

MICROBIOLOGICAL METHODS

Validation of a New Enzyme-Linked Immunosorbent Assay to Detect the Triggering Proteins and Peptides for Celiac Disease: Interlaboratory Study

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The performance of Gluten-Tec® (EuroProxima, Arnhem, The Netherlands) was tested through an interlaboratory study in accordance with AOAC guidelines. Gluten-Tec is a competitive ELISA that detects an immunostimulatory epitope of α -gliadin in dietary food for celiacs. Fifteen laboratories, representing 14 different countries, announced their interest in taking part in this study. Of the 12 laboratories that sent the results within the established timeframe, two submitted inappropriate standard curves and were excluded from the statistical analysis. Four different food matrices (rice-based baby food, maize bread, chocolate cake mix, and beer) were selected for preparing the test samples. Two gliadin extraction procedures were used: the conventional 60% ethanol, and a new method based on the reducing reagent dithiothreitol. The 38 samples (19 blind duplicates) tested in this study were prepared by diluting the different extracts in order to cover a wide range of gliadin levels. Both sample extraction and dilution were performed by EuroProxima; the present interlaboratory study was focused only on testing the ELISA part of the Gluten-Tec kit protocol. Repeatability values (within-laboratory variance), expressed as RSD_w, ranged from 6.2 to 25.7%, while reproducibility values (interlaboratory variance), expressed as RSD_R, ranged from 10.6 to 45.9%. Both statistical parameters were in the acceptable range of ELISAs under these conditions, and the method will be presented to the Codex Alimentarius as a preferred method for gluten analysis.

ELISA, recommended by the Codex Alimentarius for gliadin determination in dietary foods for celiacs, only detects intact (nonhydrolyzed) gliadins. In addition, these kits are calibrated with a mixture of intact gluten proteins (both gliadins and glutenins) extracted with 60% ethanol from 20 different wheat varieties, called the Prolamin Working Group standard (2), which is not a true standard as it cannot be reproducibly generated.

To overcome these shortcomings, the Leiden University Medical Center (Leiden, The Netherlands), in cooperation with EuroProxima (Arnhem, The Netherlands), has developed Gluten-Tec®, a novel competitive ELISA that detects a well-characterized T-cell stimulatory epitope of α -gliadin (α -20, amino acid sequence RPQQPYP) in wheat, and homolog sequences in barley (hordein), rye (secalin), and their crossbred varieties (3). A synthetic peptide is used for calibration, which allows an accurate and reproducible standardization. Moreover, not only intact, but also hydrolyzed proteins can be detected.

The purpose of this study was to test the performance of the ELISA part of the Gluten-Tec kit through an interlaboratory study, in accordance with AOAC guidelines (4). Two other aspects were of special interest: to elucidate which method should be used for the extraction of gliadin in food samples; and to test the performance of the method over a wide range of gliadin concentrations.

Materials and Methods

Collaborators

Fifteen laboratories took part in this study. All laboratories were familiar with gluten testing and/or performing of ELISAs.

ELISA

Gluten-Tec ELISA kit (EuroProxima, Cat. No. 5171GT) is a competitive immunoassay based on an mAb that detects a T-cell stimulatory epitope present in α -gliadin (α -20, amino acid sequence RPQQPYP; 5) and homolog sequences present in barley (hordein), rye (secalin), and their crossbred varieties (3). Both intact and small protein fragments, resulting from the hydrolyzation of intact proteins, can be detected. The α 20-gliadin concentration is calculated from a calibration curve, using a synthetic peptide that contains the α 20-gliadin epitope (GPFRRPQQPYPB).

All reagents required for the immunoassay

Gluten is a large protein family that can be subdivided into gliadins and glutenins. Although multiple immunostimulatory epitopes involved in celiac disease have been described in both groups, the majority belong to gliadin (1). The most frequently used commercially available ELISAs for gliadin analysis are based on the R5 monoclonal antibody (mAb). The sandwich version of the R5

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(including the standard) were contained in the Gluten-Tec ELISA kit:

(a) One microtiter plate (12 strips of eight wells each) coated with wheat gliadin.

(b) Two adhesive films (plate sealers).

(c) One vial with 40 mL of sample dilution buffer (SDB; ready to use).

(d) Seven vials with 1 mL/vial of different concentrations of the peptide standard (0, 0.25, 0.5, 1, 2, 4, and 8 ng/mL).

(e) One vial with 1 mL of positive control (1.5 ng/mL of the peptide standard; ready to use).

(f) One vial with 30 mL of rinsing buffer (20x). In order to prepare a 1x solution, 25 mL 20x rinsing buffer was added to 475 mL distilled or deionized water (not supplied with the kit). When ready, the diluted solution remained stable at 2–8°C.

