Journal of Food Protection, Vol. 78, No. 6, 2015, Pages 1167–1177
doi:10.4315/0362-028X.JFP-14-546

Detection and Quantification of Gluten during the Brewing and Fermentation of Beer Using Antibody-Based Technologies

RAKHI PANDA,[1] HANS F. ZOERB,[2] CHUNG Y. CHO,[1] LAUREN S. JACKSON,[3] AND ERIC A.E. GARBER[1]*

[1]U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Regulatory Science, Division of Bioanalytical Chemistry, College Park, Maryland 20740; [2]Department of Food Science, University of Wisconsin–Madison, Madison, Wisconsin 53706; and [3]U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Safety, Division of Food Processing Science and Technology, Bedford Park, Illinois 60501, USA

MS 14-546: Received 18 November 2014/Accepted 23 January 2015

ABSTRACT

In 2013 the U.S. Food and Drug Administration (FDA) defined the term ''gluten-free'' and identified a gap in the analytical methodology for detection and quantification of gluten in foods subjected to fermentation and hydrolysis. To ascertain the ability of current enzyme-linked immunosorbent assays (ELISAs) to detect and quantify gluten in fermented and hydrolyzed products, sorghum beer was spiked in the initial phases of production with 0, 20, and 200 μg/ml wheat gluten, and samples were collected throughout the beer production process. The samples were analyzed using five sandwich ELISAs and two competitive ELISAs and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with Western analysis employing four antibodies (MIoBS, R5, G12, and Skerritt). The sensitivity of the MIoBS ELISA (0.25 ppm) enabled the reliable detection of gluten throughout the manufacturing process, including fermentation, when the initial concentration of 20 μg/ml dropped to 2 μg/ml. The R5 antibody–based and G12 antibody–based sandwich ELISAs were unable to reliably detect gluten, initially at 20 μg/ml, after the onset of production. The Skerritt antibody–based sandwich ELISA overestimated the gluten concentration in all samples. The R5 antibody–based and G12 antibody–based competitive ELISAs were less sensitive than the sandwich ELISAs and did not provide accurate results for quantifying gluten concentration. The Western analyses were able to detect gluten at less than 5 μg/ml in the samples and confirmed the results of the ELISAs. Although further research is necessary before all problems associated with detection and quantification of hydrolyzed and fermented gluten are resolved, the analytical methods recommended by the FDA for regulatory samples can detect ≥20 μg/ml gluten that has undergone brewing and fermentation processes associated with the manufacture of beer.

In 2013, the U.S. Food and Drug Administration (FDA) defined gluten-free *(26)*:

> the food bearing the claim does not contain an ingredient that is a gluten-containing grain (e.g., spelt wheat); an ingredient that is derived from a gluten-containing grain and that has not been processed to remove gluten (e.g., wheat flour); or an ingredient that is derived from a gluten-containing grain and that has been processed to remove gluten (e.g., wheat starch), if the use of that ingredient results in the presence of 20 parts per million (ppm) or more gluten in the food (i.e., 20 milligrams (mg) or more gluten per kilogram (kg) of food); or inherently does not contain gluten; and that any unavoidable presence of gluten in the food is below 20 ppm gluten (i.e., below 20 mg gluten per kg of food). … some food matrices, such as fermented or hydrolyzed foods, may lack currently available scientifically valid methods that can be used to accurately determine if these foods contain < 20 ppm gluten.

The gluten-free regulation does not require that gluten derived from wheat, rye, or barley be distinguished nor does it include oats as a source of gluten. Several commercial enzyme-linked immunosorbent assay (ELISA) test kits suitable for the detection of gluten are available. At the time the gluten-free regulation was drafted, only the R5 monoclonal antibody–based and Morinaga Institutes of Biological Sciences (MIoBS) polyclonal antibody–based sandwich ELISAs (R5-sand and MIoBS-sand, respectively) had been validated by multiple laboratories at levels suitable for regulatory enforcement, extensively evaluated in research studies, and officially recognized by other governments or governmental agencies *(1, 4, 10, 13, 16, 17, 27)*. Since the 2013 gluten-free regulation, the G12 monoclonal antibody–based, A1-G12 monoclonal antibody–based, and Skerritt monoclonal antibody–based sandwich ELISAs (G12-sand, A1-G12-sand, and Skerritt-sand, respectively) have been validated, extensively studied, and adapted to be suitable for detection of 20 ppm of gluten *(2, 18, 19, 21, 22, 25, 28)*. In addition, two competitive ELISAs, based on the R5 and G12 monoclonal antibodies (R5-comp and G12-comp), have been commercialized *(12, 14, 18, 19)*.

Immunodiagnostic and mass spectrometric assays have been used to study barley-based beers and the presence of

* Author for correspondence. Tel: 240-402-2224; Fax: 301-436-2332; E-mail: eric.garber@fda.hhs.gov.

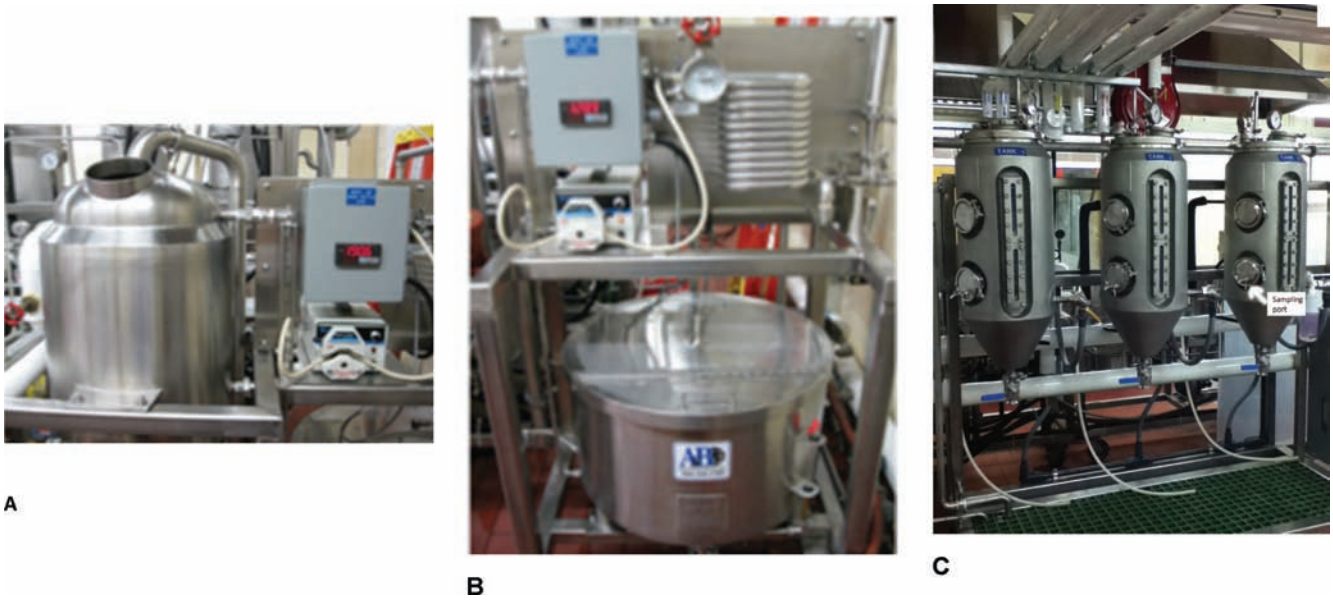

FIGURE 1. *Brewing facilities. (A) Brew kettle, (B) whirlpool and heat exchanger, and (C) fermentors with sampling port indicated by arrow.*

gluten-derived peptides *(5–7, 11, 15, 20, 23, 24).* These studies have provided much information but have not provided quantitative information about how the brewing process affects gluten in beer. When starting the brewing process with grains that contain gluten, the concentration of free gluten depends on the grain malting process, how the wort is prepared and lautered (clarified), the fermentation step, and any final treatments such as filtration. In contrast, when starting the brewing process with sorghum syrup, the concentration of gluten is directly controlled by the amount of gluten added to the brew.

