FOOD COMPOSITION AND ADDITIVES

The Validation of the RIDA®QUICK Gliadin for AOAC Research Institute

*Performance Tested Method*SM **101702**

Abstract

RIDA®QUICK Gliadin is an immuno-chromatographic test for the detection of gluten in foods, on surfaces, and in Cleaning-in-Place (CIP) waters. This test kit has been adopted as Final Action AOAC INTERNATIONAL *Official Methods of Analysis*SM **2015.16** for gluten in corn products. The assay is based on the monoclonal antibody R5, which recognizes gluten in wheat, barley, and rye. Four different surfaces were contaminated with a gliadin material and analyzed by a direct swabbing of the surface with the dip-stick. The outcome was an $LOD_{95\%}$ concentration of the assay between 1.6 and 3.0 µg/100 cm^2 gluten. For CIP waters that contain cleansing reagents, 100% positive results were obtained for minimum gluten concentration between 50 and 100 ng/mL. If the CIP water does not contain these reagents, the minimum detectable gluten level is 10 ng/mL. The independent validation study consisted of a method comparison study of recovery from a CIP solution and from a stainless-steel surface. The test kit was evaluated at six different concentration levels for both matrices, with 20 or 30 replicates per concentration level. The probability of detection was calculated for each contamination level. Additionally, the $LOD_{95\%}$ concentration was estimated for each matrix analyzed.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*SM. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

(**a**) *Target analytes*.—Gluten is composed of prolamins that can be extracted by 40–70% of 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% according to Codex Alimentarius standards (1). The monoclonal antibody R5 reacts with the gliadin-fraction from wheat and corresponding prolamins from rye and barley. Among others it detects the potentially toxic sequence QQPFP, which occurs repeatedly in the prolamin molecules of wheat, rye, and barley.

(**b**) *Matrices/surfaces*.—For this validation, RIDA®QUICK Gliadin was tested with the following surfaces: stainless steel, sealed ceramic, plastic, and silicone rubber. Three chemically different cleansing reagents and water were tested as a simulation of Cleaning-in-Place (CIP) waters.

(**c**) *Claims*.—The RIDA®QUICK Gliadin detects gluten with an $LOD_{95\%}$ of 1.6–3.0 µg/100 cm^2 gluten depending on the surface. The minimum detectable gluten concentration in cleansing reagents containing CIP waters is between 50 and 100 ng/mL, whereas CIP water with no reagents allows gluten detection at about 10 ng/mL. No cross-reacting substance has been identified by the manufacturer. Parallel measurements in various matrices using the quantitative RIDASCREEN® Gliadin [AOAC INTERNATIONAL *Official Methods of Analysis*SM (OMA) **2012.01**] and the RIDA®QUICK Gliadin showed accurate detection of the claimed analytes by the dip-stick format. There is no high-dose hook effect for wheat, rye, and barley.

General Information

Gluten is a mixture of prolamin and glutelin proteins present in wheat, rye, and barley. The use of wheat flour and gluten in foodstuff is extremely common because of their useful effects on, e.g., texture, moisture retention, and flavor. Beside its technological intended use, gluten can also be found on surfaces of production facilities and could therefore contaminate gluten-free food products during production.

Celiac disease is a permanent intolerance to gluten that results in damage to the small intestine and is reversible when gluten is avoided by diet. The Codex Alimentarius Commission has stipulated in the "Codex Standard for Foods for Special

Dietary Use for Persons Intolerant to Gluten" (1) the limit value for gluten-free food at 20 mg/kg gluten. The official type I method for quantitative gluten determination according to the Codex Alimentarius is an ELISA that uses the R5 antibody (Mendez). This requirement is fulfilled by the sandwich ELISA RIDASCREEN® Gliadin (Art. Nr. R7001). The test strips of RIDA®QUICK Gliadin also contain this antibody and show an excellent correlation with the official method, the R5-ELISA RIDASCREEN® Gliadin.

Definitions

(**a**) *Probability of detection (POD)*.—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given concentration. POD is concentration-dependent.

(**b**) $LOD_{95\%}$ *concentration*.—Concentration of the analyte that results in a POD of 95% in a given matrix. For estimation of this assay performance characteristic, a POD curve covering POD = 0 to POD = 1 should be determined with a minimum of 10 replicates. For curve fitting, a 4-parameter sigmoid curve evaluation may be used.

Principle

The dip-stick consists of different zones (Figure 1). Prolamins in the sample solution will be "chromatographed" above the "maximum line" and react with the R5-antibody coupled to a red latex microsphere. The "maximum line" indicates to the user the maximal liquid level of the sample solution.

The "result window" contains a small band of immobilized R5 antibody. When the red test band (T) and a second blue control band (C) occur, the reaction was valid and positive for gluten. If no red test band occurs, the test is negative for gluten. In the event the blue control band does not occur, the test is invalid.

Results are read visually only. Generally, the higher the analyte level in the sample the stronger the red color of the test band will be (until a maximum of color is reached).

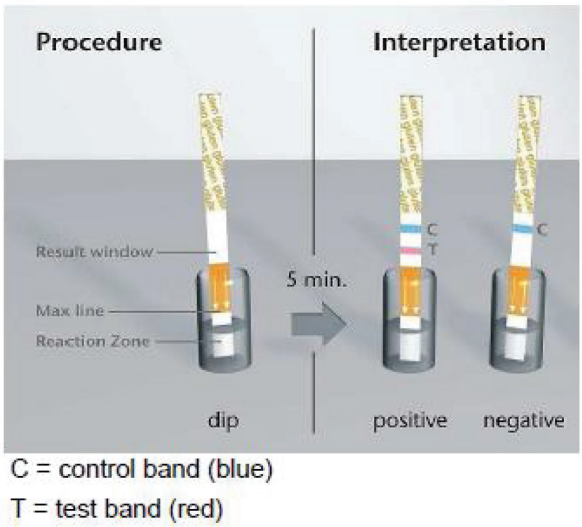

Figure 1. Schematic presentation of the test principle and the subsequent interpretation of the possible results (invalid results not shown).

Materials and Methods

Test Kit Information

(**a**) *Kit name*.—RIDA®QUICK Gliadin.

(**b**) *Cat. No*.—R7003. Remark: The same dip-stick is also available as single-packaged dip-sticks (Cat. No. R7004 and R7005). R7004 contains the same buffer vial (60 mL) with the same composition as R7003. R7005 contains 25 buffer vials with pre-aliquoted buffer (0.5 mL each) ready to use for swabbing of surfaces. This buffer also has the same composition as R7003 and R7004. The production of the dip-sticks is in all three cases identical.

