Separation of gliadins from wheat flour by capillary gel electrophoresis: optimal conditions

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Abstract:
Introduction. Gliadin proteins are one of the gluten fractions. They are soluble in alcoholic solution and divided into four groups (α + β, γ, ω1.2, and ω5-gliadins). In this paper gliadins were extracted from wheat flour, and optimal conditions for their separation were determined.

Study objects and methods. The separation was performed by capillary gel electrophoresis on Agilent apparatus, CE 7100 (a capillary with an inner diameter of 50 µm, a total length of 33 cm, and an effective length of 23.50 cm). In order to determine the optimal conditions, different solvent concentrations (50, 60, and 70% ethanol), capillary temperatures (20, 25, 30, 35, and 40°C), and electrode voltages (−14.5, −16.5, −17.5 and −18.5 kV) were applied. Migration time and relative concentration of each protein molecules within gliadin fractions in the electrophoregram were analysed using Agilent ChemStation Software.

Results and discussion. The optimal conditions for gliadin separation were: solvent 70% (v/v) ethanol, capillary temperature of 25°C, and electrode voltage of −16.5 kV. Under these conditions, the total proteins were identified as Xav = 23.50, including α + β gliadin fraction (Xav = 7.50 and relative concentration RC = 28.29%), γ-gliadins (Xav = 5.00, RC = 26.66%), ω1.2-gliadins (Xav = 4.33, RC = 14.93%), and ω5-gliadins (Xav = 6.67, RC = 30.98%).

Conclusion. The results of the research 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 effect of gluten during processing.

Keywords: Proteins, wheat, extraction, ethanol, electrophoresis, gluten

INTRODUCTION

Gliadin proteins represent one of the gluten fractions. Most gliadin proteins are present as monomers. They affect the viscosity and extensibility of wheat flour [1, 2]. Gliadins are divided into four groups, namely α, β, γ, and ω-gliadins. This division is based on mobility at low pH, i.e. in acidic conditions of A-PAGE electrophoresis medium (acid polyacrylamide gel electrophoresis). Based on research that was later conducted on amino acid sequences, α and β gliadins were classified in the same group (α/β) [3–5].

Modern methods, such as two-dimensional electrophoresis and high-pressure liquid chromatography with reversed phase, allow the separation of gliadin fractions into more than a hundred components. Based on the analysis of amino acid sequences (complete and partial), amino acid composition and molecular weight, gliadins are divided into: ω5, ω1.2, α + β and γ [3, 6–8]. ω-gliadins are characterized by a high content of glutamine, proline and phenylalanine. These amino acids together make up about 80% of the total ω gliadin composition. ω5-gliadins have a higher molecular weight (≥ 50 000 Da) than ω1.2 (≤ 40 000 Da). Most ω gliadins

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lack cysteine, so there is no possibility of disulfide binding. These proteins consist of repetitive sequences that are rich in glutamine and proline [3, 9, 10].

Molecular weights of α + β and γ-gliadins overlap (≤ 28 000–35 000 Da). The content of glutamine and proline is much lower compared to ω-gliadin. They differ in tyrosine content. Each of the two types has an N- and a C-terminal region [3, 11]. The N-terminal region (40–50% of total proteins) consists of repeating amino acid sequences that are rich in glutamine, proline, phenylalanine, and tyrosine. The repeating sequences of α + β gliadin are dodecapeptides. They are repeated five times. A typical unit of γ-gliadin is repeated up to 16 times. They are interspersed with additional remains [12, 13]. Within the C-terminal region α + β and γ-gliadins are homologous. The sequences are not repeating. They contain less glutamine and proline than the N-terminal region and have a more common composition. α + β and γ gliadins contain six or eight cysteine residues. These residues are located in the C-terminal region. They form intramolecular disulfide bonds [14, 15]. Although the content of total gliadin proteins depends on the type of wheat and growth conditions (soil, climate, fertilization), α + β and γ-gliadins are the highest components. Ω-gliadins are the highest components. Ω-gliadins contain six or eight cysteine residues. These residues are located in the C-terminal region. They form intramolecular disulfide bonds [14, 15]. Although the content of total gliadin proteins depends on the type of wheat and growth conditions (soil, climate, fertilization), α + β and γ-gliadins are the highest components.

Gluadin extraction. We analyzed gliadins in wheat flour samples (ash content: max 0.55%, moisture max: 15%, acidity: max 3, protein content 9.8 g/100 g) purchased on the market of the Republic of Srpska, Bosnia and Herzegovina by capillary gel electrophoresis.

Extraction of gliadin proteins was performed according to a modified Osborne method, as described by Lookhart and Bean [21]. After the albumins and globulins were removed (extraction was performed 3 times with 8 mL of deionized water each, it was obtained in laboratory conditions, on the apparatus Siemens water Technologies W3T199551, Siemens Ultra Clear, at a conductivity of 0.055 mS/cm and at a temperature of 20°C and 3 times with 8 mL of 2% solution of NaCl, NaCl, Lach-Ner, Czech Republic, high purity, ≥ 99.00%) gliadin was extracted with 8 mL of ethanol of different concentrations (50, 60 and 70% v/v, refined REAHEM, 96% v/v ethyl alcohol, Srbo Bran, quality corresponds to the quality property for ethyl alcohol, contains a minimum of 96% v/v ethanol). Samples were homogenized on a vortex (Advanced Vortex Mixer ZX3, 3000 rpm) for 30 min. The samples were then centrifuged in a centrifuge (Rotina 380 R, Hettich Zentrifugen) for 5 min at 1000 rpm. The resulting supernatant was poured into a normal 25 mL vessel, and after the third extraction the normal vessel was made up to final volume with ethanol of various concentrations (50, 60 and 70% v/v). The precipitate was then washed with deionized water.

