FOOD COMPOSITION AND ADDITIVES

Determination of Gluten in Processed and Nonprocessed Corn Products by Qualitative R5 Immunochromatographic Dipstick: Collaborative Study, First Action 2015.16

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In September 2013, the AACC International (AACI) Protein Technical Committee (now Protein and Enzymes Technical Committee) initiated a collaborative study of a method for the qualitative analysis of intact gluten in processed and nonprocessed corn products, using an R5 immunochromatographic dipstick system. It was validated to demonstrate that potential gluten-free products contain gluten lower than the Codex threshold of 20 mg/kg gluten. The results of the collaborative test with 18 participants confirmed that the method is suitable to detect gluten contaminations that are clearly lower than the threshold. It is recommended that the method be accepted by AOAC as Official First Action.

With a population prevalence of 0.4 to 1.2% in Europe, North America, Australia, and the Middle East (1), celiac disease (CD) is considered one of the most common food intolerances. CD is an immune-mediated inflammatory disease of the upper small intestine in genetically predisposed individuals, and it is triggered by the ingestion of dietary gluten (2). In the context of CD, gluten is defined as a protein fraction from wheat, rye, barley, or their crossbred varieties and derivatives thereof, to which some persons are intolerant, and it is insoluble in water and 0.5 mol NaCl/L (3). Gluten is composed of prolamins that can be extracted by 40–70% ethanol and by alcohol-insoluble glutelins that can only be extracted under reducing and disaggregating conditions at elevated temperatures. The prolamins from wheat, rye, and barley are called gliadins, secalins, and hordeins, respectively, and the prolamin content of gluten is generally taken as 50% (3). The only known effective treatment for CD is a lifelong gluten-free diet, which is based on the avoidance of gluten-containing cereals and should contain less than 20 mg gluten/day to prevent a relapse of intestinal damage (4). To guarantee the safety of gluten-free products for CD patients, a threshold of 20 mg/kg gluten for gluten-free foods is required by the Codex Alimentarius and legislation, e.g., in the United States by the U.S. Food and Drug Administration, Department of Health and Human Services (5), and in Europe by the European Commission (6). Specific and sensitive analytical methods are therefore needed for food quality control. Immunochemical methods are currently recommended for the quantitative and qualitative determination of gluten in foods (3). Sandwich and competitive ELISA formats based on the R5 monoclonal antibody (7) were successfully validated as AACCI approved method 38-50.01 for intact gluten (8) and 38-55.01 for partially hydrolyzed gluten (9), respectively. Additionally, the R5 sandwich ELISA was laid down as a Codex Alimentarius Type I method for the analysis of gluten (10) and has been adopted by AOAC INTERNATIONAL as First Action *Official Method of Analysis*[SM] status **2012.01**. The R5 antibody raised against ω-secalins primarily recognizes the epitope QQPFP, which is present in gliadins, secalins, and hordeins and occurs in many peptides that are toxic or immunogenic for CD patients (11–13).

Immunochromatographic assays, usually available in dipstick or lateral-flow format, provide rapid, qualitative results indicating the presence or absence of the substance to be determined. The RIDA® QUICK Gliadin dipstick based on the R5 antibody is intended as a swab test of potentially contaminated surfaces and to check for gluten contamination of raw materials after ethanol extraction or a test of processed materials after Cocktail extraction (14).

An international collaborative study was set up to validate the R5 dipstick (RIDA QUICK Gliadin) for qualitative gluten detection in raw and processed corn food products as an AACCI-approved method. The study was carried out as collaboration

Received January 19, 2016. Accepted by SG March 16, 2016.
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The method was approved by the Expert Review Panel on Food Allergens.

The Expert Review Panel on Food Allergens invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or to methodfeedback@aoac.org.

[1] Presented at the AACC annual meeting in Providence, RI (October 7, 2014) and the Prolamin Working Group meeting in Nantes, France on September 25–27, 2014 by Katharina Sherf (née Konitzer).

DOI: 10.5740/jaoacint.16-0017

between the Prolamin Working Group (PWG) and the AACCI. It was coordinated by Katharina Scherf (née Konitzer; German Research Center for Food Chemistry, vice-chair of the AACCI Protein Division, and co-chair of the AACCI Protein and Enzymes Technical Committee) and 18 participating laboratories.

