Evaluating the Performance of Gluten ELISA Test Kits: The Numbers Do Not Tell the Tale

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ABSTRACT

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A wide variety of enzyme-linked immunosorbent assays (ELISAs) are commercially available for gluten detection in food, including new formats and assays with antibodies against relevant gluten epitopes. Nevertheless, problems persist to accurately determine the gluten content of products. In this study, the performance of a set of 14 ELISA kits for gluten detection, representative of the current ELISA methods available on the market, was evaluated. These tests were used to determine gluten content in a series of relevant food matrices varying in complexity. Our results show that, currently, there is no single ELISA method that can accurately detect and quantify gluten in all different matrices. This includes the current type I method R5 as recommended by Codex Alimentarius. We conclude that further improvements are urgently needed and recommend focusing on competitive formats, improving extraction methods, and the detection of relevant gluten peptides (in order of priority).

Approximately 1% of the global population is afflicted with celiac disease (CD), also known as gluten intolerance (Lionetti and Catassi 2011). When a genetically susceptible individual is exposed to gluten, it leads to an inflammatory response in the small intestine, which damages the mucosa. Symptoms can vary between patients and may include abdominal pain, diarrhea, and complaints related to malabsorption of nutrients. Although several options for treatment are being investigated, the only treatment at this moment is lifelong adherence to a strict gluten-free diet (McAllister and Kagnoff 2012). This underpins the importance of tools to help secure the absence of gluten in food ingredients and food products that are intended to be gluten-free.

Gluten refers to the storage proteins found in wheat, rye, barley, and crossbreeds thereof. Oats seem to be safe for the majority of CD patients, although this is still the subject of debate (Pulido et al. 2009; Comino et al. 2011). Gluten can be divided into prolamin and glutelin groups, based on their ability to form larger disulfide-linked polymeric aggregates. Gliadins, the prolamins from wheat, are considered to be most harmful, although sequences found in secalins (rye), hordeins (barley), and glutenins (wheat) have been proven to be damaging for CD patients as well (Camarca et al. 2012). To help CD patients maintain their gluten-free diet, regulations have been set in place to define what gluten-free exactly means, so products can be labeled accordingly. In Europe, the European Commission adopted Commission Regulation 41/2009, which was replaced by Commission Regulation 1169/2011 by the end of 2014 (Commission of the European Communities 2009; European Commission 2011). These regulations define two thresholds for gluten. A product that contains less than 20 mg/kg of gluten can be labeled "gluten-free," and a product that contains 20–100 mg/kg of gluten can be labeled "very low gluten." In practice, only the gluten-free label is relevant for CD patients, because most of the patients risk relapse of symptoms and progression of the disease when exposed to more than 10 mg of gluten per day (Catassi et al. 2007). Therefore, it is important that the gluten content of foods can be accurately quantified to check ingredients, formulate food products within the gluten-free limits, and label them correctly.

Currently, the method of choice for gluten detection in food is the enzyme-linked immunosorbent assay (ELISA). Since 2006, the R5 method is the type I method to quantify gluten in food, according to the Codex Alimentarius Commission (2014). However, despite this standard method being available, problems in gluten detection still remain, mainly because of difficulties in extraction and the lack of a standardized reference material for calibration (Diaz-Amigo and Popping 2013; Bruins Slot et al. *in press*). Many food matrices remain difficult to measure either owing to interference of the food matrix with antibody binding, cross-reactivity, or problems in extracting the gluten from the food matrix (Wieser 2008; Mena et al. 2012; Török et al. 2014). It is perhaps for this reason that a range of new and alternative options to the R5 method have become available. These alternative methods differ in, for example, the format, antibody, extraction, and calibration. Examples are the G12 and α-20 antibody-based kits; both target confirmed harmful epitopes, leading producers to suggest that these newer methods will replace the current type I method in time (Morón et al. 2008; Mujico et al. 2012). Furthermore, competitive methods have become available that are also able to detect smaller gluten fragments. In principle, these competitive methods are more suited than the standard non-competitive sandwich-type ELISA methods to detect hydrolyzed gluten that occurs in products such as beer.

Thus far, only a limited number of comparative studies for gluten ELISA kits have been performed. Geng et al. (2008) investigated effects of food matrices and extraction procedures on the detection of gluten. Recovery of gluten spiked in gluten-free bread mix and guar gum was determined by using five different sandwich ELISAs and one lateral flow device, after extracting either with ethanol or a cocktail solution containing detergent and reducing agents. Large differences were seen between the test kits and between the extraction methods, with the cocktail solution extracting up to twice the amount of gluten compared with ethanol. Allred and Ritter (2010) also looked at the differences in extraction between ethanol and cocktail solution and compared the difference in affinity for gliadin and glutenin of the R5 and 401/21 antibody. They found that the cocktail solution containing 2-mercaptoethanol resulted in a better extraction of gluten than ethanol, in some cases for both the gliadin and the glutenin fraction. The test kits based on the 401/21 antibody had a higher affinity for glutenin compared with gliadin. Furthermore, analysis of 40 unknown samples for gluten content showed more than 20-fold differences in results between the four different test kits, which were all performed with the extraction procedure recommended by their manufacturers. Sharma (2012) compared the reactivity of antibodies from six sandwich ELISAs to different wheat protein fractions. The results were in agreement with those from Allred and Ritter, as far as they concerned the higher affinity of the 401/21 antibody for the glutenin fraction over the gliadin fraction. After spiking gluten-free maize flour with either gluten or wheat flour, the six kits reported more than 30-fold

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differences in recovery. Schwalb et al. (2011) compared four sandwich ELISAs for their recovery of different commercially available gliadin and gluten reference materials, as well as for detection of gluten-containing cereals. The difference between an R5 sandwich and an R5 competitive method was also examined in these cereals. Again, large differences in recoveries were seen between the different sandwich ELISAs. The competitive method was shown to detect 26% more gluten in wheat, 49% more gluten in rye, and 82% more gluten in barley, compared with the sandwich method. More recently, Bugyi et al. (2013) compared the recovery of Prolamin Working Group (PWG) gliadin reference material in dry cookie ingredients, cookie dough, and baked cookies by seven sandwich ELISAs. Although the names of the test kits were blinded in the results section, the differences in recovery between the kits were clear, ranging up to almost 20-fold. These comparative studies make clear that large differences exist in both qualitative and quantitative detection of the available test kits. However, these studies have focused on only a limited number of the currently available test kits. Schwalb et al. (2011) were the only ones to include at least one competitive ELISA but did not compare samples containing gluten fragments. The α-20 antibody has not been included in any of these comparative studies yet. As for the samples, most of these studies use either spiked flour or purified gluten. This is a basic setup that gives little information about how the different test kits would perform in more realistic food matrices. Geng et al. (2008) included two different ingredient mixes, bread mix and powdered guar gum, but both of these did not pose a challenge to the test kits. Bugyi et al. (2013) compared the recovery of PWG gliadin reference material from two more challenging matrices, dough and cookie, with the recovery from dry powder mix. Both food matrices contained sugar, fat, and salt, and in case of the cookies, the reference material had undergone the baking process. In dough, the recovery of the reference material was comparable to that in dry powder mix for most kits, although some test kits reported a difference in recovery up to 20%, either higher or lower, compared with the dry powder mixture. After baking the cookies, however, the recovery of the reference material was lower compared with the dry powder mix for all kits, up to 70% for some of the kits.

