INVESTIGATION OF DIFFERENT EXTRACTION CONDITIONS ON THE EFFICIENCY OF GLIADIN EXTRACTION AND DETERMINATION BY ELISA METHOD*

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Gluten refers to a complex mixture of gliadins and glutenins. It can cause numerous foodborne disorders. In sensitive individuals gluten can lead to celiac disease (CD), wheat sensitivity and allergy. Gliadin proteins are one of the gluten fractions. The aim of this paper was to examine how different conditions, mixing time (2.5, 5, 7.5, and 10 min) of the sample with the most commonly solvent 70% (v/v) ethanol and 70% (v/v) isopropanol and incubation time (15, 20, 25, and 30 min) affect the efficiency of gliadin determination with the ELISA method. A commercial kit was used to determine gliadin concentrations, and absorbance was measured at 450 nm, using the ELISA reader. Based on the obtained results, the optimal mixing time of the sample with the solvents was 5 minutes and the incubation time was 25 minutes. Under these conditions, the extraction efficiency is the best, i.e., the highest gliadin concentration is obtained. The results of research can be of fundamental importance in the study of gluten proteins and the impact of technological procedures on their change and the possibility of reducing the allergic effect.

Keywords: gliadins, mixing and incubation time, ELISA.

Introduction

Wheat feeds one-quarter of the annual worldwide demand for plant proteins. It has been the source of nutrition since the dawn of human civilization. But it is also responsible for numerous foodborne disorders [1, 2]. Gluten refers to a complex mixture of gliadin and glutenin proteins that serves as fuel for multiple disorders [3, 4]. Gluten-intake in sensitive individuals can lead to celiac disease (CD), wheat sensitivity and allergy with celiac disease (CD) being the most prevalent gastrointestinal disorder [5]. The prevalence of CD is approximately 1% in regions populated by individuals of European origin. Due to the increase in popularity of a western style diet, which is gluten-rich, the diagnosis of CD is increasing globally [5-7]. The only treatment is a lifelong gluten-free diet [8]. According to the Codex definition, any food product containing gluten > 20 mg/kg is not gluten-free [9].

The solubility of gluten differs depending on the degree of aggregation. The monomeric protein is dissolved in alcohol (gliadins), and the polymeric forms are dissolved in alcoholic solutions with the addition of a reducing agent. The gliadin content in gluten is generally determined to be approximately 50% [10, 11].

Various analytical methods are available to determine or verify the gluten content, such as enzyme-linked immunosorbent assay (ELISA), liquid chromatography with mass

spectrometry detection, molecular biological techniques (polymerase chain reaction) [12]. The most commonly used methods in routine analysis are ELISA methods. These methods are based on immunochemical reactions, in which antibodies recognize one or more short peptide sequences (epitopes) of gluten proteins. These reactions result in very specific and sensitive determinations [13-15].

The Codex Alimentarius Commission proposes enzyme-linked immunosorbent assay (ELISA) as an analytical method to achieve gluten-free product labeling. This technique includes sandwich and competitive methods. Most commercial ELISA test kits are based on monoclonal antibodies. ELISA is very expensive, and the reproducibility of the results varies depending on the ELISA test kit type [15, 16].

Commercially available ELISA tests provide partially different strategies for the determination of gluten concentration as they apply different antibodies, extraction procedures, and calibrating materials. ELISA methods cannot distinguish whether gluten originates from wheat, rye, and barley. Thus, it is unknown if a positive finding by ELISA may be due to the presence of wheat, barley, or rye. Current commercial ELISA kits for the detection of gluten are calibrated against wheat material (commonly gliadin) [16-18].

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Given that the number of people with celiac disease and allergy to gluten is increasing day by day, the aim of this paper was to examine how different extraction conditions (mixing time of the sample with the most commonly solvent 70% v/v ethanol and 70% v/v isopropanol and incubation time) affect the efficiency of gliadin determination by ELISA method.

