



Collaborative study of a new developed ELISA kit for gluten determination

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Abstract

A collaborative study in 8 laboratories to validate a new ELISA method for quantitative gluten determination in foods was performed. The study included 17 samples: 4 gluten-free mixes, maize bread with dietary fiber, rolls, 3 samples of milled buckwheat grains, rice, maize, 3 samples of rice spiked at wheat and 3 samples of maize spiked at wheat. The ELISA method is based on two monoclonal and one polyclonal antibody developed in Immunotech a Beckman Coulter Company. The kit produces no false positive results or no cross-reactivity with oat, rice, maize and buckwheat. The standard used for ELISA kit was obtained from Working Group on Prolamin Analysis and Toxicity. Repeatability (*r*) and reproducibility (*R*) of the method were determined on levels varied from 22 to 294 mg/kg. Relative standard deviations ranged from 4.1 to 30 % for RSD_r and from 18 to 46 % for RSD_R. Sensitivity of the kit to the wheat flour contamination was estimated. All participants obtained ELISA kit with standard procedure for use and protocol for result calculation. Two real samples (bakery bread mix and buckwheat flour) were found to be contaminated with some gluten source and are unacceptable for celiac patients. The gliadin in spiked samples at level of wheat 0.1 g/100 g; 0.5 g/100 g and 1 g/100 g was detected as positive in all laboratories too. This ELISA kit could be probably recommended in the future for control of gluten-free foods in the Czech Republic.

Key words: Test, antibody, celiac patient, wheat, gluten, food.

Introduction

Celiac disease (CD), a permanent intolerance to wheat gliadins and related prolamins (barley hordeins, rye secalins, oat avenins) is caused by interaction of these proteins with the intestinal mucosa leading to permanent mucosal damage⁵. The prevalence of CD in Europe was quoted to be 1:1000 ten years ago⁴. However, with the advent of sensitive serological tests for population screening, the prevalence of CD may be 1 in 200 or even greater in several European countries^{1,3}. The people suffering from celiac disease have to refrain from foods containing celiac toxic prolamins for life, if they want to avoid symptoms. This means, that celiac patients need completely gluten-free foods. It is supposed to be about 10.000 diagnosed celiac patients in the Czech Republic (and about 30.000 hidden celiac patients). The position of celiac patients was not satisfactory before the Velvet revolution in the year 1989 in the Czech Republic. Most patients or the parents of the children had to prepare their diet themselves. Only a few food products could be purchased in the supermarkets. Since that time the situation has been changed and improved. Several producers now offer a broad assortment of gluten-free food products. Those food products are available through supermarkets, dietary foods shops or they are sent by mail-order service. The Celiac Society of Czech Republic (CSCR) was established in the year 1998. The society provides different activities to the members to help them with diet, choice of the safe food. The society gives lectures and meetings with the specialists and summer or winter camp for children. The society brings together more than 200 families. The research project supported by National Agency for Agriculture was proposed in co-operation with Celiac Society of Czech Republic and other participants. It

started in June 2001. The fundamental aim of the project is the systematic and regular gluten determination in gluten-free foods provided by Food Research Institute Prague. The development of a new sensitive, reliable ELISA kit and its validation is one of the objectives of the research project².

Materials and Method

Samples: Gluten-free mixes, rolls, maize bread with dietary fiber, buckwheat grains, buckwheat broken grains and buckwheat flour were purchased in the dietary foods shop. Rolls, maize bread with dietary fiber, buckwheat grains and buckwheat broken grains were milled. The rotor-speed mill "Pulverisette 14" (Fritsch GmbH, Manufacturers of Laboratory Instruments, Idar-Oberstein, Germany) was used for the milling of the samples. Rice and maize for preparing of the spiked samples were milled too. The spiked samples were prepared by addition of 0.1 g; 0.5 g and 1 g of wheat flour per 99.9 g; 99.5 g; and 99.0 g of rice or maize. All samples were mixed carefully in glass flasks. The samples were distributed together with the ELISA kit for collaborative study. List of samples is in Table 1.

Standard: PWG gliadin standard was prepared and characterized by Working Group on Prolamin Analysis and Toxicity. The standard was isolated from 28 most common European wheat varieties by ethanolic extraction after pre-extraction of albumins and globulins using salt solution. The ethanolic extracts were concentrated by means of ultrafiltration and lyophilized. The nitrogen content of the product was 87.7% (Kjeldahl) and 89.7% (Dumas).

Table 1. Sample overview.