The objective of this study was to evaluate the performance of a representative set of commercially available gluten ELISAs. To ensure a comprehensive challenge for all test kits included, not only raw materials and spiked samples but also different types of realistic food samples were selected to examine the effects of cross-reactivity, food matrices, interfering ingredients, and extractability. In this way, we aimed to unravel the strengths and weaknesses of current gluten ELISAs, including the current type I method.

MATERIALS AND METHODS

Test Kits. First, an inventory of the ELISA methods for gluten detection, available worldwide in early 2013, was made through extensive online and literature research. From a total of 23 ELISAs, three kits were not considered: the TRANSIA Plate Gluten test kit (BioControl Systems, Bellevue, WA, U.S.A.) was excluded because of its relatively high limit of detection (LoD) compared with the other kits available; the Gliadin ELISA test kit (Immunotech Beckman Coulter, Brea, CA, U.S.A.) was excluded because it is not commercially available; and the Wheat Protein ELISA test kit (Morinaga Institute of Biological Sciences, Yokohama, Japan) was excluded because it detects wheat protein instead of gluten. The RIDASCREEN FAST Gliadin test kit was excluded in favor of the RIDASCREEN Gliadin test kit (R-Biopharm, Darmstadt, Germany), which is the recommended type I method. The Veratox for Gliadin test kit was excluded in favor of the Veratox for Gliadin R5 test kit (Neogen, Lansing, MI, U.S.A.). Manufacturers of the remaining 18 kits were contacted to participate in the study. Of these, 12 different ELISAs were freely supplied (companies mentioned in the acknowledgments) and three ELISAs were purchased. The Gliadin/Gluten test kit (Immunolab, Kassel, Germany) and the Gluten/Gliadin test kit (Diagnostic Automation, Calabasas, CA, U.S.A.) were found to be the same detection method sold under different names by different producers, and only Gliadin/Gluten test kit was included. The Gluten ELISA test kit (Astori Lab, Flero, Italy), TRANSIA Plate Prolamins test kit (BioControl Systems), and Gliadin assay (ELISA Systems, Windsor, Australia) could not be included in the study because we did not receive a reply from the provider; as a result, acquisition of the kits was not possible. The TRANSIA Plate Prolamins test kit is a sandwich R5 method, the Gliadin assay is a sandwich 401/21 method, and the Gluten ELISA is a competitive polyclonal method. All these methods were covered by other test kits that were included in this study; therefore, the representativeness of the selected group was not compromised by the absence of these three test kits. The 14 different ELISA kits used during this evaluation are summarized in Table I.

Reference Material. The gliadin reference material from the PWG (PWG gliadin) was kindly provided by the chairman of the PWG. This is a well-characterized material made of a mixture of 28 European wheat varieties that has been produced to be used as

TABLE I
List of Commercial ELISA Test Kits Evaluated in This Study[a]

Test Kit	Manufacturer	Format	Antibody	LoD	LoQ	Calibration	Lot Number
GlutenTox ELISA Competitive	Biomedal	Competitive	G12	1.5	1.5	Gliadin	111247
GlutenTox ELISA Sandwich	Biomedal	Sandwich	G12	0.3	0.3	Gliadin	190148
Gluten-Check ELISA	Bio-Check (UK) Ltd.	Sandwich	401/21	0.25	2.5	PWG Gliadin	7407
ALLER-TEK Gluten ELISA Assay	ELISA Technologies	Sandwich	401/21	2.5	2.5	Gluten	GLU140924-24
GLUTEN-TEC ELISA	EuroProxima	Competitive	α-20	2.5	2.5	Synthetic α-20 peptides	PN6902
Gliadin/Gluten	Immunolab	Sandwich	Polyclonal	0.3	2.0	Gliadin	GLI-130
GlutenAlert ELISA	InCura	Competitive	Polyclonal	1.0	2.5	NIST Gluten	GA-14349312
Ingezim Gluten	Ingenasa	Sandwich	R5	1.5	1.5	Gliadin European Standard	280514
Ingezim Gluten Hidrolizado	Ingenasa	Direct	R5	0.125	0.125	Gliadin European Standard	040414
BioKits Gluten Assay	Neogen	Sandwich	401/21	0.5	1.5	Gluten extracts, Kjeldahl analyzed	205489
Veratox for Gliadin R5	Neogen	Sandwich	R5	2.0	2.5	Gliadin	208566A
RIDASCREEN Gliadin	R-Biopharm	Sandwich	R5	1.5	2.5	PWG Gliadin	13434
RIDASCREEN Gliadin Competitive	R-Biopharm	Competitive	R5	1.36	5.0	Hydrolyzed wheat, rye, barley mixture	13174
AgraQuant Gluten G12	Romer Labs	Sandwich	G12	1.0	2.0	Gluten	GU1011-1409

[a] Limit of detection (LoD) and limit of quantification (LoQ) expressed as mg/kg of gliadin, as stated in the relevant user manual accompanying each kit or derived from the lowest gluten/gliadin standard provided with the test kit. PWG = Prolamin Working Group, and NIST = National Institute of Standards and Technology. Manufacturer locations: Biomedal (Sevilla, Spain); Bio-Check (UK) Ltd. (St. Asaph, U.K.); ELISA Technologies (Gainesville, FL, U.S.A.); EuroProxima (Arnhem, the Netherlands); Immunolab (Kassel, Germany); InCura (Casalmaggiore, Italy); Ingenasa (Madrid, Spain); Neogen (Lansing, MI, U.S.A.); R-Biopharm (Darmstadt, Germany); and Romer Labs (Runcorn, U.K.).

a reference material in antibody-based assays (van Eckert et al. 2006).

