AACCI Approved Methods Technical Committee Report: Collaborative Study on the Immunochemical Determination of Intact Gluten Using an R5 Sandwich ELISA

Peter Koehler,[1] Theresa Schwalb,[1] Ulrike Immer,[2] Markus Lacorn,[2] Paul Wehling,[3] and Clyde Don[4]

ABSTRACT

In 2008, the AACC International Protein Technical Committee (now the Protein and Enzymes Technical Committee) initiated a collaborative study of a method for determining gluten in selected foods using an R5 antibody sandwich ELISA system. The method has been approved as AACCI Approved Method 38-50.01. The new method has been validated for testing foods to determine that they conform to the newly defined Codex threshold of 20 mg of gluten/kg in total for gluten-free products.

Gluten is a protein fraction found in wheat, rye, barley, oats, and their crossbred varieties and derivatives thereof to which some persons are intolerant, and it is insoluble in water and 0.5 mol NaCl/L (4,6). Prolamins are gluten fractions that can be extracted with 40–70% ethanol. The prolamins gliadin, secalin, hordein, and avenin are found in wheat, rye, barley, and oats, respectively (4). The prolamin content of gluten is generally taken as 50% (4). The toxicity of gluten protein in oats is still under discussion, and the Codex standard notes that the allowable level for oats in foods not contaminated with wheat, rye, or barley may be determined at the national level. In foods labeled as "gluten-free," the gluten level must not exceed 20 mg/kg of food (4,6). Foods processed to reduce their gluten content to a level ranging from >20 to 100 mg/kg may not be labeled as "gluten-free"; labeling is regulated at the national level (e.g., could be labeled "very low gluten"). From these regulations it is obvious that effective test methods are needed to accurately determine the gluten concentration in foods and raw materials (4–6).

The Working Group on Prolamin Analysis and Toxicity (PWG) focused on improving the ELISA methodology for gluten analysis because the existing methods were inadequate with respect to sensitivity and reliability (10). A collaboration between the PWG and the research group headed by Enrique Méndez at the University of Madrid led to improved ELISA methods that use both sandwich and competitive assay systems and are based on the monoclonal R5 antibody. This antibody is directed toward the epitope glutamine-glutamine-proline-phenylalanine-proline (QQPFP) in gliadins, hordeins, and secalins. Immunochemical testing was accompanied by mass spectrometry, HPLC, SDS-PAGE, and capillary electrophoresis analyses (7,8,12). The monoclonal R5 antibody allows quantification of prolamins, the alcohol-soluble fraction of gluten. To convert it to a gluten concentration, the prolamin concentration is multiplied by a factor of 2.

In 2000, the PWG conducted the first collaborative study, which included 12 samples (controls, heated/nonheated spiked samples, and naturally contaminated samples) and 20 participating laboratories. At the request of consumer advocates and considering the results of this study, the R5 sandwich ELISA method (the "Mendez method") was endorsed in 2006 as a Type 1 method by the Codex Committee on Methods of Analysis and Sampling (3).

Following the guidelines of the AACC International (AACCI) Approved Methods Technical Committee (9), an international collaborative study was set up to validate the R5 sandwich ELISA method (RIDASCREEN Gliadin R7001, R-Biopharm) for gluten/prolamin quantitation in raw and processed food materials as an AACCI Approved Method. The study was carried out as a collaboration between the PWG and AACCI. It was coordinated by Peter Koehler (German Research Center for Food Chemistry, chair of the PWG, and member of the AACCI Protein and Enzymes Technical Committee) in close collaboration with Clyde Don (chair of the AACCI Protein and Enzymes Technical Committee). The analytical performance of this method is reported in this article.

ELISA Kit and Calculation Software

The R5 sandwich ELISA kit (RIDASCREEN Gliadin R7001) for the quantitation of gluten in raw and processed foods and the software (RIDASOFT Win Z9999, R-Biopharm) for constructing calibration curves (cubic spline fitting) and calculating prolamin concentrations from measured optical densities (OD) were used.