(**c**) *Ordering information*.—*United States*.— R-Biopharm Inc., 870 Vossbrink Dr., Washington, MO 63090, Tel: 877-789-3033, Fax: 269-789-3070, e-mail: sales@r-biopharm.com. *Worldwide*.—R-Biopharm AG, An der neuen Bergstrasse 17, D-64297 Darmstadt, Germany, Tel: +49-(0)6151-802-0, Fax: +49-(0)6151-8102-20, e-mail: sales@r-biopharm.de. Further information is available at the web site address: www.r-biopharm.com.

Test Kit Components

(**a**) *The test kit consists of dip-sticks (25 x), sample diluent, test tubes (30 x), disposable pipettes (25 x), and evaluation card (1 x).*

(**b**) *Reagent one*.—25 x dip-sticks in a tube.

(**c**) *Reagent two*.—30 x empty test tubes.

(**d**) *Reagent three*.—25 x disposable pipettes.

(**e**) *Reagent four*.—sample diluent (60 mL), ready to use, transparent-capped bottle.

(**f**) *Reagent five*.—1 x evaluation card.

Additional Supplies and Reagents

Variable 200–1000 µL micropipettes.

Standard Solutions and Spike Solutions

The starting material used for preparation of in-house control solutions and spike solutions was identical. The preparation of the so-called "Working Group on Prolamin Analysis and Toxicity (WGPAT) Gliadin" is described elsewhere (2). It was stored at −20°C in a lyophilized form until reconstitution. For spiking purposes, the material was reconstituted in 60% aqueous ethanol, resulting in a concentration of 1 mg/mL gliadin. The spike solution was diluted appropriately to the desired concentration. The solution is stable for 4 weeks at 2–8°C.

For validation of CIP waters, an alternative gluten isolate (G5004; Sigma-Aldrich, St. Louis, MO) was used. The material was analyzed by Eurofins (Leipzig, Germany) for the protein content. Analysis according to the Kjeldahl procedure resulted in a protein content of 82.96 g/100 g protein. The material was dissolved in 60% ethanol at a protein concentration of 1 mg/mL. The spike solution was diluted appropriately to the desired concentration. The solution is stable for 4 weeks at 2–8°C. Due to the lack of a reference method, the protein content was assumed to be the gluten content. The most likely small difference between the total protein content and the (unknown) gluten content is negligible, because a binary method was investigated.

The material was also analyzed by Katharina Scherf (Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany) for their gliadin and glutenin contents using a validated HPLC

method. The results were as follows: gliadins: 48.41 g/100 g and glutenins: 24.75 g/100 g. The contents for gliadins and glutelins determined by HPLC were not taken into account because they were not measured by a reference method. The ratio between gliadins and glutenins was quite normal (Scherf, pers. communication). During independent laboratory testing, this gluten material from Sigma-Aldrich (G-5004; Lot number SLBR5510V) was also used.

General Preparation

Sample diluent.—The sample diluent is ready to use. Bring the solution to room temperature (20–25°C) before use. Make sure that the buffer is not contaminated with gluten during use.

Sample Preparation

(**a**) *General recommendation*.—(*1*) Airborne cereal dust and used laboratory equipment may lead to gliadin contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.

(*2*) If necessary, check for gliadin contamination of reagents and equipment with the RIDA®QUICK Gliadin (Art. No. R7003).

(**b**) *Sample preparation procedure: surfaces*.—Swab with the lower end (reaction zone) of a dry dip-stick thoroughly and with constant pressure over a sampling area of 10 × 10 cm. Wear gloves during the swabbing procedure.

(**c**) *Sample preparation: CIP waters*.—CIP waters can be used directly (see dip-stick testing).

Assay

(**a**) *General recommendations for good test performance*.—(*1*) This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed. No quality guarantee is accepted after expiration of the kit (see expiration label). Do not interchange individual reagents between kits of different lot numbers.

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

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

(*4*) Use control food samples (e.g., R7012 for Cocktail extraction; distributed by R-Biopharm AG).

(**b**) *Dip-stick testing*.—(*1*) *Surfaces*.—Place the dip-stick vertically into the test tube filled with sample diluent after swabbing a surface. The arrow on the dip-stick should point down (*see* Figure 1). Do not immerse the dip-stick beyond the maximum line. *CIP waters with cleansing reagent*.—Place 500 µL sample diluent in the test tube and add 50 µL CIP water. Place the dip-stick vertically into the test tube. The arrow on the dip-stick should point down (*see* Figure 1). Do not immerse the dip-stick beyond the maximum line. *CIP waters without cleansing reagent*.—Place 250 µL sample diluent in the test tube and add 250 µL CIP water. Place the dip-stick vertically into the test tube. The arrow on the dip-stick should point down (*see* Figure 1). Do not immerse the dip-stick beyond the maximum line.

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

(*3*) For documentation and prolonged storage, the upper part of the dip-stick marked with "Gluten" together with the test bands should be cut off.

Interpretation and Test Result Reporting

(**a**) *Dip-stick evaluation*.—(*1*) *Positive result*.—If two colored complete bands (test band in red and control band in blue) are visible in the result window (*see* Figure 1) after 5 min, the sample is positive. In the case of testing a surface, a positive complete test band can sometimes show a nonuniform intensity due to the inhomogeneous distribution of gluten on the dip-stick after swabbing.

(*2*) *Negative result*.—If only the blue control band is visible in the result window (*see* Figure 1) after 5 min, the sample is negative.

(*3*) *Invalid result*.—If no bands or only parts of the red band (incomplete line) or a missing blue control band occur after 5 min, the test is invalid and should be repeated using a new dip-stick.

(**b**) *Result reporting*.—(*1*) *Positive result*.—A surface contains more than 2–4 µg/100 cm^2 gluten; a cleansing reagent containing CIP water contains more than 50–100 ng/mL gluten; a CIP water without cleansing reagent contains more than 10 ng/mL.

(*2*) *Negative result*.—A surface contains less than 2–4 µg/100 cm^2 gluten; a cleansing reagent containing CIP water contains less than 50–100 ng/mL gluten; a CIP water without cleansing reagent contains less than 10 ng/mL.

(**c**) *Result interpretation*.—(*1*) The test strip has been developed for the detection of traces of gluten.

(*2*) A negative result does not necessarily indicate the absence of the target compound; it may be not homogeneously distributed or the level of gluten on the product is below the limit of detection.

