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THE INFLUENCE OF EXTRACTION CONDITIONS AND CHROMATOGRAPHIC SEPARATION ON THE ABILITY OF IDENTIFYING GLIADINS FROM THE WHEAT FLOUR

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Abstract

Cereal proteins play an important role in the humans' and animals' diet, due to their nutritional composition and functional properties in food production. One of these proteins is gluten. It contains protein components that are present as monomers and interconnected by disulfide bonding - polymers. Based on their solubility in an aqueous alcohol, gluten proteins are divided into soluble gliadins and insoluble glutenins. Apart from the beneficial nutritional composition and importance in food production, gluten also causes adverse health effects in susceptible individuals, such as celiac disease.

In this paper, gliadin proteins have been analyzed in wheat flour by high-pressure liquid chromatography of reversed phases (RP-HPLC). The influence of different concentrations of ethanol (30%, 40% and 50%) and column temperature (40 ^{0}C, 45 ^{0}C and 50 ^{0}C) was investigated to achieve better chromatographic separation and identification of gliadin proteins. The chromatographic separation of gliadin proteins was carried out on the column Zorbax 300 SB-C3 (Agilent) and the Agilent Technologies 1260 Infinity HPLC apparatus has been used.

After extraction gliadin proteins with 30% (v/v) ethanol, the number of identified proteins was 20, after extraction with 40% (v/v) ethanol was 21 and with 50% (v/v) ethanol was 24, at a temperature of 40 ^{0}C. By increasing the column temperature (45 ^{o}C and 50 ^{o}C), the number of identified proteins after extraction with 30% ethanol was 17 and 20, after extraction with 40% ethanol was 25 and 24, and after extraction with 50% ethanol was 26 and 24.

Based on the obtained results, the largest number of gliadin proteins was identified by extraction with 50% ethanol and at a column temperature of 45 ^{0}C. By increasing the column temperature to 50 ^{o}C, the number of identified proteins decreases.

***Key words**: Gliadins, Ethanol, High-pressure liquid chromatography of reversed phases (RP-HPLC).*

1. Introduction

Cereals represent an important source of protein in humans and animals diet. Wheat grains contain 8 - 15% protein. The protein content in wheat varies, with genetic origin having the greatest influence on it [1]. Wheat is one of the most important agricultural crops. According to the FAO, around 750 million tons of wheat are produced annually in the world [2]. Gluten is a wheat protein which has functional properties that allow the usage of wheat in bread production. It represents about 90% of the total wheat protein. Gluten consists of two fractions: gliadins and glutenins [3, 4].

The classification of wheat proteins is based on solubility in various solvents. Mammon *et al.,* [5], have reported the following classifications: albumin and globulins (soluble in water and salt solution), gliadins (soluble in aqueous alcohol) and glutenins (soluble in diluted

acids and bases). Wieser, [6], considers that gliadins are half of the total gluten content, while the other half consists of the glutenins.

Gliadins are monomeric polypeptides with a molecular weight ranging between 28,000 Da and 50,000 Da [7, 8, and 9]. Glutamine (35%) and proline (15%) are amino acids which dominate in gliadins content and because of that they are also known as prolamines. Because of this, gliadins are highly resistant to the proteolytic enzymes action and degradation within the gastrointestinal tract [10]. Gliadins can have different primary molecular structure (amino acid composition) and different molecular weight. Therefore, they are divided into four fractions: ω_5, $\omega_{1,2}$, α+β, and γ gliadins. Omega gliadins have a higher molecular weight and greater content of glutamine and proline residues than α+β, and γ gliadins. α+β and γ gliadins have a similar molecular weight, as well as the glutamine and prolin content, but differ in the content of other amino acids (for example, tyrosine) [10].

Glutenins represent proteins of higher molecular weight compared to gliadins. They contain free thiol groups, which can form disulfide bridges, which interconnect the glutenin molecules [11]. In this way, polymer molecules are formed. Glutenins are divided into: subunits with high molecular weight (HMW) with a molecular weight ranging between 70,000 Da and 90,000 Da and subunits with low molecular weight (LMW) with molecular weight ranging between 20,000 Da and 45,000 Da [12, 13].

Consumption of foods containing proteins of some cereals, in people sensitive to gluten fractions, causes celiac disease (CD) and non-celiac wheat sensitivity (NCWS). Celiac disease is an autoimmune disorder and affects about 0.7 - 2.0% of the population [14]. It is characterized by: damage of the small intestine, chronic diarrhea, abdominal pain, weight loss, fatigue, anemia, and weight loss. Non-celiac wheat sensitivity (NCWS) occurs in 6% of the western population [15]. Alpha-gliadins (α-gliadins) are the dominant factor in gluten-toxicity. Due to the high proline content, this protein fraction is resistant to proteolytic degradation in the stomach and intestines [16]. Recent studies have shown that the number of disorders caused by gluten is increasing and it represents a health problem worldwide [17]. According to London *et al.,* [18], and Zingorne *et al.,* [19], for people who suffer from celiac disease (CD) lifelong gluten-free diet is the only available therapy.

Taking into account the number of people whose sensitivity to gluten is on the rise, there is a need for fast, accurate and precise methods for determination and quantification of gluten in food products. Different analytical methods are used for this purpose [20]. A large number of researchers in the world are working to optimize existing methods and develop new methods for the detection and quantification of gluten in food products. All these efforts aim to make the methods available to anyone interested in the food chain and to ensure that sensitive people are maximally informed and protected against adverse effects.

