2017 AOAC OFFICIAL METHODS BOARD AWARDS

2014 - 2016 RESEARCH INSTITUTE GLIADIN & GLUTEN METHODS TO BE REVIEWED FOR

2017 METHOD OF THE YEAR

OFFICIAL METHODS OF ANALYSIS OF AOAC INTERNATIONAL

METHOD OF THE YEAR
OMB may select more than one method in this category each year.

Selection Criteria
The minimum criteria for selection are:

a. The method must have been approved for first or final action within the last three years.
b. Generally, some unique or particularly noteworthy aspect of the method is highlighted as making it worthy of the award, such as innovative technology or application, breadth of applicability, critical need, difficult analysis, and/or range of collaborators.
c. The method demonstrates significant merit in scope or is an innovative approach to an analytical problem.

Selection Process:

a. AOAC staff lists all eligible methods for consideration and forwards that list with supporting documentation (e.g. ERP chair recommendation(s)) to the Chair of the Official Methods Board (OMB).
b. The Chair forwards the list along with any supporting information to the members of the OMB.
c. The OMB selects the Method of the Year. The winner is selected by 2/3 vote. If necessary, the OMB chair may cast tie-breaking vote.

Award
An appropriate letter of appreciation and thanks will be sent to the author(s) of the winning method. The corresponding author will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award. All authors will be acknowledged at the annual meeting, will receive an award and a letter of appreciation. The name of the winner(s), with supporting story, will be carried in the announcement in the *ILM*.

TABLE OF CONTENTS FOR METHODS

RI GLUTEN - SOLE-SOURCE OR PROPRIETARY METHODS REVIEWED IN 2014 – 2016

FOOD COMPOSITION AND ADDITIVES

Partially Hydrolyzed Gluten in Fermented Cereal-Based Products by R5 Competitive ELISA: Collaborative Study, First Action 2015.05

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In 2008, the AACC International Protein Technical Committee (now Protein and Enzymes Technical Committee) initiated a collaborative study of a method for determining gluten in fermented products, using an R5 competitive ELISA system. The method has been approved as AACCI Approved Method AACCI 38-55.02. The new method has been validated for testing fermented foods and beverages to determine that they conform to the Codex threshold of 20 mg of gluten/kg in total for gluten-free products. It is recommended that the method be accepted by AOAC as Official First Action.

Gluten is a protein fraction found in wheat, rye, barley, oats, and their crossbred varieties and derivatives thereof, to which some persons are intolerant; it is insoluble in water and NaCl solutions with a concentration of 0.5 M (1, 2). Prolamins are gluten fractions that can be extracted with 40–70% ethanol. The prolamins gliadin, secalin, and hordein are found in wheat, rye, and barley, respectively (1). The prolamin content of gluten is generally taken as 50% (1). In foods labeled as "gluten-free," the gluten level must not exceed 20 mg/kg of food (1–3). Foods processed to reduce their gluten content to a level ranging from 20 to 100 mg/kg may not be labeled "gluten-free"; labeling is regulated on a national level (e.g., could be labeled "very low gluten"). From these regulations, it is obvious that effective test methods are needed to determine the gluten concentration in food, beverages, and raw materials.

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 (4). 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 raised against the ω-type of rye prolamins (ω-secalins) is directed toward the epitope glutamine-glutamine-proline-phenylalanine-proline (QQPFP) in gliadins, hordeins, and secalins. The R5 ELISA is commercially available in two versions, as a sandwich ELISA for intact gluten proteins with at least two binding epitopes and as a competitive ELISA for partially hydrolyzed gluten (gluten peptides), which need only one epitope for binding. While the sandwich ELISA has been studied extensively (4, 5) leading to its approval as AACCI Method 38.50.01 (6, 7) and AOAC *Official Method*℠ **2012.01** (First Action), the competitive R5 ELISA method has not been validated so far. The R5 sandwich ELISA is not as suitable as the competitive ELISA format towards partially hydrolyzed gluten due to the fact that the sandwich ELISA needs two binding sites (8). The competitive assay is the method of choice for measuring partially hydrolyzed gluten in foods.

Scope of the Method

The RIDASCREEN® Gliadin competitive enzyme immunoassay quantitates gluten by measurement of peptide fragments of prolamins from wheat (gliadins), rye (secalin), and barley (hordein). To convert this result to gluten, the conversion factor of 2 set by the Codex Alimentarius is used. The antibody binds to the short amino acid sequence QQPFP and to related sequences, which exist as motifs on all the prolamin subunits (9). Some of these sequences are potentially celiac immuno-stimulatory (10, 11). Samples are extracted by a simple sample preparation and can then be analyzed within 40 min. The standard calibration curve covers gluten concentrations in a sample of 10 to 270 mg/kg. For production of a standard material and for spiking, prolamins (gluten measurement) from rye and barley were isolated and checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and RP-HPLC. For wheat, the existing PWG gliadin isolate was used. In a second step, secalins, hordeins, and gliadins were digested with pepsin and trypsin and further characterized by RP-HPLC (8). The protein content of these materials was determined according to the Dumas method.

The calibrators for the R5 competitive ELISA use pepsin-trypsin digested prolamin fractions from wheat, rye, and barley in equal proportion by mass. The multiplication factor of 2 (included in the standards) has been used to convert the prolamin into gluten (1).

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The method was approved by the Expert Review Panel for Food Allergens-Gluten as First Action.
The Expert Review Panel for Food Allergens-Gluten 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 to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Collaborative Study

Study Design

Following the guidelines of AOAC INTERNATIONAL *Official Methods* (12) and AACC International (13), an international collaborative study was set up to validate the R5 competitive ELISA (R-Biopharm RIDASCREEN® Gliadin competitive R7021; Darmstadt, Germany) for gluten quantitation in fermented foods and beverages 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; chairman of the PWG and member of the Protein and Enzymes Technical Committee of AACCI) in close collaboration with Clyde Don (chair of the Protein and Enzymes Technical Committee of AACCI).

Collaborators

All laboratories participating in the collaborative study were required to be familiar with immunological tests and, if possible, with competitive ELISA tests. They were advised to use a separate test room for the collaborative study due to the low LOD and the possibility of contamination. To check the samples, test requirements, and documentation and to identify critical points, a precollaborative study with four laboratories within 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 proceeded as scheduled. Laboratories were given 6 weeks to perform the analyses (August 1 to September 15, 2011). Sixteen laboratories were selected (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.

Description of Samples

The following samples were prepared or obtained for the collaborative study:

(**a**) *Beer*.—Gluten-free.
(**b**) *Beer*.—30 mg/kg gluten (15 mg hordeins/kg).
(**c**) *Beer*.—100 mg/kg gluten (50 mg hordeins/kg).
(**d**) *Starch syrup*.—Gluten-free.
(**e**) *Starch syrup*.—Naturally wheat gluten-contaminated.
(**f**) *Sourdough*.—70 mg/kg gluten (35 mg secalins/kg).
(**g**) *Sourdough*.—150 mg/kg gluten (75 mg secalins/kg).

All ingredients, except barley prolamin hydrolysate, contaminated starch syrup, and rye sourdough, were confirmed to be free of gluten contamination before use by means of the R5 competitive ELISA, which was also used in this collaborative study.

Peptic-Tryptic (PT) Hordein Digest

Grains from the barley cv. "Barke" were milled into white flour (ash content 0.50–0.60% in dry matter) using a laboratory mill and a 0.2 mm sieve. Flour (200 g) was dispersed twice in 600 mL light petroleum (boiling range 40–60°C) and stirred for 30 min at room temperature (RT; approximately 20°C). The solvent was removed, and the residue was air-dried overnight on a filter sheet. A 50 g amount of defatted flour was extracted stepwise with 3 × 200 mL buffer (NaCl concentration: 0.4 M, $KNaHPO_4$ concentration: 0.067 M, pH 7.6) followed by 3 × 200 mL 60% (v/v) aqueous ethanol by homogenizing in a centrifuge vessel for 5 min at RT. Each suspension was centrifuged for 30 min at 3550 × *g* and 4°C, and the supernatants were decanted and combined. The combined ethanol extracts were dialyzed against tap water containing acetic acid at a concentration of 0.01 M and freeze-dried providing the hordein fraction (= barley prolamin). The protein compositions of the hordein fractions were analyzed by SDS-PAGE. The hordein pattern was dominated by the γ-hordeins. C-hordeins were less pronounced, and D-hordeins homologous to high-MW glutenin subunits of wheat were absent. The further characterization by RP-HPLC revealed γ-hordeins at a proportion of 61%, C-hordeins at 35%, and only 5% nonidentified peaks. Therefore, it can be concluded that the protein content (84.3 g/100 g) of this isolate is 95% hordein.

Hordein (0.5 g) was suspended in 10 mL distilled water, and the pH was adjusted to 1.8 with 1.0 M HCl (14). Then, 2.5 mg pepsin (Merck, Darmstadt, Germany; No. 7192) was added, and the suspension was stirred for 4 h at 37°C. After adjusting the pH to 7.8 with 1.0 M NaOH, 2.5 mg trypsin (Merck, No. 24579) was added. After further stirring for 4 h at 37°C the pH was adjusted to 4.5 with 1.0 M HCl and the suspension was centrifuged at 4000 × *g* for 20 min at RT. The supernatant was decanted and freeze-dried, providing the peptic-tryptic (PT) hordein digest. The characterization with SDS-PAGE revealed that proteins with an MW of more than 14 kDa were absent. As expected, RP-HPLC chromatograms showed complex peptide patterns. Protein content of the PT hordein digest was 74.0 ± 0.5% (8). The crude protein contents (N × 5.7) of hordein and the PT hordein digest were determined according to Dumas using an FP-328 combustion instrument (Leco, St. Joseph, MI) and EDTA (N = 9.59%) for calibration.

The PT digest does not represent all hydrolysis processes. There are many additional factors, including temperature and time, that can affect the accuracy of the assay. Users should confirm method performance for their specific processes.

Beer

Beer as a typical fermented product that is analyzed by the R5 competitive ELISA was chosen as a sample. Gluten-free beer ("Beer up," malt´n´more trading GmbH, Grieskirchen, Austria) made from sorghum was used as a zero sample and as base material, which was spiked to a defined hordein concentration with the PT hordein digest. The advantage of this was that samples with exactly defined hordein content determined by an independent analytical method (Dumas analysis) were available. Based on the fact that the N-contents of both the PT hordein digest and the hordein had been determined, the amount of added digest corresponded to the amount of hordein used for its preparation. This was crucial for the determination of the recovery. Briefly, a defined amount of PT hordein digest was

added to the gluten-free beer and stirred for 24 h at RT in order to guarantee a homogeneous distribution in the sample.

Sourdough

A sourdough with defined gluten content was prepared by mixing dried, gluten-free quinoa sourdough with an appropriate amount of dried rye sourdough (both from Ernst Böcker GmbH & Co. KG, Minden, Germany) and shaking overhead for 3 h. The rye sourdough was from an approach in which the company tried to digest as much gluten as possible by lactic acid bacteria (fermentation time 72 h). The starting material was pure rye flour. Two sourdough samples with 70 and 150 mg/kg gluten were prepared. The R5 competitive ELISA was used to determine the gluten content of the rye sourdough (2690 mg/kg gluten) as well as the gluten contents of the quinoa/rye sourdough mixtures, which were used as samples in this study. Since one would expect rye gluten concentrations of about 44 g/kg in rye flour (8), more than 90% of gluten was not any longer detectable by the competitive ELISA after fermentation by lactic acid bacteria.

Starch Syrup

One sample of starch syrup was a commercial gluten-free product ("Stayley® 300 Corn Syrup," Tate & Lyle, London, UK), and the other sample was a wheat starch syrup contaminated with gluten from an anonymous industrial supplier. The gluten contamination was detected by means of the R5 competitive ELISA. The analysis provided a gluten concentration of approximately 10 mg/kg.

Homogeneity of Samples

All samples were checked for homogeneity before they were packaged in air-tight bottles and accepted for the collaborative study. This was done by taking 10 representative 1 g aliquots (1 mL for beer) from 10 different parts of the bulk sample and then analyzing by the R5 competitive ELISA. The CV for the gluten-containing samples was 10.1% or less for sourdough and 18.0% or less for beer. The naturally contaminated starch syrup showed higher variation (±22.3%) due to its low gliadin concentration near the LOQ. All samples were accepted for the collaborative study. Gluten-free samples 1 and 4 were considered homogeneous, because all analyses provided values below the LOQ (<10 mg/kg gluten). Both samples showed optical density (OD) values scattering around the zero calibrator provided (CVs of ODs were around ±6%; n = 10).

Presentation of Samples to Laboratories

Following the AOAC collaborative study guidelines, two independent blinded replicates for each sample were provided to the participating laboratories. Each sample was extracted using 60% (v/v) ethanol and analyzed in duplicate in one analytical run. Fourteen samples were analyzed by each laboratory. The high polyphenol content in the beer samples required a different extraction. These samples were specifically labeled and were extracted with 60% (v/v) ethanol containing 10% (w/v) fish gelatin.

Samples and ELISA kits were shipped to participants at a temperature of about 4°C. Each of the samples was labeled according to the sample code for identification (laboratory code plus number). Participants were requested to return a receipt acknowledgment form to indicate receipt and conditions of the shipped samples. They were also directed to follow the storage advice for samples and kits.

Analysis and Data Reporting

The method was written in AACCI style and was provided to each laboratory with instructions to follow the method as written with no deviations. Laboratories were directed to pay particular attention to cases where samples had to be repeated by further dilution and how dilutions were to be carried out. All OD values had to be recorded in a ready-to-use Excel (Microsoft Corp., Redmond, WA) worksheet. Participants were asked to use the RIDA®SOFT calculation software for cubic spline curve fitting; the software was provided with the kit. Final data from the laboratories were sent to the Study Coordinator.