Samples preparation for analysis at GCE. Prior to analysis samples were diluted with sample buffer (SDS-MW sample buffer, PA 800 plus, Beckman Coulter, USA), so that the total volume was 95 µL and the concentration was 1 mg/mL. Then 2 µL of internal standard (10 kDa, PA 800 plus, Beckman Coulter, United States) and 5 µL of 2-mercaptoethanol (high purity, 99.00%, Sigma-Aldrich Chemie GmbH, Germany) were added. The samples were then heated on a thermo-shaker (Thermo-Shaker, TS-100, Biosan) at 100°C for 3 min. After cooling to room temperature for 5 min, the samples were ready for analysis by capillary gel electrophoresis (Agilent, CE 7100).

Preparation SDS-MW standard for analysis by capillary gel electrophoresis. Prior to the preparation standard, based on the recommendation of the kit manufacturer, the standard was taken to room temperature for 15 min after removal from the refrigerator. It was then carefully stirred on a vortex (Advanced Vortex Mixer ZX3, 3000 rpm) for a few seconds. After that, 10 µL of standard (SDS-MW standard, PA 800 plus, Beckman Coulter, United States) was pipetted into the vial. Then 85 µL of buffer (SDS-MW sample buffer, PA 800 plus, Beckman Coulter, USA) and 2 µL of internal standard (10 kDa, PA 800 plus, Beckman Coulter, USA) were added. Then 5 µL of 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Germany, high purity, 99.00%) was added. Then, it was heated on a thermo-shaker (Thermo-Shaker, TS-100, Biosan), at a temperature of 100°C for 3 min. After heating, the standard vial was cooled to room temperature over 5 min. Prepared in this way, the standard was ready for analysis.

Gluadin proteins separation by capillary gel electrophoresis. Separation of gliadin proteins by capillary gel electrophoresis was performed on an Agilent apparatus, CE 7100, with a capillary inner
diameter of 50 µm, a total length of 33 cm, and an
effective length of 23.50 cm. The SDS-MW analysis kit,
PA 800 plus (2015 Beckman Coulter, USA) was used
for separation. SDS gel buffer (0.2% SDS, pH = 8) was
used to fill the capillary. The kit contains the following
chemicals: SDS-MW gel buffer (0.2% SDS, pH = 8),
SDS-MW sample buffer (100 mM Tris-HCl, pH = 9,
1% SDS), internal standard (10 kDa), external standard
(10 to 225 kDa), acid wash solution (0.1N HCl), base
wash solution (0.1N NaOH), as well as two capillaries
57 cm long, 50 µm ID. According to the manufacturer’s
instructions, the kit is stored at room temperature after
opening, except for the internal and external standards,
which are stored at a temperature of 2–6°C. Preparation
of the capillary electrophoresis (CE) instrument was
done according recommendations Agilent Technolo-
gies [22–24].

Statistical data processing. Statistical data
processing was performed in IBM SPSS, Statistics 26.
Descriptive statistical analysis calculated the average
value, standard deviation and 95% confidence interval
of the average value. Variance analysis of different
groups was used to evaluate the effect of solvent
concentrations, capillary temperature and electrode
voltage on the number of detected proteins and the
relative concentration of each gliadin proteins.

RESULTS AND DISCUSSION
In order to determine molecular weights unknown
proteins, a calibration curve was obtained using 7
proteins in SDS-MW size standard.

Electrophoregram, the migration time, and the
calibration curve of MW standard proteins of known molecular
weight (10, 20, 35, 50, 100, 150 and 225 kDa)
are presented in Fig. 1, Table 1, and Fig. 2, respectively.
The proteins were separated by capillary gel
electrophoresis (CE, Agilent, CE 7100, internal capillary
diameter 50 µm, total capillary length 33 cm, effective
capillary length 23.50 cm, capillary temperature 25°C,
voltage –16.5 kV (reverse mode), duration of analysis
30 min, and absorbance measured at 220 nm).

The ratio of molecular weights (log MW) and
migration time (t) of proteins is represented by the
equation $y = 0.08168x - 0.00098$, where $y$ represents
logMW and $x$ represents the migration time of pro-
teins (t). $R^2$ shows the correlation coefficient (0.9847).

A calibration curve was used to estimate the
molecular weight of unknown proteins. The coefficient
of correlation shows a high dependence of the logarithm
of the molecular weight of the protein and the migration
time of the protein.

The number of proteins in each gliadin fraction and
their relative concentration were obtained based on the
total number of identified proteins and the total relative
concentration.

Table 2 shows descriptive indicators of total proteins
and the number of gliadin proteins after extraction with
different concentrations of ethanol.