Scope of the Method

RIDA QUICK Gliadin is used for the qualitative analysis of gluten in nonprocessed and processed corn food products that are declared "gluten-free." The immunochromatographic dipstick system detects intact prolamins from wheat (gliadins), rye (secalins), and barley (hordeins). The used R5 monoclonal antibody recognizes, among other things, the potentially immune-stimulatory sequence QQPFP, which occurs repeatedly in the prolamin proteins. Samples are extracted by 60% ethanol (nonprocessed food) or by Cocktail solution (processed food), are analyzed within 5 min, and are evaluated visually. The system was developed to detect gluten clearly below the threshold of 20 mg/kg and shows no high-dose hook effect.

Collaborative Study

Study Design

Following the AOAC guidelines, which are published as Appendix D (15) and Appendix N (16), an international collaborative study was set up to validate the R5 immunochromatographic dipstick (R-Biopharm RIDA QUICK Gliadin R7003) for qualitative gluten detection in processed and nonprocessed corn-containing foods as an AACCI-approved method. The study was carried out as a collaboration between the PWG and the AACCI. It was coordinated by Katharina Scherf (née Konitzer; German Research Center for Food Chemistry, vice-chair of the AACCI Protein Division, and co-chair of the AACCI Protein and Enzymes Technical Committee) in collaboration with Peter Koehler (German Research Center for Food Chemistry; chairman of the PWG and member of the Protein & Enzymes Technical Committee of AACCI) and Clyde Don (chair of the Protein & Enzymes Technical Committee of AACCI). Because this collaborative test is the first one following the new AOAC Appendix N, the study design was discussed and revised by Paul Wehling (AOAC statistician) in advance to ensure that the number of replicates and the number of concentration levels were sufficient. The collaborative test was split into two parts (A and B) to prevent mix-up of samples and procedures resulting from the different extractions. The total number of 40 samples per part is a compromise between the number of replicates and the number of concentration levels on the one hand, and the number of samples that a participant could manage within an acceptable time on the other hand. This compromise was partly compensated for by the high number of participants.

Collaborators

To qualify for participation in the collaborative test, all laboratories were required to have previous experience with immunological tests, such as ELISA, and to be familiar with the analytical procedure. Use of a separate room for the collaborative study was recommended because of the possibility of gluten contamination and the low detection limit. The laboratories were given 4 weeks each to perform the analyses for part A (April 1–30, 2014) and for part B (May 1–31, 2014). Eighteen laboratories (designated A to W) were chosen to participate: one each in Argentina, Austria, Belgium, Canada, Finland, Hungary, Ireland, Italy, Sweden, Switzerland, and the United Kingdom; three in Germany and four in the United States (*see* also *Acknowledgments*).

Samples and Sample Preparation

The main challenge for the validation of a qualitative method is the low amount of information per sample after analysis compared to a quantitative method. Therefore, a high number of replicate samples have to be analyzed. In general, the outline of the study followed the AOAC guidelines for validation of qualitative binary chemistry methods (Appendix N).

The following samples were prepared for part A of the collaborative study:

Sample 1.—Corn flour, containing gluten at 1.76 mg/kg.

Sample 2.—Corn flour, containing gluten at 4.84 mg/kg.

Sample 3.—Corn flour, containing gluten at 11.0 mg/kg.

Sample 4.—Corn flour, containing gluten at 18.8 mg/kg.

All concentrations were determined using the RIDASCREEN® Gliadin R7001 (R-Biopharm; AOAC First Action *Official Method of Analysis* status and Type I method according to the CODEX Alimentarius). Results are provided as mg/kg gluten by using the conversion factor of 2, which is mentioned in Codex Standard 118-1979. Sample 1 was a "gluten-free" corn flour with a gluten concentration below the LOQ (5.0 mg/kg gluten) of the method. Nevertheless, to obtain an idea of the contamination level, values were extrapolated from the calibration curve of the quantitative sandwich assay (8) and showed that a very low contamination of gluten was present (1.76 mg/kg). The corn flour samples 2–4 were prepared by mixing a naturally contaminated corn flour sample with the "gluten-free" corn flour sample 1.

The following samples were prepared for part B of the collaborative study:

Sample 5.—Cookie (processed), containing gluten at 0.38 mg/kg.

Sample 6.—Corn snack (processed), containing gluten at 6.40 mg/kg.