In this paper, authors contribute to this research, by optimizing the process of proteins extraction from food and optimizing chromatographic separation and identification proteins from different gliadin fractions. Gliadin proteins from wheat flour have been analyzed by high pressure liquid chromatography of reversed phase. The influence of different ethanol concentrations (30%, 40% and 50% v/v) on protein extraction efficiency and different temperatures (40 ^{0}C, 45 ^{0}C and 50 ^{0}C) for protein separation has been studied with the aim of faster separation and identification gliadins protein.

2. Materials and Methods

2.1 Materials

In this paper gliadin proteins from wheat flour type 500 have been analyzed and purchased on a free market. Different concentrations of ethanol (30%, 40% and 50% v/v) have been used for the extraction.

2.2 Extraction of gliadin proteins from wheat flour

Gliadin extraction from wheat flour sample (100 mg) has been carried out according to the modified Wieser *et al.,* [21], method. First, water and salt soluble proteins have been extracted. Albumin and globulin have been extracted twice with 1 mL of a solution of 0.4 mol/L NaCl (Lach-Ner, Czech Republic) with the addition of 0.067 mol/L KH_2PO_4 solution (Lach-Ner, Czech Republic), at pH = 7.6, at temperature of 20 ^{0}C.

After the supernatant of albumin and globulin has been removed, extraction of alcohol soluble proteins has been carried out (gliadins). Gliadins have been extracted three times with 0.5 mL of different concentrations of ethanol (30%, 40% and 50% v/v) at room temperature (20 ^{0}C). The samples homogenization have been performed on a vortex (Advanced Vortex Mixer ZX3, Velp scientifica) for 2 minutes, each time after the addition of the appropriate solvent. The stirring was continued then on a magnetic stirrer (Velp scientifica) for 10 minutes. After the homogenization of the samples was completed, centrifugation was carried out in a centrifuge (Hettich zentrifugen, rotina 380 R) for 15 minutes (albumin and globulin) and 20 minutes (gliadins) at 7,000 rpm. The supernatants have been supplemented to 2.0 mL with a suitable extractant solvent. Immediately, before the analysis, the sample has been filtered through a 0.45 μm membrane filter (RC syringe filters, Filtratech, France).

2.3 RP-HPLC chromatography

Reversed-phase-high performance liquid chromatography (RP-HPLC) chromatography has been carried out on the HPLC Agilent Technologies 1260 Infinity apparatus. Gliadin proteins (ω_5, $\omega_{1,2}$, α+β and γ) separation has been performed on the Zorbax 300 SB-C3 (Agilent Technologies) column, size 4.6 x 150 mm, particle size 5 µm, maintained at a temperature of 40 ^{0}C, 45 ^{0}C and 50 ^{0}C. Two mobile phases have been used: deionized water (eluent A) and 0.1% trifluoroacetic acid (TFA, Acros, France) in acetonitrile (ACN, Biosolve Chimie, France) of HPLC purity (eluent B). Gliadins have been separated by gradient elution adjusted as follows: 0 - 8.5 min. 30 - 40% B; 8.5 - 10 min. 40% B; 10 - 12 min. 40 - 43% B; 12 - 20 min. 43 - 55% B; 20 - 25 min. 30% B at 1 mL/min flow. The injection volume was 70 µL. The detection wavelength was 210 nm.

3. Results and Discussion

The gliadin standard (Gliadin from wheat gluten, MP Biomedicals, LCC) has been used as an external standard. For the purposes of chromatographic separation, the gliadin standard mass of 3.00 mg (± 0.01) was dissolved in 70% (v/v) ethanol. The gliadins have been divided into four fractions: ω_5 (retention time 3.0 - 5.5 min), $\omega_{1,2}$ (retention time 8.0 - 10.5 min) and γ gliadins (retention time 10.5 - 16.0 min).

The result repeatability has been used to determine the quality measurements. The same sample has been separated six times (6 repetitions) under identical conditions, and then a comparison of the obtained chromatograms has been performed. Figure 1 shows the gliadins chromatogram obtained during 6 repetitions of samples obtained by extraction of the protein with 50% (v/v) of ethanol, separated at a column temperature of 45 ^{0}C. Figure 1 shows that the resulting chromatograms are almost identical, that is, the repeatability of the result is good. During the statistical analysis, the standard deviation of the obtained data for the relative concentration of 6 repetitions for each obtained peak on the chromatogram, the identified protein, has been determined. As an example, in Figure 1, statistical data (Figure 1) for peak with retention time 7.87 min (SD = 0.02, CV = 0.20%) and relative concentration (Xsr = 6.98%, SD = 0.46, CV = 6.59%) (Figure 1, detail) are shown.

Figure 2 shows the appearance of all 9 combinations of gliadin proteins extracted with ethanol of different concentrations (30%, 40% and 50% v/v) and separated at a column temperature of (40 ^{0}C, 45 ^{0}C and 50 ^{0}C) in order to show the quality measurements of the obtained results.