To address these gaps in our understanding of how the brewing of beer affects gluten concentrations and how our analytical methodology can be applied to hydrolyzed and fermented gluten, wheat gluten was added to the initial step of sorghum beer production, and the concentration of gluten was measured throughout production using various ELISA test kits and Western blot analysis.

MATERIALS AND METHODS

Reagents. Wheat gluten (≥80% protein; G5004), 10 mM phosphate-buffered saline (PBS; P3813), Tween 20 (P7949), and other chemical reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) and other suppliers of analytical grade reagents as specified. PBS-Tween was prepared by adding 0.1% (vol/vol) Tween 20 to the 10 mM PBS. Sorghum syrup (100% sorghum; H022836, Cargill Co., Wayzata, MN) contained approximately 1 mg/ml protein as determined by Lowry and Bradford analyses, and Brix was measured with a handheld refractometer as 78%. Cascade hops pellets (20151A, Brew and Grow, Crystal Lake, IL) had an alpha acid concentration of 7.1% and a protein concentration of 15% by bicinchoninic acid analysis.

Production of sorghum-based beers. Sorghum-based beers were prepared from wort spiked with 0 μg/ml (gluten-free control, SB0), 20 μg/ml (SB20), and 200 μg/ml (SB200) wheat gluten before boiling. The brewing and fermentation process was conducted using pilot-scale brewing equipment (Fig. 1) at the University of Wisconsin–Madison Department of Food Science. The brewing process involved transferring 7.9 kg of sorghum syrup to a stainless steel steam-jacketed boil kettle and adding sufficient 71°C water to bring the volume to 55 liters. Subsequently, 12.5 ml of phosphoric acid (85%; Fisher Scientific, Pittsburgh, PA), 35 ml of 33% calcium chloride (Sigma-Aldrich), and 0, 1.1, or 11 g of the wheat gluten suspended in 50 ml of 95% ethanol (Quality Control Distilling Co., Bardstown, KY) were added. A second 50-ml aliquot of ethanol was used to transfer residual gluten from the measuring vessel into the kettle. After the contents of the kettle started to boil (~99°C), 58 g of Cascade hops pellets, 8 g of yeast food (Wyeast Laboratories, Inc., Odell, OR), and 30 g of nitrogen source (food grade urea and diammonium phosphate, Brew and Grow) were added. The mixture was boiled for 1 h, and the wort was then pumped into a whirlpool for clarification. The clarified wort was cooled to 21°C in a tubular heat exchanger and then transferred to a stainless steel conical fermentor. Three 125-ml bags of yeast (American Ale 1056, Wyeast Laboratories) were added to start the fermentation process. The fermenting beer was kept at 20°C for up to 14 days.

Samples were collected periodically during the production process and stored in high-density polyethylene bottles (Nalgene Nunc, Rochester, NY) at −20°C until analyzed. Samples (300 ml) were collected from the following process steps: (i) wort from the boil kettle before it reached a boil, (ii) wort from the boil kettle after it reached a boil, (iii) wort after clarification in the whirlpool, (iv) solid material (trube) obtained after clarification of wort in the whirlpool, (v) beer in the fermentor after 1 h of fermentation (addition of yeast), (vi) beer in the fermentor after 1 day of fermentation, (vii) beer in the fermentor after 7 days of fermentation, and (viii) beer in the fermentor after 8 and 12 days of fermentation for SB0 and SB20, respectively. The specific gravity, percentage of solids, and pH of samples were monitored with a hygrometer, a hand-held refractometer (Atago U.S.A., Bellevue, WA), and a pH meter, respectively.

ELISA. All beer samples were analyzed in triplicate using five sandwich ELISA test kits and two competitive ELISA test kits, with all analyses repeated on a second set of ELISA plates and standards included in each analysis as per the manufacturers'

guidelines. The properties of each of the seven ELISA test kits are listed in Table 1 with the limits of detection (LODs) and limits of quantitation (LOQs) as defined by the test kit manufacturer for gluten. To minimize variability, the samples were subjected to freeze-thawing only twice, once when the frozen samples (collected from the brewing process) were aliquoted for future ELISA analysis and when analyzed. When the ELISA did not specify a protocol for analysis of beer, the procedure recommended for problematic samples (e.g., those containing polyphenols or tannins) was used. The A1-G12-sand and G12-comp ELISAs prescribed procedures involving 200-, 500-, and 1,000-fold dilution of the samples. Accordingly, the 0 μg/ml (SB0) and 20 μg/ml (SB20) wheat gluten samples were diluted 200-fold, and the 200 μg/ml (SB200) samples were diluted 500-fold.

The concentration of gluten in the samples was determined by interpolating the responses relative to those of the test kit standards. Polynomial trendlines, chosen by correlation coefficient ($R^2 > 0.99$) and consistent with a binding process, facilitated the interpolation process. The MIoBS-sand, R5-sand, G12-sand, and R5-comp ELISAs employed third-order polynomial trendlines with average R^2 values of 0.9997, 0.9999, 0.9997, and 0.9961 for eight, six, five, and five standards of different concentrations, respectively. The A1-G12-sand, Skerritt-sand, and G12-comp ELISA responses were interpolated using second-order polynomial trendlines with average R^2 values of 0.9970, 0.9977, and 0.9912, respectively, for standard curves, each based on six different concentrations.

A multimode microplate reader (Infinite M200, Tecan, Morrisville, NC) with a quad monochrometer design (two excitation and two emission) was used to measure the responses (absorbance values) at the specified wavelengths recommended by the ELISA test kit manufacturers. The Infinite M200 is unique in its wavelength accuracy (<0.5 nm), wavelength reproducibility (<0.5 nm), and absorbance range (0 to 4).

Western analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Novex 4 to 12% bis-Tris gels (Invitrogen, Carlsbad, CA) run at 200 V for 30 min. The proteins were electrotransferred to polyvinylidene difluoride membranes at 30 V for 60 min and immunoblotted with the detector antibodies included in the MIoBS, RIDASCREEN Gliadin ELISA (R5), AgraQuant ELISA Gluten G12, and Aller-Tek Gluten ELISA (Skerritt) test kits diluted 1:10, 1:110, 1:10, and 1:50, respectively. The higher dilution of the R5 antibody was necessary because it was supplied as an 11-fold concentrate. Otherwise, only the Skerritt antibody required greater than a 10-fold dilution from the concentration used in the ELISA, which was consistent with higher than expected ELISA results for the amount of gluten present in the samples. Chemiluminescence was measured using SuperSignal West Dura Extended Duration Chemiluminescent Substrate (Pierce, Thermo Scientific, Waltham, MA) and a ChemiDoc XRS+ Imager (BioRad, Hercules, CA). Gluten standards were run with the samples on each Western blot.

RESULTS AND DISCUSSION

The main objective of this study was to determine whether the currently employed ELISA methods could reliably detect the minimum concentration of gluten present in beer that would be in violation of the gluten-free definition, i.e., 20 μg/ml gluten present at the start of the brewing process. A secondary goal was to evaluate the suitability of a hydrolyzed gluten standard and competitive ELISAs for quantifying gluten during beer production. Changes in the detectability of gluten in sorghum beer at different points in the brewing and fermentation process also were evaluated.