ELISA Kit and Calculation Software

The R5 competitive ELISA kit (R-Biopharm RIDASCREEN® Gliadin competitive R7021) for the quantitation of gluten in fermented food and the software (RIDA®SOFT Win Z9999) for constructing calibration curves (cubic spline fitting) and calculating gluten concentrations from measured ODs 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 endpoints 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 (15). In this way, a function with a continuous curvature over the entire range is obtained. 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, and higher ones (>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 Standard 1) 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). As the cubic spline model did not provide concentration values for samples below the lowest standard, a second-order polynomial curve fitting model was used to determine values for Samples 1 and 4.

AOAC Official Method 2015.05
Partially Hydrolyzed Gluten in Fermented Cereal-Based Products
R5 Competitive ELISA
First Action 2015

[RIDASCREEN® Gliadin competitive ELISA kit is used

for the analysis of fermented and hydrolyzed food (e.g., beer, starch syrup, starch, malt extract, sourdough, and soy sauce) that are declared as "gluten-free." The kit is not applicable for measurement of intact gluten.]

Caution: Stop solution contains 0.5 M sulfuric acid; avoid skin and eye contact (*see* Material Safety Data Sheet).

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal antibody that can determine hydrolyzed gluten derived from wheat, rye and barley. The antibody binds to the short amino acid sequence QQPFP and to related sequences, which exist as motifs on all the prolamin subunits (9). Some of these sequences are potentially celiac immuno-stimulatory (10, 11). Since the assay is calibrated to a prolamin hydrolysate mixture form wheat, rye, and barley, a conversion to "gluten" content is achieved by the conversion factor of 2 set by the Codex Alimentarius. No cross-reactivity has been observed to oats, maize, rice, millet, teff, buckwheat, quinoa, or amaranth. Protein fragments for gluten measurement from food are extracted by using ethanol. After centrifugation, the supernatant is used in a competitive method.

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with a constant amount of gliadin. Standards (mixture of hydrolysates from wheat, rye, and barley prolamins) or sample solutions are pipetted, and peroxidase labeled antigliadin antibody (conjugate with monoclonal R5 antibodies) is added and incubated for 30 min. During incubation, free and immobilized analyte competes for the antibody binding sites (competitive enzyme immunoassay). Any unbound enzyme conjugate is then removed by a washing step. Substrate/chromogen is added to the wells and incubated for 10 min. Bound enzyme conjugate converts the chromogen into a blue product. Addition of the stop solution causes a color change from blue to yellow. The measurement is performed photometrically at 450 nm. The absorption is inversely proportional to the gluten concentration. The response of sample extracts is compared with response observed with calibrators.

B. Apparatus

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

(**a**) *Laboratory mincer/grinder, mortar and pestle, or Ultra-Turrax.*—e.g., Mr. Magic, ds-produkte GmbH, Gallin, Germany.

(**b**) *Rotator or shaker.*—e.g., Roto Shaker Genie (Scientific Industries Inc., Bohemia, NY).

(**c**) *Centrifuge*.—e.g., Minifuge RF, Kendro, Hanau, Germany.

(**d**) *Microtiter plate reader*.—e.g., Tecan Sunrise Remote (Tecan Group, Maennedorf, Switzerland).

(**e**) *Micropipets*.—Variable 20–200 µL and 200–1000 µL.

(**f**) *Graduated pipets*.

(**g**) *Graduated cylinders*.—Up to 1000 mL, plastic or glass.

(**h**) *Centrifugal glass vials with screw tops*.

C. Reagents

Items (**a**)–(**g**) are available as a test kit (RIDASCREEN® Gliadin competitive, R-Biopharm AG). All reagents are stable at least over a period of 15 months at 2–8°C (36–46°F) from the date of manufacture. Please refer to the kit label for current expiration.

(**a**) *Microtiter plate*.—Coated with gliadin (96 wells).

(**b**) *Five standard solutions*.—Labeled 0, 20, 60, 180, and 540 ng/mL gluten, 1.3 mL each; ready to use, transparent-capped bottles.

(**c**) *Conjugate.*—Horseradish peroxidase labeled R5 antibody; 0.7 mL, as an 11-fold concentrate, red-capped bottle.

(**d**) *Red Chromogen Pro.*—Substrate/chromogen; 10 mL, ready to use, brown-capped bottle.

(**e**) *Stop solution*.—14 mL, ready to use, yellow-capped bottle.

(**f**) *Sample diluent.*—60 mL, as a 5-fold concentrate, white-capped bottle.

(**g**) *Washing buffer*.—100 mL, as a 10-fold concentrate, brown-capped bottle.

Necessary or recommended but not provided with the test kit:

(**h**) *Distilled water*.

(**i**) *Ethanol*.—99% reagent grade.

(**j**) *Fish gelatin.*—Sigma, St. Louis, MO; Part No. G-7765 or Serva, Heidelberg, Germany; Part No. 22156.

Table 2015.05. Performance statistics for overall competitive R5 ELISA results without outlier (gluten concentrations are shown)

		Sample ID[a]						
	Symbol	1	2	3	4	5	6	7
Total No. of labs	p	13	12	11	13	13	13	13
Total No. of replicates	Sum(n(L))	26	24	22	26	26	26	26
Overall mean of all data (grand mean), mg/kg	XBARBAR	2.36	26.2	119.5	1.29	10.6	48.4	145.6
Repeatability SD, mg/kg	s_r	2.31	7.92	37.2	2.03	1.73	11.2	28.4
Reproducibility SD, mg/kg	s_R	2.98	9.67	37.2	3.05	3.65	12.5	40.0
Repeatability RSD, %	RSD_r	98.0	30.2	31.2	157.3	16.3	23.1	19.5
Reproducibility RSD, %	RSD_R	126.1	36.8	31.2	236.1	34.4	25.9	27.5
Recovery, %		—[b]	87	119	—	—	69	97

[a] *See* Table 1.

[b] — = Not applicable.

D. Standard Reference Material

Not existing today.

E. Standard and Spike Solution

The starting material used for preparation of standard and spike solutions is identical. Wheat, rye, and barley were separately digested by pepsin and trypsin, the peptide fragments were mixed (for preparation of the standard solutions), and the protein content was determined according to Dumas (8). This material was stored at –20°C in lyophilized form until reconstitution. In the case of spiking beer, the hordein digest was used. The material is reconstituted in 60% aqueous ethanol and results in a prolamin concentration of 1 mg/mL. The spike solution is diluted appropriately to the desired concentration. The solution is stable for a maximum of 4 weeks at 2–8°C. The standards as part of the test kit are stabilized in an aqueous solution and are designed to be stable for a minimum of 18 months at 2–8°C. Due to the nature of the standard material, all results are only traceable to this relative anchor point. Determination of trueness is not possible since the material is not a certified reference material. Therefore, the accuracy of the assay system could be biased but is still precise.

F. General Preparation

(**a**) *Sample diluent*.—The sample diluent is provided as a 5-fold concentrate. Only the amount that is actually needed should be diluted with distilled water (e.g., 3 mL concentrate + 12 mL distilled water, sufficient for the dilution of 10 samples). This dilution is stable for 1 day. Make sure that the buffer is not contaminated with gliadin.

(**b**) *60% aqueous ethanol*.—Add 150 mL ethanol to 100 mL distilled water and shake well.

(**c**) *60% aqueous ethanol containing liquid fish gelatin at an amount of 10 g/L (e.g., Serva, Part. No. 22156 or Sigma Part. No. G-7765; solid content 45%).*—Add 30 mL distilled water into a 100 mL graduated cylinder; add 10 g fish gelatin and mix well; add 60 mL ethanol, mix, and adjust pH to 8.5 if necessary. Fill up to 100 mL with distilled water.

(**d**) *Conjugate (peroxidase labeled antibody)*.—The antibody enzyme conjugate is provided as an 11-fold concentrate. Since the diluted enzyme conjugate solution has a limited stability, only the amount that is needed for the subsequent analysis on this day should be reconstituted. Before pipetting, the conjugate concentrate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1 + 10) with distilled water (e.g., 100 µL conjugate concentrate + 1 mL water, sufficient for two microtiter strips). Take care that the water is not contaminated with gliadin.

(**e**) *Washing buffer*.—The washing buffer is provided as a 10-fold concentrate. Before use the buffer has to be diluted 1:10 (1 + 9) with water (i.e., add 100 mL buffer concentrate to 900 mL distilled water). The diluted buffer is stable at 2–8°C (35–46°F) for 4 weeks. Before dilution, dissolve any crystals that may have formed in a water bath at 37°C (99°F).

G. Sample Preparation

(**a**) *General recommendation.*

(*1*) Store samples in a cold, dry room protected from light.

(*2*) Carry out the sample preparation in a room isolated from the ELISA procedure; if only one room is available, consider the high sensitivity of the assay and check for contamination [*see* (*4*) and (*5*) below.]

(*3*) Airborne cereal dust and used laboratory equipment may lead to gliadin contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.

(*4*) Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol, **F**(**b**), also after use for the next sample.

(*5*) If necessary, check for gliadin contamination of reagents and equipment with the test strips RIDA®QUICK Gliadin (Part. No. R7003).

(*6*) Keep in mind that the solid sample can be inhomogeneous; therefore, grind a representative part of the samples very well and homogenize before weighting.

(*7*) All supernatants obtained after centrifugation can be stored in tightly closed vials in the dark at room temperature (20–25°C/68–77°F) up to 4 weeks.

(**b**) *Homogenize a representative amount of the sample (5–50 g)*.

(*1*) *Solid samples (e.g., starch)*.—Weigh 1 g representative, homogeneous sample and add 10 mL 60% ethanol solution, **F**(**b**).

(*2*) *Liquid food (e.g., starch syrup)*.—Mix 1 mL sample with 9 mL 60% ethanol solution, **F**(**b**).

(*3*) *Beer*.—Mix 1 mL sample with 9 mL 60% ethanol solution containing fish gelatin **F**(**c**). Stir the suspension before and during use.

(*4*) *Malt and hops.*—Mix 1 g sample with 10 mL 60% ethanol solution containing fish gelatin, **F**(**c**). Stir the suspension before and during use.

(**c**) *Further procedure for all samples.*—Mix thoroughly for at least 30 s (vortex) and shake well upside down or rotate on a rotator for 10 min. Centrifuge the sample (2500 × *g* at least) at room temperature (20–25°C/68–77°F) for 10 min. Dilute the supernatant 1:50 (1 + 49) with diluted sample diluent, **F**(**a**), e.g., 20 µL supernatant + 980 µL diluted sample diluent. Use 50 µL/well in the assay (*see* **H**).

H. Determination

(**a**) *General recommendations for good test performance*.

(*1*) 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.

(*2*) Bring all reagents to room temperature (20–25°C; 68–77°F) before use. The Red Chromogen Pro (substrate/chromogen) is light-sensitive; therefore, avoid exposure to direct light.

(*3*) Return all reagents to 2–8°C (35–46°F) immediately after use. Unused microwells should be returned to their original foil bag. Reseal the bag with the desiccant provided in the bag.

(*4*) Do not allow microwells to dry between working steps.

(*5*) Reproducibility in any ELISA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the ELISA test procedure.

(*6*) Avoid direct sunlight during all incubations; covering the microtiter plates is recommended.

(*7*) Red Chromogen Pro reaction should be carried out in the dark.

(*8*) Each standard and sample should be analyzed in duplicate.

(*9*) Use also gluten-free and gluten-containing (spiked) samples as test controls.

(**b**) *ELISA testing*.

(*1*) Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.

(*2*) Add 50 µL of each standard solution or prepared sample, **G**(**b**), to separate wells in duplicate.

(*3*) Add 50 µL of diluted enzyme conjugate, **F**(**d**), mix gently by shaking the plate manually, and incubate for 30 min at room temperature (20–25°C/68–77°F).

(*4*) Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µL washing buffer **F**(**e**), and pour out the liquid again. Repeat two more times.

(*5*) Add 100 µL Red Chromogen Pro (substrate/chromogen solution; brown cap) to each well. Mix gently by shaking the plate manually and incubate for 10 min at room temperature (20–25°C/68–77°F) in the dark.

(*6*) Add 100 µL stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 10 min after addition of stop solution.

I. Calculation Interpretation and Test Result Report)

(**a**) *Result calculation*.—Special software RIDA®SOFT Win (Part. No. Z9999) is available and strongly recommended for evaluation of the RIDASCREEN® product line. The calculation should be done using a cubic spline function. Extrapolation is not recommended. The prolamin concentration in an extracted sample is read from the calibration curve and given as ng/mL. To calculate the concentration of prolamins or gluten in a sample, the following equations should be used.

(*1*) *Solid samples*

$$\text{Gluten, mg/kg} = \text{Gluten concentration in extract, ng/mL} \times 500/1000$$

(*2*) *Liquid samples*

$$\text{Gluten, mg/L} = \text{Gluten concentration in extract, ng/mL} \times 500/1000.$$

Alternatively, a second order polynomial curve fitting could be used.

(**b**) *Result reporting*.—Results are reported in mg/kg for solid samples or mg/L for liquid samples.