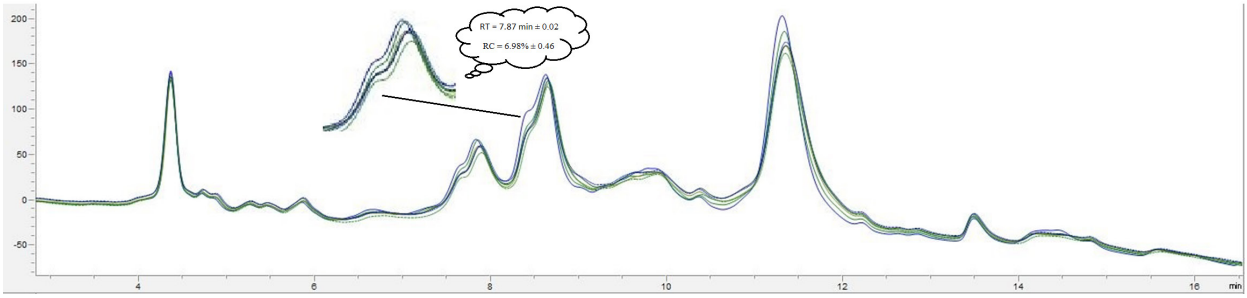

Figure 1. Repeatability of the results (chromatograms) of gliadins obtained by RP-HPLC chromatography and separated under optimal extraction and chromatographic conditions (solvent: 50% v/v ethanol, analysis time 16 minutes, column Zorbax 300 SB C3 Agilent, 6 x 150 mm, particle size 5 µm, column temperature 45 ^{0}C and pressure 80 bar)

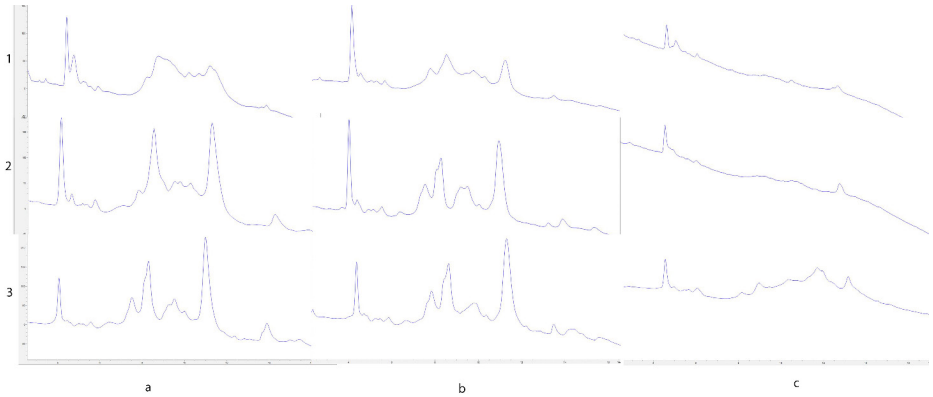

Figure 2. The appearance of the obtained chromatograms of all 9 combinations extracted with ethanol of different concentrations (1 - 30% v/v ethanol, 2 - 40% v/v ethanol, 3 - 50% v/v ethanol) and separated at a column temperature of (a - 40 ^{0}C, b - 45 ^{0}C, c - 50 ^{0}C)

Identification of the gliadin protein has been carried out according to the method described by Wieser *et al.,* [21]. By the analysis of the RP-HPLC gliadin chromatogram, the order of appearance of the gliadin protein has been determined (Figure 3A). The highest polarity, at least the retention time, have ω_5 gliadins (3.0 - 5.5 min), then $\omega_{1,2}$ (5.5 - 8.0 min), α+β (8.0 - 10.5 min) and γ gliadin (10.5 - 16.0 min) (Figure 3B).

Figure 3 shows the obtained gliadin chromatograms extracted with 50% (v/v) ethanol and separated at a temperature of 45 ºC with retention time (Figure A) and area (Figure B) of each protein. Based on the retention time, the distribution of proteins by fractions has been performed (Table 1), and the relative concentration of each protein and protein fraction has been calculated from the area (Table 2).

The total number of proteins and the number of proteins within the four gliadin fractions has been determined according to Wieser *et al.,* [21], as shown in Table 1. Subsequently, the relative concentration of individual gliadin protein fractions (Table 2) has been determined. Due to the high similarity of proteins of particular fractions and similar behavior during chromatographic separation, there is an overlapping of peaks of one number of proteins. This was the reason why the authors have chosen to show the total relative concentration of proteins for four fractions. Using the Agilent ChemStation software, the relative concentration of the individual fractions has been calculated. Proteins with a relative concentration of less than 0.1% have not been considered and are not shown in this table.

