FOOD COMPOSITION AND ADDITIVES

Quantification of Wheat, Rye, and Barley Gluten in Oat and Oat Products by ELISA RIDASCREEN® Total Gluten: Collaborative Study, First Action 2018.15

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Background: **Since its introduction to the analytical community, the R5 method to quantify gluten led to a strong improvement of the situation for the food industry and celiac patients. During recent years, some questions arose on the use of the Codex Alimentarius factor of two to convert from prolamins to gluten, an overestimation of rye and barley, inadequate detection of glutelins, and the inhomogeneous distribution of gluten in oats. These limitations of the R5 method, especially when measuring oat samples, led to AOAC *Standard Method Performance Requirement* (SMPR®) 2017.021, which was approved by stakeholders in 2017. *Objective:* We present a collaborative study of a method for the quantitative analysis of wheat, rye, and barley gluten in oat and oat products using a sandwich ELISA that is based on four different monoclonal antibodies including the R5 monoclonal anitbody. *Methods:* The sandwich ELISA detects intact gliadins and related prolamins from rye and barley, high-molecular-weight (HMW) glutenin subunits (GS) from wheat, HMW secalins from rye, and low-molecular-weight (LMW) GS from wheat. It does not detect D-hordeins from barley. Samples are extracted by Cocktail solution, subsequently followed by 80% ethanol, and analyzed within 50 min. *Results:* The measurement range is between 5 and 80 mg/kg gluten using a calibrator made out of a gluten extract from four different wheat cultivars. The results of the collaborative test with 19 participating laboratories showed recoveries ranging from 99 to 137% for all three grain sources. Relative reproducibility SDs for samples >10 mg/kg gluten ranged from 10 to 53%. *Conclusions:* The collaborative study results confirmed that the method is accurate and suitable to measure gluten from all three grain sources and has demonstrated performance on oat matrices, which meets the criteria as specified in SMPR 2017.021. Data from in-house validation experiments are available as Annex B to this publication.**

With a prevalence of 0.4–1.2% of the population in Europe, North America, Australia, and the Middle East (1), celiac disease (CD) is considered to be one of the most common food hypersensitivities. CD is an immune-mediated inflammatory disease of the upper small intestine in genetically predisposed individuals 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 is insoluble in water and 0.5 mol/L NaCl (3). Gluten is composed of prolamins that can be extracted by 40–70% ethanol and 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 per 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 recommended by the Codex Alimentarius and legislation, for example, by the Department of Health and Human Services Food and Drug Administration in the United States (5) and by the European Commission in Europe (6). Specific and sensitive analytical methods are therefore needed for food QC. Immunochemical methods are currently recommended for the quantitative and qualitative determination of gluten in foods (3). Sandwich and competitive ELISA formats (RIDASCREEN® Gliadin R-Biopharm R7001 and RIDASCREEN Gliadin competitive R-Biopharm R7021) 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

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hydrolyzed gluten (9), respectively. Additionally, the R5 Sandwich ELISA RIDASCREEN Gliadin has been endorsed as a Codex Alimentarius Type I method for the analysis of gluten (10) and has been adopted by AOAC INTERNATIONAL as Final Action *Official Method*℠ **2012.01** Final Action in 2016 (11). The competitive ELISA RIDASCREEN Gliadin competitive (R-Biopharm, R7021) has been adopted by the AOAC as *Official Method* **2015.05** (12). The R5-based Lateral Flow Device RIDA®QUICK Gliadin (R-Biopharm, R7003) has been adopted as AOAC Final Action *Official Method* **2015.16** for analysis of foods (13) and as AOAC *Performance Tested Method*℠ 101702 for surfaces and cleansing waters (14).

The R5 antibody raised against ω-secalins primarily recognizes the epitope with the amino acid sequence QQPFP, which is present in gliadins, secalins, and hordeins and occurs in many peptides that are toxic or immunogenic for CD patients (15–17).

Since its introduction, the R5 method has allowed determination and control of gluten levels in products, which subsequently led to improvement of the quality of products available to CD patients. The advantages of the method are that its response is well-characterized and well-understood. There is a deep understanding of the method performance thanks to the comprehensive initial validation, and therefore, limitations of the R5 system are well known. Additionally, with a LOQ of 5 mg/kg gluten, the method is sensitive enough to reliably control gluten-free products.

However, every analytical method has limitations. For the R5 methods, these limitations are the following: (*1*) factor of two to convert from prolamins to gluten, which is not accurate in many cases (18); (*2*) overestimation of rye and barley; and (*3*) inadequate detection of glutelins, which are not detected by the R5 except the Skerritt monoclonal antibody (19). While these were limitations of the method, they worked in favor of those suffering from CD because analytical results tended to be biased higher than true contamination levels (limitations 1 and 2). Limitation 3, the nondetectability of glutelins, is only important if a food product shows enriched proportions of glutelins to prolamins such as in wheat starch (20, 21). One additional limitation for all methods that measure gluten that was observed in past years was a high repeatability SD in some oat samples, which has been attributed to inhomogeneous distribution of gluten in oats combined with a small sample intake. Both have an impact on the analytical statement that can be made about the gluten content of the sample. Sample inhomogeneity is a sample-intrinsic problem and not a shortcoming of analytical systems. Nevertheless, it is an issue that needs to be addressed by all analytical systems quantifying gluten in oats.