A cubic spline is a curve constructed of piecewise third-order polynomials that pass through a number (m) of control points. The second derivative of each polynomial is commonly set to zero at the end points of the pieces. This provides a boundary condition that completes the system of m^{-2} equations. It produces a "natural" cubic spline and leads to a simple tridiagonal system that can be solved easily to give the coefficients of the polynomials (14). In this way a function with a continuous curvature over the entire range is obtained. In preparing descriptive statistical analysis, a third-order polynomial curve fitting is used to calculate the results for samples with <2.5 mg of gliadin/kg.

The third derivative is used as a smoothing factor in the calibration curves to determine the extent of interpolation. Lower factors lead to more approximation; higher factors (>100) lead to more interpolation of the curve function. The RIDASOFT software uses a factor of 10,000. To minimize boundary effects and allow extrapolation, two additional control points are added to the set of control points as the starting and end points, where the starting point is near zero and set to $x_0 = 0.001$ and y_0 = OD (lowest Standard1) and the virtual end point is determined by calculating the linear regression of the other control points by assuming that x_n has the same distance to x_{n-1} as x_1 has to x_0. A comparison of both calculations is provided.

Participating Laboratories

All laboratories participating in the collaborative study were required to be familiar with immunological tests and, if possible, with R5 ELISA. They were advised to use a separate test

[1] German Research Center for Food Chemistry, Freising, Germany.
[2] R-Biopharm AG, Darmstadt, Germany.
[3] General Mills, Inc., Minneapolis, MN, U.S.A.
[4] Foodphysica, Driel, The Netherlands.

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room for the collaborative study due to the low detection limit and the possibility of contamination. To check the samples, test requirements, and documentation and identify critical points, a precollaborative study with four laboratories in Europe was completed before the full collaborative study. Encouraging results were obtained in the prestudy. Only minor changes in the study design were required, and the full collaborative study went on as scheduled. The labs were given six weeks to perform the analyses (August 1 to September 15, 2011). Sixteen labs were selected to participate (designated A to P): one each in Argentina, Austria, Belgium, Canada, Finland, Hungary, Ireland, Italy, New Zealand, Sweden, and Switzerland; two in Germany; and three in the United States.

Samples and Sample Preparation

The following samples were prepared for the collaborative study:

1) Corn bread, gluten-free
2) Corn bread, 10 mg of gliadin/kg
3) Corn bread, 20 mg of gliadin/kg
4) Corn bread, 50 mg of gliadin/kg
5) Corn flour, gluten-free
6) Corn flour, naturally contaminated
7) Extruded corn snack, gluten-free
8) Extruded corn snack, 50 mg of gliadin/kg

All ingredients, except wheat flour, were confirmed to be free of gluten contamination before use by R5 sandwich ELISA, which was also used in the collaborative study.

Wheat Flour for Preparing Gluten-Containing Samples. Wheat flour from German cv. Astron was used to prepare samples with defined gliadin contents. The gliadin content of the flour was determined by an extraction–RP-HPLC method as described by Wieser et al. (15). HPLC absorbance values measured at 210 nm were converted to protein concentrations using a standard solution of gliadin from the PWG (13). The gliadin content of the wheat flour sample was 59.1 g/kg on an as-is basis.

Processing Conditions. Samples were heat treated to different extents during processing, as would be found in consumer products:

- Corn flour was used as is (not heat treated) and represented a base material for the production of gluten-free products.
- Corn bread represented a product that had been moderately heat treated.
- The corn snack represented a heavily heat-treated product.

Preparation of Corn Bread. Gluten-free corn bread was prepared as follows: 100 g of corn flour, 18 g of water, 2 mL of ethanol (60%, vol/vol), 100 g of egg albumen, 2 g of sodium chloride, 4 g of sucrose, and 2.5 g of dry yeast were mixed for 3 min, and the dough was poured into a tin. The dough was proofed for 40 min at 30°C and baked for 30 min at 230°C. After cooling, the bread (Fig. 1A) was cut into 1.5 cm pieces, lyophilized, and ground in a laboratory mill. Corn bread containing gliadin was produced by adding wheat flour (250 mg) to the formula. The resulting gluten-containing corn bread had a gliadin content of 108.2 mg/kg. Gluten-free and gluten-containing bread powders were mixed appropriately to yield "breads" that had a gliadin content of 10, 20, or 50 mg/kg.