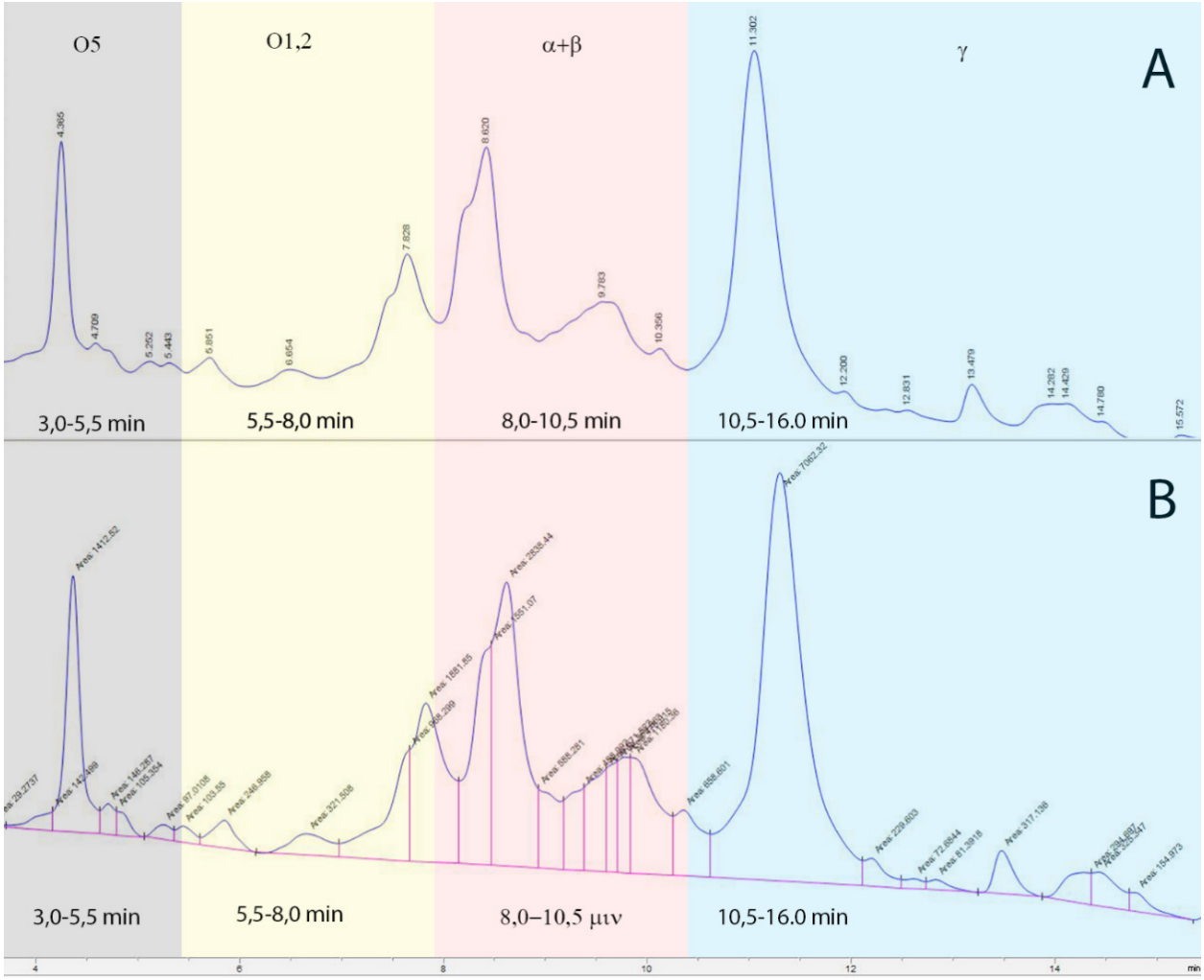

Figure 3. The obtained gliadin chromatograms extracted with 50% (v/v) ethanol and separated at a column temperature of 45 ºC with the retention time (Figures A) and with the area of each protein (Figure B)

Table 1. The total number of proteins and number of proteins separated and detected by fractions (time of separation for 16.0 minutes, column Zorbax 300 SB-C3, Agilent, size 4.6 x 150 mm, particle size 5µm, pressure 80 bar)

Solvent / conc.	Measured param.	Column temperature °C														
		40					45					50				
		Protein fractions														
		TP	ω5	ω1,2	α+β	γ	TP	ω5	ω1,2	α+β	γ	TP	ω5	ω1,2	α+β	γ
30% ethanol	X_{min}-X_{max} Xsr	18 - 21 20	5 - 6 5	2 - 4 3	2 - 4 3	8 - 10 9	15 - 17 17	4 - 5 5	3 - 3 3	1 - 3 3	6 - 7 6	18 - 21 20	4 - 4 4	4 - 6 5	3 - 4 4	7 - 7 7
	SD CV (%)	0.94 4.70	0.37 7.40	0.39 13.00	0.74 24.67	0.58 6.44	0.76 4.47	0.47 9.40	0.00 0.00	0.76 25.33	0.37 6.17	0.96 4.80	0.00 0.00	0.90 18.00	0.47 11.75	0.00 0.00
40% ethanol	X_{min}-X_{max} Xsr	20 - 21 21	4 - 6 5	3 - 4 3	4 - 5 4	7 - 9 9	24 - 26 25	7 - 7 7	4 - 5 5	5 - 6 6	7 - 8 7	21 - 25 24	4 - 4 4	3 - 4 4	2 - 4 3	11 - 14 13
	SD CV (%)	0.47 2.24	0.69 13.80	0.37 12.33	0.37 9.25	0.76 8.44	0.69 2.76	0.00 0.00	0.50 10.00	0.37 6.17	0.37 5.29	1.37 5.71	0.00 0.00	0.50 12.50	0.74 24.67	1.07 8.23
50% ethanol	X_{min}-X_{max} Xsr	23 - 25 24	6 - 6 6	2 - 3 2	6 - 7 7	8 - 10 9	25 - 28 26	7 - 9 8	3 - 3 3	6 - 9 7	8 - 9 8	21 - 27 24	7 - 7 7	2 - 3 3	4 - 7 5	7 - 10 9
	SD CV (%)	0.58 2.42	0.00 0.00	0.37 18.50	0.37 5.29	0.58 6.44	1.15 4.42	0.69 8.62	0.00 0.00	1.11 15.86	0.37 4.62	2.27 9.46	0.00 0.00	0.37 12.33	0.94 18.80	1.37 15.22