Experimental

Determination of gliadin proteins concentration by ELISA method was performed from wheat flour samples type 500 (ash content: max 0.55%, moisture max: 15%, acidity pH=3, protein content 9.8 g/100 g) purchased on the market of the Republic of Srpska, Bosnia and Herzegovina. The following chemicals were used for the determination: ethanol (Refined REAHM, 96% v/v ethyl alcohol, Srbobran, quality corresponds to the quality property for ethyl alcohol, contains a minimum of 96% v/v ethanol) and isopropanol (Lach-Ner, Czech Republic, high degree of purity, 99.90%), deionized water (obtained in laboratory conditions, on the device Simens water Technologies W3T199551, Siemens Ultra Clear, at a conductivity of 0.055 mS/cm and at a temperature of 20 °C), commercial kit (Immunolab, GmbH, Gliadin/Gluten ELISA, D-Kassel, Germany). The kit contains the following chemicals: series of gliadin standard solutions (concentrations 0, 2, 6, 20 and 60 mg/l, conjugate (anti-gliadin peroxidase), substrate (tetramethylbenzidine, TMB), stop solution (0.5 M H₂SO₄), buffer (Tris, concentrated 10x), wash solution (PBS + Tween 20, concentrated 10x), as well as 96 wells. According to the manufacturer's instructions, the kit should be stored in the refrigerator at a temperature of 2-8 °C.

Sample preparation

Wheat flour samples weighing 0.1 g (± 0.0001 g) were suspended in 10.0 ml of ethanol and isopropanol, concentration 70% (v/v). The samples were then mixed with Ultra-Turrax (IKA T25 digital, 10,000 rpm) for 2.5, 5, 7.5 and 10 minutes. Homogenized samples were centrifuged (Hettich zentrifugen, rotina 380 R) at 2 000 rpm for 10 minutes. After centrifugation, the supernatant was decanted and diluted with Tris buffer. The buffer obtained in the kit was concentrated 10 times and diluted by deionized water in ratio 1:50 v/v before use.

Determination of gliadin concentration

Gliadin standard solution (100 μ l, concentrations 0, 2, 6, 20 and 60 mg/l) and samples were pipetted into the wells. Then, an incubation was performed for 15, 20, 25 and 30 minutes at room temperature (20 °C). After incubation, the wells were rinsed with rinsing solution. The rinsing solution obtained in the kit was concentrated (10 times) and diluted 1:9 v/v with deionized water before use. 300 μ l of rinsing solution was added to the wells and this procedure was repeated three times. After rinsing, 100 μ l of conjugate (anti-gliadin peroxidase) was pipetted into the wells and incubated for 20 minutes. After the incuba-

tion was completed, the washing procedure was repeated, as already mentioned. 100 μ l of substrate was added to the wells. They were left in a dark place to react (20 minutes, 20 °C). During that period, a blue color developed. By adding 100 μ l of stop solution (0.5 M H₂SO₄), the blue color turned yellow. After mixing, the measured absorbance was read on the ELISA reader (Chromate, Awareness Technology) at 450 nm. The color was stable for 30 minutes.

The gluten concentration can be achieved when the obtained gliadin concentration is multiplied by 2, because the presence of gliadin in gluten is approximately 50%.

Statistical analysis

Statistical analysis of the results was done in IBM SPSS, Statistics 26. By descriptive statistical analysis, the average value (X_{av}), standard deviation (SD), std. error, min and max value were calculated. To assess the influence of mixing and incubation time on the gliadin concentration, analysis of variance of different groups was used, and the significance of differences between average values at significance level $p=0.05$ was assessed by subsequent Tukey tests.

Results and discussion

In order to determine the gliadin concentration from wheat flour samples, the absorbance for gliadin standard solutions was measured first (Figure 1).

Figure 1 Dependence of absorbance on gliadin standard solution concentration

Table 1 shows the descriptive indicators of gliadin concentration (mg/l) in wheat flour extracts (0.10 g $\pm$ 0.0001) obtained after extraction with 70% (v/v) ethanol and different incubation times (15, 20, 25, and 30 min).

Descriptive analysis showed that the highest gliadin concentration was obtained during the incubation time of 25 minutes (X_{av}=53.72 mg/l). The lowest gliadin concentration was obtained during the incubation time of 15 minutes (X_{av}=32.66 mg/l). Based on the results shown in Table 1, it can be seen that an increase in incubation time (15, 20, and 25 min), under the same extraction conditions, results in an increase in gliadin protein concentration, while a further increase in incubation time (above 25 minutes) leads to a decrease in concentration,

because protein deposition occurs due to coagulation, i.e., reduced particle movement [19].

One-factor analysis of variance of different groups revealed that there was a statistically significant difference in gliadin concentration. The Tukey's test of actual dif-

ferences showed that the gliadin concentration obtained during the incubation of 15 minutes was statistically significantly different from the concentration obtained during the incubation of 20, 25 and 30 minutes.