Validation Studies

General Remarks

The manufacturer's in-house validation scheme followed the OMA Appendix N (3), the special guidance document of the AOAC Allergen Community for quantitative gluten methods (4), and long-lasting practical experiences of the manufacturer.

One important point in validating a qualitative method is to blind-code the contaminated surfaces and CIP waters before measurement. This was guaranteed by splitting the analysis in two parts. (*1*) One technician contaminated the surfaces or spiked the CIP waters and blind-coded all surfaces or CIP waters, and (*2*) another technician performed the dip-stick analysis and documented the read-out of the results in a blinded form prepared by the first technician. Before starting the validation process, both technicians obtained a proper familiarization and training phase.

Selectivity Study: Target Compounds

The WGPAT gliadin material was used as a characterized reference point because its source of origin is described and the contents of gliadin and glutenin were measured independently by chromatography (2). Furthermore, wheat, rye, and barley flour were analyzed. They were supplied and characterized for their variety and their protein contents by Katharina Scherf and Peter Koehler (Deutsche Forschungsanstalt für Lebensmittelchemie,

Freising, Germany). For wheat, rye, and barley, the varieties were Akteur, Conduct, and Marthe, respectively, and the total protein contents were 13.13, 4.03, and 6.71%, respectively.

Flours were extracted both with 60% ethanol and Cocktail/80% ethanol. WGPAT gliadin was dissolved both in 60% ethanol and Cocktail/80% ethanol. The resulting solutions were further diluted with both 60% ethanol and Cocktail/80% ethanol to obtain different concentrations. The final dilution step was in sample dilution buffer as described in the test kit insert. For each target compound and extracted solution, five replicates per concentration were tested. At this stage, no more replicates were included because only the detectability of the system against pure claimed target compounds should be checked.

Gliadin concentration for WGPAT gliadin was calculated according to protein content of WGPAT gliadin and dilution. Prolamin concentrations of flour extracts (ethanol and Cocktail extracted) were determined by measurement in the sandwich ELISA RIDASCREEN® Gliadin R7001 (reference method in Codex Alimentarius containing the monoclonal antibody R5 and AOAC Final Action OMA **2012.01**). Table 1 shows the results of different target compounds extracted with 60% ethanol.

These results indicate that the dip-stick detects all three claimed target compounds with a comparable sensitivity. As expected, there is no difference between the WGPAT gliadin and wheat. The small difference of about 2 between the sensitivities for WGPAT-gliadin and wheat on one hand and rye and barley on the other hand are perhaps the result of different behavior of each matrix extract during the immuno-chromatographic process (e.g., flow rate).

After Cocktail extraction, wheat, rye, barley, and the WGPAT material showed comparable sensitivities (Table 2). The variation of results for the 2 mg/kg concentration is due to the fact that this concentration is close to the POD_{50}. One false-positive result was detected for "barley." Of a total of 316 tested blank samples during this in-house study, this was the rare case in which a false-positive test result occurred.

Using the conversion factor of 2 recommended by Codex Alimentarius (1), the dip stick detects gluten at concentrations between 2 and 4 mg/kg using the ethanol extraction and 8 mg/kg using the cocktail extraction procedure. These values were verified with incurred and spiked samples (AOAC Final Action OMA **2015.16**).

Table 1. Results for target compounds (extracted with 60% ethanol; five replicates per concentration); the sample concentration given in mg/kg is the concentration of prolamins in the extract (determined by the reference ELISA) multiplied with the dilution factor during sample preparation. It is therefore a simulation of a gluten-free matrix containing the target compounds at these concentrations

Extract, µg/mL	Sample, mg/kg prolamin	PWG[a], POD	Wheat, POD	Rye, POD	Barley, POD
0.00	0.0	0.0	0.0	0.0	0.0
0.05	0.5	0.0	0.0	0.0	0.0
0.10	1.0	1.0	0.8	0.0	0.2
0.20	2.0	1.0	1.0	0.8	1.0
0.40	4.0	1.0	1.0	1.0	1.0
0.80	8.0	1.0	1.0	1.0	1.0

[a] PWG = WGPAT gliadin reference preparation.

Table 2. Target compounds (extracted with Cocktail; five replicates per concentration); the sample concentration given in mg/kg is the concentration of prolamins in the extract multiplied with the dilution factor during sample preparation. It is therefore a simulation of a gluten-free matrix containing the target compounds at these concentrations

Extract, µg/mL	Sample, mg/kg prolamin	PWG[a], POD	Wheat, POD	Rye, POD	Barley, POD
0.00	0.0	0.0	0.0	0.0	0.2
0.025	1.0	0.0	0.0	0.0	0.0
0.05	2.0	0.4	0.4	0.2	0.0
0.10	4.0	1.0	1.0	1.0	1.0
0.20	8.0	1.0	1.0	1.0	1.0
0.40	16.0	1.0	1.0	1.0	1.0

[a] PWG = WGPAT gliadin reference preparation.

High-Dose Hook Study

To ensure that no high-dose hook effects occur at high concentrations, 1 g wheat flour (*var. Akteur* and one commercial flour), rye flour (*var. Conduct* and one commercial flour), and barley flour (*var. Marthe* and one commercial flour) were extracted with 10 mL 60% ethanol. In parallel, 0.25 g flour was extracted each with 2.5 mL Cocktail solution and 7.5 mL 80% ethanol. All resulting extracts (50 µL) were diluted according to the test kit insert with 500 µL sample diluent and analyzed with the dip-stick device. The optical evaluation revealed that all dip-sticks were positive. In some cases, the positive band appears as a broadened and/or cloudy band. Assuming a protein content of 10% for each grain and a gluten content of 80% in the protein fraction, these "samples" contained around 80 000 mg/kg gluten (corresponding to 40 000 mg/kg prolamins).