Table 1. Gluten concentrations determined by R5 competitive ELISA by all participating laboratories (raw data)

	Gluten concentration, mg/kg[a]													
	1[b]		2		3		4		5		6		7	
	Repeat													
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	2.13	5.80	23.6	20.5	111.6	93.9	4.47	7.73	7.60	8.62	46.7	47.2	152.9	170.0
B	1.46	2.66	40.8	13.8	151.4	127.4	2.98	2.13	10.6	5.10	38.8	53.0	163.6	122.8
C	5.30	10.6	34.2	82.2	192.2	107.6	6.12	1.90	12.8	12.6	47.2	67.4	181.4	143.4
D	0.74	1.77	23.8	28.6	175.2	97.6	–3.35	–3.41	9.80	11.0	33.0	60.2	106.4	107.6
E	6.45	20.4	72.4	50.4	24.6	204.0	23.5	17.6	20.4	29.4	68.6	72.8	251.0	244.2
F	–5.46	–3.99	14.6	27.0	124.0	160.0	–5.15	–5.55	9.20	6.80	47.0	51.4	128.8	151.6
G	6.06	4.30	32.4	32.0	216.2	208.2	3.29	–2.34	15.0	14.0	46.8	85.4	192.8	203.0
H	7.02	1.56	44.4	26.2	145.6	32.8	5.79	3.17	20.5	16.1	38.8	31.0	94.6	88.9
I	–0.65	–1.33	22.2	13.8	101.2	64.4	-0.89	–0.62	5.44	4.22	35.8	45.0	118.4	75.0
J	–1.50	1.14	21.2	20.0	121.8	128.8	–0.73	–1.63	7.40	8.00	45.6	58.3	132.9	139.2
K	16.3	14.8	50.0	44.8	216.7	308.6	21.1	9.70	33.5	22.4	87.5	80.0	348.0	30.2
L	1.69	–0.33	39.8	49.0	224.8	228.8	–1.83	3.39	13.2	11.6	64.0	67.2	171.6	244.6
M	–0.66	4.13	19.9	19.3	129.4	133.6	–2.27	–0.62	10.0	8.60	36.1	39.6	161.7	120.4
N	0.04	0.76	34.2	18.4	97.0	108.6	1.84	4.41	10.8	9.20	43.4	44.6	117.6	154.4
O	1.57	0.41	19.1	16.5	110.7	136.6	–0.11	1.26	11.7	8.20	51.3	46.3	152.8	164.6
P	5.96	0.84	25.4	24.8	149.4	111.2	1.54	1.33	12.6	10.8	38.2	46.2	194.8	111.2

[a] The calculation of the concentrations of the gluten-containing samples 2, 3, 5, 6, and 7 was done on the basis of a cubic spline function using the RIDA®SOFT Win software; the statistics of the gluten-free samples 1 and 4 were calculated on the basis of a second-order polynomial function; values for blinded samples are given as repeat 1 or repeat 2.

[b] Sample 1, gluten-free beer; sample 2, beer spiked at 30 mg/kg; sample 3, beer spiked at 100 mg/kg; sample 4, gluten-free starch syrup; sample 5, naturally contaminated wheat starch syrup; sample 6, sourdough containing gluten at 70 mg/kg; and sample 7, sourdough containing gluten at 150 mg/kg.

Table 2. Gluten concentrations determined by R5 competitive ELISA after eliminating laboratories E, F, and K

	Gluten concentration, mg/kg[a]													
	1[b]		2		3		4		5		6		7	
	Repeat													
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	2.13	5.80	23.6	20.5	111.6	93.9	4.47	7.73	7.6	8.62	46.7	47.2	153.0	170.0
B	1.46	2.66	40.8	13.8	151.4	127.4	2.98	2.13	10.6	5.1	38.8	53.0	163.6	122.8
C	5.30	10.6	34.2[c]	82.2[c]	192.2	107.6	6.12	1.90	12.8	12.6	47.2	67.4	181.4	143.4
D	0.74	1.77	23.8	28.6	175.2	97.6	–3.35	–3.41	9.8	11.0	33.0	60.2	106.4	107.6
G	6.06	4.30	32.4	32.0	216.2[d]	208.2[d]	3.29	–2.34	15.0	14.0	46.8	85.4	192.8	203.0
H	7.02	1.56	44.4	26.2	145.6	32.8	5.79	3.17	20.5	16.1	38.8	31.1	94.6	88.9
I	–0.65	–1.33	22.2	13.8	101.2	64.4	–0.89	–0.62	5.4	4.2	35.8	45.0	118.4	75.0
J	–1.50	1.14	21.2	20.0	121.8	128.8	–0.73	–1.63	7.4	8.0	45.6	58.3	132.9	139.2
L	1.69	–0.33	39.8	49.0	224.8[d]	228.8[d]	–1.83	3.39	13.2	11.6	64.0	67.2	171.6	244.6
M	–0.66	4.13	19.9	19.3	129.4	133.6	–2.27	–0.62	10.0	8.6	36.1	39.6	161.7	120.4
N	0.04	0.76	34.2	18.4	97.0	108.6	1.84	4.41	10.8	9.2	43.4	44.6	117.6	154.4
O	1.57	0.41	19.1	16.5	110.7	136.6	–0.11	1.26	11.7	8.2	51.3	46.3	152.8	164.6
P	5.96	0.84	25.4	24.8	149.4	111.2	1.54	1.33	12.6	10.8	38.2	46.2	194.8	111.2

[a] The calculation of the concentrations of the gluten-containing samples 2, 3, 5, 6, and 7 was done on the basis of a cubic spline function using the RIDA®SOFT Win software; the statistics of the gluten-free samples 1 and 4 were calculated on the basis of a second-order polynomial function; values for blinded samples are given as repeat 1 or repeat 2.

[b] For samples 1–7 *see* Table 1.

[c] Means outlier according to the Cochran test.

[d] Means outlier according to the double Grubbs' test.

J. Criteria for Acceptance of the Standard Curve

The shape of the standard curve is shown in the quality assurance certificate enclosed in the test kit. Absorbances may vary between different runs (e.g., due to different temperatures or analysts). However, the shape of the standard curve should be similar to the one given in the quality assurance certificate.

Minimum requirements are as follows:

(*1*) OD at 450 nm for standard 1 higher than 0.8.

(*2*) OD values for standards should continuously decrease with higher concentrations, especially when comparing standard 1 (0 ng/mL) and standard 2 (20 ng/mL).

(*3*) An OD value for standard 1 that is much higher than the OD value stated in the certificate could be an indication of errors during pipetting or incubation.

Results and Discussion

Collaborative Study Results

After finishing the analysis, each participant sent the data to the Study Coordinator. These results are given in Table 1. After statistical analysis of the data set, three problem laboratories were identified. Further review found Laboratory F did not run the calibrators in duplicate determinations as directed. Laboratory E found no difference between calibration standards S1 and S2, and as a consequence, a high OD difference between standards S4 and S5 led to an unusual curve shape. An interview with Laboratory E also revealed technical problems during sample preparation. Laboratory K had a variation in the calibration curve that was too high, and an interview revealed the possibility of gluten contamination in the laboratory and incorrect pipetting. As a result of these deviations, all data from Laboratories E, F, and K were excluded from the statistical evaluation.

For sample 5 (naturally contaminated syrup), all values were calculated by cubic spline. Due to the fact that some OD values were below the OD values of standard 2 (10 ng/mL prolamin; corresponds to concentration of 10 mg/kg in the sample), these values were extrapolated by the software. For the gluten-free samples 1 and 4 the RIDA®SOFT Win software returned only a result of <10 mg/kg, and extrapolation led to unrealistic values. To be able to use the results of the analysis of the gluten-free samples 1 and 4 in the performance statistics, estimates of concentration values for these samples were required. For this purpose, the calibration curves were constructed by using a second-order polynomial model and used to recalculate the results for samples 1 and 4 (7). This calibration provided an estimate of concentrations for the gluten-free samples (Tables 1 and 2).

Statistical Analysis and Discussion

The remaining data of 13 laboratories are shown in Table 2 and were used to calculate the necessary statistics. Only three outlying values were identified according to AOAC INTERNATIONAL guidelines (12). These are indicated in Table 2 by the superscripts "c" (for a Cochran outlier) and "d" (for a double Grubbs' outlier). The performance statistics without outliers are shown in Table 2015.05.

From the measured overall mean concentrations of the gluten-containing samples, recovery rates were calculated.

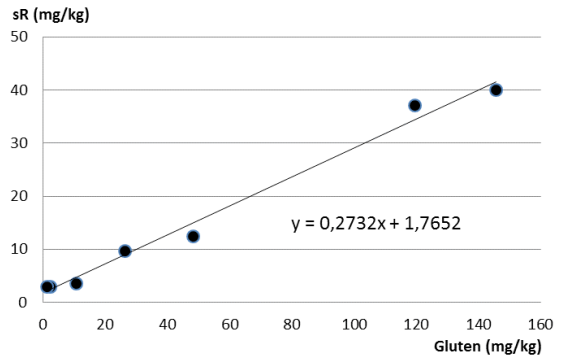

Figure 1. Plot of reproducibility (y-axis) versus the global mean observed gluten concentration for the interlaboratory study (x-axis).

The recovery values for samples 2, 3, 6, and 7 were 87, 119, 69, and 97%, respectively. The range of recoveries complies with acceptable recovery rates suggested by Abbott et al. (16) for spiked food samples, incurred samples, and/or difficult matrixes. For sample 5 (naturally contaminated starch syrup), no recovery rate could be calculated because the initial gluten content was not known. For sample 6 (sourdough spiked with 70 mg/kg), the mean recovery for all laboratories was 69%. Since the recovery for sample 7 (sourdough at 150 mg/kg) was 97%, the lower recovery could not be attributed to the matrix or the homogenization before the collaborative test. It could be speculated that a systematic error occurred during mixing the gluten-free quinoa sourdough with a rye sourdough because only minute amounts of the rye sourdough were weighed and mixed. The repeatability RSD (RSD_r) was comparable for all gluten-containing samples, ranging from 16 to 32%. This was also the case for sample 5 (naturally contaminated starch syrup), which had an average concentration of 10.6 mg/kg gluten, which was close to the LOQ specified by the manufacturer. Although the RSD_R was somewhat higher, it was limited to a maximum RSD_R of 37%. According to Abbott et al. (16), the LOD is calculated from the equation in Figure 1 at 10.6 mg/kg. The mean concentration of the blank samples was not included into this calculation since the uncertainty of this estimation is very high, and furthermore, very low gluten contaminations cannot be excluded.

Discussion

The immunochemical method for competitive gluten quantitation that was evaluated by the collaborative study described in this report is designed for the detection of the gluten content in syrups and fermented foods. In these samples, gluten is present as fragments generated by partial hydrolysis due to the action of peptidases. The method should be able to detect gluten fragments in concentrations well below 20 mg/kg gluten according to the Codex Alimentarius (1), European Union regulation 41/2009 (2), and the U.S. Food and Drug Administration (3). The assay described in this study has been shown to be more reliable for this type of samples than the sandwich version (AACCI Method 38-50.01), which is designed for quantitating nonhydrolyzed gluten (8). The analytical range of this method is estimated to be from 10.6 to 150 mg/kg.

Conclusions

The collaborative study has shown that the competitive R5 ELISA is capable of analyzing gluten fragments at concentrations starting at 10.6 up to 150 mg/kg. The competitive R5 assay enabled quantitation below and above gluten concentrations of 20 mg/kg.

The PT digest does not represent all hydrolysis processes. There are many additional factors, including temperature and time, that can affect the accuracy of the assay. Users should confirm method performance for their specific processes.

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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

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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).

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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*.

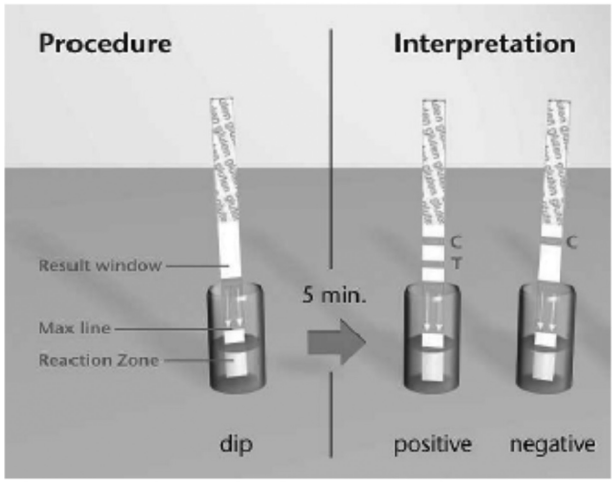

Figure 2015.16. Schematic presentation of the test principle and the subsequent interpretation of the possible results (invalid results not shown).

(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.

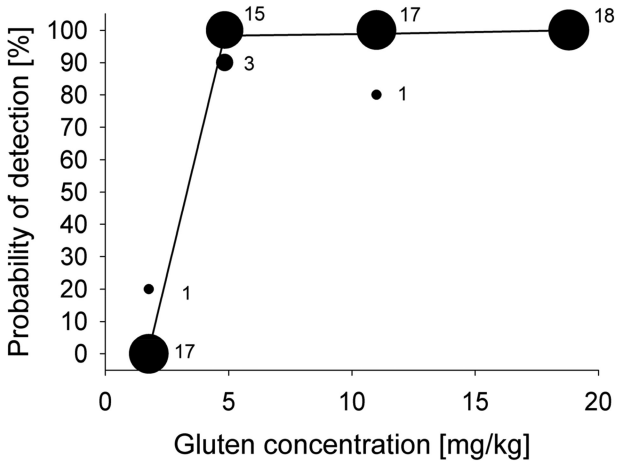

Figure 1. POD observed by each of 18 participating laboratories for samples extracted with ethanol (part A) between 1.76 and 18.8 mg/kg gluten. Number stated at each circle means number of laboratories with the same POD. Areas of circles are proportional to number of laboratories.

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

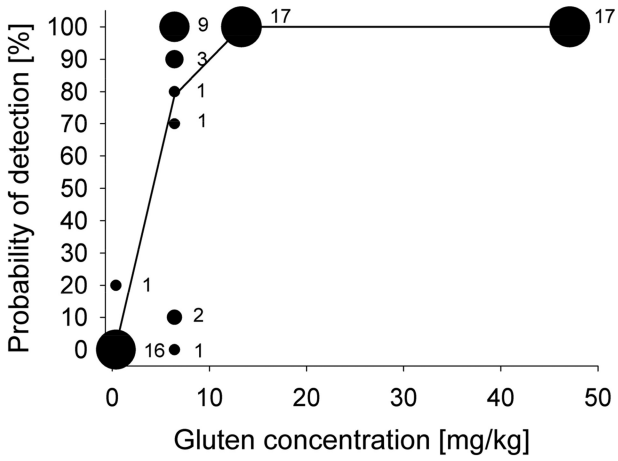

Figure 2. POD observed by each of 18 participating laboratories for samples extracted with Cocktail solution (part B) between 0.38 and 47.1 mg/kg gluten. Number stated at each circle means number of laboratories with the same POD. Areas of circles are proportional to number of laboratories.