Sample 7.—Corn snack (processed), containing gluten at 13.3 mg/kg.

Sample 8.—Corn snack (processed), containing gluten at 47.2 mg/kg.

The processed snack samples 6–8 were prepared by mixing a snack sample (spiked at 100 mg gluten/kg before processing) with a "gluten-free" snack sample. Both samples were already used in the collaborative test of the RIDASCREEN Gliadin (R7001), which was published including a description of the preparation of these samples (8). Because the "gluten-free" snack sample showed a low contamination level during the collaborative test in 2012, a commercial gluten-free cookie (sample 5) was used instead as a "zero-gluten" sample for the study of the RIDA QUICK Gliadin dipstick. The value for sample 5 was extrapolated from the calibration curve (8).

All materials were prepared by grinding to ensure all materials passed a 40-mesh screen and were combined methodically to ensure homogeneity. The complete sample was mixed for 2 h, sieved through a 40-mesh screen, and then mixed again. Samples were packaged for delivery into foil pouches at an amount of 0.7 g for processed samples and 2.8 g for nonprocessed samples.

Homogeneity of Samples

Homogeneity was tested using the R5 sandwich ELISA (RIDASCREEN Gliadin, R-Biopharm, R7001). The determination of homogeneity was performed according to the IUPAC recommendations for proficiency tests (17). The SD (s_p) was derived from the Horwitz equation to calculate a deviation that is dependent on the concentration. In brief, 10 bags were randomly chosen and two subsamples were taken from each bag. After analyzing all samples (in sum 20), the calculation was performed as described in the IUPAC guideline. All samples turned out to be homogenous according to the guidelines.

Presentation of Samples to Laboratories

Following the collaborative test guidelines of AOAC and in accordance with AOAC Appendix N, 10 blinded replicates for each sample were provided to each participating laboratory. As already stated, the number of replicates is a compromise between statistics and the workload for each participant.

The samples were marked with a laboratory-specific letter (A–W), an "E" for ethanol extraction or a "C" for Cocktail extraction, and a randomized number from 1 to 40. Each laboratory obtained its own coding (different randomized numbers for each laboratory).

Method and Qualitative Evaluation

The method was written in AACCI style and was provided to each laboratory with the instructions to follow the method as written with no deviations. All results obtained by visual inspection had to be recorded in a ready-to-use Excel sheet. The final data from the laboratories were sent to the study coordinator.

Before analyzing the blind-coded samples, each participant was asked to perform checks for contamination and to become familiar with the test method. The latter was necessary because the qualitative nature of the obtained result made a later check for sample mix-up or improper testing very difficult.

Checks for contamination.—Possible sources of contamination during sample preparation and the test evaluation include the laboratory equipment, such as containers and surfaces, the Cocktail solution, the 60 or 80% ethanol solution, and the dilution buffer. To check for these possible sources, the participants were asked to perform two experiments before starting to analyze the blind-coded samples. (*1*) The dilution buffer (containing Cocktail and/or ethanol) was checked for gluten contamination. (*2*) A swab test of the laboratory bench across a sampling area of about 10 × 10 cm using the dipstick was performed. If both tests were negative, the participants were allowed to proceed with the analysis. No participant reported a positive result to the study coordinator.

Training and familiarization with the test.—Because of the fact that outlier detection after performing the analysis is complicated, the participants obtained a training video and two sets of assay controls with known concentrations to check their own performance. One set was for part A (available as R7010; R-Biopharm) and the other one was for part B (available as R7012; R-Biopharm). To standardize the results, the test kit manufacturer inserted an evaluation card in the test kit.

Finally, each blind-coded sample was extracted once and was analyzed according to the test kit instruction. In total, 80 samples had to be analyzed by each laboratory. Each sample had to be marked positive or negative or invalid. In case of an invalid result (missing control line or incomplete target line), retesting of the sample was requested. No participant reported an invalid result to the study coordinator.

Method

Gluten is measured in food containing wheat, rye, and barley. Gluten is detected in processed and nonprocessed corn products by qualitative R5 immunochromatographic dipstick.

AOAC Official Method 2015.16
Gluten in Processed and Nonprocessed Corn Products
Qualitative R5 Immunochromatographic Dipstick
First Action 2015

[Presented by Katharina Scherf (née Konitzer) at the American Association of Cereal Chemists (AACC) annual meeting, Providence, RI, October 7, 2014, and the Prolamin Working Group meeting, Nantes, France, September 25–27, 2014.]