ELISAs. Five sandwich ELISAs and two competitive ELISAs were used to measure the gluten concentration in sorghum beer spiked with 0, 20, and 200 μg/ml gluten at the initial phase of production. Liquid samples of wort or beer obtained at key stages of production, i.e., at the beginning of the boiling step, after the boiling step, after clarification, and throughout fermentation, were analyzed for gluten. ELISAs were also used to quantify gluten in the solids obtained after clarification of the wort. Tables 2, 3, and 4 summarize the results obtained from the ELISAs of the sorghum beer samples. Absorbance values of samples and calibrants at lower concentrations are presented in Table 2. To avoid overextrapolation of the calibration curve and misinterpretation of responses less than the average absorbance of the first nonzero standard (avS1), Table 2 presents these absorbance responses in comparison with the background absorbance as defined as the average zero standard plus (or minus for the competitive ELISAs) three times the standard deviation (SD) of the zero standards (Bkgd $\pm$ 3SD). Table 3 summarizes the average coefficient of variation (CV) of the absorbance responses generated by the various samples, with each sample analyzed in triplicate on duplicate plates. These CV percentages are the variances of the absorbance responses generated by the samples unaltered by the mathematical processing, which occurs when data are interpolated against curved trendlines. The interpolated concentrations as determined by the various ELISAs are presented in Table 4. In addition to calibrating the absorbance responses against the test kit standards supplied with a particular ELISA, the responses generated by the samples analyzed using the R5-comp ELISA were also interpolated against the R5-sand test kit standards. A similar cross-comparison was done with the R5-comp test kit standards and the samples analyzed using the R5-sand ELISA (data not shown).

Sandwich ELISAs. Table 2 demonstrates the ability of MIoBS-sand to reliably detect the presence of 20 μg/ml added wheat gluten (SB20) in wort from before boiling through completion of the fermentation process. The absorbance values generated by the samples exceeded both the Bkgd + 3SD and the avS1 by more than sixfold and more than eightfold, respectively (Table 2). The samples derived from wort containing 200 μg/ml wheat gluten (SB200) generated responses with the MIoBS-sand ELISA greater than the most concentrated standard (16 μg/ml gluten) and were characteristic of a saturated assay. As such, the accuracy of the assay for quantifying gluten in the SB200 samples is questionable, but the samples exceeding 16 ppm and displaying responses characteristic of considerably greater concentrations were not (Table 4).

With the R5-sand ELISA, the gluten concentrations measured for nearly all of the SB20 samples were below the LOQ. The two exceptions were the samples taken at the start of the boil and from the solids after the whirlpool (Table 2).

TABLE 1. *Gluten ELISA test kit properties as specified by the manufacturers*

Test kit	Abbreviation[a]	Manufacturer	Antibodies[b]		Extraction	Dilution	Gluten concn (μg/g or μg/ml [ppm])[c]			
			Capture	Detector			LOD	LOQ	S1[d]	Upper limit
Wheat Protein ELISA Kit	MIoBS-sand	Morinaga Institute of Biological Sciences	Polyclonal	Polyclonal	Sodium dodecyl sulfate, β-mercaptoethanol	400	0.24[e]	0.25	0.25	16
RIDASCREEN Gliadin	R5-sand	R-Biopharm, AG	R5 Mab	R5 Mab	Mendez cocktail (guan·HCl/β-mer) and nonfat dry milk, dilute to 60% ethanol	500	3	5	5	80
AgraQuant ELISA Gluten G12	G12-sand	Romer Labs	G12 Mab	G12 Mab	Fish gelatin w proprietary, diluted to 60% ethanol	400	2	4	4	200
GlutenTox ELISA Sandwich	A1-G12-sand	Biomedal Diagnostics	A1 Mab	G12 MAb	Proprietary solution	200[f]		0.6	0.6	10
Aller-Tek Gluten ELISA	Skerritt-sand	ELISA Technologies	Skerritt Mab	Skerritt Mab	Proprietary additive in 40% ethanol	100		5	5	80
RIDASCREEN Gliadin Competitive	R5-comp	R-Biopharm, AG	Gliadin (Ag)	R5 MAb	60% ethanol, fish gelatin	500	2.6	10	10	270
GlutenTox ELISA Competitive	G12-comp	Biomedal Diagnostics	Gliadin (Ag)	G12 MAb	Proprietary solution	200[f]		3	3	48

[a] sand, sandwich ELISA; comp, competitive ELISA.
[b] Antibodies used in the ELISAs. MAb, monoclonal antibody; Ag, antigen used to precoat competitive ELISA plates.
[c] As stated by the test kit manufacturer, concentrations in the analytical sample when diluted according to kit instructions.
[d] The lowest (first) nonzero standard supplied with the test kit.
[e] Units of wheat protein, converted to units of gluten using manufacturer's claimed sensitivity of 0.3 μg/g protein, using 0.8 g of gluten per gram of protein.
[f] Protocol states that samples are to be diluted 200-, 500, or 1,000-fold. Standards supplied for 1.56, 7.5, 25, and 120 ng/ml gliadin are equivalent to 0.6, 3, 10, and 48 μg/ml gluten, respectively, in samples diluted 200-fold and 1.56, 7.5, 25, and 120 μg/ml gluten, respectively, in samples diluted 500-fold.

TABLE 2. *ELISA absorbance (optical density) values of sorghum beer spiked with 20 μg/ml gluten*[a]

ELISA	Background response			S1		Start boil	End boil	Clarify	1 h ferment	1 day ferment	7 days ferment	Final	Solids
	Plate 1[b]	Plate 2	Bkgd ± 3SD[c]	μg/g[d]	OD								
MIoBS-sand	0.047 ± 0.012	0.045 ± 0.003	0.08	0.5	0.12	2.28	1.14	0.51	0.50	0.58	0.53	0.62	2.93
R5-sand	0.055 ± 0.007	0.054 ± 0.003	0.08	5	0.16	0.41	0.07	0.07	0.07	0.10	0.09	0.11	0.42
G12-sand	0.069 ± 0.002	0.064 ± 0.002	0.08	4	0.24	0.52	0.21	0.15	0.13	0.16	0.15	0.17	0.42
A1-G12-sand	0.068 ± 0.003	0.081 ± 0.007	0.10	0.6	0.08	0.13	0.10	0.10	0.08	0.08	0.08	0.08	0.10
Skerritt-sand	0.146 ± 0.010	0.143 ± 0.013	0.18	5	0.29	1.50	1.73	2.48	2.49	3.23	3.08	3.41	3.89
R5-comp	1.038 ± 0.062	0.96 ± 0.083	0.71[e]	10	1.01	0.63	0.77	0.85	0.90	0.90	0.85	0.89	0.57
G12-comp	1.171 ± 0.015	1.196 ± 0.027	1.12	3	0.97	1.14	1.15	1.14	1.18	1.16	1.21	1.17	1.18

[a] Optical density (OD) values were compared with background to avoid problems encountered with trendlines extrapolated beyond the first nonzero standard (S1).
[b] Average OD value (of triplicates) ± standard deviation of 0 μg/g gluten in sorghum beer (samples designated A, B, and C).
[c] Used the data from the plate that calculated the worst Bkgd ± 3SD value, rounded to the 100th place, typically one or two significant figures.
[d] Calculated to include recommended dilutions.
[e] Subtract three times the standard deviation (3SD) from the average absorbance values for competitive ELISAs; used worst case, plate 2 (0.96 − [3 × 0.083]).

