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COMPARATIVE EXTERIOR MEASURES OF PRAMENKA SHEEP RAISED IN THREE LOCALITIES IN BOSNIA AND HERZEGOVINA

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

The aim of the research was to measure the basic external characteristics of Pramenka sheep (ridge height, from the ground to the highest ridge point, lower back height, from the ground to the highest lower back point, hull length, chest width, chest depth, chest circumference, hull circumference, shin circumference, body weight) with the aim of comparing the measured values in order to assess the impact of breeding areas on them. Domestic kind Pramenka (Kupres strain, Vlašić strain) were used in this research. There were 36 sheep in the experimental group, and the same number in the control group. Experiments were performed on long-term purebred herds of Pramenka on three private farms in the Una-Sana Canton, 2 municipalities of Cazin, 1 municipality of Bihać, as well as on one private farm in Central Bosnia (area of the municipality of Travnik), and one in the municipality of Kupres (Livno Canton). Based on the presented average values of the external properties of Pramenka sheep and their variations for all examined localities, we can conclude the following: that the sheep are longer in relation to their height and that the Pramenka is of medium physical development, that the differences in body measures in the examined areas are greatly influenced by the origin of certain breeds of Pramenka sheep (Kupres strain, Vlašić strain), as well as the quality of pastures and unequal access to food. By comparing our results with the results of other authors who examined the exterior of other strains of Pramenka (from region in Croatia: Rab, Lika, Pag, Istria) in our wider environment concluded that Vlašić Pramenka is the largest strain of Pramenka in this area.

KEYWORDS: sheep, exterior, measures

INTRODUCTION

Sheep farming in Bosnia and Herzegovina, along with cattle breeding, is one of the main branches of livestock production. In 1990 in Bosnia and Herzegovina, the number of sheep was about 1.3 million, and in 1960 it was even larger and amounted to about 2.2 million. This indicates the fact of a significant decline in 30 years, even by 41%. In the last period the number of sheep suffered even bigger fall, and since the list has not been made for a long time, it is not possible to say with certainty how many sheep there are today in BiH.

The characteristics of sheep breeding in developed countries is that with intensification of agriculture, the number decreases, but therefore increases production. Although the number of sheep in BiH has decreased, however, there was no significant increase in production.

From the earliest times natural meadows have been most successfully exploited with sheep. They

are together the greatest extent absolute sources of food for sheep, in relation to other species of domestic animals. That is why hill and mountain areas, economically speaking, can be most successfully used by sheep breeding. The existing population of sheep in BiH in a high percentage is Pramenka with a large number of strains (around 80%), and in smaller number are represented by various types of crossbreeds (around 20%). Production capabilities of domestic Pramenka are low, which implies a small body weight of adults, low production of meat and milk, and low yield of unwashed coarse wool. Some strains make even the tiniest sheep in the world, such as let's say, Pramenka from the area of Stolac in Herzegovina.

Exterior presents external appearance, that is, development and connection of individual parts of the body, then the size of the whole body and its overgrowth and the color of the wool in sheep, as well as everything else that can be seen on them and can be measured and evaluated (horns, hooves, etc.). Exterior

assessment is of particular point when it comes to breeding choice [1]. Nikolić et al. [2] a good assessment of the exterior is considered to be a powerful means which a reliable picture of their breeding value can be created. The exterior of domestic animals can be assessed in three ways, and those are: by measuring the development of individual parts of the body and determining the body measurements of the animal with the help of special accessories for this purpose, by assessing the appearance of the said parts by photographing the animals. The animals should stand on a flat and firm surface during the measurement. Body dimensions are taken in the following order: height, length, depth, width and volume. The measurement is performed on the left side of the animal. The measurement of domestic animals is of special importance because only by measuring can reliable and accurate data on the development and relations of individual parts of the body and the animal as a whole be established. The data obtained by measurements are entered in the appropriate registers. Based on the data obtained by measuring animals, it is possible to monitor their growth from birth to full physical development.

A large number of authors have investigated the exterior characteristic of some strains of Pramenka and crossbreed. The works of following authors stand out in particular: [3], [4], [5]. Pramenka as a breed makes up about 80% of the sheep stock in BiH. It is grown in hilly mountain areas, where food is not available in winter. In many areas it finds itself in search of food as a nomadic animal. It is characterized by high resistance to disease. It is suitable for long distance and good use of lawns. It belongs to the group of long-tailed Balkan Pramenka. There are large number of strain that differ from each other in age and production characteristics, which is the result of action of existing environmental factors. The division into strains was performed according to the area of cultivation and variability at external and production characteristics. The most famous strains are: from Vlašić region (Dupska, Travnik), Kupres strain, Privor strain, Duvanjski strain, Glamoč strain, Gatac strain, Ključ strain, Stolac strain, Podvelež strain and others [3]. The hull is rectangular in shape, with length of the hull being greater in relation to the height of ridge. The height of the ridge in sheep ranges on average from 63.5-66.9, and length of the hull from 67.7-69.8 cm. The depth of the chest is 28.4-36.5 cm, the width of the chest is 18.8-23.5 cm, while the circumference of the chest is 67.0-94.5 cm. The body weight of sheep ranges from 20.0-50.0 kg, and rams up to 70.0 kg, noting that the upper limits of variability may be higher [6], [7], [8], [9], [10], [11], [12], [13].

MATERIALS AND METHODS

The paper presents the results of measurements and analysis of exterior properties. Domestic strains of Pramenka (Kupres, Travnik) were used for research. There were 36 sheep in the group of experimental animals. The experiments were performed on long-term purebred herds of Pramenka on three private farms in Una-Sana Canton, two in the municipality of Cazin (Mutnik I i Mutnik II), one in the municipality of Bihać (Gata), as well as one on private farm in Central Bosnia (Travnik municipality) and one in Kupres municipality (Livno Canton). The results of measurements and analysis of external properties of sheep of the examined breed using standard methods and methods of measurements are presented. Ecological factors together with the hereditary characteristics of the animal shape the types or breeds suitable for the existing environmental conditions. Therefore, judging the exterior is the oldest way to assess breeding value. Every trait we want to select must be measurable.

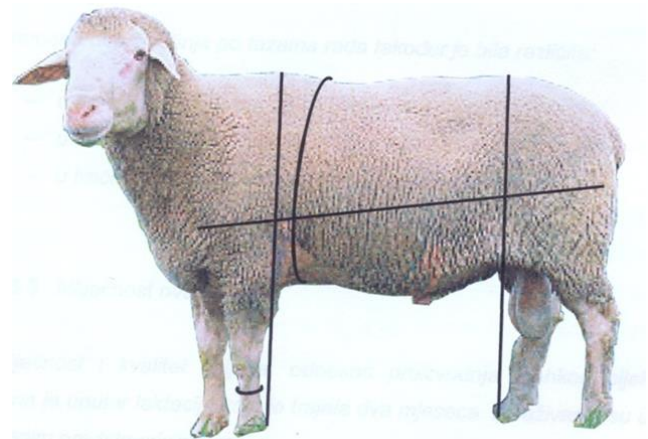


Figure 1. Marked places where body measurements are taken in sheep

For the development of the breeding program it is especially important to know the phenotypic and genotypic parameters of each trait, and especially those traits that are inherited. We try to use them as heterosis effect- since the body structure represents a certain form of its biological mass, so there is a certain connection between it and production characteristics. To assess the body of an animal, each part of the body is assessed. In this paper, measurements of individual body parts were performed to assess the exterior using a Lydthin rod and tape. A total of 180 heads were measured at the mentioned 5 locations. The following measurements were made:

- Height of the ridge, from the ground to the highest point.

- Trunk length, oblique distance from another edge of the shoulder-scapular joint to the posterior point of the static bone.
- Chest width, measured just behind the shoulder blade.
- Chest depth, vertical distance from the top of the ridge to the lower edge of the sternum.
- The circumference of the breasts, the circumference of the carcass behind the shoulder blades (tape), the circumference of the shin, measured at the thinnest point of the shin (tape).
- Body weight, weighing the body of the animal with a livestock scale.

RESULTS AND DISCUSSION

Table 1. Values of exterior measures of examined sheep from 5 localities.

Locality	Body weight (kg)	Shin circumference (cm)	Chest width (cm)	Chest circumference (cm)	Chest depth (cm)	Chest length (cm)	Cross height (cm)	Reef height (cm)
Gata	65,27	8,47	22,73	90,60	36,88	71,63	67,86	67,89
Mutnik I	65,44	9,16	24,77	93,20	39,67	74,98	70,26	70,63
Mutnik II	66,25	9,99	26,88	95,19	43,26	78,91	73,21	73,74
Vlašić	69,52	10,40	29,55	98,49	44,53	80,99	75,31	75,72
Kupres	57,95	8,27	21,62	86,43	33,74	69,39	69,39	64,56

Locality Based on the presented average values and their conclusion that the sheep are longer in relation to their height. We also conclude that Pramenka is of medium physical development, but it is more developed than most Mediterranean sheep breeds, but also physically inferior in relation to meat and meat - wool sheep breeds. If we compare these results with the results of about 30 years ago [6], we can see a larger body frame of today's breeds Pramenka expressed through all established body measures (height, length, depth, circumference and body weight). This can be attributed to the genetic influence of larger genotypes and better nutritional conditions in the examined areas.

Based on the presented results in the mentioned areas of research, we can conclude that the differences in body measures in the examined areas are greatly influenced by the origin of certain breeds - strains of sheep breed Pramenka (Kupres strain, Vlašić strain) as well as the quality of pastures and unequal access to food.

In particular, we can notice the difference between the examined areas of Kupres and Vlašić where it is observed that sheep from the area of Kupres are lower than other sheep from other areas of research, as well as that they are shorter and less body weight. Pavić et al. [14] state similarly. However, if we compare the obtained results of the examined sheep with sheep of Croatian sheep breeds, we conclude that ours are much more developed with an emphasis on differences in

breast width (about 30%) and body weight (about 20%), and this is especially reflected in the works [15], [16], [9], [17], [12], [18], [19], [13]. However, based on the obtained results, our pramenka from Kupres has a significantly smaller body frame than the Croatian Istrian sheep [20], [21]. Similar results to our research are given by Omanović [8] and Ivanković et al. [22] when it comes to the exterior of the Kupres pramenka.

When it comes to sheep of the Vlašić strain pramenka, the results of our research in comparison with the research of other authors [23] show small deviations in the following body measures: ridge height (76, 92 cm [23]; 76.42 cm [13]), body length (84.69 cm: 81.65 cm), shin circumference (10.34 cm: 10.44 cm) and body weight (74.89 kg: 70.01 kg). By comparing the results of Pavić et al. [14] with our results of research of the exterior of Travnik pramenka we come to the conclusion that our results differ significantly, which is confirmed by the following values of body measures: sheep body weight 70.74 kg, ridge height 66.76 cm, body length 73.86 cm, shin circumference 8.68 cm.

By comparing our results with the results of other authors who examined the exterior of other strains of pramenka (from region in Croatia: Rab, Lika, Pag, Istria) in our wider environment, we conclude that Vlašić pramenka is the largest strain of pramenka in this area.

Table 2. Values of exterior measures of examined rams from 5 localities.

Locality	Body weight (kg)	Shin circumference (cm)	Chest width (cm)	Chest circumference (cm)	Chest depth (cm)	Chest length (cm)	Cross height (cm)	Reef height (cm)
Gata	74,30	10,32	33,11	101,32	47,41	84,16	78,76	79,14
Mutnik I	73,82	10,42	32,96	101,21	47,36	84,09	78,56	79,00
Mutnik II	79,84	11,16	35,80	105,08	50,96	87,04	81,54	81,90
Vlašić	78,35	10,68	33,62	102,72	48,36	86,42	79,16	79,58
Kupres	79,86	9,54	27,04	96,14	41,96	78,18	72,66	73,12

Based on the presented average values and their variations for all localities, we can conclude that the rams are longer in relation to their height. We also conclude that rams of the Pramenka breed are, as a rule, larger in body frame and have a stronger constitution than sheep, which was confirmed by this research.

Comparing the body measurements between sheep and rams of the Pramenka breed by research sites, we can conclude the following: at the Gata site in the municipality of Bihać, rams are 13% taller than sheep, 13.5% longer, 27.6% wider and 12% taller. %; at the locality Mutnik I in the municipality of Cazin rams are higher by 10%, longer by 10.5%, wider by 23% and higher body weight by 10.3%; at the locality Vlašić rams are higher by 4%, longer by 3.5%, wider by 10.8% and higher body weight by 10.6%; at the locality Kupres rams are higher by 11.5%, longer by 11%, wider by 10.4% and significantly higher body weight by 20% and at the locality Mutnik II in the municipality of Cazin rams are higher by 9.7%, longer by 9.1 %, wider by 24% and higher body weight by 16.8%. Similar results are stated by Ivanković et al. , [22], where the rams of Kupres pramenka were 8.5% taller than the sheep, 10% longer, 14.8% wider and 36% taller.

The rams of our domestic pramenka, which were measured, compared to others of the same or different breeds have a significantly larger body frame, are longer and have higher body weights, such as Indian Marwari rams [24], Tibetan rams [25], rams of Lika pramenka [18], rams of Dubrovnik ore [15], Krk sheep [16], Pag sheep [9], Cres sheep [17], Rab sheep [12] and Dalmatian pramenka [19]. However, we can conclude that the rams of Kupres pramenka are significantly less developed than the rams of Travnik pramenka, which is confirmed by the results of other authors [14], [23]. Also, the rams of the Kupres pramenka are less developed than the rams of the Istrian sheep [20] and the rams of the cigaja [21].

In the examinations, Omanović [8], states similar results for the Kupres pramenka, but significantly

different results for the exterior of the Merinolandschaf (Württemberg) rams. The rams of the Wirtmeberg sheep are significantly more developed than the rams of our domestic pramenka, as follows: the height of the crest averages 83.00 cm, the height of the cross 83.25 cm, body length 85.75 cm, chest depth 35.94 cm, chest circumference 113.25 cm, chest width 27.37 cm, shin circumference 10.96 cm and body weight 116.56 kg.

CONCLUSION

1. Based on the presented average values of external properties of Pramenka sheep and their variations for all examined localities, we can conclude the following:

- That sheep are longer in relation to their height and that Pramenka is of medium physical development.
- That the differences in body measures in the examined areas are greatly influenced by the origin of certain breeds - sheep strains of the Pramenka breed (Kupres strain, Vlašić strain), as well as the quality of pasture areas and unequal access to nutrition.
- By comparing our results with the results of other authors who examined the exterior of other strains of pramenka (from region in Croatia: Rab, Lika, Pag, Istria) in our wider environment, we conclude that Vlašić pramenka is the largest strain of pramenka in this area.

2. Based on the presented average values and their variations for all localities, we can conclude that rams are longer in relation to their height, and also, we conclude that rams of the Pramenka breed are generally larger in body frame and stronger constitution than sheep, which was confirmed by this study.

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BUCKWHEAT IN THE NUTRITION OF COCK LAYING AS A FACTOR OF EGG QUALITY

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

The subject of this paper is the research of the influence of different ratios of buckwheat, in concentrated feed, on the qualitative values of laying hen eggs. The study was conducted in four groups of laying hens: one control and three experimental, which were formed with respect to different proportions of buckwheat in meals. Within the first group of laying hens, a concentrated feed mixture with 10% relative share of buckwheat was used, within the second group with 20% relative share of buckwheat and within the third group with 30% relative share of buckwheat, while in the control group standard concentrated food was used. Based on the conducted research, it can be concluded that buckwheat in the meal of laying hens has positive effects on quality, physical properties, sensory properties and frequency of carrying. It was shown that there are statistically significant differences in mean values for the following variables: protein content in egg white, protein content in egg yolk (%), fat content in egg yolk (%), where the highest value was recorded in the third group of laying hens. Also, statistically significant differences were found in terms of shell weight (g), shell thickness (mm), yolk diameter (mm) and egg white pH. The highest average frequency of egg laying was found in the first group and the lowest average frequency of egg laying was in the control group. The general conclusion is that buckwheat can be used in poultry feed, because it has a much greater positive than negative effect on the production and quality characteristics of eggs for consumption.

KEYWORDS: Buckwheat, Laying hens, Quality, Eggs

INTRODUCTION

Chicken eggs are intended for human consumption or use in the food industry. Number of eggs produced in Bosnia and Herzegovina in the period 2006-2020 fell from 724 million to 673 million pieces [1]. The biological and nutritional value of eggs is irreplaceable. Eggs are an excellent source of nutrients, so one egg provides 4.5-6 g of protein, all essential amino acids in the right proportions, also the egg contains unsaturated fatty acids. Although eggs are high quality food, their nutritional value, ie their quality can vary significantly, which depends on a number of factors, such as hen genetics, way of keeping laying hens, storage conditions, age of laying hens, and especially on nutrition. The composition and quality of the meal affects the weight of the eggs, the quality of the shell, the internal quality of the eggs, their chemical composition, etc. Therefore, special attention should be paid to the composition and quantity of meals for laying hens.

MATERIALS AND METHODS

The research was conducted in 4 groups of laying hens: one control and three experimental, where experimental groups were formed on the basis of different proportions of buckwheat (10%, 20% and 30%) in concentrated food, and the control was fed a standard concentrated meal.

The qualitative properties of eggs, the content of Ca and Mg in the shell (%), the protein content in the egg white and the content of proteins, fats, ash and minerals (iron, phosphorus and potassium) in the yolk, and the physical properties of eggs were examined: weight, egg length, egg width, shell mass, yolk mass, yolk pH, egg white mass and pH.

The protein content of egg yolk and egg white was determined by the Kjeldahl method, which is based on the destruction of organic matter by heating with sulfuric acid, where all protein and non-protein nitrogen (except nitrate and nitrite) is converted into ammonium sulfate. The addition of sodium hydroxide releases ammonia, which is pre-distilled into a certain amount of boric acid, and the resulting sodium borate

is treated with a solution of 0.02 mol / l sulfuric acid. The amount of protein is expressed as a percentage.

The fat content of the yolk was determined by the Weibl and Stoldt procedure. The method is used to determine the fat in liquid, frozen and dried egg yolk and melange. The egg sample is hydrolyzed with hydrochloric acid, and the released fats are extracted with petroleum ether. The fat was separated again and the quantity was expressed as a percentage of the mass, calculated on the sample.

The ash content in the yolk was determined by a method based on drying and burning the samples at a certain temperature to a constant mass. The process of this method was performed by crushing, homogenizing and drying the sample. After that, the samples were burned annealed to a constant mass which was weighed. The amount of ash is expressed as a percentage.

The method for determining the metal content in the yolk and magnesium in the egg shell consists of two phases. In the first phase, the extraction of metals was performed using a gold ball (aqua regia), and in the second phase, their content in the extract was determined by atomic absorption spectrometry (AAS). The extraction of metals in the gold smelter was performed according to the ISO 11464 standard, which specifies the method of extraction with the gold smelter of trace elements in different products. According to the principle of this standard, the dried samples were then extracted with an acid mixture. The AAS flame technique method was used for this research. This method determined the content of metals and magnesium.

Testing of calcium in the shell was performed according to the method BAS EN ISO 13805: 2002, IDT. The sample was dried, homogenized and treated with nitric acid and hydrogen peroxide. The samples were heated in a microwave oven for digestion in two steps. Calcium content readings were performed on a flame photometer and on AAS.

The results of physical properties were obtained using measuring instruments for length and weight values. Thus, length measures of height, width, shell thickness, diameter and height of yolks and egg whites were performed with a digital movable scale (caliper) whose accuracy is 0.01 mm and are expressed in millimeters (mm), while weight measures such as mass whole egg, the mass of the contents (yolks and whites), the mass of the shell obtained by measuring with a digital technical scale to the nearest 0,01 g and expressed in grams (g).

The pH of egg yolks and egg whites was determined with a pH meter type HANNA HI 8424 with pre-adjustment (calibration) of the instrument by immersing the pH electrode in a buffer solution (neutral) whose pH value is 7. pH values of egg yolks and egg whites were read on the display.

For all observed characteristics, the data collected in the research were analyzed by the statistical program SPSS 17.0.

RESULTS AND DISCUSSION

The obtained results in terms of the tested properties are shown in the following tables.

Table 1. Protein content in egg whites and yolks

Proteins (%)	Group of laying hens			
	Control	I experimental	II experimental	III experimental
Egg white	11,11	10,26	10,54	11,35
Yolk	16,51	15,76	15,96	16,64

From the presented data it can be seen that egg whites contain less protein than egg yolks, and that the addition of buckwheat to the rations of laying hens was not important for the protein content of egg whites and egg yolks. According to Nemanic and Beric [2], the average protein content in egg white is 10.60, and in egg yolk 16.6%, which agrees with our results. The same authors state that the fat content in egg yolk is 32.6%, while our results are slightly lower, but it can be noticed that the addition of buckwheat to the meals

of laying hens increases the fat content in egg yolk, and that the differences between groups were statistically significant. Petersen [3] state that the ash content in egg yolk is 1.70%. Our results are somewhat higher, but there were no statistical significant differences between the control and experimental groups, ie the addition of buckwheat did not result in significant changes in the ash content in the yolk (Table 2).

Table 2. Fat and ash content in egg yolk

%	Group of laying hens			
	Control	I experimental	II experimental	III experimental
Fats	29,43	30,02	30,40	30,67
Ash	1,75	1,72	1,78	1,76

Table 3. shows the content of basic minerals in egg yolk (iron, phosphorus and potassium).

Table 3. Mineral content in egg yolk

Mineral (mg/kg)	Group of laying hens			
	Control	I experimental	II experimental	III experimental
Iron	57,83	47,70	60,29	49,42
Phosphorus	6480,32	6546,19	6760,77	7031,03
Potassium	964,16	772,76	835,65	900,79

The richness of buckwheat in minerals led to an increase in the mineral content in the yolk. Thus Peterson [3] according to Naber and Bergquist (1997) states that the iron content in egg yolk was 47.70 mg, and in our study it was significantly higher (60.29 mg/kg, in the experimental group II.) Delilovic [4] states that the potassium content is 448 mg / kg, while our results are significantly higher, which indicates that the addition of buckwheat has a significant effect.

Also, the literature data show that the phosphorus content in egg yolk is 5.080 mg/kg, while in our research it was significantly higher.

As for the quality of the egg shell, it was determined on the basis of the content of minerals, calcium and magnesium, and the results are shown in Table 4.

Table 4. Calcium and magnesium content in egg shell

	Group of laying hens			
	Control	I experimental	II experimental	III experimental
Ca (%)	38,09	36,74	38,03	38,28
Mg (mg/kg)	1.736,68	2.206,67	1.991,26	1.732,20

The addition of buckwheat to the rations of laying hens did not have a significant effect on the calcium content in the shell, while the addition of 10% buckwheat to the meals resulted in a significant increase in magnesium in the shell. Shen [5] states that the calcium content in the shell was 36.4% and magnesium 0.33%.

Investigating the existence of differences in average values between groups

After the descriptive-statistical parameters, we determined the existence of statistically significant differences in the mean values of the observed characteristics between the groups of laying hens. To select the appropriate test, we present below the results of the Kolmogorov-Smirnov test on whether the observed characteristics follow a normal distribution.

Table 5. Kolmogorov-Smirnovljevi test

Observed features	Kolmogorov-Smirnov Z	Significance (p)
Protein content in egg white (%)	0,622	0,834
Protein content in egg yolk (%)	0,518	0,951
Fat content in egg yolk (%)	0,527	0,944
Ash content in egg yolk (%)	0,661	0,775
Iron content in egg yolk (mg / kg)	0,884	0,415
Potassium content in egg yolk (mg / kg)	0,502	0,962
Phosphorus content in egg yolk (mg / kg)	0,825	0,504
Calcium content in shell (%)	1,541	0,017
Magnesium content in the shell (mg / kg)	0,809	0,530

The results of the mentioned test support the conclusion that all observed characteristics-variables listed in the table follow the normal distribution, except for the variable calcium content in the shell (%) which does not follow the normal distribution since $p < 0.05$ is the level of significance at which it is testing

performed. Below for the variables that follow the normal distribution, the results of the variance analysis of the F-test, which is used to test hypotheses about the difference of the means of three or more sets, are presented.

