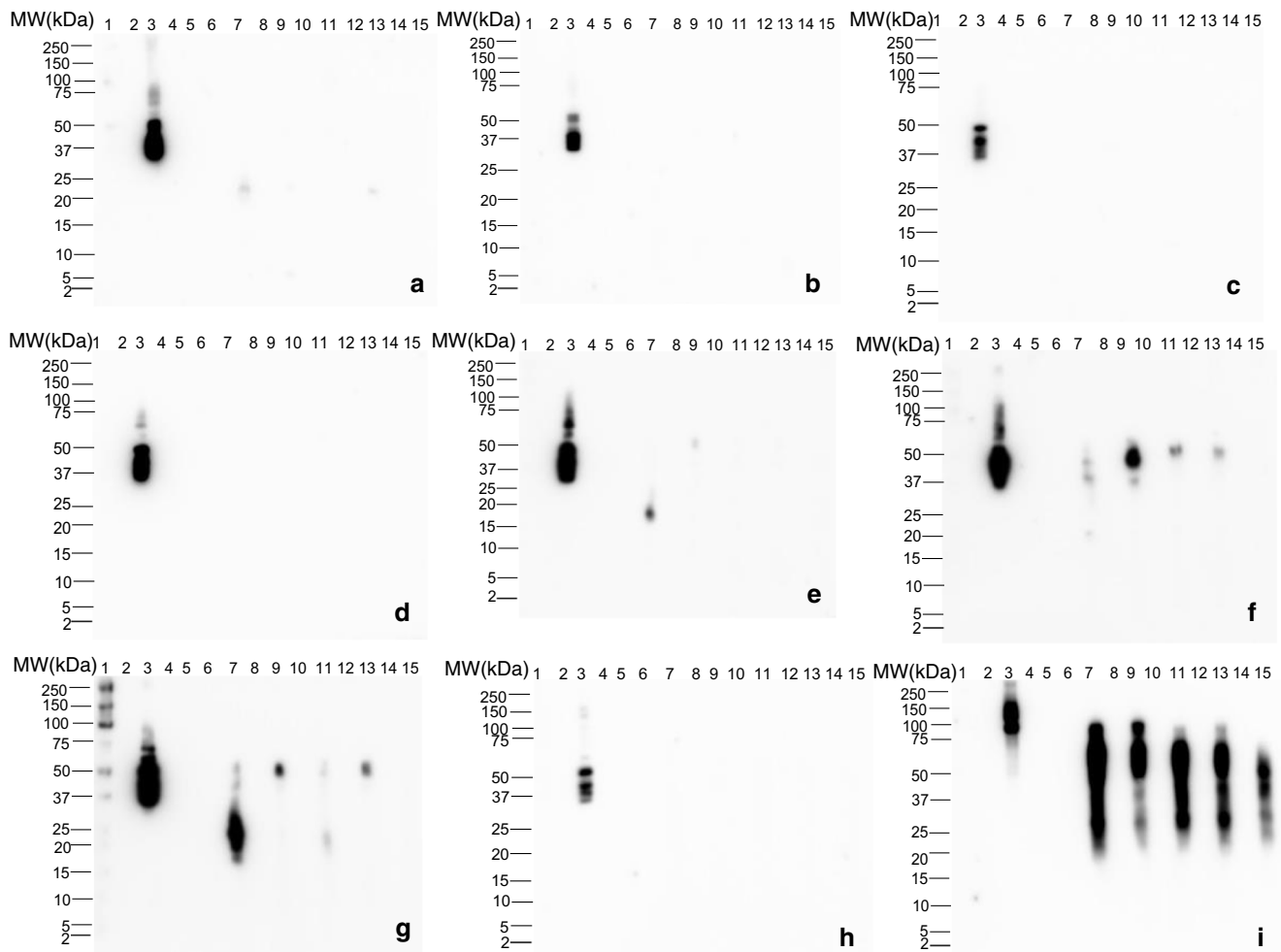


Fig. 1 (A) Western blot band patterns of barley beers (P, A, E, J, B, FA), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), and AllerTek Gluten (i) ELISA kits. Lane information for western blot: lane 1, molecular weight marker; lane 2, empty; lane 3, 2.5 µg/mL intact gluten standard; lane 4, empty; lane 5, barley beer P; lane 6, empty; lane 7, barley beer A; lane 8, empty; lane 9, barley beer E; lane 10, empty; lane 11, barley beer J; lane 12, empty; lane 13, barley beer B; lane 14, empty; lane 15, gluten-free beer

FA. (B) Western blot band patterns of barley beers (H, I, G, K, D, N), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), and AllerTek Gluten (i) ELISA kits. Lane information for western blot: lane 1, molecular weight marker; lane 2, empty; lane 3, 2.5 µg/mL intact gluten standard; lane 4, empty; lane 5, barley beer H; lane 6, empty; lane 7, barley beer I; lane 8, empty; lane 9, barley beer G; lane 10, empty; lane 11, barley beer K; lane 12, empty; lane 13, barley beer D; lane 14, empty; lane 15, barley beer N

B**Fig. 1** (continued)

shown in Fig. 2B. The western blot band intensity profile of all the 12 wheat beers, presented as different MW range and expressed in terms of $\mu\text{g/mL}$ gluten has been depicted in ESM Fig. S3. As observed in the western blot images and the band intensity profile, 10 out of 12 wheat beers resulted in very high intensity bands over a wide range of MW (ranging from as low as 20 kDa to as high as 250 kDa). Differences in band recognition pattern and intensities were observed for the same sample with different gluten-specific antibodies. For example, barley beer WC (lane 11, Fig. 2B), displayed low intensity bands with the Microbiologique Gluten antibody (g) and the GlutenTox G12 antibody (b) compared to other antibodies. Two ale style wheat beers, WI (lane 5, Fig. 2A) and WF (lane 7, Fig. 2A), resulted in comparatively low intensity bands. For wheat beer WI (lane 5, Fig. 2A), no bands were observed with the MIOBS polyclonal antibody (h) and the Skerritt antibody (i), low to moderate intensity bands ranging from 5 to 60 kDa were observed with the two G12 antibodies (a and b), the two R5 antibodies derived from the sandwich

ELISA kits (c and e), the Veratox for Gliadin, cat # 8480 antibody (f), and the Microbiologique Gluten antibody (g). Interestingly only the R5 antibody derived from the RIDASCREEN competitive R5 ELISA kit (d) resulted in high intensity bands in the range of 15–75 kDa. For wheat beer WF (lane 7, Fig. 2A), no bands were observed with the MIOBS polyclonal antibody (h). Low to moderate intensity bands (from approximately 20–60 kDa) were observed with all other antibodies, except for the R5 antibody derived from the RIDASCREEN competitive R5 ELISA kit (d), which resulted in high intensity bands from approximately 20 to 50 kDa. As observed with the barley beers, the results from the analysis of wheat beers indicated that some gluten-specific antibodies were more effective at detecting fermented-hydrolyzed gluten proteins/peptides present in wheat beers compared to the others. Appearance of proteinaceous bands at both high and low MW in wheat beers indicated the presence of both intact gluten and peptides derived from both gliadin and glutenin in wheat beers. A recent mass spectrometry study also indicated

the presence of both intact gluten and gliadin/glutenin-derived peptides in commercial wheat beers [36].

Sourdough breads

The five sourdough breads (BE, BB, BG, BC, BF) analyzed by western blot displayed intense bands with all nine HRP-antibody conjugates (Fig. 3 and ESM Fig. S4). Bands were observed both at high and low MW ranging

from 20 to 250 kDa. Similar to the wheat beers, differences were observed in the band profiles and intensities of the bands with different gluten-specific antibodies (e.g., a band at 20 kDa in sourdough bread BG (lane 9, Fig. 3) was generated by only four antibodies (a, e, f, and h) at varying intensities). As expected, the gluten-free sourdough bread (BI) did not result in any bands with any of the nine HRP-antibody conjugates (lane 15, Fig. 3).

