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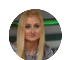
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CHROMATOGRAPHIC SEPARATION OF GLUTENIN WITH HIGH MOLECULAR WEIGHT FROM WHEAT FLOUR

Vesna Gojković Cvjetković[1*], Radoslav Grujić[2], Željka Marjanović-Balaban[3]

[1]Faculty of Technology Zvornik, University of East Sarajevo, Karakaj 34a, 75400 Zvornik, Bosnia and Herzegovina
[2]State High School of Medical Science, Prijedor, Nikole Pašića 4a, 79101 Prijedor, Bosnia and Herzegovina
[3]Faculty of Forestry Banja Luka, University of Banja Luka, Bulevar Vojvode Stepe Stepanovića 75a, 78000 Banja Luka, Bosnia and Herzegovina

[*]e-mail: vesna.gojkovic@yahoo.com

Abstract

Glutenins with high molecular weight (HMW glutenins) are one of the glutenin's fractions. They play a key role in the formation of the gluten elasticity property and contribute to the formation of large glutenin polymers. The aim of this study was to investigate the effect of solvent type and column temperature on the chromatographic separation of HMW glutenins from wheat flour.

For HMW glutenins extraction, 50% (v/v) ethanol, 1-propanol and isopropanol was used to which Tris-HCl (0.05 mol/L, Ph = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added. The high performance liquid chromatography (HPLC) method (HPLC Agilent Technologies 1260 Infinity, Zorbax 300SB-C3 column) was used for protein separation at a column temperature 40, 45 and 50 ^{0}C. Absorbance measurements were performed at 210 nm and 280 nm.

The effect of the solvents tested on the separation of HMW glutenins was shown by determining the number of observed peaks (proteins) on the chromatogram and calculating the relative concentration of HMW in the total number of glutenins from wheat flour (RC). After the extraction of glutenin proteins with 50% (v/v) ethanol, the highest number of proteins at 210 nm was observed when the column temperature was 45 ^{0}C and 50 ^{0}C (Xsr = 6.17 and RC1 = 17.76% and RC2 = 27.07%) and the lowest number at a column temperature of 40 ^{0}C (Xsr = 4, RC = 13.43%). By extraction with 50% (v/v) 1-propanol, the highest number of proteins was observed at column temperatures of 40 ^{0}C and 45 ^{0}C (Xsr = 5.17 and RC1 = 28.22% and RC2 = 31.70%, respectively) and the lowest number at 50 ^{0}C (Xsr = 4.67, RC = 34.68%) and by extraction with 50% (v/v) isopropanol the highest number of proteins was observed at a column temperature of 50 ^{0}C (Xsr = 7.17, RC = 23.61%) and the lowest number at 45 ^{0}C (Xsr = 5.83, RC = 10.67%). After the extraction of glutenin proteins with 50% (v/v) ethanol and detection at a wavelength of 280 nm, the highest number of proteins was observed at a column temperature of 45 ^{0}C (Xsr = 8.33, RC = 36.49%), and the lowest at 40 ^{0}C (Xsr = 5.50, RC = 32.57%). In the case of protein extraction with 50% (v/v) 1-propanol, the highest number of HMW glutenins was observed at 40 ^{0}C (Xsr = 7.83, RC = 61.62%) and the lowest at 50 ^{0}C (Xsr = 4.67, RC = 39.18%). When extraction was performed with 50% (v/v) isopropanol, the highest number of proteins was observed at a column temperature of 45 °C (Xsr = 7.33, RC = 21.66%), and the lowest number at a column temperature of 40 ^{0}C and 50 ^{0}C (Xsr = 7.00, RC1 = 28.64%, RC2 = 34.22%).

Based on the obtained results it can be seen that the highest number of proteins was observed with 50% (v/v) ethanol (Xsr = 8.33).

***Key words**: RP-HPLC, Zorbax C3 column, HMW glutenins.*

1. Introduction

Glutenins, in addition to gliadins, represent one of the gluten protein fractions. Both fractions are present in approximately equal amounts. They differ in their

functional properties. The glutenin fraction is responsible for the viscoelastic properties of dough and gluten [1]. Glutenin proteins are insoluble in alcohols. Unlike gliadins, which are monomers, glutenins are polymers. They are formed polypeptides binding by disulfide bonds. They are hard soluble proteins. However, by the action of strong reducing agents (β-mercaptoethanol and dithioerythritol), intermolecular disulfide bonds are broken. As a result, glutenin subunits that are soluble in aqueous alcohol are released, similar to gliadins [2, 3, and 4].

The molecular weight of glutenin varies from 500,000 to 10 million and more million daltons [5]. Part of glutenin belongs to the biggest proteins found in nature. The biggest polymers called glutenin macropolymers make the greatest contribution to the dough property. Their amount in wheat dough (approximately 20 - 40 mg/g) correlates with the strength and volume of the dough [6].

Glutenins consist of two subunits. These are glutenins with low molecular weight (LMW-GS) and glutenins with high molecular weight (HMW-GS). High molecular weight glutenins (HMW-GS) belong to smaller components within gluten proteins. They make up about 10% of the total gluten content. These components are responsible for the elasticity of gluten. They affect the formation of larger glutenin polymers. They are rich in proline, glycine and glutamine [7].