(Applicable for RIDA QUICK Gliadin for the qualitative analysis of gluten in nonprocessed and processed corn food products that are declared as "gluten-free.")

Caution: Ethanol is a highly flammable vapor. Keep away from heat, hot surfaces, sparks, open flames, and other ignition sources. Do not smoke. Keep container tightly closed. Store in a well-ventilated place and keep cool. For Cocktail solution containing 2-mercaptoethanol, which is toxic, work under a chemical fume hood, avoid skin and eye contact, and wear protective gloves and clothing (*see* MSDS, attached as separate documents or delivered by the manufacturer in the case of ethanol).

A. Principle

The dipstick consists of different zones (Figure **2015.16**). Analytes in the sample solution will be "chromatographed" above the "maximum line" and react with the R5-antibody coupled to a red latex microsphere. The "maximum line" indicates to the user the maximal liquid level of the sample solution.

The "result window" contains a small band of immobilized R5 antibody ("T"; red line after positive reaction) and a second line that turns blue when the reaction is valid. Results are read visually only. Generally, the higher the analyte level in the sample the stronger the red color of the test band (until a maximum of color is reached).

B. Apparatus

Apparatus specified here has been tested in the laboratory; equivalent apparatus may be used.

(a) *Laboratory mincer/grinder, pestle and mortar, or Ultra-Turrax*.

(b) *Scale*.

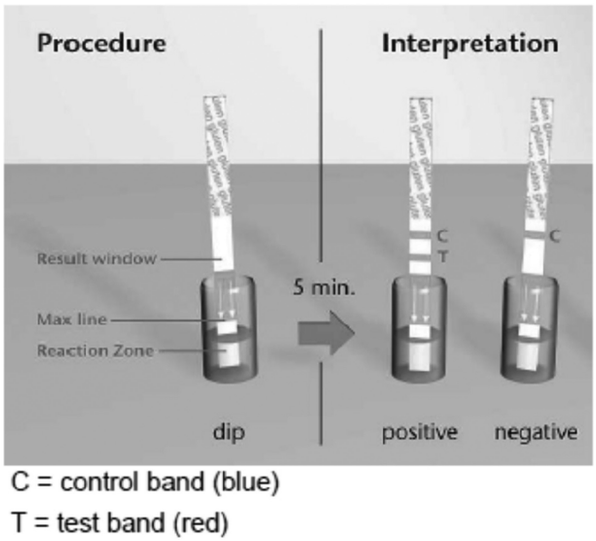

Figure 2015.16. Schematic presentation of the test principle and the subsequent interpretation of the possible results (invalid results not shown).

(c) *Graduated cylinders (plastic- or glassware)*.
(d) *Graduated pipets*.
(e) *Shaker*.—e.g., Roto Shaker Genie, Scientific Industries Inc.
(f) *Water bath*.—Temperature controlled 50°C (e.g., GFL, Burgwedel, Germany).
(g) *Centrifugal glass vials with a screw top*.
(h) *Centrifuge*.—e.g., Minifuge RF, Kendro, Hanau, Germany.
(i) *Paper filter*.
(j) *Micropipets*.—Variable 20–200 µL and 200–1000 µL.

C. Reagents

Items **(a–e)** are available as a test kit (RIDA QUICK Gliadin, R-Biopharm AG). All reagents are stable at least throughout a period of 18 months from date of manufacture at 2–8°C. Please refer to kit label for current expiration.
(a) *25 × dipsticks in a tube*.
(b) *30 × empty test tubes*.
(c) *25 × disposable pipets*.
(d) *Sample diluent (60 mL), ready to use, transparent capped bottle*.
(e) *1× evaluation card*.

Necessary but not provided with the test kit:
(f) *Distilled water*.
(g) *Ethanol, 99% reagent grade*.
(h) *Cocktail (patented)*.—R7006 (R-Biopharm AG, Germany); ready to use.
(i) *Skim milk powder (food quality)*.