(g) Two vials with 15 mL/vial of dilution buffer (ready to use).

(h) One vial with 0.1 mL biotinylated mAb against α 20-gliadin. Due to the limited stability of the diluted solution, the biotinylated mAb was supplied 100 times concentrated (100x). In order to get 1x solution, 60 μ L of 100x biotinylated mAb was diluted in 6 mL of dilution buffer (sufficient for one plate).

(i) One vial with 0.2 mL streptavidin coupled to horseradish peroxidase (HRP). Because of the limited stability of the diluted solution, streptavidin-HRP was supplied 100 times concentrated (100x). In order to get 1x solution, 120 μ L 100x streptavidin-HRP was diluted in 12 mL of dilution buffer (sufficient for one plate).

(j) One vial with 12 mL tetramethylbenzidine (TMB) substrate solution (ready to use).

(k) One vial with 12 mL sulfuric acid stop solution (ready to use).

The antibody-antigen reaction is the basis of Gluten-Tec. The gliadin peptides present in the standard/sample compete with the coated gliadin for binding to the biotinylated mAb against α 20-gliadin. The biotinylated mAb bound to the coated gliadin will be marked with streptavidine-HRP, an enzyme that converts the colorless TMB substrate solution into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorption is inversely proportional to the gliadin peptide concentration present in the standard/sample.

Food Matrixes

The food allergen working group recommends that minimum requirements for any validation study include two matrixes (6). The present study included four different food matrixes, covering a wide range of hydrolyzed and/or heat-treated food products (Table 1).

(a) The two rice-based baby foods, one nonspiked and the other spiked with 5% wheat-based baby food, were prepared by Nestlé Research Centre (Nestlé S.A., Lausanne, Switzerland). These baby foods, mixtures of water and flour, were heat-processed by steam injection at 120–130°C for 20–30 s. At the end of this process, the products remained on the line at 100°C for approximately 5–10 min. The rice baby food was dried and then ground to 100 μ m in order to resemble the granulometry of the spiking agent. To guarantee as much homogeneity as possible, the mixing was carried out under dry conditions.

(b) The incurred maize bread sample was prepared by the group of Peter Köehler (Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany). The gliadin content of

the wheat flour cv. Astron was determined by an extraction/RP-HPLC-method as previously described (7). HPLC absorbance areas at 210 nm were converted into an amount of protein by calibration with standard solutions of gliadin from the Prolamin Working Group (2). With this method, the gliadin content of the wheat flour cv. Astron was calculated as 5152 mg/kg. A maize bread was prepared as follows: 100.0 g corn flour, 18 g water, 2 mL ethanol (60%, v/v), 100 g egg albumen, 2 g sodium chloride, 4 g sucrose, 2.5 g dry yeast, and 100 mg wheat flour (cv. Astron) were mixed for 3 min and filled into a tin. The dough was proofed (allowed to rise) for 40 min at 30°C and baked for 30 min at 230°C. After cooling, the bread was cut into pieces of 1.5 cm, lyophilized and ground in a laboratory mill. The resulting lyophilized and ground maize bread sample contained 44.2 mg gliadin/kg.

(c) Two chocolate cake mixes, one containing gluten and the other gluten-free, were acquired from a retail source. A laboratory contracted by the Food Analysis Performance Assessment Scheme prepared the spiked sample, mixing 10 g of gluten-containing chocolate cake mix with 3990 g of gluten-free chocolate cake mix (0.25%) and using a Winkworth tumbler mixer (Reading, Berkshire, UK) overnight to ensure the production of homogenous materials.

(d) The beer (canned Palm beer, made from barley malt) was purchased in a food supermarket.

Gliadin Extraction Methods

Two extraction procedures were used:

(1) The conventional 60% ethanol. This method is only recommended for raw materials, where all gliadin types are in their original monomeric form and, therefore, are still soluble in alcohol. The sample (1 g) was weighed into a 15 mL tube, and 10 mL 60% ethanol was added. The sample was mixed thoroughly by vortexing for 10–60 s (until total dispersion of the sample) and incubated for 1 h at room temperature with agitation. The tube was centrifuged for 15 min at 2000 $\times$ g at room temperature, and the supernatant transferred to a clean tube.