Because of these limitations to the R5 method, a group of oat processors and test kit manufacturers founded an initiative through AOAC INTERNATIONAL in 2016. The resulting *Standard Method Performance Requirement* (SMPR®) 2017.021 was adopted by stakeholders in 2017 (22). The method acceptance criteria given in the SMPR are that mean recoveries for gluten from wheat, rye, and barley in oats and oat products must be between 50 and 200%. Another important requirement in the SMPR is the availability of "reference materials" with wheat or rye or barley gluten concentrations of 10 or 20 mg/kg in oats, including a blank material. These reference materials can be used to allow for more precise comparison of methods in the future.

The new sandwich ELISA RIDASCREEN Total Gluten (R-Biopharm, R7041) presented here employs a combination of four monoclonal antibodies including the R5 to detect the majority of gluten fractions from wheat, rye, and barley, including glutelins. It is calibrated to a wheat gluten preparation in the range of 5 to 80 mg/kg gluten. In consequence, no conversion factor of two is needed to convert from prolamin to gluten. For extraction, the test portion was increased to 1 g compared with 0.25 g for *Official Method* **2012.01** to account for inhomogeneity of gluten in oats. The test portion may be increased even more if needed. When increasing test portion mass, the amount of Cocktail (patented; R-Biopharm R7006 and R7016) and ethanol must be increased proportionately. For this new method, Cocktail and 80% ethanol are incubated with the sample simultaneously and not consecutively as for former methods. The recovery of the reference materials introduced by SMPR 2017.021 was found to be within the acceptance criteria prescribed in the SMPR. A collaborative test using oats and oat products was performed in September 2018 with 19 participants worldwide (Australia, Austria, Canada, Finland, Germany, Hungary, Ireland, Italy, Sweden, United States, and United Kingdom). The study was coordinated by Katharina Scherf (Leibniz-Institute for Food Systems Biology at the Technical University of Munich).

Scope of Method

RIDASCREEN Total Gluten is used for the quantitative analysis of wheat, rye, and barley gluten in oat flour, groats, oat flakes, and oat cereals that are declared as "gluten-free." The sandwich ELISA detects intact gliadins and related prolamins from rye and barley, high-molecular-weight (HMW) glutenin subunits (GS) from wheat, HMW secalins from rye, and low-molecular-weight (LMW) GS from wheat. It does not detect D-hordeins from barley. Samples are extracted by Cocktail solution/80% ethanol and analyzed within 50 min. The measurement range is between 5 and 80 mg/kg gluten using a calibrator made out of a gluten extract from a mixture of four wheat cultivars.

Collaborative Study

Study Design

Following the AOAC guidelines, which are published as Appendix D (23) and Appendix M (24), an international collaborative study was set up to validate the RIDASCREEN Total Gluten for quantitative gluten measurement in oat and oat-based foods as an *Official Method*. Before the participants were allowed to analyze the collaborative test samples, they needed to show analytical competency by analyzing three control samples and the buffer (pretest). The main experiment consisted of 21 different samples that were analyzed as duplicates in a blinded manner. To allow a uniform calculation of results, participants were asked to deliver raw optical densities (OD) data only. Calculation of results was done by the study director using two different curve fitting procedures.

Collaborators

In order to qualify for participation in the collaborative test, all laboratories were required to have previous experience with ELISA and be familiar with the analytical procedure. It was recommended to use a separate room for the collaborative study because of the possibility of gluten contamination and the low detection limit. The laboratories were given 4 weeks

each to perform the pretest and the main experiment afterward. Nineteen laboratories (designated A to U) were chosen to participate: one each in Australia, Austria, Finland, Hungary, Ireland, Italy, Sweden, and the United Kingdom; two in Canada; four in Germany; and five in the United States.

Samples and Sample Preparation

To characterize the ELISA for trueness, the following reference materials mentioned in AOAC SMPR 2017.021 were analyzed: (*1*) wheat flour in oat flour, 10 mg/kg gluten; (*2*) Rye flour in oat flour, 10 mg/kg gluten; (*3*) barley flour in oat flour, 10 mg/kg gluten; and (*4*) oat flour, unspiked. The determination of the gluten content of wheat, rye, or barley was performed as described by Wehling and Scherf (25). Because it is well known that a gluten contamination in oats and oat products is often not homogenous, the following material from a former collaborative test was used (8): (*5*) wheat flour contaminated corn-based processed snack, 82 mg/kg gluten. This material was mixed with the corn-based processed snack material that was naturally contaminated at a low level from the same collaborative test (8): (*6*) mixture of corn-based processed snacks, approximately 41 mg/kg gluten; and (*7*) rice flour, naturally contaminated at a very low gluten level. The numbering for samples 1–7 will be consistently used throughout this document (*see* Tables **2018.15A** and **2018.15B**).

