



# Gliadin proteins from wheat flour: the optimal determination conditions by ELISA

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## Abstract:

**Introduction.** The number of people with celiac disease is rapidly increasing. Gluten, is one of the most common food allergens, consists of two fractions: gliadins and glutenins. The research objective was to determine the optimal conditions for estimating gliadins by using enzyme-linked immunosorbent assay (ELISA).

**Study objects and methods.** The experiment involved wheat flour samples (0.10, 0.20, 0.25, 0.50, and 1.0 g) suspended in different solvents (ethanol, methanol, 1-propanol, and isopropanol) of different concentrations (40, 50, 60, 70, 80, and 90% v/v). The samples were diluted with Tris buffer in ratios of 1:50, 1:100, 1:150, and 1:200. The gliadin test was performed using a Gliadin/Gluten Biotech commercial ELISA kit (Immunolab).

**Results and discussion.** The optimal conditions for determining gliadin proteins that provided the highest gliadin concentration were: solvent – 70% v/v ethanol, extract:Tris buffer ratio – 1:50, and sample weight – 1.0 g.

**Conclusion.** The obtained results can be of great importance to determine gliadin/gluten concentrations in food products by rapid analysis methods.

**Keywords:** Extraction, gluten, gliadins, wheat flour, enzyme-linked immunosorbent assay (ELISA)

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## INTRODUCTION

Gluten is the one of the most common food allergens. According to the Codex Alimentarius, gluten is defined as a protein fraction of wheat, rye, barley, oats, their cross varieties, and derivatives, which some people are sensitive to [1, 2]. Gliadins and glutenins are two fractions present in approximately equal amounts in gluten [3].

Gliadins are represented by monomers. Due to the high content of glutamine and proline, these proteins are also called “prolamins” [4, 5]. They are not soluble in water as a result of strong hydrophobic interactions and the presence of disulfide bonds, only in aqueous alcohol [6, 7].

Gliadin proteins are divided into four groups ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\omega$  gliadins) on the basis of mobility in acidic conditions of acid polyacrylamide gel electrophoresis (A-PAGE). Some recent research on amino acid

sequences refer  $\alpha$  and  $\beta$  gliadins to the same group ( $\alpha/\beta$ ) [8, 9]. By amino acid sequences (complete and partial), amino acid composition, and molecular weight, gliadins are divided into:  $\omega 5$ ,  $\omega 1,2$ ,  $\alpha+\beta$ , and  $\gamma$  gliadins [10, 11]. As for  $\omega$  gliadins, they have a high content of glutamine, proline, and phenylalanine. They are divided into  $\omega 5$  ( $\approx 50\ 000$  Da) and  $\omega 1,2$  gliadins ( $\approx 40\ 000$  Da).

In  $\alpha+\beta$  and  $\gamma$  gliadins, the content of glutamine and proline is much lower than in  $\omega$  gliadins. The molecular weights of these fractions overlap ( $\approx 28\ 000$ – $35\ 000$  Da). They differ in the content of several amino acids (tyrosine). Both fractions contain the N- and C-terminal regions [12, 13].

Although the content of total gliadin proteins depends on the type of wheat and growth conditions (soil, climate, fertilization, etc.),  $\alpha+\beta$  and  $\gamma$  gliadins are the largest components, while  $\omega$  gliadins are present in smaller amounts [14, 15].

**Table 1** Absorption of gliadin standard solutions at 450 nm

Concentration of gliadin standard solutions, ppm	0	2	6	20	60
Absorbance (450 nm)	0.208 ± 0.02	0.365 ± 0.04	0.598 ± 0.01	1.421 ± 0.08	2.588 ± 0.17

Gluten is a common concern for people around the world, especially in the United States, where nearly one-third of the population have to reduce the intake of this protein. Numerous studies have been conducted on the adverse reactions of gluten and its impact on the health of certain population groups [16–18].

