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Development of an Incurred Cornbread Model for Gluten Detection by Immunoassays

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ABSTRACT: Gluten that is present in food as a result of cross-contact or misbranding can cause severe health concerns to wheat-allergic and celiac patients. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and lateral flow device (LFD), are commonly used to detect gluten traces in foods. However, the performance of immunoassays can be affected by non-assay-related factors, such as food matrix and processing conditions. Gluten (0−500 ppm) and wheat flour (20−1000 ppm) incurred cornbread was prepared at different incurred levels and baking conditions (204.4 °C for 20, 27, and 34 min) to study the accuracy and precision of gluten measurement by seven immunoassay kits (three LFD and four ELISA kits). The stability and immunoreactivity of gluten proteins, as measured by western blot using three different antibodies, were not adversely affected by the baking conditions. However, the gluten recovery varied depending upon the ELISA kit and the gluten source used to make the incurred cornbread, affecting the accuracy of gluten quantification (BioKits, 9−77%; Morinaga, 91−137%; R-Biopharm, 61−108%; and Romer Labs, 113−190%). Gluten recovery was reduced with increased baking time for most ELISA kits analyzed. Both the sampling and analytical variance increased with an increase in the gluten incurred level. The predicted analytical coefficient of variation associated with all ELISA kits was below 12% for all incurred levels, indicative of good analytical precision.

KEYWORDS: *Gluten, ELISA, immunoassay, incurred food, thermal processing*

INTRODUCTION

Gluten commonly refers to a complex heterologous group of proteins found in the grains of wheat, rye, and barley. It is composed of alcohol-soluble prolamins and acid-/alkali-soluble glutelins. The glutenins are generally extracted in the presence of reducing agents to dissociate large polymers linked by disulfide bonds. The prolamins in wheat, rye, and barley are known as gliadin, secalin, and hordein, respectively. The prolamins are monomeric proteins with intramolecular disulfide bonds, whereas glutelins are multimeric with both intra- and intermolecular disulfide bonds, forming a large and insoluble protein complex.[1] With increased knowledge of gluten proteins and molecular similarities between gliadin and glutenin, the new classification of gluten includes three groups: (1) sulfur-rich (α/β, γ-gliadin, low-molecular-weight glutenin subunits), (2) sulfur-poor (ω-gliadin), and (3) high-molecular-weight (high-molecular-weight glutenin subunits) prolamins.[2] These gluten groups are well-tolerated by most, but dependent upon the type of gluten protein, they may cause IgE-mediated food allergy or cell-mediated celiac disease in sensitive individuals. According to some estimates, 0.4% adults and 0.5−1% of the U.S. population suffers from wheat allergy and celiac disease, respectively.[3,4] The lack of a cure for food allergy and celiac disease requires the sensitive individuals to follow a strict diet without any gluten/wheat.

Gluten is extensively used in the food industry because it imparts unique functional properties, such as viscoelasticity, in foods. Most gluten consumption in humans comes from gluten-containing grains when used in form of baked foods (wheat and rye) or malt and beer (barley). Small amounts are also consumed when gluten extracted from grains are used as food additives. However, traces of gluten can sometimes be present in foods as a result of cross-contact during food processing and misbranding. This unintentional presence of gluten goes unnoticed for the majority but can cause life-threatening reactions to wheat-allergic and celiac patients upon ingestion. Regulatory measures are warranted to address the issue of gluten contamination in foods and conformity to the labeling mandate. To safeguard the health of gluten-sensitive individuals and ensure food safety, Codex Alimentarius (CODEX STAN 118-1979), European Commission Regulation (EC 41/2009), and more recently, the U.S. Food and Drug Administration (FR Doc. 2013-18813) have set 20 ppm as the maximum limit of gluten in foods labeled as "gluten-free".

Immunoassays are routinely used to detect food allergen and gluten contamination in foods. Several immunoassay formats,

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such as enzyme-linked immunosorbent assay (ELISA) and lateral flow device (LFD), are available for quantitative and qualitative detection of gluten, respectively. LFDs can also be employed as a quantitative tool if the band intensity can be measured in the dynamic range. Although these assays have a common goal of gluten detection, there are various technical differences (such as antibody specificity, target analyte, sample extraction buffer, extraction time/temperature, calibration standard, and unit of measurement) that may result in differing gluten estimations.[5−7] Moreover, the type of food matrix and the various processing conditions used during food manufacture may cause variations in immunoassays by changing protein conformation, resulting in altered protein extraction efficiency and antibody binding. Thermal processing (e.g., baking, boiling, frying, and roasting) causes various chemical and physical modifications (e.g., Maillard reaction and protein denaturation/aggregation), resulting in changes in food allergen detection by ELISA.[8,9] A decrease in protein solubility and increased IgE binding in the insoluble fraction were observed with increased heating time in peanuts.[10] Similarly, gluten proteins may still be present in the processed food to cause food allergy and celiac disease, but the inability to extract them completely can result in false negative or underestimation of the gluten content by immunoassays and jeopardize consumer safety.

The use of gluten-spiked food samples provides information that is helpful and suitable for the raw material testing, but such samples are not enough to study the performance of the analytical assay because it does not mimic the actual gluten contamination in the food industry. The preparation of incurred food samples, where the food is intentionally contaminated by the allergen/gluten followed by processing to a final product, is an ideal way to estimate recovery and validate analytical assays. The aim of this study was to develop an incurred cornbread model that can be used for evaluating the accuracy and precision in gluten quantification and assess the effect of the baking time on gluten detection. Incurred samples were made with gluten as well as wheat flour to imitate the type of contamination that may occur in the food industry. The accuracy and precision of four commercial ELISA kits were evaluated by determining recovery and variance/coefficient of variance, respectively.