Descriptive analysis showed that the highest number of proteins (23.50) was obtained after extraction with
70% ethanol, by the method of Lookhart and Bean. The
lowest number of proteins was obtained by extraction
with 50% ethanol (18.67). One-factor analysis of the
variance of different groups showed that there was
a statistically significant difference in the number of

| Molecular weight (MW), kDa | log MW | t, min   |
|---------------------------|-------|---------|
| 10                        | 1.00  | 13.36 ± 0.21 |
| 20                        | 1.30  | 15.77 ± 0.18 |
| 35                        | 1.54  | 18.13 ± 0.26 |
| 50                        | 1.70  | 20.15 ± 0.29 |
| 100                       | 2.00  | 24.25 ± 0.10 |
| 150                       | 2.18  | 26.78 ± 0.36 |
| 225                       | 2.35  | 29.41 ± 0.15 |

Table 1 Migration time of proteins with known molecular
weight separated by capillary gel electrophoresis
proteins, $F(2.15) = 23.70$, Sig. = 0.000. The highest number of within $\alpha + \beta$ gliadin fractions was obtained after extraction with 60% ethanol (7.67). The lowest number of those proteins was obtained after extraction with 50% ethanol (6.00). A statistically significant difference was found in the number of proteins, $F(2.15) = 8.58$, Sig. = 0.003.

Extraction with 50 and 60% ethanol produced the highest and the lowest number of proteins within the $\gamma$-gliadins (5.33 and 4.67, respectively). There was no statistically significant difference in the number of proteins, $F(2.15) = 1.15$, Sig. = 0.342. The highest amount of $\omega1.2$-gliadins was obtained after extraction with 60% ethanol (5.17), while the lowest after extraction with 50 and 70% ethanol (4.33). One-factor variance analysis showed no statistically significant difference, $F(2.15) = 2.19$, Sig. = 0.146. The highest number of $\omega5$-gliadins was obtained after extraction with 70% ethanol (6.67). The lowest amount was observed after extraction with 50% ethanol (3.83). A statistically significant difference in the number of proteins was found, $F(2.15) = 6.77$, Sig. = 0.008.

According to Table 2, an increasing ethanol concentration increased total proteins, increased and then slightly decreased $\alpha + \beta$ gliadin fraction, decreased and then increased $\gamma$-gliadins, increased and then decreased $\omega1.2$-gliadins, and increased $\omega5$ gliadin fractions.

Table 3 shows descriptive indicators of the total relative concentration and the relative concentration of gliadin proteins after extraction with different concentrations of ethanol.

Descriptive analysis showed the highest relative protein concentration of $\alpha + \beta$ gliadin fractions after extraction with 50% ethanol (31.25%) and the lowest concentration after extraction with 60% ethanol (17.69%). One-factor variance analysis revealed a statistically significant difference in the relative protein concentration, $F(2.15) = 174.13$, Sig. = 0.000.

Table 2 shows descriptive indicators of total proteins and gliadin proteins separated by fractions (Agilent, CE 7100, capillary inside diameter 50 µm, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, voltage –16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm).

| Ethanol, % (v/v) | N | Xav | SD | Std. error | 95% confidence interval of average | Min | Max |
|-----------------|---|-----|----|------------|-----------------------------------|-----|-----|
|                 |   |     |    |            | Lower bound                       |     |     |
| Total number of proteins | 50 | 6  | 18.67 | 1.21 | 0.49 | 17.40 | 19.94 | 17  | 20  |
|                  | 60 | 6  | 23.00 | 1.67 | 0.68 | 21.24 | 24.76 | 21  | 25  |
|                  | 70 | 6  | 23.50 | 1.05 | 0.43 | 22.40 | 24.60 | 22  | 25  |
| $\alpha + \beta$ gliadins | 50 | 6  | 6.00 | 0.63 | 0.26 | 5.34  | 6.66  | 5   | 7   |
|                  | 60 | 6  | 7.67 | 0.82 | 0.33 | 6.81  | 8.52  | 7   | 9   |
|                  | 70 | 6  | 7.50 | 0.84 | 0.34 | 6.62  | 8.38  | 6   | 8   |
| $\gamma$ gliadins | 50 | 6  | 5.33 | 0.82 | 0.33 | 4.48  | 6.19  | 4   | 6   |
|                  | 60 | 6  | 4.67 | 0.82 | 0.33 | 3.81  | 5.52  | 4   | 6   |
|                  | 70 | 6  | 5.00 | 0.63 | 0.26 | 4.34  | 5.66  | 4   | 6   |
| $\omega1.2$ gliadins | 50 | 6  | 4.33 | 0.82 | 0.33 | 3.48  | 5.19  | 3   | 5   |
|                  | 60 | 6  | 5.17 | 0.75 | 0.31 | 4.38  | 5.96  | 4   | 6   |
|                  | 70 | 6  | 4.33 | 0.82 | 0.33 | 3.48  | 5.19  | 3   | 5   |
| $\omega5$ gliadins | 50 | 6  | 3.83 | 0.98 | 0.40 | 2.80  | 4.87  | 3   | 5   |
|                  | 60 | 6  | 5.50 | 1.22 | 0.50 | 4.21  | 6.79  | 3   | 6   |
|                  | 70 | 6  | 6.67 | 0.52 | 0.21 | 5.12  | 7.22  | 5   | 7   |

ANOVA (TP) $F(2.15) = 23.70$, Sig. = 0.000, eta square = 84.78/111.61 = 0.76
ANOVA ($\alpha + \beta$) $F(2.15) = 8.58$, Sig. = 0.003, eta square = 10.11/18.94 = 0.53
ANOVA ($\gamma$) $F(2.15) = 1.15$, Sig. = 0.342 > 0.05
ANOVA ($\omega1.2$) $F(2.15) = 2.19$, Sig. = 0.146 > 0.05
ANOVA ($\omega5$) $F(2.15) = 6.77$, Sig. = 0.008, eta square = 12.33/26.00 = 0.47
difference in relative concentration was found, F(2.15) = 111.01, Sig. = 0.000. The relative concentration of ω1.2-gliadins was the highest after extraction with 70% ethanol (14.93%) and the lowest after extraction with 60% ethanol (4.82%). The one-factor analysis of variance showed a statistically significant difference in the relative concentration, F(2.15) = 472.47, Sig. = 0.000.