Preparation of Corn Snack. Corn snack samples were produced in a pilot-scale twin-screw extruder at a barrel temperature of 170°C (last stage). A corn flour blend (45.4 kg) was used as the starting material for the gluten-free sample. For the gluten-containing sample, wheat flour (38.4 g) was mixed with the corn flour blend (45.4 kg) prior to processing. The resulting gluten-containing sample had a gliadin content of 50 mg/kg. Snack samples (Fig. 1B) were ground to a fine powder in a laboratory mill.

Homogeneity of Samples. All samples were checked for homogeneity before they were packaged in airtight bottles and accepted for the collaborative study. This was done by taking 10 representative 1 g aliquots from the bulk sample and using R5 sandwich ELISA to analyze the aliquots. Samples were considered homogeneous if the coefficient of variation of the 10 de-

Table I. Gliadin concentrations determined using R5 sandwich ELISA and cubic spline calibration model (RIDASOFT Win Z9999 software)[a]

	Gliadin Concentration (mg/kg) of Duplicate Analyses of Samples															
	Gluten-free Corn Bread		Corn Bread, Gliadin at 10 mg/kg		Corn Bread, Gliadin at 20 mg/kg		Corn Bread, Gliadin at 50 mg/kg		Gluten-free Corn Flour		Naturally Contaminated Corn Flour		Gluten-free Corn Snack		Corn Snack, Gliadin at 50 mg/kg	
Lab[b]	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	<2.5	2.8	7.1	10.4	17.7	17.6	38.2	47.5	<2.5	<2.5	11.7	3.5	6.6 dg	6.5 dg	57.5	41.7
B	<2.5	<2.5	8.7	9.8	17.9	<2.5 o	34.1	43.9	<2.5	<2.5	<2.5 o	2.7	3.7	3.3	45.0	46.7
C	<2.5	<2.5	8.0	7.5	14.8	11.7	34.8	32.9	<2.5	<2.5	4.0	2.8	3.0	2.9	32.2	30.6
D	<2.5	<2.5	11.7	13.6	19.9	19.8	36.4	36.6	<2.5	<2.5	12.7	5.0	4.7	3.4	32.5	27.6
E	<2.5	<2.5	8.1	7.6	18.8	18.6	50.4	58.0	<2.5	<2.5	4.6	5.7	4.2	3.7	48.3	41.4
G	<2.5	<2.5	11.4	12.1	22.1	20.8	34.1	34.8	<2.5	<2.5	5.4	6.5	4.8	4.3	66.8	54.0
H	<2.5	<2.5	9.6	9.1	18.0	21.2	48.0	39.4	<2.5	<2.5	3.6	4.4	3.9	3.7	42.9	44.3
I	<2.5	<2.5	7.9	7.7	15.1	12.3	30.4	34.9	<2.5	<2.5	4.6	<2.5 o	3.6	3.1	58.4	57.0
J	<2.5	<2.5	8.9	9.7	19.9	17.7	49.8	52.3	<2.5	<2.5	5.0	5.1	4.2	4.7	56.8	52.0
K	<2.5	<2.5	10.3	8.6	21.4	18.5	48.8	42.7	<2.5	<2.5	5.4	5.4	3.5	4.3	35.5	45.1
L	<2.5	<2.5	10.8	10.9	20.8	20.3	52.4	52.5	<2.5	<2.5	7.0	6.1	5.2	4.9	59.4	55.4
M	<2.5	<2.5	9.6	8.2	17.6	16.9	37.9	36.3	<2.5	<2.5	4.8	3.0	3.4	3.2	33.8	33.3
N	<2.5	<2.5	7.2	6.8	10.6	14.6	34.9	24.7	<2.5	<2.5	2.4	3.2	<2.5 dg	<2.5 dg	25.5	28.9
O	<2.5	<2.5	7.5	7.3	13.9	14.8	40.0	32.8	<2.5	<2.5	<2.5 o	4.2	20.9 c	2.6 c	34.3	36.6
P	3.4	<2.5	9.4	8.7	17.5	18.6	48.5	50.0	<2.5	<2.5	3.2	4.1	4.1	2.9	53.3	31.9

[a] Outliers are indicated by letters following values: o = out; dg = double Grubbs; and c = Cochran.
[b] Laboratory F was excluded because it did not run the calibrations in duplicate determinations as directed.

terminations was 15% or less (determined based on previous R-Biopharm validation data). All samples fulfilled the criteria, except the naturally contaminated corn flour. The analyses for homogeneity also revealed that the gluten-free corn snack was contaminated with gluten at a very low concentration of ≈5 mg of gliadin/kg. The source of contamination was most likely the production line.