■ MATERIALS AND METHODS

Materials. The gluten and wheat flour (SRM 1567a) used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and National Institute of Standards and Technology (NIST, Gaithersburg, MD), respectively. The food ingredients used in the preparation of cornbread were purchased from a local grocery store. The commercial sandwich ELISA kits used in the study included RIDASCREEN Gliadin (R7001; R-Biopharm AG, Darmstadt, Germany), wheat protein ELISA kit (181GD; Morinaga Institute of Biological Science, Inc., Yokohama, Japan), BioKits gluten assay kit (802002Y; Neogen Corp., Lansing, MI), and AgraQuant Gluten G12 (COKAL0200; Romer Labs UK, Ltd., Cheshire, U.K.). The LFD/dip-stick assays used were RIDAQUICK Gliadin (R7003; R-Biopharm AG, Darmstadt, Germany), Gluten (Gliadin) lateral flow kit (10186; Morinaga Institute of Biological Science, Inc., Yokohama, Japan), and AgraStrip Gluten G12 (COKAL0200AS; Romer Labs, Inc., Union, MO). The horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG and goat anti-mouse IgG antibodies were from Jackson ImmunoResearch (West Grove, PA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Anti-gluten Antibodies. Specific peptides synthesized on the basis of the known amino acid sequence of α-gliadin (CQYPSGPGFFQPSQQNP) and B-hordein (CRMPQLIARSQML) were conjugated to maleimide-activated KLH (Thermo Scientific, Rockford, IL). A cysteine residue was added at the N terminus of the peptide for the purpose of KLH conjugation. These antigens were each suspended in saline mixed 1:1 with complete Freunds adjuvant and subcutaneously injected in multiple sites on the shaved back of rabbits. After three boosters, rabbits were bled and the collected serum was stored at −20 °C until further use. Monoclonal antibody A1 (anti-gliadin 33-mer) was purchased from Biomedal S.L. (Spain).

Incurred Cornbread Preparation. The recipe for cornbread preparation incurred with incremental levels of gluten and wheat flour is presented in Figure 1. Batter was prepared by mixing the dry ingredients in a Hobart mixer using a paddle attachment for 1 h followed by the addition of liquid ingredients and further mixing at medium speed for 15 min. From several batches containing added gluten or wheat flour, six 25 g subsamples of batter were obtained from various locations in the mixing bowl. Each of these subsamples was analyzed by the wheat protein ELISA kit (Morinaga) to ensure batch homogeneity. In all cases, the relative standard deviations for the wheat protein content for the six subsamples were <5%, indicating that the batches were homogeneous with respect to gluten or wheat flour content. Batches of 500 g batter were poured into a 9 in. round non-stick baking pan and baked in an oven at 204.4 °C (400 °F) for 20 min. Select gluten and wheat flour incurred batter were also baked at 204.4 °C for 27 and 34 min. Moisture loss during baking was measured by subtracting the weight of cornbread after cooling to room temperature from the original batter weight. The cornbread was ground in a food processor, and the respective batter was stored at −20 °C until further use.

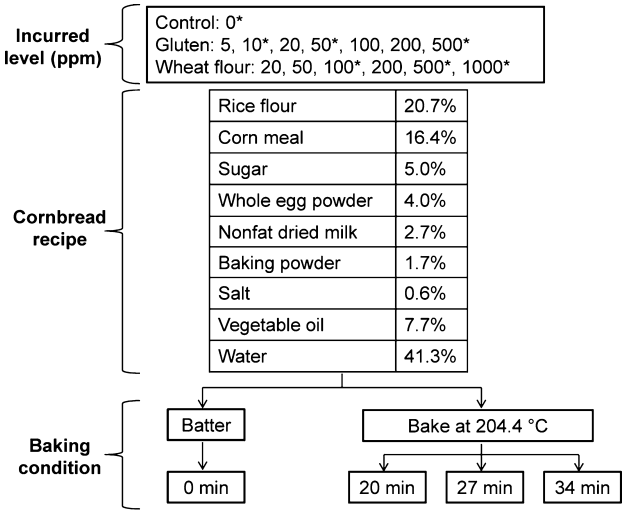

Figure 1. Schematic diagram for the preparation of gluten and wheat flour incurred cornbread. Incurred levels marked by an asterisk were baked for 27 and 34 min.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Western Blotting. Soluble proteins from cornbread samples (0.5 g) were extracted in 50 mM Tris-HCl at pH 7.5 containing 1% SDS and 2% (v/v) β-mercaptoethanol (β-ME) [1:10 (w/v) sample/buffer ratio] for 1 h at room temperature (RT), followed by centrifugation (3000*g* for 20 min) at RT. The protein content of the supernatant was measured by a 660 nm protein assay (Pierce, Rockford, IL). Protein stock solutions of 1 mg/mL were made using SDS−PAGE sample buffer [50 mM Tris-HCl at pH 6.8, 1% SDS, 30% glycerol, 0.01% bromophenol blue, and 2% (v/v) β-ME] and heated in a boiling water bath for 10 min before loading an appropriate amount on 4−12% Novex Bis-Tris gel (Invitrogen) run at a constant voltage (100 V) until the dye reached the gel bottom. The gel was either stained with Coomassie Brilliant Blue R or used to transfer polypeptides onto a 0.2 μm nitrocellulose membrane. The transferred polypeptides were visualized by Ponceau S stain, and the