Considering that the number of people with gluten intolerance has been increasing in the last decade, the research objective was to examine the optimal conditions for determining the concentration of gliadin by a rapid enzyme-linked immunosorbent assay method (ELISA).

### STUDY OBJECTS AND METHODS

The research featured wheat flour type 500 samples with maximal ash content – 0.55%, maximal moisture – 15%, maximal acidity – 3, and protein content – 9.8 g/100 g. The samples were purchased on the market of the Republic of Srpska, Bosnia and Herzegovina.

The gliadin test involved the following chemicals: ethanol (Refined REAHEM, 96% v/v ethyl alcohol, Srbobran), methanol (Lach-Ner, Czech Republic, high purity, ≥ 99.99%), 1-propanol Lach-Ner, Czech Republic, high purity, ≥ 99.00%), and isopropanol (Lach-Ner, Czech Republic, high purity, 99.90%). The deionized water was obtained in laboratory conditions using a Water Technologies device W3T199551 (Siemens Ultra Clear) at a conductivity of 0.055 mS/cm and temperature of 20°C.

The commercial kit (Immunolab, GmbH, Gliadin/Gluten ELISA, D-Kassel, Germany) contained the following chemicals: a series of gliadin standard solutions (concentrations 0, 2, 6, 20, and 60 ppm), a conjugate (anti-gliadin peroxidase), a substrate (tetramethylbenzidine, TMB), a stop solution (0.5 M H<sub>2</sub>SO<sub>4</sub>), a buffer (Tris), and a wash solution (PBS + Tween 20), plus 96 wells. According to the manufacturer's instructions, the putty is to be stored in the refrigerator at 2–8°C.

**Sample preparation.** The wheat flour samples (1.0, 0.5, 0.25, 0.20, 0.10 g ± 0.0001 g) were suspended in 10.0 ml of solvent (ethanol, methanol, isopropanol, and 1-propanol) of different concentrations (40, 50, 60, 70, 80, and 90% v/v). The samples were homogenized with an Ultra-Turrax homogenizer (IKA T25 digital, 10 000 rpm) for 5 min. The samples were then centrifuged (Hettich zentrifugen, rotina 380 R) at 2000 rpm for 10 min. After centrifugation, the supernatant was drained and diluted in a ratio of 1:50 with 10x concentrated Tris buffer, which had been diluted before use.

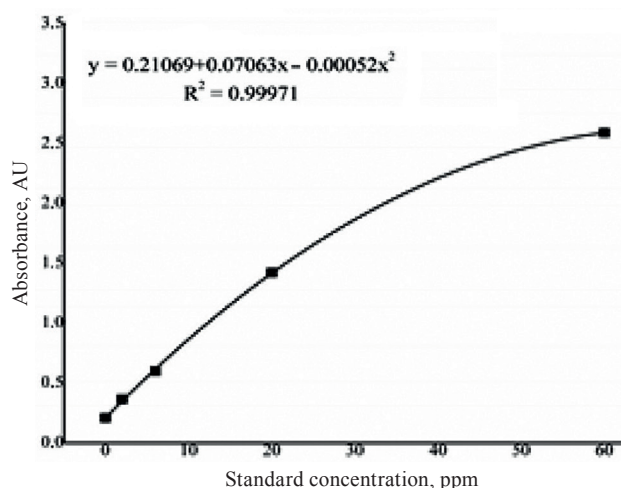
**Determination gliadin proteins by ELISA.** The samples and 100 µL of gliadin standard solution (concentrations 0, 2, 6, 20, and 60 ppm) were pipetted

into wells, followed by incubation for 20 min at room temperature. The rinsing solution was concentrated (10x) and diluted 1:9 with distilled water. The wells were rinsed with 300 µL of the rinsing solution by adding it into the wells; the procedure was repeated three times. After washing, 100 µL of the conjugate (anti-gliadin peroxidase) was pipetted into the wells and incubated for 20 min. Then, the washing procedure was repeated, and 100 µL of the substrate was put into the wells. To react, they were left in a dark place for 20 min at 20°C until the content of the well turned blue. Upon adding 100 µL of the stop solution (0.5 M H<sub>2</sub>SO<sub>4</sub>), the blue color turned yellow. After mixing, the absorbance was measured using an ELISA reader (Chromate, Awareness Technology) at 450 nm. The color was stable after 30 min.