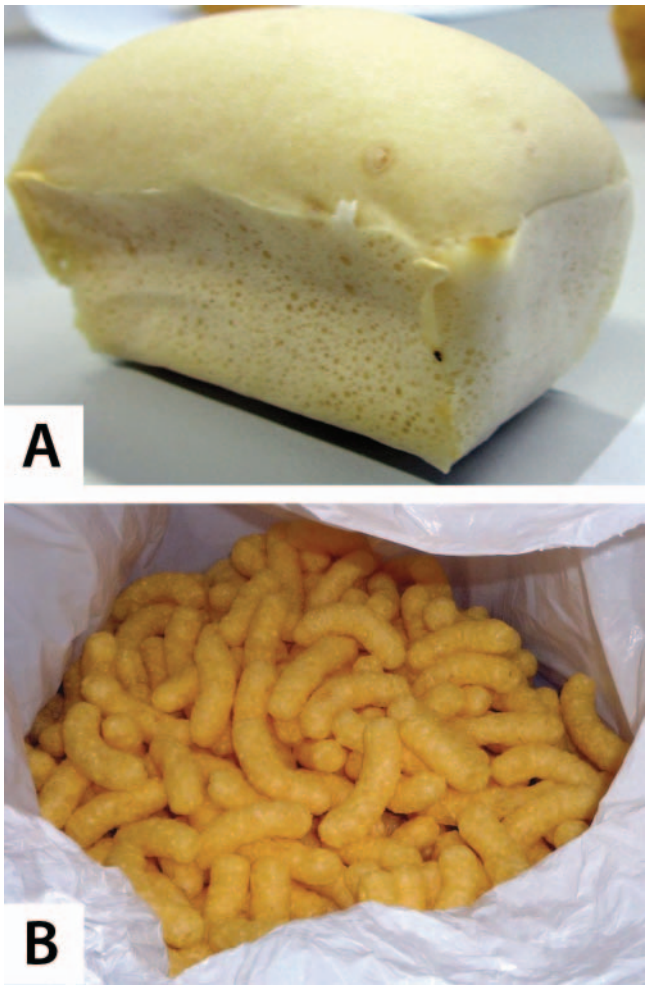

Fig. 1. Corn bread (**A**) and corn snack (**B**) samples used in this study.

Presentation of Samples to Labs

Following the AACCI collaborative trial guidelines, two independent blinded replicates for each sample were provided to the participating laboratories. Each sample was extracted using the cocktail procedure and analyzed in duplicate in one analytical run. Sixteen samples were analyzed by each laboratory.

Method

The method was written in AACCI style and was provided to each lab with instructions to follow the method as written with no deviations. The labs were directed to pay particular attention to cases in which samples had to be repeated by further dilution and how the dilutions were to be carried out. All OD values had to be recorded in a ready-to-use Excel (Microsoft Corp.) worksheet. Participants were asked to use RIDASOFT calculation software for cubic spline curve fitting; the software was provided with the kit. The final data from the laboratories were sent to the study coordinator.

Results of the Collaborative Study

The quantitative results from all participants ("raw data") are compiled in Tables I and II. Fifteen of sixteen laboratories provided results that were suitable for statistical analysis. Only laboratory F had to be excluded from the statistical evaluation because it did not run the calibrations in duplicate determinations as directed. Table I contains the results after calibration using a cubic spline function (obtained using RIDASOFT software). Table II contains the results after calibration using a third-order polynomial function. The quantities are expressed as milligrams of gliadin per kilogram of sample.