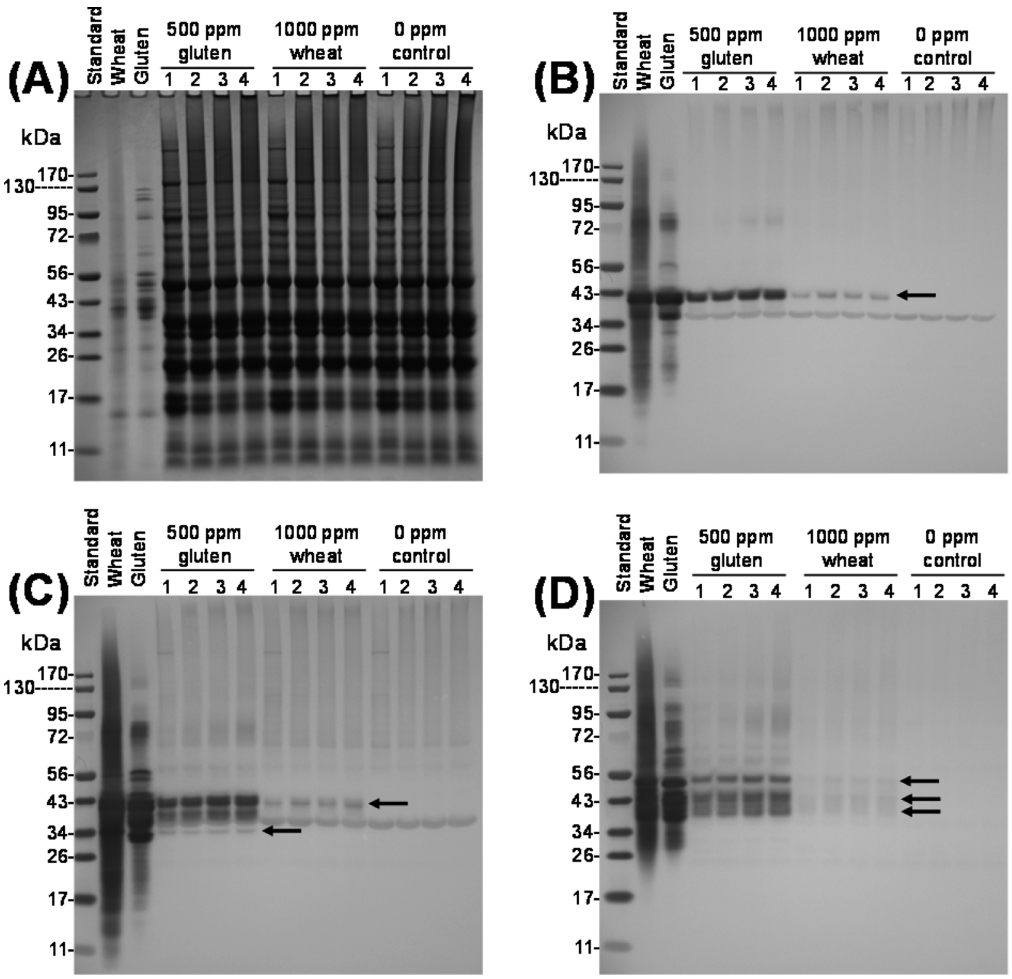

Figure 2. (A) Coomassie stain and (B, C, and D) western blots of control and incurred cornbread proteins using antibodies against (B) α-gliadin peptide, (C) B-hordein peptide, and (D) gliadin 33-mer peptide. The cornbread protein load in each lane was 20 μg, while the wheat and gluten protein load was 3 μg. 1, 2, 3, and 4 are cornbread baked for 0, 20, 27, and 34 min, respectively. The arrow indicates gluten polypepides.

membrane was blocked with 5% non-fat dried milk (NFDM) in tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at RT. After washing for 5 min with TBS-T, the membrane was incubated overnight at 4 °C with an appropriate dilution of primary antibody prepared in TBS-T containing 5% NFDM (anti-α-gliadin peptide, 5000×; anti-B-hordein peptide, 5000×; and A1 anti-gliadin 33-mer, 10000×). After washing thrice with TBS-T for 15 min each, the membrane was incubated for 1 h at RT with appropriate HRP-labeled secondary antibody. The membrane was again washed thrice with TBS-T for 15 min each, and the reactive polypeptides were developed using a CN/DAB substrate kit (Thermo Scientific, Rockford, IL).

LFD. Two samples each of incurred batter and cornbread baked for 20 min were weighed and extracted according to the recommendation of the manufacturer. Incurred levels tested were 0, 5, 10, 20, and 50 ppm gluten and 20, 50, 100, 200, and 500 ppm wheat flour. The supernatants were mixed (1:1, v/v) to minimize the variation because of sampling. An appropriate amount of supernatant was used in the lateral flow test with 10 different LFD strips to estimate the variation within the strips. Because the cornbread is heat-processed food, the RIDAQUICK Gliadin (R-Biopharm) was used with the cocktail solution, which increased the limit of detection (LOD) to 20 ppm gluten. The LOD for the Gluten (Gliadin) lateral flow kit (Morinaga) and AgraStrip Gluten G12 (Romer Labs) was 5 ppm.

ELISA. Four samples for each incurred batter and cornbread were analyzed in four aliquots (n = 16). The amount of sample (1 g) used for Morinaga and BioKits was as recommended by the manufacturer. However, the sample size (0.5 g) for R-Biopharm and Romer Labs kits was increased to reduce sampling error. Extraction buffer volumes were also increased accordingly to maintain the recommended extraction conditions. The extraction and ELISA were carried out as per the instructions of the kit manufacturer. The optical density in each well of the ELISA plate was measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA), and a four-parameter fit was plotted using the SoftMax Pro 5.4 software to calculate the gluten/wheat protein concentration based on the standard curve. The concentrations were normalized to account for the moisture loss during baking. Because the kits measure gluten or wheat proteins, the recovery in wheat flour incurred samples was quantified using an adjusted gluten/wheat protein concentration, which was calculated by determining the total protein content (micro-Kjeldahl method, with a conversion factor of 5.7) in wheat flour (12.55%) and assuming that 80% of total wheat proteins is gluten (10.04%). For example, the adjusted gluten and wheat protein concentrations of the 100 ppm wheat flour incurred sample will be 10.04 and 12.55 ppm, respectively.