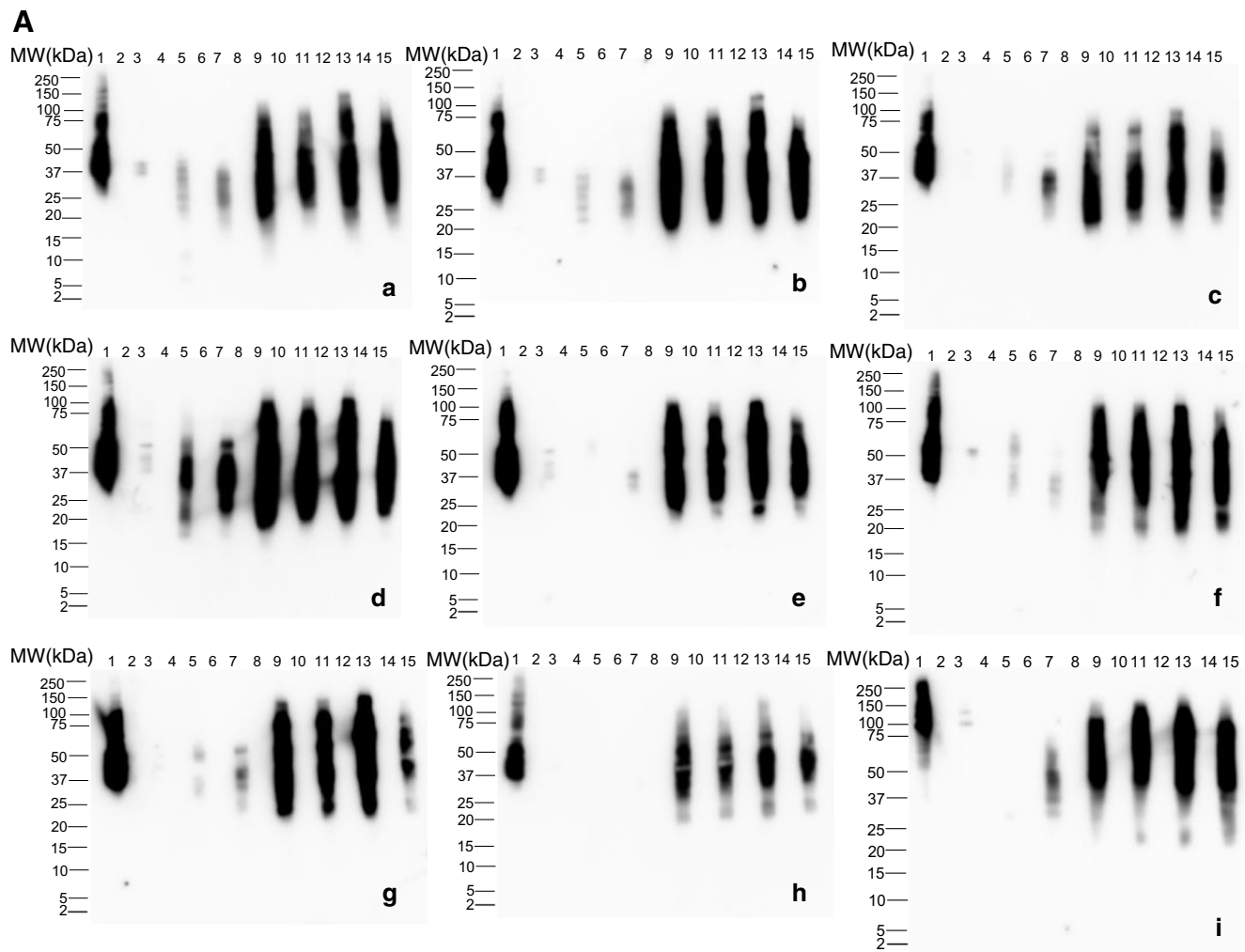


Fig. 2 (A) Western blot band patterns of wheat beers (WI, WF, WS, WK, WH, WG), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MIOBS) Gliadin (h), and AllerTek Gluten (i) ELISA kits. Lane information for western blot: lane 1, 100 µg/mL intact gluten standard; lane 2, molecular weight marker; lane 3, 2.5 µg/mL intact gluten standard; lane 4, empty; lane 5, wheat beer WI; lane 6, empty; lane 7, wheat beer WF; lane 8, empty; lane 9, wheat beer WS; lane 10, empty; lane 11, wheat beer WK; lane 12, empty; lane 13, wheat beer WH; lane 14, empty; lane 15, wheat beer WG. (B) Western

blot band patterns of wheat beers (WJ, WB, WP, WC, WN, WL), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MIOBS) Gliadin (h), and AllerTek Gluten (i) ELISA kits. Lane information for western blot: lane 1, 100 µg/mL intact gluten standard; lane 2, molecular weight marker; lane 3, 2.5 µg/mL intact gluten standard; lane 4, empty; lane 5, wheat beer WJ; lane 6, empty; lane 7, wheat beer WB; lane 8, empty; lane 9, wheat beer WP; lane 10, empty; lane 11, wheat beer WC; lane 12, empty; lane 13, wheat beer WN; lane 14, empty; lane 15, wheat beer WL

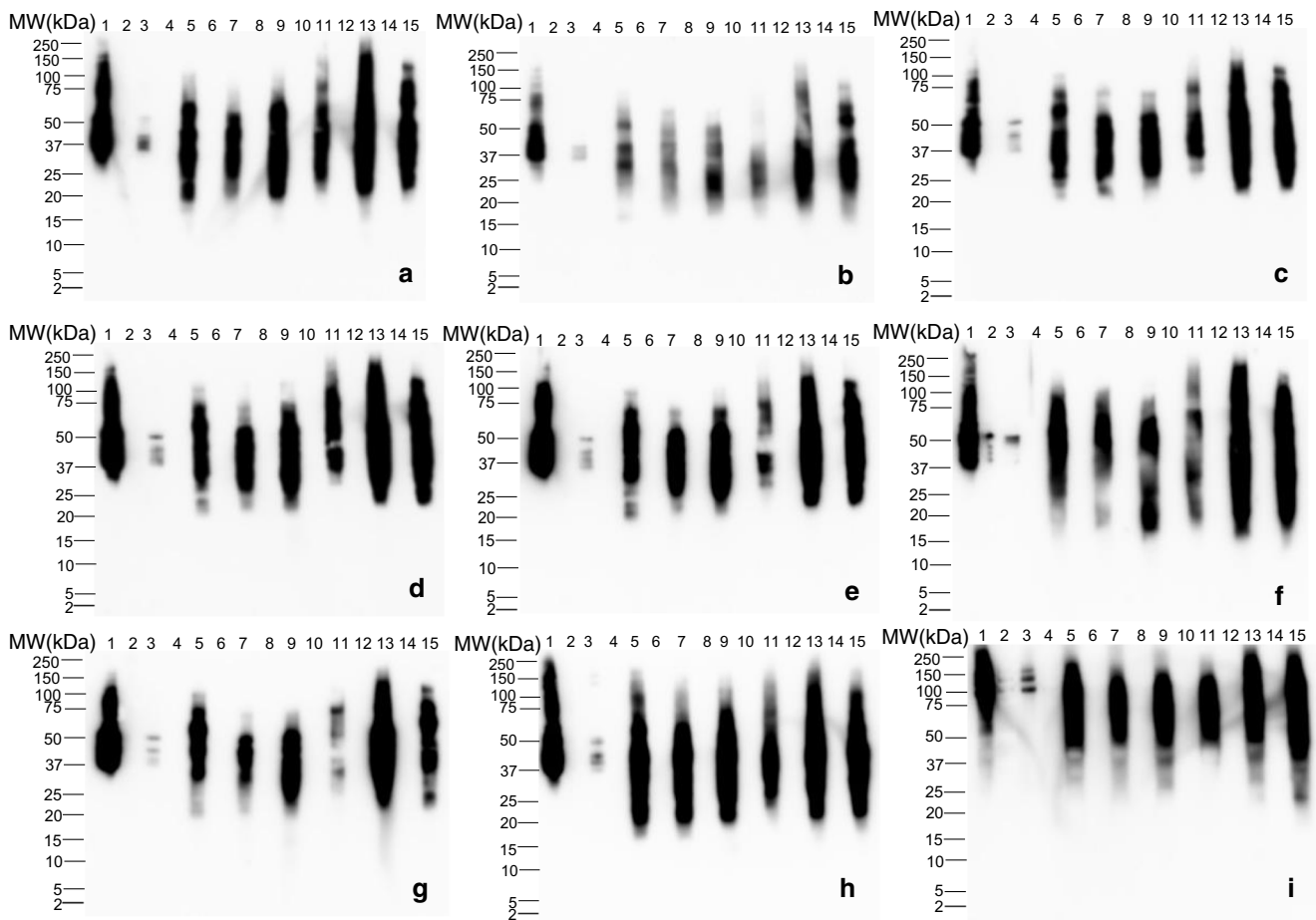


Fig. 2 (continued)

Soy-based sauces

Western blot analysis of all 12 soy sauces using all nine HRP-antibody conjugates employed in the multiplex competitive ELISA failed to visualize protein bands characteristic of the intact gluten (ESM Figs. S5 and S6). However, a low intensity protein band was observed for soy sauce SF (lane 15, Fig. 4A) at approximately 25 kDa with the deamidated gliadin specific Microbiologique Gluten antibody (g). Deamidation can occur during the fermentation process and has been shown to drastically reduce the affinity of the Skerritt, G12 and the R5 antibodies towards gluten peptides and proteins [37]. Therefore, the recognition of the 25 kDa peptide in soy sauce SF by only the deamidated gluten-specific antibody is not surprising (Fig. 4A). Soy sauce SF does not list wheat as an ingredient and is labeled gluten-free. The presence of the 25 kDa peptide band in soy sauce SF may indicate inadvertent gluten contamination and incomplete proteolysis during fermentation. The chances that the 25 kDa band is a non-specific interaction, though unlikely, cannot be ruled out since it is unknown whether soy sauce SF contained any unique components or was produced using a process not common to the

other soy sauces. To definitively identify the source of the 25 kDa band would require research (e.g., mass spectrometry) beyond the scope of this study. As observed in the western blot images of some of the barley beers with the Microbiologique Gluten antibody (g, Fig. 1B), five marker proteins (250, 150, 100, 50, and 37 kDa) also become visible during the chemiluminescent imaging of the soy sauces with the Microbiologique Gluten antibody. This observation again indicates the possible low-level cross-reaction of the Microbiologique Gluten antibody with some of the marker proteins used in the western blot (Fig. 4A).

None of the six Worcestershire sauces showed any protein bands at the same MW as the intact gluten standard (Fig. 4B; ESM Fig. S7). A 17 kDa band was visualized in Worcestershire sauce WoB (lane 7, Fig. 4B) with the AgraQuant Gluten G12 antibody (a). WoB contains wheat as an ingredient and the western blot binding indicates presence of gluten-derived peptides in this Worcestershire sauce. Worcestershire sauce WoE (lane 13, Fig. 4B) generated western blot bands at approximately 17 kDa with both the Neogen Veratox antibodies (e and f). Further, binding was observed at higher MW with the AgraQuant Gluten G12 antibody (a), potentially indicating