(2) A new method based on the reducing reagent dithiothreitol (DTT), indicated for the extraction of all gluten proteins (the monomeric gliadins and the polymeric glutenins) of heated and unheated food samples. The sample (1 g) was weighed into a 15 mL tube and 5 mL Extraction Buffer A (20 mM DTT, 60% ethanol, 50 mM Tris-HCl, pH 8.0) was added. The sample was mixed thoroughly by vortexing for 10–60 s (until total dispersion of the sample) and incubated for 30 min at 60°C in a water bath. The tube was centrifuged for 15 min at 2000 $\times$ g at room temperature, and the supernatant was transferred to a clean tube. One volume of Extraction Buffer B (110 mM iodoacetamide, 50 mM Tris-HCl, pH 8.0) was added to the supernatant, mixed thoroughly by vortexing (5–10 s), and incubated for 30 min at room temperature and in darkness.

Both rice baby foods were extracted with 60% ethanol and with the DTT-based extraction method. The extracts of the sample spiked with 5% wheat were diluted with SDB to obtain extract dilutions equivalent to lower levels of contamination (0.4, 0.2, 0.1, 0.05, and 0.025%). The nonspiked sample (0%) was used as negative control. In a similar way, the incurred maize bread sample, containing 44.2 mg gliadin/kg, was extracted with 60% ethanol and DTT. Both extracts were diluted 1/2 with SDB, to obtain new samples with a gliadin content of 22.1 mg/kg.

Table 1. α 20-gliadin content of the 38 test samples included in the collaborative study (average from parallel determination, $\mu\text{g/kg}$); average ($\mu\text{g/kg}$) and RSD (%) of each couple of blind duplicates

Sample	Spiked/nonspiked	Extraction	Blind duplicates	Lab A	Lab B	Lab C	Lab D	Lab F	Lab G	Lab I	Lab J	Lab K	Lab L
Rice baby food													
1	Nonspiked	EtOH	TS15	< LOQ ^a	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			TS21	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			Average	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			RSD (%)	—	—	—	—	—	—	—	—	—	—
2	Nonspiked	DTT	TS19	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			TS30	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			Average	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			RSD (%)	—	—	—	—	—	—	—	—	—	—
3	Spiked 0.4% wheat baby food	EtOH	TS02	> HCL ^b	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			TS32	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			Average	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			RSD (%)	—	—	—	—	—	—	—	—	—	—
4	Spiked 0.4% wheat baby food	DTT	TS04	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			TS28	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			Average	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			RSD (%)	—	—	—	—	—	—	—	—	—	—
5	Spiked 0.2% wheat baby food	EtOH	TS18	676	> HCL	> HCL	744	762	> HCL	> HCL	550	742	728
			TS38	656	> HCL	> HCL	696	662	784	> HCL	621	> HCL	732
			Average	666	> HCL	> HCL	720	712	784	> HCL	585	742	730
			RSD (%)	2	—	—	5	10	—	—	9	—	0
6	Spiked 0.2% wheat baby food	DTT	TS10	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	715	> HCL	> HCL
			TS24	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL
			Average	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	> HCL	715	> HCL	> HCL
			RSD (%)	—	—	—	—	—	—	—	—	—	—
7	Spiked 0.1% wheat baby food	EtOH	TS05	353	546	460	451	446	462	643	274	359	454
			TS25	304	590	470	445	470	477	604	310	390	377
			Average	328	568	465	448	458	469	624	292	374	416
			RSD (%)	11	5	1	1	4	2	4	9	6	13

Table 1. (continued)

Sample	Spiked/nonspiked	Extraction	Blind duplicates	Lab A	Lab B	Lab C	Lab D	Lab F	Lab G	Lab I	Lab J	Lab K	Lab L
8	Spiked 0.1% wheat baby food	DTT	TS16	531	763	> HCL	754	652	820	> HCL	683	680	661
			TS34	534	782	> HCL	667	671	658	> HCL	502	589	656
			Average	533	772	> HCL	710	661	739	> HCL	592	634	658
			RSD (%)	0	2	—	9	2	16	—	22	10	1
9	Spiked 0.05% wheat baby food	EtOH	TS09	183	270	305	259	281	300	489	149	208	225
			TS35	160	282	327	186	317	222	366	< LOQ	207	220
			Average	172	276	316	223	299	261	427	149	208	223
			RSD (%)	9	3	5	23	9	21	20	—	0	2
10	Spiked 0.05% wheat baby food	DTT	TS01	317	538	473	443	455	427	588	222	353	419
			TS29	273	491	449	374	353	395	433	293	320	331
			Average	295	514	461	408	404	411	511	258	336	375
			RSD (%)	11	6	4	12	18	6	22	19	7	17
11	Spiked 0.025% wheat baby food	EtOH	TS12	< LOQ	183	160	< LOQ	< LOQ	153	246	< LOQ	< LOQ	< LOQ
			TS20	< LOQ	193	173	< LOQ	< LOQ	141	216	< LOQ	< LOQ	< LOQ
			Average	< LOQ	188	166	< LOQ	< LOQ	147	231	< LOQ	< LOQ	< LOQ
			RSD (%)	—	3	5	—	—	6	9	—	—	—
12	Spiked 0.025% wheat baby food	DTT	TS07	173	236	261	188	190	293	419	183	196	215
			TS23	< LOQ	189	271	193	185	249	346	177	197	215
			Average	173	212	266	191	188	271	382	180	197	215
			RSD (%)	—	15	2	2	2	12	13	2	1	0
Maize bread													
13	Incurred 44.2 mg/kg gliadin ^c	EtOH	TS03	179	317	321	234	142	346	516	176	195	154
			TS37	189	204	308	160	199	257	337	220	< LOQ	215
			Average	184	260	314	197	170	302	426	198	195	184
			RSD (%)	4	31	3	26	24	21	30	16	—	24
14	Incurred 44.2 mg/kg gliadin ^c	DTT	TS14	273	308	421	403	302	491	624	354	317	365
			TS27	261	313	403	424	317	418	536	268	238	308
			Average	267	310	412	413	309	455	580	311	278	336
			RSD (%)	3	1	3	4	4	11	11	19	20	12