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*.

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FOOD COMPOSITION AND ADDITIVES

Gluten in Rice Flour and Baked Rice Products by G12 Sandwich ELISA: First Action 2014.03

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The Protein and Enzymes Technical Committee of American Association of Cereal Chemists initiated a collaborative study to confirm whether the G12 antibody-based sandwich ELISA test kit is able to detect gluten in the lower mg/kg (ppm) level. Twenty laboratories investigated 24 heat-treated and non-heat-treated blind-coded samples with incurred gluten levels up to 100 mg/kg. The method has been validated for testing foods to conform to the defined Codex thresholds for gluten in gluten-free products at less than 20 mg gluten/kg. The collaborative study showed that low levels of gluten could be detected by G12 Sandwich ELISA with reproducibility RSD_R of 32% and repeatability RSD_r of 16%. Incurred samples showed a recovery between 62 and 135%. It is recommended that the method be accepted by AOAC as Official First Action.

AgraQuant® Gluten G12 is a sandwich ELISA for quantification of gluten from wheat, rye, barley, and cross-bred varieties in various foodstuffs. The G12 antibody utilized in the test kit binds to the celiac toxic amino acid sequence QPQLPY and related sequences in rye and barley (1, 2). A homogenized sample is extracted with ethanol and a proprietary extraction solution containing reducing agents. The gluten determination is based on a microtiter plate coated with specific monoclonal G12 antibody. Gluten is detected with a peroxidase-labeled G12 antibody. The determination can be done in 60 min. Ready-to-use standards of the ELISA test kit are calibrated against the Working Group on Prolamin Analysis and Toxicity (WGPAT) gliadin standard material and cover a range from 4 to 200 mg gluten/kg sample (*see* Figure 1). The preparation of ready-to-use standards was described at Halbmayr-Jech et al. (3).

Single-laboratory validation (SLV), performed by Romer Labs UK Ltd in May 2011, determined an LOD of 2 mg gluten/kg sample and an LOQ of 4 mg gluten/kg sample (*see* Table 1) as well as a recovery rate ranging from 90 to 145% (*see* Table 2) for the Gluten G12 Sandwich ELISA assay. Coefficient of variation for repeatability and lot-to-lot variation (reproducibility) was 15% or less determined within the SLV (*see* Tables 3–5). The AgraQuant Gluten G12 kit furthermore produced results similar to those assigned values for the current Codex type I approved R5 Mendez method in three Food Analysis Performance Assessment Scheme (FAPAS) rounds in 2011 (*see* Table 6).

The Gluten G12 Sandwich ELISA assay has been evaluated in a collaborative study with 20 participants. The main target for an allowable immunogenic gluten method according to the Codex Alimentarius is that it should have a detection limit of 10 mg/kg or below (4). This paper reports the findings of the collaborative study and discusses the results in relation to current thresholds (20 mg/kg) for gluten-free products.

Collaborative Study

Study Design

The study was conducted on 12 different food samples prepared in the laboratory of the Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany. Blind-coded samples in duplicate, ELISA test kits including extraction solution, method instructions, and result reporting sheets were sent to all participating laboratories.

Collaborators

The collaborative study was coordinated by Clyde Don, Foodphysica, Driel, The Netherlands. Twenty laboratories from the food producing industry, universities, governments, contract

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The method was approved by the Expert Review Panel for Food Allergens-Gluten as First Action.
The Expert Review Panel for Food Allergens-Gluten 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 to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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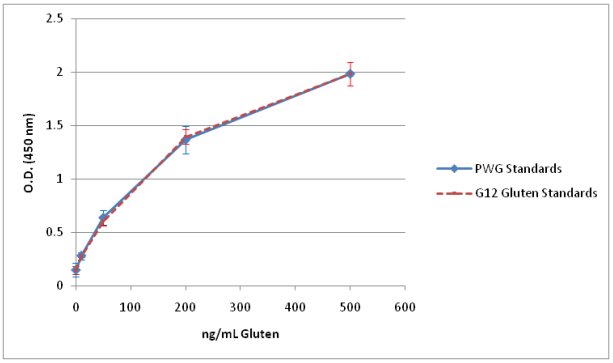

Figure 1. Calibration curve of monoclonal G12 ELISA: Six replicates each of the Vital wheat gluten and PWG gliadin standards were run on the AgraQuant Gluten G12 test kit. Error bars indicate 2 × SD of standard.

laboratories, and kit suppliers from Europe, United States, Canada, Australia, and New Zealand participated in the collaborative study. All collaborators are listed in the *Acknowledgments* section.

Description and Preparation of Samples

The following 12 samples were prepared for the collaborative study: gluten-free rice flour, rice flour containing 10 mg gluten/kg, rice flour containing 20 mg gluten/kg, rice flour containing 100 mg gluten/kg, gluten-free chocolate cake, chocolate cake containing 10 mg gluten/kg, chocolate cake containing 20 mg gluten/kg, chocolate cake containing 100 mg gluten/kg, crisp bread containing 4.5 mg gluten/kg, crisp bread containing 15 mg gluten/kg, crisp bread containing 24 mg gluten/kg, and crisp bread containing 102 mg gluten/kg. Initial target concentrations of the crisp bread samples had been 0, 10, 20, and 100 mg/kg, but a gluten contamination occurred during the preparation of these samples. The contamination was independently confirmed with another antibody-based ELISA, giving further reason to allow a re-estimation of gluten content of respective samples.

All ingredients except wheat flour were confirmed to be free of gluten contamination before use by means of the G12 Sandwich ELISA, which was also used in this collaborative study.

The gliadin content of wheat flour of the German cultivar 'Genius' was determined by an extraction/RP-HPLC method as described by Wieser et al. (5). HPLC absorbance values measured at 210 nm were converted to protein concentration using a standard solution of reference gliadin from the Prolamin Working Group (6). The gliadin content of the wheat flour sample was 67.8±0.16 g/kg (n = 3) on an "as is" basis. The gluten content of the wheat flour was calculated according to Codex (gluten = 2 × prolamin) and was 135.6 g/kg.

Samples were heat-treated to a different extent during processing as found in consumer products. Rice flour was used "as is" (not heat-treated) and represented a base material for the production of gluten-free rice based products. Gluten-free rice flour was provided by General Mills (Minneapolis, MN). Gluten-containing stock rice flour with a gluten concentration of 200 mg/kg was prepared by mixing wheat flour into rice flour and subsequently diluting the mixture with rice flour. Gluten-containing rice flour samples were prepared as follows: 10 mg/kg, 17.5 g stock rice flour was mixed with 332.5 g gluten-free rice flour; 20 mg/kg, 35 g stock rice flour was mixed with 315 g gluten-free rice flour; and 100 mg/kg, 175 g stock rice flour was mixed with 175 g gluten-free rice flour. Mixtures were shaken in an overhead shaker for at least 1 h.

Chocolate cake represented a product that had been moderately heat-treated, but with typical chocolate components that are known to be challenging for ELISA tests. Gluten-free chocolate

Table 1. Calculation of LOD from single-laboratory validation data: 47 replicates of buffer blanks were run over 10 individual AgraQuant Gluten G12 assays. The LOD was determined by calculating the mean OD of the 0 mg/kg standard + 3 SD and then reading this value back off the standard curve. The lower LOQ was determined by the lowest standard of concentration.

Standard, mg/kg	Mean (OD)	SD (OD)	CV, % (OD)	Mean + 3 SD (OD)	LOD, mg/kg
0	0.14	0.03	21.15	0.23	2.00

Table 2. Spike recovery data from single-laboratory validation data: samples were tested both in their original state and spiked with 10 mg/kg of Vital wheat gluten extract. Percentage recovery was calculated against a positive control spiked into extraction buffer. Recovery of 10 mg/kg spike was achieved from a range of processed food samples within an acceptable range (90–145%). The addition of gelatin to the extraction solution significantly increased the extraction efficiency from chocolate

		Romer extraction solution		
Sample	No spike	Spike (10 ppm gluten)	Spike CV, %	Recovery, %
Crisps	<4	12.6	1.35	134.0
Chocolate	<4	<4	NA[a]	NA
Chocolate + gelatin	<4	10.3	1.84	109.6
Cheesy corn snack	<4	8.5	5.33	90.4
Paprika	<4	10.8	0.16	114.9
Chicken	<4	9.7	2.44	103.2
Yogurt	<4	9.4	0.88	100.0
Curry sauce	<4	12.4	0.31	131.9
Margarine	<4	13.6	7.00	144.7
Positive control	NA	9.4	1.31	100.0

[a] NA = Not applicable.

Table 3. Single-laboratory validation data on repeatability using a single kit: 10 replicates of the standard curve were run using a single AgraQuant Gluten G12 test kit. Mean OD values, SD, and CV are shown below. All CV values for intra-assay analysis were less than 15%, meeting the manufacturer's QC criteria

Standard, mg/kg	Mean (OD)	SD (OD)	CV, % (OD)
0	0.138	0.018	12.80
4	0.359	0.035	9.88
20	0.698	0.058	8.34
80	1.340	0.073	5.43
200	1.877	0.109	5.82

Table 4. Single-laboratory validation data on repeatability using different kits of the same batch: 10 individual AgraQuant Gluten G12 assays containing all the standards were run. Mean OD values, SD, and CV are shown below. All CV values for interassay analysis were less than 15%, meeting the manufacturer's QC criteria

Standard, mg/kg	Mean (OD)	SD (OD)	CV, %
0	0.12	0.01	10.79
4	0.28	0.04	14.26
20	0.67	0.05	7.89
80	1.29	0.13	10.33
200	2.00	0.17	8.70

cake was prepared by mixing one bag (425 g) of gluten-free cake mix (Betty Crocker Gluten-Free Cake Mix, General Mills) with 237 mL water, 112 g baking fat (Sanella, Unilever, Hamburg, Germany), and three eggs with a hand mixer at high speed for 5 min. The mass was poured into a round baking tin [diameter (Ø) = 25 cm] and baked in an oven at 170°C for 45 min. The cake was subjected to cooling for 1 h, sliced with a knife, and air-dried at room temperature (22°C) overnight (16 h). The air-dried cake was then lyophilized and ground with a household grinder (Model 836.820 1, Privileg, Fürth, Germany). Chocolate cake with a gluten concentration of 200 mg/kg (stock chocolate cake) was produced as described, except that cake mix containing wheat flour cv. Genius was used. The amount of wheat flour in the cake mix (275 mg gluten/kg) was adjusted to provide a final gluten concentration of 200 mg/kg in the chocolate cake.

Gluten-containing chocolate cake samples for the study were prepared as follows: 10 mg/kg, 25 g stock chocolate cake was mixed with 475 g gluten-free chocolate cake; 20 mg/kg, 50 g stock chocolate cake was mixed with 450 g gluten-free chocolate cake; and 100 mg/kg, 250 g stock chocolate cake was mixed with 250 g gluten-free chocolate cake. Mixtures were shaken in an overhead shaker for at least 1 h.

The rice-based crisp bread represented a more heavily heat-treated sample. Gluten-free crisp bread was prepared by mixing 270 g of gluten-free rice flour (*see* above) and 2.7 g NaCl with 270 mL of ice-cold water using a hand mixer at high speed (air incorporation). The mass was distributed in two round baking tins (25 cm diameter) to yield a dough layer of approximately 1 cm. The dough surface was perforated with a needle, and the dough was baked at 230°C for 30 min, then turned upside down and baked for another 30 min. After cooling overnight, the bread was lyophilized and ground to a fine powder using a mortar and pestle. Crisp bread containing 200 mg gluten/kg (stock crisp bread) was produced as described, except that gluten-containing stock rice flour (200 mg gluten/kg, *see* above) was used.

Gluten-containing crisp bread samples for the study were prepared as follows: 10 mg/kg: 17.5 g stock crisp bread was mixed with 332.5 g gluten-free crisp bread; 20 mg/kg, 35 g stock crisp bread was mixed with 315 g gluten-free crisp bread; and 100 mg/kg, 175 g stock crisp bread was mixed with 175 g gluten-free crisp bread. Mixtures were shaken in an overhead shaker for at least 1 h.

The analyses of homogeneity (*see* below) revealed that the gluten-free crisp bread was contaminated with gluten at a very low concentration of about 4.5 mg gluten/kg. This may have happened during production of the crisp breads, in particular during the grinding and sifting steps. Therefore, the target gluten concentrations of the crisp bread samples (0, 10, 20, and 100 mg/kg) were corrected to the gluten concentrations that were in fact present (4.5, 15, 24, and 102 mg/kg).

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 each bulk sample and then analyzing by the G12 Sandwich ELISA. Ideally, the CV of the 10 determinations should be 15% or less. Most samples with gluten concentration above 4 mg/kg complied with this, except the chocolate cake samples containing a low concentration (≤20 mg/kg) of incurred gluten showed a CV of 21%. This was considered allowable for a sample like chocolate cake containing below 20 mg/kg gluten, because in an earlier study a beer (>20 mg/kg) and a starch syrup (<20 mg /kg) sample were accepted with a CV of 18–22% (7, 8).

Shipment

Two independent blinded replicates for each sample were provided to the participating laboratories. The coded sample vials contained 1 g of sample. Samples were shipped together with ELISA kits, instructions, and result sheet to participating laboratories.