As for γ-gliadin fractions, they were found in the highest concentration after extraction with 60% ethanol (47.45%) and the lowest after extraction with 70% ethanol (30.98%). There was a statistically significant difference in the relative concentration, F(2.15) = 104.83, Sig. = 0.000.

Based on the obtained results (Table 3), an increasing ethanol concentration decreased and then increased the relative concentration of α + β, γ- and ω1.2-gliadins and increased and then decreased that of ω5-gliadins.

Table 4 shows descriptive indicators of the total number of proteins and number of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated at a capillary temperature of 20, 25, 30, 35 and 40°C.

ANOVA (α + β) F(2.15) = 174.13, Sig. = 0.000, eta square = 609.67/635.93 = 0.96
ANOVA (γ) F(2.15) = 111.01, Sig. = 0.000, eta square = 301.93/322.33 = 0.94
ANOVA (ω1.2) F(2.15) = 472.47, Sig. = 0.000, eta square = 394.02/400.27 = 0.98
ANOVA (ω5) F(2.15) = 104.83, Sig. = 0.000, eta square = 851.07/911.96 = 0.93

According to the results obtained, it can be seen that with increasing capillary temperature, total proteins increased, then decreased and increased slightly again. α + β gliadin fractions decreased, then increased and decreased slightly again. As for γ-, ω1.2- and ω5-gliadins, their fractions increased, then decreased and increased slightly again.

Table 5 shows descriptive indicators of the total relative concentration of proteins and relative concentration of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated at different capillary temperatures.

According to the data, the highest relative concentration of α + β gliadin fractions was obtained after extraction with 70% ethanol and a capillary temperature of 40°C (47.55%). The lowest concentration was observed at 35°C (27.22%). One-factor variance analysis revealed a statistically significant difference

|                | Ethanol, % (v/v) | N   | RC, %   | SD    | Std. error | 95% confidence interval of average | Min   | Max   |
|----------------|-----------------|-----|---------|-------|------------|-----------------------------------|-------|-------|
| Total relative  |                 |     |         |       |            |                                    |       |       |
| concentration   |                 |     |         |       |            |                                    |       |       |
| 50             | 6               | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100 | 100 |
| 60             | 6               | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100 | 100 |
| 70             | 6               | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 100 | 100 |
| α + β gliadins  |                 |     |         |       |            |                                    |       |       |
| 50             | 6               | 31.25 | 1.30 | 0.53 | 29.89 | 32.61 | 29.07 | 32.87 |
| 60             | 6               | 17.69 | 1.27 | 0.52 | 16.36 | 19.03 | 16.11 | 19.61 |
| 70             | 6               | 28.29 | 1.40 | 0.57 | 26.82 | 29.76 | 26.06 | 30.09 |
| γ gliadins     |                 |     |         |       |            |                                    |       |       |
| 50             | 6               | 27.72 | 1.15 | 0.47 | 26.51 | 28.92 | 26.50 | 29.57 |
| 60             | 6               | 18.55 | 0.97 | 0.40 | 17.53 | 19.57 | 17.21 | 19.99 |
| 70             | 6               | 26.66 | 1.35 | 0.55 | 25.25 | 28.08 | 24.75 | 28.13 |
| ω1.2 gliadins  |                 |     |         |       |            |                                    |       |       |
| 50             | 6               | 5.21  | 0.34 | 0.14 | 4.85 | 5.56 | 4.84 | 5.66 |
| 60             | 6               | 4.82  | 0.21 | 0.09 | 4.59 | 5.04 | 4.58 | 5.08 |
| 70             | 6               | 14.93 | 1.04 | 0.43 | 13.84 | 16.03 | 13.03 | 15.95 |
| ω5 gliadins    |                 |     |         |       |            |                                    |       |       |
| 50             | 6               | 36.16 | 0.70 | 0.29 | 35.42 | 36.90 | 34.97 | 37.01 |
| 60             | 6               | 47.45 | 1.37 | 0.56 | 46.01 | 48.89 | 45.81 | 49.39 |
| 70             | 6               | 30.98 | 3.13 | 1.28 | 27.70 | 34.27 | 29.55 | 37.36 |
in the relative concentration, $F(4.25) = 193.61$, Sig. = 0.000. The relative concentration of $\gamma$-gliadins was the highest at 20°C (43.88%) and the lowest at 30°C (24.48%). A statistically significant difference in the relative concentration of different groups was $F(4.25) = 210.31$, Sig. = 0.000. A capillary temperature of 35°C led to the highest relative concentration within the $\omega_{1.2}$-group (27.21%), while 30°C provided the lowest (14.03%). There was a statistically significant difference in the relative concentration, $F(4.25) = 165.39$, Sig. = 0.000. The highest relative concentration of $\omega_{5}$-gliadins was obtained after extraction with 70% ethanol and at a capillary temperature of 25°C (30.98%) and the lowest at 20°C (5.42%). The effect of capillary temperature on relative protein concentration within $\omega_{5}$ gliadin fraction was examined by one-factor analysis of variance. A statistically significant difference in the relative concentration within the fraction was found, $F(4.25) = 195.85$, Sig. = 0.000.