*TP – the total number of detected proteins, Xmin – minimum value, Xmax – maximum value , Xsr – average value, SD – standard deviation, CV (%) – coefficient of variation

Table 2. The relative concentration of total proteins and protein fractions (separation time for gliadin proteins - 16.0 min, column: Zorbax 300 SB-C3, Agilent, dimension 4.6 x 150 mm, particle size 5µm, pressure 80 bar)

Solvent / conc.	Measured param.	Column temperature											
		40				45				50			
		Protein fractions											
		ω5	ω1,2	α+β	γ	ω5	ω1,2	α+β	γ	ω5	ω1,2	α+β	γ
30% ethanol	X_{min} X_{max} Xsr	13.75 - 15.09 14.43	6.51 - 7.44 6.80	44.16 - 45.92 45.12	32.21 - 35.31 33.62	16.79 - 18.34 17.62	9.89 - 14.48 12.11	39.61 - 41.63 40.47	27.18 - 30.82 29.79	19.60 - 23.12 21.21	12.54 - 15.01 13.91	27.17 - 28.44 27.95	34.57 - 39.18 36.93
	SD CV (%)	0.52 3.60	0.30 4.41	0.59 1.31	1.01 3.00	0.47 2.67	1.39 11.48	0.59 1.46	1.21 4.06	1.37 6.46	0.83 5.97	0.51 1.82	1.51 4.09
40% ethanol	X_{min} X_{max} Xsr	9.66 - 11.72 10.24	8.20 - 11.73 9.67	34.45 - 42.90 37.81	38.86 - 44.73 42.28	12.06 - 13.74 12.81	12.63 - 18.05 15.20	28.30 - 36.00 33.71	34.43 - 41.32 38.28	23.34 - 29.70 25.60	5.75 - 8.38 6.78	30.24 - 34.56 32.77	31.60 - 37.28 34.84
	SD CV (%)	0.70 6.84	1.46 15.01	2.69 7.11	2.23 5.27	0.58 4.53	2.09 13.75	2.57 7.62	2.30 6.01	2.44 9.53	0.84 12.24	1.46 4.45	1.89 5.42
50% etanol	X_{min} X_{max} Xsr	7.41 - 8.58 7.85	12.81 - 15.05 13.89	41.53 - 45.07 43.64	32.82 - 36.85 34.62	10.01 - 11.36 10.71	10.71 - 13.90 12.38	33.60 - 38.65 37.25	37.43 - 45.41 39.67	6.98 - 10.78 8.42	8.72 - 10.67 9.52	22.35 - 36.59 31.37	44.86 - 56.50 50.69
	SD CV (%)	0.40 5.09	0.86 6.19	1.07 2.45	1.44 4.16	0.47 4.39	1.18 9.53	1.71 4.59	2.64 6.65	1.31 15.56	0.73 7.67	4.84 15.43	4.11 8.11

*RC – relative concentration, Xmin – minimum value, Xmax – maximum valuet, Xsr – average value, SD - standard deviation, CV (%) – coefficient of variation

Different concentrations of ethanol 30%, 40% and 50% (v/v) have been used for gliadin extraction. During the gliadin proteins extractions with 30% (v/v) ethanol and at a column temperature of 40 ^{0}C, the number of detected proteins ranged from 18 - 21 (Xsr = 20, SD = 0.94, CV = 4.70%) (Table 1). By increasing the concentration of ethanol to 40% and 50% (v/v) and under the same separation conditions, the total number of detected proteins ranged from 20 - 21 (Xsr = 21, SD = 0.47, CV = 2.24%), respectively from 23 - 25 (Xsr = 24, SD = 0.58, and CV = 2.42%). The largest number of proteins has been detected by extraction of 50% (v/v) ethanol and has amounted to 24, and the smallest during extraction with 30% (v/v) ethanol and has amounted to 20. With an increasing ethanol concentration, there was an increase in the number of extracted proteins.

After extraction gliadin proteins with ethanol (30%, 40%, 50% v/v) and chromatographic separation, the following fractions have been detected: ω_5, $\omega_{1,2}$, α+β and γ gliadins. By extracting the gliadin proteins with 30% (v/v) ethanol, the number of detected proteins within the ω_5 gliadin fraction ranged from 5 - 6 (Xsr = 5, SD = 0.37, CV = 7.40%). During the extraction of gliadin proteins with 40% (v/v) ethanol the number of proteins within this fraction ranged from 4 - 6 (Xsr = 5, SD = 0.69, CV = 13.80%). The number of detected proteins after extraction with 50% (v/v) ethanol, within the ω_5 gliadin fractions ranged from 6 - 6 (Xsr = 6, SD = 0.00, and CV = 0.00%). The next protein fraction that is detected is the fraction $\omega_{1,2}$ gliadins. By extraction of gliadin with 30% (v/v) ethanol, the number of proteins within this fraction ranged from 2 - 4 (Xsr = 3, SD = 0.39, CV = 13.00%), and by extraction with 40% (v/v) ethanol 3 - 4 (Xsr = 3, SD = 0.37, CV = 12.33%). The number of proteins within this fraction extracted with 50% (v/v) ethanol ranged from 2 - 3 (Xsr = 2, and SD = 0.37, CV = 18.50%). After extraction gliadin proteins with 30% (v/v) ethanol, the number of detected proteins within the fraction α+β gliadins ranged from 2 - 4 (Xsr = 3, SD = 0.74, CV = 24.67%), and by extraction with 40% (v/v) ethanol from 4 - 5 (Xsr = 4, SD = 0.37, CV = 9.25%). The number of detected proteins within α+β gliadins, extracted with 50% (v/v) ethanol ranged from 6 - 7 (Xsr = 7, SD = 0.37, CV = 5.29%). The number of proteins detected within the γ gliadins fraction during extraction with 30% (v/v) ethanol ranged from 8 - 10 (Xsr = 9, SD = 0.58, CV = 6.44%), and by extraction with 40% (v/v) ethanol from 7 - 9 (Xsr = 9, SD = 0.76, CV = 8.44%). Within the fraction of γ gliadins, the number of proteins that appeared on the chromatogram when the extraction was performed with 50% (v/v) ethanol ranged from 8 - 10 (Xsr = 9, SD = 0.58 and CV = 6.44%).