Analysis and Data Reporting

Participants were requested to follow the instructions and to extract each sample using the test kit's standard procedure and to analyze in duplicate in one analytical run. If changes had been made to the analytical protocol, they had to be reported in the "comments" box of the result sheet. The samples were analyzed

Table 5. Lot-to-lot variation (reproducibility): three different kit batches of the AgraQuant Gluten G12 test kit were run, GU1001-1106, GU1002-1108, and GU1003-1111. Mean OD values, SD, and CV are shown below. All CV values for interbatch analysis were 15% or less, meeting the manufacturer's QC criteria

Standard, mg/kg	GU1001-1106	GU1002-1108	GU1003-1111	Mean (OD)	SD (OD)	CV, %
0	0.10	0.10	0.09	0.10	0.01	6.97
4	0.26	0.21	0.21	0.23	0.03	11.55
20	0.72	0.57	0.58	0.63	0.08	13.45
80	1.39	1.13	1.09	1.21	0.16	13.65
200	2.11	1.65	1.60	1.79	0.28	15.92

Table 6. Samples from FAPAS Proficiency Test 2781 (February 2011), 2792 (June 2011), and 2795 (October 2011) were analyzed during the single-laboratory validation by Romer Labs using the AgraQuant Gluten G12 test kit. Test materials from Round 2781 were cake mix to be analyzed for gluten. Test materials were prepared using a gluten and wheat free chocolate cake mix, to which a gluten and wheat containing cake mix was added. Test materials from Round 2792 were prepared by mixing infant soya formula with wheat flour. Test materials from Round 2795 were prepared by combining cake mix with wheat flour. Analysis of the FAPAS 2781, 2792, and 2795 proficiency samples using the AgraQuant Gluten G12 test kit produced very similar results to those assigned values for the R5 Mendez method (data from the R-Biopharm kit). The R5 Mendez method is currently the Codex Type I approved method for gluten analysis (4)

		Assigned value		
	AgraQuant Gluten G12 test kit, mg/kg gluten	R5 ELISA–R-Biopharm R7001, mg/kg gluten	Veratox ELISA–Neogen, mg/kg gluten	
FAPAS 2781 A	<4	Negative	Negative	
FAPAS 2781 B	22.6	27.4	42.6	
FAPAS 2781 C	95.6	91.6	120.7	
		R5 ELISA–R-Biopharm R7001, mg/kg gluten	AR5 ELISA–R-Biopharm R7002, mg/kg gluten	
FAPAS 2792 A	119.8	134.2	141.0	
FAPAS 2792 B	<4	Negative	Negative	
		R5 ELISA–R-Biopharm R7001, mg/kg gluten	R5 ELISA–R-Biopharm R7002, mg/kg gluten	Assigned value Ingenasa–R5 ELISA 30.GLU.K2, mg/kg gluten
FAPAS 2795 A	51.3	58.5	43.4	71.4
FAPAS 2795 B	<4	Negative	Negative	Negative

by each laboratory. All optical density (OD) values had to be recorded in a ready-to-use Excel sheet. The participants used the calculator, which was provided with the Excel sheet. The model was a simple linear point-to-point calculation. The final data from the laboratories were sent to the study coordinator. A statistical evaluation was performed according to AOAC guidelines (9, 10).

AOAC Official Method 2014.03
Gluten in Rice Flour and Rice-Based Food Products
G12 Sandwich ELISA
First Action 2014

(Applicable for determination of gluten in rice flour and rice-based unprocessed and processed foods as evaluated in the multilaboratory study.)

Caution: Wear protective gloves and safety glasses. The stop solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water (*see* Material Safety Data Sheet). The extraction solution contains chemicals which are harmful to health. Perform sample extraction under a chemical hood and avoid contact with skin. Dispose of all materials, containers, and devices appropriately after use.

See Table **2014.03A** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal G12 antibody that can determine gluten derived from wheat, rye, barley, and cross-bred varieties. The G12 antibody binds to the celiac toxic amino acid sequence

Table 2014.03A. Performance statistics for the overall G12 sandwich ELISA results

		Sample ID[a]											
Parameter	Symbol	1	2	3	4	5	6	7	8	9	10	11	12
Total No. laboratories	*P*	17	18	18	18	16	18	18	16	17	18	18	18
Total No. replicates	Sum [n(L)]	34	36	36	36	32	36	36	32	34	36	36	36
Overall mean of all data (grand mean; mg/kg)	xbarbar	1.6	13.5	26.2	101.2	0.1	6.2	13.1	63.5	4.1	14.9	26.6	112.7
Repeatability SD, mg/kg	s_r	0.8	2.5	8.1	14.8	1.2	1.2	1.3	5.1	1.9	1.5	4.3	20.4
Reproducibility SD, mg/kg	s_R	1.9	4.0	11.6	31.8	1.2	1.8	2.5	13.5	2.8	4.5	8.9	33.2
Repeatability RSD, %	RSD_r	48.2	18.5	30.7	14.7	2348	19.2	10.2	8.0	46.2	10.4	16.2	18.1
Reproducibility RSD, %	RSD_R	115.8	29.6	44.2	31.4	2348	28.3	19.1	21.2	69.0	30.3	33.6	29.4
Bias (mg/kg) observed-nominal		1.6	3.5	6.2	1.2	0.1	–3.8	–6.9	–36.5	–0.4	–0.1	2.6	10.7
Recovery, % = observed/nominal × 100			135.0	131.0	101.2		62.0	65.5	63.5	91.1	99.3	110.8	110.5

[a] 1 = Gluten-free rice flour; 2 = rice flour 10 mg gluten/kg; 3 = rice flour 20 mg gluten/kg; 4 = rice flour 100 mg gluten/kg; 5 = gluten-free chocolate cake; 6 = chocolate cake 10 mg gluten/kg; 7 = chocolate cake 20 mg gluten/kg; 8 = chocolate cake 100 mg gluten/kg; 9 = crisp bread 4.5 mg gluten/kg; 10 = crisp bread 15 mg gluten/kg; 11 = crisp bread 24 mg gluten/kg; and 12 = crisp bread 102 mg gluten/kg.

Table 2014.03B. Cross-reactivity of the G12 antibody (G12 antibody shows no cross-reactivity to various nuts, oils, seeds, starches, or gluten-free grains)

Food category	Food sample	Romer extraction solution, mg/kg gluten	Gluten, %
Gluten-containing grains	Wheat flour	72222	7.2
	Barley (Cumion)	292390	29.2
	Durum wheat	15733	1.6
	Spelt (Ostro)	81926	8.2
	Rye (Capitan)	41577	4.2
Naturally gluten-free grains	Soya bean	<4	
	Soya mince	<4	
	Buckwheat	<4	
	Rice flour	<4	
	Quinoa	<4	
	Corn kernels	<4	
	Teff flour	<4	
	Millet	<4	
Oats	Bastion	4.3	
	00-61 Cn	7.4	
	Brachan	<4	
	Husky	6.3	
	Fusion	6.6	
Nuts	Pecan	<4	
	Walnut	<4	
	Almond	<4	
	Cashew	<4	
	Macadamia	<4	
	Peanut	<4	
	Hazelnut	<4	
	Pine nut	<4	
	Pistachio	<4	
Seeds	Golden linseed	<4	
	Brown linseed	<4	
	Poppy	<4	
	Sesame	<4	
	Mustard	<4	
Oils	Hazelnut oil	<4	
	Walnut oil	<4	
	Vegetable oil	<4	
	Sunflower oil	<4	
Starches	Tapioca starch	<4	
	Wheat starch	<4	
	Potato starch	<4	
Miscellaneous	Amaranth	<4	

QPQLPY and related sequences in rye and barley. The antibody detects prolamins in nonheated and heated food by using a specific proprietary extraction solution. No cross-reactivity has been determined to maize, rice, teff, millet, buckwheat, quinoa, amaranth, and soy (*see* Table **2014.03B**).

Gluten is extracted from samples using proprietary extraction solution containing reducing agents followed by ethanol extraction. After centrifugation the supernatant is used in a sandwich enzyme-linked immunoassay. When incubated on monoclonal antibody-coated microwells, the analyte is forming an antibody-antigen complex. After a washing step, an enzyme-conjugated monoclonal antibody is applied to the well and incubated. After a second washing step, an enzyme substrate is added and blue color develops. The intensity of the color is directly proportional to the concentration of gluten in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with a primary absorbance filter of 450 nm (OD450). The optical densities of the samples are compared to the standards and an interpolated result is determined.

B. Apparatus

The apparatus specified has been tested. Equivalent apparatus may be used.

(**a**) *Osterizer blender*.—Used for homogenization of sample (Sunbeam-Oster, Ft. Lauderdale, FL).

(**b**) *Centrifuge tubes*.—50 mL for extraction (Star Labs International GmbH, Hamburg, Germany).

(**c**) *Glassware*.—Wash bottle (1000 mL) and graduated cylinders.

(**d**) *Water bath*.—Grant Sub Aqua 12 (Grant Instruments, Cambridgeshire, UK).

(**e**) *Stuart roller mixer*.—Bibby Scientific Ltd (Staffordshire, UK).

(**f**) *Bench top centrifuge*.—Sigma 1-14 (Sigma Laborzentrifugen, Osterode am Harz, Germany).

(**g**) *Centrifuge tubes*.—2 mL; for sample dilution (Star Labs International GmbH).

(**h**) *Micropipet*.—Accurately delivering 100 µL ± 1%.

(**i**) *Microtiter plate reader with a 450 nm filter*.—Thermo Fisher Scientific (Shanghai, China).

C. Reagents

The following items (**a**)–(**i**) are available as a test kit (AgraQuant Gluten G12 ELISA®, Romer Labs UK Ltd, Runcorn, UK). All reagents are stable for 12 months from date of manufacture at 2–8°C (36–46°F). Refer to kit label for current expiration.

(**a**) *Antibody-coated microwell strips*.—Monoclonal antibodies are coated in 20 mM phosphate buffered saline (PBS) onto a set of 12 eight-microwell strips (NUNC, Roskilde, Denmark).

(**b**) *Gluten ready-to-use standards (antigen)*.—Five vials containing 1.2 mL of each gluten G12 standard (0, 4, 20, 80, and 200 mg/kg labeled as ppm), prepared by vital wheat gluten dissolved in 60% ethanol at a concentration of 1 mg/mL. Solution is further diluted in 20 mM PBS–Tween (0.9% sodium chloride, 0.07% Tween 80) containing 0.25% fish gelatin (Sigma) to 0,

10, 50, 200 and 500 ng/mL gluten, calibrated to the WGPAT gliadin (86% highly purified gliadin from 40 different European wheat varieties).

(**c**) *Conjugate solution (peroxidase-labeled antibody, ready-to-use)*.—One bottle containing 13 mL.

(**d**) *Substrate solution (stabilized peroxide substrate and 3,3',5,5'-tetramethyl-benzidine in a dilute buffer solution)*.—One bottle containing 15 mL.

(**e**) *Stop solution (1 N H_2SO_4)*.—One bottle containing 15 mL.

(**f**) *Diluent buffer*.—One bottle containing 20 mL of 5× concentrated diluent buffer. Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.07% Tween 80) with 0.25% fish gelatin (Sigma) and 0.01% Proclin as a preservative.

(**g**) *Wash buffer*.—One bottle containing 60 mL of 10× concentrated wash buffer. Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.05% Tween 20) with 0.01% Proclin as preservative.

(**h**) *Extraction solution*.—One bottle containing 105 mL of ready-to-use proprietary extraction solution containing reducing agents.

(**i**) *Fish gelatin*.—One sachet containing 10 g.

Additional reagents needed, but not provided with the test kit:

(**a**) *Distilled or deionized water*.

(**b**) *Ethanol*.—80% (v/v).

D. General Instructions

Due to the sensitivity of the assay, a gluten-free environment must be maintained. It is preferable to perform the assay in a separate room from that used for sample preparation and extraction. Make sure balance and the surrounding space, as well as equipment such as spatulas, are clean. Cleaning can be done by using a 70% alcoholic solution. Spatula should be cleaned after each sample weighing by a 70% alcoholic solution.

Store kit at 2–8°C (35–46°F) and let all components equilibrate to 20–25°C (68–77°F) before use.

Include ready-to-use standards in duplicates to each run of samples. Use separate pipet tips for each standard and each sample extract to avoid cross-contamination.

It is recommended that an eight-channel pipettor is used to perform the assay. No more than 48 samples and standards total should be run in one experiment when using an eight-channel pipettor (24 when samples and standards are added in duplicate, e.g., six test strips). If using only single-channel pipets, it is recommended that no more than a total of 16 samples and standards are analyzed in one experiment (eight when standards and samples are added in duplicate, e.g., two test strips).

E. Preparation of Components Delivered with the Kit

(**a**) *Sample dilution buffer*.—Dilute diluent buffer concentrate 1:5 with distilled water (e.g., add 20 mL of concentrated diluent buffer to 80 mL distilled water). Dilution buffer may be used within 24 h, if stored at 4°C.

(**b**) *Wash buffer*.—If a precipitate is formed during storage of the wash buffer concentrate, the concentrate should be warmed up until it is dissolved. Dilute wash buffer concentrate 1:10 with distilled water (e.g., add 10 mL of concentrated wash buffer to 90 mL distilled water). Wash buffer may be used within 1 week, if stored at 4°C.

F. Sample and Test Portion Preparation

Obtain a representative sample and homogenize a minimum of 5 g in a mortar or blender as fine as possible. Weigh out 0.25 g of homogenized sample into a vial with a minimum 10 mL capacity, which can be tightly sealed. For chocolate-containing samples, additionally add 0.25 g of powdered fish gelatin. Add 2.5 mL extraction solution (under a fume/chemical hood), close vials, and mix vigorously on a vortex. Visually check for clumps, and continue mixing until samples are well dispersed in the extraction solution.

Incubate at 50°C (122°F) for 40 min in a water bath. Allow the extracts to cool to room temperature and add 7.5 mL of 80% ethanol; mix well. Shake for a total of 60 min at room temperature (20–25°C/68–77°F) with a rotary shaker. (After about 30 min in the rotator, check the vials visually if all sample material has suspended in the liquid. If clumps have formed, vortex and let the vials rotate for the second 30 min to complete the extraction procedure).

Centrifuge samples for 10 min at 2000 × *g* to obtain a clear aqueous layer between the particulate sediment and supernatant. Note, in some cases, a thin fatty layer creaming on top of the supernatant. Collect the aqueous supernatant (extract) and transfer into a new vial. Dilute supernatant at least 1:10 (0.1 + 0.9 mL) with prediluted sample dilution buffer (depending on the expected prolamin content of the sample). If prediluted samples are not immediately used for determination by ELISA, close vials and keep in the dark at room temperature (20–25°C/68–77°F) for a maximum of 7 days until ELISA experiments.