Table 1. Descriptive indicators of gliadin protein concentration (mg/l) measured in wheat flour extracts depending on incubation time, solvent 70% (v/v) ethanol, ELISA reader Chromate Awareness Technology

Incubation time (min)	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
15	6	32.66	1.22	0.50	31.38	33.95	31.03	34.16
20	6	48.36	1.27	0.51	47.03	49.69	46.69	49.73
25	6	53.72	1.81	0.74	51.82	55.61	51.60	56.04
30	6	50.57	1.57	0.64	48.92	52.22	48.36	52.61
ANOVA		F(3,20)=238.47, Sig.=0.000, eta square=1580.78/1624.97=0.97						

Table 2. Descriptive indicators of gliadin protein concentration (mg/l) measured in wheat flour extracts depending on the incubation time, solvent 70% (v/v) isopropanol, ELISA reader Chromate Awareness Technology

Incubation time (min)	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
15	6	34.86	1.71	0.70	33.06	36.66	32.98	37.87
20	6	53.75	1.64	0.67	52.03	55.48	51.81	56.14
25	6	55.11	1.73	0.70	53.30	56.93	51.73	56.48
30	6	43.85	2.20	0.90	41.54	46.15	42.44	48.24
ANOVA		F(3,20)=159.77, Sig.=0.000, eta square=1612.51/1679.79=0.96						

Table 2. shows the descriptive indicators of gliadin concentration (mg/l) in wheat flour extracts ($0.10 \text{ g} \pm 0.0001$) obtained after extraction with 70% (v/v) isopropanol and different incubation times (15, 20, 25, and 30 min).

Descriptive analysis showed that the highest gliadin concentration was obtained during the incubation time of 25 minutes ($X_{av}=55.11 \text{ mg/l}$). The lowest gliadin concentration was obtained during the incubation time of 15 minutes ($X_{av}=34.86 \text{ mg/l}$) (Table 2).

One-factor analysis of variance of different groups revealed that there was a statistically significant differ-

ence in gliadin concentration. Subsequent comparison with the test of actual differences showed that the gliadin concentration obtained during the incubation of 15 minutes was statistically significantly different from the concentration obtained during the incubation of 20, 25 and 30 minutes.

Table 3 shows the descriptive indicators of gliadin concentration (mg/l) in wheat flour extracts (sample weight $0.10 \text{ g} \pm 0.0001$) after extraction with 70% (v/v) ethanol and different mixing times of the samples (2.5, 5, 7.5, and 10 min).

Table 3. Descriptive indicators of gliadin protein concentration (mg/l) measured in wheat flour extracts, solvent 70% (v/v) ethanol, mixing time of samples with solvent 2.5, 5, 7.5, and 10 min, ELISA reader Chromate Awareness Technology

Mixing time (min)	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
2.5	6	46.92	2.85	1.16	43.93	49.91	44.41	52.37
5	6	53.92	1.60	0.65	52.24	55.60	51.60	56.04
7.5	6	51.27	0.91	0.37	50.31	52.23	49.75	52.23
10	6	50.84	2.67	1.09	48.05	53.64	47.87	55.82
ANOVA		F(3,20)=10.76, Sig.=0.000, eta square=150.07/243.08=0.62						

Descriptive analysis showed that the highest gliadin concentration was obtained during the mixing time of the solvent with the sample of 5 minutes ($X_{av}=53.92 \text{ mg/l}$). The lowest gliadin concentration was obtained during a mixing time of 2.5 minutes ($X_{av}=46.92 \text{ mg/l}$).

One-factor analysis of variance of different groups

revealed that there was a statistically significant difference in gliadin concentration. Subsequent comparison showed that the gliadin concentration obtained during mixing for 2.5 minutes was statistically significantly different from the concentration obtained during mixing of 5, 7.5 and 10 minutes.

Based on the obtained results, it can be explained that by mixing time for 5 min, the best homogenization of the sample is achieved. Other tested mixing times do not have a large effect on the results.

Descriptive analysis in the table 4 showed that the

highest gliadin concentration was obtained during the mixing time of the solvent with the sample of 5 minutes ($X_{av}=55.11$ mg/l), and the lowest during the mixing time of 2.5 minutes ($X_{av}=42.10$ mg/l).