In the following, preparation of blank and contaminated oat products at General Mills Medallion Labs (Minneapolis, MN) is described.

Method of Preparation

Preparation of Heat-Treated (HT) Wheat, Rye, and Barley

Wheat-HT (variety Carberry from Agriculture and Agri-Food, Swift Current SK, Canada), rye-HT (variety Hazlet from Carrington Research Station), and barley-HT (variety Tradition from Carrington Research Station) were HT with steam to simulate enzyme deactivation and flavor development steps used in industrial oat processing. Fifty grams of each of the three grain types were placed into trays, which were then placed inside larger pots that contained 1 cm water. The water placed into the bottom of the pots did not directly contact the seeds. Pots were placed in an oven at 225°F (107°C) for 130 min followed by 140 min at 400°F (204°C). After steaming, the seeds were dried at 300°F (149°C) for 60 min. Barley was also used untreated for incurring flaked oats.

Obtaining Oat Groats-Blank

Kilned groats from General Mills' oat mill (Fridley, MN) were run on a paddy table to remove residual hulls. These groats were further purified by running them through a single channel Buhler Sortex Type A color sorter equipped with two visible wave length and two IR wave length detectors. Finally, the groats were hand sorted to remove remaining foreign grains.

Preparation of Oat Flour-Blank and Naturally Incurred Groats

Several hundred pounds of oat groats-blank were passed through a Fitzmill Industrial Impact Mill with a screen opening of 2700 µm. All material was passed a second time with an 800 µm opening screen.

Preparation of Oat Flour-Very High (VH)

A total of 4540 g of oat groats-blank were mixed with 15.0 g wheat-HT, 15.0 g rye-HT, and 30.0 g barley-HT. This mixture was twice passed through the same Fitzmill Industrial Impact Mill, also with screen sizes of 2700 and 800 µm.

Preparation of Oat Flour-High

A total of 38.0 pounds (17 252 g) of oat flour-blank were blended with 1003 g of oat flour-VH.

Preparation of Oat Cereal-Blank

Oat cereal-blank was prepared from oat flour-blank using a Buhler 42 mm laboratory extruder with a single 0.159 in. die hole and a high shear screw configuration proprietary to General Mills. Dry feed rate was 900 g/min with 50 g/min of steam injection and 75 g/min water injection. The cereal was directly expanded off the die face and cut into spheres. The cereal was dried under hot forced air to approximately 2% moisture.

Preparation of Oat Cereal-High

Oat cereal-high was made under the same conditions using the oat flour-high on a Buhler 42 mm extruder.

Preparation of Flaked Oats (Blank and High)

Oat groats-blank was added to a continuous steaming screw with a residence time of 13 min. The screw was fed atmospheric pressure steam, and the pipe walls were heated to prevent condensation. At the end of the steaming screw, groats fell into a 5" laboratory flaking roll with a gap set to 0.012 in. The initial output of the flaking roll was collected to serve as flaked oats-blank. To make the gluten containing flaked oats-high, 2.3 g of nonheated barley were added to the steaming screw in a single spike, and the flaking rolls were run until a total of 4360 g flakes containing this barley were collected. All flaked oats were dried at 225°F (107°C) for 30 min. The steaming screw cannot be run empty, so it was stopped with significant groats still inside. Although the residence time analysis of the screw predicts that all 2.3 g of barley would end up in flaked oats-high, this could not be verified by inspection of the seeds remaining in the screw.

Subsampling and Homogenization

To ensure uniform material, subsamples were pulled from the larger samples and homogenized for 45 s in the food processor. In detail, 1500 g oat flakes-blank, 1500 g oat flakes-high, 2000 g oat cereal-blank, 1783 g oat cereal-high, 3708 g oat flour-blank, and 500 g oat flour-VH were homogenized. These were used for preparation of the collaborative test samples as described in Table 1. All samples (pure or mixtures) were placed in the food processor again and received an additional 45 s of blending.

All samples were packaged for delivery into foil pouches at an amount of 5 g at Trilogy Analytical Laboratories (Washington, MO).

Table 1. Preparation of collaborative test samples and their grain contents given as milligrams wheat, rye, or barley grain per kilogram oat

Sample	Label	Preparation	Barley, mg/kg	Wheat, mg/kg	Rye, mg/kg
8	Flaked oats-blank	1000 g oat flakes-blank	0	0	0
9	Flaked oats-low	500 g oat flakes-high plus 500 g oat flakes-blank	265	0	0
10	Flaked oats-high	1000 g oat flakes-high	530	0	0
11	Cereal-blank	1000 g oat cereal-blank	0	0	0
12	Cereal-low	500 g oat cereal-blank plus 500 g oat cereal-high	179	90	90
13	Cereal-medium	250 g oat cereal-blank plus 750 g oat cereal-high	269	134	134
14	Cereal-high	533 g oat cereal-high	358	179	179
15	Flour-blank	1000 g oat flour-blank	0	0	0
16	Flour-low	783 g oat flour-blank plus 9.9 g oat flour-VH	83	41	41
17	Flour-medium	975 g oat flour-blank plus 25 g oat flour-VH	165	83	83
18	Flour-high	950 g oat flour-blank plus 50 g oat flour-VH	330	165	165
19	Groats-blank	Naturally incurred	NA[a]	NA	NA
20	Groats-low	Naturally incurred	NA	NA	NA
21	Groats-high	Naturally incurred	NA	NA	NA

[a] NA = Not applicable.