Each wheat variety contains 3 - 5 subunits of HMW-GS. HMW glutenins are divided into x and y types. Their molecular weight ranges from 83,000 - 88,000, and 67,000 - 74,000 Da [8]. The high molecular weight glutenins (HMW-GS) consists of 3 structural regions. These are: non-repeating N-terminal (A) containing 80 - 105 residues, repeating central (B) containing 480-700 and C terminal region (C) containing 42 residues [9]. Regions A and C are characterized by the presence of damaged residues and the presence of most all residues. Region B contains repetitive hexapeptides with inserted hexapeptides and tripeptides.

Despite the similarities in the structure and sequences of amino acids, there are also significant differences that determine the functionality of glutenin polymers. X-type subunits have slower motility on SDS-PAGE gels and higher molecular weight than y-type subunits [7].

Considering the importance of HMW glutenin in the functional properties of gluten and dough, the aim of this study was to chromatographically separate high molecular weight glutenins (HMW-GS) and to investigate how solvent type, column temperature, and detection wavelength influence the separation and identification efficiency.

2. Materials and Methods

2.1 Materials

Glutenin proteins were extracted from wheat flour type 500 purchased from the market of Bosnia and Herzegovina. 50% (v/v) ethanol, 1-propanol and isopropanol were used for the extraction.

2.2 Methods

2.2.1 Glutenin extraction

The extraction of glutenin proteins was performed according to a modified method of Wiser *et al.*, [10], and Gojkovic *et al.*, [11]. First, albumin and globulins (soluble in aqueous and salt solution), then gliadins (soluble in alcohol) were extracted from wheat flour. After these protein fractions were removed, and glutenins were extracted. Glutenins were extracted under a nitrogen atmosphere at 60 ^{0}C with 50% (v/v) aqueous ethanol (REAHEM, Srbobran), isopropanol (Lach-Ner, Czech Republic) and 1-propanol (Lach-Ner, Czech Republic) in which Tris-HCl (0.05 mol/L, Sigma-Aldrich, United States, pH = 7.5), urea (2 mol/L, Sigma Aldrich, Germany) and dithioerythritol (1%, ACROS Organics, Switzerland) were added, at a room temperature, 20 ^{0}C.

The extraction was performed twice with 1 mL of appropriate solvent each. Each time after the extraction solvent was added, the sample was homogenized on a vortex (Advanced Vortex Mixer ZX3, Velp scientifica) for 2 minutes. Then stirring was continued on a magnetic stirrer (Velp scientifica) for 10 minutes. After homogenization was completed, centrifugation of the samples in a centrifuge (Hettich zentrifugen, rotina 380 R) was carried out for 20 minutes at 7000 rpm. The supernatants were made up to 2 mL with an appropriate extraction solvent. Samples were filtered through a 0.45 μm membrane filter (RC syringe filters, Filtratech, France) before analysis began.

2.2.2 Reversed-phase HPLC (RP-HPLC) chromatography

RP-HPLC chromatography was performed on an HPLC Agilent Technologies 1260 Infinity apparatus. The separation of glutenin proteins was performed on a Zorbax 300 SB-C3 column (Agilent Technologies), size 4.6 x 150 mm, with a particle size of 5 μm. The column temperature was 40, 45 and 50 ^{0}C. Two mobile phases were used. These are deionized water and 0.1% trifluoroacetic acid (TFA, Acros, France) in acetonitrile (ACN; Biosolve, Chimie, France). The flow rate was 1 mL/min. The injection volume was 70 μL and the detection wavelength was 210 and 280 nm, respectively.

2.2.3 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 column temperature on the number of detected proteins and the relative concentration of HMW glutenins.

3. Results and Discussion

The number of detected HMW glutenin proteins and their relative concentration is calculated relative to total proteins and total relative concentration.

Table 1 shows descriptive indicators of the number of HMW glutenin proteins, after extraction with solvents of different concentrations (50% v/v ethanol, 50% v/v 1-propanol and 50% v/v isopropanol, in which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1%) was added, at different column temperatures (40, 45 and 50 ^{0}C).

After the extraction of glutenin proteins with 50% (v/v) ethanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the number of HMW glutenin proteins observed was Xav = 4.00 (SD = 0.00, CV=0.00%), at a column temperature of 45 ^{0}C Xav = 6.17 (SD = 0.75, CV = 12.15%) and at a column temperature of 50 ^{0}C Xav = 6.17 (SD = 0.41, CV = 6.64%).

Descriptive analysis showed that the highest number of detected proteins within HMW glutenins was at a column temperature of 45 ^{0}C, when the absorbance was read at a wavelength of 210 nm (Xav = 6.17, SD = 0.75) and with an average error of estimate values of Std. error = 0.31 and 95% confidence interval of the average 95% CI: from 5.38 to 6.96, with a range of values from a minimum of 5.00 to a maximum of 7.00 proteins and at a column temperature of 50 ^{0}C (Xav = 6.17, SD = 0.41) and with an error of average Std. error = 0.17 and 95% confidence interval of the average 95% CI: from 5.74 to 6.60, with a range of values from minimum 6.00 to maximum 7.00 proteins. The lowest number of detected proteins within the HMW glutenin fractions was obtained at a column temperature of 40 ^{0}C (Xav = 4.00, SD = 0.00) and with an error in the average Std. error = 0.00 and 95% CI: 4.00 to 4.00 and a range of values from a minimum of 4.00 to a maximum of 4.00 proteins.

The influence of column temperature at a constant solvent concentration (50% v/v ethanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) was examined by one-factor analysis of variance of the different groups, glutenin proteins separation time and absorbance measurement) to the number of proteins extracted within the HMW glutenin fractions. It was found that there was a statistically significant difference in the average number of detected proteins, F(2.15)=38.41, Sig=0.00. This difference, measured by the eta square indicator is large (eta square=18.78/22.44=0.84) (Table 3.1).