D. Standard Reference Material

Not currently available

E. General Preparation

(a) *Sample diluent*.—The sample diluent is ready to use. Bring the solution to room temperature (20–25°C) before use. Make sure that the buffer is not contaminated with gluten during use.
(b) *60% Aqueous ethanol*.—Add 150 mL ethanol to 100 mL distilled water and shake well.
(c) *80% Aqueous ethanol*.—Add 200 mL ethanol to 50 mL distilled water and shake well.
(d) *Cocktail (patented)*.—The Cocktail is ready to use (C).

F. General Recommendation for Sample Preparation

(a) Store samples in a cold and dry room protected from light. Ensure that no cross-contamination takes place.
(b) Carry out the sample preparation in a room isolated from the dipstick procedure.
(c) Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol (E) and also after use for the next sample.
(d) Airborne cereal dust and used laboratory equipment may lead to gluten contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.
(e) If necessary, check for gluten contamination of reagents and equipment with the RIDA QUICK Gliadin (Art. No. R7003).
(f) Keep in mind that solid samples can be inhomogeneous, therefore grind a representative part of the samples very well and homogenize before weighing.
(g) The sample extraction with ethanol should only be used for raw material that were surely not heated and not processed.
(h) All supernatants obtained after centrifugation can be stored in a tightly closed vial in the dark at room temperature (20–25°C) for up to 4 weeks.

G. Sample Preparation

Homogenize a representative amount of the sample (minimum 50 g; preferably 200 g).
(a) *Nonprocessed samples*.—(*1*) *Solid samples*.—Weigh 1 g of a representative, homogeneous sample in a vial and add 10 mL 60% ethanol solution (E). For soy-containing products additionally add 1 g skim milk powder (C).
(*2*) Mix thoroughly for at least 30 s (vortex). Centrifuge the sample (2500 *g* at least) at room temperature (20–25°C) for 10 min; alternatively, let the sample settle down and/or filtrate. Dilute 50 µL supernatant with 500 µL sample diluent (E) in the test tubes (C) and subsequently proceed with H.
(b) *Processed samples*.—(*1*) Weigh 0.25 g of a representative, homogeneous sample (pasty or solid) into a vial and add 2.5 mL Cocktail solution (E).
(*2*) Close the vial and mix well (vortex) to suspend the sample. Incubate the vial for 40 min at 50°C in the water bath. Let the sample cool and add 7.5 mL 80% ethanol (E). Close the vial and shake for 1 h upside down or by a rotator at room temperature (20–25°C). Centrifuge the sample (2500 *g* at least) at room temperature (20–25°C) for 10 min; alternatively, let the sample settle down and/or filtrate. Dilute 50 µL supernatant with 500 µL sample diluent (E) in the test tubes (C) and subsequently proceed with **H**.

H. General Recommendations for Good Test Performance

(a) This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed.

No quality guarantee is accepted after expiry of the kit (*see* expiry label). Do not interchange individual reagents between kits of different lot numbers.

(b) Special attention should be directed to the interpretation of positive and negative outcomes (use of evaluation card and control samples).

(c) Bring the dipsticks to room temperature (20–25°C) before first use (after first use, store at room temperature). The dipsticks are very sensitive to humidity, which could turn the test useless. For this reason, keep the strips away from humidity.

(d) Use also gluten-free and gluten-containing samples as test controls (e.g., R7010 for ethanol extraction and R7012 for Cocktail extraction; both products are distributed by R-Biopharm AG, Germany). If the negative assay control sample is evaluated as positive, then a contamination of the laboratory or laboratory equipment is likely.

(e) It is recommended to compare the extraction efficiency of ethanol with the Cocktail (patented; R7006) in the case of unknown samples.

I. Dipstick Testing

(a) Place the dipstick vertically into the test tube filled with the diluted sample extract. The arrow on the dipstick should point down (*see* also Figure **2015.16**). Do not immerse the dipstick beyond the maximum line.

(b) Take out the stick after exactly 5 min (±10 s) and evaluate the result using the evaluation card (C).

(c) For documentation and prolonged storage, the upper part of the dipstick marked with "Gluten," together with the test bands, should be cut off.

J. Dipstick Evaluation

(a) *Positive result*.—If two colored bands (test band in red and control band in blue) are visible in the result window (*see* Figure **2015.16**) after 5 min, the sample is positive for gluten.

(b) *Negative result*.—If only the blue control band is visible in the result window (*see* Figure **2015.16**) after 5 min, the sample is negative for gluten.