1	Potato dumpling mix
2	Bakery bread mix
3	Dumpling mix
4	Buckwheat pancake mix
5	Maize bread with dietary fiber
6	Rolls
7	Buckwheat grain
8	Buckwheat broken grain
9	Buckwheat flour
10	Rice flour
11	Rice flour with wheat flour at 0.1 g/100g
12	Rice flour with wheat flour at 0.5 g/100 g
13	Rice flour with wheat flour at 1.0 g/100g
14	Maize flour
15	Maize flour with wheat flour at 0.1 g/100 g
16	Maize flour with wheat flour at 0.5 g/100 g
17	Maize flour with wheat flour at 1.0 g/100 g

PWG gliadin standard was characterized by various methods (MALDI-TOF MS, RP-HPLC, SE-HPLC, ELISA method, SDS-PAGE) ⁶. RP-HPLC revealed the proportion of gliadin types: α -gliadin (40.4 %), γ -gliadins (48.7 %), ω 1,2-gliadins (6.2 %) and ω 5- gliadins (4.7 %). SE-HPLC was performed to determine the proportions of high-molecular-weight (HMW) gliadins, monomeric gliadins and albumins/globulins. The distribution of the PWG standard on these three fractions were 28.9 %, 67.7 % and 3.7 % respectively⁷.

Chemicals: Ethanol 96% p.a., the use of ethanol denatured with methanol, up to 10% vol. is possible as well; the use of industrial spirit or ethanol denatured with other additives (petrol, pyridine) is not possible; Distilled or deionized water.

ELISA kit: New developed ELISA kit for gluten determination in food products and raw materials is based on two monoclonal and one polyclonal antibody developed in Immunotech a Beckman Coulter Company.

Format : two step ELISA 96 wells

Pre-analytical step: extraction in 40% ethanol and centrifugation.

Basic analytical parameters:

Detection limit: 3.00 ng/ml (e.g. 3 mg/kg for dilution 1:100)

Range of calibration curve: 20 – 320 ng/ml

Cross-reactivity with other cereals:	wheat	100%	rye	100%
	spelt	100%	barley	20-30%
	oat	0%	rice	0%
	maize	0%	buckwheat	0%

Calibration standard: PWG gliadin

Results and Discussion

Gluten-free foods and raw material: Results obtained by participating laboratories are laid down in Table 2. Nine samples of gluten-free foods or raw material were analyzed in collaborative study. Analyses of 3 “gluten-free” labeled samples (2, 4, 9) revealed definitely gliadin content higher than 100

ng/ml (100 mg/kg) or the content very close to the level 100 mg/kg. The effect of the technological processing resulted probably in gliadin contamination in the buckwheat samples (grains, broken grains, flour, pancake mix). The buckwheat flour and buckwheat pancake mix revealed a very high level consequently the technological processing. These samples can not be recommended for celiacs. Six samples (1, 3, 5, 6, 7, 8) were found to be acceptable and suitable for celiacs. All data were subjected to the statistical evaluation according to the ISO 5725. Statistical tests (Cochran, Dixon and Mandel) were used for detection of outliers. Six samples (3, 5, 7, 8, 10 and 14) were evaluated as negative – average of results were under the lowest calibration level (LCL = 20 mg/kg). Three samples (4, 13, 17) were above the highest calibration level (HCL = 320 mg/kg) and are evaluated as “very high”. For “negative” and “very high” samples statistical evaluation was not applied and calculation of repeatability and reproducibility was not done. Summary of repeatability and reproducibility results is in Table 3.

Spiked samples: Rice and maize samples were confirmed as gliadin negative, results for both samples were <20 mg/kg. All laboratories determined clear celiac positivity of the spiked samples with wheat flour levels at 0.1, 0.5 and 1.0 g/100 g. The samples spiked at wheat 0.1 g/100 g revealed higher variation of results (62 – 127 mg/kg for spiked rice; 35 – 127 mg/kg for spiked maize). These variation of results could be influenced by wheat non-homogeneity of addition of flour to the rice and maize and/or by lower precision of laboratory work. Sensitivity of detection of flour contamination is shown in Fig. 1. Detection limit of flour addition, corresponding to the detection limit of gluten determination 20 mg/kg, was 0.03 g/100 g wheat flour.

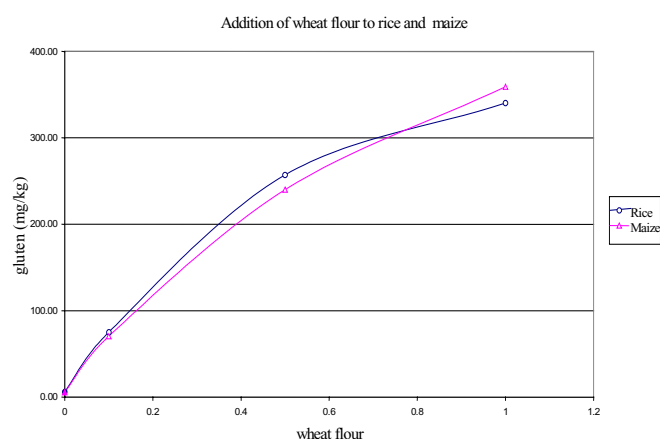


Figure 1. Sensitivity of wheat flour detection.