Flour Samples and Food Products. Whole wheat flour, whole rye flour, and barley flour were selected as gluten-containing flours and were purchased from a local mill (Windkorenmolen 'De Vlijt', Wageningen, the Netherlands). Rice flour (Dove's Farm, Hungerford, U.K.) and maize flour (Joannusmolen, Cuijk, the Netherlands) were selected as gluten-free flours and were ordered from a webshop specializing in gluten-free products (www.glutenvrijewebshop.nl). Food products were selected to present different challenges in detection. Further details of these products are presented in Table II. Biscuits were chosen to represent a product high in sugar and fat. Especially fat might interfere with extraction methods in ELISA protocols (Wieser 2008). Pasta was chosen because it is an extruded food product. Extrusion can modify gluten proteins and make extraction more difficult (Fischer 2004). For both products, one gluten-containing and one gluten-free product were selected. Soup was included because of its high salt content, which again might interfere with the kits (Wieser 2008). Beer was included because it contains mostly gluten peptides, making detection more difficult for ELISA tests (Hernando et al. 2004). Oat flour was included as a naturally gluten-free product that is often contaminated with gluten-containing cereals (Hernando et al. 2004). Finally, baby food, soy sauce, and spice mix were included because they are on the list of the Dutch Celiac Disease Society as products that are often reported by their members to give CD-related complaints, even though they carry the gluten-free label. Spice mixes typically contain large amounts of salts, peptides, and phenolic compounds that may interfere with both the extraction and detection (Wieser 2008). The same holds true for soy sauce, a fermented (i.e., partially hydrolyzed) product that is rich in Maillard reaction products. Baby food contains less than 3% fat and is free from added sugar, salt, and spices. Therefore, no high level of interfering substances is expected in this type of product. All food products were purchased from a local store (Albert Heijn, Wageningen, the Netherlands), except the oat flour, which was provided by Plant Research International, Wageningen UR (Wageningen, the Netherlands).

Spiked Samples. The protein contents of the whole wheat flour, whole rye flour, and barley flour were first determined by Kjeldahl analysis. Assuming that 80% of the total protein content is gluten, the flours contained 86, 79, and 72 g/kg of gluten for wheat, rye, and barley, respectively. Three aliquots of gluten-free rice flour were spiked separately with whole wheat flour, whole rye flour, or barley flour each at levels of 1,000 mg/kg. This resulted in flour mixtures containing 86, 79, and 72 mg/kg of gluten, respectively, which were all well above the European threshold of 20 mg/kg of gluten. The flours were mixed in liquid nitrogen with a mortar and pestle. To determine the homogeneity of the mixtures, the standard deviation and coefficient of variation (CV%) values of three to four replicates were analyzed by one of the test kits selected randomly (Gliadin/Gluten, Immunolab). Furthermore, rice flour samples were spiked with PWG gliadin reference material. To this end, the PWG gliadin was dissolved in 60% v/v ethanol and subsequently diluted in 60% v/v ethanol. Samples were spiked individually with a volume of 1% of the total extraction solvent volume, resulting in spike levels of 10 and 2.5 mg/kg. The level of 10 mg/kg of gliadin was chosen to represent the gluten threshold of 20 mg/kg, assuming that 50% of gluten consists of gliadin. The spike level of 2.5 mg/kg was chosen to represent a gliadin level around the LoQ of most of the ELISA kits included in this study.

All solid food products were homogenized by grinding and mixing with a mortar and pestle or a chopper (La Moulinette, Moulinex, Ecully, France). Liquid products were homogenized by intensive stirring with a vortex mixer. Aliquots from the baby food, soy sauce, and spice mix were spiked with 10 mg/kg of PWG gliadin reference material in the same manner as described earlier for the flours.

Evaluation Procedure. A two-step approach was used to evaluate the different ELISAs. First, the ELISAs were tested with gluten-containing and gluten-free flours, as well as gluten-free flour spiked with the PWG gliadin reference material. During this first round, the ELISAs were scored on three different points: 1) the ability to accurately quantify the gliadin reference material spike of 10 mg/kg (a positive score corresponds to a recovery between 50 and 150%, which translates to 5–15 mg/kg of gliadin); 2) no false positives (above 10 mg/kg of gliadin) in gluten-free rice and gluten-free maize flour; and 3) no false negatives (below 10 mg/kg of gliadin) in the gluten-containing wheat, rye, and barley flour samples. The test kits had to score positively on at least two out of three criteria to be included in the second round.

In this second round, we examined the performance of the test kits in food products and determined their ability to indicate the presence or absence of gluten according to the currently applied European gluten threshold of 20 mg/kg, which would translate to 10 mg/kg of gliadin. Furthermore, we examined the ability of the test kits to quantify the gliadin reference material spike of 10 mg/kg in a selection of these products. For the second round, the same criteria were used as in the first round. With respect to the recovery of the gliadin spikes, we calculated the number of necessary replicates by using a power analysis for a two-sided test. The significance level was set at 0.05, and sigma was estimated at 2.0 mg/kg. A sample size of three would result in a power of 0.99. Based on this analysis, we decided to analyze three independent samples for all flours and food products.

Analysis. All tests were performed in the laboratory of RIKILT Wageningen University and Research Centre, closely following the enclosed user manual for each kit. Extracts of all flour and food product samples were prepared in triplicate. Each sample was diluted to best fit the linear range of the test kit used and analyzed in duplicate. All ELISA plates were read on an ELx808 absorbance microplate reader (BioTek, Winooski, VT, U.S.A.) with Gen5 data analysis software (BioTek). Standard curves for each test kit were plotted and accepted when $R^2 \geq 0.95$. If the test kits reported results

TABLE II
Food Products Used in Evaluation

Type of Food	Manufacturer	Product	Product/EAN[a]	Label
Baby food	Nutricia	Olvarit 8+ stoofpotje	8712400003193	Gluten-free
Beer	Amstel Brewery	Amstel pilsener	8712000032029	Contains gluten
Biscuit	Verkade	Knappertjes	8710412236172	Contains gluten
Biscuit, gluten-free	Schär	Butterkeks	8008698003077	Gluten-free
Oat flour	Plant Research International, Wageningen UR	Cultivar 'Gigant Lelystad'	…	Gluten-free
Pasta	Albert Heijn	AH spaghetti	8710400182061	Contains gluten
Pasta, gluten-free	Schär	Spaghetti	8008698004005	Gluten-free
Soup	Honig	Koninginnesoep	8714700219007	May contain traces of wheat
Soy sauce	Saitaku	Tamari soy sauce	5060194790076	Gluten-free
Spice mix	Albert Heijn	AH kruidenmix gehakt	8710400572626	Gluten-free

[a] EAN = international article number.

in mg/kg of gluten, this value was divided by two to obtain the results in mg/kg of gliadin. Results for the spiked samples were tested with a one-sample *t* test, two-tailed, against the spike value of 10 mg/kg. Results were found significant when $P < 0.05$. According to Abbott et al. (2010), recoveries of 80–120% are ideal for ELISAs, and recoveries of 50–150% are still acceptable for difficult matrices. For that reason, significant results for the spiked samples were only considered relevant if they fell outside the 80–120% range (flour) or the 50–150% range (baby food, spice mix, and soy sauce).