### RESULTS AND DISCUSSION

Table 1 shows the absorbance of the gliadin standard solutions at the concentrations of 0, 2, 6, 20, and 60 ppm at a wavelength of 450 nm. The results made it possible to calculate the dependence of the absorbance on the protein solution concentration, as illustrated by the calibration curve (Fig. 1). The correlation coefficient ( $R^2 = 0.9997$ ) showed a high dependence of the absorbance on the concentration of standard gliadin solutions.

Table 2 shows descriptive indicators of gliadin concentration (ppm) values in extracts obtained from wheat flour samples after extraction with different concentrations of ethanol. During the extraction, which lasted for 20 min, the samples were mixed after every



**Figure 1** Dependence of absorbance on the concentration of gliadin standard solutions

**Table 2** Descriptive indicators of gliadins measured in wheat flour extracts at different solvent concentrations (sample weight 1.0 g ± 0.0001, solvent ethanol)

Ethanol concentration, %	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
40	6	85.42	3.40	1.39	81.86	88.99	78.68	87.61
50	6	88.83	3.33	1.36	85.34	92.32	83.66	93.38
60	6	102.23	2.65	1.08	99.44	105.02	98.71	105.21
70	6	104.15	2.06	0.84	101.99	106.32	100.93	107.21
80	6	75.74	1.63	0.67	74.03	77.45	73.67	78.41
90	6	69.47	3.72	1.52	65.56	73.38	62.92	73.69
ANOVA	F(5.30) = 137.58, Sig. = 0.000, eta square = 5781.29/6033.41 = 0.96							

**Table 3** Descriptive indicators of gliadins in wheat flour extracts at different solvent concentrations (sample weight 1.0 g ± 0.0001, solvent methanol)

Methanol concentration, %	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
40	6	83.29	4.85	1.98	78.20	88.38	74.13	88.26
50	6	88.70	2.02	0.83	86.58	90.83	86.65	91.81
60	6	89.51	3.26	1.33	86.09	92.93	83.98	93.41
70	6	95.49	2.69	1.10	92.67	98.31	91.23	99.33
80	6	73.77	2.81	1.15	70.83	76.72	70.52	78.03
90	6	73.81	3.22	1.31	70.43	77.18	69.04	78.33
ANOVA	F(5.30) = 44.48, Sig. = 0.000, eta square = 2360.62/2679.04 = 0.88							

5 min. The obtained extracts were diluted with Tris buffer in a ratio of 1:50.

A descriptive analysis showed that the highest gliadin concentration was obtained after extraction with 70% ethanol (104.15 ppm). Extraction with 90% ethanol demonstrated the lowest gliadin concentration (69.47 ppm). A one-factor analysis of variance of different groups revealed a statistically significant difference in the gliadin concentration at  $F(5.30) = 137.58$  and  $\text{Sig.} = 0.000$ .

Table 2 shows that the increased solvent concentration between 40 and 70% affected the efficiency of gliadin protein extraction from wheat flour samples: the protein concentration increased. However, a further increase in the solvent concentration (80 and 90%) reduced the extraction efficiency: gliadin protein concentration was lower than in the case of 70% ethanol.

Table 3 illustrates the descriptive indicators of gliadin concentration (ppm) after extraction with methanol of different concentrations.

The highest concentration of gliadins was obtained after extraction with 70% methanol (95.49 ppm), while 80% methanol showed the lowest concentration (73.77 ppm). A one-factor analysis of variance of different groups showed a statistically significant difference in the gliadin concentrations at  $F(5.30) = 44.48$  and  $\text{Sig.} = 0.000$  (Table 3).