Based on the obtained results (Table 5), it can be seen that with increasing capillary temperature, the relative concentration of $\alpha + \beta$ gliadins decreased, then increased, decreased, and increased again. Within $\gamma$-gliadins, the relative concentration decreased, then increased, and decreased again. The relative concentration of $\omega_{1.2}$-gliadins increased, then decreased, increased again and finally decreased. Within the $\omega_{5}$ gliadin fractions, the relative concentration increased and then decreased.

Table 6 shows descriptive indicators of total proteins and the number of gliadin fractions (70% ethanol, Agilent, CE 7100, capillary inside diameter 50 µm, total capillary length 33 cm, effective capillary length 23.50 cm, voltage −16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm).

| Column temperature, °C | N | Xav | SD | Std. error | 95% confidence interval of average | Min | Max |
|------------------------|---|-----|----|------------|----------------------------------|-----|-----|
| **Total number of proteins** | | | | | | | |
| 20                     | 6 | 20.83 | 1.47 | 0.60 | 19.29 - 22.38 | 19 | 23 |
| 25                     | 6 | 23.50 | 1.05 | 0.43 | 22.40 - 24.60 | 22 | 25 |
| 30                     | 6 | 21.67 | 1.75 | 0.71 | 19.83 - 23.50 | 19 | 23 |
| 35                     | 6 | 18.83 | 1.47 | 0.60 | 17.29 - 20.38 | 16 | 20 |
| 40                     | 6 | 19.33 | 1.03 | 0.42 | 18.25 - 20.42 | 18 | 21 |
| **$\alpha + \beta$ gliadins** | | | | | | | |
| 20                     | 6 | 10.00 | 1.09 | 0.45 | 8.85 - 11.15 | 8 | 11 |
| 25                     | 6 | 7.50  | 0.84 | 0.34 | 6.62 - 8.38  | 6 | 8  |
| 30                     | 6 | 8.50  | 0.84 | 0.34 | 7.62 - 9.38  | 7 | 9  |
| 35                     | 6 | 8.83  | 0.75 | 0.31 | 8.04 - 9.62  | 8 | 10 |
| 40                     | 6 | 8.67  | 0.82 | 0.33 | 7.81 - 9.52  | 8 | 10 |
| **$\gamma$ gliadins** | | | | | | | |
| 20                     | 6 | 4.00  | 0.00 | 0.00 | 4.00 - 4.00  | 4 | 4  |
| 25                     | 6 | 5.00  | 0.63 | 0.26 | 4.34 - 5.66  | 4 | 6  |
| 30                     | 6 | 4.50  | 0.55 | 0.22 | 3.93 - 5.07  | 3 | 5  |
| 35                     | 6 | 3.50  | 0.55 | 0.22 | 2.93 - 4.07  | 3 | 4  |
| 40                     | 6 | 3.67  | 0.52 | 0.21 | 3.12 - 4.21  | 3 | 4  |
| **$\omega_{1,2}$ gliadins** | | | | | | | |
| 20                     | 6 | 2.67  | 0.52 | 0.21 | 2.12 - 3.21  | 2 | 3  |
| 25                     | 6 | 4.33  | 0.82 | 0.33 | 3.48 - 5.19  | 3 | 5  |
| 30                     | 6 | 3.17  | 0.41 | 0.17 | 2.74 - 3.60  | 3 | 4  |
| 35                     | 6 | 2.67  | 0.52 | 0.21 | 2.12 - 3.21  | 2 | 3  |
| 40                     | 6 | 3.17  | 0.41 | 0.17 | 2.74 - 3.60  | 3 | 4  |
| **$\omega_{5}$ gliadins** | | | | | | | |
| 20                     | 6 | 4.50  | 0.84 | 0.34 | 3.62 - 5.38  | 4 | 6  |
| 25                     | 6 | 6.67  | 0.52 | 0.21 | 5.12 - 7.22  | 5 | 7  |
| 30                     | 6 | 4.83  | 1.17 | 0.48 | 3.61 - 6.06  | 3 | 6  |
| 35                     | 6 | 3.50  | 0.55 | 0.22 | 2.93 - 4.07  | 3 | 4  |
| 40                     | 6 | 4.33  | 0.82 | 0.33 | 3.48 - 5.19  | 3 | 5  |

ANOVA (TP) $F(4.25) = 11.02$, Sig. = 0.000, eta square = 84.33/132.17 = 0.64
ANOVA ($\alpha + \beta$) $F(4.25) = 6.24$, Sig. = 0.001, eta square = 19.13/38.30 = 0.50
ANOVA ($\gamma$) $F(4.25) = 9.01$, Sig. = 0.000, eta square = 9.13/15.47 = 0.59
ANOVA ($\omega_{1,2}$) $F(4.25) = 9.08$, Sig. = 0.000, eta square = 11.13/18.80 = 0.59
ANOVA ($\omega_{5}$) $F(4.25) = 5.63$, Sig. = 0.002, eta square = 14.87/31.37 = 0.47

