FOOD CHEMICAL CONTAMINANTS

Determination of Gliadin as a Measure of Gluten in Food by R5 Sandwich ELISA RIDASCREEN® Gliadin Matrix Extension: Collaborative Study 2012.01

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Abstract

Background: According to Codex Alimentarius, food products containing less than 20 mg/kg gluten can be labeled as "gluten-free." Since 2002, the R5 antibody method allowed determination of gluten levels and led to a huge improvement of products available to celiac disease (CD) patients.

Method: The R5-containing test kit RIDASCREEN® Gliadin in combination with the cocktail solution was endorsed as Codex Type 1 Method in 2006 based on a collaborative study with corn-based bread, rice-based dough, wheat starches, rice, and corn flour. In 2012, the method was approved as First Action Official MethodSM 2012.01 with an “in foods” claim. For Final Action in 2016, the matrix claim was reduced to rice- and corn-based matrixes.

Objective: Therefore, R-Biopharm decided to start a collaborative study to demonstrate the wide applicability of Official Method 2012.01 for the quantitative analysis of gliadin in soy, starches, pseudo cereals, legumes, spices, juice, nut nougat crème, cream cheese, pesto, meat, vegetarian meat alternative, cookies, dessert, cake, fish, bread, candies, and potatoes.

Materials for incurring were the MoniQA wheat flour and the PWG gliadin preparation.

Results: Gliadin levels ranged from 3.4 up to 27.4 mg gliadin per kg. The results of the collaborative study with 14 participating laboratories showed recoveries ranging from 80 to 130%. Relative reproducibility standard deviations for contaminated samples were between 9.8 and 27.7%.

Conclusions: The collaborative study results confirmed that the method is accurate and suitable to measure gliadin in important gluten-free food matrixes.

Highlights: The title and applicability statement of Official Method 2012.01 were changed as proposed.
With a prevalence of 0.4 to 1.2% of the population in Europe, North America, Australia, and the Middle East (1), CD is considered 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. This protein fraction is insoluble in water and 0.5 mol/L NaCl solution, respectively (3). Following a traditional definition, gluten is composed of prolamins that can be extracted by 40–70% of ethanol, and alcohol-insoluble glutenins 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. The prolamin content of gluten is generally taken as 50% (3), which will lead to a significant overestimation of the gluten content if prolamin is chosen as the analytical basis (4).

The only known effective treatment for celiac disease (CD) is a lifelong gluten-free diet, which is based on the avoidance of gluten-containing cereals. The diet should contain less than 20 mg gluten intake per day to prevent a relapse of intestinal damage (5). 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, e.g., in Europe by the European Commission (6) and in the United States by the Department of Health and Human Services—Food and Drug Administration (7). Therefore, specific and sensitive analytical methods are needed for food quality control. Immunochemical methods are currently recommended for the quantitative and qualitative determination of gluten in foods (3).

The R5 monoclonal antibody raised against α-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 (8–10).

To understand the rationale for the additionally collaborative test, one needs to roll up the history of the R5 method and Official MethodSM 2012.01 especially. Since its introduction in 2002, the R5 method allowed determination and control of gluten levels in products with a yet unmet sensitivity (limit of quantification of 5 mg/kg gluten), which subsequently led to a huge improvement in the quality of products available to CD patients.

The test kit RIDASCREEN® Gliadin was endorsed as Codex Type 1 Method in 2006 based on a collaborative study performed by the Working Group on Prolamin Toxicity and Analysis. In 2011, an additional collaborative study was performed with RIDASCREEN Gliadin. In 2012, the system was approved as First Action Official Method 2012.01 with an “in foods” claim. For the decision on Final Action in 2016, the matrix claim was reduced to only rice- and corn-based matrixes despite the fact that data on proficiency test rounds with several additional matrixes were presented. In March 2019, the method developer tried again to include the “proficiency test round matrixes” into the intended use of the method, but at that time no decision was made. In May 2019, AOAC and AACC proposed at a CCMAS meeting to limit the scope of Official Method 2012.01 to corn- and rice-based matrixes and to add Official Method 2018.15 as the method for gluten in oats in the Codex Alimentarius.

Already the reduction of the scope of Official Method 2012.01 by AOAC in 2016 led to severe irritations in the analytical community, especially for laboratories accredited toward ISO 17025. Since the reasons for this reduction were not clearly communicated by AOAC, laboratories became unsure about the applicability of the method for other food matrixes. Some laboratories had been using the method according to First Action Official Methods for all kinds of different foods for years and now questioned the analytical correctness of previous analyses. An additional reduction of the scope of Official Method 2012.01 at Codex Alimentarius would lead to a more confusing situation for laboratories and finally also for CD patients comparable to times before 2002. Therefore, R-Biopharm decided to start a new collaborative study to demonstrate the applicability of Official Method 2012.01 for all kinds of different foods and to re-examine the scope for Official Method 2012.01.

After prolific consultations with CD patient societies from the United States and Europe, a list of 19 different matrixes from 16 different food categories was prepared. Four ERD Gluten members approved the collaborative test protocol in October 2019. Meanwhile, in November 2019, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) decided that “The Committee noted that it was premature to consider the proposed methods as presented in CX/NFSDU 19/41/2, Appendix I, Part C as research is still ongoing to determine the most appropriate method for determination of gluten. The Committee agreed to wait for the completion of ring trial tests and to consider this matter at a future date when more information became available.” Until finalizing this manuscript no new discussions either at CCMAS (Codex Committee on Methods of Analysis and Sampling) or CCNFSDU were perceived.

Here, the collaborative study of Official Method 2012.01 for the quantitative analysis of gliadin in soy, starches, pseudo cereals, legumes, spices, juice, nut nougat crème, cream cheese, pesto, meat, vegetarian meat alternative, cookies, dessert, cake, fish, bread, candies, and potatoes is presented. Due to the high number of different matrixes and the total number of samples for each participant, it was decided to test each matrix at one concentration level and only a few matrixes additionally as blank matrixes (see Table 1). The different contamination levels were chosen to bracket the gluten threshold level of 20 mg/kg.