Under these conditions, the protein extraction was more effective when the methanol concentration was 40–70%, while a further increase in the concentration of methanol (80 and 90%) reduced the extraction efficiency.

Table 4 shows the descriptive indicators of gliadin concentrations (ppm) after extraction with 1-propanol of different concentrations.

**Table 4** Descriptive indicators of gliadins in wheat flour extracts at different solvent concentrations (sample weight 1.0 g ± 0.0001, solvent 1-propanol)

1-propanol concentration, %	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
40	6	97.36	1.92	0.78	95.35	99.37	93.59	98.90
50	6	98.40	1.99	0.82	96.30	100.49	95.57	100.98
60	6	101.16	2.01	0.82	99.05	103.27	97.70	103.06
70	6	94.33	1.91	0.78	92.32	96.33	91.19	96.79
80	6	96.40	1.88	0.77	94.43	98.37	93.38	98.70
90	6	84.97	1.75	0.72	83.13	86.81	83.29	88.18
ANOVA	F(5.30) = 51.45, Sig. = 0.000, eta square = 941.55/1051.34 = 0.89							

**Table 5** Descriptive indicators of gliadins in wheat flour extracts at different solvent concentrations (sample weight 1.0 g ± 0.0001, solvent isopropanol)

Isopropanol concentration, %	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
40	6	83.65	7.63	3.12	75.64	91.66	73.18	92.36
50	6	92.77	3.80	1.55	88.79	96.75	86.35	97.22
60	6	92.27	3.72	1.52	88.36	96.18	87.31	97.97
70	6	103.35	2.97	1.21	100.23	106.46	98.81	107.23
80	6	93.29	3.69	1.51	89.42	97.17	86.45	97.27
90	6	85.24	3.38	1.38	81.69	88.79	78.80	88.75
ANOVA	F(5.30) = 14.72, Sig. = 0.000, eta square = 1476.98/2079.01 = 0.71							

**Table 6** Descriptive indicators of gliadins in wheat flour extracts diluted with different Tris buffer concentrations (sample weight 1.0 g ± 0.0001, solvent 70% ethanol)

Extract:Tris buffer ratio	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
1:50	6	104.15	2.06	0.84	101.99	106.32	100.93	107.21
1:100	6	95.08	0.96	0.39	94.07	96.08	93.29	95.89
1:150	6	89.06	2.88	1.18	86.04	92.09	83.68	91.27
1:200	6	84.35	2.87	1.17	81.33	87.36	79.48	87.88
ANOVA	F(3.20) = 80.62, Sig. = 0.000, eta square = 1314.04/1422.70 = 0.92							

**Table 7** Descriptive indicators of gliadins in wheat flour extracts diluted with different Tris buffer concentrations (sample weight 1.0 g ± 0.0001, solvent 70% isopropanol)

Extract:Tris buffer ratio	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
1:50	6	103.35	2.97	1.21	100.23	106.46	98.81	107.23
1:100	6	84.87	1.47	0.60	83.33	86.42	83.69	87.69
1:150	6	74.24	2.23	0.91	71.89	76.58	70.40	76.84
1:200	6	65.95	3.25	1.33	62.53	69.36	61.12	70.30
ANOVA	F(3.20) = 235.73, Sig. = 0.000, eta square = 4691.03/4823.70 = 0.97							

The highest concentration of gliadins was obtained after extraction with 60% 1-propanol (101.16 ppm), while 90% 1-propanol resulted in the lowest concentration (84.97 ppm). A one-factor analysis of variance of different groups revealed a statistically significant difference in the gliadin concentration at  $F(5.30) = 51.45$  and  $\text{Sig.} = 0.000$  (Table 4).