Table 6. Results of F-test variance analysis

Observed features	Control group	The first group	Another group	Third group	F	df1	df2	P
	$\mu \pm \sigma$	$\mu \pm \sigma$	$\mu \pm \sigma$	$\mu \pm \sigma$				
Protein content in egg white (%)	11,11 ± 0,67	10,26 ± 0,78	10,54 ± 0,22	11,35 ± 0,19	4,40	3	16	0,019
Protein content in egg yolk (%)	16,51 ± 0,10	15,76 ± 0,25	15,96 ± 0,29	16,64 ± 0,37	12,39	3	16	0,000
Fat content in egg yolk (%)	29,43 ± 0,87	30,02 ± 0,41	30,40 ± 0,52	30,67 ± 0,38	4,33	3	16	0,021
Ash content in egg yolk (%)	1,75 ± 0,03	1,72 ± 0,01	1,78 ± 0,05	1,76 ± 0,01	2,77	3	16	0,076
Iron content in egg yolk (mg / kg)	57,83 ± 2,62	47,70 ± 1,11	60,29 ± 2,20	49,42 ± 1,61	49,17	3	16	0,000
Potassium content in egg yolk (mg / kg)	964,16 ± 56,34	772,76 ± 13,98	835,65 ± 31,15	900,79 ± 28,61	26,41	3	16	0,000
Phosphorus content in egg yolk (mg / kg)	6.480,32 ± 306,42	6.546,19 ± 151,88	6.760,77 ± 406,84	7.031,03 ± 453,99	2,53	3	16	0,094
Magnesium content in the shell (mg / kg)	1.736,68 ± 72,92	2.206,67 ± 175,47	1.991,26 ± 154,70	1.732,20 ± 70,83	16,00	3	16	0,000

According to the results of the test on the difference of mean values of the observed characteristics between groups of laying hens, we conclude that there is a statistically significant difference of mean values between groups of laying hens in the following variables: protein content in egg white (%), protein content in egg yolk (%), egg yolk (%), where the highest average value was recorded in

the group of laying hens in whose diet a concentrate mixture with 30% buckwheat was used; yolk iron content (mg / kg) with the highest average in the second group of laying hens in whose diet a concentrate mixture with 20% buckwheat was used, yolk potassium content (mg / kg) where the highest percentage was recorded in the control group and shell magnesium content (mg / kg) where the highest

percentage was recorded in the first group of laying hens where a concentrate mixture with 10% buckwheat was used.

For a variable that does not follow the normal distribution, the results of the Kruskal Wallis test, ie

the test on the difference (equality) of arithmetic means of three or more sets, are presented below.

Table 7. Kruskal Wallis test

Variable	A group of respondents	Number of respondents	Rank middle	Kruskal Wallis test		
				Chi-Square	df	P
Calcium content in shell (%)	Control group	5	12,70	13,579	3	0,004
	The first group	5	3,00			
	Another group	5	10,00			
	Third group	5	16,30			
	Total	20				

According to the results in the table and the results of the Kruskal Wallis test, there is a statistically significant difference between the four groups of laying hens, with the variable calcium content in the shell (%), since $p < 0.05$. According to the rank values, the highest average value of calcium content in the shell is in the third group where a concentrate mixture with 30% buckwheat was used, then in the control,

second and lowest average values in the first group of laying hens where a concentrate mixture with 10% buckwheat was used.

Investigations of differences in physical characteristics among groups of coca carriers

Table 8. Physical properties of eggs

Characteristic	Group of laying hens			
	Control	I experimental	II experimental	III experimental
Egg mass (g)	63,05	63,45	63,73	64,27
Egg length (mm)	56,28	56,88	56,36	56,66
Egg width (mm)	44,52	44,49	44,77	45,06
Egg shell mass (g)	7,59	7,66	7,31	6,53

As for the weight of eggs from the presented results, it can be seen that it was approximate in all tested hens, which means that the addition of buckwheat to the meals of laying hens did not affect this property. Buckwheat had a positive effect on egg length. Supić et al. [6] state that the average length of eggs for consumption is about 57 mm, which agrees with our results.

The same authors [6] state that the average width of eggs for consumption is about 42 mm. Our results

were somewhat higher, but there was no difference within the examined groups, and it can be concluded that the addition of buckwheat did not affect this property. Supić et al. [6] state that the average weight of an eggshell is about 11 g. Our results were significantly lower, which indicates that the addition of buckwheat to meals has a negative effect on the weight of the shell, ie the share of buckwheat in the meal decreases.

Table 9. Mass and pH value of egg yolks and egg whites

Characteristic	Group of laying hens			
	Control	I experimental	II experimental	III experimental
Yolk mass (g)	14,34	14,29	14,87	16,61
Egg white mass (g)	40,14	40,34	40,46	40,05
pH of the yolk	6,41	6,44	6,38	6,32
pH of the egg white	8,58	8,69	8,77	8,83

Buckwheat has a positive effect on the mass of egg yolks. Perić and Birkhold [7] state that the pH of fresh eggs is around 7.5, and in our research the pH was significantly lower, which indicates that the addition

of buckwheat to the meals of laying hens leads to a decrease in the pH of eggs. Adding buckwheat to meals did not have a significant effect on egg white mass and pH.

Table 10. Difference of physical properties, statistical parameters

Observed features	Kolmogorov-Smirnov Z	Significance (p)
Egg mass (g)	0,611	0,850
Egg length (mm)	0,412	0,996
Egg width (mm)	0,966	0,308
Egg shell mass (g)	1,009	0,261
Yolk mass (g)	0,467	0,981
pH of the yolk	0,743	0,639
Egg white mass (g)	0,770	0,593
pH of the egg white	0,654	0,786

Since the p-values of all observed characteristics-variables are greater than 0.05 (5%) levels of significance, we conclude that each of the variables follows a normal distribution.

The following table shows the results of testing hypotheses on the difference of means between three or more sets using analysis of variance, ie F-test.

Table 11. Results of F-test variance analysis

Observed features	Control group	The first group	Another group	Third group	F	df1	df2	P
	$\mu \pm \sigma$	$\mu \pm \sigma$	$\mu \pm \sigma$	$\mu \pm \sigma$				
Egg mass (g)	63,05 ± 2,28	63,45 ± 2,95	63,73 ± 4,46	64,27 ± 4,23	0,203	3	36	0,894
Egg length (mm)	56,28 ± 1,35	56,88 ± 1,26	56,77 ± 2,28	56,40 ± 1,69	0,289	3	36	0,833
Egg width (mm)	44,52 ± 0,60	44,49 ± 0,88	44,77 ± 1,10	45,17 ± 1,15	1,088	3	36	0,367
Egg shell mass (g)	7,59 ± 0,40	7,66 ± 0,65	7,31 ± 0,64	6,53 ± 0,88	6,125	3	36	0,002
Yolk mass (g)	14,34 ± 0,89	14,29 ± 1,62	14,87 ± 1,05	16,61 ± 1,26	7,775	3	36	0,000
pH of the yolk	6,41 ± 0,19	6,44 ± 0,14	6,38 ± 0,10	6,32 ± 0,16	1,077	3	36	0,371
Egg white mass (g)	40,14 ± 2,42	40,34 ± 1,95	40,46 ± 4,61	40,05 ± 3,41	0,034	3	36	0,991
pH of the egg white	8,58 ± 0,14	8,69 ± 0,27	8,77 ± 0,14	8,83 ± 0,15	3,330	3	36	0,030

Based on the test results of hypotheses on the difference of mean values between groups of laying

hens, we conclude that there is a statistically significant difference of mean values between four

groups of laying hens in the following variables: shell mass (g) with the highest average value in the first group, yolk mass) with the highest average value in the third group and pH of the egg whites with the highest average values in the third group.

CONCLUSION

From the obtained results, it can be concluded that the addition of buckwheat to the meals of laying hens has a positive effect on a number of qualitative and quantitative properties of eggs (protein content, mineral content, egg mass, as well as egg shell quality). In addition, such meals are tastier, laying hens are happy to consume them, and there is less food breakdown, and thus less harmful impact on the environment.

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ELEMENTS OF ULTRASONIC FLOWMETER INSTALLATION, MODEL OMNI TDI 200H WITH TEST WORK RESULTS

PROFESSIONAL PAPER

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ABSTRACT:

Ultrasonic flowmeter works on the principle of transmitting and returning ultrasonic waves that pass through the liquid, ie. fluid, and is used to measure the speed of fluid movement through a pipeline or some other structural form of fluid movement. By knowing the velocity of the fluid and the cross-sectional area of the pipeline or other system, the value of the volumetric or mass flow can be obtained indirectly. Fluid flow measuring device model OMNI TDI 200H owned by the laboratory of the Biotechnical Faculty was used for the first time to measure the flow of purified water at the outlet of the plant device of the training center "Grmeč", built as a pilot plant. The paper will describe the role and importance of flow measurement by the conventional method and present the results of the trial operation of an ultrasonic flow meter, model OMNI TDI 200H.

KEYWORDS: ultrasonic meter, installation elements, flow

INTRODUCTION

Nowadays, due to the greater complexity of technical systems in the manufacturing and process industry, we perform measurements, and the act of measurement itself can be presented as an experimental process of obtaining one or more values of a measured quantity. The measurement aims to determine the value of the measured quantity, while the purpose of each measurement is to obtain the value of the measured quantity. By comparing the measurement results about the given quantities, we control the process quantities, for which we use many information and technical aids. Measurement of non-electric quantities by electrical measuring procedures is realized with the help of measuring transducers, which convert non-electric to electric quantities. Thus, e.g. measures displacement, velocity, angular velocity, acceleration, mechanical power, pressure, flow, fluid level, temperature, volume, time, humidity, flow rate and composition of gases and liquids, pH value, etc. Converting non-electrical quantities into electrical ones is a very important step in process control and regulation systems because in this way different physical quantities can be measured by one method or one type of instrument, very small changes of measured non-electric quantity can be displayed and measured, very fast changes of non-electric size and perform remote measurements (online).

FLOW AS A PHYSICAL SIZE

Flow measurement, as a variable basic quantity, determines the energy and material balances of process changes (flow chart) based on which the movement of materials and fluids at the inlet, through conversion operations, as well as at the outlet of the process is monitored. The measurement of the flow of fluids, gases, multiphase liquids, and suspensions is complex, subject to numerous errors, and therefore a large number of different measuring procedures have been developed for accurate and reliable measurement. Basic flow values are given through the following expressions [1]:

- volume / volume flow Q_V [m³/h], [l/s]
- mass flow Q_M [kg / s], [t/h], [kg / s]
- molar flow Q_m [mol/h], [mol/s]

Mass flow is the mass of a fluid that passes a point in a unit of time and can be given through the equation:

$$Q_M = \frac{\Delta m}{\Delta t}, [\text{kg/s}] \dots\dots\dots (1)$$

Otherwise, the mass flow in the pipe is determined by the limit of the ratio of the mass of the fluid flowing through the cross-section of the pipe in the time interval when the interval becomes infinitesimally small:

$$Q_M = \frac{dm}{dt}, [\text{kg/s}] \dots \dots \dots (2)$$

Where is:

Δm - change in mass flowing through a space (eg inside a pipe), [kg]

Δt - duration of fluid flow flowing through a cross-section, [s]

Ultrasonic waves with frequencies from 100 kHz (for gases) to 2 MHz (for liquids) are used to measure the volume flow. The relationship between frequency f , speed of sound c , and wavelength λ can be given by the equation [2]:

$$f = \frac{c}{\lambda}, [\text{Hz}] \dots \dots \dots (3)$$

Where is:

f - frequency, [Hz]

c - speed of sound, [m/s]

λ - wavelength, [m]

Because ultrasonic signals can also penetrate solid material, in addition to placing the transducer inside a pipeline or other object, they can be placed on the outside of the pipe offering completely non-contact ultrasonic measurement, eliminating chemical compatibility problems, pressure limitation, and pressure loss. Its functionality is not affected by (a) whether the measuring object is transparent or opaque, metallic or non-metallic, liquid, rigid or powdery, (b) external environmental influences. However, the quality of the device can be affected by the acoustic properties of fluids, changes in temperature, density, viscosity, porosity, and the content of suspended particles. One of the technological quantities that is important for the operation of the water purification system, and thus the plant device, is the volume flow through the pipe, based on the obtained speeds [3]. By knowing the velocity of the fluid and the cross-sectional area of the pipeline or other system, and the density of the fluid, it is possible to calculate both the volumetric flow and the mass flow mathematically. From the continuity equation, we calculate the volume flow through the pipe. Volume flow and mass flow are determined by the following equations:

$$Q_V = \frac{\Delta V}{\Delta t}, [\text{m}^3/\text{s}] \dots \dots \dots (4)$$

$$Q_M = \rho \cdot v_t \cdot A = \rho \cdot Q_V [\text{kg/s}] \dots \dots \dots (5)$$

Where are:

ΔV - change in volume / volume of fluid [m^3], flowing through a cross section (eg pipes)

Δt - duration of fluid flow flowing through a cross-section, [s]

ρ - fluid density, [kg/m^3]

v_t - velocity of fluid in the pipe, [m/s]

A - pipe cross section, [m^2]

Q_V - volume flow, [m^3/s]

ABOUT FLOW MEASURING DEVICE MODEL OMNI TDI 200H

GENERAL CHARACTERISTICS OF THE DEVICE

Today, there are several ultrasonic flow measurement methods in use, of which the following stand out: the transient time measurement method, the pulse method, the cross method, and the Doppler effect flow measurement method. A flow meter based on the passage of time uses two transducers that act as both an ultrasonic transmitter and a receiver. A measuring transducer is a technical element that converts the output size of a sensor into an analog physical quantity suitable for transmission and visual display, while the sensor is a technical element that directly receives and registers changes in the process and gives a response at its output that is analogous to the input physical measured quantity.

Ultrasonic meters are used to measure the flow of various suspensions, fluids with added air, and fluids with little or a lot of suspended particles. They can be used in measurements related to sewerage infrastructure, activated sludge, groundwater, chemical suspensions, as well as for fluids containing particles of solid material, which is the case with sanitary wastewater of the training center "Grmeč". Ultrasonic flowmeter works on the principle of transmitting and returning ultrasonic waves that pass through the liquid, ie. fluid, and is used to measure the velocity of movement v_t [m/s]. These devices are also the most popular devices that measure the flow inside the pipeline, used in pipelines with a diameter of 2 to 300 [cm], and for flow rates from 1 to 10 [m/s]. Ultrasound is produced by piezoelectric crystals. Piezo elements send and receive sound (range from 40 to 300kHz). The speed of ultrasound c is different in some media.

For example, in water it is 1500 [m/s], in metals 3000 - 6000 [m/s], in glass 5500 [m/s], in styrofoam only 500 [m/s]. This is taken into account in the sensor design phase because the sensor is built for a specific purpose. When there is no flow, the travel time ($Q_V=0$) upstream and downstream is the same ($\Delta t=0$), ie. the frequencies of the ultrasonic wave sent to the pipe and its reflection is the same. When we have a flow ($Q_V>0$), the upstream waves will travel more slowly and will need more time than the downstream wave,

ie. the frequencies and times of the transmitted and reflected waves are different ($\Delta t > 0$) (Figure 1). As the

fluid moves faster, the difference between upstream and downstream time increases.

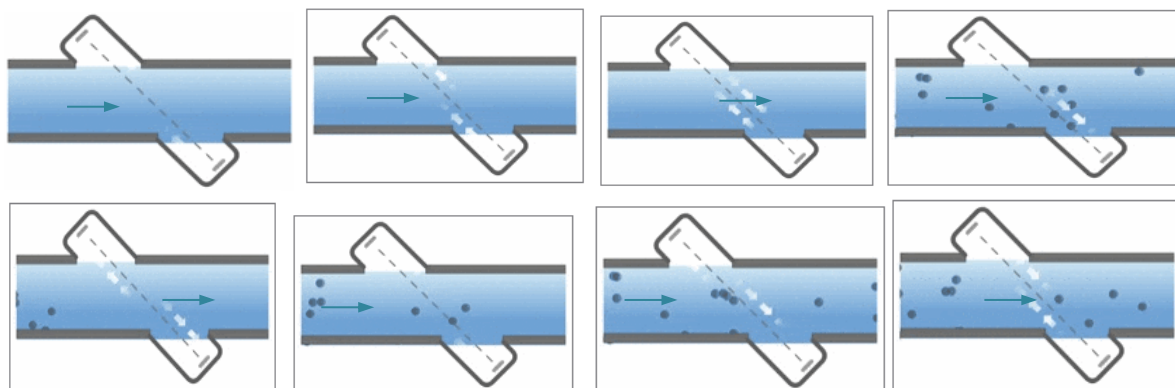


Figure 1. Illustration of the movement of a fluid through a pipe without and with impurities with the directions of propagation of waves sent downstream and reflected along with the flow of fluid

TECHNICAL CHARACTERISTICS OF THE OMNI TDI-200H HANDHELD ULTRASONIC FLOW METER

The measured flow can be displayed in several units of mass and volume display: [l/s], [l/min], [l/h], [m³/s], [m³/min], [m³/h]. The measurement results are obtained based on data on the internal cross-section of the pipe D , the distance between the transmitter and

receiver d , and the positions of the transducer about the pipe (ϑ - sound wave propagation angle relative to the direction of the fluid velocity vector v) (Figure 4). Unlike most flow meters, ultrasonic measuring devices do not include moving parts, allowing for more flexible and maintenance-free operation, as is the case with the OMNI TDI 200H flow meter.



Figure 2. OMNI TDI-200H Handheld Ultrasonic Flow Meter, set of parts, examples of transducer installation [4]

Details OMNI-TDI-200H [4]

The compact, light-weight design makes the Omni TDI-200H handheld flowmeter truly portable. The main unit weighs only 514g. The rugged case allows the flowmeter to be used in harsh environment. The unique clamp-on fixture design makes the installation very simple and no special skills or tools are required. Due to the non-intrusive nature of the clamp-on technique, there is no pressure drop, no moving parts, no leaks and no contamination.

Portable, Ultrasonic, Non-invasive, Clamp-on Flow Meter. Built-in datalogger for over 2,000 lines of data. Power Supply: Built-in rechargeable battery.

Technical data and performance:

Linearity 0.5%

Repeatability 0.2%

Accuracy $\pm 1\%$ of reading at rates > 0.6 ft/s. $\pm 0.5\%$ with on-site calibration.

Response Time 0-999 seconds, user-configurable

Velocity $\pm 0.03 - \pm 105$ ft/s ($\pm 0.01 - \pm 30$ m/s), bi-directional
 Power Supply: Built-in rechargeable battery.
 Mains adaptor 110-240V AC. Output: RS232.
 Supplied with TDI-S1 (for pipe diameter 15-100mm), TDI-M1 (50-700mm) and TDI-L1 transducers (300-6000mm),
 Pipe Material All metals, most plastics, concrete, lined pipe
 Rate Units Meter, Feet, Cubic Meter, Liter, Cubic Feet, USA Gallon, Imperial Gallon, Oil Barrel, USA Liquid Barrel, Imperial Liquid Barrel, Million USA Gallons. User configurable.
 Totalizer 7-digit totals for net, positive and negative flow
 Liquid Types Virtually all clean liquids and liquids with minor solids
 Security Setup Lockout. Access code needed to unlock
 Display 4x16 letters
 Computer Interface RS232, baud-rate: 75 to 115,200bps.
 Supplied Transducers As requested
 Transducer Cable Standard 10m. Contact sales for longer cables, up to 500m

Power 3 x AAA Ni-mH built-in batteries. When fully recharged it will last over 10 hours of operation.
 100-240vAC Adapter supplied for charging and continuous use.
 Data Logger Over 2000 lines of data
 Operating temperature range 0-90 °C.
 Handset Weight 1.2lbs (514g)

TRANSMITTER AND RECEIVER MOUNTING METHODS

Depending on the cross-section of the pipe, the transducers can be placed on the pipe in two ways: next to each other or on opposite sides of the pipe (Figure 3) depending on the size of the inner diameter of the pipe. The transducers can be mounted in the V-method for pipe diameters from 1" to 12", in which case the ultrasound crosses the pipe twice, in the W-method for pipe diameters less than 1", in which case the ultrasound crosses the pipe four times, or in the Z method for pipe diameters greater than 12", in which case the transducers are mounted on the opposite side of the pipe and the ultrasound crosses the pipe only once.

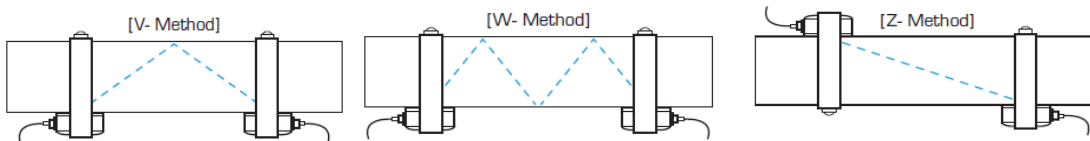





Figure 3. Methods of installing a flow transducer on a pipe according to the recommendation of the manufacturer of the ultrasonic device [4] method depends on the characteristics of the pipe and fluid. The Omni TDI-200H has pairs of transducers that can measure flows in pipes from 15 [mm] to 6,000 [mm] at temperatures between 0 and 90 [°C].

According to the design of the plant device, the diameter of the outlet pipe is $D = 65$ [mm] (2 0.5 ") and in the test work of flow measurement, the Z method will be applied. The choice of installation

Table 1. Transducer Options[5]

		
Type S Small size transducer (magnetic for pipe size 0.5"-2" (DN15-DN100 mm))	Type M Medium size transducer (magnetic for pipe size 2"-8" (DN50-DN700 mm))	Type L Large size transducer (magnetic for pipe size 11"-40" (DN300-DN6,000 mm))

CALCULATION PARAMETERS OF ULTRASONIC METER BASED ON PASSAGE TIME

When the flow meter is operating, the two transducers emit and receive an ultrasonic signal that

travels first downstream and then travels upstream. Because ultrasound travels faster downstream than upstream, a time difference (Δt) occurs. As long as there is a change in the amount of suspension or water, ie. as long as we know the time

of passage downstream and upstream, we can calculate the time difference and then the fluid flow rate (v_t) and the flow volume (QV). The passage time flow meters measure the difference in the passage time of ultrasonic waves in the direction of fluid flow and the direction opposite to the fluid flow through the pipe (Figure 4).