(c) *Invalid result*.—If no bands occur after 5 min, the test is invalid and should be repeated using a new dipstick.

K. Result Reporting

(a) *Positive result*.—A nonprocessed sample contains more than 5.0 mg/kg gluten. A processed sample contains more than 8.0 mg/kg gluten.

(b) *Negative result*.—A nonprocessed sample contains less than 5.0 mg/kg gluten. A processed sample contains less than 8.0 mg/kg gluten.

L. Result Interpretation

(a) The test strip has been developed for the detection of traces of gluten.

(b) A negative result does not necessarily indicate the absence of gluten as the gluten may not be homogenously distributed or the level of gluten in the product is below the LOD.

(c) The LOD is dependent on sample type and extraction efficiency.

(d) In case of a positive result, the RIDASCREEN Gliadin (Art. No. R7001) should be used for quantification. This test kit is also AOAC Research Institute and AOAC First Action *Official Method of Analysis* status validated.

M. Criteria for Acceptance of a Result

(a) Accept results if quality control samples (R7012, R7013, or spiked samples) are evaluated correctly.

(b) Appearance of test line and control line should be according to the evaluation card.

Results and Discussion

Collaborative Study Results

All participants reported to the study director that no contamination occurred in their laboratories and that all control samples were evaluated in the expected way.

The results for each sample and each laboratory are shown in Table 1 (ethanol extraction) and Table 2 (Cocktail extraction). Every laboratory analyzed 10 replicates for each concentration. Especially for the ethanol extraction, the results were uniform and 14 of 18 laboratories showed no false positives or false negatives. From the remaining four laboratories, only one laboratory assigned 2 of 10 blank samples as false positives. The other three laboratories found one false negative for the low concentration and only one laboratory found two false negatives

Table 1. Numbers of positive samples detected using the R5 dipstick after ethanol extraction[a]

		Sample 1 (negative)	Sample 2 (low)	Sample 3 (medium)	Sample 4 (high)
Gluten, mg/kg		1.76	4.84	11.0	18.8
Laboratory code	Total	Positive	Positive	Positive	Positive
A	10	0	10	10	10
B	10	0	10	10	10
D	10	0	10	10	10
E	10	0	10	10	10
F	10	0	10	10	10
G	10	0	10	10	10
H	10	0	10	10	10
I	10	0	9	10	10
L	10	0	10	10	10
M	10	0	9	8	10
N	10	0	10	10	10
O	10	0	10	10	10
P	10	0	10	10	10
R	10	0	10	10	10
S	10	0	9	10	10
T	10	0	10	10	10
U	10	0	10	10	10
W	10	2	10	10	10

[a] Data by each of the 18 participating laboratories; each laboratory obtained 10 blinded replicates for each concentration level.

Table 2. Numbers of positive samples detected using the R5 dipstick after Cocktail extraction[a]

		Sample 5 (negative)	Sample 6 (low)	Sample 7 (medium)	Sample 8 (high)
Gluten, mg/kg		0.38	6.4	13.3	47.1
Laboratory code	Total	Positive	Positive	Positive	Positive
A	10	2	7	10	10
B[b]	10	1	10	10	9
D	10	0	9	10	10
E	10	0	1	10	10
F	10	0	10	10	10
G	10	0	10	10	10
H	10	0	10	10	10
I	10	0	9	10	10
L	10	0	8	10	10
M	10	0	10	10	10
N	10	0	10	10	10
O	10	0	10	10	10
P	10	0	10	10	10
R	10	0	10	10	10
S	10	0	0	10	10
T	10	0	9	10	10
U	10	0	1	10	10
W	10	0	10	10	10

[a] Data by each of the 18 participating laboratories; each laboratory obtained 10 blinded replicates for each concentration level.

[b] Data set of Laboratory B was not included in the statistical calculation because two samples were apparently exchanged.

for the medium concentrated sample. It should be kept in mind that the concentration of the blank sample was clearly below the LOQ of the quantitative ELISA method, but still detectable. At these low concentrations, an inhomogeneity is not impossible and, therefore, a few false positives (2 of 180 samples) could be expected from this viewpoint.