Statistical Analysis. The data are expressed as the mean ± standard deviation. Accuracy of the kits was evaluated by estimating the percent recovery and linear regression analysis of measured versus incurred gluten levels. A slope of 1.0 denotes exact correlation and 100% recovery. Variance was used to evaluate the precision using the Proc Nested procedure in SAS.[11] The total variance was divided into sampling and analytical variances.

RESULTS AND DISCUSSION

Stability of Gluten in the Incurred Cornbread. An increase in baking time from 0 to 34 min caused changes in the SDS−PAGE profiles of cornbread samples as revealed by the decrease in band intensity at 15−17 and 90−130 kDa and a simultaneous increase in protein aggregates that did not enter the gel (Figure 2A). Because the amount of gluten is very low to be visualized by the Coomassie stain, the stability of gluten in incurred cornbread was assessed by western blot using three different anti-gluten antibodies (panels B−D of Figure 2). The anti-α-gliadin peptide antibody reacted strongly with ∼40 kDa polypeptide that was found in all cornbread samples baked for different times and incurred with 500 ppm gluten and 1000 ppm wheat flour but absent in the 0 ppm control cornbreads (marked by an arrow in Figure 2B). Similarly, the anti-B-hordein peptide antibody and anti-gliadin 33-mer peptide antibody exhibited strong reactivity to ∼34−43 and ∼35−55 kDa gluten polypeptides in the gluten/wheat flour incurred cornbread samples, respectively. The intensity of gluten reactivity with these antibodies was lower in 1000 ppm wheat flour incurred samples because the effective gluten content would amount to ∼100 ppm, which is one-fifth of the gluten content in 500 ppm gluten incurred cornbread. The presence of a similar intensity of gluten reactive bands at different baking times when probed with different antibodies indicates gluten protein stability and that the immunoreactivity is not significantly altered during baking. Immunostability of proteins upon thermal processing has been reported for other plant proteins, including food allergens, such as tree nuts[12,13] and peanut.[14]

Gluten Detection in Cornbread by LFD. LFDs are one of the rapid methods for gluten detection. Early evaluation of gluten contamination in foods can prevent further processing and expensive recalls. However, a false positive/negative on LFD can cause unnecessary cost if non-validated devices are used. Cornbread incurred with various levels of gluten (0−50 ppm) or wheat flour (20−500 ppm) and baked at 0 and 20 min were evaluated for gluten by three different LFDs (Table 1). Out of 10 strips used at each incurred level and baking time, only one faint false positive was observed in the 0 ppm control using the Morinaga LFD. The faint false positive may be the result of non-specific binding. Among the gluten incurred samples, the detection limit observed for Morinaga and R-Biopharm LFD was 5 and 10 ppm, respectively, for both unbaked (0 min) and baked (20 min) cornbread. However, the detection limit for Romer Labs LFD was 5 ppm for unbaked and 20 ppm for baked samples. Similarly, for the wheat flour incurred samples, the Morinaga LFD detected gluten in both unbaked and baked samples at as low as 20 ppm. The detection limit of the R-Biopharm LFD was 200 ppm using baked or unbaked wheat flour incurred samples. Gluten was detected at the 100 ppm level using Romer Labs LFD with wheat flour incurred samples, but the detection limit increased after (20 min) baking.

Table 1. Number of Positive Strips out of 10 Tested LFD Strips at Various Gluten and Wheat Flour Incurred Levels Using Three Different Lateral Flow Tests

		Morinaga		R-Biopharm		Romer Labs	
cornbread	incurred level (ppm)	0 min	20 min	0 min	20 min	0 min	20 min
control	0	0	1	0	0	0	0
gluten incurred	5	10	10	0	0	9	0
	10	10	10	10	10	10	0
	20	10	10	10	10	10	10
	50	10	10	10	10	10	10
wheat flour incurred	20	10	10	0	0	0	0
	50	10	10	0	0	0	0
	100	10	10	0	0	10	0
	200	10	10	10	10	10	5
	500	10	10	10	10	10	10

The Morinaga LFD uses anti-gliadin polyclonal antibodies to detect gluten, while the R-Biopharm and Romer Labs LFDs employ R5 and G12 monoclonal antibodies, respectively. Among the LFDs tested, the detection limit of Morinaga and R-Biopharm LFDs appeared to be unaffected by the baking conditions used for cornbread preparation. Because the LFDs are mostly used for qualitative detection and results are interpreted by visual inspection, very scare information is available on the validation of such immunoassays. Recently, a Skerritt antibody-based dipstick immunoassay called EZ Gluten test has been developed and performance-tested for detection of gluten at as a low as the 10 ppm level using incurred cooked dough and spiked rice flour, beer, and dog food.[15] Further studies are warranted on the validation of LFD for gluten detection and possible quantitation.

Gluten Recovery in Cornbread by ELISA. Four ELISA kits, each employing a different antibody for gluten detection, were used for gluten recovery in incurred cornbread samples: BioKits, Skerritt (401/21) monoclonal; Morinaga, anti-gluten polyclonal; R-biopharm, R5 monoclonal; and Romer Labs, G12 monoclonal antibody. The recovery of gluten can be quantified from the measured (M) and actual incurred level (I) of gluten by the formula: recovery (%) = $100 + [100(M - I)/I]$. The mean gluten recovery measured by four different ELISA kits across all incurred levels for gluten and wheat flour incurred cornbread samples is presented in Figure 3. Dependent upon the baking conditions and source of gluten, the recoveries varied with the kit: BioKits (9−77%), Morinaga (91−137%), R-Biopharm (61−108%), and Romer Labs (113−190%). The difference in the recoveries between the kits is expected because they vary in detection antibody, target gluten epitope, calibration standard, and assay features (such as extraction buffer, extraction time, and temperature).[7] The Skerritt antibody was developed to be specific for the ω-gliadin fraction but also recognizes the glutenin fraction.[16] The R5 and G12 antibodies were raised against rye secalin extract[17] and a 33-mer gliadin peptide,[18] respectively, and each recognizes different epitopes on gliadin. Also, some ELISA kits use gliadin as a calibration standard, and a conversion factor of 2 is applied to convert gliadin to the total gluten concentration. This conversion factor is arguable and may vary with cereal grain species and variety.[19] Development of a universal standard reference material for gluten will help harmonize the validation of gluten detection methods and reduce the variability among different immunoassays.