Selectivity Study: Nontarget Compounds

The nontarget selectivity panel was slightly modified but still according to Koerner *et al.* (4) and represents the opinion of the AOAC Allergen community. Some commodities were added to the list because of long-lasting experiences of the method developer. An important part of these commodities is used to compose alternative food for celiac patients. All nontarget compounds are checked to be gluten-free by the Gliadin sandwich ELISA (AOAC Final Action OMA **2012.01**). In total, 85 commodities were tested. The commodities were extracted once like a sample both with 60% ethanol or Cocktail/80% ethanol and were tested in the RIDA®QUICK Gliadin with two replicates. No cross-reactivity exists against almond, cashew, chestnut*, coconut, hazelnut, pistachio, walnut*, poppy seed, sesame, sunflower kernel, amaranth, arrowroot, buckwheat*, oat, millet*, brown rice, corn starch, white rice, flax seed, sweet rice (basmati), tapioca, teff, potato flour, quinoa, black bean, fava bean, garbanzo beans, chickpea, lentils, lima bean, lupine, white bean, yellow pea, green beans, green peas flour, romano bean*, seedlings of carob, sorghum*, soya flour, soya milk, soya protein, coriander*, cinnamon*, black caraway*, pepper*, spices mixture*, basil*, anise*, curcuma*, fennel*, ginger*, garlic*, cumin*, curry mixture*, caraway*, marjoram*, nutmeg*, cloves*, chili pepper*, black pepper*, salt*, brown mustard*, yellow mustard*, pineapple, papaya, apricot, fig,

apple fiber, sugar beet syrup, guar gum, carrageen, xanthan, turkey, chicken, beef, pork hash, sausage, casein, egg powder, gelatin (porcine), skim milk powder, coffee*, cocoa*, orange juice, and tea*. All materials marked with an asterisk were extracted with addition of skim milk powder.

It was reported that several soy milk samples from the market tested positive but contained no gluten. Therefore, 10 different soy milk samples from German retailers were tested in parallel by the reference quantitative gluten ELISA RIDASCREEN® Gliadin (AOAC Final Action OMA **2012.01**) and the qualitative dip-stick format. There was no gluten contamination measurable or even detectable using both methods.

Matrix Study

(**a**) *Surfaces (contaminated).*—Amounts of gluten on the surface were set by the gliadin protein content of the WGPAT material and according to the dilution of the spiking solution prepared from the WGPAT gliadin. The spiking solution was diluted with 60% ethanol and a volume of 100 µL was pipetted directly on the surface. Care was taken in pipetting the volume of 100 µL in order to cover the 10 × 10 cm area only. Five different amounts including zero were repeated for 20 times in a random blinded pattern. After drying in a gluten-free environment at room temperature for 1 h, the different materials were swabbed.

(1) Stainless steel.—Four stainless-steel plates with an area of 50 × 50 cm (1 mm thickness) were used for all experiments. According to EN10020, the number is 1.4301, also known as S30400 according to the Unified Numbering System in the United States. It consists of 0.08% C, 18.5% Cr, 9% Ni, and 0% Mo beside Fe.

Before starting the validation experiment, the material was cleaned with 60% ethanol and areas of 10 × 10 cm were marked with a minimum margin of 2 cm to the next area with a pencil. In total, each plate was divided into 16 areas. To check for effectiveness of the cleaning procedure, five randomly chosen areas of 10 × 10 cm were swabbed and further tested as described. Results were in all cases negative even after the first use and subsequent cleaning with 60% ethanol of the plates.

After swabbing, the plates were cleaned with 60% ethanol and checked for gluten again. 10 × 10 cm areas were marked again, and the areas were contaminated again with gliadin until all repeats were performed.

Besides no false-positives, the concentration range with negative and positive results was between 0.25 and 1.0 µg/100 cm^2 gliadin (Figure 2). Starting with 2 µg/100 cm^2 gliadin (4 µg/100 cm^2 gluten), all results were positive.

Figure 2 shows the POD response curve, which was used to evaluate the $LOD_{95\%}$ concentration. Using a 4-parameter curve fitting a $LOD_{95\%}$ concentration of 1.5 µg/100 cm^2, gliadin (3.0 µg/100 cm^2 gluten) was estimated.

(2) Plastics.—Quadratic sterile petri dishes with an area of 12 × 12 cm (17 mm height) were used for all experiments. The material was polystyrene, which is suitable for food production. The dishes were supplied by Carl Roth (Karlsruhe, Germany; www.carlroth.com; item number PX67.1).

Before starting the validation experiment, the material was cleaned with 60% ethanol and areas of 10 × 10 cm were marked with a pencil. To check for effectiveness of the cleaning procedure, five randomly chosen dishes were swabbed and further tested as described. Results were in all cases negative. Plates were used only once.

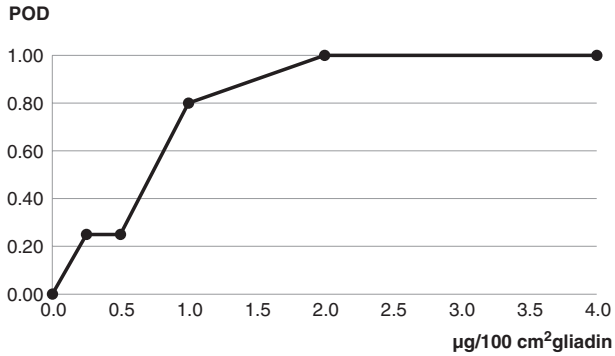

Figure 2. POD for surface stainless steel (swabbing procedure); 20 replicates per gliadin amount.

The results of the validation experiments showed only one false-positive out of 20 replicates (Figure 3). The concentration range with negative and positive results was between 0.25 and 1.0 µg/100 cm^2 gliadin. At 1 µg/100 cm^2 gliadin, 95% of all results were positive, and at 2 µg gliadin/100 cm^2 (4 µg/100 cm^2 gluten), all results were positive.

Figure 3 shows the POD response curve, which was used to evaluate the $LOD_{95\%}$ concentration. Using a 4-parameter curve fitting a $LOD_{95\%}$ concentration of 0.8 µg/100 cm^2, gliadin (1.6 µg/100 cm^2 gluten) was estimated.

(3) Silicone rubber.—Transparent silicone rubber sheets with an area of 120 × 60 cm (1 mm thickness) were used for all experiments. The material is suitable for food-producing facilities. According to ASTM D2240, the hardness of the material is 60 ± 5 shore A.

Before starting the validation experiment, the material was cleaned with 60% ethanol and areas of 10 × 10 cm were marked with a minimum margin of 2 cm to the next area with a pencil. In total, each plate was divided into 36 areas. To check for effectiveness of the cleaning procedure, five randomly chosen areas of 10 × 10 cm were swabbed and further tested as described. Results were in all cases negative.

Besides no false-positives out of 20 replicates, the concentration range with negative and positive results was between 0.25 and 0.5 µg/100 cm^2 gliadin (Figure 4). Starting with 1 µg gliadin/100 cm^2 (2 µg/100 cm^2 gluten), all results were positive.