After the extraction of glutenin proteins with 50% (v/v) 1-propanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the number of detected proteins within HMW glutenins fraction was Xav = 5.17 (SD = 0.41, CV = 7.93%), at a column temperature of 45 ^{0}C Xav = 5.17 (SD = 0.75, CV = 14.51%) and at a column temperature of 50 ^{0}C Xav = 4.67 (SD = 0.82, CV = 17.56%).

Descriptive analysis showed that the highest number of detected proteins within HMW glutenins was obtained after extraction with 50% (v/v) 1-propanol to

Table 1. Descriptive indicators for HMW glutenin proteins (solvent 50% v/v ethanol, 1-propanol and isopropanol, RP-HPLC Agilent Technologies 1260 Infinity, Zorbax 300 SB-C3 Agilent column, column size 4.6 x 150 mm, particle sizes 5 µm and column pressure 80 bar, glutenin proteins separation time 21 min, measuring the absorbance at 210 nm

Column	Temp. (^{0}C)	N	Average value	Std. dev.	Std. error	95% confidence interval of average		Min	Max
						Lower Bound	Upper bound		
50% (v/v) ethanol	40	6	4.00	0.00	0.00	4.00	4.00	4	4
	45	6	6.17	0.75	0.31	5.38	6.96	5	7
	50	6	6.17	0.41	0.17	5.74	6.60	6	7
	Total	18	5.44	1.15	0.27	4.87	6.02	4	7
50% (v/v) 1-propanol	40	6	5.17	0.41	0.17	4.74	5.60	5	6
	45	6	5.17	0.75	0.31	4.38	5.96	4	6
	50	6	4.67	0.82	0.33	3.81	5.52	4	6
	Total	18	5.00	0.69	0.16	4.66	5.34	4	6
50% (v/v) isopropanol	40	6	6.00	0.00	0.00	6.00	6.00	6	6
	45	6	5.83	0.41	0.17	5.40	6.24	5	6
	50	6	7.17	0.41	0.17	6.74	7.60	7	8
	Total	18	6.33	0.69	0.16	5.99	6.67	5	8
ANOVA (HMW, 50% v/v ethanol)	F(2.15) = 38.41, Sig. = 0.00, eta square = 18.78/22.44 = 0.84								
ANOVA (HMW, 50% v/v 1-propanol)	F(2.15) = 1.07, Sig. = 0.37 > 0.05								
ANOVA (HMW, 50% v/v isopropanol)	F(2,15) = 28.50, Sig. = 0.00, eta square = 6.33/8.00 = 0.79								

which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) was added, at a column temperature of 40 ^{0}C, when the absorbance was read at a wavelength of 210 nm (Xav = 5.17, SD = 0.41) and with an error in the average Std. error = 0.17 and 95% confidence interval of the average 95% CI: from 4.74 to 5.60, with a range from a minimum of 5.00 to a maximum of 6.00 proteins, and at a column temperature of 45 ^{0}C (Xav = 5.17, SD = 0.75), with an error of average Std. error = 0.31 and 95% confidence interval of the average 95% CI: from 4.38 to 5.96, with a range from a minimum 4.00 to a maximum of 6.00 proteins. The lowest number of detected proteins within HMW glutenins was obtained at a column temperature of 50 ^{0}C (Xav = 4.67, SD = 0.82), with an error in the average Std. error = 0.33 and 95% CI: 3.81 to 5.52 and with range from a minimum 4.00 to maximum 6.00 proteins.

The influence of column temperature at a constant solvent concentration (50% v/v 1-propanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was addded) was investigated by one-factor analysis of variance of different groups, glutenin proteins separation time and absorbance measurement) to a number of detected proteins within HMW glutenins. It was found that there was no statistically significant difference in the average number of detected proteins, F (2.15) = 1.07, Sig = 0.37 > 0.05.

After the extraction of glutenin proteins with 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the number of detected proteins within the HMW glutenin fractions was Xav = 6.00 (SD = 0.00, CV = 0.00%), at a column temperature of 45 ^{0}C Xav = 5.83 (SD = 0.41, CV = 7.03%) and at a column temperature of 50 ^{0}C Xav = 7.17 (SD = 0.41, CV = 5.72%).

Descriptive analysis showed that the highest number of detected proteins within the HMW glutenin fractions after extraction with 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) was added, at a column temperature of 50 ^{0}C, when the absorbance was read at a wavelength of 210 nm (Xav = 7.17, SD = 0.41) and with an error of estimate of the average Std. error value = 0.17 and 95% confidence interval of average 95% CI: 6.74 to 7.60, with a range from a minimum of 7.00 to a maximum of 8.00 proteins. The lowest number of detected proteins within HMW glutenins was obtained at a column temperature of 45 ^{0}C (Xav = 5.83, SD = 0.41) and with an error of estimate of the average Std. error = 0.17 and 95% CI: from 5.40 to 6.26 and a range from the minimum 5.00 to the maximum 6.00 proteins.

The influence of column temperature at a constant solvent concentration (50% v/v isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) was investigated by one-factor analysis of variance of different groups, glutenin proteins separation time and absorbance measurement) to a number of detected proteins. It was found that there was a statistically significant difference in the average number of detected proteins F(2.15) = 28.50, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square = 6.33/8.00 = 0.79) (Table 1).