Transceiver pairs are used for the measurement, each of which contains a piezoelectric crystal. The fluid flow rate in the pipe [6]:

$$v_t = \left(\frac{t_2 - t_1}{t_2 \cdot t_1} \right) \cdot \left(\frac{d}{2 \cos \vartheta} \right) \dots\dots\dots (6)$$

Where is:

t_1 - time of passage of the ultrasonic wave in the direction of fluid flow, [s]

t_2 - time of passage of the ultrasonic wave in the direction opposite to the direction of fluid flow, [s]

d - ultrasonic wavelength, the ie distance between transceivers, [m]

ϑ - the angle of the passage of the ultrasonic wave about the longitudinal axis of the pipe, [radians]

Δt - a difference in ultrasonic wave passage times, [s]

ϱ - the angle between transmitted ultrasonic beam and fluid velocity vector, [radians]

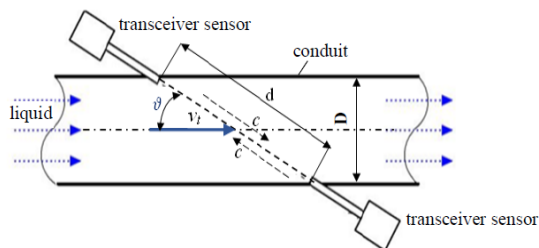


Figure 4. Principle of operation of the ultrasonic transducer with transmitter and receiver outside the pipe, Z method

When there is no flow, the frequencies of the ultrasonic wave sent to the pipe and its reflections are the same, ie. the velocity of the fluid is zero and the time difference (Δt) is zero. If we denote by the sign c the speed of propagation of the ultrasonic wave, we have such a state that the duration of the acoustic wave through the current of the liquid is shorter in relation to the time of return with the current of the liquid. If the angle ϑ enclosed by the direction of the acoustic wave with the direction of the velocity of the fluid (Figure 4) is equal to zero, then we obtain the following expressions for times [6]:

$$t_1 = \frac{d}{c + v_t} ; \quad t_2 = \frac{d}{c - v_t} \dots\dots\dots (7)$$

The difference in duration is the time spent:

$$\Delta t = t_2 - t_1 = \frac{2d \cdot v_t}{c^2 - v_t^2} \dots\dots\dots (8)$$

Provided that v_t is much less than the speed of propagation of the acoustic wave c (for water $c \approx 1500$ m/s) then it is:

$$\Delta t = \frac{2d \cdot v_t}{c^2} \Rightarrow v_t = \frac{c^2}{2d} \cdot \Delta t \dots\dots\dots (9)$$

With the ultrasonic flow meter, the Omni TDI-200H transducers are not in contact with the fluid but are placed in one of the given positions and on the outside of the pipe, and are located at a certain angle. When transmitting sound signals in the transmission and the return, ie. down and along with the fluid flow, a phase difference φ occurs which is taken as the basis for flow measurement:

$$\varphi = \omega \cdot \Delta t \Rightarrow \Delta t = \frac{\varphi}{\omega} \approx \frac{2d \cdot v_t}{c^2} \dots\dots\dots (10)$$

Where is:

c - speed of acoustic wave propagation in the pipe, [m/s]

ω - frequency of the ultrasonic wave, [Hz]

As the fluid moves faster, the difference in frequency increases linearly. It is important to point out that ultrasonic devices that measure flow work with a frequency that is higher than 50 [kHz] to several [Mhz]. If the angle ϑ which closes the direction of the acoustic wave with the direction of the velocity of the fluid is greater than zero then we use the following expression for the time difference:

$$t_1 = \frac{d}{c + v_t \cdot \cos \vartheta} ; \quad t_2 = \frac{d}{c - v_t \cdot \cos \vartheta} \dots\dots\dots (11)$$

$$\Delta t = t_2 - t_1 = \frac{2d \cdot v_t \cos \vartheta}{c^2 - v_t^2 \cos^2 \vartheta} \dots\dots\dots (12)$$

$$\Delta t = \frac{2d \cdot v_t \cos \vartheta}{c^2}$$

In case $v_t \ll c$ then: \Rightarrow
so the velocity of the fluid is:

$$v_t = \frac{c^2}{2d \cdot \cos \vartheta} \cdot \Delta t \dots\dots\dots (13)$$

For a pipe of circular cross-section and by setting the equation of volumetric flow with the entry of the corresponding values, we will obtain the equation for volume flow [6]:

$$Q_v = A \cdot v_t = \frac{D \cdot \pi \cdot c^2}{8d \cdot \cos \vartheta} \cdot \Delta t \quad [\text{m}^3/\text{s}] \dots\dots\dots (14)$$

Where is:

D - inner diameter of the pipe, [m]

MUNICIPAL WASTEWATER FLOW ANALYSIS IN N.C. "GRMEČ"

CONSTRUCTED WETLAND AS A RESEARCH OBJECT

When designing constructed wetlands for wastewater treatment, the characteristics of the wastewater to be treated up to the limit values prescribed by the normative acts for the given waters have been taken into account. The main characteristics of wastewater take into account the concentration of solutes and solid organic compounds, ie. biochemical oxygen demand (BOD), suspended solids, nitrogen, and phosphorus compounds, heavy metals, pathogenic bacteria, and/or viruses. Technical data of the constructed wetland in N.C. "Grmeč": active area field I 4 [m²], field II 16 [m²], total: 20 [m²], active length 10 [m] and average depth: 0.75 [m]. Device design involves determining the hydraulic capacity, load level, retention time (retention), plant species [7]. The hydraulic retention time of HRT [day] can be calculated by the following formula [7]:

$$HRT = \frac{V}{Q_v} = \frac{L \cdot B \cdot (d_m \cdot n + d_t)}{Q_v} = \frac{A \cdot d_m \cdot (n + d_t)}{Q_v} \dots \dots \dots (15)$$

where is:

V - the volume of water in the constructed wetland, [m³]

Q_v - mean flow through the constructed wetland, [m³ / day]

A - surface of the constructed wetland, [m²]

L - length of the constructed wetland, [m]

B - width of the device, [m]

d_m - thickness of the medium through which water passes, [m]

d_t - depth of water from the surface of the medium, [m]

n - porosity, [-]

Hydraulic capacity can be defined as the ability of a plant device to purify a certain volume of wastewater at a given time. This period is called the hydraulic retention time (HRT), depending on the amount of pollution and the given level of purification.

FLOW MEASUREMENT BY VOLUMETRIC METHOD

The method for determining the flow is usually chosen depending on the conditions in which the flow is measured and the required accuracy. In this case, a volumetric method was used to measure water flow. Volumetric flow measurement is simple, but also

relatively accurate, so it is often used to calibrate other instruments. Volumetric measurement is based on measuring time (t), so that the amount of water represented through the flow (Q_v), which flows through a system, fills a vessel of exactly known dimensions, ie volume (V). The flow is defined by equation 3. To drain the purified water from the constructed wetland, a round plastic pipe with a diameter of $D = 60$ [mm] is placed under a smaller drop to ensure free flow into the natural watercourse. Constructed wetlands are mainly used as a function of the second stage of purification and in most cases, before reaching the billets (bodies of the constructed wetland), the wastewater is subjected to preliminary and/or primary treatment. During the experimental work, 12 samples of wastewater and 12 samples of purified water were taken. Examination of samples was done in the laboratory of the Biotechnical Faculty. According to the work plan for processing purified water samples and measuring the flow of communal water on the constructed wetland N.C. "Grmeč" there was a variation in the flow that changed during the day and week, depending on the daily load, ie the number of users (students and other persons).

MEASUREMENT RESULTS AND DISCUSSION

One of the important parameters for efficient operation of the constructed wetland is the control of the amount of wastewater at the inlet and treated water at the outlet of the device [8]. In a constructed wetland, water flows almost horizontally at low speed and the length of the entire system. During the research period, the flow varied, and based on the measured values and process parameters (Table 2), it was calculated that the total effective field volume, in which water will flow, represents approximately 30[%] of the total volume and amounts to 4.20 [m³].

The retention time in the plant device itself is 84 [h], and in the sedimentation tank for municipal wastewater as well as in the sedimentation tank for laboratory wastewater is 50 [h/sedimentation tank]. The total retention time is thus 134 [h], ie 5.58 [days], which is sufficiently long retention of water, which is the basis for the treatment of municipal wastewater, up to the required limit values of wastewater emissions. Table 2 shows the average values of municipal wastewater flow in the teaching center "Grmeč" by volumetric method during the year.

The hydraulic retention or retention time of water (HRT) in the constructed wetland was 5 days, but in the summer the retention time increased to 6 days due to evapotranspiration. This period depends on the amount of pollution and the given level of purification.

The parameters were monitored for 5 days, with the flow varying depending on the weekly workload of students in the lecture halls and laboratory. The flow ranged from 4.34×10^{-6} [m³/h] to 2.86×10^{-5} [m³/h]. The final recipient was the stream Drobinica, which is

located near the training center. During the operation of the plant device, it was not clogged and the hydraulic conductivity was very good and stable.

Table 2. Average results of measuring the flow of municipal wastewater in the teaching center "Grmeč" by volumetric method during the year

Days of the week	Flow [m ³ /s] average			
	Spring	Summer	Autumn	Winter
Monday	$1,33 \times 10^{-5}$	$2,49 \times 10^{-5}$	$2,53 \times 10^{-5}$	$2,48 \times 10^{-5}$
Tuesday	$2,01 \times 10^{-5}$	$1,98 \times 10^{-5}$	$4,13 \times 10^{-6}$	$2,60 \times 10^{-5}$
Wednesday	$1,26 \times 10^{-5}$	$2,60 \times 10^{-5}$	$4,73 \times 10^{-6}$	$2,86 \times 10^{-5}$
Thursday	$7,02 \times 10^{-6}$	$5,62 \times 10^{-6}$	$2,66 \times 10^{-5}$	$4,34 \times 10^{-6}$
Friday	$2,72 \times 10^{-5}$	$3,1 \times 10^{-5}$	$3,17 \times 10^{-5}$	$1,86 \times 10^{-5}$

FLOW MEASUREMENT WITH ULTRASONIC DEVICE OMNI TDI 200H

Preparation of measuring devices



Figure 5: Flow measurement with an Omni TDI 200H ultrasonic meter on site

Before starting work, the device is prepared for measurement to avoid interruptions or irregularities in operation. For these reasons, field (location) and technical preparations for working with the device have been previously made, following the device manufacturer's instructions [9]. Figure 5 shows the measurement of water flow at the outlet pipe of the plant with an on-site ultrasonic meter Omni TDI 200H.

After mounting the converter, the cable connections were connected and the device was connected to the power source, after which the data for configuring the measuring sensor was entered, i.e. setting parametric quantities, which provided the conditions for the start of the trial measurement. Table 3 shows the measurement results and the average values during the day. The distance between the transducers (sensors) is $d = 60$ [mm].

Table 3. Measurement results

Days	Monday	Tuesday	Wednesday	Thursday	Friday	Note
Measured flow values, average [m ³ /h]	0,120	0,009	0,019	0,110	0,118	During the day, the water outlet from the pipe was interrupted
Addition:	Non-contact flow measurement. Period: autumn, October 2020, average values during the day. The measurement was done during the day from 9 ⁰⁰ to 14 ⁰⁰ .					

Measurement results with measurement problems

The flow unit is determined based on the flow value. At lower flows, it is better to set the measuring device in [m³/h] whose value index is 3.6 times higher than when measured in [l/s] [10]. To measure correctly

with any ultrasonic flow meter, care must be taken that the pipe must be filled with water. The flow measurement technology is based on the conventional flow measurement method, but also the control flow measurement at the outlet of the device using

ultrasonic measurement technique gives significant results. The flow measurement results are shown in Tables 2 and 3. Problems in the trial operation of this device are variable measurement results, minor interruptions of measurements, or "silence" of the device, which can be explained by the appearance of interruptions in the flow of water, ie. discontinuous intensity of water flow through the pipe, possibly insufficiently purified water or a problem in installation and assembly. Measurement results are not officially taken as valid indicators. For correct flow measurement with any ultrasonic flow meter, care must be taken that the pipe must be filled with water. This issue as well as other observed problems can be solved by activating the services of an authorized service technician regarding the operation of the device on-site in the given field conditions and through maintenance. By eliminating the observed shortcomings in the coming period and with the support of an authorized service technician, it is to be expected that the device will work efficiently.

CONCLUSION

The constructed wetland for wastewater treatment was built in 2018 on the land surface of the Teaching Center "Grmeč" of the Biotechnical Faculty in Bihać, to treat wastewater of various origins through research and conduct experiments. Omni TDI-200H Handheld Ultrasonic Flow Meter was procured through the project "Efficiency testing of constructed wetland with different types of wastewater", a project funded by the Cantonal Ministry of Construction, Physical Planning and Environmental Protection of the USC (2016). During the trial operation of the device, it was concluded that any change in flow, and indirectly changes in the operation of the constructed wetland, affect the intensity, consistency, and amount of purified water, which directly affects the quality of flow meters. For correct flow measurement with any ultrasonic flow meter, care must be taken that the pipe must be filled with water. This issue, as well as other observed problems, will be resolved through the services of an authorized service technician regarding the operation of the device on-site in the given field conditions and through maintenance. Due to problems in the operation of the flow measuring device, the

current volumetric flow measuring method is currently used to obtain more accurate results. Research on a pilot plant constructed wetland has shown that the device can work efficiently at different flows. By regulating the inflow of water to the device, optimal results can be obtained and at the same time meet the standards and legal regulations related to wastewater.

ACKNOWLEDGMENT

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ELECTROCHEMICAL REMOVAL OF HEXAVALENT CHROMIUM BY ELECTROCOAGULATION

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Chromium in wastewater is discharged from tannery, electroplating, metal finishing, dyeing industry and many other industries. Presence of hexavalent chromium (Cr(VI)) in the environment is a considerable concern because it is non-biodegradable, highly toxic and carcinogenic. There are several treatments for the removal of chromium from wastewater, but the most common method involves reduction to its less toxic trivalent state. In this paper electrocoagulation was used as treatment for removing of hexavalent chromium from synthetic prepared wastewater initial concentration 50 mg/L, in presence of sodium chloride as supporting electrolyte (1 g/L). The treatment was performed in a batch electrochemical reactor 250 mL capacity and with possibility of constant stirring. It was examined the impact of electrode materials and current density (5, 10, 20 mA/cm²) on Cr(VI) removal efficiency. The examination showed that iron has higher removal efficiency compared to aluminium. Also, it was observed high efficiency at very begin of treatment and at low current density. For 20 minutes of treatment it was achieved almost complete Cr(VI) removal at current density of 5 mA/cm² by using iron electrodes ($E=99.8\%$).

KEYWORDS: removal efficiency, current density, iron anode

INTRODUCTION

Surface water and groundwater are recipients of various types of industrial wastewater. Organic matter of different toxicity and biodegradability and various heavy metals are the most common pollutants in wastewater. Heavy metals differ from most organic pollutants because they are not biodegradable and over time they only accumulate in certain systems [1]. Industrial pollution is the main source of all heavy metals that occur in natural surface waters. Due to its wide application in various industrial technologies, chromium (Cr), as an element from the group of heavy metals is a one of pollutant found in industrial wastewater.

Chromium can appear in two oxidation states, Cr(III) and Cr(VI). There are significant differences in physicochemical properties and toxicity between these two oxidation states. At neutral pH, Cr(III) is easily precipitated and forms Cr(OH)₃ which is nearly insoluble in water. Cr(VI) is much more soluble in a wide range of pH values, which increases its mobility and distribution. The toxic effect of Cr(VI) is estimated to be 100 to 1000 times higher than that of Cr(III) [2]. Trivalent chromium is naturally present in the environment and is one of the essential heavy metals necessary for the normal functioning of living

organisms. The main sources of Cr(VI) emissions are mainly anthropogenic. The most common sources are pigment and paint production, galvanizing and corrosion protection plants, glass production, cement and ceramics industry, and perhaps the largest source of hexavalent chromium emissions (in some countries) - the leather industry, where large amounts of this pollutant are formed in the leather tanning process. Due to this, some members of the European Union have completely banned the operation of tanneries near water bodies [3,4,5,6].

There are different methods for hexavalent chromium removal from aqueous solutions. It can be removed either in the form of Cr(VI) or converted to less toxic Cr(III) and precipitated in the form of insoluble Cr(OH)₃. Methods used for Cr(VI) removal are ion exchange, chemical precipitation, adsorption, biosorption, coagulation, and electrochemical treatments (electrocoagulation, electroreduction, electrodeionization) [2,4]. The choice of technology depends on the efficiency of the Cr(VI) removal method, investment and maintenance costs of the technology and complexity of the process.

Electrochemical methods have become frequently used in environmental technology. Electrocoagulation (EC) is an electrochemical process most commonly

used in wastewater treatment. EC implies the formation of coagulants in situ by electrolytic dissolution of the electrode made of aluminum or iron, in an electrochemical reactor. The metal ions formed on the anode by hydrolysis create a large number of different compounds that are effective coagulants for the removal of pollutants. At the cathode, hydrogen is released which often carries flocculated particles to the solution surface. The EC reactor primarily produces colloidal aggregation of coagulants (increased size) and gas bubbles which are small, if the applied current is low [7].

In EC processes, Cr(VI) ions are first reduced to Cr(III) ions at the cathode and then combine with the generated OH⁻ ions and precipitate as insoluble Cr(OH)₃. The reaction mechanism of the EC process in the case of using an iron anode is formation of Fe(OH)₃ or Fe(OH)₂ on anode and evolution of hydrogen gas on cathode. Chromium is isolated in the form of a precipitated Cr(OH)₃ or is adsorbed on iron hydroxide particles [8,9]. When aluminum electrodes are used in the EC process, the process of removing hexavalent chromium takes place mainly by the adsorption mechanism of Cr(VI) on amorphous Al(OH)₃ [5].

The efficiency of the EC process of chromium wastewater depends on the applied current density, treatment duration, used electrodes and connection modes, electrolyte pH and composition, initial pollutant concentration [5, 8-18]. Since Fe and Al hydroxides are very good coagulants that have an affinity to adsorb different organic and inorganic compounds, the EC process is proved to be effective in simultaneous removal of several different pollutants, together with Cr(VI) (reducing the BOD and COD, simultaneous removal of heavy metal ions, turbidity) [5,12].

The main advantages of the EC process and its use in wastewater treatment are the reduced amount of chemical reagents required for precipitation, the reduced amount of sludge formed in the process. The Fe and Al hydroxide flocs formed in the electrolysis process have a large particle size, are stable and easy to separate from treated water, and the material used for this process, metal iron and aluminum plate, are available and relatively inexpensive [15,16]. In this paper different process parameters on EC of synthetic prepared wastewater containing Cr(VI) was examined.

MATERIALS AND METHODS

Experimental part of the research is contained by the application of EC for removing of hexavalent chromium from simulated wastewater.

Electrochemical batch reactor (Fig. 1) is made of polypropylene of capacity 250 cm³ with possibility of constant mixing (300 rpm/min), which contains two electrodes of the same dimensions (area), $A=24\text{ cm}^2$, and distance between electrodes, $d=20\text{ mm}$. Electrodes were connected to programmable DC power supply (GW INSTEK, PSP-2010; 20V, 10A). Before each treatment electrodes were cleaned and degreased.

Used electrode materials are made out of metals known compositions, and comply with prescribed standards, respectfully, steel (EN10130-91), stainless steel (EN 1.4301/AISI 304) and aluminum (Al 99.5/EN AW-1050 A). Steel and aluminium were used as anode and stainless steel as cathode. For the experimental purpose it was used commercially available chemicals *p.a.* (Lachner, Czech): 99,5% sodium chloride, NaCl, 35% hydrochloric acid, HCl, acetone, (CH₃)₂CO, 97% sodium hydroxide, NaOH and 99,5% potassium chromate, K₂CrO₄.

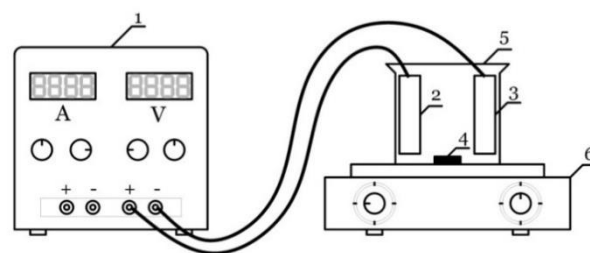


Figure 1. Schematic view of electrochemical reactor: 1 – source of electric power; 2 – anode; 3 – cathode; 4 – magnetic stir bar; 5 – electrochemical cell; 6 – magnetic stirrer)

All the experiments were performed at an ambient temperature and with synthetic wastewater volume of 250 cm³. Initial wastewater concentration was 50 mg/L of Cr(VI) and it was added sodium chloride (0.5 or 1 g/L) as supporting electrolyte. Initial pH was 5.13 (without adjustment) and electric conductivity was 2.5 mS/cm. Before each treatment, current density was set at desirable value (5; 10 and 20 mA/cm²).

After each EC process, treated synthetic wastewater was filtered through filter paper, Filtres Fioroni, France (Ref.:0015A00007; size: 125 mm, qty.: 1000) and it was collected formed sludge. The samples of wastewater before and after treatment were analyzed on the following parameters: pH, conductivity (κ) and Cr(VI) concentration. The chromium concentration before and after treatment was determined spectrophotometrically ($\lambda_{max}=540\text{ nm}$) on UV-VIS spectrophotometer (Perkin Elmer, Lambda 25) according to standard methods [19] and pH and κ are determined on the multimeter (Consort C861). The IR spectrum of the resulting sludge was

analyzed by Fourier transform infrared spectroscopy (FTIR) in the range of 400–4000 cm^{-1} (Bruker, Tensor 27).

In order to improve the EC treatment, from three in practice represented current regime it was used pulsed current regime. The regime is a schematic represented in Figure 2. A pulsed current regime is defined by the cathode current density (j_k), time cathode deposition (t_k), anodic current density (j_a), and time of anodic dissolution (t_a). The period of pulsed current waves, (T), is the sum of the time of cathode deposition (t_k) and time of anodic dissolution (t_a) [20].

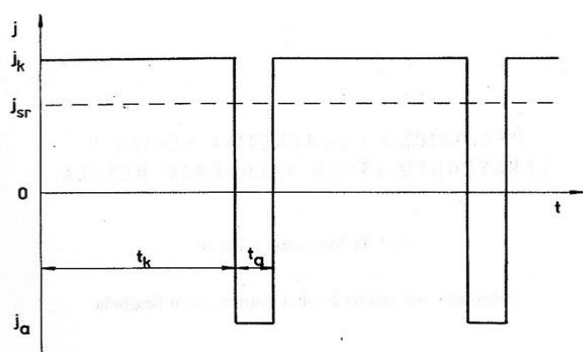


Figure 2. Schematic view of pulsed current regime [20]

RESULTS AND DISCUSSION

Results of the EC process are shown through chromium removal efficiency $E_{\text{Cr(VI)}} (\%)$ calculated by following equation:

$$E_{\text{Cr(VI)}} = \frac{\gamma_i - \gamma_f}{\gamma_i} \times 100 [\%] \quad (1)$$

where are γ_i and γ_f the initial and the final concentration of Cr(VI) expressed in mg/L.

The energy consumed to remove pollutants unit is one of the most important technological performance indicators of electrochemical reactor, because it affects the overall cost of treatment. Specific energy consumption (W_{sp}) is calculated by the following equation:

$$W_{sp} = \frac{\int_0^{\theta} IE_r dt}{3600 \times 1000m} \left[\frac{kWh}{kg_{\text{pollutant}}} \right] \quad (2)$$

where: E - voltage (V), I - current (A), t - time (h), m - mass of removed chromium (kg).

This research investigates the impact of: anode material, stirring, electrolysis duration, current density, cathode material, supporting electrolyte concentration and reverse current regime.

During wastewater treatment, special attention is paid to the choice of electrode material. In the case of EC,

the choice of anode material is reduced to a choice between an iron and an aluminum electrode. In the first set of experiments, electrode pairs (anode - cathode) made of steel (Fe - Fe) and aluminum (Al - Al) were used. Iron electrodes achieve higher Cr(VI) removal efficiency compared to aluminum electrodes, under the same experimental conditions, which is in accordance with previous research [17,18]. In 30 minutes of treatment with Al-Al electrode pair it was achieved 43.9% removal efficiency, while with Fe-Fe pair, 97.9% removal of chromium was achieved in 5 only minutes of treatment (Figure 3).

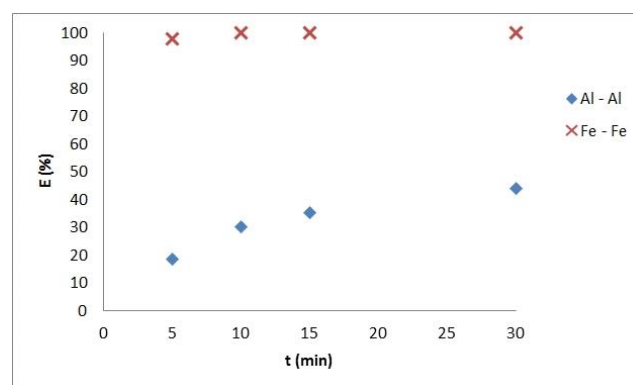


Fig. 3. The impact of anode material on Cr(VI) removal efficiency at different electrolysis time ($j=20 \text{ mA/cm}^2$, $\gamma_{\text{Cr(VI)}}=50 \text{ mg/L}$, $\gamma_{\text{NaCl}}=1 \text{ g/L}$ stirring 300 rpm)

After this experiments it was examined whether the stirring has an impact on the efficiency of chromium removal. For 15 minutes of treatment with stirring (300 rpm), an efficiency of 71.9% was achieved, while without stirring under the same conditions, the efficiency was 54.1%. In further research, stirring was performed since it was confirmed that the removal chromium efficiency is diffusion controlled.

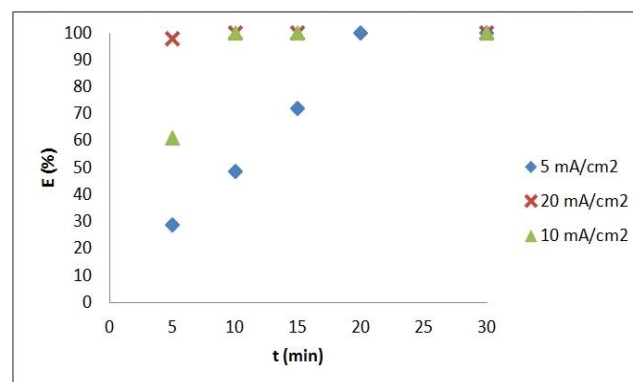


Fig. 4. The impact of current density on Cr(VI) removal efficiency at different electrolysis time ($\gamma_{\text{Cr(VI)}}=50 \text{ mg/L}$, $\gamma_{\text{NaCl}}=1 \text{ g/L}$ stirring 300 rpm)

Figure 4 shows Cr(VI) removal efficiency at different current densities (5; 10; 20 mA/cm²) and different treatment time. The treatment is characterized by high efficiency at the very beginning of the process, since complete removal of chromium was achieved in 30 minutes at all tested current densities. At the lowest current density (5 mA/cm²), the efficiency increases linearly with increasing treatment time and reaches a maximum at 20 min (99.8%). Since at the lowest current density the energy consumption is the lowest and a good removal efficiency is achieved, current density of 5 mA/cm² was used in further research.