Figure 4 shows the POD response curve, which was used to evaluate the $LOD_{95\%}$ concentration. Using a 4-parameter curve fitting a $LOD_{95\%}$ concentration of 0.8 µg/100 cm^2, gliadin (1.6 µg/100 cm^2 gluten) was estimated.

(4) Sealed ceramics.—White sealed ceramic tiles with an area of 15 × 15 cm (5 mm thickness) were used for all

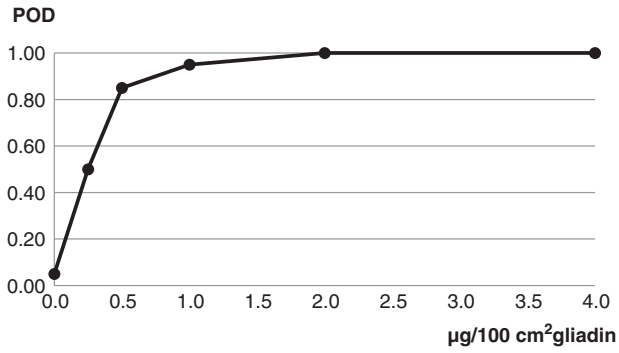

Figure 3. POD for surface plastic (swabbing procedure); 20 replicates per gliadin amount.

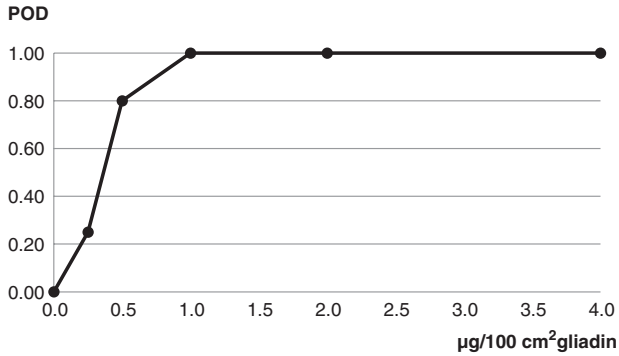

Figure 4. POD for surface silicone rubber (swabbing procedure); 20 replicates per gliadin amount.

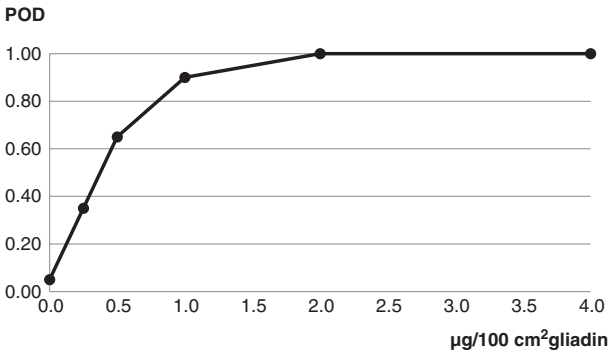

Figure 5. POD for surface sealed ceramic (swabbing procedure); 20 replicates per gliadin amount.

experiments. The tiles were produced by Lasselsberger s.r.o. (Pilsen, Czech Republic; www.rako.eu; W1301) and obtained from a local retailer in Germany.

Before starting the validation experiment, the tiles were cleaned with 60% ethanol and areas of 10 × 10 cm were marked. To check for effectiveness of the cleaning procedure, five randomly chosen tiles were swabbed and further tested as described. Results were in all cases negative, even after the first use and subsequent cleaning with 60% ethanol of the tiles.

After swabbing, the tiles were cleaned with 60% ethanol. Areas of 10 × 10 cm were marked again, and the areas were contaminated again with gliadin until all repeats were performed.

Besides only one false-positive out of 20 replicates, the concentration range with negative and positive results was between 0.25 and 1.0 µg/100 cm^2 gliadin (Figure 5). Starting with 2 µg/100 cm^2 gliadin (4 µg/100 cm^2 gluten), all results were positive.

Figure 5 shows the POD response curve, which was used to evaluate the $LOD_{95\%}$ concentration. Using a 4-parameter curve fitting a $LOD_{95\%}$ concentration of 1.4 µg/100 cm^2, gliadin (2.8 µg/100 cm^2 gluten) was estimated.

(**b**) *CIP waters (contaminated).*—Amounts of gluten in the CIP waters with and without cleansing solutions were set by the protein content of the Sigma gluten material. Respective volumes of a gluten stock solution containing 1 mg/mL total protein were pipetted into the diluted cleansing reagents or water. Five different concentrations including zero were repeated for 20 times in a random blinded pattern. Each spiked solution was diluted with sample diluent directly before analysis according to the procedure described above. Four different CIP waters were tested according to recommendations from a gluten-free food producer from Austria.

(1) Mikro-Quat Classic.—According to the manufacturer Ecolab, the cleansing reagent is used to remove fat and strong contaminations. It shows antimicrobial activities. Mikro-Quat Classic contains alkyldimethylphenylammonium chloride, dodecyldimethylammonium chloride, ethanol-amine, and fatty alcohol ethoxylates. The cleansing reagent was diluted to the manufacturer's recommendation at a level of 1%.

No false-positive results out of 20 replicates were found (Figure 6). The concentration range with negative and positive results was between 25 and 50 ng/mL gluten. For concentrations of 100 and 200 ng/mL, only positive results were obtained.

Figure 6 shows the POD response curve, which was used to evaluate the $LOD_{95\%}$ concentration. Using a 4-parameter curve fitting an $LOD_{95\%}$ concentration of 66 ng/mL, gluten was estimated.

(2) Acifoam VF10.—The manufacturer Johnson Diversey describes Acifoam VF10 as suitable to remove scale and protein films. It contains phosphoric acid, alkylphenylsulfonic acid, (2-methoxymethyl-ethoxy)propanol, and sodium cumenesulfonate. The cleansing reagent was diluted to the manufacturer's recommendation at a level of 10%.

The rate of false-positive results out of 20 replicates was zero (Figure 7). Negative and positive results were observed at 25 ng/mL gluten, whereas for concentrations at or higher than 50 ng/mL, only positive results were obtained. At 200 ng/mL, two false-negative results were obtained. Because results from all surfaces and other cleansing reagents at high concentrations were consistent, it can be assumed that in the case of Acifoam VF10, a technical error occurred.

Figure 7 shows the POD response curve. In this case, a 4-parameter curve fitting was not successful, so an $LOD_{95\%}$ concentration worst-case scenario of 50 ng/mL (100% positive results) was assumed.