The recoveries varied with the source of gluten used for the preparation of incurred samples. For example, the recoveries using the Morinaga and R-Biopharm kits were lower when using the wheat flour incurred cornbread compared to gluten incurred cornbread, whereas the opposite was observed for the BioKits. Similar variations in gluten recovery by various ELISA kits were also observed when corn flour was spiked with gluten

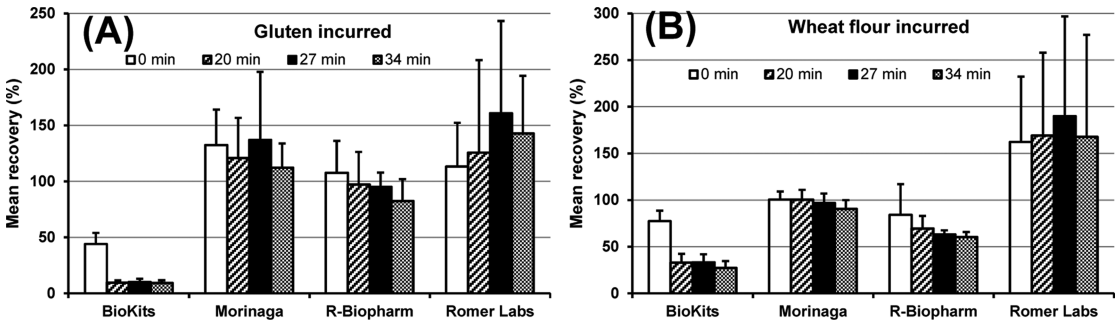

Figure 3. Average gluten recovery in cornbread incurred with (A) gluten and (B) wheat flour measured by different ELISA kits.

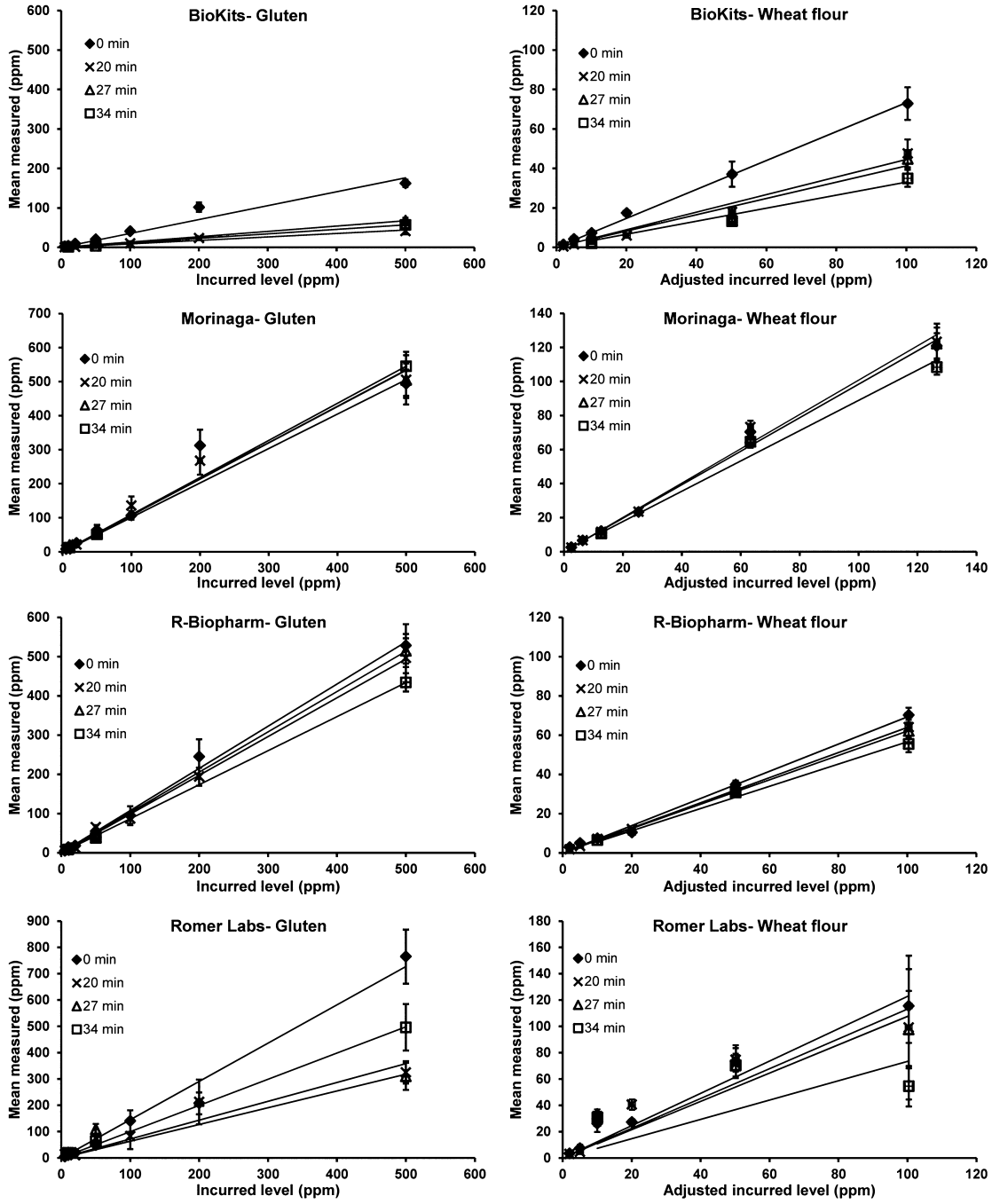

Figure 4. Correlation between measured and incurred levels of (left panels) gluten and (right panels) wheat flour in cornbread baked at different temperatures using different ELISA kits.