Statistical Analysis and Discussion

Outliers were identified using the Cochran and Grubbs tests according to AOAC International (AOACI) guidelines (2). The performance statistics without outliers are given in Table III (cubic spline) and Table IV (third-order polynomial function). The

Table II. Gliadin concentrations determined using R5 sandwich ELISA and third-order polynomial calibration model (Excel software)

	Gliadin Concentration (mg/kg) of Duplicate Analyses of Samples[a]															
	Gluten-free Corn Bread		Corn Bread, Gliadin at 10 mg/kg		Corn Bread, Gliadin at 20 mg/kg		Corn Bread, Gliadin at 50 mg/kg		Gluten-free Corn Flour		Naturally Contaminated Corn Flour		Gluten-free Corn Snack		Corn Snack, Gliadin at 50 mg/kg	
Lab[b]	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	1.36 c	2.30 c	7.4	11.1	17.5	17.4	38.3	47.2	0.90	1.67	12.3	3.0	6.7 dg	6.6 dg	57.8	41.2
B	0.14	0.18	8.7	10.0	18.2 c	0.0 c	34.8	44.0	0.18	0.32	2.2	2.5	3.4	3.0	45.2	46.7
C	0.23	0.07	8.1	7.7	14.8	12.1	35.1	33.2	0.24	0.30	3.9	2.6	2.8	2.7	32.5	31.0
D	0.17	–0.04	11.5	13.4	20.0	20.0	36.9	37.2	–0.03	–0.08	12.5	5.0	4.7	3.5	33.4	28.2
E	0.33 c	1.48 c	8.3	7.8	18.6	18.6	47.1	66.3	2.38 g	2.06 g	4.6	5.9	4.1	3.7	54.1	56.3
G	0.38	0.47	10.8	11.4	22.9	21.4	34.2	35.0	0.37 c	6.31 c	5.5	7.7	5.1	4.5	51.6	51.6
H	0.57	0.65	9.9	9.4	17.9	21.1	41.2	39.4	0.74	0.82	3.3	4.2	3.7	3.4	45.3	46.8
I	0.13	0.10	7.8	7.6	15.1	12.2	30.9	49.2	0.07	–0.01	4.6	1.6	3.7	3.2	59.3	57.8
J	3.22	0.50	9.0	9.8	20.1	17.9	49.7	52.2	0.43	0.57	5.0	5.1	4.0	4.6	57.1	52.1
K	0.44	0.47	10.5	8.7	22.0	19.0	50.2	43.9	0.51	0.41	5.0	5.0	3.0	3.9	35.7	46.5
L	0.37	0.39	10.6	10.7	20.9	20.5	53.9	54.1	0.37	0.43	7.0	6.2	5.3	5.0	60.8	56.8
M	0.30	0.22	9.5	8.1	17.7	17.0	37.9	36.5	0.07	0.04	4.9	3.0	3.4	3.3	34.2	33.7
N	0.21	0.38	7.1	6.7	9.9	14.2	35.4	26.2	0.29	–0.61	2.8	3.4	0.6 dg	1.9 dg	26.6	29.9
O	0.32	0.27	7.3	7.0	14.4	15.4	41.5	28.6	0.26	0.42	2.0	3.8	21.9 c	2.4 c	34.6	36.5
P	3.67 c	–0.16 c	9.0	8.4	17.4	18.6	48.9	50.5	–0.37	0.04	3.3	4.4	4.4	3.1	54.1	32.6

[a] Outliers are indicated by letters following values: g = Grubbs; dg = double Grubbs; and c = Cochran.
[b] Laboratory F was excluded because it did not run the calibrations in duplicate determinations as directed.

number of outliers was identified from the total number of replicates and total number of laboratories in both Tables III and IV.

Table I shows the data after calibration with a cubic spline model. The gluten-free corn flour (sample 5) and gluten-free bread (sample 1) contained <2.5 mg of gliadin/kg (<5 mg of gluten/kg). In the software, the limit of quantification (LOQ) for gliadin was set at 2.5 mg/kg. The gluten-free corn snack (sample 7), which was prepared from gluten-free corn flour, contained a trace of gluten. This was probably caused by contamination in the extruder. The gluten concentrations in the other samples were within the expected ranges.

To gain an impression of the limit of detection (LOD) of the method using the AOACI calculation (overall mean plus 3.3-fold reproducibility standard deviation) (2), calculation of numbers for the blank samples was necessary. This was done using a third-order polynomial curve fitting. The resulting values for each laboratory and sample are given in Table II. As expected, some of the reported values were negative because the values fluctuated around zero.