In order to examine the influence of cathode materials, as the cathode was used steel (Fe) and stainless steel (SS). Figure 5 shows removal efficiency with cathodes of Fe (Fe:Fe) and SS (Fe:SS), where slightly higher efficiency is observed by using a cathode made of stainless steel. Iron electrodes can be successfully replaced by cathodes of stainless steel which make process more efficient and easier to maintain.

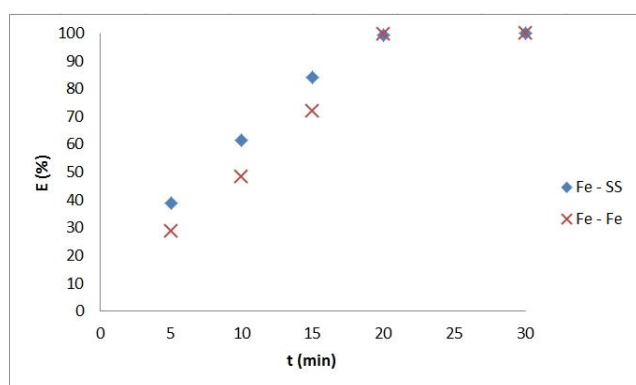


Fig. 5. The impact of cathode material on Cr(VI) removal efficiency at different electrolysis time ($j=5$ mA/cm², $\gamma_{\text{Cr(VI)}}=50$ mg/L, $\gamma_{\text{NaCl}}=1$ g/L stirring 300 rpm)

The supporting electrolyte has an important role in electrochemical wastewater treatments. If the wastewater, which is an electrolyte in this case, does not have sufficient conductivity, a supporting electrolyte is used to increase the ionic conductivity and reduce energy consumption [7]. In this paper NaCl was tested as supporting electrolyte in two concentrations: 0.5 and 1 g / L. The experiments showed that there was no significant difference in the achieved efficiencies, and for 20 min of treatment the efficiency was 98.4% with 0.5 g/L NaCl, and 99.3% with 1 g/L NaCl. Lower supporting electrolyte concentrations reduce the economic costs of the EC process.

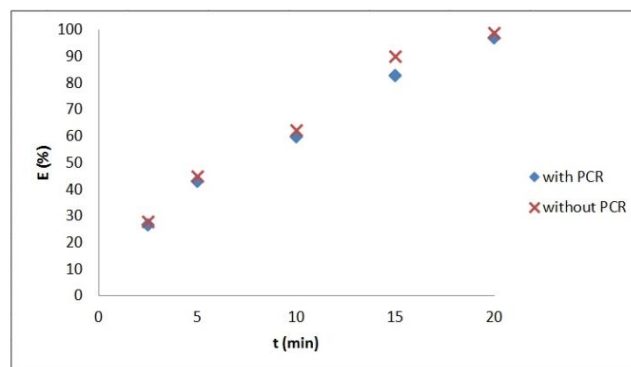


Fig. 6. The impact of pulsed current regime on Cr(VI) removal efficiency at different electrolysis time for electrode pair Fe-Fe ($j=5$ mA/cm², $\gamma_{\text{Cr(VI)}}=50$ mg/L, $\gamma_{\text{NaCl}}=0.5$ g/L stirring 300 rpm)

In order to prevent passivation of the electrodes, it may be necessary to replace them after a certain time. Another way to prevent passivation is to apply a reverse current regime (pulsed current regime) in which after a certain time the polarity of the electrodes changes. A change in the polarity of the electrodes is also desirable due to the uniform electrolytic dissolution of the sacrificed anodes. Figure 6 shows a slight increase in removal efficiency without the application of the pulsed current regime (PCR). This behavior is a sign that during electrolysis there is no significant passivation and "soiling" of the electrodes, so PCR regime does not have a positive impact on the efficiency of the process. Considering that the treatment time was short and it was a synthetically prepared wastewater, it is assumed that PCR would have a positive effect in longer treatments of real (more loaded) wastewater.

Based on the results shown in Figure 4, it can be seen that almost complete removal of chromium (>97%) is achieved in 5 min with the application of the highest current density (20 mA/cm²), in 10 min with current density of 10 mA/cm², and in 20 min with the application of the lowest current density (5 mA/cm²). Figure 7 shows specific energy consumption for these best achieved efficiencies. The lowest energy consumption (per mass of removed pollutant $W_{sp}=2.82$ kWh/kg_{Cr(VI)} or per volume of treated wastewater 0.66 kWh/m³) was at the lowest current density and high efficiency was achieved (99.8%) in a relatively short treatment time, which is favorable from the economic aspect of the process. Also, for current density of 20 mA/cm², energy consumption is not high, but only because the treatment time was short. In the case of loaded waters (higher initial chromium concentrations), the treatment time would be longer, and thus the energy consumption. In the paper of Kobya et al. [13] energy consumption was 1.20 kWh/m³ for 30 minutes of

treatment with iron electrodes at current density of 30 mA/cm² (concentration of Cr(VI) in galvanizing rising water was 32 mg/L). Dermentzis et al. [8] achieved 99.9 % Cr(VI) removal efficiency from electroplating wastewater ($\gamma_{Cr(VI)}=500$ mg/L) for 50 minutes of treatment with iron electrodes at 40 mA/cm² and energy consumption amounts 46 kWh/m³.

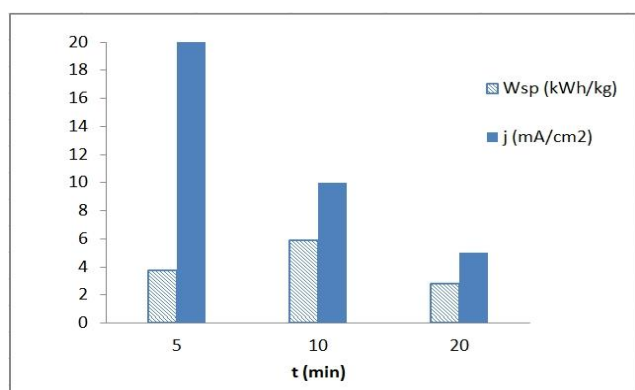


Fig. 7. Energy consumption, time and current density during EC with Fe-Fe electrode pair ($\gamma_{Cr(VI)}=50$ mg/L, $\gamma_{NaCl}=1$ g/L stirring 300 rpm, $E_{Cr(VI)}\approx 100\%$)

The main advantage is that EC produces only 50% of the sludge chemical process, which shows more benign properties for the environment [2]. Although Cr(VI) was reduced to less hazardous Cr(III) during the EC process, the resulting sludge is certainly a problem in terms of further disposal. In this study, the sludge formed after the EC process was collected and analyzed by FTIR spectroscopy. It has been observed that with aluminium electrodes formed sludge was more voluminous, compared to sludge formed with iron electrodes.

Figures 8 and 9 show FTIR spectrum of the sludge generated after EC treatment with aluminium and iron electrodes. It can be observed that both spectrums have broad peaks in the range of 3200 - 3500 cm⁻¹, which are associated with OH⁻ groups originate from Al and Fe hydroxides generated in this process [21,22]. Peak at 1634 cm⁻¹ (Fig. 9) can confirmed presence of nondissociated H₂O from Cr(OH)₃·H₂O [23, 24], since according to the described mechanism, the removal of chromium takes place through the adsorption of chromium on Fe(OH)₃, but also through the precipitation of Cr(OH)₃. Peaks appearing in the range of 460 - 540 cm⁻¹ (Fig. 8, Fig. 9) confirm the presence of Al and Fe, since these peaks are characteristic of Al-O and Fe-O bonds [25].

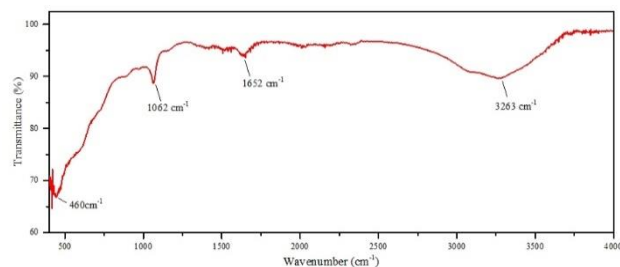


Fig. 8. FTIR spectrum of the sludge generated after treatment with aluminium electrodes

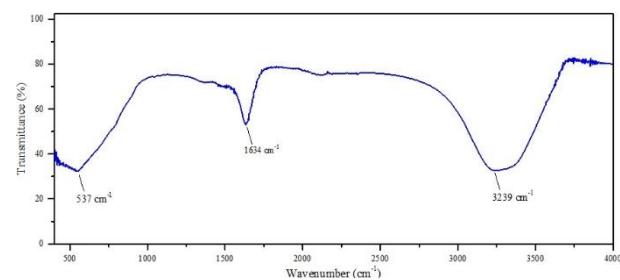


Fig. 9. FTIR spectrum of the sludge generated after treatment with iron electrodes

CONCLUSIONS

Research has confirmed that the electrocoagulation process is very effective for removing hexavalent chromium from wastewater. Iron anodes show significantly higher efficiency compared to aluminum anodes, while the choice of cathode material does not have a great impact on the chromium removal efficiency, but only on the economic feasibility of the process. The tested initial concentration required only 20 min for almost complete removal of chromium, at a current density of 5 mA/cm², where the energy consumption was $W_{sp}=2.82$ kWh/kg_{Cr(VI)} / 0.66 kWh/m³.

ACKNOWLEDGMENT

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KINETICS OF DOLOMITE GRINDING IN A LABORATORY BALL MILL

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Grinding is one of the most widely used methods of obtaining solid particles of controlled/ desired distribution systems in all branches of industry. Enlargement of this process is often based on experience and on trial and error methods that requires a large number of experiments. The development of mathematical models enabled the transfer of results to a larger scale in similar systems. The proposed researches were conducted in a laboratory scale. The modeling of the grinding process by the population balance included a description of the kinetics of dolomite grinding by kinetic parameters and the development of models that enabled the estimation of kinetic parameters on the basis of the particle size, the geometric characteristics of the mill and the process parameters. Rajamani and Herbst model is suggested for the development of selection function in a ball mill under given conditions process. The selection function was determined based on the change in the proportion of unbroken material over time for eight size intervals in four mills of different volumes, using one-size interval method. Change of unbroken material content with time is linear. The specified dependence suggests first-order breakage kinetics. The selection function was then described by the Rajamani Herbst model. Laboratory-level researches and the development of mathematical models for transferring results to a larger scale is a potential way of reducing energy consumption.

KEYWORDS: ball milling, kinetic, grinding, mathematical model

INTRODUCTION

Grinding is the process of reducing the size of a raw material for its direct use or mineral raw material for the concentration process, or other form of technological processing. As the size decreases, the surface area of the raw material increases. Therefore, it can be said that grinding is a process that leads to the creation of new surfaces.[1]

Apart from its technological importance which is reflected in the reduction of its size and the release of minerals, grinding also has important economic importance. About 4.5-5% of produced electricity in the world is spent on the grinding of various materials. Grinding is a very expensive process. Considering the energy consumption and the equipment cost, improvements in the grinding process are of great economic and therefore environmental importance.

The most well-known method of controlled production of these sizes is grinding in ball mills. This process is the subject of much research today if considered high energy consumption in the grinding process and its versatile application in industry.[2,3] One of the research directions is focused on studying the legality of the grinding process and, on the basis of them, defining mathematical models of grinding that

represent the basis for optimization and automation of the grinding process.[4,5]

Optimal design and control of the grinding process requires a good selection of a mathematical model that will allow the prediction of material behavior in the grinding process. The most commonly used tool to simulate the grinding process are population balances that describe the change in mass of particles of a given size in a given time. In conditions of discontinuous grinding, the population balance means a description of the process by the basic functions: by a selection function that defines the probability of fracture of a given size material, and by a fracture function that defines the distribution of fracture products.[6,7]

The selection function and the fracture function can be determined experimentally on a small scale and used for the product properties prediction on an industrial scale.

These functions depend on the process conditions and the particle size distribution of the input current of the material. They are experimentally determined on a small scale. However, their direct determination in large volume mills is almost impossible. Therefore, it is necessary to extrapolate the results obtained on a smaller scale. The particle size distribution of the grinding products depends on a number of factors: characteristics of the input material, mill speed, type,

size and number of grinding bodies, distribution of grinding body sizes. Process parameters affect kinetic parameters: selection function and fracture product distribution function.[8]

RAJAMANI HERBST MODEL

$$\ln\left(\frac{S_j}{S_1}\right) = \alpha_1 \cdot \ln\left(\frac{x_j}{x_1}\right) + \beta_1 \cdot \left[\ln\left(\frac{x_j}{x_1}\right)\right]^2 \dots\dots\dots (1)$$

For a wide range of size intervals, Rajamani and Herbst used a polynomial function to estimate the selection function.[9,10]. This model actually represents a second-degree polynomial with coefficients, where parameter α_1 denotes particle size for maximum selection function (turning point), and parameter β_1 denotes sharpness of turning point for larger particles. Using the experimental data, the specified parameters are determined. If there is a significant deviation from the literary model proposed to describe the selection function from the experimental data, a model to adequately describe the change in the selection function with particle size must be sought.

When choosing a mathematical model, the model with shorter duration of the experiment is chosen.

AUSTIN MODEL

$$S_j = S_1 \cdot \left(\frac{x_j}{x_1}\right)^\alpha \dots\dots\dots (2)$$

If the diameter of the ball mill is appropriately selected and the particle size input is less than 1mm, the second part of the Rajamani and Herbst model can be neglected, and the function becomes the Austin model.[8]

In the expression x_j , upper limit of interval marked as the j -interval, and α is a model parameter that depends on the material properties and the grinding conditions.

SNOW MODEL

$$\frac{S_j}{S_{max}} = \left(\frac{x_j}{x_{max}}\right)^c \exp\left(-\frac{x_j}{x_{max}}\right) \dots\dots\dots (3)$$

Snow introduced a mathematical model that describes the change in selection function with maximum selection function and particle size.[11,12] This model is often used with certain corrections, that is, it is not applied without additional corrections.

MATERIALS AND METHODS

TESTED SYSTEM

Experimental researches were conducted on a model dolomite sample Samoborka d.o.o.Zagreb. Dolomite is a part of sedimentary carbonate rocks of about 10%, consisting mainly of dolomite minerals. Theoretically, dolomite contains 54.35% CaCO_3 and 45.65% MgCO_3 impurities such as Si-, Al- and Fe and oxides. For commercial purposes, the proportion of these impurities should not exceed 7%, since then its industrial use becomes questionable. The sample was divided into eight one-particle intervals defined by the mesh diameter of the sieve.

Table 1. One-particle intervals

Size interval	Size range, μm
M1	1700 – 1180
M2	1180 – 850
M3	850 – 600
M4	600 – 425
M5	425 – 300
M6	300 – 212
M7	212 – 150
M8	150 – 106

The particle size intervals are chosen in a way that the ratio of the upper and lower interval sizes is always $\sqrt{2}$ as defined by the ASTM sieve standard.

GRINDING MACHINES (BALL MILLS)

The grinding of the material in the ball mill occurs primarily due to the kinetic energy consumption of the normal ball impact. The most effective action of the balls in the mill is achieved at the speed of rotation of the mill, for which the total kinetic energy of the normal impact of the ball in the unit of time is maximum. Starting from these assumptions, theoretical expressions were obtained for the optimum rotation speed of ball mills in terms of the kinetic energy of the balls. The optimum speed is reached at 60 to 80% of the critical speed. Various shapes (balls, bars, prisms, cubes, etc.) can be used as grinding balls.[13] The best results with regard to the efficiency of grinding were given by rods and balls. Apart from the wear resistance, an important feature of the bars and balls is their even wear, i.e. retaining their original shape.

The filling of the mill with the grinding balls is characterized by a filling coefficient (ϕ). The grinding balls rotate together with the pot. Centrifugal force and gravitational force affect the ball (grinding balls) in the mill:

$$F_c = m \cdot \omega^2 \dots \dots \dots (4)$$

$$F_g = m \cdot g \dots \dots \dots (5)$$

F_p - centrifugal force (N)

F_g -gravitational force (N)

g -gravitational acceleration (m/s²)

ω -angular velocity (°/s)

m -mass (kg)

At the moment of equalization of centrifugal force and force of gravity, material centrifugation occurs, and the speed at which this occurs is called the critical speed.[14]

$$n_{kr} = \frac{42,3}{d_u^{0,5}} \dots \dots \dots (6)$$

n_{kr} - critical speed of the ball mill (°/s)

d_u - the inner diameter of the ball mill vessel (m)



Figure 1. Ball mill

ANALYSIS BY SIEVES

The particles size distribution samples grinded over a shorter period of time was determined by analysis by sieves For this purpose, sieves whose mesh aperture is shown in the table and defined by the ASTM standard were used. In this way, the proportion of particles in a given particle size interval is obtained. For comparison with the computationally obtained distribution of particle sizes, the experimental values were translated into cumulative form.[15]

DETERMINING THE SELECTION FUNCTION (S-FUNCTION)

The selection function, S_i , is also called the specific fracture rate. It is the mass fraction of size particles that is selected and broken in a unit of time.[16,17] Represents the probability that a particle

of size x_i is selected for breaking and broken to a size smaller than the lower limit of the interval i (x_{i+1}).

The most common methods for determining the selection function are, - a one-size intervals method in which the loss of material from the initial j interval is monitored[18] - a radioactive labeling method in which the particle's path is traced through size classes during the grinding process. It is determined by the one-size intervals method.[15] The sample is divided into narrow particles size intervals. According to the ASTM sieve standardization, the ratio of the upper and lower bounds of the interval is $\sqrt{2}$ or $\sqrt[4]{2}$. Each interval is grinded separately and the proportion of unbroken interval material j in time is monitored. Assuming that the fracture kinetics of individual particle sizes is a first order process, the velocity of disappearance of that size is proportional to the mass of the same. With the application of the one-size interval method, the function of formation is lost. The rate of disappearance of particles from the initial interval can be described by the expression. [5,13,18,19]

$$\frac{dw_i(t)}{dt} = -S_1 \cdot w_1(t) \dots \dots \dots (7)$$

Where S_1 is the constant of proportionality and is called the specific fracture rate. With the integration of the expression in the range from 0 to t , the following equation is obtained:

$$w_1(t) = w_1(0) \cdot \exp(-S_1 \cdot t) \dots \dots \dots (8)$$

Which logarithmically transforms into a form that forms the basis for determining the specific fracture rate of a given size.

$$\ln w_1(t) = \ln w_1(0) - S_1 \cdot t \dots \dots \dots (9)$$

Since, according to the one-size interval method, all particles are located in initial interval i , $w_i(0)=1$, equation (9) goes to the simplest form of equation of the direction whose direction coefficient represents the value of the specific fracture velocity:

$$\ln w_1(t) = -S_1 \cdot t \dots \dots \dots (10)$$

If the specific fracture rate is constant, the fracture kinetics is of the first order, which means that the accumulation of particles in the pot has no particular influence on the specific fracture rate. Based on the above expression, by the method of one-size intervals, the values of the selection function (specific fracture velocity) of all 8 particle size intervals ($i = 1 \dots 8$) were determined.

RESULTS AND DISCUSSION

Based on the one-size intervals method, the values of the selection function are determined, assuming that there is no their change in time, that is, it is a first order process. The selection function is determined by the

change in the proportion of unbroken material in time. (Fig.2) shows the experimental results on the basis of which the selection function was determined for the mill 400 ml and for the size interval 1700-1180 μm . In this way, we determined the selection function for all size intervals in all four mill volumes.

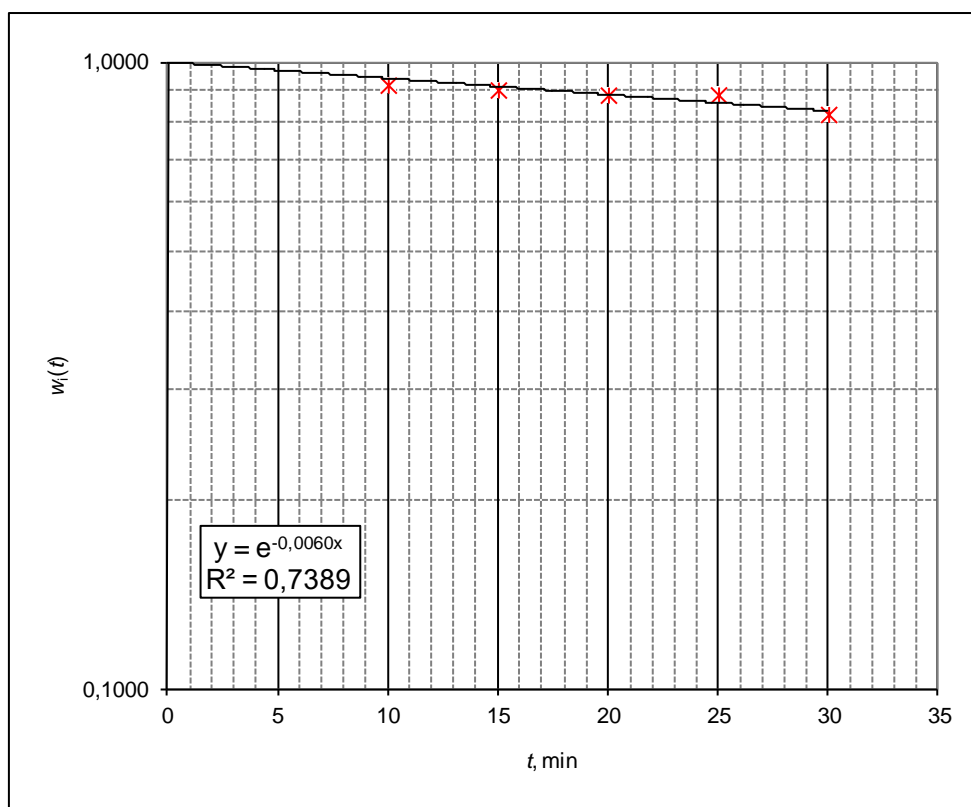


Figure 2. Change in the proportion of unbroken material in time; mill 400ml, 1700-1180 μm

Table 2. The view of all obtained values of the selection function.

S_j, s^{-1}	$x_i, \mu\text{m}$								
	1180	850	600	425	300	212	150	106	
V, m^3	0,0004	1,00E-04	2,22E-04	4,72E-04	5,98E-04	6,67E-04	1,17E-03	8,80E-04	4,85E-04
	0,001	1,77E-04	2,48E-04	3,75E-04	5,48E-04	5,53E-04	8,40E-04	7,42E-04	3,77E-04
	0,0017	2,12E-04	2,58E-04	3,12E-04	3,90E-04	3,83E-04	6,48E-04	4,57E-04	2,43E-04
	0,002	2,25E-04	2,63E-04	3,27E-04	3,57E-04	3,38E-04	5,72E-04	3,78E-04	2,10E-04

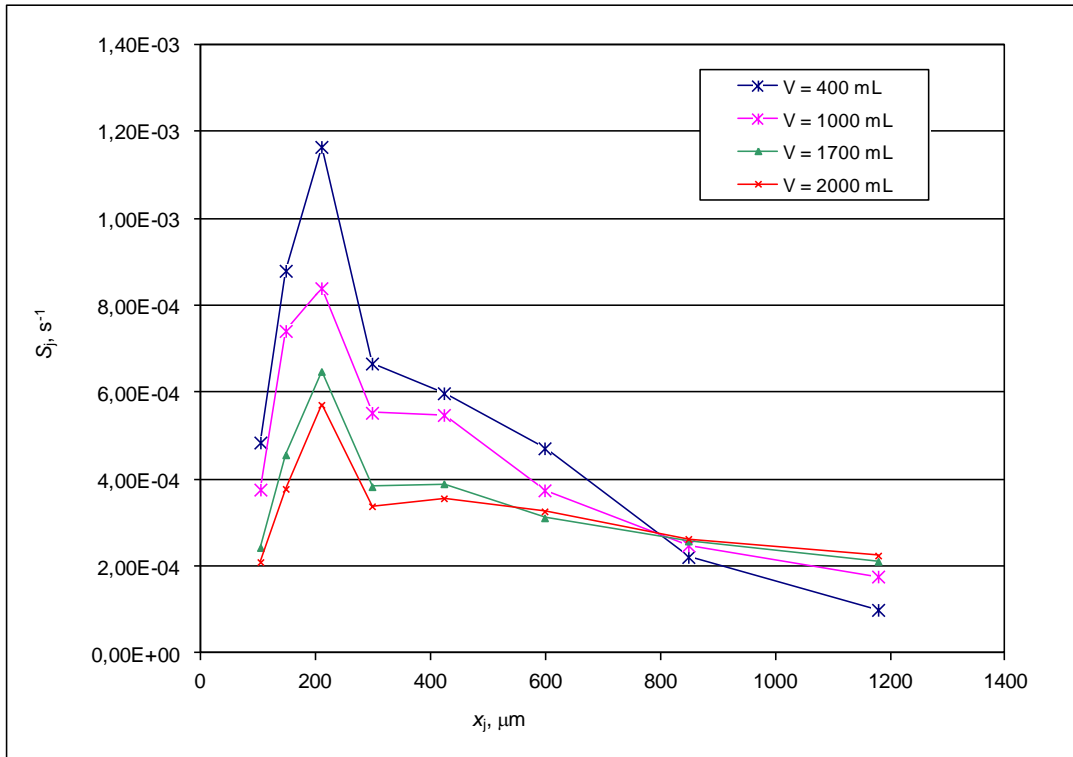


Figure 3. Dependence of the selection function on the particle size.