Table 2. Linear Regression Analysis of Correlation between Measured and Incurred Levels Presented in Figure 4

baking time (min)	linear regression analysis[a]	BioKits		Moringa		R-Biopharm		Romer Labs	
		gluten	wheat flour	gluten	wheat flour	gluten	wheat flour	gluten	wheat flour
0	slope (R^2)	0.3526 (0.95)	0.7329 (1.00)	1.0685 (0.94)	0.9841 (0.99)	1.0745 (0.99)	0.6931 (0.99)	1.4551 (0.98)	1.2283 (0.96)
20	slope (R^2)	0.0877 (0.97)	0.4452 (0.98)	1.0659 (0.98)	1.0075 (0.99)	0.9872 (1.00)	0.6389 (1.00)	0.7178 (0.91)	1.1314 (0.83)
27	slope (R^2)	0.135 (1.00)	0.4129 (0.94)	1.0113 (1.00)	0.983 (1.00)	1.0271 (1.00)	0.6203 (1.00)	0.636 (0.86)	1.0756 (0.58)
34	slope (R^2)	0.1139 (1.00)	0.3304 (0.97)	1.0903 (1.00)	0.8883 (0.98)	0.8674 (1.00)	0.5656 (0.99)	0.9964 (1.00)	0.7345 (−1.60)

[a]The intercept is considered as zero for the linear regression analysis.

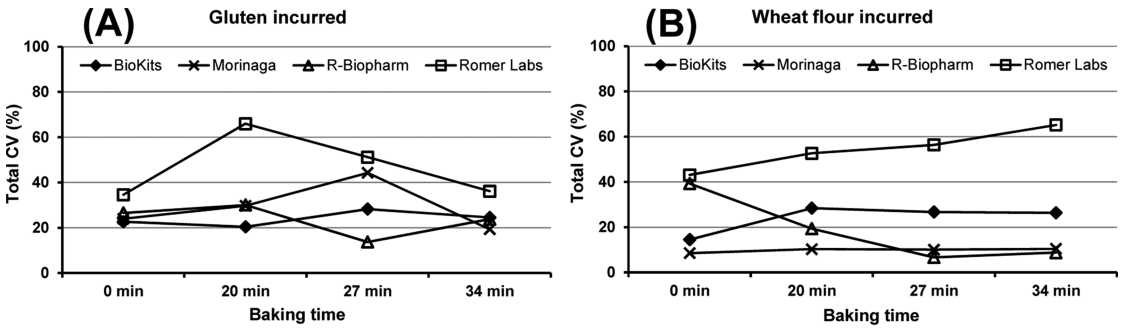

Figure 5. Effect of the cornbread baking time on total CV across all incurred levels using different ELISA kits.

or wheat flour.[5] The matrix effect may be responsible for different recoveries in spiked versus incurred samples. Higher gluten recoveries with ELISA were reported by Bugyi et al.[6] in a 10 mg/kg gliadin incurred cookie model matrix when gliadin recovery of the dry component mixture was compared to that of the cookie dough. This discrepancy was attributed to the effect of margarine and water added to the dry mixture.

The accuracy of the ELISA method can be measured by linear regression analysis from the slope of the straight line when plotting the incurred level (I) on the x axis and measured level (M) on the y axis. A slope close to 1 denotes the most accurate results, whereas the values higher or lower than 1 are a result of overestimation and underestimation of guten levels, respectively. The correlation between the measured and incurred levels of gluten/wheat flour in cornbread is shown for all ELISA kits in Figure 4. Assuming that the intercept is (0,0), i.e., ELISA measuring 0 ppm at an incurred level of 0 ppm, the slope (s) can be obtained by the equation $M = sI$. The slopes and the coefficient of determination (R^2) values for the plots shown in Figure 4 are listed in Table 2. On the basis of the slopes, the Morinaga kit was the most accurate using either gluten or wheat flour incurred samples. Gluten was underestimated by the BioKits kit (Table 2). There was a good correlation between measured versus incurred levels with all kits using cornbread baked for 0 min (R^2 = 0.94−1.00) and 20 min (R^2 = 0.83−1.00), but poor correlation was observed with Romer Labs for wheat flour incurred cornbread baked for 27 min (R^2 = 0.58) and 34 min (R^2 = −1.60). The poor fit may be due to the lack of enough data points because only three incurred levels were selected for longer baking times. A concentration-dependent variation in gluten quantitation by ELISA has been observed using gluten-spiked oat flour[20] or buckwheat flour.[21] A decrease in gluten recovery by the R-Biopharm and BioKits ELISA kits at higher levels of gluten (1000 ppm compared to 20 and 100 ppm) in buckwheat flour was attributed to the extraction saturation effect.[21]