Homogeneity of Samples

Homogeneity of the reference materials (samples 1–3) was tested at R-Biopharm using the RIDASCREEN Total Gluten with a test portion of 1 g. In brief, 10 bags were randomly chosen and analyzed. All samples turned out to be homogenous because the CVs were 12% for wheat and rye, while the barley-containing sample was at CV of 15%. Snack B and Snack C (samples 5 and 6) were tested for homogeneity in 2012 (8).

All other materials were not checked for homogeneity before sending them to the participants. Because it is known that gluten is not homogeneously distributed in oats, it was decided to include this uncertainty in the total precision estimates. By doing this, a more realistic value will be obtained.

Presentation of Samples to Laboratories

Following the collaborative test guidelines of AOAC, two blinded replicates for each sample were provided to each participating laboratory. The samples were marked with a laboratory-specific letter (A to U) and a randomized number from 1 to 42. Each laboratory obtained its own coding (different randomized numbers for each laboratory).

Method and Measurement of Samples

The method was written in AOAC style and was provided to each laboratory with the instructions to follow the method as written with no deviations. Before analyzing the blind-coded samples, each participant was asked to become familiar with the test method by analyzing three control samples and the test kit dilution buffer. All OD values obtained had to be recorded in a ready-to-use Excel sheet. The final data from the laboratories were sent to the study director. The participants were advised to analyze samples 1–21 in run 1, while samples coded 22–42 were analyzed in run 2. To facilitate the calculation later on, the participants were asked to use a fixed pipetting scheme on the microtiter plate.

AOAC Official Method 2018.15
Gluten from Wheat, Rye, and Barley in Oats and Oat Products by Quantitative Sandwich ELISA RIDASCREEN® Total Gluten
First Action 2018

[RIDASCREEN Total Gluten is a sandwich enzyme immunoassay to quantify gluten proteins from wheat, rye, and barley in oat and oat products within a measurement range from 5 to 80 mg/kg gluten. The assay calibrators were made from a total gluten extract of four wheat cultivars. Results are traceable to the reference oat samples described in AOAC SMPR 2017.021.]

Caution: Ethanol is highly flammable and 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. The Cocktail (patented) contains 2-mercaptoethanol, which is toxic. The stop solution contains sulphuric acid, which is caustic; work under a chemical fume hood, avoid skin and eye contact, and wear protective gloves and clothing (*see* Material Safety Data Sheet attached as separate documents or delivered by the manufacturer in case of ethanol).

For interlaboratory study results, *see* Tables **2018.15A** and **2018.15B**.

A. Principle

The basis of the test is the antigen-antibody reaction. The wells of the microtiter plate are coated with specific monoclonal antibodies against gluten proteins. By adding the standard or sample solution to the wells, present gluten proteins will bind to the specific antibodies. The result is an antibody-antigen complex. In a washing step, components not bound are removed. Then, antibodies conjugated to peroxidase (enzyme conjugate) are added. This antibody

Table 2018.15A. Performance characteristics using data from four-parameter logistic regression analysis for curve fitting[a]

	Samples 1–10[b]										Samples 11–21[c]										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Labs, *n*	19	18	17	15	18	19	16	19	19	19	19	19	19	19	17	19	19	19	19	19	18
Replicates, *n*	38	36	34	30	36	38	32	38	38	38	38	38	38	38	34	38	38	38	38	38	36
Mean, mg/kg	10.8	13.7	11.0	0.9	62.1	33.4	4.0	10.0	32.8	47.9	3.1	21.0	15.9	27.2	2.1	6.3	12.9	22.0	7.2	13.5	20.3
s_r, mg/kg[d]	2.29	1.88	1.40	0.96	5.31	4.62	0.79	9.68	5.88	7.12	1.51	5.91	3.87	5.80	2.23	2.70	5.34	5.99	1.93	2.86	2.22
s_R, mg/kg[e]	2.29	2.05	1.96	0.96	7.35	6.82	2.52	9.99	7.27	7.96	2.20	6.15	4.68	6.88	2.23	3.44	5.79	6.75	2.31	2.92	3.67
RSD_r, %[f]	21.1	13.7	12.7	103.9	8.5	13.8	19.8	96.7	17.9	14.9	48.4	28.2	24.3	21.3	107.2	42.6	41.3	27.2	26.6	21.2	10.9
RSD_R, %	21.1	15.0	17.8	103.9	11.8	20.4	63.0	99.8	22.2	16.6	70.5	29.3	29.4	25.3	107.2	54.3	44.7	30.6	31.9	21.7	18.1

[a] For raw data from each participant, including outlier calculation, refer to Annex A Tables A3 and A4.