Conclusions

Collaborative study approved, that a new developed ELISA kit is suitable for determination of gliadin and corresponding prolamins in gluten-free food products and raw materials.

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Table 2. Results of participating laboratories gliadin content (mg/kg).

Sample	Laboratory															
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	9.8	9.8	3.7	3.7	40.1	151.7	7.4	7.4	3.8	3.8	1.4	1.4	5.8	6.4	0.0	0.0
2	79.7	93.4	136.3	134.2	101.7	208.8	81.9	108.3	147.2	120.6	583.9	670.3	81.6	78.4	106.8	86.8
3	19.8	18.8	21.7	15.7	43.2	112.4	18.8	16.4	14.4	14.4	15.9	16.8	22.0	17.9	51.9	63.1
4	514.6	514.6	424.0	424.0	440.9	428.4	34.4	35.6	337.1	463.9	395.1	395.1	468.1	468.1	432.0	432.0
5	13.6	13.6	6.1	5.7	33.4	17.9	12.5	11.5	6.3	6.6	7.5	6.2	7.6	8.1	0.1	0.0
6	24.1	24.8	18.2	23.6	109.7	81.1	21.3	23.7	31.3	35.0	18.3	20.1	24.5	23.0	16.1	10.0
7	10.9	11.4	5.3	7.7	11.6	11.6	8.7	8.0	9.2	8.9	4.3	5.4	9.9	8.3	0.3	0.1
8	16.5	15.8	22.5	17.5	13.9	45.9	9.3	9.3	8.9	8.8	11.4	12.2	9.8	11.2	0.2	0.1
9	420.1	394.7	279.9	290.8	308.6	281.5	243.5	255.7	277.8	272.3	267.3	260.4	5.8	5.8	277.0	281.7
10	9.5	9.5	5.4	7.3	11.6	11.6	7.4	7.4	3.8	3.8	1.6	1.8	6.0	10.0	0.7	0.0
11	70.4	61.4	61.3	62.8	111.5	143.0	34.8	31.6	96.3	85.4	67.6	69.0	70.9	76.9	70.2	90.8
12	491.4	394.0	241.7	364.9	401.7	330.8	73.0	69.6	208.1	189.8	202.8	209.0	177.8	210.8	307.8	341.2
13	585.4	585.4	372.8	424.0	371.6	440.9	205.2	164.9	220.0	224.2	281.9	241.2	361.3	335.6	311.5	319.2
14	11.8	11.3	5.5	5.2	12.5	19.4	9.0	8.0	4.1	4.4	3.9	2.8	6.6	6.0	0.0	0.0
15	67.8	62.1	106.3	114.9	95.0	167.0	32.8	37.4	58.1	55.8	105.8	89.0	78.4	64.1	51.9	63.1
16	502.4	486.3	248.2	233.4	214.5	379.6	16.0	14.6	185.8	190.9	670.3	48.3	177.8	210.8	269.6	231.5
17	585.0	585.0	395.7	371.1	288.1	440.9	125.3	125.9	337.1	367.9	320.4	310.5	335.6	368.0	357.4	432.0

Table 3. Repeatability and reproducibility.

		Sample number						
		6	15	11	2	16	12	9
Average (mg/kg)	y	22.42	70.54	75.23	111.84	240.10	257.15	293.65
Number of accepted results		7.00	7.00	8.00	7.00	7.00	8.00	7.00
Number of outliers		1.00	1.00	0.00	1.00	1.00	0.00	1.00
	s_r^2	7.64	62.00	118.83	1123.90	2525.68	1260.51	145.50
	s_r	2.76	7.87	10.90	33.52	50.26	35.50	12.06
	RSD_r	0.12	0.11	0.14	0.30	0.21	0.14	0.04
Relative standard deviation of repeatability (%)	RSD_r	12.33	11.16	14.49	29.98	20.93	13.81	4.11
Repeatability limit	r	7.74	22.05	30.52	93.87	140.72	99.41	33.77
	n_{ij}	2.00	2.00	2.00	2.00	2.00	2.00	2.00
	sd_j^2	74.10	1323.26	1445.24	1694.24	41107.41	27252.99	5463.09
between-laboratory variation	sL_j^2	33.23	630.63	663.20	285.17	19290.87	12996.24	2658.80
reproducibility variation	sR_j^2	40.87	692.63	782.04	1409.07	21816.54	14256.75	2804.29
	sR_j	6.39	26.32	27.96	37.54	147.70	119.40	52.96
	RSD_R	0.29	0.37	0.37	0.34	0.62	0.46	0.18
Relative standard deviation of reproducibility (%)	RSD_R	28.52	37.31	37.17	33.56	61.52	46.43	18.03
Reproducibility limit	R	17.90	73.69	78.30	105.11	413.57	334.32	148.28
	r/R	0.43	0.30	0.39	0.9	0.34	0.30	0.23

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