Table 1. (continued)

Sample	Spiked/nonspiked	Extraction	Blind duplicates	Lab A	Lab B	Lab C	Lab D	Lab F	Lab G	Lab I	Lab J	Lab K	Lab L
15	Incurred 22.1 mg/kg gliadin ^c	EtOH	TS17	< LOQ	216	254	166	< LOQ	259	369	< LOQ	148	159
			TS31	< LOQ	< LOQ	196	< LOQ	< LOQ	208	291	162	< LOQ	< LOQ
			Average	< LOQ	216	225	166	< LOQ	233	330	162	148	159
			RSD (%)	—	—	18	—	—	15	17	—	—	—
			TS11	161	273	236	229	217	273	415	159	164	189
16	Incurred 22.1 mg/kg gliadin ^c	DTT	TS36	< LOQ	227	272	< LOQ	194	199	277	< LOQ	< LOQ	189
			Average	161	250	254	229	205	236	346	159	164	189
			RSD (%)	—	13	10	—	8	22	28	—	—	0
			Chocolate cake mix										
			TS08	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
17	Nonspiked	EtOH + Gel + PVP	TS26	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			Average	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
			RSD (%)	—	—	—	—	—	—	—	—	—	—
			TS13	340	424	453	442	422	471	613	383	325	381
			TS33	344	506	470	391	451	415	604	326	372	435
18	Spiked 0.25% gluten-containing chocolate cake mix	EtOH + Gel + PVP	Average	342	465	461	417	437	443	608	355	349	408
			RSD (%)	1	12	3	9	5	9	1	11	10	9
			Beer										
			TS06	161	183	218	165	190	221	223	141	179	209
			TS22	150	< LOQ	221	< LOQ	141	206	193	156	178	179
19	Nonspiked	—	Average	156	183	220	165	166	214	208	148	178	194
			RSD (%)	5	—	1	—	21	5	10	7	0	11

^a < LOQ = Below the LOQ.^b > HCL = Above the highest calibration level (HCL = 8 ng/mL; 800 µg/kg for sample dilution 1:100).^c Total gliadin (ω-, α- and γ-gliadin) as determined by HPLC (values expressed in mg/kg).