(3) Divosan Extra VT55.—The antimicrobial cleansing reagent Divosan Extra VT55 from Johnson Diversey contains alkyldimethylphenylammonium chloride and sodium hydroxide. The reagent was diluted to the manufacturer's recommendation at a level of 1.8%.

No false-positive results out of 20 replicates were found (Figure 8). Negative and positive results were observed at 25 ng/mL gluten, whereas for concentrations at or higher than 50 ng/mL, only positive results were obtained. Figure 8 shows

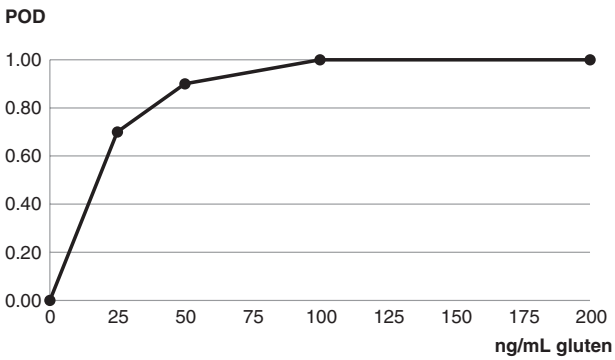

Figure 6. POD for cleansing reagent Mikro-Quat Classic; 20 replicates per gluten amount.

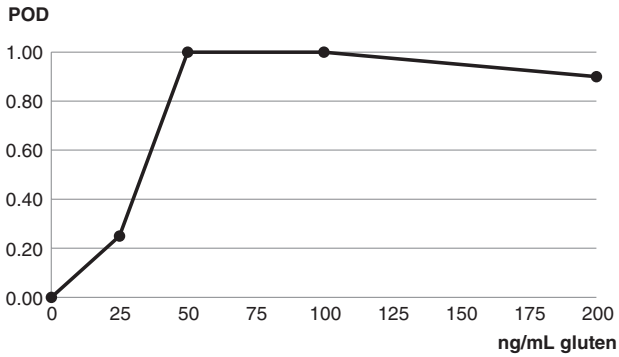

Figure 7. POD for cleansing reagent Acifoam VF10; 20 replicates per gluten amount.

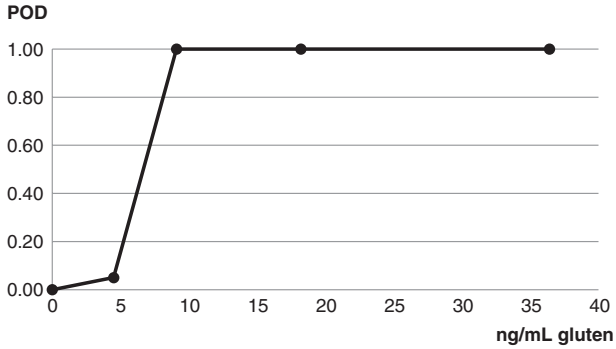

Figure 9. POD for water with no cleansing reagent; 20 replicates per gluten amount.

the POD response curve. In this case, a 4-parameter curve fitting was not successful, so an $LOD_{95\%}$ concentration worst-case scenario of 50 ng/mL (100% positive results) was assumed.

(4) Water.—In the case of pure water, the ratio between sample volume and sample diluent (buffer) can be increased so that a lower LoD is expected (*see* paragraph dip-stick testing).

The rate of false-positive results out of 20 replicates was zero (Figure 9). Negative and positive results were observed at 4.5 ng/mL gluten, whereas for concentrations at or higher than 9.1 ng/mL, only positive results were obtained. Figure 9 shows the POD response curve. In this case, a 4-parameter curve fitting was not successful, so an $LOD_{95\%}$ concentration worst-case scenario of 9.1 ng/mL (100% positive results) was assumed.

(5) Hypofoam VF6.—This cleansing reagent produced by Johnson Diversey contains sodium hydroxide, sodium hypochlorite, and surfactants. It was diluted to the manufacturer's recommendation at a level of 16.7%. No data are shown because all spiked samples came out negative. It was assumed that the high pH value of the diluted reagent in combination with the very high concentration of the strong oxidizing agent sodium hypochlorite caused these problems. Titrating the pH value to neutral values and subsequent spiking with gluten exerted positive results (the oxidizing strength of hypochlorite diminishes at lower pH). In contrast, spiking of the basic reagent solution and subsequent pH adjustment also resulted in negative results. Therefore, it can be concluded that under practical conditions, this cleansing reagent destroys gluten very quickly (probably by oxidation) and antibody recognition is not possible any longer. If the resulting oxidized breakdown products still exert immunotoxic reaction in celiac patients is not clear.

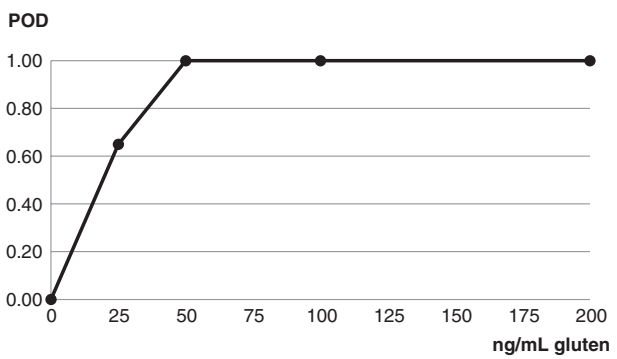

Figure 8. POD for cleansing reagent Divosan Extra VT55; 20 replicates per gluten amount.

Robustness

According to OMA Appendix N (3), no ruggedness testings are required when validating a qualitative method. Nevertheless, two collaborative tests were already performed with this system using an unprocessed and a processed food matrix. Ruggedness from collaborative tests included random variation of results due to different participants, temperatures, time, and volumes. Because the outcome of both collaborative tests was excellent (5, 6), the influence of these varying parameters in a lab should be small. Nevertheless, two parameters were tested systematically for their ruggedness, incubation time and incubation temperature.

For testing of robustness for the incubation time and temperature, it was not necessary to use naturally contaminated samples. Therefore, a blank rice meal was spiked with different amounts of WGPAT gliadin to obtain concentrations of 0.5 to 2 mg/kg gliadin. All components used for extraction and dip-stick analysis were brought to the respective temperatures prior to extraction and analysis. Thus, a complete change in laboratory temperature was simulated. Using these samples for ethanol extraction and subsequently for dip-stick evaluation (n = 10 for each variation), it was revealed that there is no significant influence of a change of temperature between 16 and 30°C (Table 3).