Effect of the Baking Time on Gluten Detection. The temperature causes various physical and chemical modifications in proteins, which may affect the ELISA quantification by reducing extraction efficiency and/or changing the antigen−antibody interaction. To evaluate the effect of thermal processing on the gluten detection by ELISA, we compared the gluten detected in unbaked (0 min) to those baked for 20 min. Also, select gluten incurred cornbread was baked for 27 and 34 min. The gluten recovery from incurred cornbread samples varied with the ELISA kit and length of baking. In general, the recovery decreased with an increase in the baking time, with some exceptions in Morinaga and Romer Labs kit. The Morinaga kit was least affected by the baking time, as seen by the minimal changes in the recovery (Figure 3) and accuracy (Figure 4 and Table 2). BioKits had a lower recovery and accuracy in the unbaked (0 min) cornbread, which reduced further with an increase in the baking time. Although the gluten recovery by R-Biopharm was close to 100%, a trend of reduced recovery and accuracy was observed with increased baking time. The changes in gluten recovery by Romer Labs did not show any trend with baking time but unexpectedly exhibited higher average recovery in baked samples compared to unbaked samples. This could be due to sampling variation or high standard deviation in average gluten recovery. An adverse effect on gluten recovery in the cookie matrix as a result of thermal processing has been reported earlier. Bugyi et al.[6] noted lower gliadin content in baked cookies compared to cookie dough with seven commercial ELISA kits analyzed. A study by Gomaa et al.,[22] using gluten incurred cookies, also reported a decrease in gluten recovery with an increase in the baking time and a decrease in the cookie size by two different ELISA kits.

The gluten structure is modified during baking or other thermal processes because of polymerization and sulfydryl−disulfide bond exchange.[23,24] These changes along with other chemical modifications (e.g., Maillard reaction) may result in inaccurate ELISA gluten quantification. However, processing effects can be minimized by various factors, such as (1) extraction buffer, chaotropic and reducing agent will help dissociate protein aggregation and increase protein solubility; (2) target epitope, an antibody targeting a linear epitope will

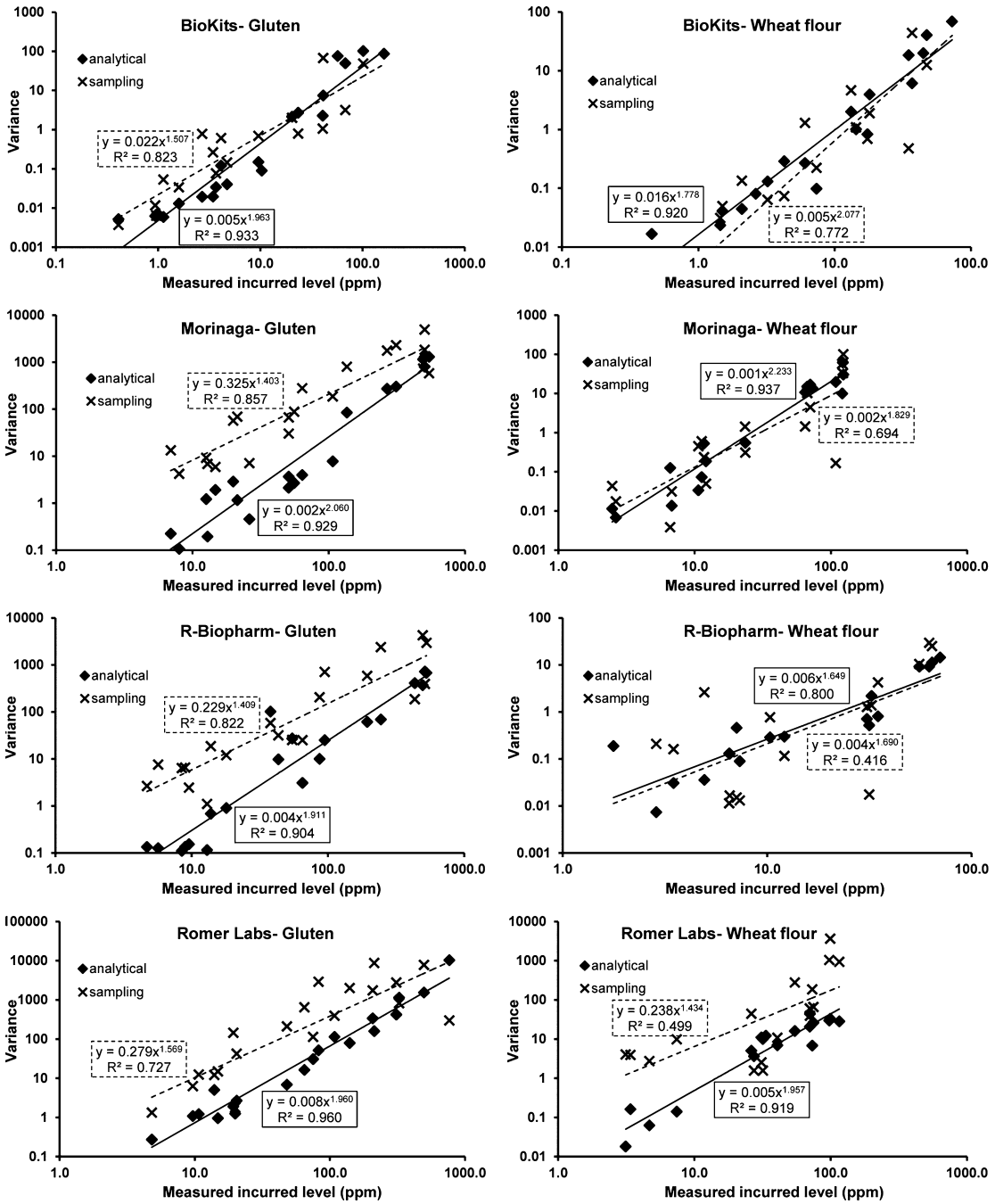

Figure 6. (Solid lines) Analytical and (dashed lines) sampling variance associated with measured gluten in cornbread incurred with (left panels) gluten and (right panels) wheat flour using different ELISA kits. The variance value of zero is not included on the log scale.

have better antigen−antibody interaction compared to those targeting nonlinear epitopes because the latter may be destroyed during baking; and (3) incurred standard or control, the incurred standard or control can account for the decrease in gluten recovery following thermal processing. The difference in any of these factors may contribute to changes in recovery after baking with an individual ELISA kit.