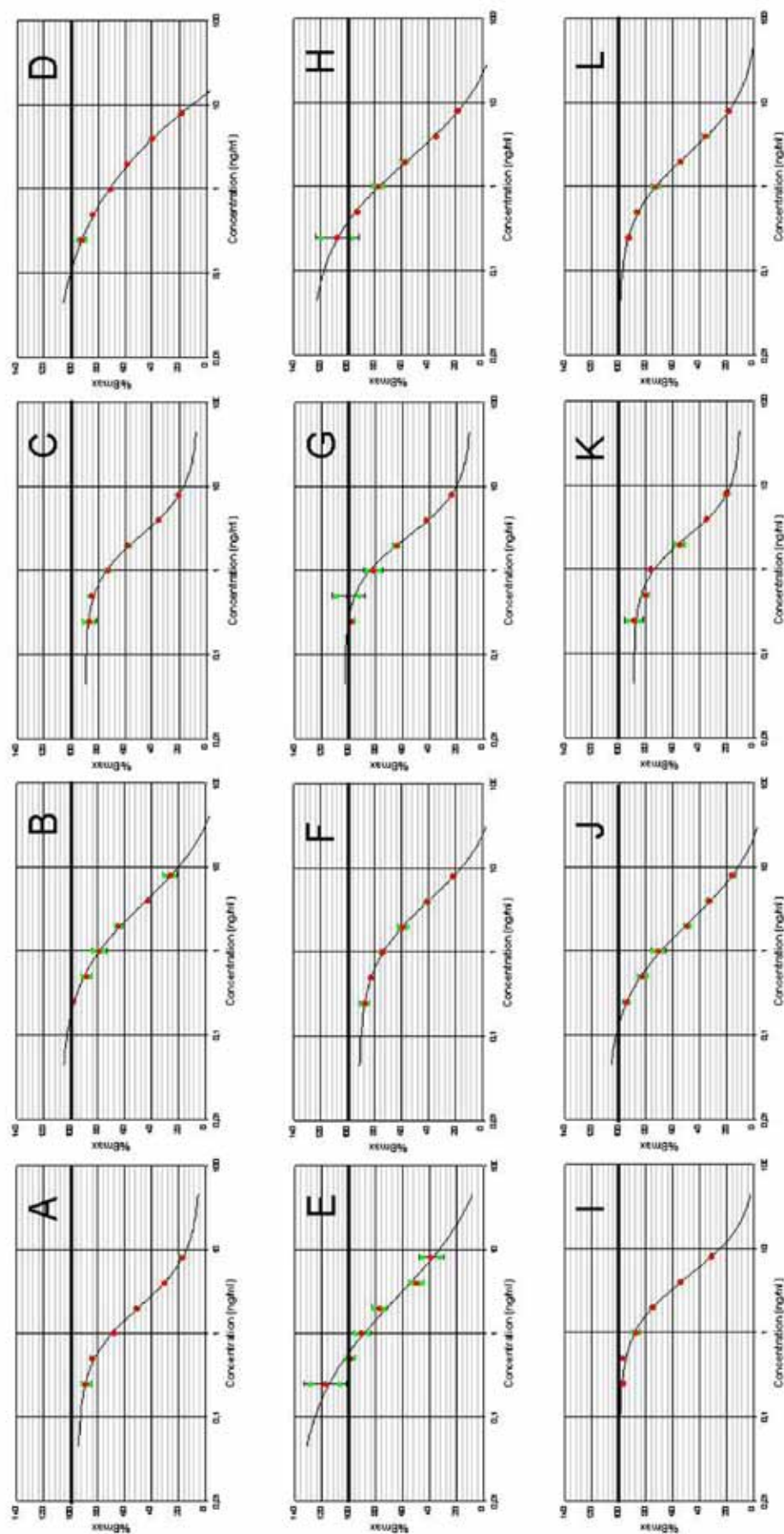


Figure 1. Calibration curves of the 12 laboratories that sent the results within the established timeframe.

Table 2. Repeatability and reproducibility of the Gluten-Tec ELISA kit; results of the statistical evaluation (AOAC, 2005) of the data of the collaborative study

Matrix	Sample	Spiked/nonspiked	Extraction	Mean, µg/kg	Repeatability		Reproducibility	
					RSD _r (%)	HorRat _r	RSD _R (%)	HorRat _R
Rice baby food	1	Nonspiked	EtOH	< LOQ ^a	—	—	—	—
	2	Nonspiked	DTT	< LOQ	—	—	—	—
	3	Spiked 0.4% wheat baby food	EtOH	> HCL ^b	—	—	—	—
	4	Spiked 0.4% wheat baby food	DTT	> HCL	—	—	—	—
	5	Spiked 0.2% wheat baby food	EtOH	> HCL	—	—	—	—
	6	Spiked 0.2% wheat baby food	DTT	> HCL	—	—	—	—
	7	Spiked 0.1% wheat baby food	EtOH	444	6.2	0.34	23.1	1.29
	8	Spiked 0.1% wheat baby food	DTT	650	9.3	0.55	10.6	0.63
	9	Spiked 0.05% wheat baby food	EtOH	255	15.1	0.77	33.7	1.73
	10	Spiked 0.05% wheat baby food	DTT	397	13.6	0.75	17.9	0.98
	11	Spiked 0.025% wheat baby food	EtOH	* ^c	—	—	—	1.87
	12	Spiked 0.025% wheat baby food	DTT	226	10.1	0.51	29.9	1.50
Maize bread	13	Incurred 44.2 mg/kg gliadin ^d	EtOH	239	25.5	1.29	40.2	2.04
	14	Incurred 44.2 mg/kg gliadin ^d	DTT	367	10.7	0.58	27.6	1.50
	15	Incurred 22.1 mg/kg gliadin ^d	EtOH	170	25.7	1.24	45.9	2.21
	16	Incurred 22.1 mg/kg gliadin ^d	DTT	211	21.4	1.07	32.9	1.64
Chocolate cake mix	17	Nonspiked	EtOH+Gel+PVP	< LOQ	—	—	—	—
	18	Spiked 0.25% gluten-containing chocolate cake mix	EtOH+Gel+PVP	428	7.7	0.43	19.0	1.05
Commercial beer	19	Nonspiked	—	179	11.9	0.58	17.8	0.87