The number of positive results for the sample with a gliadin concentration of 1 mg/kg rises when the incubation time was increased from 4 to 6 min (Table 3). This effect is only visible in a very small concentration range because at 0.5 mg/kg, no sample was positive (except one outlier), whereas at 2 mg/kg, all samples were positive irrespective of their incubation time. Nevertheless, for best reproducibility, the test kit manual strongly emphasizes that the readout should be performed exactly after 5 min.

Lot-to-Lot Consistency

Three lots were checked for lot-to-lot consistency with matrix-containing samples (Table 4) and 20 replicates per concentration. Results for ethanol extraction in Table 4 reveal that all three lots are comparable. There is only a very small variation for the POD values at 1 mg/kg gliadin using the ethanol extraction. Results are identical for 0.0, 0.5, and 2.0 mg/kg. Comparable results were obtained when a processed cookie

Table 3. Comparison of three different incubation temperatures (16, 23, 30°C) and three different incubation times (4, 5, 6 min) for the dip-stick procedure (10 repeats for each condition and concentration); WGPAT gliadin preparation was used for spiking

Gliadin, mg/kg	Temperature, °C	POD	Time, min	POD
0.50	16	0.0	4	0.0
	23	0.1	5	0.1
	30	0.0	6	0.0
1.00	16	0.2	4	0.2
	23	0.4	5	0.4
	30	0.2	6	0.6
2.00	16	1.0	4	1.0
	23	1.0	5	1.0
	30	1.0	6	1.0

sample after Cocktail extraction was used (Table 4). Due to the higher dilution of the extracted sample before measurement, the concentration that indicates the extent of variation is at 2 mg/kg, compared with 1 mg/kg for the sample extracted with ethanol. Again, all three lots were highly comparable.

Independent Validation Study

Design of Experiment

The evaluation consisted of evaluating stainless steel (10 × 10 cm test area) and a CIP solution (Mikro-Quat Classic). Each matrix was evaluated for six levels of contamination, with 20 or 30 replicates per level as outlined in Table 5.

The stainless steel and the Cleaning-in-Place reagent were artificially contaminated with gluten. For the inoculation of the stainless steel, the gluten concentration was achieved by preparing a stock solution of 1.205 mg/mL Sigma Gluten (G5004; Lot number SLBR5510V) in 60% ethanol to a concentration of 1.0 mg/mL gluten. The stock solution was then diluted with 60% ethanol to the following concentrations of gluten: 2.5, 5, 10, 20, and 40 µg/mL. During the inoculation of

Table 4. Lot-to-lot comparability for rice flour spiked with WGPAT gliadin and extracted with 60% ethanol and for a cookie spiked with WGPAT gliadin and extracted with Cocktail solution; results for 20 replicates per concentration level are given

	Gliadin, mg/kg				
Lot No.	0.0	0.5	1.0	2.0	4.0
	Ethanol				
E 94	0.0	0.0	1.0	1.0	—[a]
E 93	0.0	0.0	0.45	1.0	—
E 95	0.0	0.0	0.75	1.0	—
	Cocktail				
E 94	0.0	—	0.0	0.1	1.0
E 93	0.0	—	0.0	0.0	1.0
E 95	0.0	—	0.0	0.1	1.0

[a] Not analyzed

Table 5. Matrices and target levels

Matrix	Contaminant	Target level	Replicates
Stainless steel	Gluten	0.0 µg/100 cm^2	20
		0.25 µg/100 cm^2	30
		0.5 µg/100 cm^2	30
		1.0 µg/100 cm^2	30
		2.0 µg/100 cm^2	30
		4.0 µg/100 cm^2	20
Cleaning-in-Place (Mikro-Quat Classic)	Gluten	0.00 µg/mL[a]	20
		0.013 µg/mL[a]	30
		0.025 µg/mL[a]	30
		0.05 µg/mL[a]	30
		0.10 µg/mL[a]	20
		0.20 µg/mL[a]	20

[a] Gluten concentration before final dilution with Cleaning-in-Place reagent.

stainless steel, replicate 100 µL aliquots of each concentration were placed onto separate 10 × 10 cm test areas. For the un-inoculated control test areas, a 100 µL aliquot of 60% ethanol was added onto each surface area. Each test area was spread evenly and allowed to dry at room temperature (20–25ºC) for at least 1 h, in a gluten-free environment.

Prior to the inoculation of the CIP solution, the Cleaning-in-Place solution was diluted in water to a 1% volume. The stock solution, prepared as previously described, was diluted with 1% CIP to the following concentrations of gluten: 0.0125, 0.025, 0.05, 0.1, and 0.2 µg/mL.

Calculation of POD and Estimation of LOD_{95}

As per criteria outlined in Appendix N: ISPAM Guidelines for Validation of Qualitative Binary Chemistry Methods (3), fractional positive results were obtained for each matrix. For the analysis of each test matrix, the POD was calculated as the number of positive outcomes divided by the total number of trials (7). The POD, along with 95% confidence interval, was calculated for each concentration level. The POD versus the gluten concentration was plotted in order to report the LOD_{95} for each matrix. A 4-parameter sigmoid curve (8) for curve fitting was used.

Results for Stainless Steel

Figure 10 depicts the graphical presentation of POD versus concentration with 95% confidence intervals. In detail, the following results were obtained for each contamination level:

0.0 µg/100 cm^2.—For stainless steel, the 0.0 µg/100 cm^2 concentration level produced 0 observed positive sample results. A POD value of 0.00 was obtained with 95% confidence interval at 0.00–0.16.

0.25 µg/100 cm^2.—For stainless steel, the 0.25 µg/100 cm^2 concentration level produced 15 observed positive sample results. A POD value of 0.50 was obtained with 95% confidence interval at 0.33–0.67.

0.5 µg/100 cm^2.—For stainless steel, the 0.5 µg/100 cm^2 concentration level produced 21 observed positive sample results. A POD value of 0.70 was obtained with 95% confidence interval at 0.52–0.83.

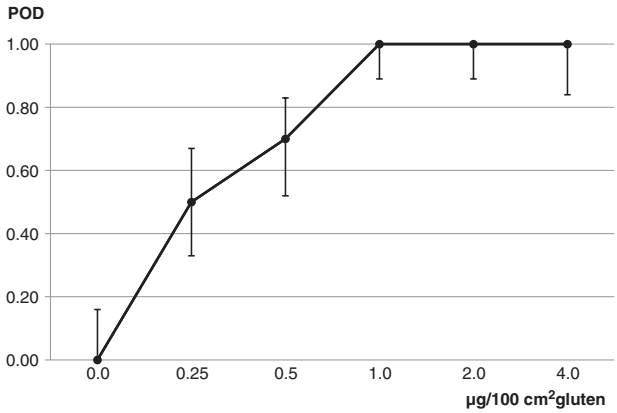

Figure 10. POD versus concentration for the detection of gluten on stainless steel.