[b] Samples 1–10 are as follows: (*1*) wheat flour in oat flour, 10 mg/kg gluten; (*2*) rye flour in oat flour, 10 mg/kg gluten; (*3*) barley flour in oat flour, 10 mg/kg gluten; (*4*) oat flour, naturally contaminated approximately at 2 mg/kg gluten; (*5*) wheat flour contaminated corn-based processed snack, 82 mg/kg gluten; (*6*) mixture of corn-based processed snacks, approximately 41 mg/kg gluten; (*7*) rice flour; contaminated at a very low gluten level; (*8*) flaked oats blank; (*9*) flaked oats low; and (*10*) flaked oats high.

[c] Samples 11–21 are as follows: (*11*) cereals-blank, (*12*) cereals-low, (*13*) cereals-medium, (*14*) cereals-high, (*15*) flour-blank, (*16*) flour-low, (*17*) flour-medium, (*18*) flour-high, (*19*) groats-blank, (*20*) groats-low, and (*21*) groats-high.

[d] s_r = Precision of repeatability.

[e] s_R = Precision of reproducibility.

[f] RSD_r = Repeatability relative SD.

Table 2018.15B. Performance characteristics using data from quadratic curve fitting[a]

	Samples 1–10[b]										Samples 11–21[c]										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Labs, *n*	19	18	18	18	18	19	16	19	19	19	19	19	19	19	17	19	19	19	19	19	17
Replicates, *n*	38	36	36	36	36	38	32	38	38	38	38	38	38	38	34	38	38	38	38	38	34
Mean, mg/kg	9.9	13.2	10.4	1.1	63.6	35.2	3.5	9.1	34.2	50.0	2.5	21.2	15.8	28.1	1.7	5.5	12.4	22.6	6.4	13.0	19.7
s_r, mg/kg[d]	2.43	2.12	1.74	1.19	4.40	5.32	0.70	9.26	7.03	7.19	1.37	6.44	4.46	6.47	1.99	2.92	5.76	6.64	1.90	3.09	1.98
s_R, mg/kg[e]	2.43	2.22	2.34	1.21	6.35	8.02	2.45	9.53	8.31	7.93	1.74	6.92	5.52	7.80	1.99	3.39	6.59	7.78	2.31	3.39	3.15
RSD_r, %[f]	24.5	16.1	16.7	111.1	6.9	15.1	20.0	102.2	20.5	14.4	54.5	30.4	28.3	23.0	115.3	52.6	46.6	29.4	29.9	23.8	10.1
RSD_R, %	24.5	16.9	22.4	113.6	10.0	22.8	70.4	105.2	24.3	15.8	69.1	32.7	35.0	27.8	115.3	61.0	53.3	34.4	36.3	26.2	16.0

[a] For raw data from each participant, including outlier calculation, refer to Annex A Tables A3 and A4.

[b] Samples 1–10 are as follows: (*1*) wheat flour in oat flour, 10 mg/kg gluten; (*2*) rye flour in oat flour, 10 mg/kg gluten; (*3*) barley flour in oat flour, 10 mg/kg gluten; (*4*) oat flour, naturally contaminated approximately at 2 mg/kg gluten; (*5*) wheat flour contaminated corn-based processed snack, 82 mg/kg gluten; (*6*) mixture of corn-based processed snacks, approximately 41 mg/kg gluten; (*7*) rice flour; contaminated at a very low gluten level; (*8*) flaked oats blank; (*9*) flaked oats low; and (*10*) flaked oats high.

[c] Samples 11–21 are as follows: (*11*) cereals-blank, (*12*) cereals-low, (*13*) cereals-medium, (*14*) cereals-high, (*15*) flour-blank, (*16*) flour-low, (*17*) flour-medium, (*18*) flour-high, (*19*) groats-blank, (*20*) groats-low, and (*21*) groats-high.

[d] s_r = Precision of repeatability.

[e] s_R = Precision of reproducibility.

[f] RSD_r = Repeatability relative SD.

conjugate is bound to the antibody-antigen complex. An antibody-antigen-antibody complex (sandwich) is formed. Substrate/chromogen is added after removal of any unbound enzyme conjugate in a washing step. Bound enzyme conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm against air. The absorption is proportional to the gluten protein concentration in the sample.

B. Antibody Characteristics

All major gluten fractions from wheat, rye, and barley are detected. In detail, these fractions are gliadin-fractions from wheat and corresponding prolamins from rye and barley, HMW-glutenin-subunit proteins from wheat, HMW secalins from rye, and LMW-glutenin-subunit proteins from wheat. D-hordeins (barley glutelins) are not detected.

C. Apparatus

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

(**a**) *Microtiter plate spectrophotometer*.—450 nm.
(**b**) *Centrifuge, centrifugal vials*.
(**c**) *Water bath*.—50°C (122°F).
(**d**) *Shaker*.
(**e**) *Graduated pipet*.—10 and 50 mL.