Scope of method

RIDASCREEN Gliadin is suitable for the quantitative measurement of intact gliadin as a measure of gluten in unprocessed
and processed matrixes from important gluten-free food categories, including rice- and corn-based products, soy, starches, pseudo cereals, legumes, spices, juice, nut nougat crème, cream cheese, pesto, meat, vegetarian meat alternative, cookies, dessert, cake, fish, bread, candies, and potatoes. The sandwich ELISA quantifies intact gliadin from wheat and also intact related proteins from rye and barley. This method is not accurate for quantification of fermented or hydrolyzed gluten.

Collaborators
In order to qualify for participation in the collaborative test, all laboratories were required to have previous experience with ELISA, and to be familiar with the analytical procedure. It was recommended to use a separate room for the collaborative study due to the possibility of gluten contamination and the low detection limit. The laboratories were given the period from November 2020 to January 2021 to perform the experiments. Fourteen laboratories (designated A to O, without G) were chosen to participate: one each in Austria, Finland, Ireland, Italy, two in Canada, four in Germany, and four in the United States. One laboratory from the United States obtained sample set G but could not guarantee proper storage over the weekend. It was decided to send an additional sample set O to them and to discard sample set G.

Samples and Sample Preparation
During the preparation of this collaborative test, European and US CD patient societies were asked to deliver the most important gluten-free food categories according to their view. The result was a list of 16 different food categories (Table 1). Afterwards, recipes for 19 different food matrices were developed by the method developer paying special attention to the feasibility of production, achievable sample homogeneity, and stability. Due to its relevance, the category “cereals and products thereof” will be represented by three different matrixes (cookies, cake, bread). The 14 recipes were sent to the Hochschule Geisenheim University for independent production. MoniQA wheat flour (13) with defined gliadin and gluten levels of 7.26 and 10.60% (personal communication with Katharina Scherf), respectively, and the PWG gliadin preparation were used for incurring the matrixes (14). After preparation, samples were aliquoted, blinded, and checked for homogeneity by the Hochschule Geisenheim University.

In the following, the ingredients, processing, and contamination levels of each matrix at the Hochschule Geisenheim University are described. The Hochschule Geisenheim University provided a detailed report on the production of each matrix as a confidential document to the ERP Gluten. The contamination was added to the matrixes prior to the processing in all cases. Loss of water or other components during the preparation of each matrix was taken into account by weighing experiments throughout the production process. The concentrations in Table 1 were chosen to bracket the threshold of 20 mg gluten per kg of food.

### Table 1. List of gluten-free food categories and the representative sample matrix; all matrixes were incurred using MoniQA wheat flour (column “Gliadin (gluten)” therein gluten content is given in brackets). Additionally, some matrixes were incurred using PWG gliadin (column “PWG gliadin”).

<table>
<thead>
<tr>
<th>Category</th>
<th>Sample name</th>
<th>Gliadin (gluten)</th>
<th>PWG gliadin</th>
<th>In-house testing, mg/kg</th>
</tr>
</thead>
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<tr>
<td>Starch</td>
<td>Starch</td>
<td>27.36 (39.95)</td>
<td>–a</td>
<td>Blank, 6.84, 13.69, 27.37</td>
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<td>Pseudo cereals</td>
<td>Pseudo cereals</td>
<td>3.43 (5.00)</td>
<td>–</td>
<td>Blank, 6.84, 13.69, 27.37</td>
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<tr>
<td>Legumes</td>
<td>Legumes</td>
<td>27.36 (39.95)</td>
<td>20.00</td>
<td>Blank, 6.84, 13.69, 27.37</td>
</tr>
<tr>
<td>Soy</td>
<td>Soy</td>
<td>Blank and 3.43 (5.00)</td>
<td>–</td>
<td>6.84, 13.69, 27.37</td>
</tr>
<tr>
<td>Spices and their mixtures</td>
<td>Spices</td>
<td>6.86 (10.01)</td>
<td>5.00</td>
<td>Blank, 12.08, 24.15</td>
</tr>
<tr>
<td>Mayonnaise, sauces, vegetables</td>
<td>Pesto</td>
<td>13.70 (20.00)</td>
<td>10.00</td>
<td>Blank, 6.86, 24.51</td>
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<tr>
<td>Cereals and products thereof</td>
<td>Cake</td>
<td>20.51 (29.95)</td>
<td>15.01</td>
<td>Blank, 6.84, 13.69, 27.38</td>
</tr>
<tr>
<td>Potatoes and products thereof</td>
<td>Gnocchi</td>
<td>10.27 (15.00)</td>
<td>–</td>
<td>Blank, 6.85, 13.70, 27.39</td>
</tr>
<tr>
<td>Potatoes and products thereof</td>
<td>Gnocchi (MW)</td>
<td>10.27 (15.00)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Egg products, ice cream, milk, and products thereof</td>
<td>Crema</td>
<td>Blank and 10.67 (19.96)</td>
<td>10.00</td>
<td>7.01, 28.02</td>
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<td>Cheese and products thereof</td>
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<td>Blank and 20.55 (30.00)</td>
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<td>6.84, 13.69, 27.37</td>
</tr>
<tr>
<td>Spread</td>
<td>Nougat*</td>
<td>Blank and 20.55 (30.00)</td>
<td>–</td>
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</tr>
<tr>
<td>Soft drinks, vinegar, fruits</td>
<td>Juice*</td>
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<td>5.00</td>
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</tr>
<tr>
<td>Fish and products thereof</td>
<td>Cod</td>
<td>Blank and 27.75 (40.51)</td>
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<tr>
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<td>Candies</td>
<td>Caramel</td>
<td>27.40 (40.00)</td>
<td>–</td>
<td>Blank, 6.85, 13.69</td>
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</table>

*a – No gliadin added.

*b – Samples with an * had to be extracted with the addition of skim milk powder. In-house testing for additional levels is given in the last two columns on the right (only MoniQA wheat flour materials were tested). Gnocchi (microwaved, MW) were not analyzed in-house due to the similarity to gnocchi.
### Table 2. Results from in-house experiments to characterize additional concentration levels for the matrices used in the collaborative test

<table>
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<tr>
<th>Gliadin target, mg/kg</th>
<th>Mean, mg/kg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SD, mg/kg&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CV, %&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Recovery, %&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
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<td>0.34</td>
<td>2.7</td>
<td>91</td>
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</tbody>
</table>

<sup>a</sup>Performance characteristics mean (n = 6 replicates).

<sup>b</sup>SD – Standard deviation.