The Cocktail extraction procedure ends up with a 4-fold higher dilution compared to the ethanol extraction. Therefore it was not surprising that the low concentrated sample showed a higher variation compared to the ethanol extraction. Laboratory B had to be excluded because it was obvious from the raw data (Excel sheet sent to the study coordinator) that a blank sample had been mixed up with a sample containing the high concentration. Nevertheless, 9 of 17 laboratories reported no false-negative or false-positive results. Only one laboratory found false-positive results. In total, 2 of 170 samples were detected as false positive. This rate is the same as for the ethanol extraction method. It is interesting to see that for the low-concentrated sample (6.4 mg/kg), laboratories could be separated into two groups reporting either 70 up to 100% correct detection or 0 to 10% correct results. It seems that the visual inspection results in a clear individual cut-off "color" for a positive sample and not—as speculated from a hypothetical point of view—a variation within the fractional range. In conclusion, it will be difficult to find or prepare a sample within the fractional range as requested by AOAC Appendix N.

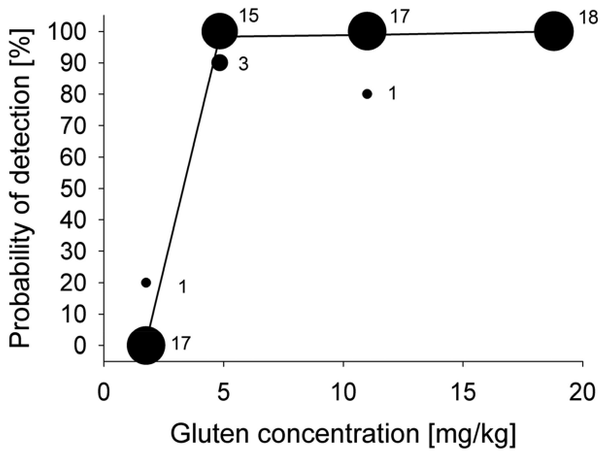

Figure 1. POD observed by each of 18 participating laboratories for samples extracted with ethanol (part A) between 1.76 and 18.8 mg/kg gluten. Number stated at each circle means number of laboratories with the same POD. Areas of circles are proportional to number of laboratories.

A graphical way to show the results for both collaborative tests appears in Figure 1 (ethanol extraction) and Figure 2 (Cocktail extraction). In these figures, the probability of detection (POD) is plotted against the concentration. Note that only 10% increments are possible for the POD in this figure. The bigger the area of the circle, the more laboratories reported this POD, as indicated by the number next to the circles.

Statistical Analysis and Discussion

Following the AOAC Appendix N for the validation of qualitative methods, some method performance characteristics were calculated and are shown in Tables 3 and 4 for both collaborative tests. Reproducibility SD was in the range between 0.00 and 0.18 after ethanol extraction and between 0.00 and 0.36 after Cocktail extraction. Repeatability SD was between 0.00 and 0.13 (ethanol extraction) and 0.00 and 0.21 (Cocktail extraction). A nonprocessed sample containing 4.8 mg/kg

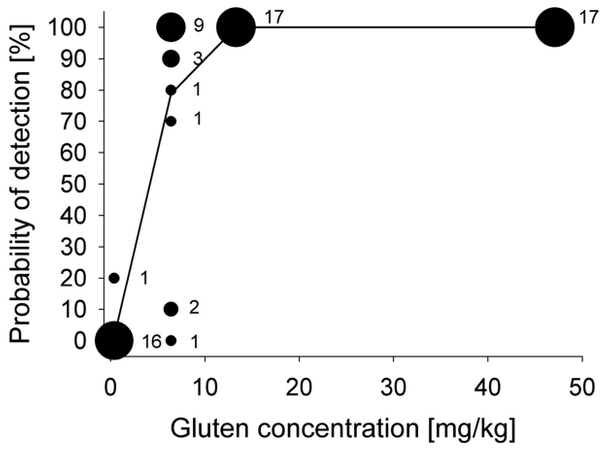

Figure 2. POD observed by each of 18 participating laboratories for samples extracted with Cocktail solution (part B) between 0.38 and 47.1 mg/kg gluten. Number stated at each circle means number of laboratories with the same POD. Areas of circles are proportional to number of laboratories.