RESULTS

Homogeneity of Flour Samples. Flour mixtures used in this study were considered homogenous if the CV% was below 20%. Our analysis showed CV% values of 6.2, 7.8, and 14.1% for whole wheat flour, rye flour, and barley flour mixtures, respectively, indicating the flour mixtures were suitable for use in the study.

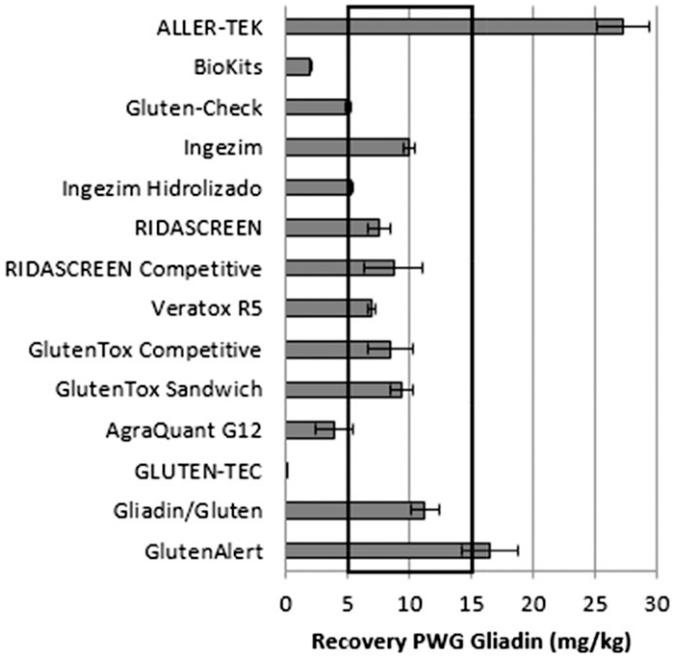

Fig. 1. Recoveries of 10 mg/kg of Prolamin Working Group (PWG) gliadin reference material in gluten-free rice flour. Error bars represent 95% confidence interval. Box represents accepted recovery range of 50–150%, corresponding to 5–15 mg/kg of PWG gliadin reference material.

Round One: Performance in Simple Matrices. In the first round, the recovery of the PWG reference material in gluten-free rice flour was determined for each kit. The first criterion of recovering the gliadin reference material in the accepted range of 50–150% was met by nine out of 14 kits (Fig. 1). The GLUTEN-TEC test kit did not detect the 10 mg/kg spike at all. The BioKits and AgraQuant G12 test kits reported low recoveries of the gliadin reference material, 20% ($P < 0.001$) and 39% ($P < 0.05$), respectively. The ALLER-TEK ($P < 0.01$) and GlutenAlert ($P < 0.05$) test kits reported a recovery of more than 150%. The amount of gliadin in the flour mixtures as quantified by the different test kits is shown in Table III. The gliadin spike of 2.5 mg/kg was not accurately quantified by most of the test kits because this level falls around or below their LoQ, which is the reason we did not include this spike level in the criteria. Nevertheless, the results seem to be consistent overall, and no outliers are reported. The second criterion was no false positive results in gluten-free flours. In the gluten-free rice flour, six out of 14 test kits did not detect any gluten (values below LoD). Five out of 14 test kits reported traces of gluten below their LoQ, and two out of 14 test kits reported traces around 2 mg/kg of gliadin. Only the GlutenTox Competitive test kit gave a false positive signal for the presence of gluten (not significant). In the gluten-free maize flour, five out of 14 test kits did not detect any gluten. Six out of 14 test kits reported gluten traces below their LoQ. Ingezim Hidrolizado, GlutenTox Competitive, and GlutenAlert test kits detected traces ranging from 0.2 to 7.9 mg/kg of gliadin. The final criterion was no false negative results in the gluten-containing flour mixtures. Thirteen out of 14 test kits were able to detect more than 10 mg/kg of gliadin in the wheat flour samples. GlutenTox Competitive reported 9.6 mg/kg of gliadin. However, this signal was lower than that in gluten-free rice flour, indicating a problem in this test kit. In the rye flour samples, 12 out of 14 test kits reported more than 10 mg/kg of gliadin. Only 10 out of 14 test kits managed to report more than 10 mg/kg of gliadin in the barley flour samples.

Based on these results, 11 out of 14 test kits met the criteria set for the first round and were included in the second round. Three kits (the BioKits, GlutenTox Competitive, and GlutenAlert test kits) were excluded from further experiments.

Round Two: Effects of Food Matrices and Food Processing. Gluten test kits should not only be able to assess the gluten content of raw materials and ingredients but also the gluten content of actual food products. We therefore tested the 11 selected kits to measure the gliadin content of five different food matrices. The gliadin contents detected by these gluten test kits are shown in Table IV.

Three out of 11 test kits detected gluten traces above their LoQ in the gluten-free biscuit, and two out of 11 did so in the gluten-free

TABLE III
Quantification of Gliadin (mg/kg) in Flour Mixtures[a]

Antibody	Test Kit	PWG Reference 2.5 mg/kg	PWG Reference 10 mg/kg	Rice GF	Maize GF	Wheat Mix 43 mg/kg	Rye Mix 40 mg/kg	Barley Mix 36 mg/kg
401/21	ALLER-TEK	5.7 ± 0.2**	27.3 ± 1.1**	<2.5	<2.5	88.0 ± 6.1	52.4 ± 2.0	41.4 ± 2.2
	BioKits	<1.5***	2.0 ± 0.0***	<1.5	<1.5	12.4 ± 0.7	10.4 ± 1.2	5.5 ± 0.1
	Gluten-Check	2.7 ± 0.1	5.1 ± 0.1***	<2.5	<2.5	41.2 ± 4.4	16.6 ± 0.1	9.4 ± 0.8
R5	Ingezim	2.5 ± 0.5	10.0 ± 0.2	<1.5	<1.5	74.4 ± 10.9	70.9 ± 5.2	190.1 ± 26.7
	Ingezim Hidrolizado	1.1 ± 0.1**	5.2 ± 0.1***	<0.125	0.2 ± 0.0	77.9 ± 8.4	82.2 ± 7.7	83.7 ± 3.5
	RIDASCREEN	<1.5***	7.5 ± 0.5*	<1.5	<1.5	63.1 ± 3.5	108.0 ± 11.7	166.5 ± 12.5
	RIDASCREEN Competitive	<5.0	8.7 ± 1.2	<5.0	<5.0	30.6 ± 5.4	53.2 ± 7.7	152.2 ± 27.2
	Veratox R5	<2.5	6.9 ± 0.2*	<2.0	<2.0	46.9 ± 3.2	90.9 ± 6.8	66.5 ± 6.1
G12	GlutenTox Competitive	3.1 ± 0.7	8.4 ± 0.9	10.3 ± 1.5	7.9 ± 0.9	9.6 ± 3.1	9.3 ± 2.1	11.0 ± 1.2
	GlutenTox Sandwich	2.2 ± 0.1	9.4 ± 0.4	<0.3	<0.3	67.0 ± 3.7	76.0 ± 2.1	103.6 ± 7.8
	AgraQuant G12	<2.0	3.9 ± 0.7*	2.2 ± 0.0	<2.0	26.2 ± 1.4	16.1 ± 3.1	33.0 ± 4.2
α-20	GLUTEN-TEC	<2.5	<2.5***	<2.5	<2.5	34.4 ± 2.0	86.8 ± 11.9	103.6 ± 4.4
Polyclonal	Gliadin/Gluten	3.1 ± 0.3	11.2 ± 0.6	<2.0	<2.0	67.9 ± 2.9	14.2 ± 1.9	7.3 ± 1.6
	GlutenAlert	3.6 ± 0.3	16.5 ± 1.1*	2.0 ± 0.1	2.8 ± 0.1	126.9 ± 6.9	3.0 ± 0.2	2.8 ± 0.2