The performance statistics for the results obtained with the cubic spline calibration are shown in Table III. The relative standard deviations were between 10 and 20% for most samples. The naturally contaminated corn flour (sample 6) showed high standard deviations, which would be expected because the homogeneity tests had already shown higher variation compared with the other samples. Nevertheless, the method did recognize very low gliadin concentrations with high precision.

The third-order polynomial calibration allowed the estimation of LOQ and LOD based on the gluten-free samples (Table IV). Calculating the overall mean resulted in gliadin values of 0.3 mg/kg for the gluten-free corn flour (sample 5) and bread (sample 1). As expected, the standard deviations were high because each value resulted from one calibration curve in a laboratory. At low concentrations calibration curves were very flat, and small differences in OD resulted in high variation in the gliadin concentrations. From the data for samples 1 and 5 (gluten-free bread and corn flour, respectively), LODs of 0.86 and 1.76 mg of gliadin/kg, respectively, were calculated. This agrees with the R-Biopharm validation data values of 1.5 mg of gliadin/kg measured in six matrices (n = 10). These results confirm that the chosen cutoff of <2.5 mg/kg was higher than the LOD. As a consequence of the high standard deviations for samples 1 and 5, high HORRAT values were obtained. However, according to Thompson (11) and Abbott et al. (1) the calculation of HORRAT values is meaningless in cases of concentrations below the LOQ or zero samples. For the other samples containing gliadin at concentrations >2.5 mg/kg, the repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R) values were lower and in a similar range, irrespective of the curve-fitting procedure (cubic spline or third-order polynomial fitting).

Figure 2 shows individual gliadin concentrations obtained by 15 laboratories and the random error, according to Youden (16), of the bread sample with 10 mg of gliadin/kg (equal to 20 mg of gluten/kg). The graphs show that the standard errors were higher than those reported in Tables III and IV. The third-order polynomial calibration (Fig. 2B) yielded a higher variation than the cubic spline method (Fig. 2A). Values that are far away from the diagonal line in the Youden plot represent random errors.

Table III. Performance statistics for overall R5 sandwich ELISA results from Table I (calibration by cubic spline function using RIDASOFT Win Z9999 software)

	Sample ID[a]							
Parameter	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Total number of laboratories (P)	–	15	15	15	–	15	12	15
Total number of replicates ($Sum_{n(L)}$)	–	30	29	30	–	27	24	30
Overall mean for all data (grand mean; XBARBAR) (mg/kg)	–	9.1	17.6	41.3	–	5.0	3.9	43.6
Repeatability standard deviation(s_r) (mg/kg)	–	0.9	1.5	4.3	–	2.4	0.5	6.1
Reproducibility standard deviation (s_R) (mg/kg)	–	1.7	3.0	8.4	–	2.4	0.7	11.5
Repeatability relative standard deviation (RSD_r) (%)	–	9.6	8.5	10.3	–	48.5	11.8	13.9
Reproducibility relative standard deviation (RSD_R) (%)	–	18.7	17.3	20.3	–	48.5	17.6	26.5
HORRAT value	–	1.6	1.7	2.2	–	3.9	1.4	2.9

[a] 1: gluten-free corn bread; 2: corn bread with 10 mg of gliadin/kg; 3: corn bread with 20 mg of gliadin/kg; 4: corn bread with 50 mg of gliadin/kg; 5: gluten-free corn flour; 6: naturally contaminated corn flour; 7: gluten-free corn snack; and 8: corn snack with 50 mg of gliadin/kg.