G. Determination (Assay)

Bring all reagents to room temperature (20–25°C, 68–77°F) before use.

Use dilution of the sample extract to carry out ELISA experiments. Run standards and diluted sample extracts in duplicate. Place an appropriate number of antibody-coated microwells in a microwell strip holder. Record standard and sample positions.

Using a single-channel pipettor, add 100 µL of each ready-to-use standard or prepared sample into the appropriate well. Use a fresh pipet tip for each standard or sample. Make sure the pipet tip has been completely emptied.

Incubate at room temperature (20–25°C, 68–77°F) for 20 min. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with diluted wash buffer, and then emptying the buffer from the microwell strips. Repeat this step four times for a total of five washes. Take care not to dislodge the strips from the holder during the wash procedure. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel all of the residual buffer after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

Measure the required amount of conjugate from the green-capped bottle (about 120 µL/well or 1 mL/strip) and place in a separate container (e.g., reagent boat when using the

eight-channel pipettor). Using an eight-channel pipet, dispense 100 µL of conjugate into each well.

Incubate at room temperature (20–25°C, 68–77°F) for 20 min. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with diluted wash buffer, and then emptying the buffer from the microwell strips. Repeat this step four times for a total of five washes. Take care not to dislodge the strips from the holder during the wash procedure. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel all of the residual buffer after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

Measure the required amount of substrate from the blue-capped bottle (about 120 µL/well or 1 mL/strip) and dispense into a separate container (e.g., reagent boat for an eight-channel pipettor).

Pipet 100 µL of the substrate into each microwell using an eight-channel pipettor. Incubate at room temperature (20–25°C, 68–77°F) for 20 min in the dark.

Measure the required amount of stop solution from the red-capped bottle (about 120 µL/well or 1 mL/strip) and dispense into a separate container (e.g., reagent boat for an eight-channel pipet).

Pipet 100 µL of stop solution into each microwell using an eight-channel pipettor. The color should change from blue to yellow.

H. Reading

Eliminate air bubbles prior to reading wells as they are likely to affect analytical results.

Read the absorbance of wells with a microwell reader using a 450 nm filter. Record OD readings for each microwell.

I. Calculations

Use unmodified OD values or OD values expressed as a percentage of the OD of the 200 ppm standard to construct a dose-response curve using the five standards (0, 4, 20, 40, and 200 ppm gluten). Gluten concentration given for the standards already consider sample preparation and 1:10 dilution according to method protocol. Gluten concentrations of samples can be calculated by interpolation from this standard curve using a point-to-point calculation.

If a sample contains gluten levels higher than the highest standard (>200 ppm), the sample extract should be further diluted with dilution buffer such that the diluted sample results are in the range of 4 to 200 ppm and reanalyzed to obtain accurate results. The dilution factor must be included when the final result is calculated.

J. Criteria for Acceptance of Standard Curve

An example for the calibration curve is shown in the Certificate of Analysis included in each test kit. Higher OD values of the absorbance at 450 nm compared to the certificate may indicate insufficient washing or gluten contamination. For samples showing OD values higher than the 200 ppm standard, a further dilution and repeated analysis is recommended. The additional dilution factor must be taken into consideration during calculation.

Any coloration of the substrate solution prior to the analysis or OD value of less than 1.1 absorbance units for 200 ppm standard may indicate instability or deterioration of reagents.

Collaborator´s Comments

Participants were following the instructions and the study coordinator did not receive any comments that changes to the procedure had been made. One laboratory reported that the test kit was not cold on arrival, but the results could still be used.

Results and Discussion

Two laboratories returned result sheets that could not be used. This was due to high CV in calibration duplicates and incomplete result sheets. Negative results that were reported <LOD in the Excel calculator sheet were calculated by a linear back-extrapolation method using a linear regression curve fit for lower calibrators (0, 4, and 20 mg/kg).

Finally, the results from 18 laboratories were used for the evaluation (*see* Table 7). Outliers were identified by using the Cochran and the Grubbs tests according to AOAC guidelines (9). After removal of the outliers, the statistical performance was calculated. The results of the calculations are shown in Table **2014.03A**.

The LOD was calculated according to recommendations from AOAC (9, 10). A plot of the reproducibility SD (s_R) versus the mean for all samples ($\bar{x}$) in the dataset was created (*see* Figure 2). With this plot the LOD was calculated using the intercept of the linear regression line, which was 0.69. Using slope correction, this resulted in a calculated s_0 of 1.30 mg/kg. Since LOD = 3.3 × s_0, the LOD of the method was 4.3 mg/kg. The RSDs were between 20 and 30% for most of the gluten containing samples. The RSD_R was in a similar range as found for other ELISA methods (7, 8). The contaminated crisp bread had a higher RSD_R, but the trace of 4.5 mg/kg was close to the LOD of the method, hence a higher RSD could be expected (11). Overall, the G12 method was able to detect and quantify low gluten concentrations in these different matrixes.

According to Abbott et al. (10), recoveries between 80 and 120% are ideal for ELISA methods. Recoveries in a range between 50 and 150% are acceptable as the extended recovery range for incurred samples or difficult matrixes. For the present study, the spiked rice flour showed a recovery range of 101–135%, and the recovery for the rice-based crisp bread was 91–111%. For low levels of spiked gluten at 10 mg/kg, the G12 method is sensitive to a gluten spike with average recovery of 130%. With the gluten-incurred chocolate cake the recovery was 62–66%, which is at the lower end of acceptable recovery. Details for recoveries and biases per matrix of individual concentrations are shown in Table **2014.03A**.

The chocolate cake recipe contained eggs, fat, chocolate, and hydrocolloid (guar gum). Ingredients like egg proteins are strong thermal aggregators possibly resulting in highly insoluble covalently bonded (S-S) aggregates with incorporated gluten proteins. In general, the reducing agent in the ELISA extraction medium can usually deal with heat-aggregated gluten. The high fat content of more than 20% based on dry mass as well as the presence of polyphenols from chocolate might have promoted interactions with gluten proteins affecting gluten recovery. Furthermore, guar gum acted as a thickener during extraction

Table 7. Individual results from interlaboratory study for gluten by AgraQuant Gluten G12 Sandwich ELISA (results are stated as mg gluten/kg). Outliers determined with Cochran and the Grubbs test are marked in bold font and highlighted in grey. Laboratory B did not return a result sheet in time. Result sheets of laboratories C and J could not be used

	Sample ID[a]																							
Lab	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Sample 7		Sample 8		Sample 9		Sample 10		Sample 11		Sample 12	
A	0, 7	0, 3	14, 1	18, 3	21, 4	23, 1	107, 5	115, 5	–1, 1	0, 1	4, 6	4, 7	12, 4	10, 1	61, 0	59, 5	2, 5	3, 2	12, 0	13, 7	26, 5	24, 9	92, 1	109, 9
D	1, 1	3, 6	18, 5	17, 6	39, 8	53, 9	150, 5	162, 8	0, 9	0, 3	9, 3	7, 1	16, 2	17, 3	121.4[b]	120.7[b]	5, 8	3, 7	16, 8	18, 2	45, 0	41, 6	154, 5	159, 7
E	0, 4	0, 6	17, 7	14, 7	19, 1	48, 6	149, 1	177, 6	0, 9	–1, 2	6, 7	6, 1	12, 6	15, 9	79, 7	78, 5	0, 3	0, 5	11, 8	11, 0	17, 8	19, 9	102, 0	104, 9
F	0, 2	0, 4	11, 4	15, 6	19, 2	10, 2	58, 1	71, 2	0, 0	–2, 1	5, 3	5, 4	12, 1	10, 9	71, 2	62, 3	6, 7	2, 0	12, 0	9, 1	18, 8	18, 4	86, 7	79, 3
G	1, 2	0, 8	9, 1	14, 6	27, 3	26, 3	76, 7	70, 4	0, 5	–0, 9	7, 2	4, 9	12, 0	11, 6	52, 2	55, 4	4, 0	3, 0	13, 5	13, 8	22, 2	27, 0	103, 6	86, 5
H	–0, 2	–0, 3	7, 7	7, 2	12, 6	14, 1	64, 2	60, 8	0, 7	0, 3	5, 3	6, 1	11, 9	13, 1	60, 4	59, 9	0, 7	0, 6	9, 1	8, 9	17, 3	16, 4	73, 8	66, 3
I	2, 0	2, 8	15, 6	12, 9	22, 7	19, 0	81, 5	78, 0	–1, 3	1, 9	3, 9	7, 1	10, 1	11, 7	51, 1	39, 0	5, 5	4, 9	15, 2	15, 0	39, 3	26, 2	65, 8	100, 4
K	6, 7	5, 6	15, 7	15, 5	38, 7	29, 9	85, 6	76, 0	0, 4	–0, 8	4, 3	4, 5	8, 9	11, 2	40, 1	39, 4	13.5[b]	14.6[b]	26, 0	23, 3	36, 4	36, 8	158, 4	114, 6
L	8.9[c]	0.5[c]	22, 0	17, 5	38, 3	56, 8	120, 5	143, 2	2, 9	–1, 7	9, 8	9, 1	12, 9	15, 1	66, 2	75, 4	3, 0	4, 7	13, 4	17, 2	34, 9	21, 4	118, 5	175, 7
M	3, 9	3, 5	18, 5	17, 7	20, 3	38, 9	99, 6	81, 4	0, 6	–0, 4	5, 3	7, 6	15, 5	13, 6	70, 3	78, 8	6, 9	11, 1	21, 3	19, 7	16, 9	21, 1	154, 6	90, 3
N	1, 7	4, 7	9, 7	12, 7	18, 9	15, 0	145, 0	111, 6	0, 1	0, 9	4, 5	3,8	9,6	9,5	45,5	52,0	4,8	10,9	11,1	10,4	20,6	23,8	102,1	90,5
O	1,3	1,9	15, 9	7, 4	26, 2	36, 2	124, 5	95, 6	0.3[c]	1.5[c]	6, 8	4, 9	18, 7	13, 9	73.6[c]	108.7[c]	4, 4	1, 6	19, 3	15, 3	26, 7	27, 5	144, 8	142, 8
P	–0, 6	1, 1	7, 7	11, 1	14, 5	14, 2	74, 1	74, 5	0, 5	0, 1	7, 5	7, 0	13, 7	12, 7	71, 3	66, 1	6, 6	3, 5	12, 0	16, 4	22, 9	18, 7	75, 7	70, 8
Q	0, 3	0, 6	12, 6	10, 3	18, 9	13, 7	114, 9	108, 1	–0, 7	–1, 2	5, 3	6, 6	12, 1	13, 2	67, 7	69, 7	2, 5	2, 3	12, 6	11, 3	23, 2	20, 3	112, 9	99, 8
R	3, 3	3, 6	11, 3	15, 2	25, 2	32, 2	139, 4	93, 1	0, 3	0, 1	6, 5	6, 4	14, 1	12, 8	72, 6	70, 5	6, 4	6, 6	19, 3	19, 1	46, 0	38, 9	178, 5	152, 9
S	0, 8	1, 0	9, 9	7, 6	31, 9	17, 9	92, 3	58, 7	0, 9	0, 3	5, 7	3, 8	10, 8	10, 3	44, 1	55, 1	1, 3	2, 0	12, 4	11, 9	28, 6	18, 2	109, 2	60, 7
T	–1, 1	–1, 4	10, 1	9, 3	21, 0	22, 7	95, 2	77, 8	0, 2	0, 1	6, 6	4, 6	14, 2	14, 2	71, 8	76, 9	1, 7	1, 1	9, 8	10, 6	19, 8	20, 0	109, 7	129, 0
U	2, 5	2, 8	16, 4	17, 7	26, 5	29, 0	97, 0	109, 4	–2.28[b]	–2.74[b]	11, 0	7, 8	17, 3	17, 9	76, 9	91, 2	4, 6	8, 4	19, 6	22, 7	33, 4	41, 4	139, 6	141, 3

[a] 1 = Gluten-free rice flour; 2 = rice flour 10 mg gluten/kg; 3 = rice flour 20 mg gluten/kg; 4 = rice flour 100 mg gluten/kg; 5 = gluten-free chocolate cake; 6 = chocolate cake 10 mg gluten/kg; 7 = chocolate cake 20 mg gluten/kg; 8 = chocolate cake 100 mg gluten/kg; 9 = crisp bread 4.5 mg gluten/kg; 10 = crisp bread 15 mg gluten/kg; 11 = crisp bread 24 mg gluten/kg; and 12 = crisp bread 102 mg gluten/kg.

[b] Grubbs outlier.

[c] Cochran outlier.

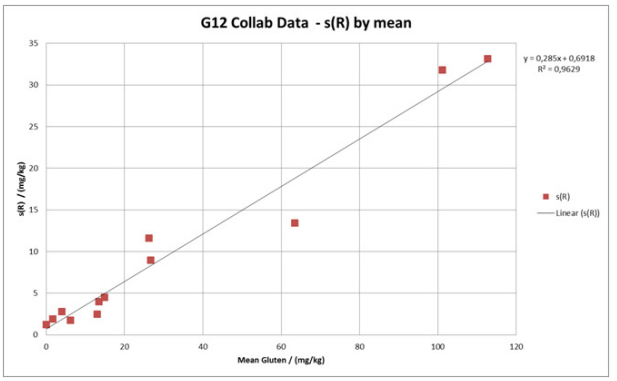

Figure 2. Plot of reproducibility SD (S_R) versus the global mean observed gluten concentration for the interlaboratory study.

and strongly increased the viscosity of the extract. Hence, a clear separation of extract aliquots was more difficult with this matrix. This may also explain the higher CV in the homogeneity tests. Due to the complexity of the cake recipe we cannot pinpoint a single reason for low recovery, but a combination of the factors mentioned is most likely. Taking this complexity into account, the method evaluated here largely complies with the guidelines and best practices for allergen ELISA methods (10). With an LOD of 4.3 mg gluten/kg, it fulfills the LOD requirement of ≤10 mg/kg of Codex Alimentarius (4).