Gliadin proteins separation by RP-HLPC chromatography has been carried out at three column temperatures: 40 ^{0}C, 45 ^{0}C and 50 ^{0}C, regardless of the solvent concentration during the extraction of gliadin from the flour. The number of detected proteins during the extraction gliadin proteins with 30% (v/v) ethanol and at a column temperature of 45 ^{0}C ranged from 15 - 17 (Xsr = 17, SD = 0.76, CV = 4.47%), and by extraction with 40% (v/v) ethanol and at this column temperature ranged from 24 - 26 proteins (Xsr = 25, SD = 0.69, CV = 2.76%). When the protein was separated at a column temperature of 45 ^{0}C, the number of detected proteins extracted with 50% (v/v) ethanol ranged from 25 - 28 (Xsr = 26, SD = 1.15, CV = 4.42%). By increasing the solvent concentration and at a column temperature of 45 ^{0}C, the number of detected proteins has increased.

Within the ω_5 gliadin fractions, the number of detected proteins after extraction with 30% (v/v) ethanol and at a column temperature of 45 ^{0}C ranged from 4 - 5 (Xsr = 5, SD = 0.47, CV = 9.40%), by extraction with 40% (v/v) ethanol ranged from 7 - 7 (Xsr = 7, SD = 0.00, CV = 0.00%) and after extraction with 50% (v/v) ethanol ranged from 7 - 9 (Xsr = 8, SD = 0.69, CV = 8.62%). When gliadin proteins were extracted with 30% (v/v) ethanol within the fraction $\omega_{1,2}$ gliadins and at a column temperature of 45 ^{0}C, the number of proteins ranged from 3 - 3 (Xsr = 3, SD = 0.00, CV = 0.00%), by extraction with 40% (v/v) ethanol from 4 - 5 proteins (Xsr = 5, SD = 0.50, CV = 10.00%) and by extraction with 50% (v/v) ethanol from 3 - 3 (Xsr = 3, SD = 0.00, CV = 0.00%). Within the α+β gliadin fractions, the number of proteins extracted with 30% (v/v) ethanol and at a column temperature of 45 ^{0}C ranged from 1 - 3 (Xsr = 3, SD = 0.76, CV = 25.33%), by extraction with 40% (v/v) ethanol and the same separation conditions the number of detected proteins ranged from 5 - 6 (Xsr = 5, SD = 0.50, CV = 10.00%). By extraction proteins with 50% (v/v) ethanol, the number of detected proteins ranged from 6 - 9 (Xsr = 7, SD = 1.11, CV = 15.86%). During the gliadin proteins extraction with 30% (v/v) ethanol and at a column temperature of 45 ^{0}C, the number of detected proteins within γ gliadins ranged from 6 - 7 (Xsr = 6, SD = 0.37, CV = 6.17%), by extraction with 40% (v/v) ethanol from 7 - 8 (Xsr = 7, SD = 0.37, CV = 5.29%), and by extraction with 50% (v/v) ethanol from 8 - 9 (Xsr = 8, SD = 0.37, CV = 4.62%).

During the extraction of gliadin proteins with 30% (v/v) ethanol and at a column temperature of 50 ^{0}C, the total number of detected proteins ranged from 18 - 21 (Xsr = 20, SD = 0.96, CV = 4.80%), by extraction with 40% (v/v) from 21 - 25 (Xsr = 24, SD = 1.37, CV = 5.71%) and by extraction with 50% (v/v) ethanol from 21 - 27 (Xsr = 24, SD = 2.27, CV = 9.46%). By increasing the solvent concentration for the extraction of gliadin proteins and at a column temperature of 50 ^{0}C the number of proteins increased, and then decreased. By analyzing the gliadin proteins, the following gliadin fractions have been detected: ω_5, $\omega_{1,2}$, α+β and γ gliadins. During the extraction of the gliadin proteins with 30% (v/v) ethanol and at a column temperature of 50^0 C, the number