<sup>c</sup>CV – Coefficient of variation.

<sup>d</sup>Recovery based on gliadin content.

<sup>e</sup>In the case of starch, the asterisk indicates a wheat contamination confirmed by PCR (SureFood ALLERGEN 4plex Cereals S7006 together with SureFood PREP Advanced S1053 for extraction; Congen, Berlin, Germany); the measured values and the recoveries were corrected for the concentration of the nonincurred starch.

<sup>f</sup>/ – Assumed to be blank.
formatting of tables later on. For concentrations of materials used in the collaborative study and for additional in-house testing levels, refer to Table 1.

(a) Starches (starch).—
(1) Ingredients.—Mixture of starch from wheat, corn, tapioca, and potato.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 27.36 mg gliadin/kg (39.95 mg gluten/kg).

(b) Pseudo cereals (pseudo cereals).—
(1) Ingredients.—Mixture of flour from amaranth, quinoa, and buckwheat.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 27.36 mg gliadin/kg (39.96 mg gluten/kg).

(c) Legumes (legumes).—
(1) Ingredients.—Mixture of flour from lentils, beans, and peas.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 27.36 mg gliadin/kg (39.96 mg gluten/kg) and 20.00 mg/kg of PWG gliadin.

(d) Soy (soy).—
(1) Ingredients.—Soy flour.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 27.36 mg gliadin/kg (39.96 mg gluten/kg).

(e) Spices (spices).—
(1) Ingredients.—Mixture of paprika, cinnamon, mustard, pepper, marjoram, and nutmeg.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 27.36 mg gliadin/kg (39.96 mg gluten/kg).

(f) Mayonnaise, sauces, vegetables, and spices (pasta).—
(1) Ingredients.—200 g dried tomatoes, 200 g sieved tomatoes, 100 g tomato paste, 50 g olive oil, two garlic cloves, 30 g ground almonds, 5 g salt, 5 g sugar, 30 g dried, finely ground basil, preservatives.
(2) Processing.—Boiling for 5 min and stirring at 80°C (176°F) for 10 min.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 35.50 mg gliadin/kg (51.83 mg gluten/kg) and 23.62 mg/kg of PWG gliadin.

(g) Cereals and products thereof (bread).—
(1) Ingredients.—150 g butter, 125 g dark chocolate, 100 g gluten-free flour mixture (corn starch, rice flour, millet flour, thickening agents [carob gum, rice whole flour]), 180 g powdered sugar, 125 g ground almonds, five eggs, 15 g baking powder.
(2) Processing.—55 min at 170°C (338°F), dried overnight at 40°C (104°F).
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 24.51 mg gliadin/kg (35.78 mg gluten/kg) and 23.62 mg/kg of PWG gliadin.

(h) Cereals and products thereof (cookies).—
(1) Ingredients.—250 g gluten-free flour mixture (see cake), 7.5 g baking powder, 2.5 g salt, 100 g sugar, one egg, 110 g corn oil.
(2) Processing.—25 min at 150°C (302°F), dried overnight at 40°C (104°F).

(i) Cereals and products thereof (bread).—
(1) Ingredients.—500 g gluten-free bread flour mixture (corn starch, rice flour, millet flour, buckwheat sour- dough powder [buckwheat, quinoa flour, starting culture], thickening agent guar gum), 7 g dry yeast, 5 g salt, 30 mL olive oil, three eggs, 300 mL water.
(2) Processing.—60 min at 180°C (356°F), dried overnight at 40°C (104°F).
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 34.08 mg gliadin/kg (49.75 mg gluten/kg).

(j) Potatoes and products thereof (gnocchi and gnocchi MW).—
(1) Ingredients.—400 g boiled potatoes, 100 g gluten-free flour mix (corn starch, rice flour, millet flour, thickening agent guar gum), two egg yolks, 0.2 g salt, preservatives.
(2) Processing.—15 min at 100°C (212°F) and additionally 2.5 min at 1500 W in a microwave oven (gnocchi MW).
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 32.09 mg gliadin/kg (46.84 mg gluten/kg) for the boiled gnocchi and 37.22 mg gliadin/kg (54.33 mg gluten/kg) for the additionally microwaved (MW) gnocchi.

(k) Egg products, ice, milk, and products thereof (crema).—
(1) Ingredients.—65 g gluten-free flour mixture (see cake), 5 g vanilla sugar, 65 g sugar, one whole egg, two egg yolks, 375 mL milk, 2.25 g gelatin, 40 g butter, 20 g ground gluten-free biscuits (corn starch, butter, corn flour, sugar, soy flour, glucose syrup, salt, rice flour, modified corn starch), preservatives.
(2) Processing.—10 min at 100°C (212°F).
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 28.02 mg gliadin/kg (40.91 mg gluten/kg) and 29.93 mg/kg of PWG gliadin.

(l) Cheese and products thereof (cheese).—
(1) Ingredients.—Cream cheese, preservatives.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 20.55 mg gliadin/kg (30.00 mg gluten/kg).

(m) Spread (nougat).—
(1) Ingredients.—Nut-nougat crème (sugar, palm oil, hazelnuts, skimmed milk powder, fat reduced cocoa, soy lecithin, vanillin), preservatives.
(2) Processing.—80°C (176°F) for 1 h under stirring.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 20.55 mg gliadin/kg (30.00 mg gluten/kg).

(n) Soft drinks, vinegar, fruits (juice).—
(1) Ingredients.—Multivitamin juice (concentrates of juice from apple, orange, mandarin, pineapple, peach, passion fruit, pear, lime, and lemon), water, fruit concentrates (banana, guava, mango, papaya) carrot juice, grape juice, vitamins, preservatives.
(2) Processing.—Mixing.
(3) Contaminating material and initial concentration.—MoniQA wheat flour at 27.37 mg gliadin/kg (39.96 mg gluten/kg) and 20.00 mg/kg of PWG gliadin.