In contrast, the SB200 samples generated responses slightly lower than the upper limit as defined by the test kit standards. Hence, the SB200 samples definitely contained gluten, and the amounts changed over time, but the quantitative accuracy is questionable (Table 4).

The responses generated by the SB20 samples when analyzed using the G12-sand ELISA exceeded the Bkgd + 3SD but were less than the avS1 threshold, except for the wort samples at the start of boil and the solids removed by the whirlpool, which exceeded both thresholds. The responses were greater than zero; however, assigning a reliable concentration was not possible (Table 2). In contrast, accurate quantification of gluten in the SB200 samples was possible because the G12-sand ELISA is calibrated through 200 μg/ml and the absorbance values generated by the samples were within the calibration curve. The gluten content of the initial SB200 sample (the wort at the start of the boil) was interpolated at 73 μg/ml; this concentration was comparable to the amounts obtained with the R5-sand and A1-G12-sand ELISAs (88 and 69 μg/ml, respectively). Similar results were also obtained with the three sandwich ELISAs for the other liquid SB200 samples. However, the responses for the solid samples collected from the whirlpool were not comparable because of the high concentrations of gluten present and the differences in the upper limits of quantification for each ELISA (Table 4).

The A1-G12-sand ELISA has an LOD of 0.6 μg/ml, as stated by the test kit manufacturer for samples diluted 200-fold. Thus, the assay was expected to be able to detect and quantify gluten in the SB20 samples. However, the Bkgd + 3SD, variability in the responses generated by the samples, and the shallow calibration curves resulted in an inability to distinguish the responses from background (Table 2). When the SB200 samples were diluted 500-fold, the liquid samples were interpolated to contain 60 to 81 μg/ml gluten, and the solids from the whirlpool contained 107 μg/g gluten; all samples contained gluten within the 2 to 120 μg/ml dynamic range of the ELISA kit (Table 4).

The Skerritt-sand ELISA generated responses indicative of a gluten concentration much higher than expected (Tables 2 and 4). Only the three SB0 samples collected before the fermentation step were comparable to expectations. The slight increase in gluten concentration in the fermented SB0 samples may reflect generation or release of an antigen from either the mixture or the yeast preparation. These results are consistent with the insignificant increase in results from the MIoBS-sand, R5-sand, G12-sand, and A1-G12-sand ELISAs for the same samples. Of the SB20 samples analyzed by the Skerritt-sand ELISA, only the first two (wort at start of boil and wort at end of boil) were within the dynamic range of the ELISA. The following two samples (clarified wort and wort after 1 h of fermentation) had absorbances of <3, and the samples collected later in the fermentation process generating absorbances <4, all within the dynamic range of the plate reader.

The overestimation of gluten by the Skerritt-sand ELISA (Table 4) may be due to how this ELISA is calibrated. According to ELISA Technologies *(8),* the standard solutions are prepared from the National Institute

TABLE 3. *Average coefficient of variation (CV) of ELISA absorbance values*

Added gluten (μg/ml)[a]	Stage of production	CV (%)[b] MIoBS-sand (3rd poly)	R5-sand (3rd poly)	G12-sand (3rd poly)	A1-G12-sand (2nd poly)	Skerritt-sand (2nd poly)	R5-comp (3rd poly)	G12-comp (2nd poly)
0	Sorghum syrup	3	2	2	8	5	9	2
	Start boil	24	1	3	4	5	6	1
	End boil	24	12	3	7	5	7	1
	Clarified	16	9	2	4	8	6	1
	1 h fermentation	3	14	7	7	9	4	3
	1 day fermentation	5	4	4	5	17	11	2
	7 days fermentation	6	7	2	7	4	6	2
	8 days, final product	4	4	2	7	7	9	1
	Solids after whirlpool	0	2	1	10	6	10	2
20	Start boil	5	6	11	3	20	7	2
	End boil	13	6	2	11	15	11	2
	Clarified	10	9	2	11	4	9	2
	1 h fermentation	2	4	4	10	10	9	2
	1 day fermentation	3	3	3	2	12	6	2
	7 days fermentation	2	3	2	3	10	12	2
	12 days, final product	1	39	2	2	6	3	1
	Solids after whirlpool	1	6	2	6	1	10	3
200	Start boil	4	7	5	4	ND[c]	6	0
	End boil	4	9	7	6	ND	15	4
	Clarified	2	2	2	2	ND	5	2
	1 h fermentation	3	3	6	2	ND	32	2
	1 day fermentation	1	4	3	6	ND	8	2
	7 days fermentation	9	3	8	67[d]	ND	11	7
	Solids after whirlpool	ND	2	3	4	ND	7	3

[a] Intact wheat gluten (G5004, Sigma-Aldrich) added before boiling.
[b] Average CV of two plates. Samples analyzed in triplicate, each replicate prepared from scratch and run on two ELISA plates of each test kit. Trendline equation applied to calculate the gluten concentration; poly, polynomial; $R^2 > 0.99$.
[c] ND, not determined because the absorbance was beyond the dynamic range.
[d] High CV reflects inclusion of an outlier; omission of the outlier decreased the CV to 26%.

of Standards and Technology Durum Wheat Reference Material using a proprietary procedure to generate the standard solutions. Therefore, the standards may preferentially not include aggregates but instead favor lower molecular weight, highly soluble proteins with fewer epitopes. However, the Skerritt antibody is selective for high-molecular-weight glutenins (Fig. 3D) *(2, 3).* Consequently, if the standards contained a smaller percentage of antigenic aggregates or multivalent units than normally present in gluten, the standard curve would overestimate the gluten concentration, especially if the standards used in this kit were prepared with the goal of containing soluble low-molecular-weight proteins, an approach used in some assays when dealing with insoluble complex analytes. All of the SB200 samples generated responses that exceeded the dynamic range and thereby were not quantifiable. However, all samples clearly contained gluten.

Competitive ELISAs. Although universally recognized reference materials are not available for hydrolyzed gluten, two competitive ELISAs, the R5-comp and the G12-comp designed to detect hydrolyzed gluten, were used to evaluate the beer samples. Competitive ELISAs detect the presence of gluten as a decrease in absorbance due to competition between gluten in the analytical sample and gluten immobilized to the microtiter plate. Anything that prevents the detector antibody from binding to the surface of the microtiter plate causes a decrease in the response (absorbance), and the sample is scored as gluten positive. To compensate, the threshold to delineate positive samples from background must be increased; therefore, the sensitivity of the assay is decreased. The SDs for results of competitive ELISAs are greater than those for the results of the sandwich ELISAs because of the poorer signal-to-noise ratio. The LOQ of the R5-comp ELISA was reported by the manufacturer as 10 μg/ml, a value consistent with failure of the calibration curve at concentrations <10 μg/ml (data not shown). This greater variance means that it is more difficult for a response to be distinguished from the background; the response must be less than the average absorbance of the zero standards minus three times the SD (Bkgd − 3SD). The R5-comp ELISA generated responses with the SB20 samples less than the avS1 but not less than the Bkgd − 3SD except for the wort samples at the start of boil and the solids obtained after whirlpool clarification. The G12-comp ELISA responses generated from the SB20 samples were indistinguishable from background, not less than either the Bkgd − 3SD or avS1 (Table 2).