(**f**) *Filter paper filters*.
(**g**) *Variable micropipets*.—20–200 and 200–1000 µL.
(**h**) *Multistepper pipet and tips for 100 µL*.
(**i**) *Eight-channel pipet and tips for 100 and 250 µL*.

D. Reagents

Items **a–g** are available as a test kit (RIDASCREEN Total Gluten; R7041, R-Biopharm Darmstadt, Germany). All reagents are stable as indicated on the label at 2–8°C (36–46°F).

(**a**) *Antibody-coated microwell strips*.
(**b**) *Standards*.—Six vials (1.3 mL each, ready to use) gluten proteins in aqueous solution.
(**c**) *Conjugate*.—One vial (11 mL, ready to use), peroxidase conjugated antibody solution.
(**d**) *Red Chromogen Pro (substrate/chromogen)*.—One vial (13 mL, stained red).
(**e**) *Stop solution*.—One vial (14 mL, contains 1 N sulfuric acid).
(**f**) *Buffer*.—One bottle (120 mL, ready to use).
(**g**) *Wash buffer*.—One bottle (100 mL, 10-fold concentrate).
(**h**) *Cocktail (patented)*.—One bottle (1000 mL, ready to use, R7016; R-Biopharm).

Items **i** and **j** are common laboratory reagents but not included in the test kit.

(**i**) *Distilled water*.
(**j**) *Ethanol (96%, p.a.)*.

E. General Instructions

Store the kit at 2–8°C (36–46°F). Let all kit components adjust to room temperature, 20–25°C (68–77°F), before use. Do not freeze any of the kit components.

Return any unused microwells to their original foil bag, reseal them together with the desiccant provided, and further store at 2–8°C (36–46°F). The substrate/chromogen is light sensitive; therefore, avoid exposure to direct light.

Carefully dilute the components included in the kit as concentrates; avoid contaminations by airborne grain dust or dirty laboratory equipment. Wear gloves during the preparation and performance of the assay. Clean surfaces, glass vials, mincers, and other equipment with 40% ethanol or 2-propanol. Carry out sample preparation in a room isolated from ELISA procedure. Check for gluten protein contamination of reagents and equipment.

Include ready-to-use standards in duplicate to each run of diluted sample extracts in duplicate. Do not reuse wells of the plate. Use separate pipet tips for each standard and each sample extract to avoid cross-contamination and preflush the tip before pipetting standard or sample extract. 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 the test kit have been standardized for use in this procedure. Do not interchange components between kits of different batches (lot numbers).

F. Preparation of Samples

Weigh a representative amount (200 g) of oats or oat products and homogenize.

(**a**) *Solid samples*.—Weigh 1 ± 0.05 g of homogenized sample to a 50 mL centrifuge tube. Add 10 mL Cocktail (patented) [**D(h)**], cap the tube vial, mix vigorously, and pay attention to obtain a homogenous suspension.

(**b**) Add 30 mL 80% ethanol [**G(b)**], close the tube, and mix well. Incubate for 40 min at 50°C in a water bath.

(**c**) Remove samples from the water bath and shake for 1 h up-side-down or by a rotator at room temperature (20–25°C/68–77°F).

(**d**) Centrifuge for 10 min at least 2500 *g* (alternatively, 2 mL of the extract can be centrifuged with high speed for 10 min in reaction caps by using a microcentrifuge). Afterward, filter the supernatant (with fluted paper filter).

(**e**) This extract can be stored at room temperature for at least 7 days.

(**f**) Dilute the sample 1:25 with buffer [**D(f)**], e.g., 40 µL extract + 960 µL buffer (1:1000 final sample dilution). Use diluted supernatant immediately in the assay within 30 min (use 100 µL per well in the assay).

(**g**) If further dilution is required, a solution consisting of 1% Cocktail (patented) [**D(h)**], 3% of 80% ethanol [**G(b)**], and 96% buffer [**D(f)**], e.g., 50 µL Cocktail (patented), 150 µL 80% ethanol, and 4800 µL buffer, should be used. Do not use the diluted samples that were already measured for further dilution because the diluted samples are stable for 30 min only. Restart the dilution using the extract obtained after filtration.

G. Preparation of Components

(**a**) *Washing buffer*.—Provided as a 10-fold concentrate [**D(g)**]. Before use, the buffer has to be diluted 1:10 (1+9) with distilled water (i.e., 100 mL buffer concentrate + 900 mL distilled water). Prior to dilution, dissolve possibly formed crystals by incubating the buffer in a water bath at 37°C (99°F). The diluted buffer is stable at 20–25°C (68–77°F) for 4 weeks.

(**b**) *Ethanol, 80%*.—Mix ethanol and water at a ratio of 4 + 1 parts (e.g., add 120 mL ethanol p.a. to 30 mL distilled water) and shake well.

H. Determination

(**a**) Bring all reagents to room temperature (20–25°C/68–77°F) before use. Carefully follow the recommended washing procedure. Do not allow microwells to dry between working steps.