The highest number of proteins was obtained after extraction with 70% ethanol, according to the method by Lookhart and Bean and electrophoretic separation at a voltage of −16.5 kV (23.50). The lowest number of proteins was obtained at −14.5 kV (14.83). It was found that there is a statistically significant difference in the number of proteins, $F(3.20) = 46.16$, Sig. = 0.000. The highest and the lowest amounts of proteins within
Table 5 Descriptive indicators of the total relative concentration of proteins and relative concentration of gliadin fractions (solvent 70% ethanol, Agilent, CE 7100, capillary inside diameter 50 µm, total capillary length 33 cm, effective capillary length 23.50 cm, voltage –16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm)

| Column temperature, °C | N | RC, % SD Std. error 95% confidence interval of average Min Max Lower bound Upper bound | α + β gliadins | γ gliadins | ω1.2 gliadins | ω5 gliadins |
|------------------------|---|------------------------------------------------|-------------------|------------|-----------------|----------------|
| Total relative concentration | 20 | 6 | 100.00 0.00 0.00 100.00 100.00 100 100 | | | | |
| 25 | 6 | 100.00 0.00 0.00 100.00 100.00 100 100 | | | | |
| 30 | 6 | 100.00 0.00 0.00 100.00 100.00 100 100 | | | | |
| 35 | 6 | 100.00 0.00 0.00 100.00 100.00 100 100 | | | | |
| 40 | 6 | 100.00 0.00 0.00 100.00 100.00 100 100 | | | | |
| α + β gliadins | 20 | 6 | 36.38 1.11 0.45 35.21 37.55 34.90 37.88 | | | | |
| 25 | 6 | 28.29 1.40 0.57 26.82 29.76 26.06 30.09 | | | | |
| 30 | 6 | 33.86 1.29 0.52 32.51 35.21 32.27 35.38 | | | | |
| 35 | 6 | 27.22 2.11 0.86 25.01 29.44 26.00 31.49 | | | | |
| 40 | 6 | 47.55 0.99 0.41 46.50 48.60 46.01 48.99 | | | | |
| γ gliadins | 20 | 6 | 43.88 1.14 0.47 42.68 45.08 42.39 45.37 | | | | |
| 25 | 6 | 26.66 1.35 0.55 25.25 28.08 24.75 28.13 | | | | |
| 30 | 6 | 24.88 1.59 0.65 23.82 27.05 23.07 27.39 | | | | |
| 35 | 6 | 29.83 1.08 0.44 28.70 30.96 28.36 31.44 | | | | |
| 40 | 6 | 47.38 0.99 0.41 46.50 48.60 46.01 48.99 | | | | |
| ω1.2 gliadins | 20 | 6 | 14.75 0.89 0.36 13.81 15.69 13.28 15.87 | | | | |
| 25 | 6 | 14.93 1.04 0.43 13.84 16.03 13.03 15.95 | | | | |
| 30 | 6 | 14.03 1.34 0.55 12.62 15.44 12.07 15.71 | | | | |
| 35 | 6 | 27.21 1.35 0.55 25.80 28.63 25.10 28.95 | | | | |
| 40 | 6 | 14.46 0.58 0.24 13.85 15.07 13.71 15.13 | | | | |
| ω5 gliadins | 20 | 6 | 5.42 0.34 0.14 5.06 5.77 5.02 5.90 | | | | |
| 25 | 6 | 30.98 3.13 1.28 27.70 34.27 29.55 37.36 | | | | |
| 30 | 6 | 27.28 2.04 0.83 25.14 29.42 24.69 29.61 | | | | |
| 35 | 6 | 12.86 1.90 0.77 10.87 14.85 10.83 15.95 | | | | |
| 40 | 6 | 10.67 1.07 0.44 9.55 11.80 9.20 12.22 | | | | |

ANOVA (α + β) F(4.25) = 193.61, Sig. = 0.000, eta square = 1593.94/1645.39 = 0.97
ANOVA (γ) F(4.25) = 210.31, Sig. = 0.000, eta square = 1448.88/1491.94 = 0.97
ANOVA (ω1.2) F(4.25) = 165.39, Sig. = 0.000, eta square = 773.27/802.49 = 0.96
ANOVA (ω5) F(4.25) = 195.85, Sig. = 0.000, eta square = 2949.03/3043.13 = 0.97

We can see that with increasing voltage, total proteins increased, then decreased and increased slightly again. Within the α + β and γ gliadin fractions, the number of proteins increased and then decreased. Within the fraction of ω1.2- and ω5-gliadins, the amount of proteins increased, then decreased and increased slightly again.

Table 7 shows descriptive indicators of the total relative concentration of proteins and relative concentration of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated by applying different electrode voltages (reverse mode).

Descriptive analysis showed that the highest relative concentration of α + β gliadins was obtained at a voltage of –17.5 kV (65.13%). The lowest concentration within this fraction was at –16.5 kV (28.29%). A statistically significant difference in the relative protein concentration was found, F(3.20) = 851.47, Sig. = 0.000. The highest and the lowest relative concentrations of γ-gliadins were obtained at –14.5 kV and at –18.5 kV (27.37 and 21.87%, respectively). A statistically significant difference in the relative protein concentration was found, F(3.20) = 20.47, Sig. = 0.000. A voltage of –14.5 kV...
Table 6 Descriptive indicators of total proteins and the number of gliadin fractions (solvent 70% ethanol, Agilent, CE 7100, capillary inside diameter 50 µm, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, duration 30 min, absorbance measured at 220 nm)