Table 2 shows descriptive indicators of the relative concentration of HMW glutenins, after extraction with different solvents (50% v/v ethanol, 50% v/v 1-propanol and 50% v/v isopropanol to which Tris-HCl

Table 2. Descriptive indicators of the relative concentration of HMW glutenins (solvent 50% v/v ethanol, 1-propanol and isopropanol, RP-HPLC Agilent Technologies 1260 Infinity, Zorbax 300 SB-C3 Agilent column, column dimensions 4.6 x 150 mm, particle sizes 5 µm and column pressure 80 bar, glutenin proteins separation time 21.0 min, measuring the absorbance at 210 nm

Column	Temp. (^{0}C)	N	Average value	Std. Dev.	Std. error	95% confidence interval of average		Min	Max
						Lower bound	Upper bound		
50% (v/v) ethanol	40	6	5.50	0.84	0.34	4.62	6.38	5	7
	45	6	8.33	1.03	0.42	7.25	9.42	7	9
	50	6	6.00	0.89	0.36	5.06	6.94	5	7
	Total	18	6.61	1.54	0.36	5.85	7.38	5	9
50% (v/v) 1-propanol	40	6	7.83	0.41	0.17	7.40	8.26	7	8
	45	6	5.83	0.98	0.40	4.80	6.87	5	7
	50	6	4.67	1.21	0.49	3.40	5.94	3	6
	Total	18	6.11	1.60	0.38	5.31	6.91	3	8
50% (v/v) isopropanol	40	6	7.00	0.00	0.00	7.00	7.00	7	7
	45	6	7.33	0.82	0.33	6.48	8.19	6	8
	50	6	7.00	0.00	0.00	7.00	7.00	7	7
	Total	18	7.11	0.47	0.11	6.88	7.35	6	8
ANOVA (HMW, 50% v/v ethanol)	F(2.15) = 16.04, Sig. = 0.00, eta square = 27.44/40.28 = 0.68								
ANOVA (HMW, 50% v/v 1-propanol)	F(2.15) = 17.76, Sig. = 0.00, eta square =30.78/43.78 = 0.70								
ANOVA (HMW, 50% v/v isopropanol)	F(2.15) = 1.00, Sig. = 0.39 > 0.05								

0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) at different column temperatures (40, 45 and 50 ^{0}C).

After the extraction of glutenin proteins from wheat flour with 50% (v/v) ethanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the relative concentration of HMW glutenin is Xav = 13.43% (SD = 1.32, CV = 9.83%), at a column temperature of 45 ^{0}C Xav = 17.76% (SD = 2.02, CV = 11.37%) and at a column temperature of 50 ^{0}C Xav = 27.07% (SD = 1.78, CV = 6.57%).

Descriptive analysis showed that the highest relative concentration of HMW glutenins after extraction with 50% (v/v) ethanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added, at a column temperature of 50 ^{0}C, when the absorbance was read at a wavelength of 210 nm (Xav = 27.07%, SD = 1.78) and with an error of estimate of the average value of Std. error = 0.73 and 95% confidence interval average values 95% CI: from 25.20% to 28.94%, with a range from a minimum of 23.57% to a maximum of 28.49%. The lowest relative concentration of HMW glutenins was obtained at a column temperature of 40 ^{0}C (Xav = 13.43%, SD = 1.32) and with an error of average Std. error = 0.54 and 95% CI: from 12.04% to 14.82% and a range from a minimum 11.30% to a maximum of 14.93%.

The influence of column temperature at a constant solvent concentration (50% v/v ethanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) was examined by one-factor analysis of variance of the different groups, glutenin proteins separation time and absorbance measurement) to the relative concentration of HMW glutenins. It was found that there was a statistically significant difference in relative concentration F(2.15) = 96.77, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square = 582.86/628.04 = 0.93) (Table 2).

After glutenin proteins extraction with 50% (v/v) 1-propanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added and chromatographic separation at a column temperature of 40 ^{0}C, the relative concentration of HMW glutenins is Xav = 28.22% (SD = 0.53, CV = 1.88%), at a column temperature of 45 ^{0}C Xav = 31.70% (SD = 2.19, CV = 6.91%) and at a column temperature of 50 ^{0}C Xav = 34.68% (SD = 2.13, CV = 6.14%).

Descriptive analysis showed that the highest relative concentration of HMW glutenins after extraction with 50% (v/v) 1-propanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1%, at a column temperature of 50 ^{0}C, when the absorbance was read at 210 nm was Xav = 34.68%, SD = 2.13, with an error of average Std. error = 0.87 and 95% confidence interval average values 95% CI: from 32.44% to 36.91%, with a range from a minimum of 31.43% to a maximum of 37.44%. The lowest relative concentration of HMW glutenins was obtained at a column temperature of 40 ^{0}C (Xav = 28.22%, SD = 0.53), with an error of average Std. error = 0.21 and 95% CI: 27.66% to 28.77% and a range from a minimum of 27.41% to a maximum of 28.93%.

The influence of column temperature at constant solvent concentration (50% v/v 1-propanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) was investigated by one-factor analysis of variance of different groups, glutenin proteins separation time and absorbance measurement) to the relative concentration of HMW glutenins. It was found that there was a statistically significant difference in relative concentration F(2.15) = 19.55, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square = 125.44/173.56 = 0.72).

After the extraction of glutenin proteins with 50% (v/v) isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added and chromatographic separation at a column temperature of 40 ^{0}C, the relative concentration of HMW glutenis is Xav = 19.14% (SD = 1.60, CV = 8.36%), at a column temperature of 45 ^{0}C Xav = 10.67% (SD = 1.84, CV = 17.24%) and at a column temperature of 50 ^{0}C Xav = 23.61% (SD = 2.98, CV = 12.62%).