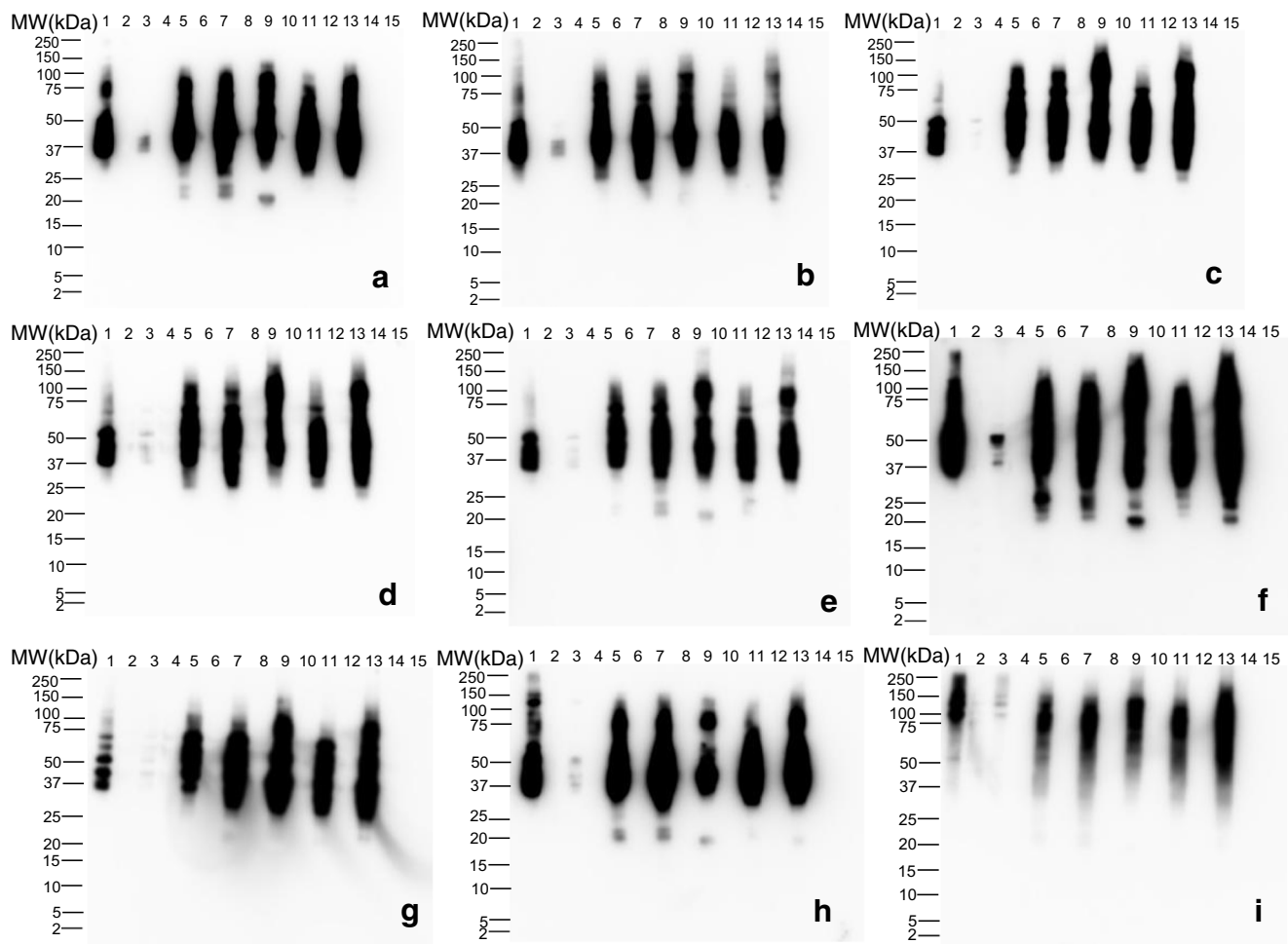


Fig. 3 Western blot band patterns of sourdough breads (BE, BB, BG, BC, BF, BI), using the detector antibodies of AgraQuant Gluten G12 (**a**), GlutenTox ELISA Competitive G12 (**b**), RIDASCREEN Gliadin (**c**), RIDASCREEN Gliadin Competitive (**d**), Neogen Veratox for Gliadin R5 (**e**), Veratox for Gliadin, cat # 8480 (**f**), Microbiologique Gluten (**g**), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (**h**), and AllerTek Gluten (**i**) ELISA kits. Lane information for western blot: lane 1,

100 µg/mL intact gluten standard; lane 2, molecular weight marker; lane 3, 2.5 µg/mL intact gluten standard; lane 4, empty; lane 5, sourdough bread BE; lane 6, empty; lane 7, sourdough bread BB; lane 8, empty; lane 9, sourdough bread BG; lane 10, empty; lane 11, sourdough bread BC; lane 12, empty; lane 13, sourdough bread BF; lane 14, empty; lane 15, sourdough bread BI

binding to aggregates. Worcestershire sauce WoE does not list wheat as an ingredient in the product label. The western blot bindings may indicate the presence of gluten-derived proteins/peptides in this Worcestershire sauce. Similar to soy sauce SF, the possibility that the visible bands in Worcestershire sauce WoE could be non-specific needs to be ruled out by further research. Analysis of the western blot reactive samples of soy sauce (SF), Worcestershire sauces (WoB and WoE) by the MioBS and RIDASCREEN gliadin sandwich ELISAs resulted in an estimated gluten content that was below the LOQ of the assays, confirming the absence of intact gluten in these samples.

Five of the six teriyaki sauces analyzed by the western blot did not show binding to any protein bands with any of the nine HRP-antibody conjugates. Teriyaki sauce TE (lane

13, Fig. 5), however, showed high intensity bands from approximately 37 to 250 kDa with all the nine antibodies (Fig. 5). Compared to the intact 2.5 µg/mL gluten standard, the binding intensity observed was estimated as > 5 µg/mL. The bands observed were at the same MW or higher compared to what was observed with the intact gluten standard, indicating the presence of some, if not all intact gluten in this teriyaki sauce. Teriyaki sauce TE resulted in a gluten content of 7.2 and 8.1 µg/mL when analyzed by the MioBS sandwich ELISA and RIDASCREEN gliadin sandwich ELISA respectively. However, to conclude that the gluten content is < 20 ppm is not possible since the preparation of teriyaki sauce entails fermentation and the likelihood of proteolysis to generate peptides that may not be detected using the sandwich ELISAs and possibly by western blots

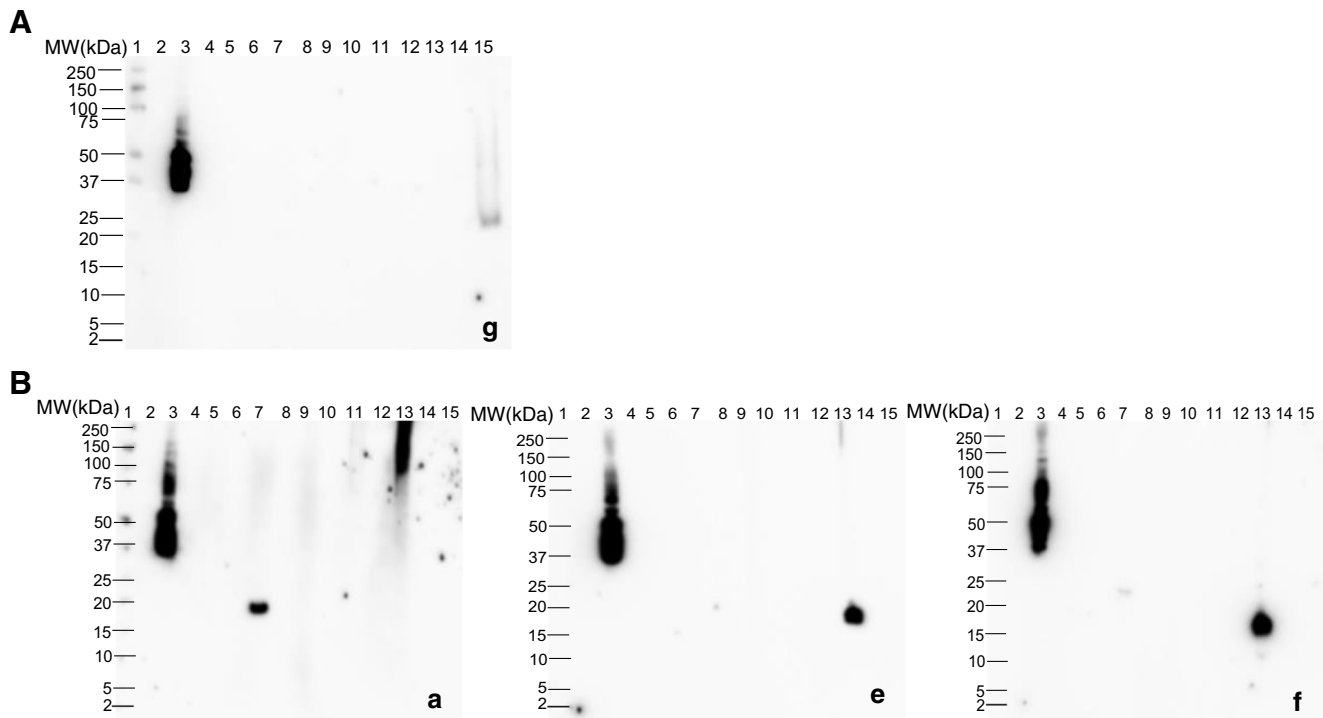


Fig. 4 (A) Western blot band patterns of soy sauces (SA, SB, SV, ST, SX, and SF), using the detector antibody of Microbiologie Gluten (g) ELISA kits. Lane information for western blot: lane 1, molecular weight marker; lane 2, empty; lane 3, 2.5 $\mu\text{g/mL}$ intact gluten standard; lane 4, empty; lane 5, soy sauce SA; lane 6, empty; lane 7, soy sauce SB; lane 8, empty; lane 9, soy sauce SV; lane 10, empty; lane 11, soy sauce ST; lane 12, empty; lane 13, soy sauce SX; lane 14, empty; lane 15, soy sauce SF. (B) Western blot band patterns of Worcestershire sauces (WoA,

WoB, WoC, WoD, WoE, WoF), using the detector antibodies of AgraQuant Gluten G12 (a), Neogen Veratox for Gliadin R5 (e), and Veratox for Gliadin, cat # 8480 (f) ELISA kits. Lane information for western blot: lane 1, molecular weight marker; lane 2, empty; lane 3, 2.5 $\mu\text{g/mL}$ intact gluten standard; lane 4, empty; lane 5, Worcestershire sauce WoA; lane 6, empty; lane 7, Worcestershire sauce WoB; lane 8, empty; lane 9, soy sauce SV; lane 10, empty; lane 11, soy sauce ST; lane 12, empty; lane 13, soy sauce SX; lane 14, empty; lane 15, soy sauce SF

(intense binding to intact gluten in western blot might have exceeded the saturation point, compromising detection of peptides).

Vinegars

Five of six vinegar samples analyzed by western blot did not show any bands with any of the nine antibodies (ESM Fig. S8). However, vinegar VB (lane 7, Fig. 6) showed bands at approximately 50–100 kDa with the Skerritt antibody (i). Bands were observed both at the same MW range as intact gluten standard as well as lower MW (Fig. 6) indicating the presence of both intact gluten and gluten-derived peptides. However, vinegar VB resulted in an estimated gluten content that was below the LOQ of the assays, when analyzed by the MIOBS and RIDACREEN gliadin sandwich ELISAs. Vinegar VB is a malt (dried barley) vinegar, and binding to the Skerritt antibody (prefers HMW glutenins) as observed in the western blot is consistent with a recent mass spectrometry study, where HMW glutenin peptides were found to be abundant in a sample of vinegar [38]. These results indicate that, similar to the other fermented-hydrolyzed products such as beers [20, 36,

39], the HMW glutenin components are resistant to proteolysis during the manufacture of vinegar.