of detected proteins within ω_5 gliadins ranged from 4 - 4 (Xsr = 4, SD = 0.00, CV = 0.00%) and by extraction with 40% (v/v) ethanol from 4 - 4 (Xsr = 4, SD = 0.00, CV = 0.00%). The number of detected proteins within ω_5 gliadins extracted with 50% (v/v) ethanol and at a column temperature of 50 ^{0}C ranged from 7 - 7 (Xsr = 7, SD = 0.00, CV = 0.00%). The next gliadin proteins fractions analyzed were $\omega_{1,2}$ gliadins. By extracting the gliadin proteins with 30% (v/v) ethanol, the number of detected proteins within this fraction ranged from 4 - 6 (Xsr = 5, SD = 0.90, CV = 18.00%) and by extraction with 40% (v/v) ethanol from 3 - 4 (Xsr = 4, SD = 0.50, CV = 12.50%). The number of extracted proteins with 50% (v/v) ethanol and at a column temperature of 50 ^{0}C ranged from 2 - 3 (Xsr = 3, SD = 0.37, CV = 12.33%). During the extraction of gliadin proteins with 30% (v/v) ethanol, the number of detected proteins within α+β gliadins ranged from 3 - 4 proteins (Xsr = 4, SD = 0.47, CV = 11.75%). By extraction the gliadin proteins with 40% (v/v) ethanol, the number of detected proteins ranged from 2 - 4 (Xsr = 3, SD = 0.74, CV = 24.67%) and by extraction with 50% (v/v) ethanol the number of proteins ranged from 4 - 7 (Xsr = 5, SD = 0.94, CV = 18.80%). The number of proteins detected within γ gliadin ranged from 7 - 7 (Xsr = 7, SD = 0.00, CV = 0.00%) - extraction with 30% (v/v) ethanol, from 11 - 14 (Xsr = 13, SD = 1.07, CV = 8.23%) - extraction with 40% (v/v) ethanol, from 7 - 10 (Xsr = 9, SD = 1.37, CV = 15.22%) - extraction with 50% v/v) ethanol.

Results obtained in this study are in agreement with data published by other researchers. Horvat *et al.,* [22], after separating gluten proteins by RP-HPLC chromatography (column C18, column temperature during separation 50 ^{0}C, separation time of 30 minutes), detected a total of 28 proteins, 1 - 3 proteins within ω_5 gliadin, 3 - 6 proteins within $\omega_{1,2}$, 8 - 10 proteins of α and 6 - 9 proteins within γ gliadins. Bietz *et al.,* [23], optimized the conditions for the gliadin extraction (solvent: 70% v/v ethanol and 55% v/v isopropanol) by HPLC method. After chromatographic separation, almost identical chromatograms were obtained. The results obtained in this paper show that the number of detected and separated proteins is the highest (26) and the best when gliadin extraction was performed with 50% (v/v) ethanol and at a column temperature of 45 ^{0}C.

Table 2 shows the data for the relative concentration of proteins classified in four gliadin fractions. By extracting the gliadin proteins using ethanol (30%, 40%, 50% v/v), the following fractions have been detected: ω_5, $\omega_{1,2}$, α+β and γ gliadins.

By extraction the gliadin proteins with 30% (v/v) ethanol, it was found that the number of proteins within ω_5 gliadin ranged from 5 - 6. Their relative concentration ranged from 13.75 to 15.09% (Xsr = 14.43%, SD = 0.52, CV = 3.60%). The relative concentration of the protein within this protein fractions after extraction with 40% (v/v) ethanol was 9.66 - 11.72% (Xsr = 10.24%, SD = 0.70, CV = 6.84%), and after extraction with 50% (v/v) ethanol, from 7.41 - 8.58% (Xsr = 7.85%, SD = 0.40, CV = 5.09%). The next protein fraction detected was the fraction $\omega_{1,2}$ gliadins. The relative protein concentration within the protein fraction of $\omega_{1,2}$ gliadins, during the extraction with 30% (v/v) ethanol ranged from 6.51 - 7.44% (Xsr = 6.80%, SD = 0.30, CV = 4,41%), by extraction with 40% (v/v) ethanol from 8.20 - 11.73% (Xsr = 9.67%, SD = 1.46, CV = 15.01%), and during the extraction with 50% (v/v) ethanol from 12.81 - 15.05% (Xsr = 13.89%, SD = 0.86, CV = 6.19%). The relative protein concentration α+β gliadins fraction depends on the solvent concentration. The relative protein concentration within this fraction during the extraction with 30% (v/v) ethanol ranged from 44.16 - 45.92% (Xsr = 45.12, SD = 0.59, CV = 1.31%), during the extraction with 40% (v/v) ethanol from 34.45 - 42.90% (Xsr = 37.81%, SD = 2.69, CV = 7.11%), and by extraction with 50% (v/v) ethanol from 41.53 - 45.07% (Xsr = 43.64, SD = 1.07, CV = 2.45%). The relative concentration of γ gliadins extracted with 30% (v/v) ethanol ranged from 32.21 to 35.31% (Xsr = 33.62%, SD = 1.01, CV = 3.00%), by extraction with 40% (v/v) ethanol from 38.86 - 44.73% (Xsr = 42.28%, SD = 2.23, CV = 5.27%) and by extraction with 50% (v/v) ethanol from 32.82 - 36.85% (Xsr = 34.62%, SD = 1.44, CV = 4.16%).

While analyzing the column temperature influence (40 ^{0}C, 45 ^{0}C and 50 ^{0}C) during the separation of the gliadin proteins by RP-HLPC chromatography on the relative concentration of gliadin protein fractions, the data shown in Table 2.