Descriptive analysis showed that the highest relative concentration of HMW glutenins was obtained after extraction with 50% (v/v) isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added and at a column temperature of 50 ^{0}C, when the absorbance was read at 210 nm (Xav = 23.61%, SD = 2.98), with an error of average value Std. error = 1.21 and 95% confidence interval of the average 95% CI: from 20.48% to 26.73%, with a range from a minimum of 18.59% to a maximum of 27.65%. The lowest relative concentration of HMW glutenins was obtained at a column temperature of 45 ^{0}C (Xav = 10.67%, SD = 1.84), with an error in the average Std. error = 0.75 and 95% CI: 8.73% to 12.60% and with range from a minimum of 8.44% to a maximum of 13.82%.

The influence of column temperature at constant solvent concentration (50% v/v isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added), glutenin proteins separation time and absorbance measurement was investigated by one-factor analysis of variance of different groups to the relative concentration of HMW glutenins. It was found that there was a statistically significant difference in relative concentration, F(2.15) = 52.51, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square=518.53/592.59 = 0.87) (Table 2).

Table 3 shows descriptive indicators of the number of HMW glutenin proteins, after extraction with different solvents (50% v/v ethanol, 50% v/v 1-propanol and 50% v/v isopropanol, to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) at different column temperatures (40, 45 and 50 ^{0}C).

After the extraction of glutenin proteins with 50% (v/v) ethanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the number of detected proteins of HMW glutenins was Xav = 5.50 (SD = 0.84, CV = 15.27%), at a column temperature of 45 ^{0}C Xav = 8.33 (SD = 1.03, CV = 12.36%) and at a column temperature of 50 ^{0}C Xav = 6.00 (SD = 0.00, CV = 0.00%).

Descriptive analysis showed that the highest number of detected proteins of HMW glutenins was obtained after extraction with 50% (v/v) ethanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added, at a column temperature of 45 ^{0}C, when the absorbance was read at 280 nm (Xav = 8.33, SD = 1.03), with an error of average Std. error = 0.42 and a 95% confidence interval of the average value 95% CI: 7.25 to 9.42, with a range from a minimum of 7.00 to a maximum of 9.00 proteins. The lowest number of HMW glutenin proteins was obtained at a column temperature of 40 ^{0}C (Xav = 5.50, SD = 0.84), with an error of average Std. error = 0.34 and 95% CI: of 4.62 to 6.38 and a range from a minimum of 5.00 to a maximum of 7.00 proteins.

The influence of column temperature at a constant solvent concentration (50% v/v ethanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added), glutenin proteins separation time and absorbance measurement) was examined by one-factor analysis of variance of the different groups to the number of HMW glutenin proteins. It was found that there was a statistically significant difference in the average number of detected proteins, F(2.15) = 16.04, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square = 27.44/40.28 = 0.68) (Table 3).

After the extraction of glutenin proteins with 50% (v/v) 1-propanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added, the number of HMW glutenin proteins at a column temperature of 40 °C was obtained Xav = 7.83 (SD = 0.41, CV = 5.24%), at a column temperature of 45 ^{0}C Xav = 5.83 (SD = 0.98, CV = 16.81%) and at a column temperature of 50 ^{0}C Xav = 4.67 (SD = 1.21, CV = 25.91%).

Descriptive analysis showed that the highest number of detected HMW glutenin proteins was obtained after extraction with 50% (v/v) 1-propanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) was added, at a column temperature of 40 ^{0}C, when the absorbance was read at 280 nm (Xav = 7.83, SD = 0.41), with an error of estimate average value Std. error = 0.17 and 95% confidence interval average values 95% CI: 7.40 to 8.26, with a range from a minimum 7 to a maximum 8 proteins. The lowest number of average HMW glutenins proteins was obtained at a column temperature of 50 ^{0}C (Xav = 4.67, SD = 1.21), with an error in the average value Std. error = 0.49 and 95% CI: from 3.40 to 5.94 and a range from a minimum of 3 to a maximum of 6 proteins.

The influence of column temperature at a constant solvent concentration (50% v/v 1-propanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% were added, glutenin proteins separation time and absorbance measurement) was investigated by one-factor analysis of variance of different groups

Table 3. Descriptive indicators of HMW glutenin proteins number (solvent 50% v/v ethanol, 1-propanol and isopropanol, RP-HPLC Agilent Technologies 1260 Infinity, Zorbax 300 SB-C3 Agilent column, column size 4.6 x 150 mm, particle sizes 5 µm and a column pressure 80 bar, glutenin proteins separation time 21.0 min, measuring the absorbance at 280 nm