1.0 µg/100 cm².—For stainless steel, the 1.0 µg/100 cm² concentration level produced 30 observed positive sample results. A POD value of 1.00 was obtained with 95% confidence interval at 0.89–1.00.

2.0 µg/100 cm².—For stainless steel, the 2.0 µg/100 cm² concentration level produced 30 observed positive sample results. A POD value of 1.00 was obtained with 95% confidence interval at 0.89–1.00.

4.0 µg/100 cm².—For stainless steel, the 4.0 µg/100 cm² concentration level produced 20 observed positive sample results. A POD value of 1.00 was obtained with 95% confidence interval at 0.84–1.00.

For stainless-steel environmental surfaces, an LOD_{95} of 1.1 µg/ 100 cm² was determined (graph not shown).

Results for CIP Solution

Figure 11 depicts the graphical presentation of POD versus concentration with 95% confidence intervals. In detail, the following results were obtained for each contamination level:

0.0 µg/mL.—For CIP solution, the 0.0 µg/mL concentration level produced 0 observed positive sample results. A POD value of 0.00 was obtained with 95% confidence interval at 0.00–0.16.

0.0125 µg/mL.—For CIP solution, the 0.0125 µg/mL concentration level produced 0 observed positive sample results. A POD value of 0.00 was obtained with 95% confidence interval at 0.00–0.11.

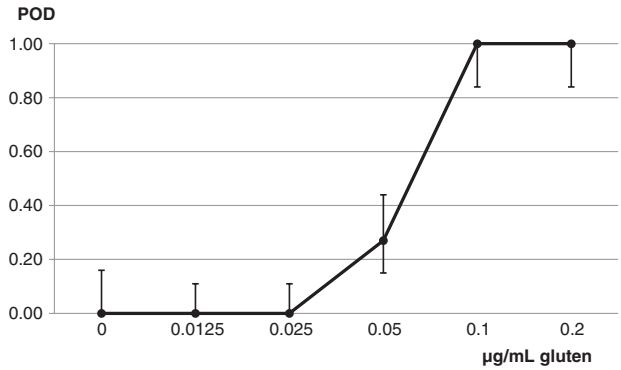

Figure 11. POD versus concentration for the detection of gluten in Cleaning-in-Place solution.

0.025 µg/mL.—For CIP solution, the 0.025 µg/mL concentration level produced 0 observed positive sample results. A POD value of 0.00 was obtained with 95% confidence interval at 0.00–0.11.

0.05 µg/mL.—For CIP solution, the 0.05 µg/mL concentration level produced 8 observed positive sample results. A POD value of 0.27 was obtained with 95% confidence interval at 0.14–0.44.

0.1 µg/mL.—For CIP solution, the 0.1 µg/mL concentration level produced 20 observed positive sample results. A POD value of 1.00 was obtained with 95% confidence interval at 0.84–1.00.

0.2 µg/mL.—For CIP solution, the 0.2 µg/mL concentration level produced 20 observed positive sample results. A POD value of 1.00 was obtained with 95% confidence interval at 0.84–1.00.

For CIP solutions, an LOD_{95} of 0.06 µg/mL was determined (graph not shown).

Discussion of In-House and Independent Laboratory Validation

The immunochromatographic dip-stick RIDA®QUICK Gliadin investigated in this validation study was demonstrated to be applicable for the detection of traces of gliadin on surfaces and in CIP waters. The in-house validation included a target and nontarget compound study, a matrix study with four different surfaces, different cleansing reagents, a lot-to-lot comparability, and ruggedness testing.

The claimed target prolamins (gliadin, secalin, and hordein) were shown to react in a comparable manner as the "reference" WGPAT gliadin preparation. The 85 nontarget compounds (oats, pseudocereals, vegetables, seeds, nuts, fruits, spices, and alternative protein sources such as egg, milk, and soy) were checked to be gluten-free (<5 mg/kg gluten) with the R5 sandwich ELISA (AOAC Final Action OMA **2012.01**). The subsequent analysis with the dip-stick revealed no positive results, which proved the selectivity of the method.

Stainless-steel, plastic, sealed ceramic, and silicone rubber surfaces contaminated with WGPAT gliadin showed corresponding gluten concentrations at which all results were positive at 4.0, 4.0, 4.0, and 2.0 µg/100 cm², respectively. Using a 4-parameter curve fitting, $LOD_{95\%}$ concentrations of 3.0, 1.6, 2.8, and 1.6 µg/100 cm², respectively, were estimated.

Three chemically different cleansing reagents were spiked with gluten and revealed 100% positive results at or above 50–100 ng/mL gluten. Spiking of clean water with gluten resulted in 100% positive read-outs at or above 9.1 ng/mL gluten.

Ruggedness testing included the analysis of variation of incubation temperature of the dip-stick analysis (16, 23, 30°C) and incubation time of the dip-stick (4, 5, 6 min). Variation of temperature was found to have no influence on the result in a way that could be critical under practical conditions. The longer the incubation time, the higher the probability of detection for a given concentration. Therefore, the test kit insert clearly recommends incubating the dip-stick for exactly 5 min. Nevertheless, this effect is only visible at very low concentrations and not at the legal threshold. All tested lots were comparable using spiked samples after ethanol or Cocktail extraction.

An independent laboratory validation study using contaminated stainless-steel surfaces and spiked CIP solutions proved that the manufacturer's claims are correct. The RIDA®QUICK Gliadin is a fast and easy-to-use

immunochromatographic test to determine the presence of gluten at a concentration as low as 0.25 µg/100cm^2 for stainless steel and 0.05 µg/mL for Cleaning-in-Place solutions.

Conclusions

In summary, the data of the in-house validation study proved that the performance claims for surfaces and CIP waters are fulfilled. Together with the information obtained by the collaborative test on food matrices that led to the AOAC Final Action *Official Method*^{SM} **2015.16** and the validation data presented in this document, the RIDA®QUICK Gliadin should be recommended as AOAC *Performance Tested Method*^{SM} for surfaces and CIP waters.

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Dedicated to Peter Köhler for his scientific service to the analytical gluten community.

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