| Voltage, kV | N  | Xav  | SD  | Std. error | 95% confidence interval of average | Min  | Max  |
|------------|----|------|-----|------------|-----------------------------------|------|------|
|            |    |      |     |            | Lower bound                       |      |      |
| Total number of proteins | –14.5 | 6 | 14.83 | 1.17 | 0.48 | 13.61 | 16.06 | 13 | 16 |
|            | –16.5 | 6 | 23.50 | 1.05 | 0.43 | 22.40 | 24.60 | 22 | 25 |
|            | –17.5 | 6 | 17.33 | 1.21 | 0.49 | 16.06 | 18.60 | 15 | 18 |
|            | –18.5 | 6 | 16.67 | 1.75 | 0.71 | 15.83 | 19.50 | 15 | 19 |
| α + β gliadins | –14.5 | 6 | 5.17  | 0.75 | 0.31 | 4.38  | 5.96  | 4 | 6 |
|            | –16.5 | 6 | 7.50  | 0.84 | 0.34 | 6.62  | 8.38  | 6 | 8 |
|            | –17.5 | 6 | 8.17  | 1.17 | 0.48 | 6.94  | 9.39  | 6 | 9 |
|            | –18.5 | 6 | 7.17  | 0.75 | 0.31 | 6.38  | 7.96  | 6 | 8 |
| γ gliadins  | –14.5 | 6 | 2.50  | 0.55 | 0.22 | 1.93  | 3.07  | 2 | 3 |
|            | –16.5 | 6 | 5.00  | 0.63 | 0.26 | 4.34  | 5.66  | 4 | 6 |
|            | –17.5 | 6 | 3.00  | 0.63 | 0.26 | 2.34  | 3.66  | 2 | 4 |
|            | –18.5 | 6 | 3.00  | 0.00 | 0.00 | 3.00  | 3.00  | 3 | 3 |
| ω1.2 gliadins | –14.5 | 6 | 3.00  | 0.00 | 0.00 | 3.00  | 3.00  | 3 | 3 |
|            | –16.5 | 6 | 4.33  | 0.82 | 0.33 | 3.48  | 5.19  | 3 | 5 |
|            | –17.5 | 6 | 2.50  | 0.55 | 0.22 | 1.93  | 3.07  | 2 | 3 |
|            | –18.5 | 6 | 3.00  | 0.63 | 0.26 | 2.34  | 3.66  | 2 | 4 |
| ω5 gliadins  | –14.5 | 6 | 4.17  | 0.75 | 0.31 | 3.38  | 4.96  | 3 | 5 |
|            | –16.5 | 6 | 6.67  | 0.52 | 0.21 | 5.12  | 7.22  | 5 | 7 |
|            | –17.5 | 6 | 3.00  | 0.63 | 0.26 | 2.34  | 3.66  | 2 | 4 |
|            | –18.5 | 6 | 4.67  | 1.03 | 0.42 | 3.58  | 5.75  | 3 | 6 |

ANOVA (TP) F(3.20) = 46.16, Sig. = 0.000, eta square = 242.33/277.33 = 0.87
ANOVA (α + β) F(3.20) = 12.50, Sig. = 0.000, eta square = 30.00/46.00 = 0.65
ANOVA (γ) F(3.20) = 26.82, Sig. = 0.000, eta square = 22.12/27.62 = 0.80
ANOVA (ω1.2) F(3.20) = 10.85, Sig. = 0.000, eta square = 11.12/17.96 = 0.62
ANOVA (ω5) F(3.20) = 12.83, Sig. = 0.000, eta square = 22.12/33.62 = 0.66

caused the highest (26.73%) and –18.5 the lowest (3.91%) relative concentration of ω1.2-gliadins. The one-factor analysis of variance showed a statistically significant difference, F(3.20) = 1316.91, Sig. = 0.000. Relative concentration of ω5-gliadins obtained at a voltage of –18.5 kV was the highest (40.30%) and at –17.5 kV the lowest (4.91%). There was a statistically significant difference in the relative concentration, F(3.20) = 549.81, Sig. = 0.000.

According to the results obtained, the increasing voltage decreased then increased and decreased again the relative concentration of α + β gliadin proteins. Within the fraction of γ- and ω1.2- gliadins the concentration decreased, and ω5-gliadins increased then decreased and increased again.

Lookhart and Bean performed separation and characterization of wheat proteins by high-pressure capillary electrophoresis (HPCE) [21]. Gliadins were extracted with 70% (v/v) ethanol. Separation of proteins was performed at a voltage of 22 kV and at a temperature of 45°C. The detection wavelength was 200 nm. Based on the obtained results, the retention time of gliadin proteins was: α gliadins 3–4 min (molecular weight according to SDS-PAGE 35–38 kDa), β 4–6 min (37–43 kDa), γ 5–6 min (43–47 kDa), and ω 6.8–10 min (48–63 kDa).

Bietz and Schmalzried analyzed gliadins from wheat by capillary electrophoresis [25]. Gliadins were extracted with ethanol and methanol of different concentrations (30, 40, 50, 60 and 70% v/v), with and without the reducing agent dithioerythritol. The temperature of the capillary ranged from 30 to 50°C, and the voltage from 8 to 12 kV. The detection wavelength was 200 nm. Capillary temperature of 40°C and voltage 10 kV showed optimal conditions. Ethanol proved to be a better solvent than methanol.

Changing ethanol concentration (50, 60, and 70% v/v), capillary temperature (20, 25, 30, 35, and 40°C), and voltage (–14.5; –16.5, –17.5, and –18.5 kV), we found that the optimal conditions for separation of gliadin proteins were 70% ethanol concentration, a capillary temperature of 25°C, and a voltage of –16.5 kV (reverse mode) (Fig. 3).