^a < LOQ = Below the LOQ.^b > HCL = Above the highest calibration level (HCL = 8 ng/mL; 800 µg/kg for sample dilution 1:100).^c Without sufficient quantitative data (fewer than eight laboratories reporting valid data).^d Total gliadin (ω-, α-, and γ-gliadin) as determined by HPLC (values expressed in mg/kg).

To reduce gliadin binding to tannins, the two chocolate cake mixes were extracted with a solution of 60% ethanol containing 5% fish gelatin (Norland Products, Cranbury, NJ) and 2% polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO). No extraction was performed with the commercial beer.

Test Samples

The commercial beer and gliadin extracts of the other food samples were diluted 100 times (final dilution) with SDB. For allergen ELISA methods, the food allergen working group has concluded that a minimum of two replicates per laboratory will optimize the statistical confidence while not imposing undue burden on study participants (6). In order to generate blind duplicates, each diluted sample was split in half, randomly coded (from TS01 to TS38), and transferred into 1.5 mL tubes (0.5 mL/tube). These 38 diluted test sample extracts, together with two Gluten-Tec ELISA kits, were sent to each laboratory in a package containing ice bricks, and were required to be stored at 2–8°C until use.

Time Schedule

For validation of the repeatability and reproducibility of the Gluten-Tec ELISA kit, an interlaboratory study, in accordance with AOAC requirements for interlaboratory studies (4), was carried out between November 2009 and January 2010. The trial was performed in 2 days: Day 1 for the analysis of a positive control sample, and Day 2 for the analysis of the test samples. Collaborators were instructed not to proceed with the analysis of the test samples unless adequate results on the positive control sample were obtained first. Each day, 3.5–4 h were required for the entire procedure, including preparation of the reagents/solutions, performance of the assay, and filling out of the reporting form.

Analytical Protocol

The quantitative measurement of gliadin levels in the 38 test samples was performed using the same batch of Gluten-Tec (KN6565). Collaborators were instructed to perform duplicate estimations of each diluted test sample extract on the same day. Following the manufacturer's instructions, 50 μ L of the standard points (0, 0.25, 0.5, 1, 2, 4, and 8 ng/mL), test samples (TS01-TS38), and positive control (C+) were dispensed into each appropriate well of the microtiter plate. A 50 μ L volume of diluted biotinylated mAb was then dispensed into each well, and the plate incubated for 1 h in the dark at 2–8°C. The contents of the wells were shaken out, the wells were rinsed three times with diluted wash solution, and residual droplets were removed. A 100 μ L volume of streptavidin-HRP enzyme complex was dispensed into each well and incubated for 1 h in the dark at 2–8°C. Removal and washing of the wells were repeated before 100 μ L TMB substrate solution was added, then incubated for 30 min in the dark at room temperature. The enzymatic reaction was terminated by adding 100 μ L sulfuric acid stop solution into each well, and the absorbance at 450 nm was determined using an ELISA microtiter plate reader.

Calculations

Upon completion of the study, a data reporting form was available to be filled with the 450 nm optical densities (ODs). Competitive ELISAs yield inverse calibration curves, where higher values of antigen in the samples or standards yield a lower amount of color change. For visual presentation, calibration curves were constructed by plotting a graph with the percentages of the standard absorbances with respect to the absorbance of a solution with 0 ng/mL (zero standard) against the logarithm of peptide concentration of the standard solutions. Gen5 software (BioTek Instruments, Winooski, VT) was used to perform 4-parameter logistic curve fits for the ELISA titration curves, and to calculate the α 20-gliadin concentration of the 38 test samples (in ng/mL). To determine the peptide concentration actually contained in the sample (in μ g/kg), the concentration read from the calibration curve was further multiplied by the final dilution factor (100).

Statistical Analysis

The analytical data obtained by this interlaboratory study were statistically evaluated according to AOAC guidelines (4).

Results

Laboratories Submitting Valid Data

Twelve laboratories sent the results within the established timeframe of 1 month, corresponding to the stability of the test samples at 2–8°C (data not shown). From these 12 laboratories, two submitted inappropriate standard curves and were excluded from the statistical analysis (Figure 1, Laboratory E and H).