Cluster analysis

The multiplex competitive ELISA previously developed was able to distinguish between differences in the protein/peptide profiles of fermented-hydrolyzed food groups [25]. A subset of the same fermented-hydrolyzed food samples, used in the multiplex ELISA, were analyzed using western blots employing the same nine antibodies. The western blot images displayed multiple differences between the barley beers from those of the wheat beers and sourdough bread protein/peptide band patterns. However, differentiation between the wheat beers and sourdough breads by qualitative analysis of the western blot images was not as obvious. To objectively evaluate whether western blots could differentiate between different categories of food, the gluten concentration of the barley beers, wheat beers, and sourdough breads were estimated from the western blot band signal intensities (utilizing the intact gluten standards and assuming linearity as previously discussed) and cluster analysis was performed. Except for

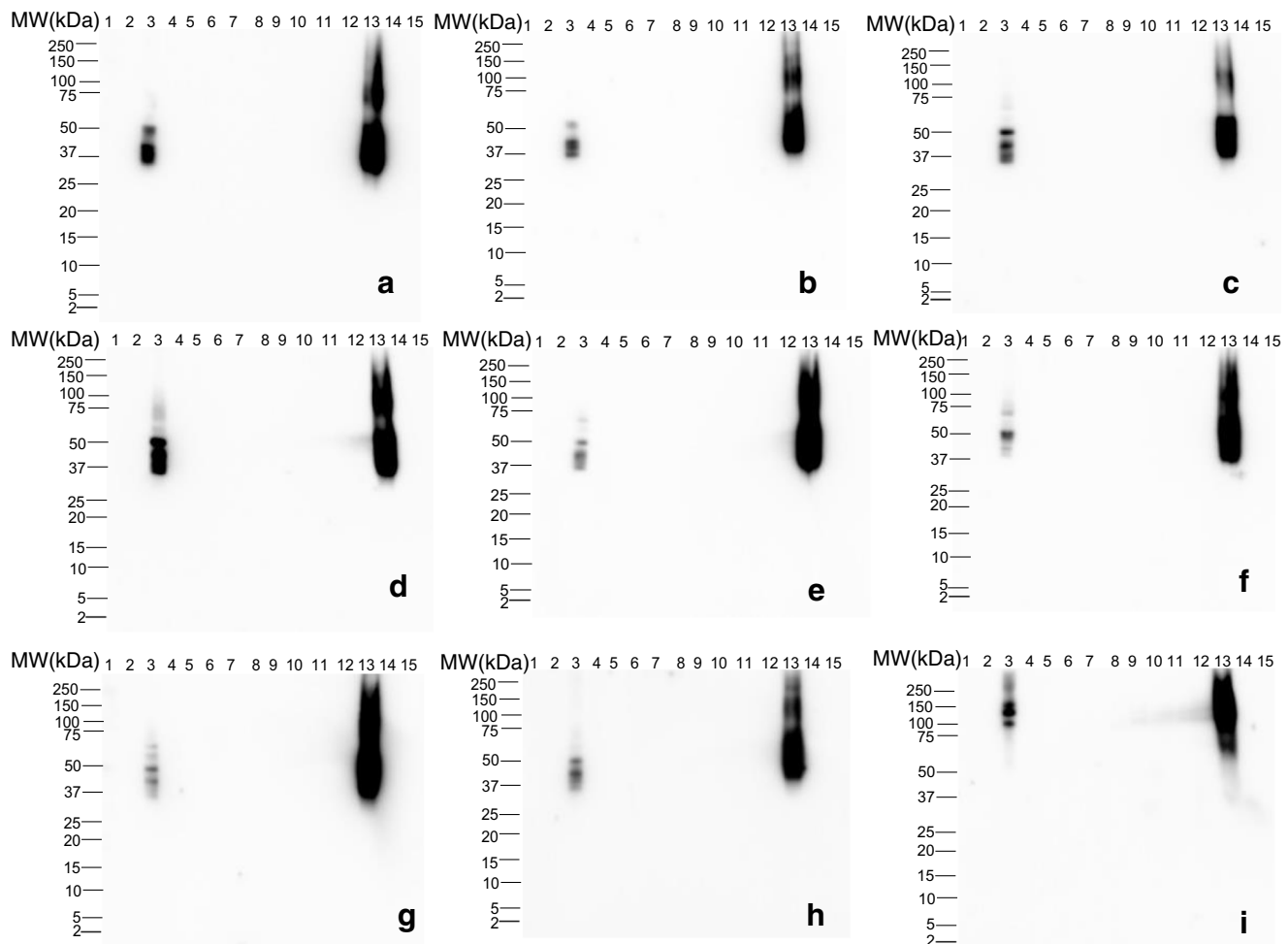


Fig. 5 Western blot band patterns of teriyaki sauces (TA, TB, TC, TD, TE, TH), using the detector antibodies of AgraQuant Gluten G12 (**a**), GlutenTox ELISA Competitive G12 (**b**), RIDASCREEN Gliadin (**c**), RIDASCREEN Gliadin Competitive (**d**), Neogen Veratox for Gliadin R5 (**e**), Veratox for Gliadin, cat # 8480 (**f**), Microbiologique Gluten (**g**), Morinaga Institute of Biological Sciences, Inc. (MIoBS) Gliadin (**h**), and

AllerTek Gluten (**i**) ELISA kits. Lane information for western blot: lane 1, molecular weight marker; lane 2, empty; lane 3, 2.5 $\mu\text{g/mL}$ intact gluten standard; lane 4, empty; lane 5, teriyaki sauce TA; lane 6, empty; lane 7, teriyaki sauce TB; lane 8, empty; lane 9, teriyaki sauce TC; lane 10, empty; lane 11, teriyaki sauce TD; lane 12, empty; lane 13, teriyaki sauce TE; lane 14, empty; lane 15, teriyaki sauce TH

teriyaki sauce TE, the western blot signal intensities of the other three positive soy-based sauces (SF, WoB, and WoE) and one vinegar (VB) sample were too low (compared to the intact gluten standard) to support gluten concentration estimation and modeling. Therefore, soy-based sauces and vinegars were excluded from cluster analysis. Cluster analysis was performed on the estimated gluten concentration values ($\mu\text{g/mL}$ gluten) as well as the relative response values obtained by the nine antibodies (relative to the Skerritt antibody). The purpose was to evaluate whether the western blot analyses could differentiate between the protein/peptide profile characteristics among the three different categories of fermented-hydrolyzed foods, not only based on the differences in the amount of protein/peptide detected, but also, based on differences in the protein/peptide ratios.

Figure 7a and b depict the constellation plots obtained by the cluster analysis of the estimated gluten concentration

values ($\mu\text{g/mL}$ gluten) and the relative response values respectively, generated by the nine antibodies in western blot. The cluster pattern of the same samples generated by the previously developed multiplex competitive ELISA has been shown in Fig. 7c, for comparison. With only a few exceptions (as described below), separate clusters were obtained for the wheat beers, the barley beers and the sourdough breads, both when cluster analysis was performed on the estimated gluten concentration values (a) as well as the relative response values (b). These results were comparable to what was observed in the multiplex competitive ELISA (v). In both the analyses (a and b), wheat beers WI and WF clustered separately from the other ten wheat beers (WI and WF showed different band recognition profile and signal intensities compared to the other wheat beers). Another exception was wheat beer WN, which clustered along with the sourdough breads when analysis was performed on the relative responses values of each antibody

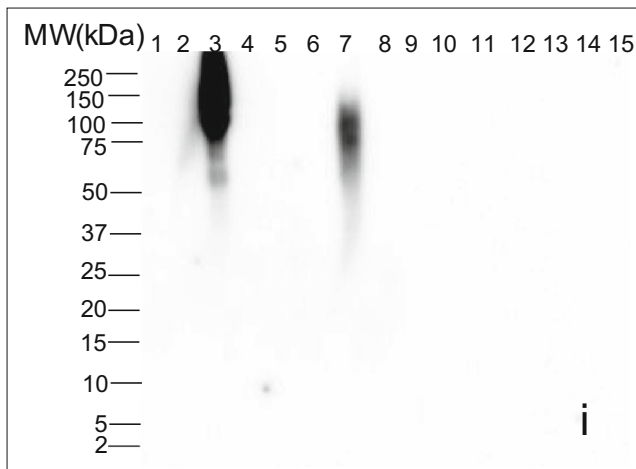


Fig. 6 Western blot band patterns of vinegars (VA, VB, VC, VD, VE, VF), using the detector antibody of AllerTek Gluten (i) ELISA kits. Lane information for western blot: lane 1, molecular weight marker; lane 2, empty; lane 3, 2.5 $\mu\text{g/mL}$ intact gluten standard; lane 4, empty; lane 5, vinegar VA; lane 6, empty; lane 7, vinegar VB; lane 8, empty; lane 9, vinegar VC; lane 10, empty; lane 11, vinegar VD; lane 12, empty; lane 13, vinegar VE; lane 14, empty; lane 15, vinegar VF

(b). The appearance of separate clusters with all the 11 barley beers, all five sourdough breads and nine wheat beers (a and b) indicate the likelihood that the fermentation and associated hydrolysis associated with the manufacturer of these food categories were different. Since the antibodies used in the western blot have specificity towards various gluten proteins (gliadin, glutenin, and deamidate gliadin), the separate clusters also reflect differences in antigenic protein/peptide profiles arising due to the manufacturing process differences. These results indicate that, similar to the previously developed multiplex competitive ELISA, western blot analyses can distinguish the antigenic profile differences among the different categories of fermented-hydrolyzed foods (Fig. 7c, [25]).

Analytical perspective on soy-based sauces and vinegars

Non-specific responses (false positives) were observed with all the soy-based sauces and vinegars that were analyzed in the previously developed multiplex competitive ELISA [25]. In this study, western blot analyses of soy-based sauces and vinegars recognized those false positive responses. Except for soy sauce SF, Worcestershire sauce WoB and WoE, teriyaki sauce TE, and vinegar VB, none of the other soy-based sauces and vinegars resulted in any binding with the nine gluten-specific antibodies (Figs. 4, 5, and 6, ESM Figs. S5-S8). The binding observed with soy sauce SF, which is gluten-free and Worcestershire sauce WoE, which does not contain wheat as an ingredient, could represent inadvertent gluten contamination in those products or the presence of proteins or non-specific substances other than gluten, which showed antibody

specificity. Therefore, further research is needed before confirming such. Western blot is not a highly sensitive method for the detection of low MW peptides that are generated during fermentation and hydrolysis. Upon proteolysis, the multivalency of the protein may be lost and the intensity of the signal per polymeric unit is reduced, thereby making it difficult for visualization of peptides by western blot [19]. Nevertheless, in our study, antibody binding to peptides was observed in beers and sourdough breads as well as in few soy-based sauces and one vinegar sample, where extreme proteolysis of gluten protein is believed to occur. Accurate quantitation of the actual amount of gluten peptides in these samples is not possible by western blot, as the binding intensity observed by the peptides cannot be compared without a suitable calibration reference standard. Further, depending on the degree of proteolysis, the interaction of peptides with antibodies may change [40, 41]. Therefore, sufficient concentration (equivalent to 20 ppm or more intact gluten) of gluten peptides may still be present in these samples despite weaker band intensities observed in the western blot.