(o) Fish and products thereof (cod).—
(1) Ingredients.—500 g cod, 50 g gluten-free flour mixture, 20 mL water, preservatives.
Meat and products thereof (meat).—

1. Ingredients.—Ground meat from beef and pork, 20 mL water, preservatives.
2. Processing.—Fried at 190°C (374°F) for 16 min.
3. Contaminating material and initial concentration.—MoniQA wheat flour at 70.77 mg gliadin/kg (103.32 mg gluten/kg)

Vegetarian meat alternatives (burger).—

1. Ingredients.—275 g gluten-free commercial vegetarian tomato burger mixture (rice flakes, rice flour, millet flakes, corn flour, corn extrudate, tomato pieces, tomato flakes, salt, basil, onions, parsnip, carrots, curcuma, pepper, olive oil), 5 g gluten-free flour mixture (see gnocchi), 350 mL water, preservatives.
2. Processing.—Fried at 190°C (374°F) for 20 min, dried overnight at 40°C (104°F).
3. Contaminating material and initial concentration.—MoniQA wheat flour at 53.48 mg gliadin/kg (78.08 mg gluten/kg) and 55.12 mg/kg of PWG gliadin.

Candies (caramel).—

1. Ingredients.—230 g sugar, 80 g water, 110 g butter, 0.2 g salt, 150 mL cream.
2. Processing.—100°C for 15 min.
3. Contaminating material and initial concentration.—MoniQA wheat flour at 35.57 mg gliadin/kg (51.92 mg gluten/kg).

Presentation of Samples to Laboratories

Following the collaborative test guidelines of AOAC INTERNATIONAL, two blinded replicates for each sample were provided to each participating laboratory. The samples were marked with a laboratory-specific letter (A to O) and a randomized number (1 to 64). Each laboratory obtained its own coding (different randomized numbers for each laboratory).

Homogeneity of Samples

Homogeneity of each material (n = 32) was tested at the Hochschule Geisenheim University using the RIDASCREEN Gliadin. In brief, five vials from each material were randomly chosen, extracted twice, and analyzed. All samples turned out to be homogeneous since the coefficients of variation (CVs) were 14% or less (results not shown). Only in the case of cookies and juice, CVs were at 18 or 20%, respectively.

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. All OD values obtained had to be recorded in a ready-to-use datasheet. The final data from the laboratories were sent to the study director. The participants were advised to analyze sample 1 to 32 in run 1, whereas samples coded 33 to 64 were analyzed in run 2. Samples with OD values higher than calibrator 6 (80 ng gliadin/mL) had to be repeated with a 1:2,000 dilution in run 3 (the regular final dilution factor is 500). To facilitate the calculation later on, the participants were asked to use a fixed pipetting scheme on the microtiter plate. As a check for any contamination at the collaborator’s site, participants were asked to analyze their skim milk powder (SMP) (added to some samples before extraction; see Table 1) and the sample dilution buffer in addition.

AOAC Official Method 2012.01
Gliadin as a Measure of Gluten in Food
by R5 Sandwich ELISA RIDASCREEN Gliadin
Based on a Specific Monoclonal Antibody to
Celiac Toxic Amino Acid Prolamin Sequences
First Action 2012
Final Action 2016

[Applicable for the quantitative measurement of intact gliadin as a measure of gluten in unprocessed and processed matrices from important gluten-free food categories including rice- and corn-based products, soy, starches, pseudo cereals, legumes, spices, juice, nut nougat crème, cream cheese, pesto, meat, vegetarian meat alternative, cookies, dessert, cake, fish, bread, candies, and potatoes. The sandwich ELISA quantifies intact gliadin from wheat and also intact related proteins from rye and barley. This method is not accurate for quantification of fermented or hydrolyzed gluten.]

Caution: Ethanol is highly flammable and vapor; keep away from heat, hot surfaces, sparks, open flames, and other ignition sources; do not smoke; keep the container tightly closed; store in a well-ventilated place and keep cool. The cocktail (patented) contains 2-mercaptopethanol, which is toxic; the stop solution contains sulfuric acid, which is caustic; work under a chemical fumehood and avoid skin and eye contact and wear protective gloves and clothing (see SDS, attached as separate documents or delivered by the manufacturer in case of ethanol).

See Tables 2012.01A, 2012.01B, and 2012.01C for the results of the interlaboratory studies supporting acceptance of the method.

Table 2012.01A. Performance characteristics RSDr, RSDR, and recovery (based on gliadin content); original table taken from (18) and reformatted

<table>
<thead>
<tr>
<th></th>
<th>Maize bread (baked)</th>
<th>Rice dough</th>
<th>Wheat starch</th>
<th>Rice flour</th>
<th>Wheat starch</th>
<th>Maize flour</th>
<th>Maize flour</th>
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</thead>
<tbody>
<tr>
<td>mg (PWG) gliadin/kg</td>
<td>168.0</td>
<td>35.0</td>
<td>79.0</td>
<td>0.0</td>
<td>147.0</td>
<td>14.0**</td>
<td>13.0*</td>
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<td>18</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>Mean, mg gliadin/kg</td>
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<td>36.8</td>
<td>74.1</td>
<td>8.3</td>
<td>34.7</td>
<td>&lt;1.5</td>
<td>126.6</td>
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<tr>
<td>RSDr, %</td>
<td>20.8</td>
<td>37.7</td>
<td>14.2</td>
<td>32.0</td>
<td>18.3</td>
<td>26.8</td>
<td>26.8</td>
</tr>
<tr>
<td>RSDR, %</td>
<td>28.6</td>
<td>40.3</td>
<td>32.4</td>
<td>41.5</td>
<td>25.6</td>
<td>35.4</td>
<td>40.7</td>
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<tr>
<td>Recovery (%)</td>
<td>84.4</td>
<td>105.0</td>
<td>93.8</td>
<td>84.6</td>
<td>86.1</td>
<td>89.3</td>
<td>108.5</td>
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</table>

*a Samples marked with an asterisk were naturally contaminated.
Table 2012.01B. Performance characteristics $s_r$, $s_R$, RSD, RSDR, and recovery (based on gliadin content)