TABLE 4. *Gluten concentration at various stages of beer production*

Added gluten (μg/ml)[a]	Stage of production	Gluten concn (μg/g)[b]							
		MIoBS-sand (3rd poly)	R5-sand (3rd poly)	G12-sand (3rd poly)	A1-G12-sand (2nd poly)	Skerritt-sand (2nd poly)	R5-comp (3rd poly)	R5-comp (sand stds) (3rd poly)[c]	G12-comp (2nd poly)
0	Sorghum syrup	0	0	−1	1	0	9	6	11
	Start boil	0	0	−1	0	0	15	7	0
	End boil	0	1	−1	1	0	14	8	5
	Clarified	0	0	−1	1	0	13	8	2
	1 h fermentation	0	1	0	1	4	10	7	0
	1 day fermentation	1	1	1	1	15	15	8	2
	7 days fermentation	1	1	0	2	8	10	8	0
	8 days, final product	1	1	0	2	8	10	7	2
	Solids after whirlpool	1	0	0	1	4	13	7	0
20	Start boil	18	14	14	10	44	21	13	7
	End boil	5	1	5	5	55	15	10	3
	Clarified	2	1	3	5	80	13	8	2
	1 h fermentation	2	1	2	2	82	15	8	1
	1 day fermentation	2	2	4	3	110	13	8	3
	7 days fermentation	2	2	3	2	106	15	9	−1
	12 days, final product	2	4	4	3	120	14	8	3
	Solids after whirlpool	32	14	12	5	150	26	16	−1
200	Start boil	40	88	73	69	146	200	72	31
	End boil	33	81	65	71	ND[d]	242	84	37
	Clarified	34	78	59	65	ND	202	73	49
	1 h fermentation	31	78	51	67	ND	224	79	36
	1 day fermentation	33	80	59	81	ND	235	81	45
	7 days fermentation	32	55	43	60	ND	177	65	30
	Solids after whirlpool	ND	147	276	107	ND	381	122	34

[a] Intact wheat gluten (G5004, Sigma-Aldrich) added before boiling.
[b] Each sample analyzed in triplicate with each ELISA using two plates. Concentration was calculated using trendlines derived from standards. Trendline equation applied to calculate the concentration; poly, polynomial; $R^2 > 0.99$.
[c] R5-comp responses interpolated using trendlines against R5-sand standards run with the samples; average $R^2 = 0.9936$.
[d] ND, not determined because the absorbance was beyond the dynamic range.

The absorbance responses for the SB200 samples were within the dynamic range of the R5-comp and G12-comp ELISAs (10 to 270 and 2 to 120 μg/ml, respectively, with the latter based on a 500-fold dilution of the samples). However, the averages CVs for the R5-comp and G12-comp ELISA responses were 12% ± 9% and 3% ± 2%, respectively (Table 3). With a CV of 12% ± 9%, it is not definite that the ±20% differences observed in the R5-comp ELISA results are significant. Although the G12-comp ELISA generated more precise responses, the concentrations of gluten detected in the SB20 and SB200 solid materials from the whirlpool were lower than expected and not consistent with the results from the other six ELISAs (Table 4).

Competitive ELISA standards. The R5-comp ELISA uses as a calibrant, a mixture of the wheat, rye, and barley hydrolysates prepared according to the method of Gessendorfer et al. *(9, 12).* This calibrant is used in the R5-comp ELISA on a mass basis, meaning that the response generated by an analytical sample is compared with the response generated by the mixture of hydrolysates on a mass per volume basis. Thus, suitability of the hydrolysate mixture as a calibrant depends on the degree of similarity between the prolamins from wheat, rye, and barley, variations between cultivars (a problem documented in the use of barley for beer production *(23)*), and the degree of similarity between the hydrolytic reactions associated with beer production (or other hydrolyzed/fermented foods) and those involved in the generation of the calibrant (e.g., protease specificity and degree of digestion). Thus, the hydrolysate mixture may not be representative of the gluten hydrolysate found in beer. The differences in the chemical nature of the calibrant and the gluten hydrolysis products in beer will likely cause inaccuracies in the calibration curve used for analytical quantitation. However, the magnitude of this inaccuracy may be acceptable when compared with other factors affecting ELISA performance and data variance. The distribution of the antigenic epitopes upon prolamin digestion may ultimately determine the suitability of the hydrolysate mixture as a reference material.

Based on the ability to distinguish the responses generated by the SB20 samples from avS1, the R5-comp ELISA is a reasonable assay for quantifying the concentration of gluten in the samples. The results of the R5-comp ELISA indicate that the initial SB20 sample (wort at the start of boil, Tables 2 and 4) contained 21 μg/ml, which is 17% higher than the gluten concentration obtained with the MIoBS-sand ELISA (18 μg/ml) and 50% higher than the 14 μg/ml detected by the R5-sand and G12-sand ELISAs. At the end of boiling, the gluten concentration in the SB20

wort samples measured with the R5-comp ELISA decreased to 15 μg/ml, and this concentration remained virtually unchanged during fermentation. This trend was similar to those observed for samples analyzed with the MIoBS-sand ELISA, although greater losses of gluten during boiling and clarification steps were observed with the MIoBS-sand ELISA (24 versus 89%). Heat-induced aggregation and thermal hydrolysis of intact gluten may have a greater impact on the MIoBS-sand ELISA than on the R5-comp ELISA.

The responses generated by the sorghum SB0, SB20, and SB200 samples were also evaluated using calibration curves generated from the R5-sand standards (Table 4). The gluten concentrations determined for the R5-sand standards that were analyzed with the R5-comp ELISA were comparable to the concentrations obtained with the R5-sand, G12-sand, and A1-G12-sand ELISAs for their respective standards, except that the samples that were normally interpolated as having 0 μg/ml gluten had 7 μg/ml gluten. This shift in the zero point is consistent with the third-order polynomial trendline ascribed to the R5-sand standards (e.g., $y = -119.02x^3 + 362.24x^2 - 385.09x + 150.55$, $R^2 = 0.9959$) becoming nonresponsive to changes in gluten concentrations below 7 μg/ml and the LOQ ascribed by the R5-comp ELISA manufacturer (10 μg/ml). The initial SB20 and SB200 samples (wort at beginning of boil) analyzed with the R5-comp ELISA calibrated using the R5-sand standards were interpolated to contain 13 and 72 μg/ml gluten, respectively; these concentrations were comparable to those obtained with the other sandwich ELISAs but were substantially lower than the 21 and 200 μg/ml gluten calculated from the R5-comp standards. Because changes to gluten should be minimal in the initial wort samples, the sandwich ELISAs should accurately measure the concentration of gluten present. The discrepancy between the concentrations of gluten indicated by the sandwich ELISAs and the R5-comp ELISA calibrated against the R5-sand standards versus the R5-comp ELISA calibrated with the hydrolysate standards can be explained in one of three ways: (i) the sandwich ELISAs are not accurately detecting the presence of intact gluten, (ii) the intact gluten standards are not appropriate for quantifying the intact wheat gluten used in this study, or (iii) the R5-comp standards overestimate the amount of intact gluten present.

The possibility that the sandwich ELISAs do not accurately detect the presence of gluten would arise if the ELISAs were not performing reliably or if the extraction protocols used for the ELISAs were inadequate. The responses of the ELISAs with their respective standards support that their performance was as expected. Thus, the question is whether the extraction protocols were adequate. The R5-comp ELISA uses an alcohol extraction protocol, whereas the R5-sand uses the extensively validated Mendez cocktail *(13, 17)*. The other sandwich ELISAs also use alcohol and reducing-denaturing conditions, and some of these extraction procedures have been validated. Therefore, it is unlikely that the extraction protocols were the cause of the discrepancy.

The explanation that the intact gluten standards are not appropriate is inconsistent with data indicating that the R5-sand standards (which are based on the standard developed by the Prolamin Working Group), the wheat protein standard of the MIoBS-sand, and the added gluten used in this study generated identical calibration curves (see Fig. 2).