Table IV. Performance statistics for overall R5 sandwich ELISA results from Table II (calibration by third-order polynomial function using Excel software)

	Sample ID[a]							
Parameter	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Total number of laboratories (P)	12	15	14	15	13	15	12	15
Total number of replicates ($Sum_{n(L)}$)	24	30	28	30	26	30	24	30
Overall mean for all data (grand mean; XBARBAR) (mg/kg)	0.3	9.1	17.7	42.0	0.3	4.7	3.8	44.3
Repeatability standard deviation(s_r) (mg/kg)	0.1	0.9	1.5	6.3	0.3	2.4	0.5	5.6
Reproducibility standard deviation (s_R) (mg/kg)	0.2	1.6	3.2	9.1	0.4	2.5	0.8	11.1
Repeatability relative standard deviation (RSD_r) (%)	26.8	10.4	8.5	14.9	82.7	50.2	12.2	12.7
Reproducibility relative standard deviation (RSD_R) (%)	56.8	18.1	18.3	21.6	144.8	53.7	20.0	25.1
HORRAT value	3.0	1.6	1.8	2.4	7.6	4.2	1.5	2.8

[a] 1: gluten-free corn bread; 2: corn bread with 10 mg of gliadin/kg; 3: corn bread with 20 mg of gliadin/kg; 4: corn bread with 50 mg of gliadin/kg; 5: gluten-free corn flour; 6: naturally contaminated corn flour; 7: gluten-free corn snack; and 8: corn snack with 50 mg of gliadin/kg.

Dots on the line show no random error, but the further the distance to the origin the higher the systematic error. The data from both calibration models show the majority of the values were close to the diagonal line; only two values were far away. However, there were also samples close to the line but outside the standard error circle. The number of dots within the standard error range was six for the cubic spline and nine for the third-order polynomial calibration due to the higher standard error for this model.

Discussion

The immunochemical method for gliadin quantitation that was evaluated in this collaborative study is designed for the detection of gluten contamination in the critical concentration range around 20 mg/kg (Codex threshold for declaring a food gluten-free) (4). In this context it is important to note that this article reports gliadin concentrations, which can be converted to gluten concentrations by multiplying by a factor of 2. Therefore, a method to quantify gluten accurately according to the established threshold requires precision at ≈10 mg of gliadin/kg. Furthermore, with the threshold for gluten-free foods set at 20 mg of gluten/kg (4,6), a more sensitive ELISA method compared with the previously developed and evaluated method (10) is required.

Abbott et al. (1) suggest recoveries between 80 and 120% as acceptable criteria for ELISA methods. For ELISA methods used for quantitating food allergens, recoveries between 50 and 150% are considered acceptable due to the frequent occurrence of difficult matrices. When these criteria are applied to the data in the current study, recoveries between 83 and 91% (mean recovery of 87%) were recorded for gluten-containing samples 2, 3, 4, and 8. Therefore, this method complies with the recovery rates suggested by Abbott et al. (1) and fulfills the validation criteria for an ELISA method.

Conclusions

This collaborative study has shown that the R5 sandwich ELISA method is capable of analyzing gliadin, and therefore gluten, in foods. Gliadin concentrations from >2.5 mg/kg (>5 mg of gluten/kg) up to 50 mg/kg (equal to 100 mg of gluten/kg) can be analyzed quantitatively. In the concentration range that is of most interest (10 mg of gliadin/kg; 20 mg of gluten/kg) for determining whether a sample is gluten-free, the method shows high precision. The collaborative study has also shown that wheat flour with a defined gliadin content can be used to produce gluten-containing materials suitable for validation tests and that heating of gliadin does not affect its reactivity with the R5 antibody.

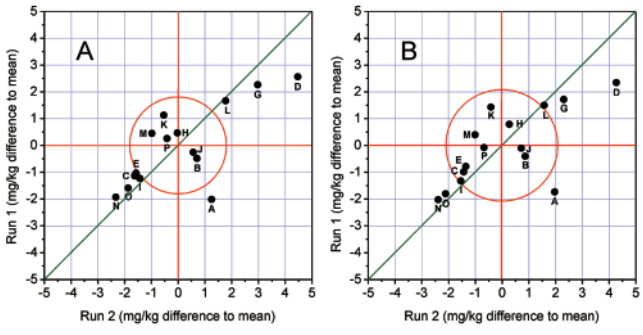

Fig. 2. Youden plot of results obtained from analysis of the bread sample containing 10 mg of gliadin/kg (equal to 20 mg of gluten/kg) using R5 sandwich ELISA after calibration with a cubic spline model (**A**) and a third-order polynomial model (**B**). The red circle represents the random error according to Youden (16), which is similar to the reproducibility standard deviation (s_R). Dots are labeled with the code of the laboratory that produced the results.

Acknowledgments

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