<table>
<thead>
<tr>
<th></th>
<th>Soy</th>
<th>Soy</th>
<th>Starch</th>
<th>Pseudo cereals</th>
<th>Legumes</th>
<th>Legumes</th>
<th>Spices</th>
<th>Spices</th>
<th>Juice</th>
<th>Juice</th>
<th>Nougat</th>
<th>Nougat</th>
<th>Cheese</th>
<th>Cheese</th>
<th>Pesto</th>
<th>Pesto</th>
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<tbody>
<tr>
<td><strong>mg gliadin/kg</strong></td>
<td>/a</td>
<td>3.43</td>
<td>27.36</td>
<td>3.43</td>
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<td>20.00</td>
<td>/</td>
<td>13.70</td>
<td>6.84</td>
<td>5.00</td>
<td>/</td>
<td>20.55</td>
<td>/</td>
<td>20.55</td>
<td>6.86</td>
<td>5.00</td>
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<td>24</td>
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<td>20</td>
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<td>26</td>
<td></td>
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<tr>
<td><strong>Mean, mg gliadin/kg</strong></td>
<td>0.049</td>
<td>4.38</td>
<td>28.6</td>
<td>4.15</td>
<td>29.7</td>
<td>19.8</td>
<td>1.02</td>
<td>11.4</td>
<td>5.49</td>
<td>3.20</td>
<td>-0.001</td>
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<td><strong>$s_r$, mg gliadin/kg</strong></td>
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<td>0.49</td>
<td>3.41</td>
<td>0.72</td>
<td>5.18</td>
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<td>0.31</td>
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<td>0.0492</td>
<td>4.83</td>
<td>1.32</td>
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<td><strong>$s_R$, mg gliadin/kg</strong></td>
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<td>0.94</td>
<td>4.68</td>
<td>0.92</td>
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<td>0.35</td>
<td>1.17</td>
<td>2.13</td>
<td>0.38</td>
<td>0.0543</td>
<td>1.74</td>
<td>0.0492</td>
<td>5.73</td>
<td>1.61</td>
<td>0.60</td>
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<tr>
<td><strong>RSD, %</strong></td>
<td>11.1</td>
<td>11.9</td>
<td>17.4</td>
<td>17.5</td>
<td>12.8</td>
<td>31.3</td>
<td>10.2</td>
<td>32.3</td>
<td>9.8</td>
<td>9.0</td>
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<tr>
<td><strong>RSDR, %</strong></td>
<td>21.4</td>
<td>16.3</td>
<td>22.2</td>
<td>20.8</td>
<td>18.0</td>
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<tr>
<td><strong>Recovery (%)</strong></td>
<td>/</td>
<td>128</td>
<td>105</td>
<td>121</td>
<td>108</td>
<td>99</td>
<td>/</td>
<td>83</td>
<td>80</td>
<td>64</td>
<td>/</td>
<td>86</td>
<td>/</td>
<td>122</td>
<td>120</td>
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</table>

*a*/ = Assumed to be blank.

Table 2012.01C. Performance characteristics $s_r$, $s_R$, RSD, RSDR, and recovery (based on gliadin content); gnocchi MW is the microwaved version

<table>
<thead>
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<th></th>
<th>Meat</th>
<th>Burger</th>
<th>Burger</th>
<th>Cookie</th>
<th>Cookie</th>
<th>Crema</th>
<th>Crema</th>
<th>Crema</th>
<th>Crema</th>
<th>Bread</th>
<th>Cake</th>
<th>Cake</th>
<th>Cod</th>
<th>Cod</th>
<th>Caramel</th>
<th>Gnocchi</th>
<th>Gnocchi MW</th>
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</thead>
<tbody>
<tr>
<td>mg gliadin/kg</td>
<td>27.43</td>
<td>13.70</td>
<td>10.00</td>
<td>20.51</td>
<td>15.01</td>
<td>/a</td>
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<td>10.00</td>
<td>/</td>
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<td>26</td>
<td>22</td>
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<tr>
<td>Mean, mg gliadin/kg</td>
<td>28.6</td>
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<td>9.60</td>
<td>26.2</td>
<td>16.3</td>
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<td>31.1</td>
<td>13.4</td>
<td>13.3</td>
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<tr>
<td>$s_r$, mg gliadin/kg</td>
<td>3.37</td>
<td>1.58</td>
<td>0.84</td>
<td>4.09</td>
<td>2.72</td>
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<td>1.24</td>
<td>0.40</td>
<td>0.48</td>
<td>0.66</td>
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<td>5.92</td>
<td>2.98</td>
<td>1.85</td>
<td>1.03</td>
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<tr>
<td>$s_R$, mg gliadin/kg</td>
<td>4.27</td>
<td>1.66</td>
<td>1.62</td>
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<tr>
<td>RSD, %</td>
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<td>8.7</td>
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<td>16.6</td>
<td>10.6</td>
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<tr>
<td>RSDR, %</td>
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<td>16.9</td>
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<td>19.6</td>
<td>13.0</td>
<td>14.7</td>
<td>10.1</td>
<td>15.6</td>
<td>14.0</td>
<td>27.7</td>
<td>13.1</td>
<td>16.0</td>
<td>11.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery, %</td>
<td>104</td>
<td>109</td>
<td>96</td>
<td>128</td>
<td>109</td>
<td>/</td>
<td>85</td>
<td>85</td>
<td>104</td>
<td>56</td>
<td>58</td>
<td>/</td>
<td>81</td>
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*a*/ = Assumed to be blank
A. Principle

The method is based on an enzyme immunoassay format using a monoclonal antibody that can determine gliadin derived from wheat and related proteins that are derived from rye and barley. The antibody binds to the celiac toxic amino acid sequence QQPFP (8–10, 16) and to related sequences, which exist as motifs on all prolamin proteins. The antibody detects prolamins in unprocessed and processed food by using an additional specific extraction method (cocktail patented). No cross-reactivity exists to oats, maize, rice, millet, teff, buckwheat, quinoa, amaranth, and 78 other common gluten-free food ingredients. An additional collaborative study of Official Method 2012.01 was published in 2013 (17).

Samples are extracted by using cocktail (patented) containing β-mercaptoethanol and guanidine hydrochloride described by García et al. (16), followed by an extraction with 80% ethanol. After centrifugation, the supernatant is used in a two-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 bond analyte. Nonimmobilized components are removed by washing steps. The response of sample extracts is compared with the response observed with calibrators.

B. Apparatus

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

- Thermomix TM31.—For sample homogenization (Vorwerk Deutschland Stiftung & Co KG, Wuppertal, Germany).
- Water bath WN8 14.—(Memmert GmbH & Co KG, Schwabach, Germany).
- Bench-top centrifuge.—Multifuge 3L-R, operating at 2500 rpm (Thermo Electron GmbH, Dreieich, Germany).
- Poly styrol tubes.—5 mL; for sample dilution (Brand GmbH, Wertheim, Germany).
- Glassware.—Wash bottle (1000 mL) and graduated cylinders.