Column	Temp. (^{0}C)	N	Average value	Std. Dev.	Std. error	95% confidence interval of average Lower bound	Upper bound	Min	Max
50% (v/v) ethanol	40	6	5.50	0.84	0.34	4.62	6.38	5	7
	45	6	8.33	1.03	0.42	7.25	9.42	7	9
	50	6	6.00	0.89	0.36	5.06	6.94	5	7
	Total	18	6.61	1.54	0.36	5.85	7.38	5	9
50% (v/v) 1-propanol	40	6	7.83	0.41	0.17	7.40	8.26	7	8
	45	6	5.83	0.98	0.40	4.80	6.87	5	7
	50	6	4.67	1.21	0.49	3.40	5.94	3	6
	Total	18	6.11	1.60	0.38	5.31	6.91	3	8
50% (v/v) isopropanol	40	6	7.00	0.00	0.00	7.00	7.00	7	7
	45	6	7.33	0.82	0.33	6.48	8.19	6	8
	50	6	7.00	0.00	0.00	7.00	7.00	7	7
	Total	18	7.11	0.47	0.11	6.88	7.35	6	8
ANOVA (HMW, 50% v/v ethanol)	F(2.15) = 16.04, Sig. = 0.00, eta square = 27.44/40.28 = 0.68								
ANOVA (HMW, 50% v/v 1-propanol)	F(2.15) = 17.76, Sig. = 0.00, eta square =30.78/43.78 = 0.70								
ANOVA (HMW, 50% v/v isopropanol)	F(2.15) = 1.00, Sig. = 0.39 > 0.05								

to the number of HMW glutenin proteins. It was found that there was a statistically significant difference in the average number of detected proteins, F(2.15) = 17.76, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square = 30.78/43.78 = 0.70).

After the extraction of glutenin proteins with 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the number of HMW glutenin proteins was obtained Xav = 7 (SD = 0.00, CV = 0.00%), at a column temperature of 45 ^{0}C Xav = 7.33 (SD = 0.82, CV = 11.19%) and at a column temperature of 50 ^{0}C Xav = 7.00 (SD = 0.00, CV = 0.00%).

Descriptive analysis showed that the highest number of detected HMW glutenin proteins was obtained after extraction with 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added at a column temperature of 45 ^{0}C, when the absorbance was read at 280 nm (Xav = 7.33, SD = 0.82), with an error of the average value Std. error = 0.33 and 95% confidence intervals of average values 95% CI: 6.48 to 8.19, with a range from minimum 6 to maximum 8 proteins. The lowest number of HMW glutenin proteins was obtained at a column temperature of 40 and 50 ^{0}C (Xav = 7.00, SD = 0.00), with an error of average value Std. error = 0.00 and 95% CI: from 7 to 7 and a range from a minimum 7 to a maximum 7 proteins.

The influence of column temperature at a constant solvent concentration (50% v/v isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% were added, glutenin proteins separation time and absorbance measurement) was investigated by one-factor analysis of variance of different groups to the number of HMW glutenin proteins. It was found that there was no statistically significant difference in the average number of detected proteins, Sig = 0.39 > 0.05.

Table 4 shows descriptive indicators of the relative concentration of HMW glutenins, after extraction with different solvents (50% v/v ethanol, 50% v/v 1-propanol and 50% v/v isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added), at a different column temperatures (40, 45 and 50 ^{0}C).

After the extraction of glutenin proteins with 50% (v/v) ethanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the relative concentration of HMW glutenin is Xav = 32.57% (SD = 0.82, CV = 2.52%), at a column temperature of 45 ^{0}C Xav = 36.49% (SD = 2.38, CV = 6.52%) and at a column temperature of 50 ^{0}C Xav = 40.19% (SD = 2.21, CV = 5.50%).

Descriptive analysis showed that the highest relative concentration of HMW glutenins was obtained after extraction with 50% (v/v) ethanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added at a column temperature of 50 ^{0}C, when the absorbance was read at 280 nm (Xav = 40.19%, SD = 2.21), with an error of average value Std. error = 0.90 and 95% confidence interval of the average 95% CI: from 37.87% to 42.51%, with a range from a minimum of 36.12% to a maximum of 42.74%. The lowest relative concentration of HMW glutenins was obtained at a column temperature of 40 ^{0}C (Xav = 32.57%, SD = 0.82), with an error of average value Std. error = 0.33 and 95% CI: from 31.71 % to 33.43% and a range from a minimum of 31.25% to a maximum of 33.45%.

Table 4. Descriptive indicators of the relative concentration of HMW glutenins (solvent 50% v/v ethanol, 1-propanol and isopropanol, RP-HPLC Agilent Technologies 1260 Infinity, Zorbax 300 SB-C3 Agilent column, column size 4.6 x 150 mm, particle sizes 5 µm and a column pressure 80 bar, glutenin proteins separation time 21.0 min, measuring the absorbance at 280 nm

Column	Temp. (^{0}C)	N	Average value	Std. dev.	Std. error	95% confidence interval of average Lower Bound	95% confidence interval of average Upper bound	Min	Max
50% (v/v) ethanol	40	6	32.57	0.82	0.33	31.71	33.43	31.25	33.45
	45	6	36.49	2.38	0.97	33.98	38.99	33.54	40.13
	50	6	40.19	2.21	0.90	37.87	42.51	36.12	42.74
	Total	18	36.42	3.68	0.87	34.58	38.25	31.26	42.74
50% 1-propanol	40	6	61.62	3.19	1.30	58.27	64.97	55.96	64.91
	45	6	35.27	2.65	1.08	32.49	38.05	33.41	40.13
	50	6	39.18	2.38	0.97	36.68	41.69	37.12	42.74
	Total	18	45.36	12.23	2.88	39.28	51.44	33.41	64.91
50% (v/v) isopropanol	40	6	28.64	1.70	0.69	26.86	30.42	26.38	30.91
	45	6	21.66	3.56	1.45	17.92	25.40	17.18	26.34
	50	6	34.22	2.74	1.12	31.34	37.10	29.47	36.84
	Total	18	28.17	5.89	1.39	25.24	31.11	17.17	36.84
ANOVA (HMW, 50% v/v ethanol)	F(2.15) = 23.27, Sig. = 0.00, eta square = 174.47/233.69 = 0.76								
ANOVA (HMW, 50% v/v 1-propanol)	F(2.15) = 158.88, Sig. = 0.00, eta square = 2426.69/2541.24 = 0.95								
ANOVA (HMW, 50% v/v isopropanol)	F(2.15) = 30.86, Sig. = 0.00, eta square = 475.34/590.87 = 0.80								

The influence of column temperature at a constant solvent concentration (50% v/v ethanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% were added, glutenin proteins separation time and absorbance measurement) was examined by one-factor analysis of variance of the different groups to the relative concentration of HMW glutenins. It was found that there was a statistically significant difference in the average relative concentration, F(2.15) = 23.27, Sig. = 0.00. This difference, measured by the eta square indicator is large (eta square = 174.47/230.69 = 0.76).