Thus, the first and second explanations are not consistent with the results. The error must be the suitability of the R5-comp standards (a mixture of hydrolysates) to quantify intact gluten. Use of the R5-comp hydrolysate standards resulted in an overestimation of the gluten concentrations in the SB20 and SB200 wort samples at the start of boiling by 62 and 180%, respectively (Table 4). Calibration of the data generated by the R5-sand ELISA with the R5-comp standards resulted in an overestimation of the gluten concentrations of the SB20 and SB200 samples at the start of boiling by 78 and 240%, respectively (data not shown). The seriousness of this problem needs to be further explored and may require a more detailed understanding of the immunopathogenic sensitivity and risk associated with intact and hydrolyzed forms of gluten as it relates to the accuracy of the detection of gluten.

Western blot analysis. Figure 3 depicts the Westerns blots of standards and SB0, SB20, and SB200 samples using the MIoBS, R5, G12, and Skerritt antibodies. The MIoBS, R5, and G12 antibodies recognized the lower molecular weight prolamines, and the Skerritt antibody favored the higher molecular weight glutenins. The intensities of the various bands for the SB20 samples (lanes 9 through 12) decreased progressively for all four antibodies as a function of processing. With the intact gluten standards run at 200, 40, 20, and 10 μg/ml (lanes 2 through 5) in each of the Western blots, the concentration of gluten in samples could be estimated, and changes in antigenic gluten proteins could be observed at various stages of the brewing process. The bands in the MIoBS Western blot decreased in intensity from approximately 27 μg/ml gluten (lane 9, wort at start of boil) to an estimated 5 μg/ml gluten (lane 12, beer at end of fermentation); higher molecular weight proteins decreased to almost undetectable concentrations, and the band for the lowest molecular weight protein (40) was slightly more intense than that for the 10 μg/ml gluten standard (lane 5). The other antibodies produced similar results. The bands in the R5, G12, and Skerritt Western blots dropped similarly in intensity from 27 to 5, 30 to <10, and 17 to <10 μg/ml gluten, respectively. Only the Western blots with the MIoBS antibodies had heterogeneous losses in band intensity, which indicates differences in antigen-antibody binding constants and may be due to either epitopic differences and/or antibody differences. Antibody differences are common when working with multiple antibody populations, as occurs with polyclonal antibodies. Therefore, the heterogeneous response may be due to the fact that the MIoBS antibody is polyclonal whereas the R5, G12, and Skerritt antibodies are monoclonal.

The SB200 samples (lanes 13 through 15) had slightly lower band intensities than did the 200 μg/ml intact gluten standard (lane 2) and contained an estimated 80 to 160 μg/ml

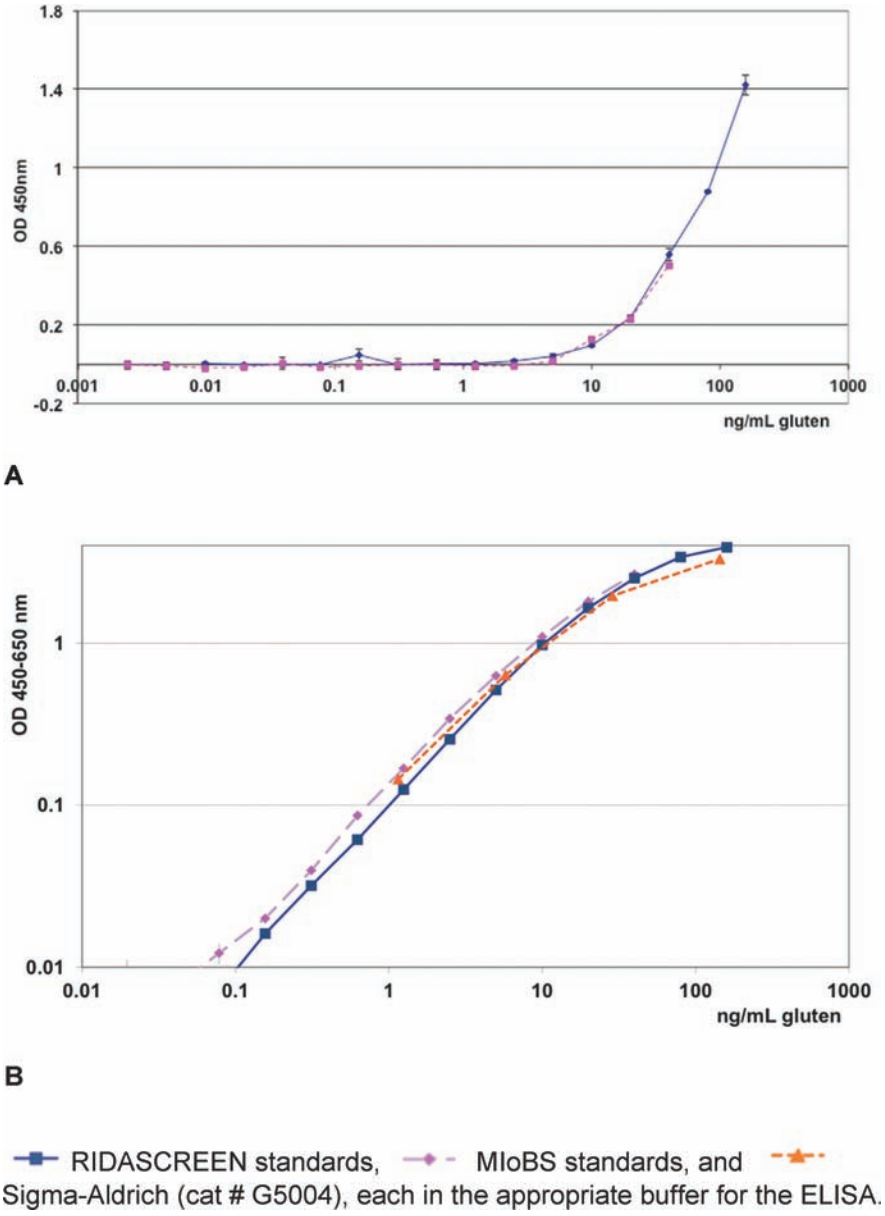

FIGURE 2. *Comparison of intact gluten standards. The RIDASCREEN R5 sandwich ELISA (A) and MIoBS sandwich ELISA (B) were used to analyze gluten standards from the RIDASCREEN R5 sandwich ELISA (—■—), the MIoBS gliadin ELISA (—◆—), and the gluten used for spiking samples (G5004, Sigma-Aldrich) (- - -▲- - -), each in the appropriate buffer for the ELISA. Because the RIDASCREEN standards were supplied as a solution, the most concentrated standard (80 ppb) was diluted in MIoBS buffer to generate the samples. The responses (optical density [OD] at 450 nm or 450–650 nm) after subtracting the background (approximately 0.04) were plotted versus the concentration of gluten in the analytical sample. The samples were run in triplicate and the error bars represent ±1 standard deviation.*

gluten based on additional images of the Western blots taken for shorter durations (data not shown). The higher gluten concentrations in the SB200 samples estimated with the Western blots compared with those estimated with the ELISAs for the same samples may reflect a greater dynamic range. However, the G12-sand ELISA should be able to accommodate samples with gluten concentrations of up to 200 μg/ml (Table 1).