C. Reagents

Items (a)–(h) are available as a test kit (RIDASCREEN Gliadin, R7001, R-Biopharm AG, Darmstadt, Germany); item (i) is available separately as cocktail (patented) in two different volumes (R7006 with 105 mL and R7016 with 1000 mL, R-Biopharm AG, Darmstadt, Germany). Refer to kit label for current expiry.

- Antibody-coated microwell strips.—Monoclonal antibodies are coated onto a set of 12 eight-microwell strips.
- Wash buffer concentrate.—100 mL/bottle, 10× concentrate.
- Peroxidase-labeled antibody.—One vial (1.2 mL, 11× concentrated).
- Gliadin standards.—Six vials (1.3 mL each, ready to use). Calibrated to the gliadin preparation of Working Group on Prolamin Analysis and Toxicity (14) (PWG gliadin, 97% highly purified gliadin from 28 different European wheat varieties).
- Substrate.—One vial, 7 mL (urea peroxide).
- Chromogen.—One vial, 7 mL (tetramethylbenzidine in methanol).
- Stop solution.—One vial, 14 mL (1 N H2SO4).
- Sample dilution buffer.—60 mL, 5× concentrate.
- Cocktail (patented).—One vial, 105 mL.

Required but not provided with the test kit:

- Skim milk powder.—Food quality; gluten-free.
- 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.

D. General Instructions

- Store kit at 2–8°C (35–46°F).
- Let all kit components warm up 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).
- Use an uncoated pre-plate if more than six strips are used in one ELISA run.
- 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 duplicates.
- 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 the 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, micromers, and other equipment with 60% ethanol.
- Carry out sample preparation in a room isolated from real dust or dirty laboratory equipment.
- Check for prolamin contaminations of reagents and equipment.

E. Preparation of Test Samples

- Grind solid samples to a powder, homogenize pasty-like sample with a spatula, and mix liquid samples.
- Weigh 0.25 g of sample or use 0.25 mL of a liquid sample in a 10 mL glass vial and add 2.5 mL cocktail (patented).
- If tannin- and polyphenol-containing samples (e.g., chocolate, chestnut, juice, coffee, cacao, sorghum, spices, or buckwheat) are prepared, add an additional 0.25 g SMP...
(food quality; gluten-free) to the sample before addition of cocktail (patented).

(d) Close vial and mix well (avoid cross-contamination).
(e) Incubate for 40 min at 50 °C (122 °F) in a water bath.
(f) Let sample cool down; then mix with 7.5 mL 80% ethanol. Close vial and shake for 1 h upside down or by a rotator at room temperature (20–25 °C/68–77 °F).

(g) Centrifuge 10 min at 2500 g at room temperature (20–25 °C/68–77 °F) and/or filter the extract.
(h) Transfer the supernatant (extract) in a screw-top vial and keep for testing.
(i) Dilute the sample 1:12.5 (1 + 11.5, 0.08 + 0.92 mL) with the prepared sample dilution buffer.
(j) Use 100 μL per well in the assay immediately after dilution (not more than 30 min).

F. Preparation of Components Delivered With Kit

(a) Sample diluent—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 15 samples). Make sure that the buffer is not contaminated with gliadin.

(b) Antibody enzyme conjugate—(Bottle with red cap.) 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—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 20–25 °C (68–77 °F) for 4 weeks.

G. Determination

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

(b) 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. Use an uncoated pre-plate if more than six strips are used in one ELISA run.

(c) Add 100 μL of each standard solution or prepared sample to separate wells and incubate for 30 min at room temperature (20–25 °C/68–77 °F).

(d) 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 diluted washing buffer and pour out the liquid again. Repeat two more times.

(e) Add 100 μL of the diluted enzyme-labeled conjugate to each well and incubate for 30 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 diluted washing buffer and pour out the liquid again. Repeat two more times.

(g) Add 50 μL substrate and 50 μL chromogen to each well. Mix gently by shaking the plate manually and incubate for 30 min at room temperature (20–25 °C/68–77 °F) in the dark.

(h) Positive wells should develop a blue color, indicating the presence of prolamins.
(i) Add 100 μL stop reagent to each well. Mix gently by shaking the plate manually. The color of positive prolamin-containing wells changes to yellow.

H. Reading

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

I. Calculations

(a) Determine the gliadin content of each set of duplicate sample wells by reference to a calibration curve measured by the actual test run.

(b) It is recommended to use the RIDASOFT WIN.NET (29996FF, R-Biopharm, Darmstadt, Germany) and a cubic spline curve fitting procedure.

(c) The standard calibration curve of the ELISA covers a range from 5 to 80 ng gliadin/mL in the calibrators. Including the sample dilution factor of 500, this corresponds to 2.5–40 mg gliadin/kg sample or 5–80 mg gluten/kg samples (see also in this chapter for calculation from gliadin to gluten).

(d) A further dilution and new detection of the sample is recommended for absorbance values > standard 6. This additional dilution factor must be taken into account for calculation.

(e) Convert the units ng gliadin/mL diluted sample to mg gliadin/kg sample as follows: Multiply the amount in ng/mL by the dilution factor. Divide the product by 1000 to achieve a unit of mg/kg. The dilution factor corresponds to the sample preparation of at least 500.

(f) Absorbance below standard 2 (5 ng gliadin/mL) implies that the sample assayed is diluted too much or that no gliadin or that gliadin below the LOQ is present in the sample.

(g) Gluten content of a sample can be calculated from the gliadin value. Gluten values can be expressed in mg/kg by multiplying the gliadin value by 2 as defined by Codex Alimentarius (3). However, recent research shows a gliadin to gluten conversion factor of 1.5 is more accurate (4, 13).

(h) A measurement result of less than 10 mg gliadin/kg assures a content of gluten below the Codex threshold of 20 mg gluten/kg.

(i) For analysis of oat and oat products, AOAC Official Method 2018.15 is recommended.

(j) For analysis of hydrolyzed or fermented gluten, AOAC Official Method 2015.05 is recommended.

J. Criteria for Acceptance of Standard Curve

(a) The course of the calibration curve is shown in the Quality Assurance Certificate, enclosed in the test kit.