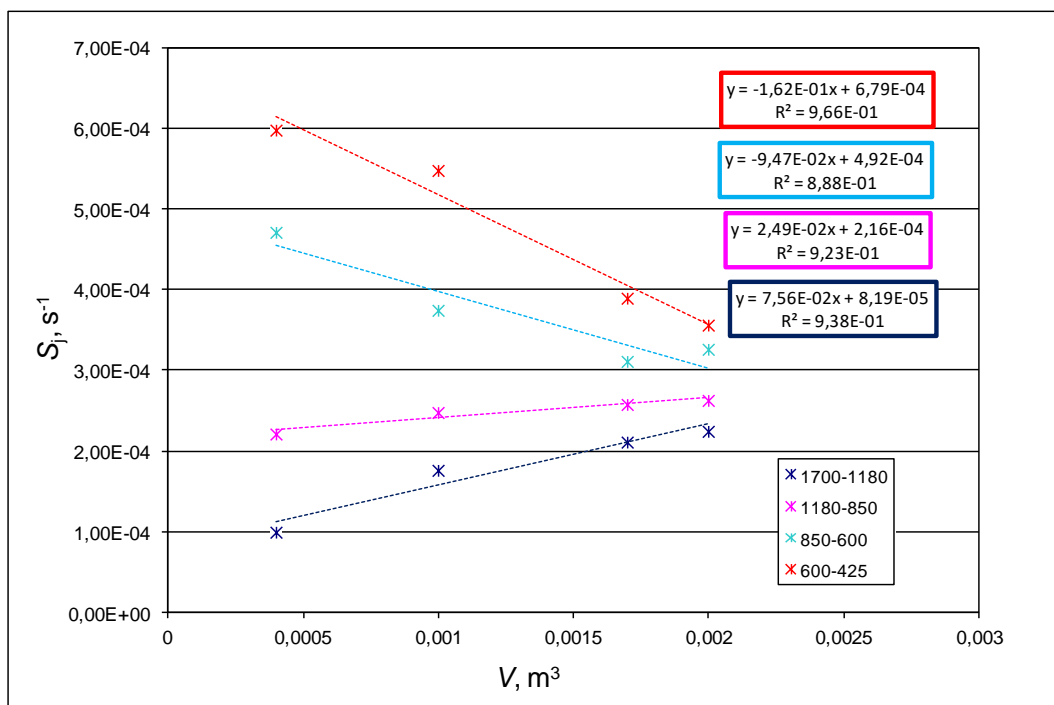


Figure 4. Dependence of the experimentally obtained values of the selection function on the volume of the mill drum; intervals M1, M2, M3 and M4.

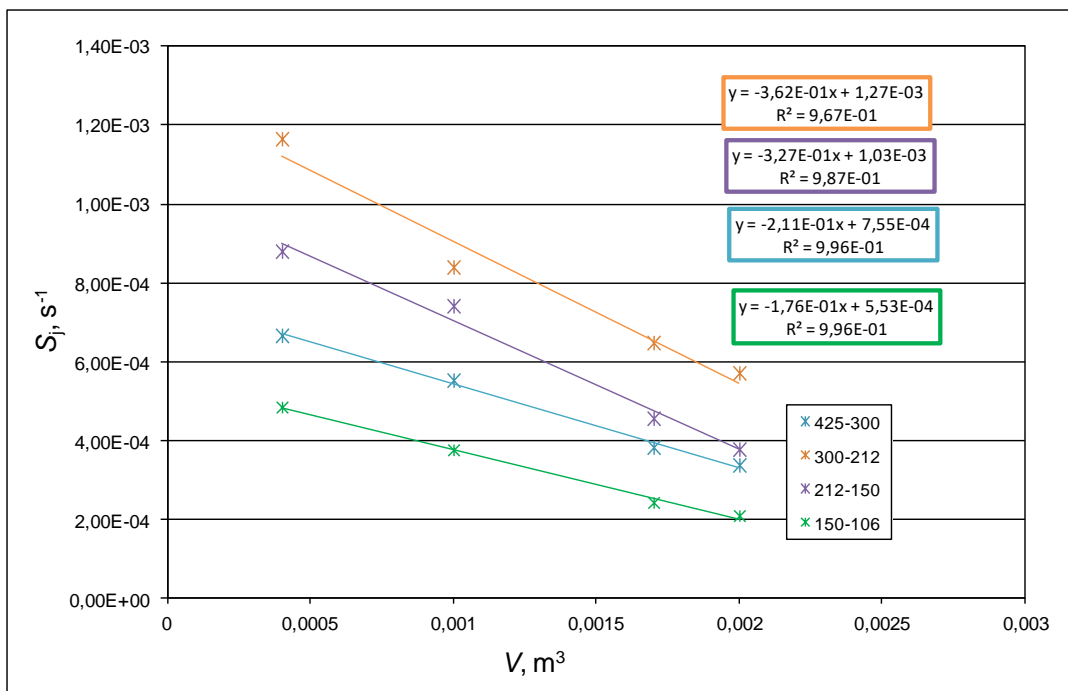


Figure 5. Dependence of the experimentally obtained values of the selection function on the volume of the mill drum; intervals M5, M6, M7 i M8

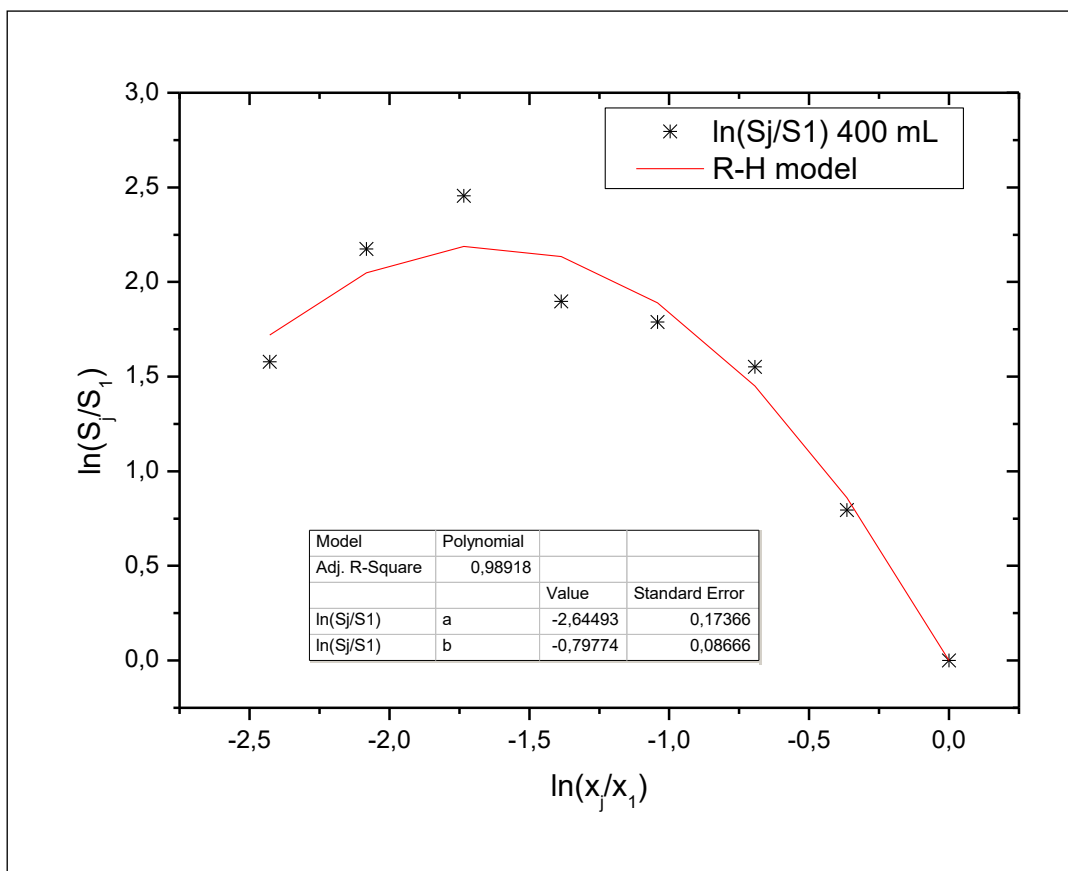


Figure 6. Describing the specific fracture velocity by the Rajamani-Herbst model, mill volume 400 mL.

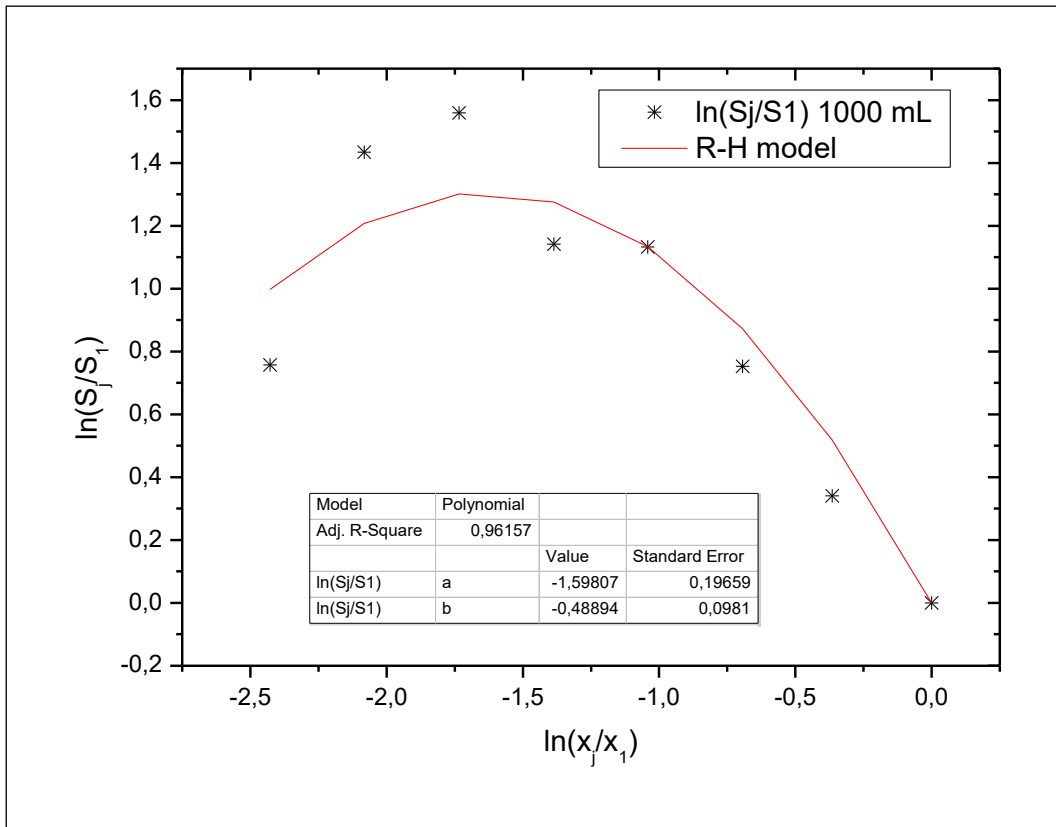


Figure 7. Describing the specific fracture velocity by the Rajamani-Herbst model, mill volume 1000 mL.

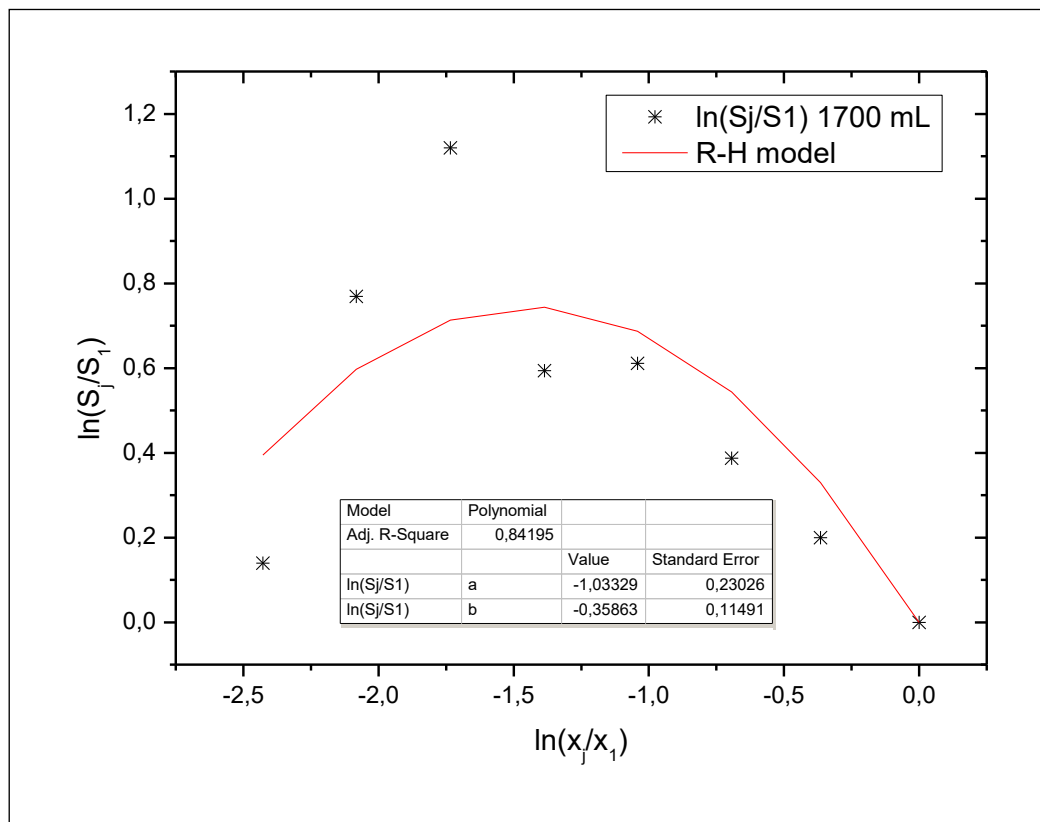


Figure 8. Describing the specific fracture velocity by the Rajamani-Herbst model, mill volume 1700 mL.

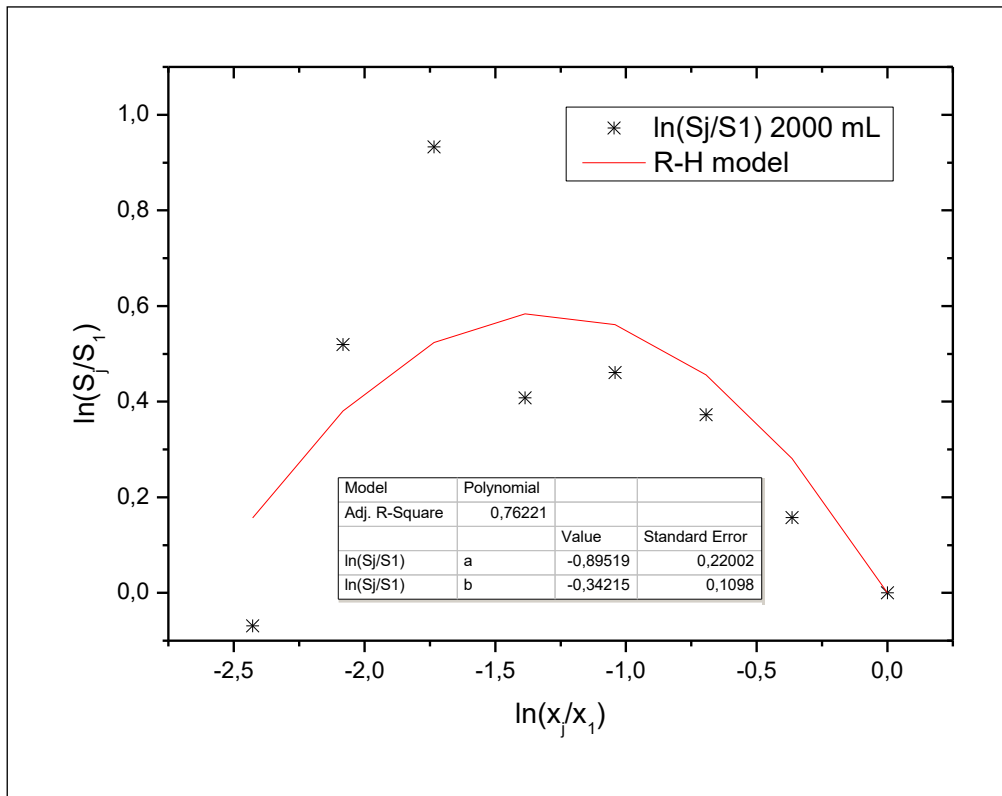


Figure 9. Describing the specific fracture velocity by the Rajamani-Herbst model, mill volume 2000 mL.

For many researchers, the focus of the work is on development and application of suitable grinding models, due to both widespread use and high energy intensity. By knowing the selection function and the distribution function of the breakage products, it is possible to predict the property of the resulting product and transfer the results to a larger scale. The samples were divided into eight particle size intervals. The study of grinding kinetics was carried out in mills of different volumes (400, 1000, 1700, 2000 ml).

Based on the results, it can be seen that the proportion of unbroken material in the initial interval changes linearly with time. (Fig.2) Which means that the kinetics of dolomite grinding in a ball mill takes places according first- order breakage kinetics. Bigger ball mills are more efficient for coarse particles because coarser particles need the higher fracture energy possessed by the larger balls. Smaller ball mills have a larger surface and are more efficient for particles smaller than 0.212 mm. (Fig. 4,5) Namely, by increasing the diameter of the grinding balls, more efficient grinding of larger particles is achieved, since larger balls result in collisions of higher specific energies. [11]

With grinding, the proportion of cracks in particles is reduced and the grinding process slows down. The particles tend to break because they are smaller and more regular. Therefore, it is advisable to use more energy, because, in that way, the particles of a more regular shape will be broken as well. The values of R^2 show a deviation from the experimental and by the model obtained values. It was shown that at the 400 and 1000 mL mills, the R-H model describes the experimental data well. (Fig. 6, 7). However, by increasing the scale, a significant deviation occurs and a correlation coefficient of 0.7622 is by no means acceptable since it is considered that further magnification would lead to even greater deviations and inability to predict selection function. (Fig. 8, 9) Since, at all volumes, the maximum values of the selection function are obtained at the same particle size (212 μ m), it is expected to achieve this maximum with further magnification. We tried to use the Snow model which requires knowledge of the maximum value of the selection function and the size at which this is achieved. However, the results showed even greater deviations and the Snow model cannot be used. With the appropriate model, the coefficients of the model can be correlated with mill volume or other

sizes (diameter, diameter of the grinding body, power).

The values of the model parameters depend on the type of raw material and the grinding conditions, which makes this process complex. Therefore, no correlation has yet been established between model parameters in laboratory and industrial mills.

CONCLUSIONS

Following the process of dry grinding of dolomite in a ball mill, the linear dependence of the change in the mass share of the unbroken material over time was determined which indicates that dolomite grinding kinetics is a first order process.

The applicability of the Rajamani and Herbst models was investigated. The Rajamani and Herbst model can be used for estimation of the selection function under given process conditions. Model parameters can be correlated with mill diameter, balls diameter. According to the results obtained, it can be concluded that it is possible to estimate the model parameters and the selection function following the proposed methodology. The disadvantage of this model is that it is limited to mill diameter up to 30 mm. The inability to predict the change in model parameters with the change in the characteristics of the material which is grinded and the conditions of grinding greatly diminishes its practical value. Despite the great effort put into improving the grinding process, its inefficiency is still significant, and the process design and magnification are based more on experiential rules than theoretical and empirical models.

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COMPARISON OF BROWN RENDZINA AND COLLUVIAL SINKHOLE SOIL IN THE AREA OF THE NATIONAL PARK "UNA" WITH SPECIAL EMPHASIS ON THE DISTRIBUTION OF CADMIUM, NICKEL AND ARSENIC

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

The paper presents the results of a comparison of brown rendzina and colluvial sinkhole at approximately the same altitudes and the same land use. The sites were located in the Kalati area within the National Park „Una“. More precisely, the places referred to the top of the sinkhole (brown rendzina - top) and the bottom of the sinkhole (colluvial soil - bottom). The main objective of the research, in addition to the physical and chemical parameters of soil quality, was to determine the distribution of the total content of cadmium (Cd), nickel (Ni) and arsenic (As). The total content of these elements were observed in composite samples at two depths of 0 – 10 and 0 – 20 cm and horizons profile. Their total content was measured by atomic adsorption spectrometry – AAS. The results were statistically analyzed using Kruskal - Walisovog test at the significance level of $p \leq 0.05$ using correlation coefficient χ^2 . The results showed a single legality of the distribution of Cd, Ni and As in samples of soil profile, while the average soil samples showed unique legality of the distribution of observed elements.

KEYWORDS: brown rendzina, colluvial soil, distribution, cadmium, nickel, arsenic

INTRODUCTION

The entire area of the National Park is located in the Federation of BiH within the Una-Sana Canton and belongs to the municipality of Bihac.

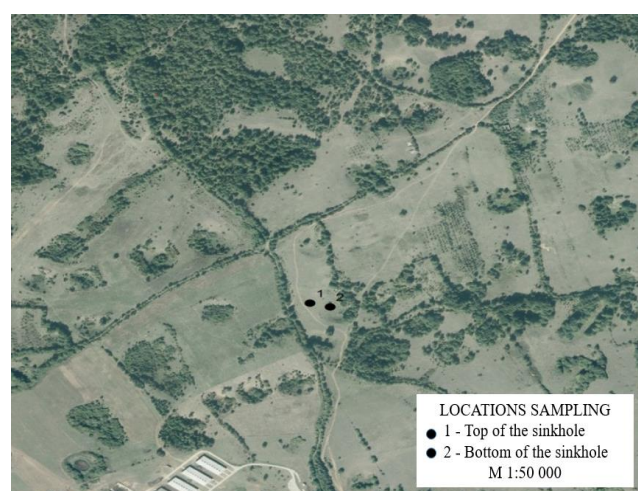


Figure 1. Locality Kalati in the National Park

National Park Una includes the area of the canyon part of the upper course of the river Una upstream from Lohovo, the area of the canyon part of the lower

course of the river Unac to its confluence with the river Una, and the interspace between Una and Unac on a total area of 19,800 ha [1]. National Park Una continues to arouse the interest of many scientists and researchers of various profiles [2]-[4]. There are researches especially from the aspect of distribution of toxic elements under the influence of wind, different way of land use and others [5]-[7]. However, the transfer factors of toxic elements can also be colluvial processes in sinkholes. The colluvial process is a geomorphological process that occurs under the direct action of gravity [8]. Sinkhole is the most diagnostic feature of karst terrains [9]. There are several types of sinkholes [10]. This paper presents the results of a comparison of different soil type variants, ie. the association of brown rendzina (top of the sinkhole – TOP) – the first variant and the colluvial soil of the sinkhole (bottom of the sinkhole – BOTTOM) – the second variant. These variants are on the same site are the same land use and with the same sampling depths of average samples. The study has placed a special emphasis on the distribution of the total content of cadmium (Cd), nickel (Ni) and arsenic (As) in the average samples (0 – 10 and 0 – 20 cm) and by depth of the soil profile. Both variants of the locality were

from the association of the soil of the brown rendzina with the colluvial soil of sinkholes, of different depths, approximately the same altitudes (501 – 509 m), the same pressure: 812,73 hPa, the same way of use – neglected natural pastures and different wind speed (TOP: 12 m/s, BOTTOM: 5 m/s). The appearance of the place is shown in Figures 1.