Table 3. Performance statistics for overall results using the R5 dipstick after ethanol extraction[a]

Gluten, mg/kg	Sample 1 (negative) 1.76		Sample 2 (low) 4.84		Sample 3 (medium) 11.0		Sample 4 (high) 18.8	
	Positive	Total	Positive	Total	Positive	Total	Positive	Total
Total (18 laboratories)	2	180	177	180	178	180	180	180
POD[b]		0.01		0.98		0.99		1.00
LCL[c]		0.00		0.95		0.96		0.98
UCL[d]		0.04		0.99		1.00		1.00
s_r[e]		0.10		0.13		0.10		0.00
s_R[f]		0.11		0.18		0.11		0.00

[a] Part A (*see also* Table 1).

[b] POD = Probability of detection.

[c] LCL = Lower limit of the confidence interval.

[d] UCL = Upper limit of the confidence interval.

[e] s_r = Repeatability standard deviation.

[f] s_R = Reproducibility standard deviation.

gluten is detected with a POD of 0.98 (confidence interval from 0.95 to 0.99), whereas a processed sample with 6.4 mg/kg gluten is detected with a POD of 0.79 (confidence interval from 0.72 to 0.84). This clearly indicates the high suitability of the assay to detect contaminated samples lower than the threshold of 20 mg/kg. A more detailed statistical analysis, especially on LOD and its prediction intervals, is available elsewhere (18).

Discussion

The immunochromatographic method that was evaluated in this collaborative study was designed to detect gluten at levels clearly less than the threshold of 20 mg/kg gluten. A qualitative method to detect gluten will only result in a yes or no answer, but a user of this system needs to know with a given confidence (*1*) what minimal concentration is present if the result is positive and (*2*) what maximum amount of gluten may be present when the result is negative. From the data it can be concluded that the immunochromatographic dipstick RIDA QUICK Gliadin is capable of detecting gluten in processed and nonprocessed samples below the threshold of 20 mg/kg. A further characterization of the analytical performance of this assay, for example, LOD are given elsewhere (18). If a trained potential user works in a gluten-free laboratory and set up a quality-control plan by using control samples, the results obtained with the described method will be comparable to the results of the participating laboratories.

Table 4. Performance statistics for overall results using the R5 dipstick after Cocktail extraction[a]

Gluten, mg/kg	Sample 5 (negative) 0.38		Sample 6 (low) 6.40		Sample 7 (medium) 13.3		Sample 8 (high) 47.1	
	Positive	Total	Positive	Total	Positive	Total	Positive	Total
Total (17 laboratories)	2	170	134	170	170	170	170	170
POD[b]		0.01		0.79		1.00		1.00
LCL[c]		0.00		0.72		0.98		0.98
UCL[d]		0.04		0.84		1.00		1.00
s_r[e]		0.10		0.23		0.00		0.00
s_R[f]		0.11		0.42		0.00		0.00

[a] Part B (*see also* Table 2).

[b] POD = Probability of detection.

[c] LCL = Lower limit of the confidence interval.

[d] UCL = Upper limit of the confidence interval.

[e] s_r = Repeatability standard deviation.

[f] s_R = Reproducibility standard deviation.

Conclusions

Results from samples extracted with ethanol were uniform among laboratories, and 14 of 18 laboratories showed no false-positives or false-negatives. For Cocktail-extracted processed samples, still 9 of 17 laboratories reported no false-negative or false-positive results. In total, 4 of 350 samples were detected as false positive. A nonprocessed sample with a concentration of 4.8 mg/kg gluten was detected with an overall POD of 0.98, whereas processed samples with gluten concentrations of 6.4 and 13.3 mg/kg resulted in POD values of 0.79 and 1.0, respectively. Because the data show that the immunochromatographic dipstick RIDA QUICK Gliadin is suitable to detect gluten clearly below the CODEX threshold of 20 mg/kg, the study director, Katharina Scherf, together with the method developers from R-Biopharm, recommends this method for First Action *Official Methods of Analysis*.

Acknowledgments

We thank Carrie Maune (Trilogy Lab, Washington, MO) for preparing the samples and Nathalie Widmann and Helene Bayer (R-Biopharm AG, Darmstadt, Germany) for homogeneity testing and blinding the samples. We thank Patricia Meinhardt (R-Biopharm Inc., Washington, MO) for proofreading the manuscript and Peter Koehler (German Research Center for Food Chemistry, Freising, Germany) and Clyde Don (Foodphysica, Driel, The Netherlands) for their helpful contribution to this successful study.

We acknowledge the participation of the following laboratories in the collaborative study:

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