(b) Absolute 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.
Statistical Analysis of Laboratories

The study director asked 14 laboratories to participate in the collaborative test. All participants delivered data sets for the 64 blind coded samples. The data set from one participant (laboratory L) showed an undefined contamination in the analytical laboratory (lowest OD values around 1.0, including the OD value for the sample dilution buffer and blank samples). Furthermore, the OD values of the calibrators also pointed to a severe contamination problem (Figure 1). With exception of the calibrator with highest concentration (80 ng/mL), there was significant difference between results from laboratory L compared to the other labs (at least \( P < 0.05; P = 0.0013 \) for standard 1; \( t \)-test assuming different variances). It was decided to exclude this data set from any further calculation. Nevertheless, Figure 1 also depicts the high quality and comparability of results for calibrators from the remaining participants. With exception of the zero calibrator, CVs below 15% were obtained (results not shown).

Another participant’s data set (laboratory H) indicated a high contamination of the SMP used for extraction of some samples (pseudo cereals, spices, juice, nougat, and cake). Laboratory H analyzed pure SMP with a mean OD value of 1.055 (\( n = 4 \) replicates) compared to a mean OD value of 0.127 ± 0.045 from all the other participants. Comparing the results of the incurred samples analyzed by laboratory H with the results of all the other participants, all samples analyzed by laboratory H using SMP showed much higher values compared to the other participants (Figure 2; \( P < 0.001 \) by paired \( t \)-test). These values were excluded from any further statistical calculations. All other samples from laboratory H were comparable to the other participants (Figure 2, linear regression; \( P = 0.91 \) by paired \( t \)-test). It was decided to use the results from those samples for statistical analysis.

Data from all other participants showed only two minor errors because these participants did not repeat one sample each, which had OD values higher than calibrator 6 and should have been analyzed again with a higher dilution (laboratory C with cookie and laboratory M with caramel). These samples were not included for further statistical analyses, as the collaborative study protocol was not followed. Several other laboratories re-analyzed samples additionally in a higher dilution although the samples were already within the calibrator range in their first ELISA measurements. In this case, only the results

![Figure 1](https://academic.oup.com/jaoac/article/105/2/442/6429280)
from the first ELISA measurements were included in the statistical analyses.

Outlier Detection and Performance Characteristics

According to AOAC Appendix D (11), data sets from collaborative tests should be checked for outlying values. Twenty-three outliers according to Cochran, Grubbs, and double Grubbs were detected with a high proportion in blank samples. In case of the blank cod samples, outlier removal was stopped after detection of one outlier according to Cochran and one double Grubbs following AOAC guidance (11). In total, 7 out of 13 participants showed no outliers at all. Seventeen outliers were detected by the Cochran test, indicating higher differences between the two replicates compared to the other labs. Only five outliers according to Grubbs and one so-called double Grubbs were detected. Four participants are responsible for more than 80% of all outlying values. Considering that the participants performed 832 extractions in total, the overall number of outliers is very small (2.8%).

After elimination of outliers, the performance characteristics precision of repeatability s(r) and precision of reproducibility s(R) were calculated using the AOAC Interlaboratory Study Workbook for Blind (Unpaired) Replicates (version 2.0) and cross-checked by an AOAC statistician in February 2021. Table 2012.01A (parts A and B) show the results of these calculations together with calculation of the mean recovery for each incurred matrix. Table 2012.01 (reformatted) shows the results of the collaborative study that led to Official Method 2012.01 First Action in 2011.

Matrixes

This collaborative test was performed to add additional matrixes to the already existing Official Method 2012.01 Final Action.

The collaborative study that led to the approval of Official Method 2012.01 in 2011 was conducted with PWG gliadin as the incurring agent and with naturally contaminated samples with an unknown source of gluten. Matrixes tested for the first collaborative study were corn-based bread and rice-based dough (contaminated with PWG gliadin), wheat starches, rice flour, and corn flour. In 2011, an additional collaborative study was performed with AOAC Official Method 2012.01 using corn bread, corn flour, and extruded corn snack as matrixes (17).

Since most unintended contaminations of supposed gluten-free food occur with flour or products made with flour, this study was predominantly conducted with matrixes incurred with MoniQA wheat flour with defined concentrations of gliadin and gluten (13, personal communication with Katharina Scherf). In order to enable comparison to the previous two collaborative studies, some matrixes (legumes, juice, pesto, burger, crema, and cake) were additionally incurred with PWG gliadin. For the present collaborative study, typical representative matrixes from important gluten-free food categories were analyzed. In total, 19 different matrixes from 16 different food categories were prepared independently at the Hochschule Geisenheim University. Since it was not possible to measure all matrixes at more than one incurred level by participants of the collaborative study, more levels were analyzed at the method developer’s site to cover the measurement range for every matrix (see Tables 1 and 2).

Additionally, soy, nougat, spices, cheese, crema, and cod were analyzed as gluten-free matrixes (Table 1). The spice mixture showed a very low contamination level of about 1 mg gliadin/kg (Table 2012.01B), which was confirmed to be a barley and rye contamination by PCR (SureFood ALLERGEN 4plex Cereals S7006 together with SureFood PREP Advanced S1053 for extraction; Congen, Berlin, Germany). It was therefore excluded from
estimating LOD and LOQ from the data obtained by 13 different
participants. Following the procedure described in AOAC
Appendix M (12), LOD and LOQ can be estimated in two different
ways. The first one is the so-called basic approach using the
mean of each matrix specific $s_R$ of all blank samples (0.061 mg/
kg) multiplied by a factor of 3.3 and 10, respectively. Following
this approach, LOD and LOQ are 0.20 mg gliadin/kg or 0.61 mg
gliadin/kg, respectively. For the second one, $s_R$ is modeled by
mean concentration, and slope and intercept of the linear re-
gression are calculated to 0.182 and 0.0793 mg/kg, respectively
(Figure 3). Using these data together with mean concentration
from all blank samples (0.015 mg/kg), LOD and LOQ are calcu-
lated to 0.40 mg gliadin/kg and 1.19 mg gliadin/kg, respectively,
by using the equation

$$LOD = \frac{\text{mean concentration} + 3.3 \times \text{intercept}}{1-1.65 \times \text{slope}}$$

The LOQ by this method is 3 times the LOD. Since the second
approach is dependent on all other samples analyzed for this
collaborative test, we prefer the basic approach described above.
Nevertheless, the LOD and LOQ stated in the instruction for use
are 0.5 mg gliadin and 2.5 mg gliadin/kg, respectively.