[a] Values noted as mean ± SEM ($n = 3$). *, **, and *** indicate P value < 0.05, 0.01, and 0.001, respectively. PWG = Prolamin Working Group, and GF = gluten-free.

pasta. However, all reported values were lower than 10 mg/kg of gliadin, which would result in a total gluten content lower than the European threshold of 20 mg/kg for both products. One out of 11 test kits reported more than 10 mg/kg of gliadin in the soup samples. This was the Gliadin/Gluten test kit, which reported a gliadin content of 13.9 mg/kg. Only two out of 11 kits were able to detect hydrolyzed gluten in the beer samples above 10 mg/kg of gliadin. These were the RIDASCREEN Competitive and GLUTEN-TEC test kits, both competitive formats. All test kits reported levels of gliadin in the oat flour samples, ranging from 0.2 to 8.9 g/kg. The RIDASCREEN Competitive test kit reported a gliadin content that was notably higher, 27.3 g/kg of gliadin.

In the biscuit samples, nine out of 11 test kits reported a gliadin content of 7–26 g/kg. The RIDASCREEN Competitive and Gliadin/Gluten test kits reported a notably lower gliadin content, 2.4 and 2.5 g/kg, respectively. In the case of pasta, we expected to find at least twice the amount of gliadin as found in biscuit, based on the formulation of these products. Six out of 11 test kits reported a gliadin content that was at least 1.5 times higher than they reported in biscuit. The GLUTEN-TEC test kit reported similar gliadin contents in the biscuit and pasta samples. The remaining four test kits reported gliadin contents notably lower than the gliadin contents in the biscuit samples.

Effects of Interfering Ingredients. The baby food, spice mix, and soy sauce contained less than 10 mg/kg of gliadin according to all test kits (Table V). In the baby food samples, one out of 11 test kits reported trace amounts of gliadin. In the spice mix samples, two out of 11 test kits reported trace amounts of gliadin, but below 10 mg/kg.

Next, the recovery of 10 mg/kg of PWG gliadin was checked in all three products. Seven out of 11 test kits met the criterion for the recovery of the spike in baby food; four out of 11 met the criterion for the spike in spice mix; and six out of 11 met the criterion for the spike in soy sauce ($P < 0.05$) (Fig. 2). The assessments with real food products revealed serious shortcomings of most of the test kits included in this study. The false negative results of GLUTEN-TEC for the reference material were seen again in this experiment, with the exception of the soy sauce samples. The low recovery of the gliadin reference material by the AgraQuant G12 test kit and the high recovery by the ALLER-TEK test kit were also in line with the results found in the first round. In baby food, all test kits except the GLUTEN-TEC test kit confirmed the presence of the reference material. However, the ALLER-TEK and Gliadin/Gluten test kits overestimated the gliadin content, with recoveries of 28.7 mg/kg ($P < 0.05$) and 19.6 mg/kg ($P < 0.05$), respectively, whereas the AgraQuant G12 test kit underreported the gliadin content at 4.3 mg/kg ($P < 0.001$). In spice mix, both the RIDASCREEN Competitive and GLUTEN-TEC test kits could not detect any gliadin reference material. The Gluten-Check, RIDASCREEN, Veratox R5, and AgraQuant G12 test kits all underestimated the gliadin content, reporting recoveries of 3.8 mg/kg ($P < 0.001$), 3.5 mg/kg ($P < 0.01$), 4.3 mg/kg ($P < 0.05$), and 4.4 mg/kg ($P < 0.05$), respectively. The only test kit overestimating the gliadin content of the spice mix samples was the ALLER-TEK test kit, reporting 15.1 mg/kg ($P < 0.05$). In soy sauce the RIDASCREEN and AgraQuant G12 test kits underestimated the gliadin content, reporting 4.7 mg/kg ($P < 0.01$) and 3.7 mg/kg ($P < 0.001$), respectively. The ALLER-TEK, GlutenTox Sandwich, and Gliadin/Gluten test kits all made an overestimation and reported gliadin contents of 29.6 mg/kg ($P < 0.05$), 15.5 mg/kg ($P < 0.001$), and 16.0 mg/kg ($P < 0.01$). Interestingly, the GLUTEN-TEC test kit was able to quantify the gliadin reference material in the soy sauce samples, whereas it was unable to do so in the other matrices tested.

DISCUSSION

Since the R5 method, the antibodies G12 and α-20 have been developed against harmful gluten epitopes. Furthermore, competitive

TABLE IV
Quantification of Gliadin (mg/kg or g/kg) in Food Products[a]