Several studies have indicated the absence of any gluten-derived peptides in soy-based sauces by means of different methods such as ELISAs, mass spectrometry, or serum IgE binding studies [26, 38, 42, 43]. The soy sauces included for western blot analysis in our previous study also resulted in no binding with four gluten-specific antibodies [25]. To scrutinize whether soy-based sauces contain no detectable gluten, effort was made to evaluate additional soy-based sauce samples by western blot in this study. The detection of gluten in some of these other sauces may be attributed to different fermentation processes or ingredients used in these soy-based sauces. Chemical hydrolysis as a reason can be ruled out, as except for Worcestershire sauce WoE, which contains chemically hydrolyzed soy sauce, all the positive soy-based sauces appear to be produced by fermentation process. The deliberate use of soy-based sauces produced by classical fermentation process, in the study by Cao et al. [26], may explain the non-detection of any gluten proteins/peptides in the sauces analyzed in that study. However, any deviations from the classical fermentation process or ingredients used may result in a difference in gluten content in the soy-based sauces.

It is possible that the proteins/peptides detected in the soy-based sauces and vinegars, analyzed in our study, contain immunopathogenic epitopes. This is particularly true in the case of vinegar VB, as a previous study reported the presence of immunopathogenic epitopes in the HMW glutenin peptides derived from a malt vinegar [38]. However, not all immunoreactive elements of gluten protein associated with CD are known due to the complex nature of the gluten protein and partial understanding of the pathogenesis of celiac disease [44, 45]. For these reasons, along with the western blot results, it can be concluded that the general perception about the safety of soy-based sauces and vinegars for patients with CD cannot

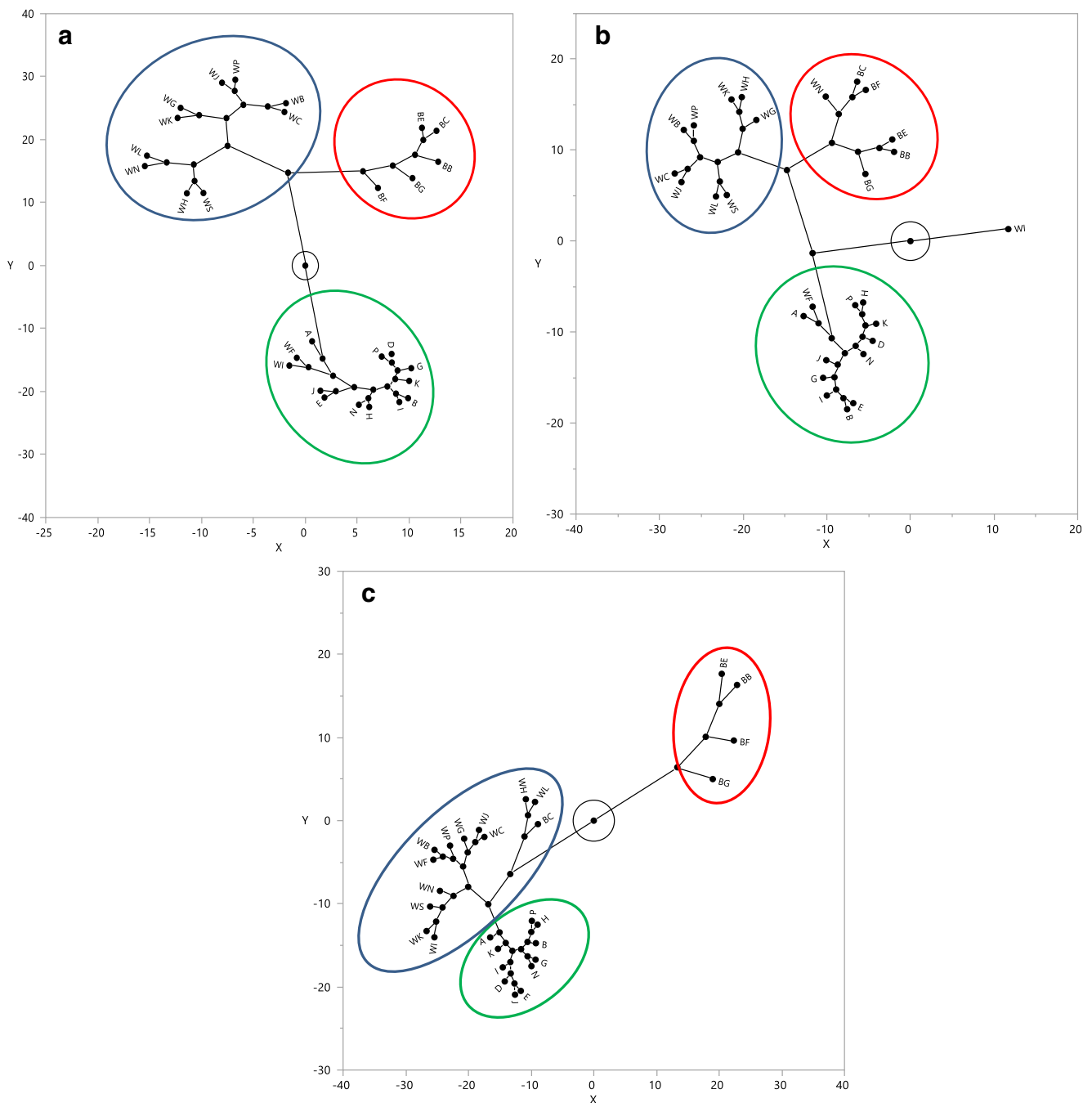


Fig. 7 Constellation plot, displaying the clusters, of the estimated gluten concentration ($\mu\text{g/mL}$) values (**a**), and the relative response values of the nine gluten-specific antibodies to that of the Skerritt antibody (**b**), obtained by western blot for 11 barley beers, 12 wheat beers, and five sourdough

bread. Cluster pattern of the same fermented-hydrolyzed samples obtained by the previously developed multiplex competitive ELISA has been shown in (**c**), for comparison purpose

be decided until a reliable method for accurate quantitation of fermented-hydrolyzed gluten becomes available, or all immunopathogenic epitopes have been identified and can be reliably detected, or biological assays/clinical studies prove the safety of the various soy-based sauces and vinegars.

In summary, the western blot analyses, by separating the antigenic proteins by size, provided novel information regarding differences in the proteolytic processes associated with the

different fermentation processes while confirming the clusters indicated by the multiplex competitive ELISA. The use of two orthogonal, complementary approaches should assist in selecting appropriate calibration standards that may be useful in the qualitative and quantitative characterization of gluten in fermented-hydrolyzed food products. Specifically, the western blot analyses make it possible to distinguish between false positive responses and the presence of gluten-derived

proteinaceous materials while, with the appropriate calibration standards, the multiplex competitive ELISA could be used to quantitate the gluten content. The complementary utility of the two approaches is illustrated by the results with the soy-based sauces and vinegars. Though extensively proteolyzed, residual gluten-derived proteinaceous materials were detected in four soy-based sauces and one vinegar, posing a potential concern for individuals with celiac disease.

Acknowledgments Appreciation is expressed to Dr. Lauren S. Jackson, FDA, for providing invaluable insights into the various manufacturing processes associated with production of fermented-hydrolyzed foods.

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

Western blot analysis of fermented-hydrolyzed foods utilizing gluten specific antibodies employed in a novel multiplex competitive ELISA

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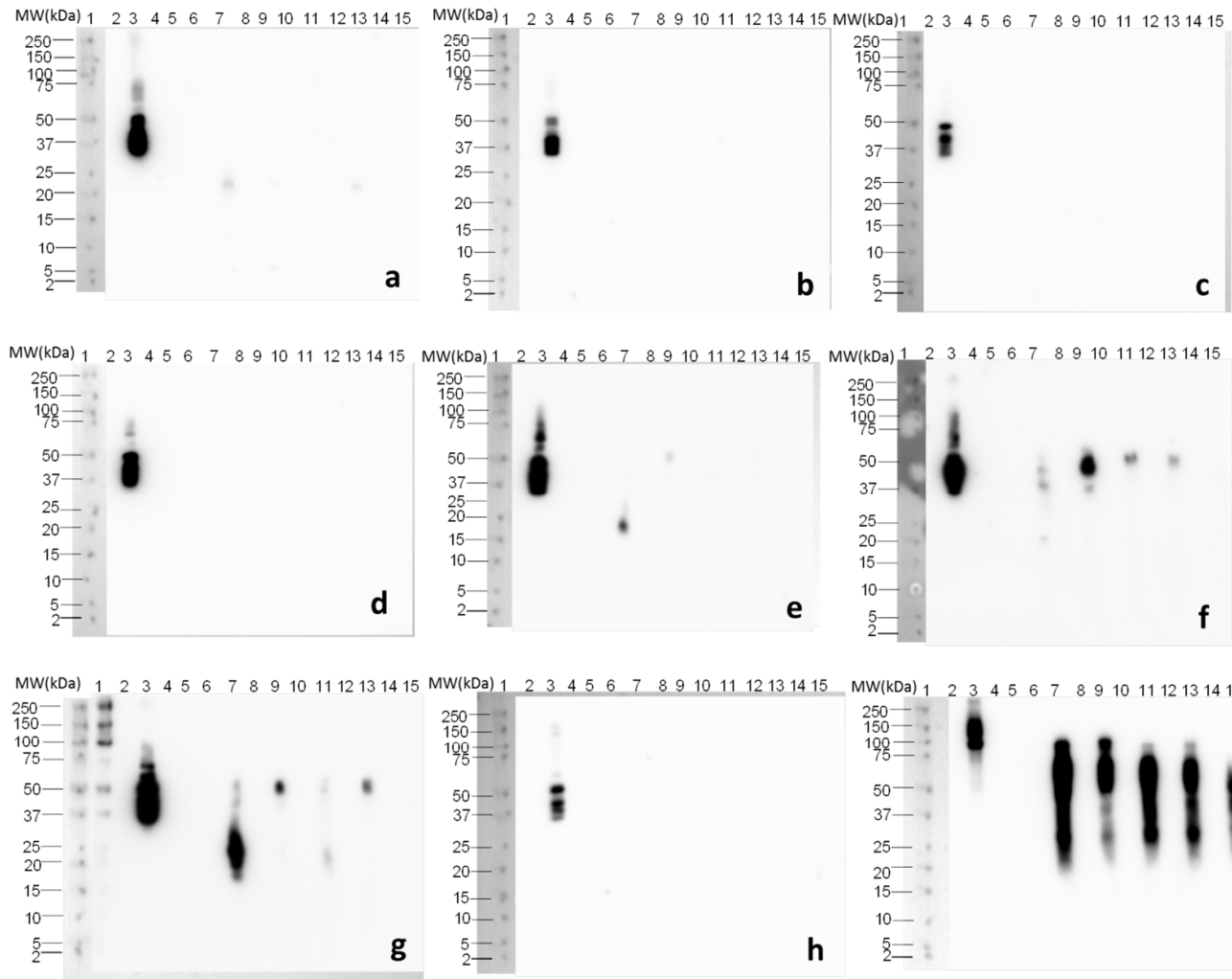