A lower solvent concentration of 1-propanol between 40 and 60% increased the efficiency of gliadin protein extraction, while the protein extraction efficiency tended to decrease with a further increase in solvent concentration (70, 80 and 90%), i.e. the concentration decreased.

Table 5 shows the descriptive indicators of gliadin concentrations (ppm) after extraction with isopropanol of different concentrations.

The highest concentration of gliadin was obtained after extraction with 70% isopropanol (103.35 ppm). Extraction with 40% isopropanol showed the lowest concentration of gliadins (83.65 ppm). A one-factor analysis of variance showed a statistically significant difference in gliadin concentrations at  $F(5.30) = 14.72$  and  $\text{Sig.} = 0.000$  (Table 5).

A higher solvent concentration of isopropanol for gliadin protein extraction between 40 and 70% increased the extraction efficiency, while further increase in the solvent concentration (80 and 90%) resulted in a lower extraction efficiency, compared to the experiment with 70% isopropanol.

Based on Tables 2–5, the best efficiency of gliadin protein extraction was achieved during the experiments with 70% ethanol and 70% isopropanol as solvents.

Table 6 demonstrates the descriptive indicators of the gliadin concentration (ppm) after extraction with 70% ethanol, followed by dilution of the extract with different Tris buffer concentrations.

The extract:Tris buffer ratios of 1:50 and 1:200 demonstrated the highest and the lowest concentration of gliadins (104.15 and 84.35 ppm, respectively). A one-factor analysis of variance of different groups showed a statistically significant difference in gliadin concentration at  $F(3.20) = 80.62$  and  $\text{Sig.} = 0.000$ . An increase in Tris buffer concentration decreased gliadins.

Table 7 shows the descriptive indicators of gliadin concentrations (ppm) in wheat flour extracts obtained after extraction with 70% isopropanol and diluted with different Tris buffer concentrations.



**Table 8** Descriptive indicators of gliadins in wheat flour extracts at different sample weights (solvent 70% ethanol, extract:buffer ratio 1:50)

Sample weight, g	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
0.10 ± 0.0001	6	48.41	1.06	0.43	47.30	49.53	46.69	49.73
0.20 ± 0.0001	6	54.67	4.40	1.80	50.05	59.28	51.51	63.30
0.25 ± 0.0001	6	55.80	3.62	1.48	52.01	59.60	52.13	61.72
0.50 ± 0.0001	6	63.94	3.64	1.49	60.12	67.77	60.37	68.91
1.00 ± 0.0001	6	104.15	2.06	0.84	101.99	106.32	100.93	107.21
ANOVA	F(4,25) = 20.85, Sig. = 0.000, eta square = 732.45/966.64 = 0.76							

**Table 9** Descriptive indicators of gliadins in wheat flour extracts at different sample weights (solvent 70% isopropanol, extract:buffer ratio 1:50)

Sample weight, g	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower Bound	Upper bound		
0.10 ± 0.0001	6	53.59	1.58	0.65	51.93	55.25	51.81	56.14
0.20 ± 0.0001	6	54.96	2.98	1.22	51.84	58.09	52.18	60.33
0.25 ± 0.0001	6	58.77	1.66	0.68	57.02	60.51	56.24	61.12
0.50 ± 0.0001	6	65.58	1.25	0.51	64.27	66.88	64.22	67.79
1.00 ± 0.0001	6	103.35	2.97	1.21	100.23	106.46	98.81	107.23
ANOVA	F(4,25) = 44.05, Sig. = 0.000, eta square = 518.69/597.20 = 0.87							

The highest concentration of gliadins was obtained in the extract diluted with Tris buffer in a ratio of 1:50 (103.35 ppm). The ratio of 1:200 showed the lowest concentration of gliadins (65.95 ppm). A one-factor analysis of variance of different groups demonstrated a statistically significant difference in the concentration of gliadins calculated by the eta square indicator at  $F(3,20) = 235.73$  and  $\text{Sig.} = 0.000$  (Table 7). An increase in Tris buffer decreased gliadin protein concentration.