Antibody	Test Kit	Biscuit, GF (mg/kg)	Pasta, GF (mg/kg)	Soup (mg/kg)	Beer (mg/kg)	Oat Flour (g/kg)	Biscuit (g/kg)	Pasta (g/kg)
401/21	ALLER-TEK	<2.5	<2.5	5.4 ± 0.7	6.1 ± 0.4	1.8 ± 0.0	25.5 ± 1.9	45.0 ± 10.4
	Gluten-Check	<2.5	<2.5	<2.5	<2.5	0.7 ± 0.0	7.6 ± 0.4	23.4 ± 1.4
R5	Ingezim	<1.5	<1.5	1.7 ± 0.3	5.5 ± 0.1	8.6 ± 0.5	16.2 ± 0.1	24.5 ± 1.8
	Ingezim Hidrolizado	<0.125	0.3 ± 0.1	0.2 ± 0.0	8.7 ± 0.2	5.8 ± 0.0	7.7 ± 0.5	18.0 ± 1.7
	RIDASCREEN	<1.5	<1.5	<1.5	<1.5	3.9 ± 0.6	21.2 ± 2.9	1.7 ± 0.1
	RIDASCREEN Competitive	<5.0	<5.0	<5.0	18.5 ± 1.1	27.3 ± 2.8	2.4 ± 0.0	7.2 ± 1.1
	Veratox R5	3.2 ± 0.0	2.6 ± 0.0	3.0 ± 0.1	4.2 ± 0.3	NT	17.4 ± 1.6	3.9 ± 0.2
G12	GlutenTox Sandwich	<0.3	<0.3	0.6 ± 0.1	0.3 ± 0.0	6.3 ± 0.3	7.3 ± 0.2	1.7 ± 0.2
	AgraQuant G12	2.6 ± 0.7	<2.0	3.3 ± 2.8	<1.0	8.9 ± 0.6	16.9 ± 0.8	6.6 ± 1.2
α-20	GLUTEN-TEC	<2.5	<2.5	4.2 ± 2.2	27.0 ± 1.7	0.2 ± 0.0	20.0 ± 1.0	17.3 ± 0.6
Polyclonal	Gliadin/Gluten	2.2 ± 0.5	<2.0	13.9 ± 0.3	2.4 ± 0.1	0.2 ± 0.0	2.5 ± 0.1	7.8 ± 0.2

[a] Values noted as mean ± SEM ($n = 3$). GF = gluten-free, and NT = not tested.

TABLE V
Recovery of Prolamin Working Group Gliadin Reference Material (PWG-R, 10 mg/kg) in Spiked Food Products[a]

Antibody	Test Kit	Baby Food	PWG-R in Baby Food	Spice Mix	PWG-R in Spice Mix	Soy Sauce	PWG-R in Soy Sauce
401/21	ALLER-TEK	<2.5	28.7 ± 2.5*	<2.5	15.1 ± 0.8*	<2.5	29.6 ± 2.8*
	Gluten-Check	<2.5	6.8 ± 0.1***	<2.5	3.8 ± 0.2***	<2.5	5.2 ± 0.5*
R5	Ingezim	<1.5	12.5 ± 0.3*	<1.5	8.3 ± 0.9	<1.5	13.2 ± 0.2*
	Ingezim Hidrolizado	0.2 ± 0.2	8.7 ± 0.3	0.7 ± 1.0	9.4 ± 0.8	<0.125	10.7 ± 0.2*
	RIDASCREEN	<1.5	5.5 ± 0.5*	<1.5	3.5 ± 0.3**	<1.5	4.7 ± 0.3**
	RIDASCREEN Competitive	<5.0	8.9 ± 0.2	5.4 ± 0.2	<1.36***	<5.0	14.3 ± 0.6*
	Veratox R5	<2.0	5.8 ± 0.1***	<2.0	4.3 ± 0.8*	<2.0	6.7 ± 0.2*
G12	GlutenTox Sandwich	<0.3	10.1 ± 0.1	<0.3	10.2 ± 1.0	<0.3	15.5 ± 0.1***
	AgraQuant G12	<1.0	4.3 ± 0.2***	<1.0	4.4 ± 0.8*	<1.0	3.7 ± 0.1***
α-20	GLUTEN-TEC	<2.5	<2.5***	<2.5	<2.5***	<2.5	7.4 ± 1.9
Polyclonal	Gliadin/Gluten	<2.0	19.6 ± 0.5*	<2.0	8.6 ± 0.8	<2.0	16.0 ± 0.5**

[a] Values noted as mean ± SEM ($n = 3$). *, **, and *** indicate P value < 0.05, 0.01, and 0.001, respectively.

formats that are able to detect hydrolyzed gluten peptides have recently become available. This study investigated a set of kits representative of what is available on the market at this time. To the best of our knowledge, this is the first time that the α-20 antibody-based kit has been included in a comparative study. Furthermore, this is the first time that the performance of different gluten ELISA kits has been compared in realistic food matrices. We have evaluated the test kits by using two different criteria. First, we determined the qualitative detection of gluten in products. A gluten-free sample should be recognized as such, without false positive results. Likewise, a gluten-containing sample should also be recognized as such, without false negative results. This is important because false negative results can lead to contaminations not being detected. False positive results, on the other hand, can lead to a gluten-free product not labeled as such, unnecessarily restricting the choice in products for CD patients. This is especially the case in Australia and New Zealand, where only products in which no gluten is detected (<LoD) can carry the gluten-free label, but also in countries that use a threshold of 20 mg/kg of gluten for gluten-free products this could become a problem. Second, we have studied the suitability of the test kits to accurately quantify gluten. For this, we have used an acceptance criteria of 50–150%, which is normally used for experiments in difficult matrices that are part of multilab validation studies (Abbott et al. 2010). One could argue that applying such a criterion is not restrictive enough, because it has been reported that interlaboratory variation can be large (Poms et al. 2005) and our tests were performed in one laboratory. However, in this study we have applied several criteria with the aim of obtaining a broad understanding of the performance of current test kits.