Fig. S1 The superimposed white light and chemiluminescent images of the western blots shown as an example for how the marker proteins were aligned in all western blot figures. Illustrated are the western blot band patterns of barley beers (H, I, G, K, D, N), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Lane information for western blot: Lane 1- Molecular weight marker, Lane 2- Empty, Lane 3- 2.5 µg/mL intact gluten standard, Lane 4- Empty, Lane 5- Barley beer H, Lane 6- Empty, Lane 7- Barley beer I, Lane 8- Empty, Lane 9- Barley beer G, Lane 10- Empty, Lane 11- Barley beer K, Lane 12- Empty, Lane 13- Barley beer D, Lane 14- Empty, Lane 15- Barley beer N

Individual band intensity profile obtained by western blot

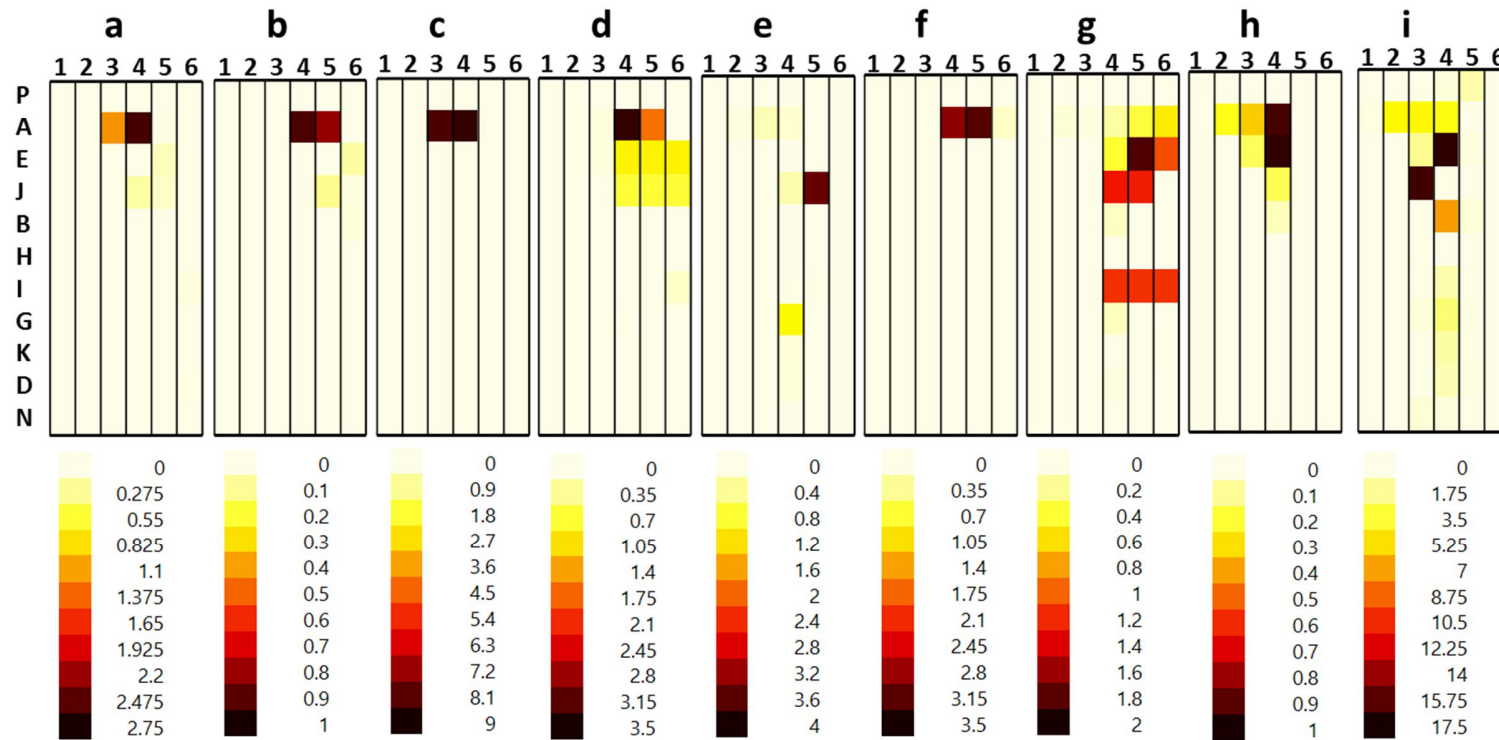


Fig. S2 Individual band intensities (expressed as ppm gluten) profile obtained by western blot for 11 barley beers (P, A, E, J, B, H, I, G, K, D, N) with the gluten specific detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MIoBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Bands recognized at MW ranges 150-250 kDa (1), 100-150 kDa (2), 60-100 kDa (3), 30-60 kDa (4), 20-30 kDa (5), 15-20 kDa (6) with intensities expressed as $\mu\text{g/mL}$ gluten has been illustrated

Individual band intensity profile obtained by western blot

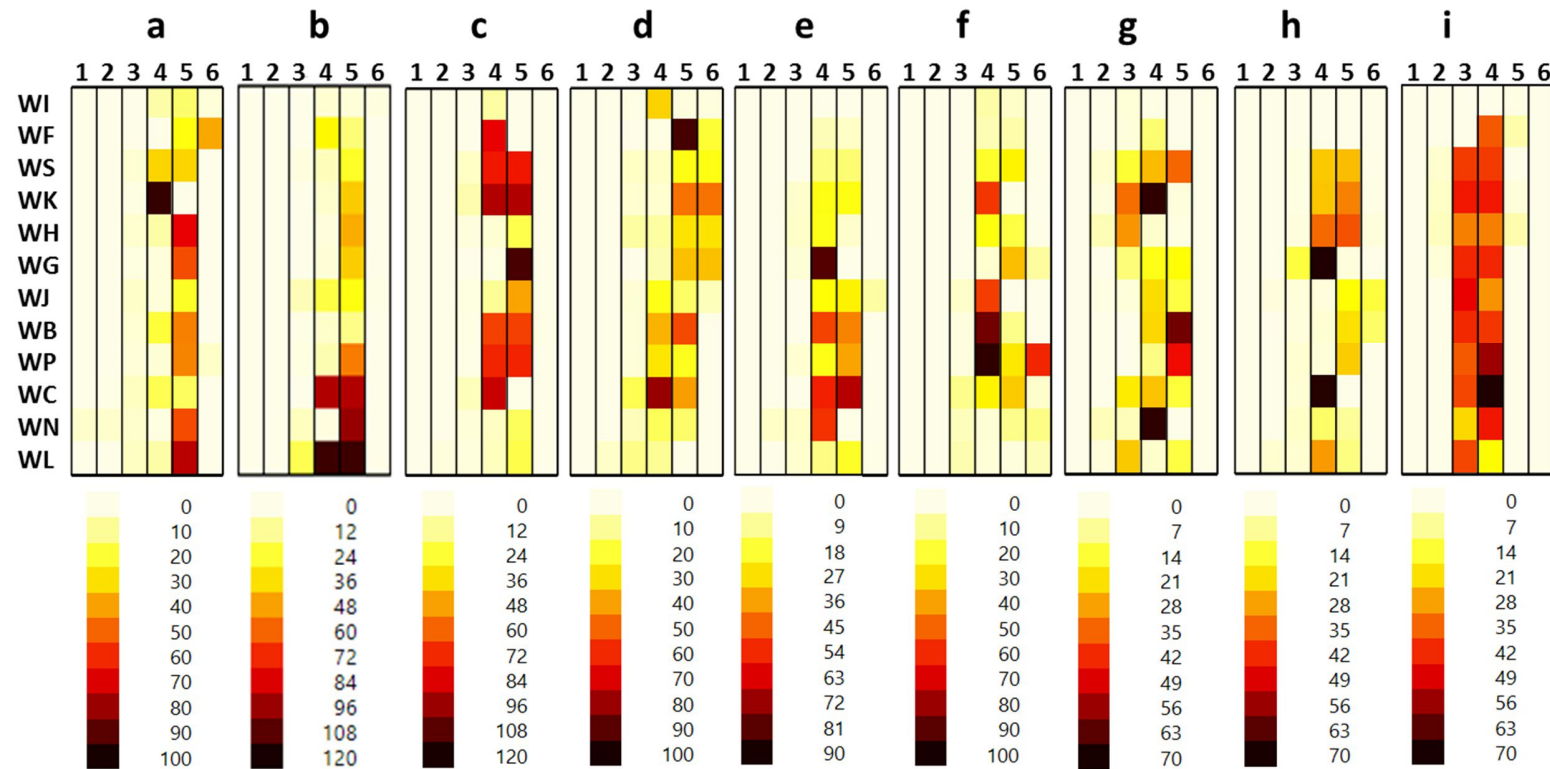


Fig. S3 Individual band intensities (expressed as ppm gluten) profile obtained by western blot for 12 wheat beers (WI, WF, WS, WK, WH, WG, WJ, WB, WP, WC, WN, WL) with the gluten specific detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MIoBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Bands recognized at MW ranges 150-250 kDa (1), 100-150 kDa (2), 60-100 kDa (3), 30-60 kDa (4), 20-30 kDa (5), 15-20 kDa (6) with intensities expressed as $\mu\text{g/mL}$ gluten has been illustrated