Seven different matrices (soy, starches, pseudo cereals,
legumes, spices, juice, and cheese) were incurred by mixing
with MoniQA wheat flour or PWG gliadin (Table 1) without any
further treatment such as heat. With exception of juice spiked
with wheat flour, none of these matrixes showed RSDR values
higher than 23%. The range of recoveries for these matrixes was
between 83% for spices and 128% for soy (Table 2012.01B). Juice
spiked with PWG gliadin had lower recoveries of around 64 and
80% spiked with MoniQA wheat flour (Table 2012.01B). Without
addition of SMP, recovery for both MoniQA wheat flour and
PWG gliadin is nearly zero, and even neutralizing the low pH
value of the juice did not help to obtain higher recoveries
(results are not part of this collaborative study and are not
shown). A possible explanation is the high level of polyphenols
and pectins in many fruits. These components tend to bind to
proteins and may thereby mask gliadin proteins present in the
juice. By adding SMP, other proteins are added in high excess,
so that the binding of the polyphenols to gliadin proteins is re-
duced. Since the MoniQA flour was never completely solved in
the juice, aggregation and sedimentation could have taken
place, which could also explain the high variation of results
among participants.

Matrixes with a moderate heat treatment were nougat (80°C
for an hour) and pesto (100°C for 5 min and 80°C for 10 min),
both with a quite high fat content. As can be seen in
Table 2012.01B, these matrixes showed excellent relative stan-
dard deviations of reproducibility of less than 20%, and in the
case of nougat it is less than 10%. Recoveries were between 83
and 120%.

Gnocchi and cod were cooked at 100°C in a boiling water
bath for 15 or 20 min, respectively. Gnocchi were also analyzed
with additional microwave treatment at 1500 W for 2.5 min due
to a report stating that the R5 antibody is not able to detect
microwaved gluten (15). It is quite clear from the results in
Table 2012.01C that neither cooking nor microwaving has an
important effect on recovery. RSDR was at or below 16% and
therefore excellent for gnocchi and 27.7% for cod. This higher
RSDR is probably due to the difficult homogenization of this ma-
trix. Tendencies toward higher recoveries in gnocchi may be
due to the high water content. In contrast to most other ma-
trixes, the gnocchi were not dried additionally after preparation,
so that the remaining moisture was quite high. In this case, wa-
ter from the sample may have evaporated and (re-)frozen on
the inner lid of the vial, resulting in an increase of analyte
concentration.

Two matrixes (caramel and crema; Table 2012.01C) were
also cooked, but due to their higher fat and sugar content com-
pared to cod and gnocchi, the cooking temperature should be

![Figure 3. Mean concentrations for each sample (y-axis) were modeled by the reproducibility deviation $s_R$ (x-axis) from all participants except laboratory L and SMP-containing samples from laboratory H; data for linear regression are shown.](https://academic.oup.com/jaoac/article/105/2/442/6429280)
higher than 100°C, especially for caramel. Again, recoveries were between 85 and 113%, and precision was excellent (RSDr better than 15%). It should be noted that caramel was delivered as chunks to the participants, which had to be heated in a 50°C water bath before weighing. All highly heated matrixes (meat, burger, cake, cookie, and bread) showed precision measures below 25% (Table 2.02.01C). Both fried matrixes and the cake showed even lower values. Recoveries were very good, with values between 96 and 128%. The chocolate cake with almonds was an exception because recoveries were unexpectedly low irrespective of the incurring material. Since the nougat also contained high levels of chocolate and the bread had similar processing as the cake but showed better recoveries, the lower recovery for the cake probably comes from the combination of a high level of cocoa and a high level of processing.

Supplementary to levels tested in the collaborative study, additional levels were analyzed by an in-house study at the method developer’s site. These results (Table 2.02.01 and B) underpin that each matrix shows comparable recoveries at different levels. In addition, the CVs were comparable at different levels and matrices, as also observed in the collaborative study (see Figure 3).

Implications
The results of this comprehensive collaborative study are even better than the results of the two preceding collaborative studies (17, 18), especially regarding the complexity of all incurred matrixes and their closeness to commercial samples. Taken these two facts together, the results clearly show that Official Method 2.02.01 is suitable for all these additional matrices from 16 different food categories (see also Figure 3). These matrixes all included relevant ingredients for all kinds of foods in various combinations and different processing levels. These included very high levels of potentially difficult ingredients such as spices, cocoa, fat, and sugar, as well as very high processing such as baking at high temperatures for almost an hour or frying at very high temperatures. The completeness of this study is to our knowledge unprecedented.

Official Method 2.02.01 is calibrated toward PWG gliadin since 2002 and showed excellent recoveries of all matrices incurred with PWG gliadin in the present collaborative study. Furthermore, the MoniQA wheat flour used for incurring all matrixes also showed excellent recoveries of the independently determined gliadin content. Official Method 2.02.01 is in general also able to determine wheat gluten with a similarly high accuracy using an appropriate conversion factor for gliadin to gluten calculation. It is known for many years that the theoretical conversion factor of 2 given by Codex Alimentarius (3) is too high. The advantage of this is that it results in higher protection of CD disease patients. However, to obtain scientifically more valid results, this factor should be reduced to a value of around 1.5 (range 1.32 to 1.66) as shown by several publications (4, 13, 19, 20). A typical example is the MoniQA wheat flour with a gluten content of 10.6% and a gliadin content of 7.26% (13, personal communication with Katharina Scherf). The factor to convert gliadin into gluten is in this case 1.46.

Conclusions
The data obtained by this (nearly) all-encompassing collaborative study show that the RIDASCREEN Gliadin is suitable to quantify gliadin from matrixes representing important gluten-free food categories.

The study director Katharina Scherf together with the method developers from R-Biopharm and the provider of test samples from Hochschule Geisenheim University recommend that the title and applicability statement of the Official Methods of AnalysisSM 2012.01 Final Action of AOAC INTERNATIONAL is changed as proposed.

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Conflict of Interest
All authors declare no conflict of interest.

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