According to pedological maps of BiH [11] and pedological interpreters (Bihać 3), soils in selected localities alternate by different types (variants) and depths. Lighter mechanical composition (TOP). In the surface layers, the mechanical composition is lighter, and at the bottom it is heavier (BOTTOM). The soils are weakly humus, poor in phosphorus (P_2O_5) and calcium carbonate ($CaCO_3$), potassium is medium (K_2O). Water capacity (K_w) is medium for air (K_a) is good. Their color is usually brown and yellow-brown.

MATERIALS AND METHODS

This paper presents results of the field research, sampling and laboratory testing. All analyzes of soil samples were determined according to standardized methods: Mechanical composition (Modified method B pipettes, ISO 11277); Structure (Method by Sekera); The rights specific gravity, Porosity (Conventional method, ISO 11508); The volume specific gravity (Gravimetric method by Kopecky, ISO 11272); Actual humidity, Capacity of soil for water and Air capacity (Gravimetric method, ISO 11465); Humus (Spectrophotometric method, ISO 10694); $CaCO_3$ (Volumetric method ISO 10693); pH (Electrometric method, ISO 10390); El.conductivity (Electrometric method, ISO 11265); P_2O_5 and K_2O (Al method, ISO 19730) and The total content of metals and metalloids in the soil (Extraction $HCl/HNO_3 = 3:1$, ISO 11466). A total of two pedological profiles were opened at the sites of research and average samples were taken from two soil depths: 0 – 10 cm and 0 – 20 cm.

Preparation of soil samples was carried out at the Biotechnical Faculty of the University of Bihać. All analyses of soil samples were carried out in the laboratory of the Institute of Soil Science, Agrochemistry and Reclamation of the Faculty of Agricultural and Food Sciences, University of Sarajevo, except for the content of arsenic that was carried out at the Faculty of Agriculture, University of Zagreb, and the Institute for Medical Research and Occupational Health in Zagreb.

Statistical analysis was performed by the Kruskal - Wallis test at the level of significance of $p \leq 0.05$. To determine the relation and distribution of Cd, Ni and

As in the soil we used the correlation coefficient (χ^2) [12]. Data were statistically analyzed using SPSS 17. The maximum allowable amounts of total contents were determined in accordance with the current regulations [13] in relation to the soil texture (MRL) and the use of soil in organic farming (MRL-OF). Used are orthophoto imagery in the scale of 1:5000 and 1:75000, topographic maps and GIS.

RESULTS AND DISCUSSION

Results of the study on the comparison of different types (variants) of soil provide:

Description of the soil profile of the brown rendzina at the top of the sinkhole (TOP) (Figure 2)

Site: Kalati – brown rendzina (top of the sinkhole – TOP) (N 44° 34.066', E 16° 03.434')

Vegetation: natural pasture

Use: pasture

Parent substrate: dolomite

Ah 0-40 cm: surface layer medium overgrown with grass roots, dark brown in color, coarsely crumbly structure and according to the texture – clay loam (sand = 28,7 %, powder = 40,5 %, clay = 30,8 %). Skeleton present.

C > od 40 cm: coarse dolomite

Description of the colluvial soil profile of the sinkhole (BOTTOM) (Figure 3)

Site: Kalati – colluvial soil (bottom of the sinkhole – BOTTOM) (N 44° 34.063', E 16° 03.448')

Vegetation: natural pasture

Use: pasture

Parent substrate: dolomite

I 0-19 cm: layer overgrown with grass roots and weeds, dark brown in color, finely crumbly structure. Texture – powdery clay loam (sand = 16.6 %, powder = 43.5 %, clay = 39.9 %)

II 19-42 cm: in the layer still evident roots of weed plants, dark yellow color, layer moist, crumbly structure and texture – powdery clay (sand = 15.3 %, powder = 41.5 %, clay = 43.2 %).

III 42-64 cm: yellow-brown color, pea-shaped structure, the layer dries a little in relation to the upper one, evident smaller parts of the skeleton with a diameter of about 2 cm, texture – powdery clay to clay (sand = 13.3 %, powder = 39.3 %, clay = 47.4 %).

IV 64-120 cm: yellow-brown color, layer moist and sticky, visible larger parts of the skeleton 5 - 8 cm, texture – clay (sand = 11.7 %, powder = 34.7 %, clay = 53.6 %).



Figure 2 – 3. Profiles of soil at the sites Kalati TOP (left) and BOTTOM (right)

Physical properties of soil at the sites Kalati of TOP and BOTTOM

The stability of the structural aggregates in the depth of the profile was very good to good. The mechanical composition in the surface layer was clay loam (TOP) and powdery clay loam (BOTTOM), in

deeper layer – clay (BOTTOM). The soils were porous, with medium soil water and moderate soil air capacities.

Mean values of real and bulk specific density also indicated that the soils were porous. Overview of the physical properties of the soil is provided in Table 1.

Table 1. The physical properties of the soil profile ($\Sigma n_{\text{TOP-BOTTOM}} = 18$)

Parameters		$X_{mv} \pm X_{se}$	min	max	V	σ
S	TOP	1.50 ± 0.22	1.00	2.00	0.30	0.54
	BOTTOM	2.33 ± 0.31	1.00	4.00	1.07	1.07
P_{sg} (g/cm ³)	TOP	2.65 ± 0.00	2.65	2.66	0.00	0.01
	BOTTOM	2.65 ± 0.01	2.62	2.68	0.00	0.02
V_{sg} (g/cm ³)	TOP	1.26 ± 0.02	1.22	1.30	0.00	0.03
	BOTTOM	1.17 ± 0.31	1.01	1.28	1.09	1.09
P (%)	TOP	50.13 ± 0.59	49.00	51.00	1.05	1.02
	BOTTOM	54.95 ± 0.86	50.0	59.90	8.26	2.87
K_w (%)	TOP	41.73 ± 0.29	41.30	42.30	0.26	0.51
	BOTTOM	43.66 ± 0.80	40.60	47.70	7.13	2.67
K_a (%)	TOP	9.40 ± 0.11	9.20	9.60	0.04	0.20
	BOTTOM	11.29 ± 0.76	8.00	16.40	6.49	2.54

TOP – brown rendzina; BOTTOM – colluvial soil; X_{mv} – mean value, X_{se} – statistical error, min – minimum value, max – maximum value, V – variance, σ – deviation, n – number of samples, S – soil structure, P_{sg} – the rights specific gravity, V_{sg} – the volume specific gravity, P – porosity, K_w – capacity of soil for water, K_a – air capacity

Chemical properties of the soil profile and average samples 0 – 20 cm at the sites Kalati of TOP and BOTTOM

In open profiles the reactions of pH values for the TOP and BOTTOM of the sinkhole ranged from neutral to moderately alkaline. In the surface layer, the contents of K_2O ranged from good to poor supply (TOP) and from medium to poor (BOTTOM). The supply of P_2O_5 was generally weak (TOP and BOTTOM). The humus content was moderate to weak

(3.9 to 1.9%). Total content of $CaCO_3$ was low with a tendency to decrease with profile depth. Mean values of measured electro-conductivity of the soil were not indicative of soil salinity at the profile.

Results of the observed chemical parameters in samples (0 – 20 cm) had similar characteristics as the parameters of the soil profile of the surface layer (Table 2 – 3).

Table 2. The chemical properties of the soil profile ($\Sigma n_{TOP-BOTTOM} = 18$)

Parameters		$X_{mv} \pm X_{se}$	min	max	V	σ
pH _{H2O}	TOP	7.61 ± 0.22	6.73	8.12	0.30	0.55
	BOTTOM	7.85 ± 0.09	7.15	8.12	0.10	0.33
pH _{KCl}	TOP	6.39 ± 0.20	5.60	6.90	0.24	0.49
	BOTTOM	6.55 ± 0.07	5.97	6.72	0.06	0.25
EC ($\mu\text{S}/\text{cm}$)	TOP	142.61 ± 5.13	126.6	161.3	158.21	12.57
	BOTTOM	153.88 ± 3.71	131.5	175	165.56	12.86
K ₂ O (mg/100g soil)	TOP	11.10 ± 2.28	6.50	20.10	31.2	5.58
	BOTTOM	10.50 ± 0.64	7.20	15.50	4.96	2.22
P ₂ O ₅ (mg/100g soil)	TOP	1.38 ± 0.17	0.88	1.85	0.17	0.41
	BOTTOM	1.92 ± 0.20	0.77	3.04	0.5	0.71
Humus (%)	TOP	2.88 ± 0.37	1.89	3.90	0.84	0.92
	BOTTOM	2.60 ± 0.18	1.64	3.50	0.38	0.62
CaCO ₃ (%)	TOP	0.22 ± 0.04	<0.01	0.23	0.01	0.10
	BOTTOM	0.24 ± 0.13	<0.01	1.47	0.47	0.22

TOP – brown rendzina; BOTTOM – colluvial soil, n – numer of samples, X_{mv} – mean value, X_{se} – statistical error, min – minimal value, max – maximum value, V – variance, σ – deviation

Table 3. The chemical properties of the average samples 0 – 20 cm ($\Sigma n_{TOP-BOTTOM} = 18$)

Parameters		$X_{mv} \pm X_{se}$	min	max	V	σ
pH _{H2O}	TOP	7.13 ± 0.12	6.63	7.40	0.09	0.30
	BOTTOM	7.23 ± 0.18	6.76	7.84	0.20	0.45
pH _{KCl}	TOP	6.00 ± 0.14	5.48	6.32	0.13	0.36
	BOTTOM	6.16 ± 0.20	5.57	6.85	0.25	0.5
K ₂ O (mg/100g soil)	TOP	16.06 ± 2.21	10.50	23.80	29.39	5.42
	BOTTOM	24.25 ± 4.30	14.20	35.80	111.1	10.5
P ₂ O ₅ (mg/100g soil)	TOP	4.10 ± 1.39	1.28	9.54	11.64	3.41
	BOTTOM	2.01 ± 0.33	1.19	3.14	0.69	0.83
Humus (%)	TOP	3.50 ± 0.28	2.84	4.28	0.49	0.69
	BOTTOM	4.00 ± 0.74	0.63	5.70	3.33	1.82
CaCO ₃ (%)	TOP	0.23 ± 0.27	0.00	0.71	0.14	0.37
	BOTTOM	0.27 ± 0.31	0.00	0.96	0.17	0.41

TOP – brown rendzina; BOTTOM – colluvial soil; n – numer of samples; X_{mv} – mean value; X_{se} – statistical error; min – minimal value; max – maximum value; V – variance; σ – deviation

Distribution of the total contents of Cd, Ni and As in average samples

In average samples at the sites of TOP and BOTTOM (0 – 10 and 0 – 20 cm), a significant difference ($p \leq 0.05$) in the total content of Cd, Ni and As was established (Table 4).

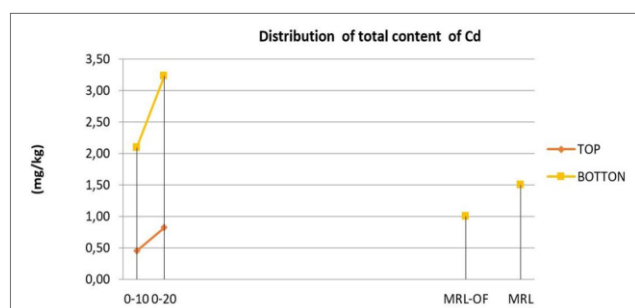
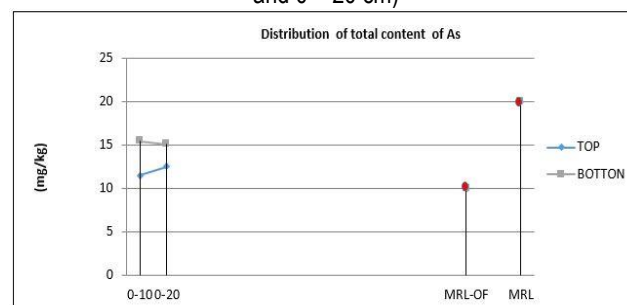
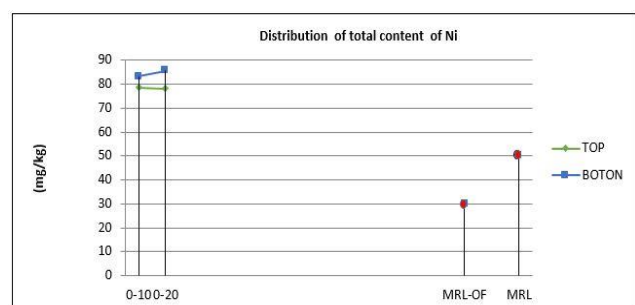
In the average samples at the sites TOP and BOTTOM (0 – 10 and 0 – 20 cm), the total mean contents Cd was lower than MRL and MRL-OF (TOP) but higher than MRL and MRL-OF (BOTTOM), the total mean contents As was between (TOP and BOTTOM) while the total mean contents Ni was above the allowable values of MRL and MRL-OF (TOP and BOTTOM). At the sites TOP and BOTTOM in depth

0 – 10 cm ($\text{Cd}_{TOP} = 0.45$ mg/kg; $\text{Cd}_{BOTTOM} = 2.09$ mg/kg; $\text{Ni}_{TOP} = 78.58$ mg/kg; $\text{Ni}_{BOTTOM} = 83.11$ mg/kg; $\text{As}_{TOP} = 11.49$ mg/kg; $\text{As}_{BOTTOM} = 15.49$ mg/kg), and the sites TOP and BOTTOM in depth 0 – 20 cm ($\text{Cd}_{TOP} = 0.82$; $\text{Cd}_{BOTTOM} = 3.23$; $\text{Ni}_{TOP} = 78.24$ mg/kg; $\text{Ni}_{BOTTOM} = 85.54$ mg/kg; $\text{As}_{TOP} = 12.54$ mg/kg; $\text{As}_{BOTTOM} = 15.13$ mg/kg). Generally, the total content of analyzed elements at the bottom of the sinkhole was higher compared to the soils at the top of the sinkhole, which confirms the fact of possible deposition due to colluvial processes or carrying soil to the bottom of the sinkhole due to gravity. See in the charts 1 – 3.

Table 4. Distribution of the total contents of Cd, Ni and As in average samples

Observed		$X_{mv} \pm X_{se}$	min	max	V	σ	χ^2	p
Depth 0 – 10 cm ($\Sigma n = 9$)								
Cd (mg/kg)	TOP	$0,45 \pm 0,02$	0,43	0,49	0,00	0,03	3,97	$p \leq 0,05$
	BOTTOM	$2,09 \pm 0,00$	2,09	2,10	0,00	0,01		
Ni (mg/kg)	TOP	$78,58 \pm 0,09$	78,40	78,68	0,02	0,15	3,97	$p \leq 0,05$
	BOTTOM	$83,11 \pm 0,02$	83,10	83,15	0,00	0,02		
As (mg/kg)	TOP	$11,49 \pm 0,02$	11,45	11,52	0,00	0,03	3,97	$p \leq 0,05$
	BOTTOM	$15,49 \pm 0,01$	15,47	15,50	0,00	0,01		
Depth 0 – 20 cm ($\Sigma n = 9$)								
Cd (mg/kg)	TOP	$0,82 \pm 0,16$	0,43	1,20	0,16	0,40	8,36	$p \leq 0,05$
	BOTTOM	$3,23 \pm 0,51$	2,09	4,44	1,57	1,25		
Ni (mg/kg)	TOP	$78,24 \pm 0,15$	77,90	78,68	0,14	0,38	8,36	$p \leq 0,05$
	BOTTOM	$85,54 \pm 1,08$	83,10	88,00	7,04	2,65		
As (mg/kg)	TOP	$12,54 \pm 0,47$	11,45	13,60	1,32	1,15	8,36	$p \leq 0,05$
	BOTTOM	$15,13 \pm 0,16$	14,74	15,50	0,15	0,39		

TOP – brown rendzina; BOTTOM – colluvial soil; n – numer of samples; X_{mv} – mean value; X_{se} – statistical error; min – minimal value; max – maximum value; V – variance; σ – deviation; χ^2 – Kruskal – Wallis coefficient; p – level of significance

**Chart 1.** Distribution of the total content of Cd (0 – 10 and 0 – 20 cm)**Chart 2.** Distribution of the total content of Ni (0 – 10 and 0 – 20 cm)**Chart 3.** Distribution of the total content of As (0 – 10 and 0 – 20 cm)

Distribution of the total content of Cd, Ni and As in the soil profiles

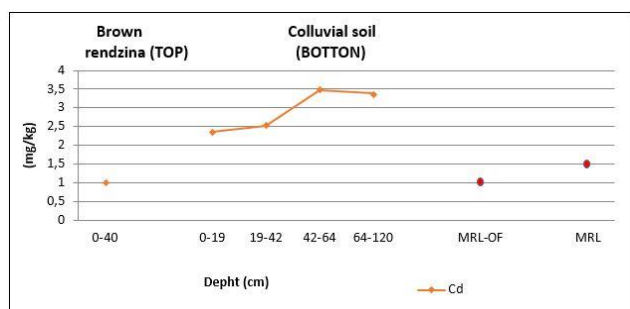
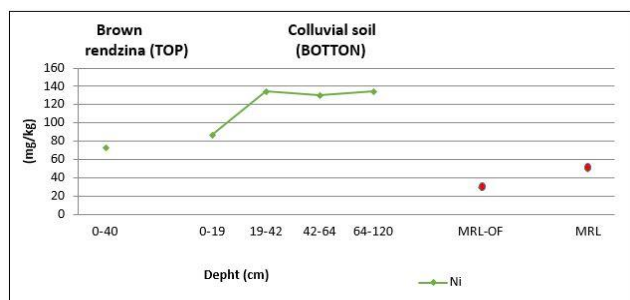
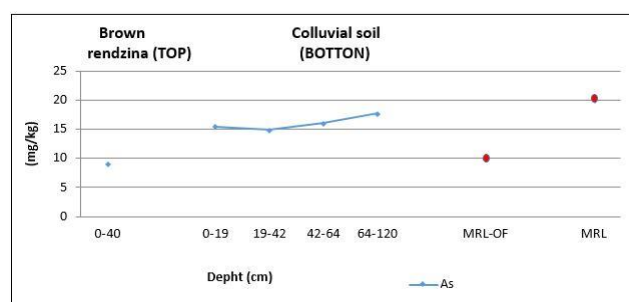
Results of the analysed profiles TOP and BOTTOM sites of the same use (natural pasture) have shown significant differences ($p \leq 0.05$) in concentrations of Cd, Ni and As (Table 5).

Table 5. Distribution of the total content of Cd, Ni and As in the soil profiles ($\Sigma n_{TOP-BOTTOM} = 18$)

Observed		$X_{mv} \pm X_{se}$	min	max	V	σ	χ^2	p
Cd (mg/kg)	TOP	0.99 ± 0.06	0.85	1.15	0.02	0.14	11.41	$p \leq 0.05$
	BOTTOM	2.94 ± 0.15	2.35	3.50	0.27	0.52		
Ni (mg/kg)	TOP	72.37 ± 1.99	67.70	76.90	23.76	4.87	11.36	$p \leq 0.05$
	BOTTOM	122.35 ± 6.20	87.10	138.21	462.13	21.49		
As (mg/kg)	TOP	9.01 ± 0.67	7.45	10.58	2.69	1.64	11.38	$p \leq 0.05$
	BOTTOM	16.35 ± 0.38	14.90	18.30	1.79	1.34		

TOP – brown rendzina; BOTTOM – colluvial soil; n – numer of samples; X_{mv} – mean value; X_{se} – statistical error; min – minimal value; max – maximum value; V – variance; σ – deviation; χ^2 – Kruskal – Wallis coefficient; p – level of significance

Observed in general in the examined profiles at the TOP and BUTTOM sites, the total mean contents of Cd, Ni and AS in the profile at the bottom of the sinkhole was higher compared to the soil profile at the top of the sinkhole (Chart 4 - 6). In the examined profiles, the total mean Cd content was 66% higher, nickel 40% higher, and arsenic 46% higher at the bottom of the sinkhole (colluvial soil) compared to its top (brown rendzina). The total contents of Cd and Ni tended to increase and exceeded the allowable values (MRL and MRL-OF) while the content of As was between MRL and MRL-OF.

**Chart 4.** Distribution of the total content of Cd in soil profiles**Chart 5.** Distribution of the total content of Ni in soil profiles**Chart 6.** Distribution of the total content of As in soil profiles

CONCLUSIONS

At the sites of TOP and BOTTOM, according to the physical and chemical parameters of soil, stability of structural aggregates declines from good to very good; mechanical composition of the soil in the surface layer – clay loam (TOP) and powdery clay loam (BOTTOM), and in the deeper ones – clay (BOTTOM). The soils were porous, with medium soil water and moderate soil air capacities. In general, the pH values at the sites of TOP and BOTTOM ranged from neutral to moderately alkaline ($pH_{TOP \text{ in } H_2O}$ 6.73 – 8.12; $pH_{TOP \text{ in } KCl}$ 5.60 – 6.90; $pH_{BOTTOM \text{ in } H_2O}$ 7.15 – 8.12; $pH_{BOTTOM \text{ in } KCl}$ 5.97 – 6.72) and average samples ($pH_{TOP \text{ in } H_2O}$ 6.63 – 7.40; $pH_{TOP \text{ in } KCl}$ 5.48 – 6.32; $pH_{BOTTOM \text{ in } H_2O}$ 6.76 – 7.84; $pH_{BOTTOM \text{ in } KCl}$ 5.57 – 6.85).

In the average samples (0–10 and 0–20 cm) at the sites, total mean content of Cd at the top of the sinkhole was lower than the permitted levels of MRL-OF and MRL, but at the bottom of the sinkhole the Cd content was higher than the permitted levels ($Cd_{TOP} = 0.43 - 1.20$ mg/kg; $Cd_{BOTTOM} = 2.09 - 4.44$ mg/kg).

The total mean content of Ni at the top and bottom of the sinkhole was above the permitted levels ($Ni_{TOP} = 77.90 - 78.68$ mg/kg; $Ni_{BOTTOM} = 83.10 - 88.00$ mg/kg), while the total mean content As was between the permitted levels ($As_{TOP} = 11.45 - 13.60$ mg/kg; $As_{BOTTOM} = 14.74 - 15.50$ mg/kg).

In the surface layer, content of P_2O_5 was low, while the K_2O content ranged from weak to moderate as in humus. Total content of $CaCO_3$ was low with a tendency to decrease with profile depth ($CaCO_{3TOP} < 0.01 - 0.23\%$; $CaCO_{3BOTTOM} < 0.01 - 1.47\%$). The measured levels of soil electrical conductivity were not indicative of the soil salinity ($EC_{TOP} 126.6 - 161.3 \mu S/cm$; $EC_{BOTTOM} 131.5 - 175.0 \mu S/cm$).

In the distribution of Cd, Ni and As in average samples (0 – 10 and 0 – 20 cm) at the top and bottom of the sinkhole, it was found that there is a significant difference ($p \leq 0.05$) in the total content of Cd, Ni and As.

In general, in the average samples (0 – 10 and 0 – 20 cm) there was a unique tendency to distribute (increase) the total content of Cd, Ni and As at the top and bottom of the sinkhole. This confirms the fact that due to colluvial processes, Cd, Ni and As are deposited at the bottom of the sinkhole. In the profiles at the sites (TOP and BOTTOM), significant differences ($p \leq 0.05$) were determined in the total content of Cd, Ni and As. At the sampling sites at Kalati at the top and bottom of the sinkhole, it was found that the total mean content of Cd, Ni and As at the bottom of the sinkhole was higher compared to the top of the sinkhole, with Cd by 79% with Ni by 9% and As by 9%.

In this research in the area of the National Park „Una“, a possible accumulation of Cd, Ni and AS was determined, which takes place under the influence of colluvial processes. Colluvial processes under the influence of gravity or relief carry the surrounding material from the top of the sinkhole to the bottom of the sinkhole.

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SELECTIVE SEPARATION AND REMOVAL OF LEAD(II) ION FROM MULTICOMPONENT SYSTEM THROUGH BLMs SUPPORTED BY FATTY ACIDS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Selective separation and removal of a metal species from complex matrices of other species is of critical importance in separation sciences and especially in industrial processes. The selectivity and efficiency of Pb(II) ion removal from an aqueous solution in the presence of Ni(II), Zn(II), Co(II), Cu(II), and Cd(II) ions as competing ions in a multi-component aqueous source phase through bulk liquid membrane system were investigated. The influence of the palmitic and oleic acid as supporting surfactants in the membrane phase on the removal of Pb(II) ions was also analyzed. Dicyclohexano-18-crown-6 dissolved in chloroform, proved to be a selective and efficient carrier for Pb(II) ions. In the optimized procedure, 3 hours of equilibrium time was required to achieve the maximum removal efficiency. It was found, that removal of Pb(II) ions was achieved without significant reduction in the efficiency compared to the liquid membrane transport of a single-component system. None of these cations interfered with Pb(II) removal. The presence of the oleic and palmitic acid in the membrane phase increase the content of Pb(II) removed to the receiving phase. In optimal transport conditions, more than 97% efficiencies for the removal of Pb(II) were obtained for a system supported by oleic acid. The excellent efficiency and high degree of selectivity for the Pb(II) ion removal by the proposed BLM system, reveals its potential application for the selective removal, concentration, and purification of lead ions from its different mixtures.