After the extraction of glutenin proteins with 50% (v/v) 1-propanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) was added and chromatographic separation at a column temperature of 40 ^{0}C, the relative concentration of HMW glutenins is Xav = 61.62% (SD = 3.19, CV = 5.18%), at a column temperature of 45 ^{0}C Xav = 35.27% (SD = 2.65, CV = 7.51%) and at a column temperature of 50 ^{0}C Xav = 39.18% (SD = 2.38, CV = 6.07%).

Descriptive analysis showed that the highest relative concentration of HMW glutenins was obtained after extraction with 50% (v/v) 1-propanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added, at a column temperature of 40 ^{0}C, when the absorbance was read at 280 nm (Xav = 61.62%, SD = 3.19) with an error of average value Std. error = 1.30 and 95% confidence intervals of average values 95% CI: from 58.27% to 64.97%, with a range from a minimum of 55.96% to a maximum 64.91%. The lowest relative concentration of HMW glutenins was obtained at a column temperature of 45 ^{0}C (Xav = 35.27%, SD = 2.65), with an error in the average value Std. error = 1.08 and 95% CI: 32.49% to 38.05% and a range of values from a minimum of 33.41% to a maximum of 40.13%.

The influence of column temperature at a constant solvent concentration (50% v/v 1-propanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added, glutenin proteins separation time and absorbance measurement) was investigated by one-factor analysis of variance of different groups to the relative concentration of HMW glutenins. It was found that there was a statistically significant difference in the average relative concentration, F(2.15) = 158.88, Sig. = 0.00. This difference, as measured by the eta square indicator is large (eta square = 2426.69/2541.24 = 0.95) (Table 4).

After the extraction of glutenin proteins with 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and chromatographic separation at a column temperature of 40 ^{0}C, the relative concentration of HMW glutenin is Xav = 28.64% (SD = 1.70, CV = 5.93%), at a column temperature of 45 ^{0}C Xav = 21.66% (SD = 3.56, CV = 16.44%) and at a column temperature of 50 vC Xav = 34.22% (SD = 2.74, CV = 8.01%).

Descriptive analysis showed that the highest relative concentration of HMW glutenins was obtained after extraction with 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and at a column temperature of 50 ^{0}C, when the absorbance was read at 280 nm (Xav = 34.22%, SD = 2.74), with an error of the average value Std. error = 1.12 and a 95% confidence interval of the average 95% CI: from 31.34% to 37.10%, with a range from a minimum of 29.47% to a maximum of 36.84%. The lowest relative concentration of HMW glutenins was obtained at a column temperature of 45 ^{0}C (Xav = 21.66%, SD = 3.56), with an error in the average value Std. error = 1.45 and 95% CI: 17.92% to 25.40% and a range of values from a minimum of 17.18% to a maximum of 26.34%. The influence of a column temperature at constant solvent concentration (50% v/v isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added, glutenin proteins separation time and absorbance measurement) was investigated by one-factor analysis of variance of different groups to the relative concentration of HMW glutenins. It was found that there was a statistically significant difference in the average relative concentration, F(2.15) = 30.86, Sig = 0.00. This difference, measured by the eta square indicator is large (eta square = 475.34/590.87 = 0.80).

Wieser *et al.*, [10], quantified gluten proteins in wheat flour by high-pressure reversed phase liquid chromatography (RP-HPLC). The glutenins were extracted twice with 1 ml solution of 50% (v/v) 1-propanol + 2 mol/L urea + 0.05 mol/L Tris-HCl (pH = 7.5) + 1% dithioerythritol (DTE) in a nitrogen stream. The glutenin proteins were separated into two columns on a C8 and C18 column, at a column temperature of 50 and 70 ^{0}C, and at a wavelength detection of 210 and 220 nm. Glutenin separation was satisfactory on both columns. The temperature of the column of 50 ^{0}C was shown to be better because at the column temperature of 70 ^{0}C, there is an overlap. The wavelength detection of 210 nm had a higher sensitivity than at 220 nm. Glutenin proteins were eluted on the basis of hydrophobicity, in the following order ωb gliadins, HMW and LMW glutenin subunits.

As part of a comparative study of different varieties of wheat, Wieser, [12], quantitatively and quantitatively identified gluten proteins. The glutenins were extracted 2 times with 1 ml of 50% (v/v) 1-propanol with the reducing agent dithioerythritol (DTE). The chromatographic separation was performed on a C8 silica gel column. The wavelength detection was 210 nm. Based on the obtained results, the retention time of HMW glutenin subunits was 18 - 27 minutes, LMW glutenin subunit > 27, ωb gliadins 12 - 18. The relative

concentration of LMW glutenin subunits was 10.00 - 25.10%, HMW glutenin subunits 2.60 - 9.30% and ωb gliadins 0.30 - 1.30%.