Our results are in agreement with Lookhart and Bean and Bietz and Schmalzried [21, 25]. Although the mentioned authors separated gliadin proteins by using different techniques of capillary electrophoresis, 70% ethanol proved to be the optimal solvent, which lines up with our results [21]. The gliadin proteins in this work were separated in less than 10 min, which is in agreement with Lookhart and Bean [21].
CONCLUSION

Based on the results obtained, the optimal conditions for gliadin separation were 70% ethanol concentration, a capillary temperature of 25°C, and a voltage of –16.5 kV (reverse mode). Under these conditions, total proteins were 23.5, including α + β gliadin proteins (Xav = 7.50, relative concentration 28.29%), γ-fractions (Xav = 5.00, RC = 26.66%), α1.2-gliadins (Xav = 4.33, RC = 14.93%), and α5-gliadins (Xav = 6.67, RC = 30.98%).

The results obtained in this paper can greatly contribute to the prevention of the incorrect declaration of the “gluten free” products, reduction of health risks for people who are sensitive to gluten proteins, as well as the cost of treating the ones with celiac disease.

Table 7 Descriptive indicators of the relative concentration of proteins and relative concentration of gliadin fractions (solvent 70% ethanol, Agilent, CE 7100, capillary inside diameter 50 µm, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, duration 30 min, absorbance measured at 220 nm)

| Voltage, kV | N | RC, % | SD | Std. error | 95% confidence interval of average | Min | Max |
|-------------|---|-------|----|------------|-----------------------------------|-----|-----|
| -14.5       | 6 | 100.00| 0.00| 0.00       | 100.00                           | 100 | 100 |
| -16.5       | 6 | 100.00| 0.00| 0.00       | 100.00                           | 100 | 100 |
| -17.5       | 6 | 100.00| 0.00| 0.00       | 100.00                           | 100 | 100 |
| -18.5       | 6 | 100.00| 0.00| 0.00       | 100.00                           | 100 | 100 |
| α + β gliadins | -14.5 | 35.42 | 1.27 | 0.52 | 34.09 | 36.75 | 33.06 | 36.81 |
| α + β gliadins | -16.5 | 28.29 | 1.40 | 0.57 | 26.82 | 29.76 | 26.06 | 30.09 |
| α + β gliadins | -17.5 | 65.13 | 1.90 | 0.78 | 63.13 | 67.13 | 61.63 | 67.05 |
| α + β gliadins | -18.5 | 34.08 | 0.73 | 0.29 | 33.31 | 34.85 | 33.09 | 34.95 |
| γ gliadins   | -14.5 | 27.37 | 1.03 | 0.42 | 26.29 | 28.45 | 26.10 | 29.22 |
| γ gliadins   | -16.5 | 26.66 | 1.35 | 0.55 | 25.25 | 28.08 | 24.75 | 28.13 |
| γ gliadins   | -17.5 | 25.51 | 1.24 | 0.51 | 24.20 | 26.81 | 23.81 | 26.96 |
| γ gliadins   | -18.5 | 21.87 | 1.61 | 0.66 | 20.18 | 23.56 | 20.02 | 24.38 |
| α1.2-gliadins | -14.5 | 26.73 | 0.74 | 0.30 | 25.95 | 27.51 | 25.98 | 28.10 |
| α1.2-gliadins | -16.5 | 14.93 | 1.04 | 0.43 | 13.84 | 16.03 | 13.03 | 15.95 |
| α1.2-gliadins | -17.5 | 5.34 | 0.37 | 0.15 | 4.95 | 5.73 | 4.99 | 5.89 |
| α1.2-gliadins | -18.5 | 3.91 | 0.50 | 0.20 | 3.39 | 4.43 | 3.21 | 4.52 |
| α5-gliadins  | -14.5 | 10.66 | 0.75 | 0.31 | 9.87 | 11.45 | 9.92 | 11.98 |
| α5-gliadins  | -16.5 | 30.98 | 3.13 | 1.28 | 27.70 | 34.27 | 29.55 | 37.36 |
| α5-gliadins  | -17.5 | 4.91 | 1.01 | 0.41 | 3.85 | 5.97 | 3.99 | 6.73 |
| α5-gliadins  | -18.5 | 40.30 | 0.88 | 0.36 | 39.37 | 41.22 | 38.88 | 41.25 |

ANOVA (α + β) F(3.20) = 851.47, Sig. = 0.000, eta square = 4935.49/4974.13 = 0.99
ANOVA (γ) F(3.20) = 20.47, Sig. = 0.000, eta square = 107.68/142.75 = 0.75
ANOVA (α1.2) F(3.20) = 1316.91, Sig. = 0.000, eta square = 2000.54/2010.67 = 0.99
ANOVA (α5) F(3.20) = 549.81, Sig. = 0.000, eta square = 5014.18/5074.98 = 0.99

Figure 3 Electrophoregram of gliadin proteins extracted from wheat flour using 70% (v/v) ethanol and separated by capillary gel electrophoresis at a capillary temperature of 25°C and at a voltage of –16.5 kV
In addition, the results of the research are 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 effect of individuals gluten proteins, during processing.

**CONTRIBUTION**

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

**CONFLICT OF INTEREST**

The authors declare no potential conflict of interest.

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