KEYWORDS: liquid membrane transport; Pb(II) ion removal; dicyclohexano-18-crown-6; oleic acid; palmitic acid

INTRODUCTION

Lead, Pb(II) is a toxic metal that is mainly introduced into the environment through various industrial applications such as batteries, fuels, paints, constructions, and foundries. Because of its toxicity and non-biodegradable nature, controlling lead in the environmental samples is of major importance [1]. Due to the increasing industrial use of lead and its serious hazardous effect on human health, the investigation of new methods for selective removal, concentration, and purification of Pb(II) ion from its mixtures is of special interest. A variety of separation methods such as chemical precipitation, ion exchange, adsorption, membrane filtration, coagulation-flocculation, flotation, solvent extraction, and liquid membrane transport have been utilized to remove toxic heavy metal ions from the environmental and biological systems [2]. New technologies in wastewater treatment are precisely membrane processes. Liquid membrane is known as a green technology because of its green characteristics such as being eco-friendly and its low consumption of organic

solvent [3]. A liquid membrane system involves an organic liquid membrane that serves as a semipermeable barrier between two aqueous phases, the source phase (SP) and receiving phase (RP). Macrocycles as extractants were successfully used for metal ions separation in solvent extraction and as ion carriers in transport through liquid membranes. Along with cryptands, crown ethers are an important class of ion carriers [4,5]. With regard to molecular recognition and selective binding, macrocyclic and chelating ligands are extensively studied in the transport of alkali and alkaline earth metal cations across liquid membranes [6,7]. The interaction of the ligand and metal cation depends on the nature of the metal cation but also depends on the number, distance, and orientation of the donor atoms of the ligand that are structurally accessible to the complexed cation [8]. The Pb(II) ions are removed from the source phase (SP) to the receiving phase (RP) via a membrane phase (MP). The movement of charged species (carrier-metal complex) through the hydrophobic liquid membrane is accomplished by the presence of a large

lipophilic anion, such as picrate, in the source phase [9]. In this technique, the extraction and stripping steps are combined into a one-step process and, due to its simplicity, low cost, and efficiency, it plays an important role in biology, chemistry, and separation sciences [10,11,12].

Surfactant systems have been recognized as very useful alternatives for improving analytical methodologies and development of new concepts in analytical chemistry [13]. The addition of a long chain fatty acid, reduces the degree of carrier loss and also could have a cooperative effect in the uphill transport of the metal cation through the ligand membrane. A possible reason for this cooperative behavior would be the existence of some proton donor–acceptor interaction between the lipophilic fatty acid (as proton donor) and the donor atoms of the ligand (as proton acceptor), which can impart a greater degree of lipophilicity to the ligand–metal ion complex, in order to facilitate the cation transport through the liquid membrane [14]. It has been shown that the addition of long chain fatty acids to the membrane phase could not only overcome the carrier loss but also provide a cooperative effect in the uphill transport of metal ions through the liquid membrane via imparting a greater lipophilicity to the carrier species [15]. It is well-known that the transport selectivity of mixture ions are significantly dependent on the nature of the macrocyclic ligand and fatty acids.

In this paper, we studied the efficiency of Pb(II) removal from an aqueous solution in the presence of Ni(II), Zn(II), Co(II), Cu(II), and Cd(II) ions as competing ions in a multi-component aqueous source phase through the bulk liquid membrane system. We propose an environmental removal technique for heavy metal ions based on selective and spontaneous transfer supported by fatty acids. In the presence of picrate ion as a hydrophobic counter ion in the source phase and disodium-EDTA ion as a selective stripping agent and Triton X-100 as a suitable synergistic co-stripping agent in the receiving phase, the Pb(II) ion found to transport selectively and quantitatively across the chloroform bulk liquid membrane in 3 h. The addition of fatty acids to the membrane phase leads to better transport efficiency.

MATERIALS AND METHODS

For every transport experiment, two aqueous solutions and one non-aqueous solution (membrane), were prepared, as follows.

Source solutions were prepared from:

- Standard Pb(II), Ni(II), Zn(II), Co(II), Cu(II) and Cd(II) solution (1000 mg/L), Merck

- Picric acid ($C_6H_3N_3O_7$), $c = 1 \cdot 10^{-3}$ mol/L, 99%, Kemika
- Formic acid buffer solution (pH=3), prepared from HCOOH and NaOH (g.r., Merck)

Membrane solution were prepared from:

- Organic solvents: chloroform ($CHCl_3$)
- Macrocyclic ligands: dicyclohexano-18-crown-6 (DCH18C6), 18-crown-6 (18C6)
- Fatty acids: oleic, palmitic, stearic acids

Receiving solution were prepared from:

- Triton X-100 surfactant ($1,4 \cdot 10^{-3}$ mol/L)
- Disodium-EDTA ($1 \cdot 10^{-3}$ mol/L)
- Acetate buffer solution (pH=5), prepared from CH_3COOH (purris. p.a., Fluka) and NaOH (g.r., Merck)

TRANSPORT PROCEDURE

Cylindric glass container, i.e. "transport cell", with inner diameter of 5 cm and central glass tube (2 cm in diameter), have been used for this study.

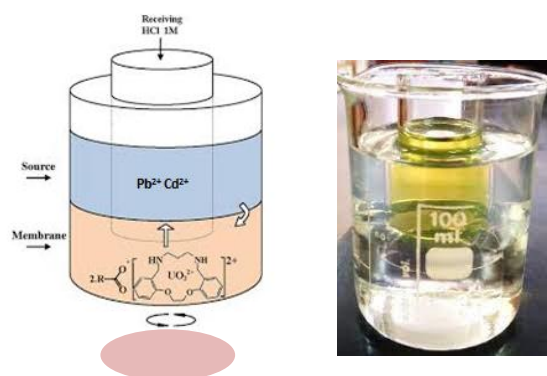


Figure 1. a) Scheme of a cylindrical glass vessel, "Transport Cell"; b) Homemade transport cell used in experiments [16]

Central tube provides physical separation of two aqueous phases: source phase (SP), and receiving phase (RP). Membrane phase (MP) lies under the aqueous phases and connects them. The source phase (SP) contained 10 mL of a mixture of tested Pb(II) ions ($1 \cdot 10^{-3}$ mol/L, $1 \cdot 10^{-4}$ mol/L) and metal cations Ni(II), Zn(II), Co(II), Cu(II), Cd(II) ($1 \cdot 10^{-3}$ mol/L, $1 \cdot 10^{-4}$ mol/L). The receiving phase (RP), which is outside the central tube, contained a stripping agent. The membrane phase (MP) contained a suitable ligand ($1 \cdot 10^{-3}$ mol/L) dissolved in organic solvent; the membrane layer lies beneath the aqueous phases and connects them. The membrane phase is mixed with a magnetic stirrer so that under these conditions the contact surfaces between the aqueous phases are

straight and precisely defined [17]. pH measurements of aqueous solutions were performed using the pH meter (GLP31 Crison Instruments). Quantification of metal ions removed during the transport experiments was obtained by Flame Atomic Absorption Spectrometry technique, using the instrument Perkin Elmer AAnalyst 200.

RESULTS AND DISCUSSION

The results of the measured content of removed Pb(II) ions in the final phase, for the among different mixture, was tested by performing the transport of Ni(II), Zn(II), Co(II), Cu(II), Cd(II) metal cations containing Pb(II) ions in their mixtures. From the results shown in Figure 1. there is no serious interference and a significant reduction in the efficiency compared in the transport process of Pb(II) ions, from different mixtures with the liquid membrane transport of a single-component system. The highest efficiency removed Pb(II) ions was achieved for a single-component system, approximately 60 % removed Pb(II) ions compared to other mixtures of metal ions. Dicyclohexano-18-crown-6 (DC18C6) due to its very lipophilic character and its corresponding cavity size for selective complexation with Pb(II) ion proved to be a selective and efficient carrier for Pb(II) removal via BLM.

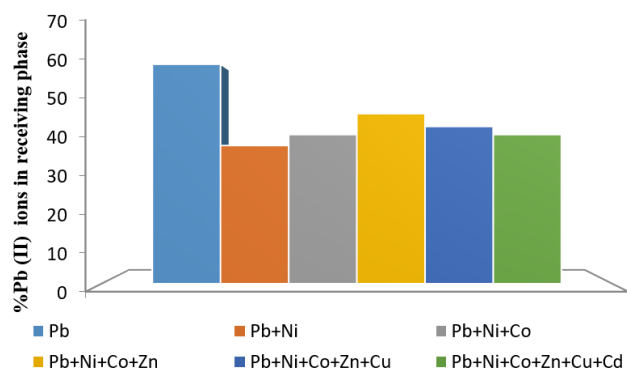


Figure 2. Amount of Pb(II) ions removed from various mixtures through BLMs; SP: $1 \cdot 10^{-3}$ mol/L mixture of metal ions and $1 \cdot 10^{-3}$ mol/L picrates; MP: organic solvent chloroform contained $1 \cdot 10^{-3}$ mol/L of DCH18C6; RP: Triton X-100 surfactant ($1,4 \cdot 10^{-3}$ mol/L) disodium-EDTA ($1 \cdot 10^{-3}$ mol/L)

It was found that a major role of the fatty acids is to contribute to the transport process by increasing in lipophilicity of crown-cation complex through some proton-donor and proton-acceptor interactions between surfactant (as proton donor) and oxygen atoms of ligand (as proton acceptor) which can facilitate the cation transport through liquid membrane and also serve to inhibit any bleeding of partially

species from the organic membrane phase into either of the aqueous phases. To evaluate the influence of adding a fatty acids on Pb(II) ion removal; stearic, palmitic and oleic acid were added into the membrane phase.

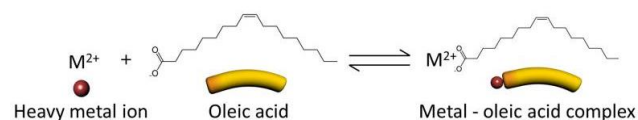


Figure 3. Schematic illustration of the spontaneous phase transfer-mediated selective removal of heavy metal ions using oleic acid

The influence of the palmitic and oleic acid as surfactant ($1 \cdot 10^{-3}$ mol/L) in the membrane phase containing a known concentration of the carriers; 18C6 and DCH18C6 ($1 \cdot 10^{-3}$ mol/L) and organic solvents (chloroform) on the removal of Pb(II) ions was also investigated. The results are summarized in Figure 4. As it is evident, the efficiency of Pb(II) ion transport increases in the presence of the surfactants with 18-crown-6 ether as a ligand for metal ions in chloroform as liquid membrane. But, in the case with DCH18C6 as a ligand for metal ions, the transport decreases in the presence of the surfactants, therefore, the cooperative behavior in the presence of these long-chain hydrocarboxylic acids is decreased. This may be due to the formation of hydrogen bonds between the donor atoms of the ligand and the acidic proton of the carboxylic acids in these solvent systems which results in formation of a weaker complex between the Pb(II) ion and the ligand in the presence of these fatty acids. Another possibility is the formation of micelles of the fatty acids in the membrane phase which trap the ligand-cation complex and, therefore, the transport rate of Pb(II) decreases.

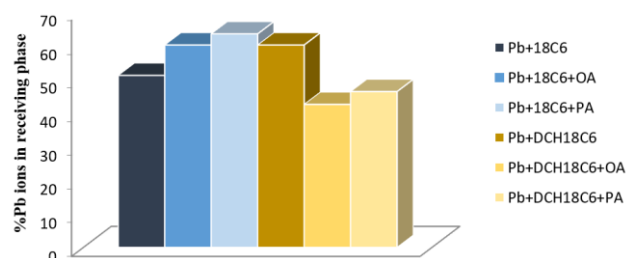


Figure 4. Comparison of the Pb(II) removal efficiencies in presence of oleic acid (OA) and palmitic acid (PA) (source phase: $1 \cdot 10^{-3}$ mol/L mixture of metal ions and $1 \cdot 10^{-3}$ mol/L picrates; organic solvent chloroform contained 18C6 and DCH18C6 ($1 \cdot 10^{-3}$ mol/L); receiving phase: Triton X-100 surfactant ($1,4 \cdot 10^{-3}$ mol/L) disodium-EDTA ($1 \cdot 10^{-3}$ mol/L)

The efficiency of Pb(II) removal from an aqueous solution in the presence of Ni(II), Zn(II), Co(II), Cu(II) ions as competing ions in a multi-component aqueous source phase through the bulk liquid membrane system in the presence of oleic acid were investigated. The effect of Pb(II) ions concentration in the source phase on Pb(II) ions removal under optimal experimental conditions was investigated and the results are shown in Figure 5. As seen, the removal efficiency is increased with decreasing concentration of Pb(II) ions in the source phase. It may be due to the carrier loading effect in the membrane [18]. Optimal transport conditions were obtained for a system with decreasing concentration supported by oleic acid. The excellent efficiency and high degree of selectivity for the Pb(II) ions showed a system with lowest concentration of Pb(II) ions in source phase with more than 97% removed Pb(II) ions (Figure 5).

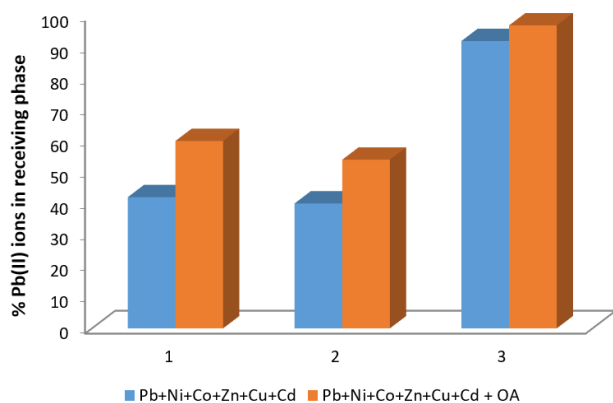


Figure 5. Efficiencies of the Pb(II) removal in presence of oleic acid (OA) for varying concentration of metal mixtures source phase: (1) $1 \cdot 10^{-3}$ mol/L, (2) $2,5 \cdot 10^{-4}$ mol/L and (3) $1 \cdot 10^{-4}$ mol/L M^{2+} ; organic solvent chloroform contained $1 \cdot 10^{-3}$ mol/L DC18C6 with oleic acid $1 \cdot 10^{-3}$ mol/L, receiving phase; Triton X-100 surfactant ($1,4 \cdot 10^{-3}$ mol/L) disodium-EDTA ($1 \cdot 10^{-3}$ mol/L)

Considering that the system with the lowest concentration of Pb (II) ions, achieved the highest level of removal of these metal ions, with DCH18C6 supported by oleic acid, (Figure 5), these results were compared with the same competitive transport systems in the presence of stearic and palmitic acid. Selective separation and removal of Pb (II) ions were efficient in BLM systems supported by this fatty acids. The efficiency of removed Pb (II) ions increases in the presence of fatty acids following the order: palmitic < stearic < oleic acid (65 < 77.4 < 97%).

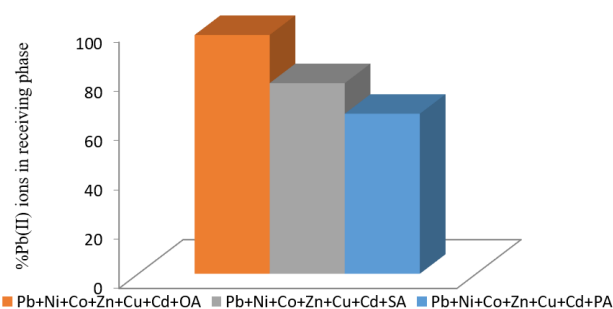


Figure 6. Efficiencies of the Pb(II) removal in presence of oleic acid (OA), palmitic acid (PA), stearic acid (SA) source phase, M^{2+} conc. ($1 \cdot 10^{-4}$ mol/L),

50 mL organic solvent chloroform contained $1 \cdot 10^{-3}$ mol/L DC18C6 with oleic, palmitic and stearic acid ($1 \cdot 10^{-3}$ mol/L); receiving phase: Triton X-100 surfactant ($1,4 \cdot 10^{-3}$ mol/L) disodium-EDTA ($1 \cdot 10^{-3}$ mol/L)

CONCLUSIONS

The BLMs experiments of Pb(II), Zn(II), Cu(II), Co(II), and Cd(II), metal cations using 18C6 and DCH18C6 as a carrier in organic membranes, supported by fatty acids, showed that it is possible to separate and removed Pb(II) ions from the different mixtures of metal ions. The transport rate of the Pb(II) cation is influenced by the nature and also the composition of the membrane solvents. Removal of Pb(II) ions from different mixtures were achieved without significant reduction in the efficiency compared to the liquid membrane transport of a single-component system. The results show that the transport rate of the Pb(II) ion increases in the presence of stearic acid, palmitic acid, and oleic acid as supported surfactants in the membrane phase. The simplicity, efficiency, and selectivity for Pb(II) ions demonstrate its potential applicability to selective removal, concentration, or purification of the Pb(II) ions from its different mixtures.

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COMPARISON AND QSAR STUDY OF THERMODYNAMIC pK_{BH^+} VALUES OF BENZOYLHYDRAZONES DETERMINED BY UV-VIS SPECTROSCOPY

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Thermodynamic dissociation constants of fifteen protonated aromatic hydrazones were determined by UV-Vis spectroscopic method, as one of the main methods for determination of pK_{BH^+} values of a weak acids and bases. The stoichiometric pK_{BH^+} values of investigated compounds were determined at three different ionic strengths (0.1, 0.25 and 0.5 mol/dm³) in acidic media (1 < pH < 7). The thermodynamic pK_{BH^+} values were evaluated graphically as an intercept with extrapolation to zero ionic strength of the curve which represents dependence of ionization constants on square root of ionic strength. The obtained results showed that there are no significant differences in the values of thermodynamic dissociation constants of investigated hydrazones with no substituent and methoxy group (-OCH₃) on hydrazide part of the molecule. The differences in the pK_{BH^+} values were noticed for hydrazones with nitro group (-NO₂) on the benzene ring of benzaldehyde. Nitro hydrazones have lower pK_{BH^+} values, probably due to the influence of the -NO₂ group present in their molecule. The obtained pK_{BH^+} values were in accordance with those for similar class of compounds. QSAR study has been made by correlation thermodynamic ionization constants with structure using various physicochemical parameters.

KEYWORDS: UV-Vis spectroscopy, benzoylhydrazones, protonation, thermodynamic dissociation constant, Characteristic Vector Analysis, QSAR, descriptors

INTRODUCTION

Hydrazones are organic compounds with an azomethine group (-NHN=CH-) in their structure. Due to the presence of this group hydrazones and their derivatives possess variety of different biological activities [1] - [5]. The introduction of different functional groups in the hydrazone molecules expands the scope of their application in organic synthesis. Because of that, there is a growing interest in the structural studies and synthesis of hydrazones as they show a broad spectrum of applications in different fields [6]. Various derivatives of hydrazones possess insecticidal activities and they are used as active ingredients for controlling agricultural pests [7]. Many chemical, physical and biological properties of organic compounds depend on the interactions of acidic and basic groups present in their molecule. As hydrazones and their derivatives have acidic and basic functional groups, their biological activity depends on the ionic form in which they exist in solution. The degree of ionization of molecules in solution at different pH values can be predicted knowing the

value of the ionization constants (pK_{BH^+}). The pK_{BH^+} is a physicochemical parameter which is important to know for developing new pharmaceutical drugs or improving the available ones [8]. Ionization constants have significant importance in optimization of analytical procedures such as acid - base titration, solvent extraction and complex formation. Taking into consideration the importance of hydrazones it is of a great interest to determine their pK_{BH^+} values. For that purpose, various analytical methods can be used. Among them, the UV-Vis spectroscopy is the most common method [9-11]. This technique has some advantages such as: simplicity, availability, low cost, possibility of analyzing compounds with low solubility, accuracy, reproducibility and so on [12]. The most important thing is that spectroscopy is a highly sensitive and suitable method for determination of pK_{BH^+} values in very dilute aqueous solutions with low compound concentration. Usage of UV-Vis spectrometry for pK_{BH^+} determination requires presence of a chromophore close to the ionization site of the compound. In that case, the spectra of the

dissociated and the ionized forms of the molecule are different [13]. The organic solvent usually has influence on the appearance on the UV-Vis spectra as well as on pK_{BH^+} values. The influence of the solvent can be eliminated by employing method of Characteristic Vector Analysis (CVA), which has been tested and proven to be applicable in the analysis of spectroscopic data [14]. The purpose of this work was examination of the protonation process of fifteen different aromatic hydrazones in perchloric acid media ($1 < pH < 7$) by UV-Vis spectrophotometry. Furthermore, the aim was calculation of the stoichiometric and thermodynamic ionization constants from the absorbance data of the experimental and reconstructed spectra. In order to see how significant statistical differences are between pK_{BH^+} values determined numerically and graphically, and between pK_{BH^+} values calculated from absorbance data obtained from experimental and reconstructed spectra, t-test and Analyses of Variance (ANOVA) test will be performed.

The second part of this work was to investigate QSAR analysis of thermodynamic ionization constants using different physicochemical (electronic, steric and hydrophobic) parameters. It is also known that the ionization constants depend upon the polarity of molecule and directly or indirectly on the intra and intermolecular forces [15,16]. The parameters accounted for the intra and intermolecular forces and steric effect definitely plays a significant role in the modeling of dissociation constants. Physicochemical parameters taken into consideration in this QSAR study are Hammett substituents parameter (σ), hydrophobic parameters ($\log P$ and π), field effect as electronic influences (F), Verloop's STERIMOL parameter L and molar refractivity (MR) as steric parameter [17,18].

EXPERIMENTAL

MATERIALS AND METHODS

The investigated hydrazones were twice purified by recrystallization from 96 % ethanol. The perchloric acid, sodium perchlorate and ethanol were of analytical grade p.a. (Merck). The ionic strength of solutions (0.1, 0.25 and 0.5 mol/dm³) was adjusted using sodium perchlorate. The blanks were prepared with the same composition as the test solutions, but without investigated hydrazone. The pH of each test solution was measured after recording the UV spectra. The UV spectra were recorded immediately after preparation of the test solutions, at room temperature.

INSTRUMENTATION

The UV-Vis measurements were carried out on a Varian Cary 50 spectrophotometer controlled by a computer and equipped with a 1 cm path length quartz cell, in the wavelength region from 190 nm to 400 nm. A digital pH meter with glass electrode was used for measurements of the pH values (pH range from 1 to 7).

STOCK AND TEST SOLUTIONS

The stock solutions were prepared in 96 % ethanol with concentration of investigated hydrazones around $1 \cdot 10^{-3}$ mol/dm³. The test solutions were prepared with concentration of investigated hydrazones around $3 \cdot 10^{-5}$ mol/dm³. The test solutions for nitrohydrazones (H₁₁-H₁₅) were prepared in redistilled water and ethanol with volume ratio 1:1 (V/V) because the solubility of investigated hydrazones in aqueous solutions is low. The stability of these solutions was satisfactory for only 24 hours of time.

QSAR ANALYSIS AND SELECTED DESCRIPTORS

The MVA (multi variable analysis) approach in QSAR analysis has been most widely and effectively used for theoretical drug design due to various physicochemical (electronic, steric and hydrophobic) parameters. The assumption can be formulated as:

$$pK_{BH^+} = A_1x + A_2y + A_3z + B$$

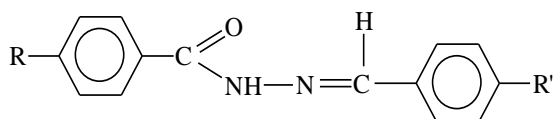
where x , y and z are molecular properties (descriptors). From the values of linear slopes A_1 , A_2 , A_3 we can see the correlation of the particular molecular properties with the activity of the investigated compounds.

Several descriptors with different characteristics were selected for QSAR analysis: classical Hammett electronic parameter of substituents (σ) [19], hydrophobic parameter (π) [19], molar refractivity (MR) [20], field effect (F) as electronic influences [19], Verloop's STERIMOL parameter for the steric interactions of the substituents (L) [19]; L is defined as the length of a substituent along the axis of its substitution to the parent skeleton. Hammett electronic parameter of substituents were calculated as sum from individual substituents values in both aromatic rings. For each hydrazone the partition coefficient ($\log P$) has been calculated [21].