Consistent with the ELISA data was the observation of very intense bands when the Skerritt antibody was used to generate Western blots. In these blots, the Skerritt antibody was used at 1/50 the concentration used in the ELISA, while the MIoBS, R5, and G12 antibodies were used at 1/10 the concentration used in their respective ELISAs. However, the increased intensity of the bands from the SB20 and SB200 samples was also observed with the gluten standards. Thus, when the intensities of the bands for the SB20 and SB200 samples were compared with those of the gluten standards (lanes 2 through 5), the gluten concentrations indicated were similar to those indicated by the MIoBS, R5, and G12 antibodies (Fig. 3). Thus, the Western blots support the hypothesis that the overestimation of gluten concentration by the Skerritt-sand ELISAs and the progressive increase in detectable gluten during brewing are probably artifacts resulting from problems with the ELISA test kit standards possibly associated with how the multivalent high-molecular-weight glutenins are calibrated and affected by the brewing process.

The inability to detect low-molecular-weight (<10) peptides using Western blot analysis is not understood. Use of higher density gels and various blot transfer conditions failed to solve the problem. Our ability to detect gluten at concentrations >10-fold lower than those routinely analyzed with SDS-PAGE may be due to the multivalency of the proteins. Upon digestion, the multivalency is lost and the intensity of the signal per polymeric unit is reduced. Whether the peptides are at a concentration sufficient to enable detection without concentrating the samples needs to be explored.

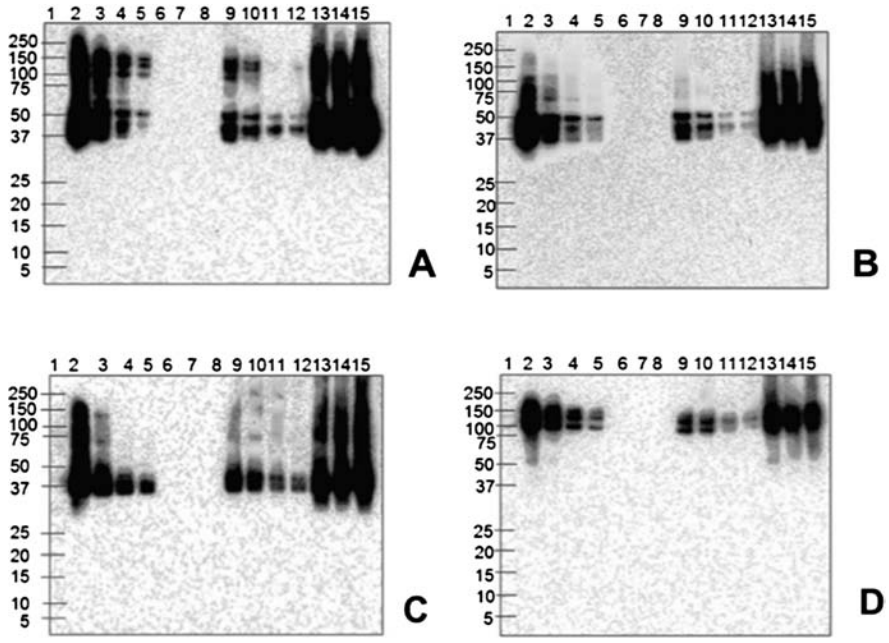

FIGURE 3. *Western blot analysis using detector antibodies: (A) Morinaga Institute of Biological Sciences (MIoBS) Gliadin, (B) R5 RIDASCREEN Gliadin, (C) AgraQuant ELISA Gluten G12, and (D) ALLER-TEK (Skerritt antibody based) Gluten sandwich ELISA test kits. Bands were separated by SDS-PAGE: lane 1, Precision Plus molecular weight marker proteins (BioRad); lane 2, 200 μg/ml gluten; lane 3, 40 μg/ml gluten; lane 4, 20 μg/ml gluten; lane 5, 10 μg/ml gluten; lane 6, blank; lane 7, sorghum beer (SB0) at start of boil; lane 8, SB0 after 1 week of fermentation; lane 9, SB with 20 μg/ml gluten (SB20) at start of boil; lane 10, SB20 at end of boil; lane 11, SB20 after 1 day of fermentation; lane 12, SB20 end product; lane 13, SB with 200 μg/ml gluten (SB200) at start of boil; lane 14, SB200 end product; lane 15, SB200 after 1 day of fermentation. The beer samples (lanes 7 through 15) were loaded with approximately 20 μg of total protein in the 10 μl loaded per lane.*

Sorghum beer manufactured with 20 μg/ml wheat gluten spiked into the wort at the initial phase of production generated definite positive responses for the presence of gluten using the MIoBS-sand ELISA, a method mentioned in the FDA gluten-free regulation *(26)*. Six of the seven ELISAs employed in this study indicated a significant decrease in detectable gluten during beer production, with most of the decrease occurring during the initial steps before fermentation, a result consistent with previous studies of barley malt–based beer using the R5-sand ELISA *(7)*. Four sandwich ELISAs indicated decreases in detectable gluten of 50 to 93% (72, 93, 64, and 50%) in the wort at the end of the boiling step. The two competitive ELISAs indicated decreases of 29 and 60% after boiling. The losses in detectable gluten during boiling and clarification are consistent with heat-induced aggregation and hydrolysis. The Skerritt-sand ELISA overestimated the initial gluten concentration at the start of boiling (40 μg/ml) and, unlike the other ELISAs, indicated a substantial increase in detectable gluten during the beer-making process (120 μg/ml gluten in fermented beer). This surprising result was not confirmed by Western blot analysis using the Skerritt antibody. Instead, a progressive decrease in antigenic proteins was observed, consistent with the other ELISAs and the Western blot analyses. These quantitative anomalies with the Skerritt-sand ELISA and how they relate to possible problems in calibration or other factors that may arise during fermentation and hydrolysis require additional research.

The two ELISA test kits whose dynamic ranges encompassed the responses generated by the SB200 samples (G12-sand and A1-G12-sand) generated comparable results, and these results were similar to those obtained with the R5-sand ELISA. The MIoBS-sand ELISA generated extrapolated results with a comparable pattern, although at approximately half the magnitude. This result was consistent with saturation and a reduced sensitivity to changes in gluten concentration. The gluten concentration of 70 to 88 μg/ml in the SB200 wort at the start of the boiling step can be explained by the observed clumping of the wheat gluten upon addition to the kettle; these clumps were removed during the clarification step.

The competitive ELISAs were unable to reliably detect and quantify gluten in various wort and beer samples. The reduced sensitivity of these ELISAs is reflected in the LOQs and SDs observed between replicates and among multiple test kits. Calibration using a mixture of wheat, rye, and barley hydrolysates prepared according to the method of Gessendorfer et al. *(9)* resulted in inaccurate quantification of intact gluten. Such quantification inaccuracy may be an acceptable approximation when analyzing hydrolyzed and fermented foods, but further research is needed before this hydrolysate can be used as a standard for gluten detection and quantification.

ACKNOWLEDGMENTS

Appreciation is expressed to the staff and faculty of the Department of Food Science (University of Wisconsin–Madison) and numerous

colleagues at the FDA Center for Food Safety and Applied Nutrition for intellectual discussions, without which this project could not have come about. The authors also acknowledge the Cargill Company for their kind donation of sorghum syrup and for providing assistance with brewing conditions.