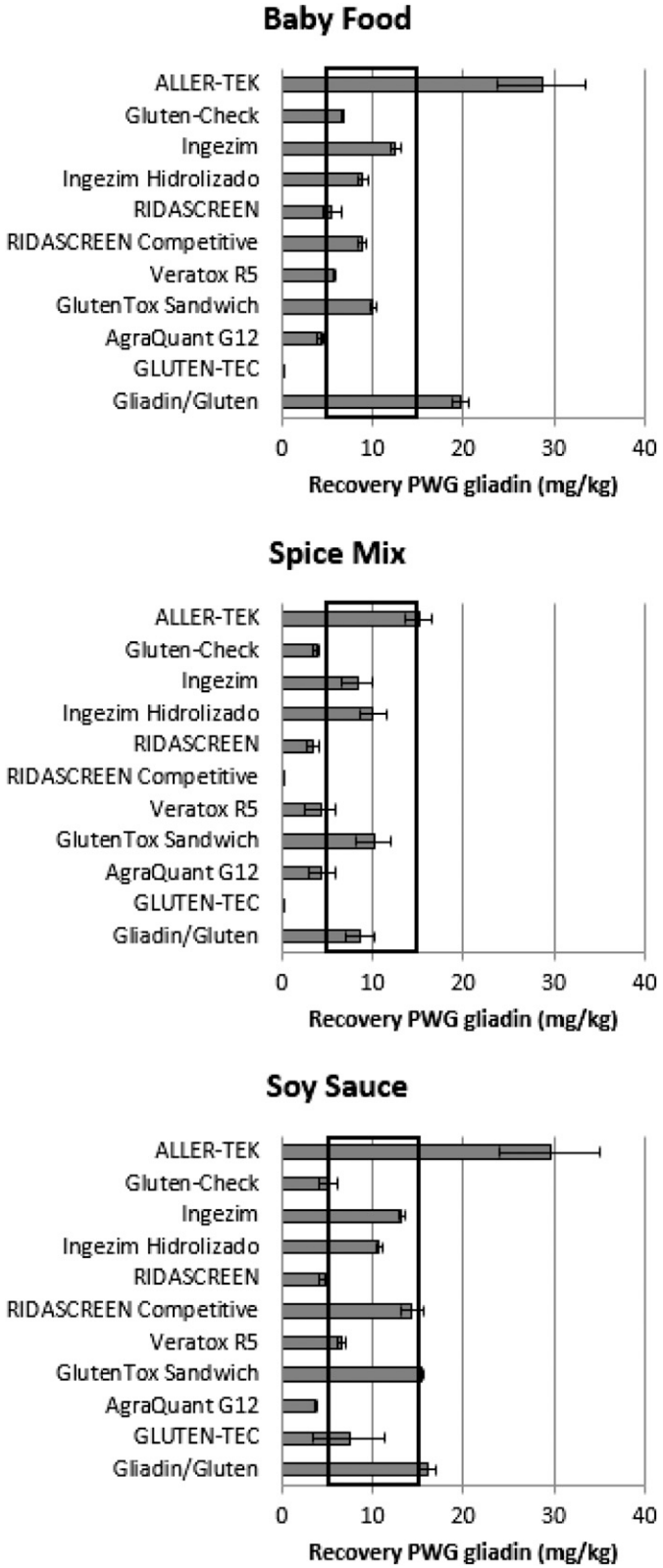

Fig. 2. Recoveries of 10 mg/kg of Prolamin Working Group (PWG) gliadin reference material in gluten-free baby food, spice mix, and soy sauce. Error bars represent 95% confidence interval. Boxes represent accepted recovery range of 50–150%, corresponding to 5–15 mg/kg of PWG gliadin reference material.

In the first round of experiments, both the BioKits and AgraQuant G12 test kits reported low recoveries of the gliadin reference material in gluten-free rice flour. In case of the BioKits test, this could be because of the test protocol prescribed. The samples need to be blended for 90 s at room temperature before further dilution. Other test kits use extraction times ranging from 10 to 100 min. Also, most other test kits extract at higher temperatures (>45°C). Lower recoveries for the BioKits test compared with other gluten ELISAs have been reported before, with recoveries as low as 3.4% (Scharf et al. 2013). This would explain why the BioKits test was unable to detect the gliadin reference material, because such a low gliadin recovery would be under the LoD of the kit. However, Sharma (2012) reported recoveries for the BioKits test of 104 and 146% for gluten reference material and wheat flour, respectively. This is possibly because Sharma used a commercially available gluten reference material from Sigma, whereas the samples from Scharf et al. were spiked with wheat flour. The choice of reference material can have a large impact on the reported gluten or gliadin recovery by test kits. This is illustrated by the recovery of the PWG gliadin reference material by the GLUTEN-TEC test kit in this study. The AgraQuant G12 test kit gave a clear positive signal in the three gluten-containing flour mixtures but failed to accurately quantify the gliadin reference material. We have no explanation for this.

All test kits detected positively the gluten-containing flour mixtures. However, the quantity of gliadin detected differed between the kits. According to the GlutenTox Competitive and GlutenAlert tests, the rye flour mixture should be labeled gluten-free if the 20 mg/kg threshold for gluten is applied. When applying the same threshold, the barley flour mixture should be labeled gluten-free according to the BioKits, Gluten-Check, Gliadin/Gluten, and GlutenAlert test kits. This is concerning because the rye flour mixture and barley flour mixture contained 79 and 72 mg/kg of gluten, respectively, which is 3–4 times higher than the current European legal limit for gluten-free products. In this respect, we observe that the choice of test kit is critical in labeling gluten-free products.

In the second round of experiments, confusing results were obtained for the oat flour samples. In principle, oat flour can be gluten-free (Londono et al. 2013). However, in practice, oats can easily become contaminated with gluten-containing cereals (Størsrud et al. 2003; Thompson 2004). This makes a proper detection of gluten in these samples extremely important. All test kits confirmed that the oat flour samples used in this study had been contaminated with gluten. This means that this particular oat flour should not be

labeled as gluten-free. In this round, two gluten-containing products were assessed as well, biscuit and pasta. Gluten is notoriously difficult to extract from both. In the case of biscuit, this is likely to be the effect of protein denaturation as a result of the baking process. In pasta, the extrusion process causes denaturation. Because the gluten content of both products is high, the question whether the test kits are able to detect any gluten is irrelevant. However, it is relevant to see to what extent gluten could be extracted and quantified. The proportion of wheat flour in pasta is significantly higher compared with biscuit, and we expected to find at least twice the amount of gliadin in pasta as in biscuit. However, half of the test kits in this round reported gliadin contents of the pasta samples to be equal to or lower than those in biscuit. This shows that the gluten content of pasta can easily be underestimated.

Our results show that the detection of the PWG spike in the spice mix and soy sauce was challenging. This result is concerning, considering that of the current type I method R5, only two out of five gave an acceptable recovery. Gluten present in such a difficult matrix can easily be overlooked. In the soy sauce samples, five out of 11 test kits reported significantly higher gliadin contents than 10 mg/kg. Furthermore, the GLUTEN-TEC test kit, which was unable to detect the gliadin reference material in all other spiked samples, gave a signal in the spiked soy sauce samples. The reason this kit gave a signal in the soy sauce samples is unknown, because the nonspiked soy sauce samples were proven to be gluten-free by all test kits included in the second round.

When looking at the first (qualitative) criterion, all test kits had problems in detecting the presence of gluten in at least one matrix. For test kits with a sandwich format, this included the detection of hydrolyzed gluten peptides, shown in the beer samples. This was to be expected, because competitive formats have specifically been developed to overcome this problem. According to the enclosed user manual, the direct format of the Ingezim Hidrolizado test kit should also be able to detect hydrolyzed gluten. Although it gave a higher signal than the sandwich kits, the Ingezim Hidrolizado test kit only reported a gliadin content of 8.7 mg/kg ($P < 0.05$) in the beer samples. This means that with the current European legislation, this beer would have incorrectly been labeled gluten-free. The two competitive formats, the RIDASCREEN Competitive and GLUTEN-TEC test kits, were able to detect hydrolyzed gluten in beer but experienced problems with gluten detection in other products.