(**b**) Do not use more than three strips (24 wells) at a time. In the case of more than three strips, a second uncoated plate (e.g., low binding from Greiner bio-one; Cat. No. 655101) should be used as a preplate to avoid a time shift over the microtiter plate. All standards and samples are pipetted into the uncoated plate (at least 150 µL) and then quickly transferred to the coated microtiter plate with an eight-channel pipet.

(**c**) It is recommended to pipet the conjugate, the substrate/chromogen, and the stop solution with a multichannel or stepper pipet to avoid a time shift over the plate.

(**d**) 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.

(**e**) Add 100 µL of each standard solution or prepared sample to separate duplicate wells and incubate for 20 min at room temperature (20–25°C/68–77°F).

(**f**) 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 the wells with 250 µL washing buffer [**G(a)**] and pour out the liquid again. Repeat two additional times.

(**g**) Add 100 µL of the ready-to-use enzyme conjugate [**D(c)**] to each well. Mix gently by shaking the plate manually and incubate for 20 min at room temperature (20–25°C/68–77°F).

(**h**) 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 the wells with 250 µL washing buffer [**G(a)**] and pour out the liquid again. Repeat two additional times.

(**i**) Add 100 µL of the red-colored substrate/chromogen solution [**D(d)**] 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.

(**j**) Add 100 µL of the stop solution [**D(e)**] to each well. Mix gently by shaking the plate manually.

I. Reading

Read the results with a microtiter plate reader. Measure the absorbance at 450 nm. Read within 10 min after addition of stop solution.

The dilution factor 1000, which results after sample preparation, has already been considered for the standard concentrations. The concentration of the sample can be directly read from the standard curve.

A further dilution and new detection of samples is necessary for absorbance readings ($A_{450\ nm}$) > standard 6. Please follow instructions given in **F(g)**. Do not use diluted samples that were already measured for further dilution.

J. Calculations

Determine the gluten content of each duplicate sample wells by reference to a calibration curve measured by the actual test run utilizing special computer software; plot absorbance of standards versus gluten content of standards. It is recommended to use the RIDA®SOFT Win (R-Biopharm AG, Z9999) with four-parameter logistic regression analysis (26). The four-parameter sigmoid curve is given by:

$$y = \frac{A - D}{1 + \left(\frac{x}{c}\right)^{B}} + D$$

where y = measurement signal; x = concentration; A = the minimum value that can be obtained (concentration is zero); B = the maximum value that can be obtained; C = the point of inflection; and D = Hill's slope of the curve.

As an alternative, a three-parameter Quadratic model can be used:

$$c \text{ (milligrams per kilogram gluten)} = a\,(OD - OD_{mean\ zero})^2 + b\,(OD - OD_{mean\ zero})$$

where $OD_{meanzero} = (OD_{zero1} + OD_{zero\ 2}) / 2$, which is the mean OD of the zero-level calibrators; b = first-order polynomial (linear slope) coefficient; and a = second-order polynomial (curvature) coefficient.

To fit, transform all OD values by subtracting the average OD of the zero calibrators from all ODs on the plate. Call these values OD'. Fit two-parameter quadratic model with no intercept (origin forced through mean zero response) with concentration as dependent variable and OD' as independent variable. To calculate unknowns, take raw OD value, subtract $OD_{meanzero}$ to obtain OD', and multiply by coefficients to obtain concentration values.

K. Criteria for Acceptance of Standard Curve

The course of the standard curve is shown in the Quality Assurance Certificate enclosed in the test kit. Absolute absorbances may vary between different runs (e.g., because of 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:

(**a**) OD at 450 nm for standard 6 higher than 1.2.

(**b**) OD values for standards should continuously increase with higher concentrations, especially when comparing standard 1 (0 mg/kg gluten) and standard 2 (5 mg/kg gluten)

(**c**) An OD value for standard 1, which is much higher than the OD value stated in the certificate, could be an indication for errors during pipetting or incubation or contamination.

Results

Collaborative Study Results

The study director asked 19 laboratories to participate in the collaborative test. All participants delivered valid data sets for the pretest, so the study director gave each participant the permission to perform the main experiment with 42 blind-coded samples. Two participants (laboratory A and C) reported one high OD value around 0.3 for the zero-calibrator duplicate, while the other duplicate was at an OD value of 0.1. It was decided to eliminate this single high value and to calculate the calibration function using the low value in duplicate. Because the results for samples from these laboratories showed no irregularities compared with other laboratories, this was noted as a random event. Two other participants (laboratory F and P) showed constantly higher background values for the zero calibrator at an OD value of 0.3 (laboratory F) and of 0.5 (laboratory P), which could be an indicator of contamination. Because the results for samples from laboratory F showed no irregularities compared with other labs, it was decided to include the laboratory in the data set. Laboratory P had a higher incidence of identified outlier values (samples 3, 4, 7, and 15; *see* Annex A Tables A1–A4), but given the fact that the results for the other samples were within the expected range, it was decided to include the data set from this participant in the statistical analyses, except in the case of identified outliers.