REFERENCES

1. Akiyama, H., K. Isuzugawa, N. Harikai, H. Watanabe, K. Iijima, H. Yamakawa, Y. Mizuguchi, R. Yoshikawa, M. Yamamoto, H. Sato, M. Watai, F. Arakawa, T. Ogasawara, R. Nishihara, H. Kato, A. Yamauchi, Y. Takahata, F. Morimatsu, S. Mamegoshi, S. Muraoka, T. Honjoh, T. Watanabe, K. Sakata, T. Imamura, M. Toyoda, R. Matsuda, and T. Maitani. 2004. Inter-laboratory evaluation studies for development of notified ELISA methods for allergic substances (wheat). *Shokuhin Eiseigaku Zasshi* 45:128–134.
2. Allred, L. K., and B. W. Ritter. 2010. Recognition of gliadin and glutenin fractions in four commercial gluten assays. *J. AOAC Int.* 93: 190–196.
3. Anjum, F. M., M. R. Khan, A. Din, M. Saeed, I. Pasha, and M. U. Arshad. 2007. Wheat gluten: high molecular weight glutenin subunits—structure, genetics, and relation to dough elasticity. *J. Food Sci.* 72:R56–R63.
4. Codex Alimentarius Commission. 2008. Codex standard for foods for special dietary use for persons intolerant to gluten. Codex Stan 118-1979. Alinorm 08/31/26. Joint FAO/WHO Food Standards Programme, Geneva.
5. Colgrave, M. L., H. Goswami, C. A. Howitt, and G. J. Tanner. 2012. What is in a beer? Proteomic characterization and relative quantification of hordein (gluten) in beer. *J. Proteome Res.* 11:386–396.
6. Comino, I., A. Real, M. L. Moreno, R. Montes, A. Cebolla, and C. Sousa. 2012. Immunological determination of gliadin 33-mer equivalent peptides in beers as a specific and practical analytical method to assess safety for celiac patients. *J. Sci. Food Agric.* 93: 933–943.
7. Dostalek, P., I. Hochel, E. Mendez, A. Hernando, and D. Gabrovska. 2006. Immunochemical determination of gluten in malts and beers. *Food Addit. Contam.* 23:1074–1078.
8. ELISA Technologies, Inc. 2014. Personal communication.
9. Gessendorfer, B., P. Koehler, and H. Wieser. 2009. Preparation and characterization of enzymatically hydrolyzed prolamins from wheat, rye, and barley as references for the immunochemical quantitation of partially hydrolyzed gluten. *Anal. Bioanal. Chem.* 395:1721–1728.
10. Gilbert, A., A. G. Kruizinga, S. Neuhold, G. F. Houben, M. A. Canela, A. Fasano, and S. Catassi. 2013. Might gluten traces in wheat substitutes pose a risk in patients with celiac disease? A population-based probabilistic approach to risk estimation. *Am. J. Clin. Nutr.* 97: 109–116.
11. Guerdrum, L. J., and C. W. Bamforth. 2012. Prolamin levels through brewing and the impact of prolyl endopeptidase. *J. Am. Soc. Brew. Chem.* 70:35–38.
12. Haas-Lauterbach, S., U. Immer, M. Richterm, and P. Koehler 2012. Gluten fragment detection with a competitive ELISA. *J. AOAC Int.* 95:377–381.
13. Immer, U., and S. Haas-Lauterbach. 2012. Gliadin as a measure of gluten in foods containing wheat, rye, and barley—enzyme immunoassay method based on a specific monoclonal antibody to the potentially celiac toxic amino acid prolamin sequences: collaborative study. *J. AOAC Int.* 95:1118–1124.
14. Koehler, P., T. Schwalb, U. Immer, M. Lacorn, P. Wehling, and C. Don. 2013. AACCI approved methods technical committee report: collaborative study on immunochemical determination of partially hydrolyzed gluten using an R5 competitive ELISA. *Cereal Foods World* 58:1–6.
15. Laube, T., S. V. Kergaravat, S. N. Fabiano, S. R. Hernández, S. Alegret, and M. I. Pividori. 2011. Magneto immunosensor for gliadin detection in gluten-free foodstuff: towards food safety for celiac patients. *Biosens. Bioelectron.* 27:46–52.
16. Matsuda, R., Y. Yoshioka, H. Akiyama, K. Aburatani, Y. Watanabe, T. Matsumoto, N. Morishita, H. Sato, T. Mishima, R. Gamo, Y. Kihira, and T. Maitani. 2006. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *J. AOAC Int.* 89:1600–1608.
17. Mendez, E., C. Vela, U. Immer, and F. W. Janssen. 2005. Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. *Eur. J. Gastroenterol. Hepatol.* 17:1053–1063.
18. Morón, B., M. T. Bethune, I. Comino, H. Manyani, M. Ferragud, M. C. Lopez, A. Cebolla, C. Khosla, and C. Sousa. 2008. Toward the assessment of food toxicity for celiac patients: characterization of monoclonal antibodies to a main immunogenic gluten peptide. *PLoS ONE* 3(5):e2294. doi:10.1371/journal.pone.0002294.
19. Morón, B., A. Cebolla, H. Manyani, M. Alvarez-Maqueda, M. Megías, M. C. Thomas, M. C. López, and C. Sousa. 2008. Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. *Am. J. Clin. Nutr.* 87:405–414.
20. Real, A., I. Comino, M. L. Moreno, M. A. López-Casado, P. Lorite, M. I. Torres, A. Cebolla, and C. Sousa. 2014. Identification and in vitro reactivity of celiac immunoactive peptides in an apparent gluten-free beer. *PLoS ONE* 9(6):e100917. doi:10.1371/journal.pone.0100917.
21. Skerritt, J. H., and A. S. Hill. 1990. Monoclonal antibody sandwich enzyme immunoassays for determination of gluten in foods. *J. Agric. Food Chem.* 38:1771–1778.
22. Skerritt, J. H., and A. S. Hill. 1991. Enzyme immunoassay for determination of gluten in foods: collaborative study. *J .Assoc. Off. Anal. Chem.* 74:257–264.
23. Tanner, G. J., M. J. Blundell, M. L. Colgrave, and C. A. Howitt. 2013. Quantification of hordeins by ELISA: the correct standard makes a magnitude of difference. *PLoS ONE* 8(2):e56456. doi:10.1371/journal.pone.0056456.
24. Tanner, G. J., M. L. Colgrave, M. J. Blundell, H. P. Goswami, and C. A. Howitt. 2013. Measuring hordein (gluten) in beer—a comparison of ELISA and mass spectrometry. *PLoS ONE* 8(2): e56452. doi:10.1371/journal.pone.0056452.
25. Torgler, C., M. A. Síglez, F. Vilchez, A. Cebolla, and C. Sousa. 2010. Analytical tools to detect gluten immunotoxic fractions in food based on monoclonal antibodies raised against the gliadin 33-mer peptide. *In* Proceedings of the 24th meeting, Working Group on Prolamin Analysis and Toxicity. Available at: http://foodsafety.biomedal.com/wp-content/uploads/2013/10/Working-group-on-prolamin-Analytical-Tools-p-47-51.pdf. Accessed 8 November 2014.
26. U.S. Food and Drug Administration. 2013. Food labeling; gluten-free labeling of foods. 21 CFR Part 101, docket FDA-2005-N-0404, RIN 0910-AG84. *Fed. Regist.* 78:47154–47179. Available at: http://www.gpo.gov/fdsys/pkg/FR-2013-08-05/pdf/2013-18813.pdf. Accessed 31 October 2014.
27. Valdes, I., E. Garcia, M. Llorente, and E. Mendez. 2003. Innovative approach to low level gluten determination in foods using a novel sandwich ELISA protocol. *Eur. J. Gastroenterol. Hepatol.* 15:465–474.
28. Yman, I. M. 1999. Gluten determination in foods by an enzyme immunoassay. Presented at Agri-Food Antibodies 99: The Society for Food and Agricultural Immunology's Fifth International Conference, Norwich, UK, 14 to 17 September 1999.