Horvat *et al.*, [13], analysed gluten proteins in wheat by RP-HPLC chromatography. Proteins were quantitatively extracted from 100 mg flour according to the method of Wieser *et al.*, [10]. The chromatographic separation was performed on a reverse phase Supelcosil LC 318 (Supelco) C18 column. The column temperature was 50 ^{0}C, the mobile phase flow was 1 mL/min, and the wavelength detection was 210 nm. The retention time for glutenin subunits was 6 - 10 minutes for ωb gliadins (number of subunits 1 - 3), for HMW glutenins 10 - 15 minutes (4 - 6 subunits) and for LMW glutenins 15 - 30 minutes (16 - 19 subunits). The relative concentration of glutenins was 36.77%.

Qian *et al.*, [14], characterized the gluten proteins from wheat by high-pressure liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization (MALDI), and the mass analyser is time-of-flight (TOF) mass spectrometry. Gluten proteins were extracted from the Canadian wheat variety. The sample was extracted by adding 6 mL of 50% (v/v) 1-propanol containing 1% dithioerythritol (DTT) in 1 g of the sample. Separation of the samples was performed on a reverse phase Zorbax SB300-C18 column. The column temperature was 60 ^{0}C and the mobile phase flow was 1 ml/min. The wavelength detection was 210 nm. 35 large peaks were identified on the HPLC chromatogram. Based on retention time, they were divided into 4 groups: ω gliadins (4 proteins), HMW glutenins (5), α+β gliadins, and LMW glutenins (22) and γ gliadins (4).

Živancev *et al.*, [15], examined the advantages and disadvantages of the lab-on-a-chip method relative to reverse-phase high-pressure liquid chromatography in the determination of gluten proteins. 9 wheat varieties were used to determine gluten proteins. Gluten proteins were extracted by the method of Wieser *et al.*, [10]. The chromatographic separation was performed on a Supelco Discovery BIO Wide Pore C18 column at a flow rate of 1 mL/min and at a column temperature of 50 ^{0}C. The wavelength detection was 210 nm. Based on the obtained results, the number of proteins within HMW glutenins ranged from 4 - 5, and within LMW glutenins from 15 - 19.

Scherf, [16], examined how the preparation of gliadins, glutenins and gluten from wheat starch samples affected their content in determination by reverse-phase high-pressure liquid chromatography (RP-HPLC) and enzyme-immunochemical method (ELISA). After gliadins, glutenins were also extracted 3 times with 0.15 mL of a solution of 50% (v/v) 1-propanol containing Tris-HCl (pH = 7.5), 0.06 mol/L (v/v) dithioerythritol and 2 mol/L urea. The glutenin supernatants were evaporated under a stream of liquid nitrogen at 60 ^{0}C for 30 minutes. The samples were analyzed by reverse-phase high-pressure liquid chromatography (RP-HPLC) on an Acclaim 300 C18 column, at a column temperature of 60 ^{0}C, a flow rate of 0.2 mL/min, and a wavelength of 210 nm. The relative concentration of glutenins was 28.3%, of which ωb gliadins was 1.2%, high molecular weight glutenin subunits (HMW) 9.4% and low molecular weight glutenin subunits (LMW) 17.7%. Glutenin proteins separation was performed for 22 minutes.

In this paper, three types of solvents were used for the extraction of glutenin proteins (50% v/v ethanol, 50% v/v 1-propanol and 50% v/v isopropanol to which Tris-HCl 0.05 mol/L, pH = 7.5, urea 2 mol/L and dithioerythritol 1% was added) as well as three column temperatures (40, 45 and 50 ^{0}C). Separation was performed on column C3. Numerous authors have generally used 50% (v/v) 1-propanol for glutenins extraction, and separation was performed on columns C8 and C18.

The results obtained in this work (when comparing after extraction with 50% v/v 1-propanol) are in accordance with the authors Qian *et al.*, [14], and Živancev *et al.*, [14], because the number of HMW glutenin proteins obtained ranged from 4 - 5.

4. Conclusions

- After the chromatographic separation of glutenin proteins and examination of the solvent type and column temperature, the following conclusions were reached.

- After the extraction of glutenin proteins with 50% (v/v) ethanol, 50% (v/v) 1-propanol and 50% (v/v) isopropanol to which Tris-HCl (0.05 mol/L, pH = 7.5), urea (2 mol/L) and dithioerythritol (1%) were added and by measuring the absorbance at 210 nm, the most proteins were obtained by extraction with 50% (v/v) isopropanol and at a column temperature of 50 ^{0}C (Xav = 7.17, RC = 23.61%) and the lowest number by extraction with 50% (v/v) ethanol and at a column temperature of 40 ^{0}C (Xav = 4.00, RC = 13.43%).

- After extraction under the above conditions and measuring the absorbance at 280 nm, the largest number of glutenin proteins was obtained by extraction with 50% (v/v) ethanol and at a column temperature of 45 ^{0}C (Xav = 8.33, RC = 36.49%), and the smallest number by extraction with 50% (v/v) 1-propanol and at a column temperature of 50 ^{0}C (Xav = 4.67, RC = 39.18%).

- Based on the results obtained, it can be seen that the highest number of HMW glutenin proteins (by measuring the absorbance at two wavelengths, 210 and 280 nm) were obtained by extraction of 50% (v/v) ethanol (Xav = 8.33), at a column temperature of 45 ^{0}C and at 280 nm.

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