Conclusions and Recommendation

This collaborative study has shown that the G12 Sandwich ELISA is capable of quantifying gluten in foods with an LOD of 4.3 mg gluten/kg. This method shows good precision and accuracy in the concentration range of most interest (20 mg/kg and above), where it has to be decided whether a sample meets guidelines for gluten content. Some matrix effects, especially with the incurred chocolate cake samples, may lower recovery as compared to spiked samples. Therefore, it may be beneficial to occasionally check recovery by using internal reference samples with known gluten content.

According to these results, it is recommended that the method be accepted by AOAC as *Official First Action*.

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FOOD COMPOSITION AND ADDITIVES

Gliadin as a Measure of Gluten in Foods Containing Wheat, Rye, and Barley—Enzyme Immunoassay Method Based on a Specific Monoclonal Antibody to the Potentially Celiac Toxic Amino Acid Prolamin Sequences: Collaborative Study

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The Working Group on Prolamin Analysis and Toxicity (WGPAT) organized a collaborative study to confirm whether the two R5 antibody-based ELISA test kits are able to detect gliadin in the lower mg/kg (ppm) level. Twenty laboratories investigated 12 blind-coded samples, spiked and naturally contaminated, to show the possibility of determining traces of gliadin in heat-treated or nonheat-treated foods by ELISA. It was shown that very small amounts of gliadin (below 100 ppm) could be detected by ELISA with a reproducibility RSD_R (37%) and a repeatability RSD_r (27%) common for ELISA under these conditions. The recovery of gliadin from the spiked samples was between 84 and 109%, based on the results of all laboratories, including those with poor performance. No false positives were found by the method ($P \leq 0.05$), but one negative sample was contaminated during the bakery process. It is recommended that the method be accepted by AOAC as Official First Action.

RIDASCREEN® Gliadin is a sandwich ELISA for the quantification of gliadin derived from wheat and related prolamins derived from rye and barley in various foodstuffs. The test is based on a microtiter plate coated with the specific monoclonal antigliadin R5-antibody (1). Bound gliadin is finally detected with a peroxidase-labeled specific antibody (R5).

A preground sample is extracted by the use of a special solvent (cocktail) sample preparation method (2) and can be analyzed in less than 100 min. The standard calibration curve of the ELISA covers a range from 2.5 to 40 mg gliadin/kg sample and is standardized against the Working Group on Prolamin Analysis and Toxicity (WGPAT) gliadin standard material. Calibration of the gliadin standard against the WGPAT gliadin standard material was conducted using a mixture of defatted wheat, rye, and barley and was pre-extracted with 0.5 M NaCl to remove albumins and globulins. The remaining material was extracted with aqueous ethanol (60% ethanol, v/v) to extract the prolamin fraction. The resulting solution was measured in different dilutions against a set of calibrators prepared from the WGPAT gliadin, which is an aqueous 60% ethanolic stock solution of 1 mg gliadin/mL. All different solutions from the homemade extract were within the 95% confidence interval of the WGPAT gliadin calibration curve.

The assay is applicable to the detection of gliadin with an LOQ of 2.5 mg gliadin/kg and an LOD of 1.5 mg gliadin/kg as well as a recovery rate of 84–109%. This method is developed to detect traces of gliadin in gluten-free food, not for quantifying the prolamin content in wheat, rye, or barley flour.

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The recommendation was approved by the Methods Committee on Microbiology as First Action. *See* "Standards News," (2012) *Inside Laboratory Management*, January/February 2012 issue.
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Appendixes are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
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Collaborative Study

Study Design

The WGPAT coordinated a large collaborative study of the R5 ELISA systems. This study was conducted to investigate standardized and reliable methods for gliadin detection in food with detection limits lower than 100 mg/kg (ppm) gliadin, corresponding to 200 mg/kg (ppm) gluten. The R5 ELISA methods are able to determine wheat, rye, and barley to 100%. The International WGPAT Collaborative Study involved two test systems (INGEZIM GLUTEN and RIDASCREEN® Gliadin kit); however, this study investigated only the RIDASCREEN Gliadin kit (3, 4).

The study was conducted on 12 different test materials (Table 1), prepared by Herbert Wieser, Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany, and Enrique Mendez, Centro Nacional de Biotecnologia, Universidad Autonoma, Madrid, Spain. All of the laboratories were sent instructions as well as analytical protocols, including extraction protocols, encoded samples, extraction solvents, ELISA kits, and report forms. The laboratories were instructed to submit the results in printed as well as in electronic form.

Collaborators

Twenty laboratories from Europe and Argentina participated in a coded form to evaluate twelve encoded samples. Seven laboratories were from the food-producing industry, six from universities (Argentina, Ireland, Germany, Italy, and Spain), five were food-investigating laboratories, and two were ELISA kit suppliers. All collaborators are listed in *Acknowledgments*. Study Director was Frits Janssen, Zutphen, The Netherlands.

Description of Samples

A total of 12 samples were evaluated (Table 1). Two bread doughs were prepared on the basis of maize (samples 1–4) and rice (samples 5–7). Four maize bread samples were produced by adding water and yeast, and three were spiked with different levels of purified gliadin (WGPAT gliadin standard). Loaves (100 g) were leavened and heated for 10 min at 240°C in small baking tins. Three samples of nonheated rice dough (mixture of flour with water) were prepared, of which two were spiked with the purified gliadin. Samples four and six represent the nonspiked basis bread dough; sample four contains maize flour, water, and yeast; sample six contains rice flour and water. Additionally, five commercial samples of gluten-free flour containing low amounts of gliadin (presumably by contamination during processing) were collected from the market (samples 8–12). All 12 samples were milled to a fine powder after drying, divided into portions, packed in plastic tubes, and coded with an alpha-numeric code. The samples were sent blind-coded in duplicates to the participants.

Purified WGPAT gliadin (containing 86% gliadins), prepared from a mix of 40 European wheat varieties, was used as the spiking material (5). The declared gliadin content in Table 1 represents the theoretical amount of gliadin added to the dough for samples 1–3, 5, and 7. Samples 8–12, collected from the market, were milled and tested several times by the R5 in-house ELISA (1) at the laboratory of E. Mendez to obtain an average value. Homogeneity was tested by the R5 in-house ELISA by E. Mendez.

Table 1. Overview of samples used in the study

No.	Gliadin level, ppm	Spiked/ contaminated	Type	Heated/ unheated
1	168	Spiked	Maize	Heated
2	35	Spiked	Maize	Heated
3	79	Spiked	Maize	Heated
4	0[a]		Maize	Heated
5	41	Spiked	Rice	Nonheated
6	0[a]		Rice	Nonheated
7	147	Spiked	Rice	Nonheated
8	14	Contaminated	Wheat starch	Nonheated
9	13	Contaminated	Rice flour	Nonheated
10	(12–15)	Contaminated	Wheat starch	Nonheated
11	<1.5		Maize flour	Nonheated
12	<1.5		Maize flour	Nonheated

[a] Non-spiked material.

Shipment

Samples and ELISA kits were shipped to the participants at ambient temperature. Each of the bags containing the samples was labeled according to the sample code for identification. Participants were requested to return a receipt acknowledgment form to indicate receipt and conditions of the shipped samples. They were also directed to follow the storage advice for samples and kits.

Analysis and Data Reporting

ELISA kits, including protocols, were sent to the participants. The extraction solution (cocktail) was provided with the kits. Participants were requested to carry out the analysis according to the leaflet of the kit supplier, extracting the samples in duplicate according to the extraction protocol, and subsequently using three dilutions (1:25, 1:50, and 1:100) of each extracted sample in two ELISA runs. The raw data of both runs had to be reported to the Study Director. All data were calculated by the RIDA®SOFT Win software to be sure that there is no influence coming from the software. The final data of the dilution row per sample was selected by a mathematical algorithm. A statistical evaluation was performed according to AOAC guidelines. For each sample there were two runs that led to two results per sample, insufficient for making outlier checks. Two laboratories (Laboratories D and F) sent back results from only one run of experiments.

AOAC Official Method 2012.01
Gliadin as a Measure of Gluten in Foods Containing Wheat, Rye, and Barley
Enzyme Immunoassay Method Based on a Specific Monoclonal Antibody to the Potentially Celiac Toxic Amino Acid Prolamin Sequences
First Action 2012

Caution: Cocktail solution necessary for sample preparation contains β-mercaptoethanol. Use a chemical hood for sample preparation. Stop solution contains 1 M sulfuric acid; avoid skin and eye contact (*see* Material Safety Data Sheet, Appendix 1).

See **Table 2012.01** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal antibody that can determine gliadin derived from wheat and related prolamins derived from rye and barley. The antibody binds to the potentially celiac toxic amino acid sequence QQPFP (6) and to related sequences, which exist as motifs on all the gliadin subunits. The antibody detects prolamins in nonheated and heated food by using an additional specific extraction method (cocktail solution). No cross-reactivity exists to oats, maize, rice, millet, teff, buckwheat, quinoa, and amaranth (*see* AOAC Research Institute validation report, Appendix 2).

Prolamins from food are extracted by using a cocktail solution, containing β-mercaptoethanol and guanidine hydrochloride

Table 2012.01. Interlaboratory study results for gliadin by RIDASCREEN® Gliadin

Material						
Matrix	Level, mg/kg	No. of labs (outliers)	Mean, mg/kg	Recovery, %	Repeatability RSD_r, %	Reproducibility RSD_R, %
Maize	168	19 (1)	141.8	84.4	20.8	28.6
Maize	35	20 (0)	36.8	105.0	37.7	40.3
Maize	79	18 (2)	74.1	93.8	14.2	32.4
Maize	0	20 (0)	8.3		32.0	41.5
Rice	41	18 (2)	34.7	84.6	18.3	25.6
Rice[a]	0		<1.5			
Rice	147	17 (1)	126.6	86.1	26.8	35.4
Wheat starch	14	20 (0)	12.5	89.3	26.8	40.7
Rice flour	13	20 (0)	14.1	108.5	37.4	38.1
Wheat starch	13.5	17 (0)	13.2	97.8	29.7	52.1
Maize flour[a]	<1.5		<1.5			
Maize flour[a]	<1.5		<1.5			

[a] Negative samples were not included in the statistical evaluation (*see Results and Discussion*).

described by Garcia et al. (1), following an extraction with 80% ethanol. After centrifugation, the supernatant is used in a second-step sandwich method. The analyte is incubated in monoclonal antibody-coated wells forming an antibody-antigen complex. In a second step, an antibody peroxidase (POD) conjugate reacts with the complex to form an antibody-analyte-antibody complex. A chromogen/substrate reaction with the immobilized POD labeled conjugate determines the bound analyte. Non-immobilized components are removed by washing between steps. The response of sample extracts is compared with response observed with calibrators.

B. Apparatus

Apparatus specified here has been tested. Equivalent apparatus may be used.

(**a**) *Grindomix GM 200*.—For sample homogenization (Retsch GmbH, Haar, Germany).

(**b**) *Water bath*.—GFL (Ges. f. Labortechnik mbH, Burgwedel, Germany).

(**c**) *Bench top centrifuge*.—Multifuge 3L-R, operating at 2500 rpm (Thermo Electron GmbH, Dreieich, Germany).

(**d**) *Glass tubes (10 mL)*.—For extraction (Brand GmbH, Wertheim, Germany).

(**e**) *Polystyrol tubes (5 mL)*.—For sample dilution (Sarstedt, Nümbrecht, Germany).

(**f**) *Microtiter plate reader with 450 nm filter*.—Tecan Deutschland GmbH (Crailsheim, Germany).

(**g**) *Micropipet*.—Accurately delivering 100 µL ± 1%.

(**h**) *Glassware*.—Wash bottle 1000 mL; graduated cylinders.

(**i**) *Rotator 3100 CMV or equivalent*.—Fröbel Labortechnik (Lindau, Germany).

C. Antibody Characteristics

Antibodies must satisfy the following criteria:

(*1*) Bind to gliadin derived from wheat and to related prolamins derived from rye and barley.

(*2*) Recognize the potential celiac toxic structure QQPFP and related sequences.

(*3*) Bind to the alpha-, beta-, gamma-, and omega-gliadin motifs in nonheated and heated food, extracted by cocktail solution.

(*4*) No binding to oats, maize, rice, teff, buckwheat, quinoa, and amaranth.

(*5*) Bind with high affinity to allow an LOD of 1.5 mg/kg gliadin or related prolamins.

(*6*) Able to build a stable POD labeled conjugate, stable for more than 1 year.

(*7*) Show reproducible affinity, sensitivity, specificity, and stability from batch to batch for more than 1 year.

(*8*) Monoclonal antibodies are preferred; polyclonal antibodies can be used if they fulfil the same specificity criteria to react with wheat, rye, and barley to 100% and have no cross-reactivity to oat, maize, teff, and others.

D. Reagents

Items (**a**)–(**i**) are available as a test kit (R-Biopharm AG). All reagents are stable for 18 months from date of manufacture at 2–8°C (36–46°F). Refer to kit label for current expiration. Equivalent antibodies may be used for (**a**) and (**c**) provided they satisfy characteristic criteria described in **C** above.

(**a**) *Antibody-coated microwell strips*.—Monoclonal antibodies are coated in 20 mM phosphate buffered saline (PBS), pH 6.0, onto a set of twelve 8-microwell strips (NUNC, Roskilde, Denmark), containing 0.01% sodium azide as preservative.

(**b**) *Wash buffer concentrate (100 mL/bottle, 10x concentrate)*.—Contains a final concentration of 20 mM PBS (0.9% sodium chloride) with 0.1% Synperonic and 0.01% Kathon as preservative.

(**c**) *Peroxidase-labeled antibody*.—One vial (1.2 mL, 11x concentrated).