The relative protein concentration within the ω_5 gliadin fractions, extracted with 30% (v/v), 40% (v/v) and 50% (v/v) ethanol and at a column temperature of 45 ^{0}C ranged from 16.79 - 18.34% (Xsr = 17.62%, SD = 0.47, CV = 2.67%), from 12.06 - 13.74% (Xsr = 12.81%, SD = 0.58, CV = 4.53%) and from 10.01 - 11.36% (Xsr = 10.71%, SD = 0.47, CV = 4.39%) respectively. The relative concentration $\omega_{1,2}$ gliadin fractions during the extraction with 30%, 40% and 50% (v/v) ethanol, at a column temperature of 45 ^{0}C, ranged from 9.89 - 14.48% (Xsr = 12,11%, SD = 1.39, CV = 11.48%), from 12.63 - 18.05% (Xsr = 15.20%, SD = 2.09, CV = 13.75%) and from 10.71 - 13.90% (Xsr = 12.38%, SD = 1.18, CV = 9.53%) respectively. The relative concentration α+β gliadin proteins extracted with 30% (v/v) ethanol and at a separation temperature of 45 ^{0}C ranged from 39.61 to 41.60% (Xsr = 40.47%, SD = 0.59 , CV = 1.46%), and proteins extracted using 40% (v/v) and 50% (v/v) ethanol and at the same temperature from 28.30 - 36.00% (Xsr = 33.71%, SD = 2.57, CV = 7.62%) and from 33.63 - 38.65% (Xsr = 37.25%, SD = 1.71, CV = 4.59%) respectively. When the gliadin proteins extracted with 30% (v/v) ethanol and at a column temperature of 45 ^{0}C, the relative concentration of γ gliadin extracted

with 30% (v/v) ethanol ranged from 27.18 - 30.82% (Xsr = 29.79%, SD = 1.21, CV = 4.06%), extracted with 40% (v/v) ethanol from 34.43 - 41.32% (Xsr = 38.28%, SD = 2.30, CV = 6,01%) and extracted with 50% (v/v) ethanol from 37.43 - 45.41% (Xsr = 39.67%, SD = 2.64, CV = 6.65%).

When the gliadin proteins separation was performed at a column temperature of 50 ^{0}C, the relative concentration of the proteins classified into the ω_5 gliadin fractions had the following values: extracted with 30% (v/v) ethanol from 19.60 - 23.12% (Xsr = 21.21%, SD = 1.37, CV = 6.46%), extracted with 40% (v/v) ethanol from 23.34 - 29.70% (Xsr = 25.60%, SD = 2.44, CV = 9.53%) and extracted with 50% (v/v) ethanol from 6.98 - 10.78% (Xsr = 8.42%, SD = 1.31, CV = 15.56%). The relative concentration of $\omega_{1,2}$ gliadins obtained after extraction with 30% (v/v) ethanol was 12.54 - 15.01% (Xsr = 13.91%, SD = 0.83, CV = 5.97%), from 5.75 - 8.38% (Xsr = 6.78%, SD = 0.84, CV = 12.24%) - extraction with 40% (v/v) ethanol and from 8.72 - 10.67% (Xsr = 9.52%, SD = 0.73, CV = 7.67%) - extraction with 50% (v/v) by ethanol. The relative concentration of α+β gliadins extracted with 30% (v/v) ethanol was 27.17 - 28.44% (Xsr = 27.95%, SD = 0.51, CV = 1.82%). By extraction of the gliadin proteins with 40% (v/v) ethanol relative concentration was 30.24 - 34.56% (Xsr = 32.77%, SD = 1.46, CV = 4.45%) and by extraction with 50% (v/v) ethanol was 22.35 - 36.59% (Xsr = 31.37%, SD = 4.84, CV = 15.43%). The relative concentration of the γ gliadin fractions obtained after the extraction samples with 30%, 40% and 50% (v/v) ethanol ranged from 34.57 - 39.18% (Xsr = 36.93%, SD = 1.51, CV = 4.09%), from 31.60 - 37.28% (Xsr = 34.84%, SD = 1.89, CV=5.42%), and from 44.86 - 56.50% (Xsr = 50.69%, SD = 4.11, CV = 8.11%) respectively. The results obtained in this study show that the relative concentration of ω_5 gliadins when gliadin was extracted with 50% (v/v) ethanol and at a column temperature of 45 ^{0}C was 10.71%, $\omega_{1,2}$ gliadins 12.38%, α+β gliadins 37.25% and γ gliadins 39.67%. These results are in agreement with the findings of other authors.

4. Conclusions

- The aim of this paper was to determine the influence of the used solvent concentration - ethanol (30%, 40% and 50% v/v) on the extraction of gliadin proteins and the influence of the column temperature (40 ^{0}C, 45 ^{0}C and 50 ^{0}C) during the chromatographic separation of proteins.

- An increase ethanol concentration (30%, 40% and 50% v/v) affects the increase in the number of separated peaks on the chromatogram and the number of detected proteins. The best results were obtained after extraction of the protein with an ethanol concentration of 50% (v/v). On that occasion, a total of 26 proteins were detected.

- The temperature during the chromatographic separation (40 ^{0}C, 45 ^{0}C and 50 ^{0}C) affects the number of obtained peaks and the number of identified proteins. During the extraction of protein from wheat flour with ethanol concentration of 50% (v/v), the best results were obtained when the separation was carried out at 45 ^{0}C.

- By extraction gliadin proteins with 50% (v/v) ethanol, and at a column temperature of 45 ^{0}C within ω_5 gliadins 8 proteins (10.71%) is detected, within $\omega_{1,2}$ 3 (12,38%) proteins, within α+β gliadins 7 proteins (37.25%) and within γ gliadins 8 proteins (39.67%).

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