Individual band intensity profile obtained by western blot

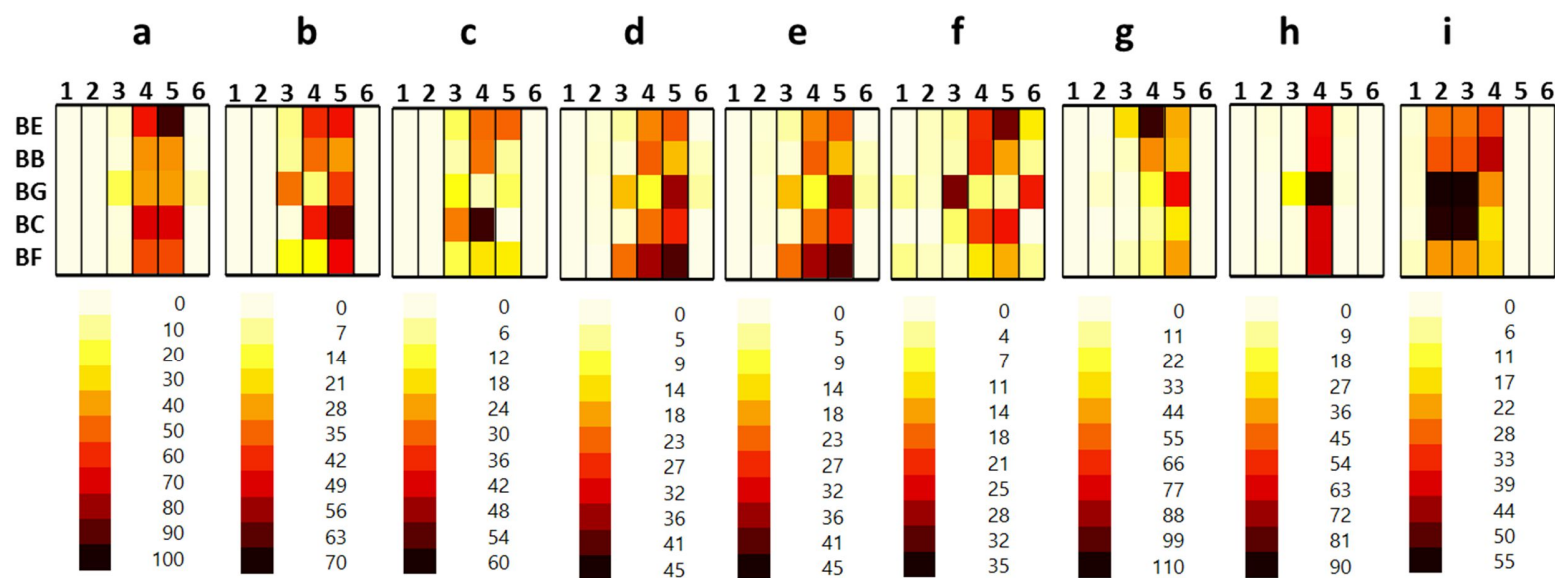


Fig. S4 Individual band intensities (expressed as ppm gluten) profile obtained by western blot for 5 sourdough breads (BE, BB, BG, BC, BF) with the gluten specific detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Bands recognized at MW ranges 150-250 kDa (1), 100-150 kDa (2), 60-100 kDa (3), 30-60 kDa (4), 20-30 kDa (5), 15-20 kDa (6) with intensities expressed as $\mu\text{g/mL}$ gluten has been illustrated

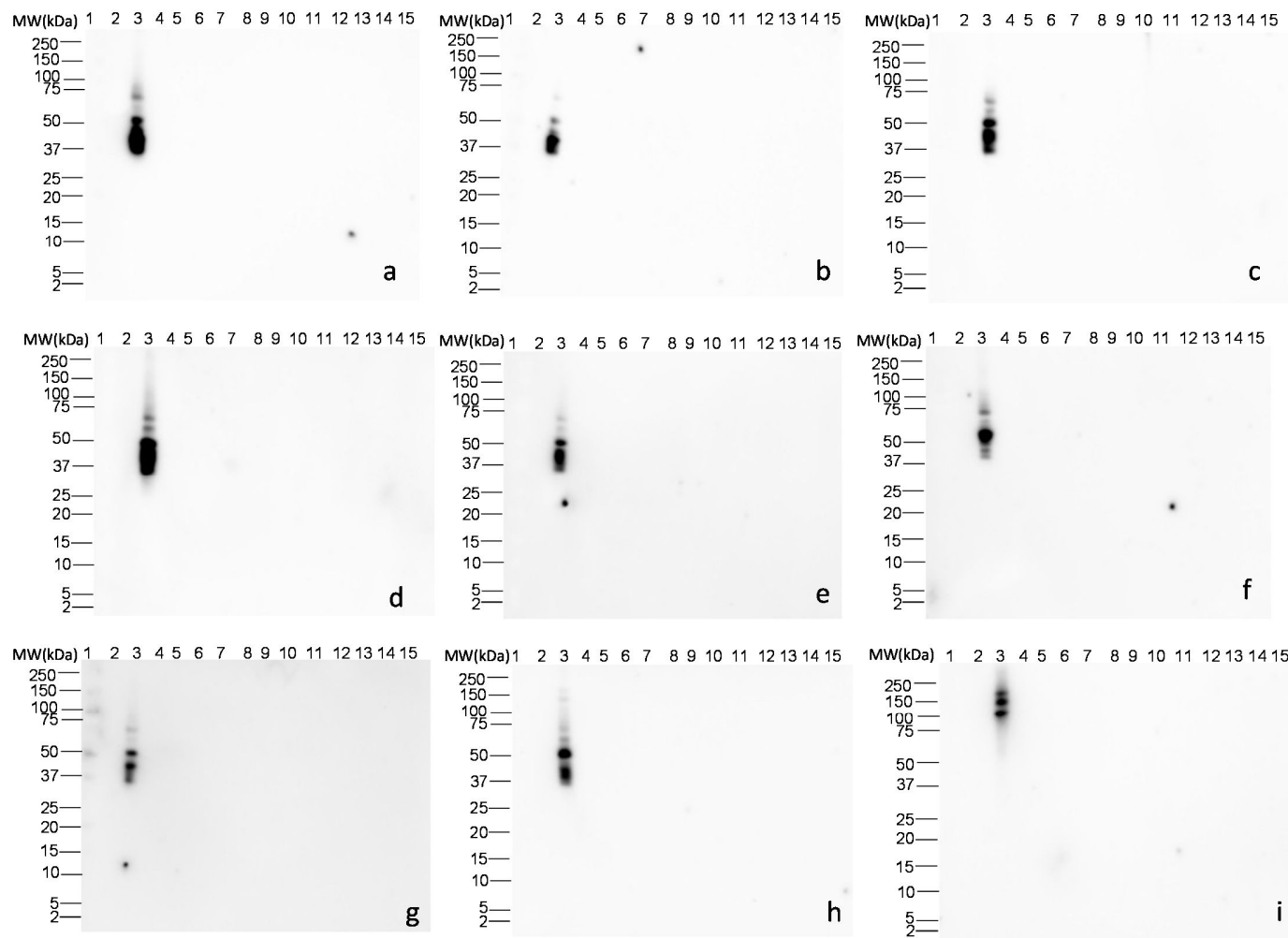


Fig S5 Western blot band patterns of soy sauces (SC, SD, SE, SI, SJ, SS), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Lane information for western blot: Lane 1- Molecular weight marker, Lane 2- empty, Lane 3- 2.5 µg/mL intact gluten standard, Lane 4- Empty, Lane 5- soy sauce SC, Lane 6- Empty, Lane 7- soy sauce SD, Lane 8- Empty, Lane 9- soy sauce SE, Lane 10- Empty, Lane 11- soy sauce SI, Lane 12- Empty, Lane 13- soy sauce SJ, Lane 14- Empty, Lane 15- soy sauce SS

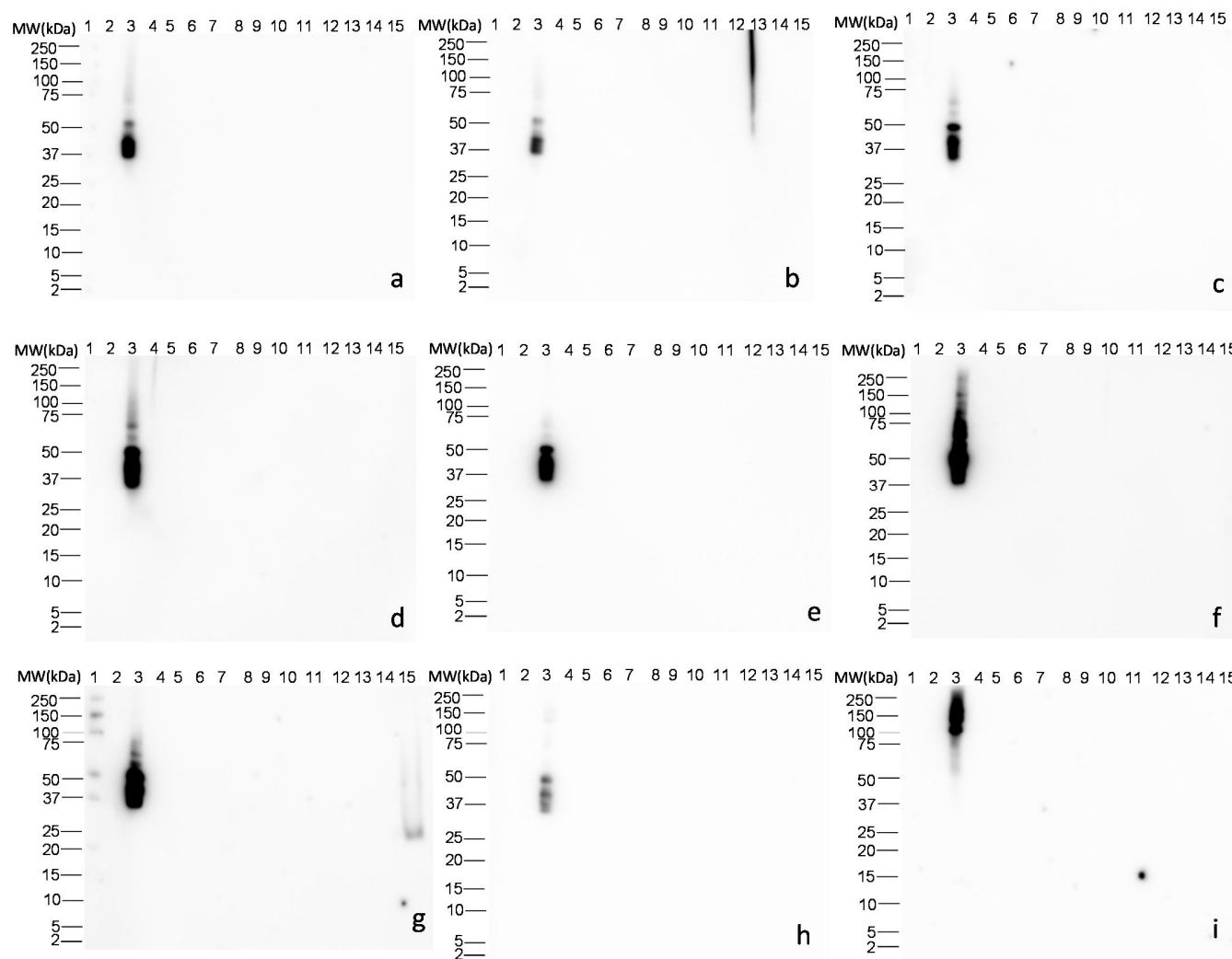


Fig S6 Western blot band patterns of soy sauces (SA, SB, SV, ST, SX, SF), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Lane information for western blot: Lane 1- Molecular weight marker, Lane 2- empty, Lane 3- 2.5 µg/mL intact gluten standard, Lane 4- Empty, Lane 5- soy sauce SA, Lane 6- Empty, Lane 7- soy sauce SB, Lane 8- Empty, Lane 9- soy sauce SV, Lane 10- Empty, Lane 11- soy sauce ST, Lane 12- Empty, Lane 13- soy sauce SX, Lane 14- Empty, Lane 15- soy sauce SF