(**d**) *Gliadin ready-to-use standards (antigen)*.—Six vials (1.3 mL each, ready to use). Prepared by Sigma gliadin or own preparation, dissolved in 60% ethanol at a concentration of 1 mg/mL. The solution is further diluted in 20 mM PBS-Tween (0.9% sodium chloride, 0.05% Tween 20) containing 0.22% fish gelatin (Sigma) to 0, 5, 10, 20, 40, and 80 ng/mL gliadin, calibrated to the Working Group on Prolamin Analysis and Toxicity (WGPAT) gliadin (86% highly purified gliadin from 40 different European wheat varieties).

(**e**) *Substrate*.—One vial, 7 mL (urea peroxide).

(**f**) *Chromogen*.—One vial, 7 mL (tetramethylbenzidine in methanol). Can be added either separately or mixed 1 + 1 with (**e**) before pipetting.

(**g**) *Stop solution*.—One vial, 14 mL (1 N H_2SO_4).

(**h**) *Sample dilution buffer (60 mL, 5x concentrate)*.—Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.05% Tween 20) with 0.22% fish gelatin (Sigma) and 0.01% Kathon as preservative.

(**i**) *Cocktail solution*.—One vial, 105 mL.

Recommended but not provided with the test kit:

(**a**) Skim milk powder (food quality).

(**b**) Three control samples (powder), one nongliadin-containing sample (rice flour) and two prolamine-contaminated maize samples (A and B, concentration given by a certificate), which can be extracted with 60% ethanol and diluted further with the sample dilution buffer to control the test from run to run.

E. General Instructions

Store the kit at 2–8°C (35–46°F). Let all kit components come to 20–25°C (68–77°F) before use.

Return any unused microwells to their original foil bag, reseal them together with the desiccant provided, and store at 2–8°C (35–46°F). The colorless chromogen is light-sensitive; therefore, avoid exposure to direct light.

Include ready-to-use standards in duplicates to each run of diluted sample extracts in duplicate. Add the diluted antibody-POD conjugate (diluted by water) to all wells. Add substrate and chromogen simultaneously. Stop the reaction with stop solution, measure in a microtiter plate reader at 450 nm versus air within 30 min after stopping the reaction. Do not reuse wells of the plate.

Use separate pipet tips for each standard and each sample extract to avoid cross-contamination.

Use a multistepper pipet for adding the conjugate, substrate/chromogen, and stop solution. Use a single tip for each of these components. Components and procedures of this test kit have been standardized for use in this procedure. Do not interchange individual components between kits of different batches (lot numbers). Do not freeze any of the kit components.

Carefully dilute the components that are included in the kit as concentrates; avoid contaminations by airborne cereal, dust, or dirty laboratory equipment. Wear gloves during preparation and performance of the assay. Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol. Carry out sample preparation in a room isolated from ELISA procedure. Check for prolamin contaminations of reagents and equipment.

F. Preparation of Test Samples

Weigh 5 g sample and grind to a powder as fine as possible to obtain maximal surface. Weigh 0.25 g of the solid ground sample or use 0.25 mL of a liquid sample in a 10 mL glass vial and add 2.5 mL cocktail. Close the vial and mix it well (avoid cross-contamination). If tannin- and polyphenol-containing samples (e.g., chocolate, chestnut, or buckwheat) are prepared, add an additional 0.25 g skim milk powder (food quality) to the sample-cocktail solution (*see* product leaflet, Appendix 3).

Incubate for 40 min at 50°C (122°F) in a water bath. Let the sample cool down; then mix it with 7.5 mL 80% ethanol. Close the vial and shake for 1 h upside down or by a rotator at room temperature 20–25°C (68–77°F). Centrifuge 10 min at 2500 × *g* at room temperature 20–25°C (68–77°F). Remove the supernatant (extract) in a screw-top vial and keep for testing.

Dilute the sample at least 1:12.5 (1 + 11.5, 0.1 + 1.15 mL) with the prepared sample dilution buffer (depending on the expected prolamin content of the sample). Dilute serially from the first dilution, if necessary mixing thoroughly each time before diluting further. Use 100 µL per well in the assay.

G. Preparation of Components Delivered with the Kit

(**a**) Sample diluent is provided as a concentrate (5-fold). Only the amount that is actually needed should be diluted 1:5 (1 + 4) with distilled water (e.g., 3 mL concentrate + 12 mL distilled water, sufficient for the dilution of 10 samples). Make sure that the buffer is not contaminated with gliadin.

(**b**) Antibody enzyme conjugate (bottle with red cap) is provided as a concentrate (11-fold). Since the diluted enzyme conjugate solution has a limited stability, only the amount that is actually needed should be diluted. Before pipetting, the conjugate concentrate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1 + 10) with distilled water (e.g., 200 µL concentrate + 2.0 mL distilled water, sufficient for two microtiter strips). Take care that the water is not contaminated with gliadin.

(**c**) Washing buffer is provided as a 10-fold concentrate. Before use, the buffer must be diluted 1:10 (1 + 9) with distilled water (i.e., 100 mL buffer concentrate + 900 mL distilled water). Prior to dilution, dissolve any crystals formed by incubating the buffer in a water bath at 37°C (99°F). The diluted buffer is stable at 2–8°C (35–46°F) for 4 weeks.

H. Determination

Bring all reagents to room temperature (20–25°C/68–77°F) before use. Do not allow microwells to dry between working steps. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run. Record standard and sample positions.

Add 100 µL of each standard solution or prepared sample to separate wells, mix 10 s manually, and incubate for 30 min at room temperature (20–25°C/68–77°F). Dump the liquid out of the wells, then tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all the wells with 250 µL diluted washing buffer and dump out the liquid again. Repeat two more times.

Add 100 µL of the finally diluted enzyme-labeled conjugate

to each well, mix 10 s manually, and incubate for 30 min at room temperature (20–25°C/68–77°F). Dump the liquid out of the wells, then tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all the wells with 250 µL diluted washing buffer and dump out the liquid again. Repeat two more times.

Add 50 µL of substrate and 50 µL of chromogen to each well. Mix gently by shaking the plate 10 s manually and incubate for 30 min at room temperature (20–25°C/68–77°F) in the dark. Positive wells should develop a blue color, indicating the presence of prolamins. Add 100 µL of the stop reagent to each well. Mix gently by shaking the plate manually. The color of positive prolamin containing wells changes from blue to yellow.

I. Reading

Read the results with a microtiter plate reader. Measure the absorbance at 450 nm. Read within 30 min against air after addition of stop solution.

J. Calculations

Determine the gliadin content of each set of duplicate sample wells by reference to a calibration curve measured by the actual test run utilizing special computer software or semilogarithmic paper; plot absorbance of standards (linear scale) versus gliadin content of standards (logarithmic scale).

The standard calibration curve of the ELISA covers a range from 2.5 to 40 mg gliadin/kg sample, which corresponds to a range of 5–80 ng/mL gliadin in the calibrators (Appendix 4).

Convert the units ng gliadin/mL diluted sample is converted to mg gliadin/kg sample in the following manner: Multiply the amount in ng/mL by the dilution factor. Divide the product by 1000 to achieve units of mg/kg. The dilution factor corresponds to the sample preparation and is usually 500; however, 1000 was used in this study. Absorbance below standard two (5 ng/mL gliadin) implies that the sample assayed is diluted too much or that no gliadin or gliadin below the LOQ is present in the sample.

Gluten content of a sample can be calculated from the gliadin value, as gliadin generally represents 50% of the proteins present in gluten. Gluten values can be expressed in mg/kg by multiplying the gliadin value by 2.

Example calculation: A sample was extracted with the recommended dilution factor of 500. The absorbance value of the sample corresponds to 10 ng/mL gliadin in the calibration curve. By multiplying the obtained value by the factor 500 leads to 5000 ng/mL, corresponding to 5 mg/kg gliadin, respectively, 0.0005% gliadin. To calculate the gluten content, multiply by factor 2, which results in 10 mg/kg gluten, respectively, 0.001% gluten. This sample is considered to be gluten-free because the gluten concentration is below 20 mg/kg gluten.

LOD was calculated by testing 10 blank samples/matrix; mean values and SD were calculated. LOD was defined as mean +3x SD.

LOQ was verified by analyzing 10 replicates of a food sample, which contains a gliadin content close to standard 2 (5 ng/mL × 500 (dilution factor) = 2.5 ppm gliadin). In parallel standard 1 (= 0 ng/mL gliadin) was measured 10 times. The variation of standard 1 (absorbance value + 3x SD) was confirmed. The mean value – 3x SD was found significantly different from zero in consideration of the CV.

K. Criteria for Acceptance of the Standard Curve

The course of the calibration curve is shown in the Quality Assurance Certificate (Appendix 4), enclosed in the test kit. In comparison with the certificate, higher values of the absorbance at 450 nm, especially for the zero calibrator, may be a result of insufficient washing or gliadin contamination. A further dilution and repeated measurement of the samples is recommended for absorbance values (450 nm) higher than standard 6. This additional dilution factor must be taken into consideration during calculation.

Indication of instability or deterioration of reagents is shown by any coloration of the chromogen solution prior to test implementation or if values of less than 0.6 absorbance units for standard 6 occur. SD of replicates should be less than 10%.

Table 2. Statistical results (expressed in mg/kg gliadin) of collaborative tests carried out at the international level in 2002 by WGPAT[a]

Sample ID	No. of labs, P	No. of replicates, Sum[n(L)]	Overall mean of all data (grand mean), $\bar{x}$	Repeatibility SD, s(r)	Reproducibility SD, s(R)	Repeatability relative SD, RSD(r)	Reproducibility SD, RSD(R)
1	19	37	141.8	29.4	40.4	20.8	28.6
2	20	39	36.3	13.7	14.6	37.7	40.3
3	18	35	74.1	10.5	24.0	14.2	32.4
4	20	39	8.3	2.64	3.43	32.0	41.5
5	18	35	34.7	6.40	8.94	18.3	25.6
7	17	33	126.6	33.9	44.8	26.8	35.4
8	20	39	12.5	3.35	5.09	26.8	40.7
9	20	39	14.1	5.26	5.37	37.4	38.1
10	17	33	13.2	3.97	6.95	29.7	52.1

[a] Twenty laboratories participated, each performing two replicates.

Test controls offered by R-Biopharm should be measured in the reported ranges from run to run.

Reference: *J. AOAC Int.* **95**, 1119(2012)

Results and Discussion

Collaborative Study Results

The data sent to the Study Director were delivered on data reporting sheets. The participants were asked to report any important observations and significant deviations of the method. No negative comments were received regarding handling and performance of the kits.

Each sample was extracted twice and diluted three times. Each dilution was measured in double determination. Each extraction was measured in a separate run (extraction 1 in run 1 and extraction 2 in run 2).

The result of the analyte gliadin, which was measured by ELISA, was expressed as mg/kg (ppm) gliadin. The raw data were calculated by the ELISA software RIDASOFT Win. The final data of the dilution row was selected by a mathematical algorithm. The results for both runs are given in Appendix 5.

The negative samples included in the set of samples were not included in the statistical evaluation. They were chosen for the study design to show that negative samples can be detected with a high probability (≥95%). Sample 6 was found negative by all 20 laboratories; for samples 11 and 12, 18 out of 19 laboratories found the sample negative.

Mean recovery of all samples (spiked and naturally contaminated samples) was 93.7%, ranging from 84 to 109%, which is excellent for ELISA. These values are based on all laboratories with exception of outliers (Tables 2 and **2012.01**), including those with poor performance. The negative samples were all well below standard 2 (<2.5 ppm gliadin), except sample 4, which obviously was contaminated during the bakery process at a low level of gliadin (mean 8.3 ppm). The contamination was proved by analyzing the added yeast alone. It was shown that the yeast preparation contained gliadin.

The contamination was distinguished from a potential false positive by analyzing the basic ingredients of the bread samples. The basic material of samples 1 to 4 was maize flour (Table 1), which was tested before baking the bread in the R5 ELISA to be noncontaminated. Afterwards, dough was made by adding water and yeast. The dough was baked in small baking tins which were purified before with 50% propanol to exclude any contamination with prolamins. The baked bread was milled to a fine powder, whereas the zero-level bread was milled in a purified, noncontaminated mill. During the collaborative study the zero sample (No. 4) was found to be slightly above the LOQ. By checking the added materials, it was recognized that the yeast used for bread making was contaminated with prolamins; and therefore, the contamination of the zero maize bread sample No. 4 was attributed to the prolamin-containing yeast in the bread.

Laboratories F and D provided only results from one run, and therefore, occur only in one run. The precision parameters of the collaborative study are presented in Tables 2 and **2012.01**. The results shown are related to the 12 samples (Nos. 1–12).

For the RIDASCREEN Gliadin kit, the mean of the RSD of repeatability ($RSD_{r)}$ was 27% and the mean of the RSD of reproducibility (RSD_R) was 37% (Table 2). Both were found in the usual range of ELISA tests. There was no influence recognized on the RSD_r and RSD_R values within the complete concentration range of the tested samples.

The Horwitz equation is based on empirical data from chromatographical and/or spectrophotometrical determinations. In contrast to these methods, samples used for antibody-based methods are often diluted before measurement, e.g., 1:500 to obtain concentrations within the range of calibration. The calibration curve in the present case covers values from 5 to 80 µg/L. Therefore, it was not possible to calculate a CV from the Horwitz equation (7, 8). At a level of 100 µg/L the calculated CV is 23%. The theoretically calculated values in the present case would be higher than 23% and fit to our data.

Conclusions and Recommendations

The test is valid to determine gliadin contamination around a 10 mg/kg (ppm) gliadin cut-off with sufficient accuracy, which is the accepted value by the Codex Alimentarius Nutrition and Food for Special Dietary Uses (NFSDU) for gluten-free food (9). Thus, the test fulfilled the criteria of the gliadin collaborative study and guarantees the sensitivity of the new limit for gluten-free food. Based upon these results, it is recommended that the method be accepted by AOAC as Official First Action.

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