Table 4. Descriptive indicators of gliadin protein concentration (mg/l) measured in wheat flour extracts, solvent 70% (v/v) isopropanol, mixing time of samples with solvent 2.5, 5, 7.5, and 10 min, ELISA reader Chromate Awareness Technology

Mixing time (min)	N	Xav	SD	Std. error	95% confidence interval of average		Min	Max
					Lower bound	Upper bound		
2.5	6	42.10	2.21	0.90	39.77	44.42	37.88	43.83
5	6	55.11	1.73	0.71	53.30	56.93	51.73	56.48
7.5	6	51.57	2.40	0.98	49.05	54.09	48.51	55.59
10	6	50.84	2.67	1.09	48.05	53.64	47.87	55.82

ANOVA $F(3,20)=35.34$, Sig.=0.000, eta square=550.56/654.43=0.84

One-factor analysis of variance revealed that there was a statistically significant difference in gliadin concentration. Tukey's test for actual differences found that the gliadin concentration obtained during mixing for 2.5 minutes was statistically significantly different from the concentration obtained during mixing for 5, 7.5 and 10 minutes.

Nicolas et al. [20] investigated the advantages of ELISA competitive technique in the determination of gliadin content in wheat flour (21 flour samples), compared to biochemical methods. Gliadin extraction was performed with 70% (v/v) ethanol. Incubation was performed for one hour and 30 minutes. Based on the obtained results, the gliadin content ranged from 23.3-52.0 mg/g depending on the wheat variety.

Mena et al. [21] determined the gluten content in food by the competitive ELISA technique. The samples were extracted with 60% (v/v) ethanol and with a solution 2-mercaptoethanol+guanidine hydrochloride in phosphate buffer. Incubation was performed on a thermoshaker for 40 minutes at 50 °C. The absorbance was measured at 450 nm.

Comparing the results obtained in this paper with other authors [20, 21], it can be seen that in this paper a shorter incubation time is needed to achieve a more efficient extraction and a higher gliadin concentration.

Conclusions

By investigating how different extraction conditions of gliadin from wheat flour, such as mixing sample with the most commonly solvents (70% v/v ethanol and 70% v/v isopropanol) for 2.5, 5, 7, and 10 minutes and different incubation times (15, 20, 25, and 30 minutes) affect the efficiency of determination of gliadin concentration with the ELISA method, the following conclusions were reached: the optimal mixing time of the sample with the both solvent was 5 minutes and the incubation time was 25 minutes. Under these conditions, the extraction efficiency was the best, i.e., the highest gliadin concentration was obtained. The results of the investigation can

be of fundamental importance in the study of gluten proteins and the influence of technological procedures on their change and the possibility of reducing the allergic reactions.

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Izvod

ODREĐIVANJE RAZLIČITIH USLOVA EKSTRAKCIJE NA EFIKASNOST EKSTRAKCIJE I ODREĐIVANJE GLIJADINA ELISA METODOM

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Gluten predstavlja složenu smešu glijadina i glutenina. Može izazvati brojne hranom izazvane poremećaje. Kod osetljivih pojedinaca dovodi do celijačne bolesti, preosetljivosti na pšenicu i alergiju. Glijadini su jedna od frakcija glutena. Cilj ovog rada je bio da se ispita kako različiti uslovi ekstrakcije, kao što su vreme mešanja uzorka (2,5; 5; 7,5 i 10 min) sa najčešćim rastvaračima kao što su 70% (v/v) etanol i 70% (v/v) izopropanol i vreme inkubacije (15, 20, 25 i 30 min) utiču na efikasnost određivanja glijadina ELISA metodom. Za određivanje je korišćen komercijalni kit, a apsorbanca je izmerena na 450 nm, koristeći ELISA čitač. Na osnovu dobijenih rezultata, kao optimalno vreme mešanja uzorka sa rastvaračem, pokazalo se vreme u trajanju od 5 min i vreme inkubacije u trajanju od 25 min. Pri navedenim uslovima, efikasnost ekstrakcije je najbolja, odnosno dobijena je najveća koncentracija glijadina. Rezultati istraživanja mogu biti od fundamentalnog značaja u proučavanju proteina glutena i uticaju tehnoloških postupaka na njihovu promjenu i mogućnost smanjenja alergijskog djelovanja.

Ključne reči: glijadini, vreme mešanja i vreme inkubacije, ELISA