Table 8 shows the descriptive indicators of gliadins (ppm) extracted from wheat flour samples of different weights with 70% ethanol as solvent. The extracts were diluted with Tris buffer in a ratio of 1:50.

The highest and lowest concentration of gliadins was observed in samples with wheat flour weights of 1.00 and 0.10 g (104.15 and 48.41 ppm, respectively). A one-factor analysis of variance of different groups showed a statistically significant difference in gliadin concentration at  $F(4,25) = 20.85$  and  $\text{Sig.} = 0.000$  (Table 8).

Table 9 shows descriptive indicators of gliadins (ppm) extracted from wheat flour samples of different weights with 70% isopropanol as solvent. The extracts were diluted with Tris buffer in a ratio of 1:50.

Samples with wheat flour weights of 1.00 and 0.10 g had the highest and the lowest gliadin concentrations (103.35 and 53.59 ppm, respectively). A one-factor analysis of variance of different groups showed a statistically significant difference in gliadin concentration at  $F(4,25) = 44.05$  and  $\text{Sig.} = 0.000$  (Table 9). An increase in the weight of the wheat flour increased the gliadin protein concentration value.

Ayob *et al.* developed an enzyme-linked immunosorbent assay (ELISA) in order to determine gliadin proteins in food [19]. They studied three gliadins extracted from wheat flour samples with 70% (v/v) ethanol. The samples were vortexed for 30 min. Prior to the analysis, they were diluted with water in different ratios (1:10, 1:100, 1:1000, and 1:10 000). The highest concentration of gliadin was obtained in the sample diluted 1:10, and the lowest – in the sample diluted 1:10 000.

Allred and Ritter determined the gliadin and glutenin content in flour and in products available on the market, using four commercial ELISA tests [20]. They extracted gliadin with 0.3 M Na-iodide and 7.5% (v/v) 1-propanol. The first test detected gluten in 29 out of 40 analyzed products, the second – in 20 products, the third – in 12 products, and the fourth in 18 products.

Gujral *et al.* determined the gliadin content by ELISA sandwich technique [21]. Gliadins were extracted with 250 mM 2-mercaptoethanol+2M guanidine hydrochloride. The scientists added 7.5 mL of 80% (v/v) ethanol to the solution. Vortex mixing was performed for 30 min. The gliadin content in wheat flour was 7.4 µg/kg.

The results obtained in this work are in conformity with the research by Ayob *et al.*, who also extracted gliadins with 70% (v/v) ethanol and detected the dependance between an increasing dilution and a lowering gliadin concentration [19].

## CONCLUSION

To determine the optimal conditions for estimating

gliadin proteins by the ELISA method, we used different solvents (ethanol, methanol, 1-propanol, and isopropanol) at different concentrations (40, 50, 60, 70, 80, and 90%) as well as varied wheat flour weights (0.10, 0.20, 0.25, 0.50 and 1.00 g) and extract:buffer ratios (1:50, 1:100, 1:150, and 1:200).

The experiments demonstrated that 70% ethanol and 70% isopropanol were the optimal solvents, which resulted in the highest gliadin concentrations. However, 70% ethanol had a better financial feasibility. 70% ethanol, a Tris buffer dilution ratio of 1:50, and a wheat flour sample weight of 1.00 g were the optimal conditions that provided the highest concentration of gliadins (104.15 ppm).

Considering the growing number of people with celiac disease, the results obtained can be of great fundamental importance in the study and determination of gliadin/gluten concentrations in food products labeled as gluten or gluten free by ELISA rapid method.

#### CONTRIBUTION

Ž. Marjanović-Balaban, V. Gojković Cvjetković, R. Grujić conceived, designed, and performed the experiments, analyzed the data, contributed reagents, materials and analytical tools, and wrote the paper.

#### CONFLICT OF INTEREST

The authors declare no potential conflict of interests regarding the publication of this article.

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