The second criterion, accurate quantification of the PWG gliadin reference material present in all spiked samples, was met by two out of 11 test kits, namely the Ingezim and Ingezim Hidrolizado test kits. We have not been able to check if the GLUTEN-TEC test kit was susceptible to matrix effects such as encountered in spice mix, because it was unable to detect the PWG gliadin reference material in nearly all samples. In further experiments with higher spiked concentrations, we were able to obtain a recovery of approximately 15%. This is an issue that will have to be addressed by the kit's manufacturer. It is still unclear why the GLUTEN-TEC test kit was not able to detect the PWG gliadin properly, because the α-20 antibody has been raised against a specific sequence present in α-gliadin that is also present in the PWG gliadin reference material (van Eckert et al. 2006; van Eckert et al. 2010).

Based on the series of samples used in our experiments, we can conclude that major hurdles still exist, preventing a robust and reliable result in a wide range of food products. These hurdles should be further studied to understand the strengths and weaknesses of the currently available gluten test kits. The first hurdle is related to the ability to detect hydrolyzed gluten. This is of paramount importance because CD patients are already triggered by small peptides containing one toxic sequence (Tye-Din et al. 2010). Only two out of 11 test kits were able to correctly identify the beer samples as gluten-containing. Many food products contain a mixture of both intact gluten proteins and gluten peptides, of which the latter part will remain undetected by most kits. This could lead to a serious underestimation of the total gluten content of these products. Competitive formats have specifically been developed to detect hydrolyzed gluten, which is a known problem in the analysis of beer samples (Haas-Lauterbach et al. 2012; Comino et al. 2013). Both competitive formats tested in this study recommend an alternative extraction protocol and alternative dilution steps when beer samples are analyzed. Effectively, this means that the test method is altered as soon as hydrolyzed gluten is expected. In beer, the presence of hydrolyzed gluten is known, but for other food products this might not always be the case. This could limit the effectiveness of the currently available methods. Another problem is seen in samples that contain interfering ingredients, such as the high salt content in the spice mix samples. High salt content can lead to the precipitation of gliadins (Meredith 1965), which would leave them undetected. Only four out of 11 test kits correctly quantified the added gliadin spike. Thus, most test kits would be unable to detect the presence of gluten in such a matrix and could, therefore, lead to incorrect labeling of these products. To minimize interference, interfering ingredients should either be removed or blocked, if possible. Removing the interfering ingredient can be done before the extraction of gluten, such as defatting samples with a high fat content (Wieser 2008). A second option is to block the interfering ingredient during the extraction process, such as adding gelatin to samples high in polyphenols to prevent binding to prolamins (García et al. 2004). Extraction will also be a problem when analyzing processed food such as pasta or baked products such as cookies. In this study, analysis of pasta samples led to an underestimation of the gluten content of 54–97%. Five out of 11 test kits reported gliadin values within the expected range, of which the ALLER-TEK, Gluten-Check, and Ingezim Hidrolizado test kits reported a gliadin value of approximately twice the amount that they had reported in biscuit. These three test kits all used a cocktail solution for the extraction of gluten. Cocktail solutions sometimes contain reducing agents that are known to improve gluten extraction from food matrices (García et al. 2005). However, other test kits that also used a cocktail solution still had difficulties extracting gluten from the pasta samples. Because the exact composition of the different extraction cocktails used by the test kits is unknown, we cannot conclude whether or not this difference in success is related to the type of cocktail solution used for extraction. In this case, the affinity of the 401/21 antibody for the heat-stable ω-gliadins, which are more extractable than other types of gliadins (Skerritt and Hill 1990), gave the ALLER-TEK and Gluten-Check test kits an extra advantage over the test kits that used other antibodies. Cross-reactivity did not seem to be a problem for most test kits, because all nonspiked gluten-free food matrices were confirmed to be gluten-free, with the exception of the oat flour. However, in the case of the oat flour samples, the high gliadin recoveries were more likely the result of cross-contamination of the samples than of cross-reactivity of the test kits.

Previously, Diaz-Amigo and Popping (2013) criticized the status of the R5 method as the only recommended method. They stated that interpreting differences in detection between the R5 method and test kits based on a different antibody as shortcomings of these other kits is unjust. We can only subscribe to their criticism, because none of the R5-based methods tested in this study were able to meet all criteria. However, the newer methods also struggled to detect gluten in various food samples. This means that improvements in gluten detection are still much needed.

When looking at future prospects in gluten detection, we suggest four key points that should be taken into account with the development of new test methods. First, the extraction process used by the test kit should be improved to overcome food matrix effects. Gluten extraction should be maximal. Ingredients from the food matrix that end up in the extract can interfere with the test method, as this study has indicated. Extraction protocols should be developed to solve both problems related to extraction and interference. The second is the format of the test kit. Because food samples can contain both gluten proteins and gluten peptides, it is

important that a test kit can detect both so the hydrolyzed peptides are not overlooked. A sandwich format is more specific because it requires the binding of two antibodies instead of one as is the case with a competitive format. However, a sandwich format will overlook gluten peptides, which are known to be harmful for CD patients. Therefore, we propose to focus new test kit development on competitive formats that allow for the detection of gluten proteins and peptides. Finally, it is important to detect relevant epitopes. For CD patients, it suffices to know whether or not a food product contains harmful gluten proteins or peptides, instead of gluten proteins in general. Both the G12 and α-20 antibodies are targeted against a relevant epitope, but results from this study suggest that these kits still encounter problems in detecting and quantifying gluten in difficult matrices. Furthermore, the α-20 antibody has been shown to fail in the detection of the PWG gliadin reference material. This indicates that this test kit has problems detecting α-20 epitopes present in the PWG gliadin mixture, a problem that should be studied further. To make sure that the chance of missing or underestimating the relevant gluten proteins and peptides becomes smaller, we propose to use a multiplex assay. In this type of assay multiple antibodies against relevant gluten epitopes are combined. This way, the strengths of several methods can be combined to overcome their individual weaknesses. Of course, all of these recommended improvements should be combined into one new assay.

CONCLUSIONS

Despite the availability of a type I detection method, many problems in gluten detection in food still remain. Our study underpins the relevance of testing commercial test kits against accepted standards and samples relevant to raw materials, complex ingredients, and finished products. The difficulties encountered by the test kits used in this study suggest that, at this moment, there is no test kit available that can accurately detect and quantify the amount of gluten present in all challenging food products. The presence of gluten can easily be overlooked or overestimated for a variety of reasons, including incomplete extraction from the food matrix, an interfering ingredient, the format of the applied test kit, or problems with antibody specificity. This is concerning, because CD patients have to rely on the correct labeling of gluten-free food to make safe food choices. To ensure correct labeling and safe products for CD patients in the future, test methods that are able to detect multiple gluten proteins and peptides simultaneously should be investigated.

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