Statistical Analysis

According to AOAC Appendix D (23), data sets from collaborative tests should be checked for outlying values. In the case of a gluten contamination in oats, it was known that the distribution of gluten is often not homogenous in a sample. Furthermore, the distribution is not normal but instead skewed toward higher concentrations. As a consequence, it was decided to use log-transformed concentrations for outlier

calculation. This will result in fewer outliers than with untransformed data, providing for more generous allowance for deviation toward high concentrations. As can be seen in Annex A (Tables A1–A4), outliers according to Cochran, Grubbs, and double Grubbs were detected. Eight out of all thirteen outlying values were due to results from laboratory D and P when analyzing the data sets calculated by the four-parameter logistic regression. For the quadratic curve fitting, 5 out of 11 outliers were from laboratory D and P. If the data sets are analyzed by sample, not more than four outliers were detected for sample 4 (oat reference material blank; four-parameter logistic regression) or three outliers for sample 7 (rice flour blank; quadratic curve fitting). AOAC Guidelines allow for up to 4 laboratories per 18 laboratories to be removed as outliers per blind duplicate pair set. In this study, no more than four laboratories were removed per sample set.

After elimination of outliers, the performance characteristics precision of repeatability and precision of reproducibility [s(R)] were calculated for both curve fitting procedures. Table **2018.15A** shows the results for the four-parameter logistic regression, while Table **2018.15B** depicts the results for the quadratic curve fitting procedure. As there are no obvious systematic differences between both curve fitting procedures regarding the precision estimates, the following discussion will focus on the four-parameter logistic regression.

Discussion

The analysis of the three reference materials with gluten contents from wheat, rye, or barley of 10 mg/kg resulted in mean values of 10.8 mg/kg for wheat (108% recovery), 13.7 mg/kg for rye (137% recovery), and 11.0 mg/kg for barley (110% recovery). The RSD_R was between 15 and 21%, which is, for a significant part, driven by the inhomogeneity of the samples (*see Homogeneity of Samples* section). Therefore, it can be concluded that the RIDASCREEN Total Gluten is not only precise but also accurate. Samples 5 and 6 are highly processed corn-based snack samples that were incurred with wheat before processing. The wheat gluten (determined by HPLC; Koehler, P., Leibniz-Institute, personal communication, 2013) content of sample 5 is 82 mg/kg. Based on this finding, the mean recovery in the collaborative study is 76%. The wheat gluten content of sample 6 is not exactly known, but using the estimated gluten content of 41 mg/kg, the recovery in the collaborative study is 81%. The homogeneity of these samples was proven during an AACCI collaborative test for AOAC *Official Method* **2012.01** (8). The precision estimates for these samples demonstrate that the ELISA procedure is highly precise because RSD_R values of 20% or lower were obtained. Most of the oat-based products showed RSD_R values at or lower than 30%, with the exception of oat flours that seems to be more inhomogeneous than the other samples. Samples 12 and 13 are likely to have been interchanged before shipping to all laboratories, as the mean measured concentration of cereals-low (sample 12) was higher than for cereals-medium (sample 13). As described in Table 1, cereals-low, cereals-medium, and cereals-high have been contaminated with equidistant concentrations and cereals-high with twice the amount than cereals-low. Assuming that the two samples were mixed up, the mean of the measured concentrations would be 15.9 (cereals-low), 21.0 (cereals-medium), and 27.2 (cereals-high), showing the described proportions of concentrations. A general switching of these samples might have occurred during production, homogenization, or blinding of the samples. Nevertheless, these samples were intended as a test for method precision, not trueness, so the intent of the collaborative study was not compromised by this switch of samples. As all oat-based products are subject to inhomogeneity, it was decided not to estimate LODs for these samples using the collaborative test results. The only true blank sample is the reference material that was not spiked (sample 4). Using the precision estimate for s(R), which is 0.96 mg/kg gluten, and the mean gluten concentration of 0.9 mg/kg, it can be calculated using the basic formula from AOAC Appendix M (24) that the LOD is around 4 mg/kg gluten. Mean gluten concentrations for samples 11 and 15 were at 3.1 and 2.1 mg/kg, respectively, but as can be seen from the values for s(R), the imprecision is high as expected. Confirmation of LOQ is described in chapter 4.1.7.2 in Annex B. The presented method thus fulfills all requirements set in the AOAC SMPR 2017.021 (Analytical range, ≤5 to ≥15 mg/kg gluten; LOQ ≤ 5 mg/kg gluten; LOD ≤ 5 mg/kg gluten; recovery of wheat, rye, and barley, 50–200%).

Conclusions

As the data show that the RIDASCREEN Total Gluten is suitable to quantify gluten from wheat, rye, or barley with a high precision, especially at concentrations between 10 and 20 mg/kg gluten, the study director Katharina Scherf together with the method developers from R-Biopharm and the provider of test samples from General Mills recommend this method for *AOAC First Action Official Methods of Analysis.*

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