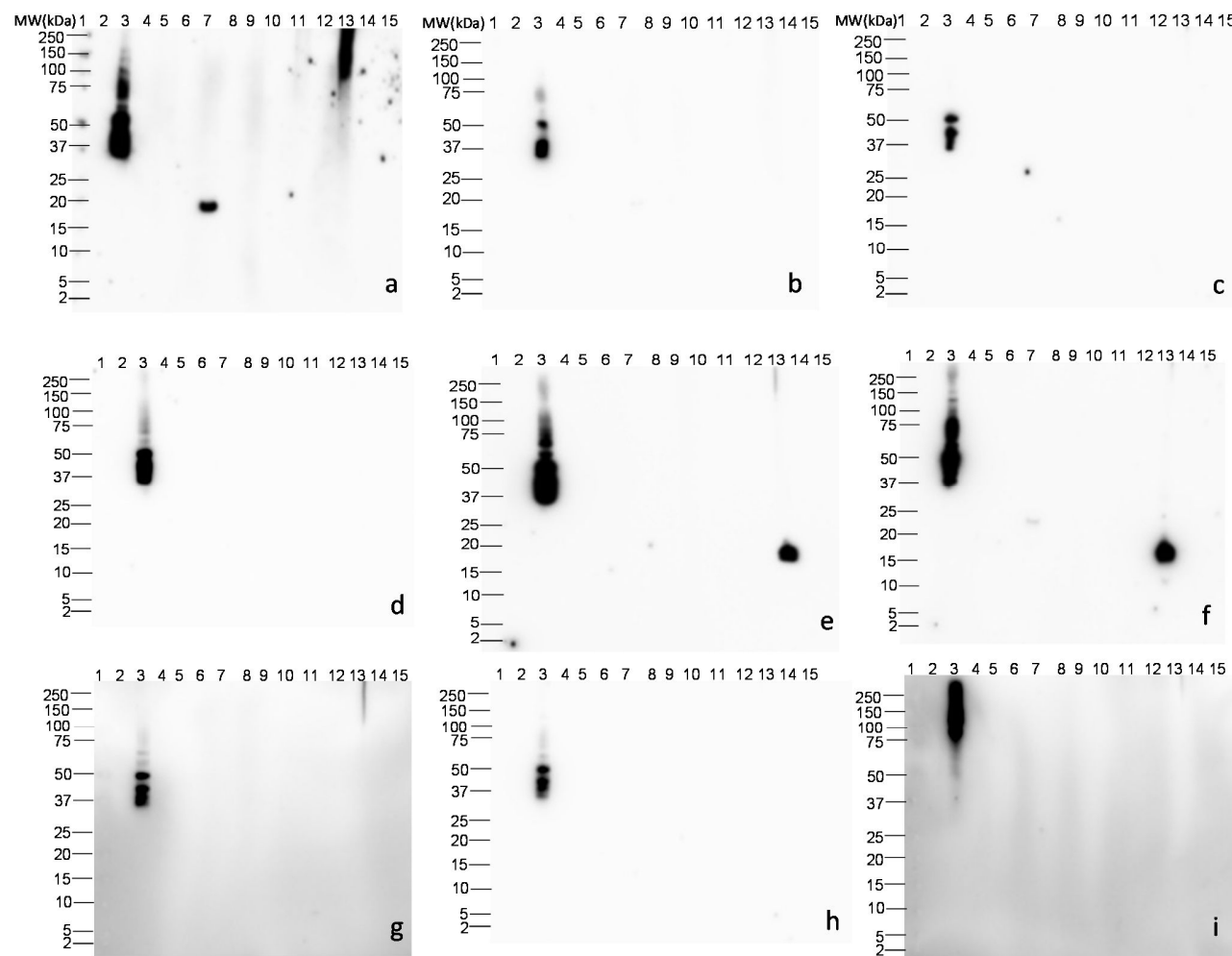


Fig S7 Western blot band patterns of Worcestershire sauce (WoA, WoB, WoC, WoD, WoE, WoF), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Lane information for western blot: Lane 1- Molecular weight marker, Lane 2- empty, Lane 3- 2.5 µg/mL intact gluten standard, Lane 4- Empty, Lane 5- Worcestershire sauce WoA, Lane 6- Empty, Lane 7- Worcestershire sauce WoB, Lane 8- Empty, Lane 9- Worcestershire sauce WoC, Lane 10- Empty, Lane 11- Worcestershire sauce WoD, Lane 12- Empty, Lane 13- Worcestershire sauce WoE, Lane 14- Empty, Lane 15- Worcestershire sauce WoF

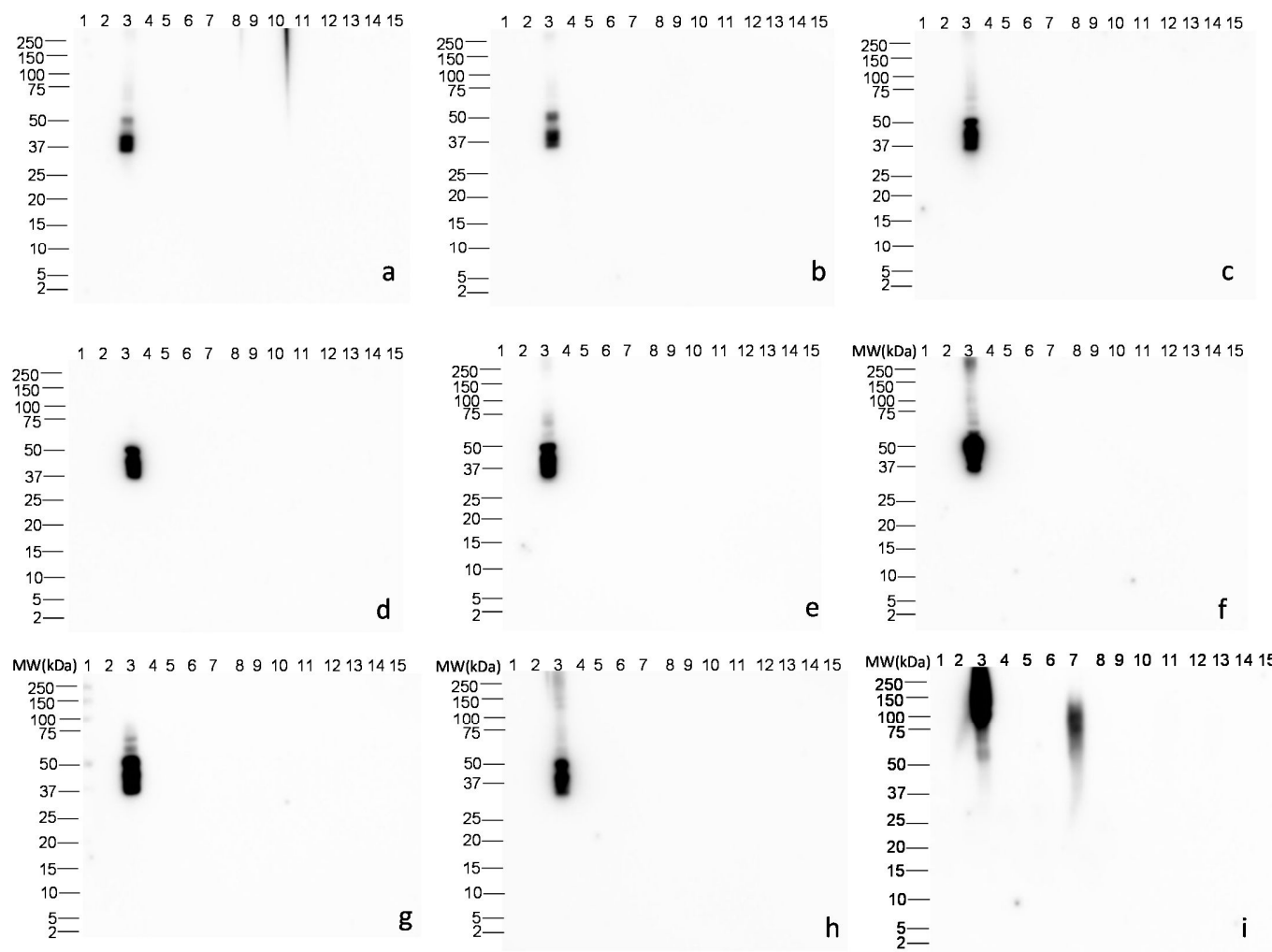


Fig S8 Western blot band patterns of vinegars (VA, VB, VC, VD, VE, VF), using the detector antibodies of AgraQuant Gluten G12 (a), GlutenTox ELISA Competitive G12 (b), RIDASCREEN Gliadin (c), RIDASCREEN Gliadin Competitive (d), Neogen Veratox for Gliadin R5 (e), Veratox for Gliadin, cat # 8480 (f), Microbiologique Gluten (g), Morinaga Institute of Biological Sciences, Inc. (MioBS) Gliadin (h), AllerTek Gluten (i) ELISA kits. Lane information for western blot: Lane 1- Molecular weight marker, Lane 2- empty, Lane 3- 2.5 µg/mL intact gluten standard, Lane 4- Empty, Lane 5- vinegar VA, Lane 6- Empty, Lane 7- vinegar VB, Lane 8- Empty, Lane 9- vinegar VC, Lane 10- Empty, Lane 11- vinegar VD, Lane 12- Empty, Lane 13- vinegar VE, Lane 14- Empty, Lane 15- vinegar VF