LOD and LOQ

The sensitivity of the Gluten-Tec ELISA kit was characterized by the LOD or LOQ at the lower end of the quantifiable analyte concentration range. Traditionally, in immunoassay, LOD is defined as 3 times the SD of the response at zero dose, expressed in concentration or mass units. It is obtained by dividing the SD of the response by the slope of the standard curve at zero dose level. Here we used the SD of the OD response of a zero matrix (nonspiked rice baby food) and the slope of the OD versus concentration relation between the two lowest standard points (0 and 0.25 ng/mL) to calculate the LOD. CC α (decision limit) is the limit at and above which it can be concluded that a sample is noncompliant, with an error probability of α . Thus, the LOD corresponds to CC α for substances for which no permitted limit has been established according to EC decision 2002/657/EC (8). CC α or LOD thus defined implies probability of a noncompliant result in a truly compliant sample of 0.14%. According to the data provided by the 10 laboratories submitting valid data, an LOD of 0.41 ng/mL (41 μ g/kg for sample dilution 1:100) was established for ethanol extracts, and 0.46 ng/mL (46 μ g/kg for sample dilution 1:100) for DTT extracts.

LOQ is the lowest concentration at which not only can the analyte be reliably detected, but at which some predefined goals for bias and imprecision are met. In the present interlaboratory study, LOQ was defined as the concentration that results in a CV of 10%, and is thus a measure of the assay's precision at low

analyte levels (without addressing bias). Assuming that the SD of the concentration of the zero matrix sample extracts as determined to calculate LOD is constant in the lowest concentration range, the LOQ values of the Gluten-Tec ELISA kit were calculated as 1.38 and 1.53 ng/mL (138 and 153 µg/kg for sample dilution 1:100) for ethanol and DTT extracts, respectively.

Analytical Results

The analytical results (in µg/kg) of the 19 blind duplicates, as measured by the 10 laboratories submitting valid data, are presented in Table 1. All laboratories reported the extracts of the nonspiked rice baby food (samples 1 and 2) and chocolate cake mix (sample 17) as below the LOD and, therefore, as negative. On the other side, both extracts of the highest spiked rice baby food (samples 3 and 4) were always above the highest calibration level (HCL = 8 ng/mL; 800 µg/kg for sample dilution 1:100). All laboratories were able to determine reliably the presence of gliadin in the spiked samples, except for the ethanol extract of the incurred maize bread containing 22.1 mg gliadin/kg (sample 15).

Detection of Outliers

According to AOAC guidelines (4), the data were first tested for the presence of outliers. This has to be done by application of two different tests: the Cochran maximum variance test, followed by Grubbs' test. Cochran's test is a test of the within-laboratory variabilities, and should be applied first. Grubbs' test is primarily a test of between-laboratory variability. No outliers were indicated by both tests and, therefore, the study maintained valid data from 10 laboratories.

Precision of the Assay

To assess precision, the data were statistically evaluated according to AOAC guidelines. The results of this evaluation for the 19 samples are presented in Table 2. Samples below the LOD (samples 1, 2, and 17), above the HCL (samples 3–6), or without sufficient quantitative data (fewer than eight laboratories reporting valid data: sample 11) were not statistically evaluated. Only 11 samples (samples 7–10, 12–16, and 18–19) could be subjected to statistical analysis (Table 2). Repeatability values (RSD_r) of these samples ranged from 6.2 to 25.7%. Reproducibility (RSD_R) values ranged from 10.6 to 45.9%. As might be expected, repeatability and reproducibility were dependent on concentration over the calibration range of the kit. Repeatability was within the acceptable range, as $HorRat_r$ values were below 1.5 in all tested samples. Reproducibility was acceptable in all but two samples (13 and 15, with $HorRat_R$ values of 2.04 and 2.21, respectively).

When DTT was used, more gliadins were extracted (between 1.2 and 1.7 times more) and lower RSD_R values were obtained (10.6–32.9 versus 23.1–45.9%) as compared to only 60% ethanol extraction. The chocolate cake mix spiked with 0.25% gluten-containing chocolate cake mix (sample 18) was clearly positive (428 µg/kg, 19.0% RSD_R). Moreover, all laboratories were capable of measuring the low content of hydrolyzed hordein present in the commercial beer (sample 19, 179 µg/kg), also with a very low RSD_R (17.8%).

Discussion

Gluten-Tec is a competitive ELISA capable of detecting native and hydrolyzed gliadins (3). In theory, even if the hydrolyzed fragments contained only one epitope, it would be sufficient to be detected by the antibody in a competitive format. The sequence recognized by the Gluten-Tec antibody (RPQQPYP) is included in a well-characterized T-cell stimulatory epitope involved in celiac disease (FRPQQPYPQ; 5).