Variation in Gluten Quantitation by ELISA. Variance and coefficient of variation (% CV) are often used to estimate the precision of the analytical assay. Overall, the range of % CV at all incurred levels and baking times for different kits was as follows: BioKits kit, 2.9−29.8 (gluten incurred) and 6.8−28.5 (wheat flour incurred); Morinaga kit, 7.7−47.6 (gluten incurred) and 2.9−8.7 (wheat flour incurred); R-Biopharm kit, 5.4−43.8 (gluten incurred) and 2.3−29.5 (wheat flour incurred); and Romer Labs kit, 8.2−59.2 (gluten incurred) and 8.1−57.7 (wheat flour incurred). The total CV (sampling plus analytical variation) at all incurred levels was compared for different baking times (Figure 5). There was no correlation observed between the CV and baking time for any of the kits. The CV for the Romer Labs kit using wheat flour incurred samples increased with an increase in the baking time, but similar results were not seen using gluten incurred samples. Khuda et al.[9] reported an increase in CV for baked cookies compared to cookie dough, which was a reflection of the

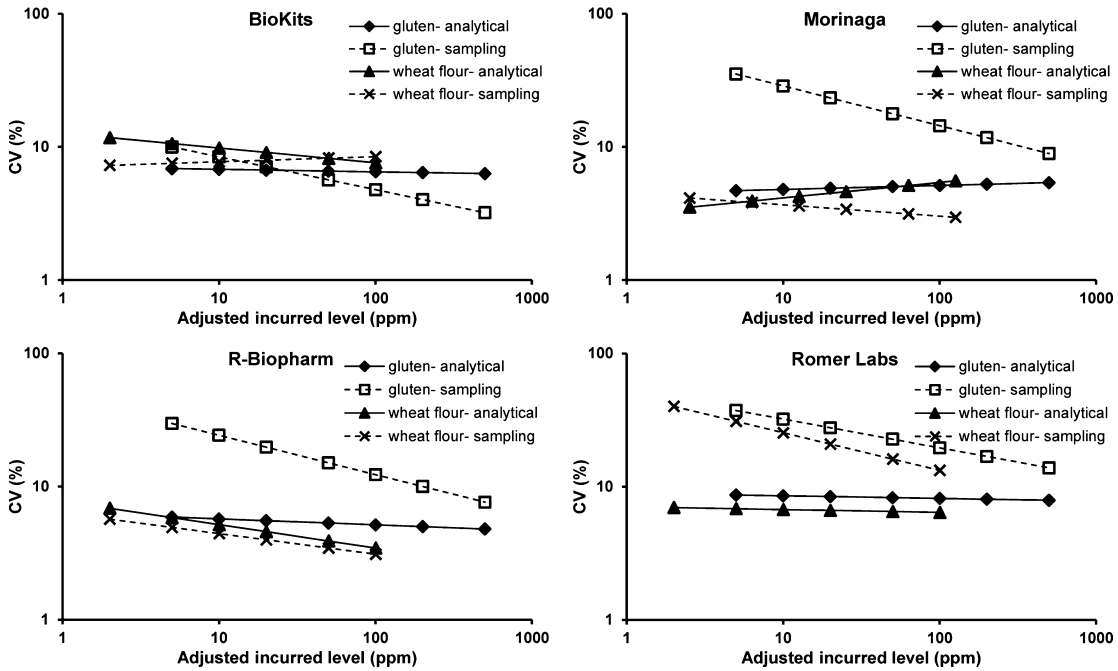

Figure 7. (Solid line) Predicted analytical and (broken line) sampling CV associated with the incurred level of gluten in cornbread using different ELISA kits. The CV is calculated from the respective variance equations in Figure 6

reduced measured amount of food allergens in the baked samples.

The total variance associated with ELISA is the sum of (1) sampling variance, which relates the measured concentration differences among four samples, and (2) analytical variance, which relates the measured concentration differences among the four aliquots of each sample.[25] The sampling and analytical variance for gluten kits using gluten and wheat flour incurred cornbread are shown in Figure 6. An increase in both types of variance is observed with an increase in the measured incurred level for all kits. This relationship is typical and has been previously reported for other food allergen ELISA kits.[9,25,26] Analytical variance was found to be lower than the sampling variance for most kits at a given incurred level with some exceptions. The R-Biopharm kit has similar analytical and sampling variances for wheat incurred cornbread, while the analytical variance was higher for BioKits (gluten incurred) and Morinaga (wheat flour incurred) kits at higher incurred levels. The variation in measured gluten by ELISA kits can be better visualized by calculating the analytical and sampling variance from the regression equations given in Figure 6 for each kit and then predicting the respective CV by the formula: % CV = $100(v^{0.5}/I)$, where v is the calculated sampling or analytical variance and I is the incurred level. The predicted values for analytical and sampling CV are plotted against the incurred level on a log−log scale (Figure 7). The predicted sampling CV decreased with an increase in the incurred level, with the effect being more pronounced in the gluten incurred cornbread for all kits and wheat flour incurred cornbread for Romer Labs kit. The predicted analytical CV associated with all kits was less than 12% for all incurred levels, exhibiting good analytical precision.

In summary, cornbread was used as an incurred food model to study the effect of baking on gluten measurement by immunoassays. The accuracy and precision of gluten quantitation was markedly affected by the ELISA kit type, baking conditions, and incurred gluten source. Although all kits showed good analytical precision, accuracy varied depending upon the kit used and length of baking time. Both accuracy and precision should be considered for validation of an ELISA assay. An increase in baking time reduced the measured gluten levels for most kits. A reduction in measured levels after baking that is below the limit of detection for a given ELISA may generate false negative results and risk the safety of gluten-sensitive individuals. Further studies are warranted to better understand the effect of food processing on gluten extraction and immunoreactivity, and develop a universal reference material for gluten to minimize the variations between immunoassays.

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Notes

The authors declare no competing financial interest.

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