The rice baby foods selected for this study were previously used in a interlaboratory study organized by the Swedish Food Administration to check the precision and limits of determination of the Ridascreen® Gliadin assay, a sandwich R5 ELISA kit manufactured by R-Biopharm (Darmstadt, Germany). The R-Biopharm kit, in combination with the Cocktail Solution, gave gliadin values twice as high as those obtained with 60% ethanol (9). Even for a short period of time, the heat-treatment of these samples had changed the proteins in such a way that only 60% ethanol was not enough to extract all gliadins (10). Therefore, a novel extraction procedure, specially designed for the Gluten-Tec ELISA kit, was developed. This method, based on DTT, extracts all gluten proteins (gliadin plus glutenin) of heated and unheated food samples.

For the validation of the Gluten-Tec ELISA kit, a two-step strategy was proposed. During the first step, the 16 rice baby foods, previously tested with the R5 ELISA in the Swedish study, were also analyzed with the Gluten-Tec antibody (11). Although a comparison between the gliadin levels determined with both kits could not be easily performed (because both ELISAs used different standards), similar sensitivities were observed (11). Both methods were capable of detecting all rice baby foods spiked with 0.1% (or more) wheat, barley, or rye baby foods.

Based on the kit's good performance, we decided to go to the second step: organization and execution of an interlaboratory study to test the repeatability and reproducibility of the ELISA part of the Gluten-Tec kit. It was performed in several international laboratories and according to AOAC guidelines. All laboratories were able to determine reliably the presence of gliadin in the spiked samples, except for the ethanol extract of the incurred maize bread sample containing 22.1 mg gliadin/kg (sample 15). This could be explained because fewer gliadins were extracted (as compared to the DTT extraction procedure) and a high variation between the participants was observed at the lower end of the standard curve.

Our definition of LOD conforms to traditional immunoassay and EC guidelines for establishing $CC\alpha$ or detection limits for substances without stated permissible levels. There are other current definitions of LOD, however. One approach is defining LOD as the sum of 1.64 times the SD of zero matrix (limit of blank; LOB) and 1.64 times the SD of a sample with concentration equal to 1.64 times the SD of zero matrix. Thus, a sample with actual concentration equal to LOD has a 5% probability of yielding a measurement result below the LOB (12). This is analogous to the notion of $CC\beta$: the concentration if present in a sample has a probability of 5% of giving a result below $CC\alpha$.

Repeatability values (within-laboratory variance), expressed as RSD_r , ranged from 6.2 to 25.7%, while reproducibility values (interlaboratory variance), expressed as RSD_R , ranged from 10.6 to 45.9%. Both statistical parameters were similar to those obtained with the sandwich R5 ELISA method for gliadin determination in dietary foods for celiacs, recommended by the

Codex Alimentarius (ALINORM 08/31/26, paragraph 64 and Appendix III), which reported RSD_r values in the range of 7–35% and RSD_R values in the range of 21–42% (13). The design of the R5 interlaboratory study included extraction and dilution of the test samples by the participants themselves, something that could have contributed to the increased RSD values.

To obtain a conversion factor between $\alpha 20$ -gliadin peptides and total gliadins, the values measured by Gluten-Tec of the incurred maize bread samples extracted with DTT (367 and 211 $\mu\text{g/kg}$) were divided by the corresponding total gliadin content, as determined by HPLC (44.2 and 22.1 mg/kg , respectively). A conversion factor of approximately 10 was calculated (i.e., 10 $\mu\text{g/kg}$ $\alpha 20$ -gliadin peptide equates approximately to 1 mg/kg gliadin). Nevertheless, food samples with different matrixes should be tested with both methods (Gluten-Tec and HPLC) in order to adequately demonstrate, with some degree of confidence, that the conversion factor between peptides and gliadin is appropriate and reproducible.

Conclusions

Gluten-Tec has been designed to overcome the drawbacks of the currently available gliadin ELISA methods. The present interlaboratory study was organized to determine its performance in laboratories having experience with ELISA methods, at levels relevant in gluten-free foods. The results confirmed that Gluten-Tec is suitable for the measurement of T-cell stimulatory epitopes over a wide range of concentration. It can clearly distinguish between negative and gliadin-containing samples (there were no false-positive results). The repeatability and reproducibility were in the normally acceptable range for ELISAs, and are comparable to those of the sandwich R5 ELISA. Moreover, the LOD is suitable for determining gliadin contamination in gluten-free products.

As good levels of precision and sensitivity were obtained, and because Gluten-Tec has a true standard, can measure both intact gliadins and fragments, and has been tested in an interlaboratory study, it will be presented to the Codex Alimentarius as a preferred method for gluten analysis.

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