RESULTS AND DISCUSSION

STRUCTURE OF INVESTIGATED HYDRAZONES

The subject of this study was fifteen benzoylhydrazones with different substituents on the benzene ring of benzaldehyde [22]. All investigated hydrazones have following substituents on the benzene ring on the hydrazone part of the molecule: –H, –CH₃, –OCH₃, –Cl, –OH. Five of them (H₁ – H₅) have no substituent on the benzene ring of benzaldehyde, five (H₆ – H₁₀) have methoxy group (–OCH₃), and five (H₁₁ – H₁₅) have nitro group (–NO₂) on the benzaldehyde group of the molecule. The structural formula of investigated hydrazones is presented in Scheme 1.



H₁-H₅: R = –H, –CH₃, –OCH₃, –Cl, –OH; R' = –H
 H₆-H₁₀: R = –H, –CH₃, –OCH₃, –Cl, –OH; R' = –OCH₃
 H₁₁-H₁₅: R = –H, –CH₃, –OCH₃, –Cl, –OH; R' = –NO₂

Scheme 1. Structural formula of investigated hydrazones

UV-VIS SPECTRA

The UV-Vis spectra of investigated hydrazones were recorded at three different ionic strengths (0.1; 0.25 and 0.5 mol/dm³). The experimental and reconstructed spectra of hydrazone H₁₃ are given in the Figs 1 and 2, respectively.

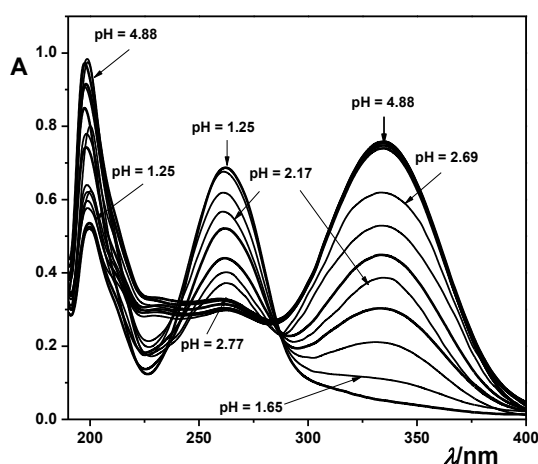


Fig. 1. Experimental UV-Vis spectra of H₁₃ ($c = 3.00 \cdot 10^{-5}$ mol/dm³) in pH region between 1.25 and 4.88 ($\mu = 0.1$ mol/dm³)

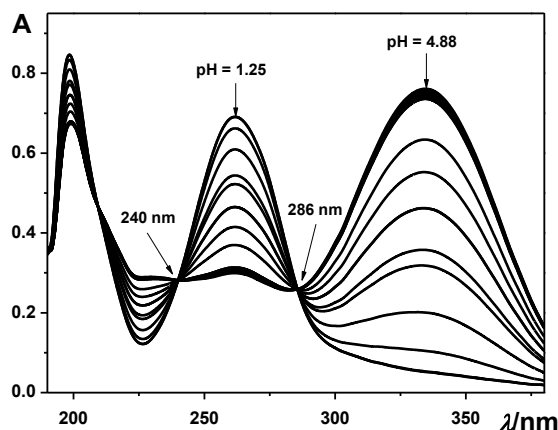


Fig. 2. Reconstructed UV-Vis spectra of H₁₃ ($c = 3.00 \cdot 10^{-5}$ mol/dm³), $\mu = 0.1$ mol/dm³

From the Fig. 1 it can be seen that in the weak acid media in the UV-Vis spectra of hydrazone H₁₃ there are two absorption bands. The second one with a maximum at 330 nm which was due to n→π* electron transitions were interesting for our further investigations [23]. In the reconstructed spectra well defined isosbestic points at 240 and 285 nm can be observed. The existence of isosbestic points confirmed that in the solution are present both neutral and protonated form of investigated hydrazones.

IONIZATION CONSTANTS

One of the main methods for determining the pK_{BH^+} values of a compound is UV-Vis spectroscopy. The stoichiometric pK_{BH^+} values were calculated by absorbance data using experimental and reconstructed spectra using the Henderson – Hasselbach equation.

$pK_{BH^+} = n \cdot pH + \log I$ (1)
 where pK_{BH^+} is the dissociation constant of the protonated form, I is the ionization ratio, and n is number of transferred protons

The ionization ratio I ($I = c(BH^+)/c(B)$) is actually the ratio between the concentration of the protonated (BH⁺) and neutral (B) form of the hydrazones. The calculations of ionization ratio were performed using the molar absorption coefficient values and the absorbance data obtained at four selected wavelengths. In accordance with Beer's law an over determined system of four equations (absorbance data) with two unknown parameters (concentration of neutral and protonated form) was obtained. Absorbance value which corresponds to the absorption maximum was used in calculations.

Furthermore, the pK_{BH^+} values were determined graphically as an intercept of the dependence of $\log I$ on pH [24]. The values of stoichiometric constants were further used for determination of the thermodynamic pK_{BH^+} values (Tables 1 and 2) as an intercept with extrapolation of the curve $pK_{BH^+} = f(\sqrt{\mu})$, to zero ionic strength (See Fig. 3).

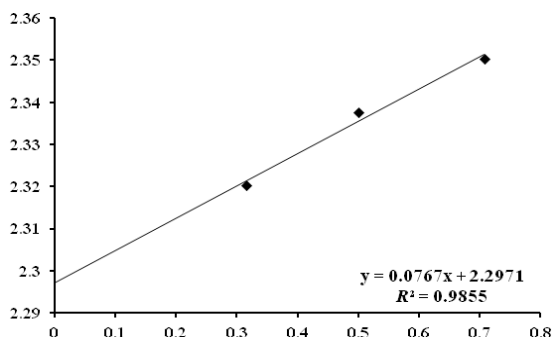


Fig. 3. The dependence of pK_{BH^+} on $\sqrt{\mu}$ for H₁₁
($c = 3.00 \cdot 10^{-5}$ mol/dm³)

The pK_{BH^+} values calculated from experimental spectra for hydrazones H₁ – H₆ were similar to the pK_{BH^+} values calculated from reconstructed spectra. It means that the influence of the solvent on the appearance of the UV-Vis spectra is insignificant. The test solutions of hydrazones H₆ – H₁₀ were prepared at the same manner as those for hydrazones H₁ – H₆, because of that it was expected that the solvent will not influence i.e. the experimental spectra of these hydrazones were not reconstructed. The volume ratio in the test solution of hydrazones H₁₁ – H₁₅ was 50 %, hence to investigate the influence of the solvent their experimental spectra were reconstructed.

The experimental spectra of hydrazones H₆ – H₁₀ were not reconstructed because there were no significant differences between experimental and reconstructed spectra for hydrazones H₁ – H₅. Test solutions of hydrazones H₁ – H₅ and H₆ – H₁₀ were prepared in the same solution.

Table 1. Thermodynamic ionization constants (pK_{BH^+}) and determination coefficient (R^2), experimental spectra

Hydrazone	numerically		graphically	
	pK_{BH^+}	R^2	pK_{BH^+}	R^2
H ₁	3.44	0.993	3.42	0.991
H ₂	3.12	0.996	3.10	0.994
H ₃	3.25	0.997	3.23	0.989
H ₄	2.56	0.989	2.58	0.999
H ₅	2.74	0.990	2.77	0.988
H ₆	3.26	0.996	3.26	0.963
H ₇	3.37	0.991	3.41	0.998
H ₈	3.46	0.99	3.48	0.986
H ₉	3.21	0.976	3.24	0.998
H ₁₀	3.10	0.999	3.08	0.961
H ₁₁	2.29	0.985	2.32	0.999
H ₁₂	2.55	0.974	2.55	0.989
H ₁₃	2.42	0.937	2.39	0.926
H ₁₄	2.05	0.999	2.08	0.999
H ₁₅	2.22	0.969	2.22	0.996

Table 2. Thermodynamic ionization constants (pK_{BH^+}) and determination coefficient (R^2), reconstructed spectra

Hydrazone	numerically		graphically	
	pK_{BH^+}	R^2	pK_{BH^+}	R^2
H ₁	3.29	0.998	3.28	0.999
H ₂	3.13	0.991	3.11	0.996
H ₃	3.21	0.993	3.19	0.996
H ₄	2.58	0.998	2.56	0.995
H ₅	2.73	0.993	2.78	0.988
H ₁₁	2.23	0.944	2.15	0.941
H ₁₂	2.29	0.998	2.35	0.963
H ₁₃	2.31	0.995	2.27	0.969
H ₁₄	2.24	0.995	2.17	0.838
H ₁₅	2.25	0.997	2.14	0.991

STATISTICAL DATA

In order to see how significant statistical differences are between pK_{BH^+} values determined numerically and graphically, and between pK_{BH^+} values calculated from absorbance data obtained from experimental and reconstructed spectra, t-test was performed with level of significance 0.05 (5 %). The obtained results showed that t critical two-tail was smaller than t statistical i.e. p critical was greater than 0.05 in following comparisons: pK_{BH^+} values determined numerically from experimental spectra, pK_{BH^+} values determined graphically from experimental spectra and pK_{BH^+} values determined numerically from reconstructed spectra. These results suggested that here were no statistical differences between mentioned pK_{BH^+} values. Insignificant statistical difference was observed between pK_{BH^+} values determined graphically from reconstructed spectra. In this case p critical was 0.049, while t critical was 2.26 and t statistical 2.27. These numbers

suggested that it is not about significant statistical difference.

On the other hand, for comparison of pK_{BH^+} values calculated for different groups of hydrazones analyses of variance (ANOVA) test was performed. The obtained results showed that there is statistical difference between hydrazones H₁ – H₅, H₆ – H₁₀ and H₁₁ – H₁₅. These differences are probably due to the substituents present in their structure.

CORRELATION ANALYSIS OF THE THERMODYNAMIC IONIZATION CONSTANTS WITH SELECTED DESCRIPTORS

Correlation matrix

It was important for further analysis to find correlation matrix for used descriptors. The data presented in Table 3 indicated that there isn't the high collinearity between selected descriptors ($R < 0.8$), indicating that these parameters could be combined to get statistically reliably QSAR models.

Table 3. Correlation matrix between selected descriptors

	σ	π	MR	F	R	log P
σ	1.00					
π	0.10	1.00				
MR	0.14	0.17	1.00			
F	0.61	-0.26	0.64	1.00		
R	0.77	0.32	-0.36	-0.03	1.00	
log P	0.37	0.90	0.07	-0.09	0.53	1.00

ii) Mono-parametric QSAR models

The relatively good mono-parametric model was obtained only for Hammett σ constants indicating the importance of this descriptor in pK_{BH^+} values

predicting. Obtained mono-parametric QSAR models are presented in Table 4, together with statistical parameters: R^2 , R^2_{adj} , SD and F-test.

Table 4. Mono-parametric QSAR models with statistical parameters: R^2 , R^2_{adj} , SD and F-test

	pK_{BH^+num}	pK_{BH^+graph}
Model	$pK_{BH^+num} = 2.9129 - 0.8075*\sigma$	$pK_{BH^+graph} = 2.9187 - 0.8023*\sigma$
R^2	0.7206	0.7205
R^2_{adj}	0.6991	0.6990
SD	0.0709	0.0700
Ftest	33.5207	33.5126
	$pK_{BH^+num-recon}$	$pK_{BH^+graph-recon}$
Model	$pK_{BH^+num-recon} = 2.8362 - 0.7674*\sigma$	$pK_{BH^+graph-recon} = 2.8249 - 0.8207*\sigma$
R^2	0.6787	0.7076
R^2_{adj}	0.6386	0.6710
SD	0.0686	0.0685
Ftest	16.9008	19.3560

Relatively same statistical values were obtained in all cases: $R^2 = 0.67 \div 0.73$; $R^2_{adj} = 0.63 \div 0.7$; SD = $0.06 \div 0.07$ and F-test = $16.0 \div 33.6$. In all mono-parametric correlation, σ value is negative is negatively correlated with pK_{BH^+num} , pK_{BH^+graph} , $pK_{BH^+num-recon}$ and $pK_{BH^+graph-recon}$.

The correlations with low R^2 values (< 0.20) are not considered being statistically significant, and data shows that rest of the chosen descriptors are not linearly correlated with the activity, and they individually can't be used for modeling the pK_{BH^+} values.

iii) Bi-parametric QSAR models

Since the mono-parametric regression resulted into relatively statistically significant models using the selected parameter (σ), an attempt for constructing of bi-parametric models were made.

Following statistically significant bi-parametric correlation were obtained:

$$pK_{BH^+num} = 2.9099 - 0.8351*\sigma + 0.2821*\pi$$

$$R^2 = 0.8124$$

$$R^2_{adj} = 0.7811$$

$$SD = 0.0516$$

$$F\text{-test} = 25.9815$$

$$pK_{BH^+graph} = 2.9155 - 0.8311*\sigma + 0.2949*\pi$$

$$R^2 = 0.8222$$

$$R^2_{adj} = 0.7925$$

$$SD = 0.0483$$

$$F\text{-test} = 27.7420$$

$$pK_{BH^+num-recon} = 2.8505 - 0.7970*\sigma + 0.2525*\pi$$

$$R^2 = 0.7746$$

$$R^2_{adj} = 0.7102$$

$$SD = 0.0550$$

$$F\text{-test} = 12.0261$$

$$pK_{BH^+graph-recon} = 2.8407 - 0.8537*\sigma + 0.2810*\pi$$

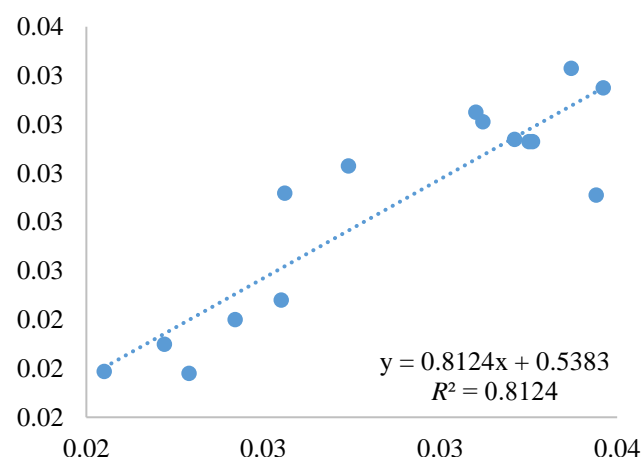
$$R^2 = 0.8157$$

$$R^2_{adj} = 0.7631$$

$$SD = 0.0494$$

$$F\text{-test} = 15.4954$$

Initial regression analysis indicated that out of the six descriptors used, only σ and π are important descriptors in predicting of pK_{BH^+} values. The positive coefficients of π in all bi-parametric QSAR models, indicates that the pK_{BH^+} values increases as the magnitude of this descriptors increases. The correlation coefficients were found to be good ($0.837 \div 0.77$) in all cases and the standard deviations were below 0.055. According the all statistical parameters, correlation between pK_{BH^+num} and σ and π is the best QSAR model.

**Fig. 1.** Plot of experimental vs. calculated pK_{BH^+} numerical values

Finally, in order to confirm our findings, pK_{BH^+num} predicted were compared with the corresponding pK_{BH^+num} values reported in Table 1. A plot obtained between the experimental and calculated pK_{BH^+num}

values is presented in Figure 1. Predictive correlation coefficient (R_{pre}), was calculated by correlating the estimated $pK_{BH^+}^{num}$ values with the experimental once. The obtained predictive correlation coefficient ($R_{pre} = 0.8124$) confirms our findings.

CONCLUSION

Ionization constants can be determined by UV-Vis spectroscopy using the absorbance data of experimental and reconstructed spectra. Reconstruction of the spectra by CVA method is useful in order to eliminate the influence of the solvent. Stoichiometric pK_{BH^+} values determined numerically and graphically at different ionic strengths were further used for evaluation of thermodynamic constants of investigated hydrazones. The obtained results showed that there are no significant statistical differences in the values of thermodynamic pK_{BH^+} values of investigated hydrazones determined from the absorbance data of experimental and reconstructed spectra, as well as for those determined numerically and graphically. The statistical differences in the pK_{BH^+} values were noticed for the hydrazones from different group $H_1 - H_5$, $H_6 - H_{10}$ and $H_{11} - H_{15}$ probably as a result of the influence of the substituents. The obtained pK_{BH^+} values were in accordance with those for similar class of compounds.

QSAR study has been made by correlation previously calculated thermodynamic ionization constants with various physicochemical parameters such: Hammett electronic parameter of substituents (σ), hydrophobic parameter (π), molar refractivity (MR), field effect (F), Verloop's STERIMOL parameter (L) and partition coefficient (log P). Only mono-parametric regression with σ resulted into relatively statistically significant model. Bi-parametric correlation between $pK_{BH^+}^{num}$ and descriptors σ and π is the best statistically QSAR model, according the selected statistical parameters. Predictive correlation coefficient ($R_{pre} = 0.8$), calculated by correlating the estimated $pK_{BH^+}^{num}$ values with the experimental once, confirm our conclusion for statistically best model.

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BIOLOGICAL ACTIVITY AND FOOD POTENTIAL OF PLANTS *RUMEX CRISPUS* L. AND *RUMEX OBTUSIFOLIUS* L. – A REVIEW

SCIENTIFIC REVIEW

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ABSTRACT:

Rumex crispus L. and *Rumex obtusifolius* L. (*Polygonaceae*) are perennial wild plants. For centuries these plants have been considered weeds in conventional agriculture. They have also been recognized as a serious problem in organic agriculture, as they are difficult to control without the use of chemical agents. However, *Rumex crispus* L. and *Rumex obtusifolius* L. are used worldwide in human nutrition and traditional medicine. They are available both in periods of abundance and in difficult times, and can be an important source of nutrients. As wild growing plants, with use in diet and traditional medicine, *R. crispus* and *R. obtusifolius* present potential sources of new therapeutic agents. This paper provides an overview of previous research for the plants *Rumex crispus* L. and *Rumex obtusifolius* L. in terms of their nutritional value, use in traditional medicine, as well as antioxidant and antimicrobial activity.

KEYWORDS: *Rumex crispus* L.; *Rumex obtusifolius* L.; antioxidant; antimicrobial; nutritional potential

INTRODUCTION

The potentials of biological resources are still intensively explored for the benefit of mankind, and for improving the quality of human life in a sustainable way. The research „bioprospecting” involve the search for valuable compounds in nature [1]. Thus, wild plants, which are traditionally used in human nutrition and treatment, become the subject of study. They represent potential sources of new and effective therapeutic agents, including new antibiotics and antioxidants. Phenolic components are considered to be the most important antioxidants of plants, so the antioxidant activity of plant material is correlated with the content of phenolic components [2]. Variability in the content of these components depends on: plant species, environmental factors and collection period [3]. It should be noted that phenols play a significant role in the fight against chronic human diseases [4].

Given that wild plant species *Rumex crispus* L. and *Rumex obtusifolius* L. (*Polygonaceae*) are still used in human nutrition, and have been used for years as medicinal plants in traditional medicine, there is a need for their more detailed study in terms of antioxidant and antimicrobial properties. There are a limited number of studies on the content of total phenols, as well as on the antioxidant and antibacterial

activity of *Rumex crispus* L. An even smaller number of studies have been conducted for the plant *Rumex obtusifolius* L. The studies conducted so far show that both plants represent a significant source of antioxidants and compounds with antibacterial effect [5], [6]. Some of the research suggests that the consumption of both plant species may be beneficial due to the high content of proteins and antioxidants [7]. These plants' potential to inhibit pathogens in food is also pointed out.

This paper provides an overview of previous research for the plants *Rumex crispus* L. and *Rumex obtusifolius* L. in terms of their nutritional value, as well as antioxidant and antimicrobial activity.

RUMEX CRISPUS L. AND RUMEX OBTUSIFOLIUS L.

Polygonaceae (the knotweed family or smartweed–buckwheat family) are a family of dicotyledonous plants under the order *Caryophyllales*. This family includes 43 genera with over 1000 species, including the genus *Rumex*. Plants of the genus *Rumex* are represented worldwide, with about 200 plant species. Plants of the family *Polygonaceae* contain a large number of chemically complex and biologically active compounds. The root and aboveground parts of plants from the *Polygonaceae*

family, including the genus *Rumex*, are traditionally used worldwide for their: psychopharmacological, antioxidant, purgative, antidiarrheal, antitumor, antidermatitis, diuretic and antiviral effects [8]. Numerous phytochemicals have been isolated from plants of the genus *Rumex* [9].

Rumex crispus L. (the curly dock, curled dock, yellow dock) is a perennial wild plant, which can reach a height of 30-100 cm (Figure 1).



Figure 1. *Rumex crispus* L.

It has a well-developed root. The tree is bare, furrowed. The leaves are large, somewhat fleshy, on a stalk as long as the leaf blade (30 cm). The lower leaves are lanceolate and wrinkled, while the upper are linear. The flowers are bisexual, up to twice as short as the flower stalk. They are gathered in whorls, and these in loose panicles. The fruit is a nut, brown in colour, about 3 mm long. It is sharpened at the top. It blooms from June to August. It is widespread in Europe, Asia, North and South America. It grows in neglected places, ruderal habitats and meadows, mostly wet or mesophilic [10].

Rumex obtusifolius L. (bitter dock, broad-leaved dock, bluntleaf dock, dock leaf or butter dock) is a perennial wild plant, up to 120 cm tall (Figure 2).

It has a spindle-shaped and strong root. The tree is erect, sometimes reddish. The leaves are large and long. They have longer stems at the bottom of the tree. The plant blooms from June to August. The red flowers are clustered in racemose inflorescence. The plant is widespread in temperate climates, thriving on moist, alkaline, humus and heavier soil [11].

For centuries, acetosa – especially *Rumex obtusifolius* L. and *Rumex crispus* L. – have been considered weeds in conventional agriculture [12], [13]. Recently, they have also been recognized as a serious problem in organic agriculture, as they are difficult to control without the use of chemical agents;

[14] they grow rapidly, are resistant to cutting (in two to three weeks, they renew a supply of hydrocarbon that they used to re-grow), live long, and are capable of producing up to 80,000 seeds per plant per year. In this way, they form a „seed bank” in the soil, which allows them to survive for up to 80 years [15].



Figure 2. *Rumex obtusifolius* L.

THE USE OF *RUMEX CRISPUS* L. AND *RUMEX OBTUSIFOLIUS* L. IN HUMAN NUTRITION

In different parts of the world, wild plants play an important role in the diet of the population. Given that they are drought resistant, they are available both in periods of abundance and in difficult times. If used in the daily diet, they can be an important source of nutrients, macro and micro nutrients [7]. Plants of the *Polygonaceae* family are used worldwide in human nutrition. Young leaves of *R. crispus* L. and *Rumex obtusifolius* L. are used in the diet as vegetable, for the preparation of sour soups or meat wrapping [11,16-18]. Of the 26 wild plants used in the diet of Anatolia inhabitants, the leaves of *R. crispus* L. turned out to have the highest protein content (11.56% per gram of dry matter) and the highest nitrogen content (1.17%), even higher N content than some cultivated plants [7]. In general, plants from the family *Polygonaceae* had the highest content of N, S and Cu, in relation to other examined families [7]. In Brazil *Rumex obtusifolius* it is considered as an unconventional food plant, and can be used as a good source of nutritional components necessary for human diet, such as lipids, proteins and carbohydrates. Root of *R. obtusifolius* presented a high content of carbohydrates (30.6%), and the leaves presented more proteins (2.7%). *R. obtusifolius* is among these plants with excellent nutraceutical properties [19]. The plants from genus *Rumex* also contain oxalic acid, which can cause kidney damage in higher concentrations.

THE USE OF *RUMEX CRISPUS* L. AND *RUMEX OBTUSIFOLIUS* L. IN TRADITIONAL MEDICINE

Herbal remedies have been traditionally used around the world for thousands of years [20]. A significant number of indigenous wild plants are used in the treatment of various diseases [21], [22], [23], [24]. Wild plants, including the genus *Rumex* (*Polygonaceae*), are widely used in traditional medicine in rural parts of eastern Anatolia (Turkey). The flowers, stem, leaves and root of these plants are commonly used in the treatment of various disorders, which include: pain, edema, eczema, digestive problems, arthritis, colds, flu, and irritability. A tonic is prepared from root, and it is also used to „cleanse” the blood. Plants of this genus show laxative, diuretic, antipyretic, and anti-inflammatory effects [25], [26]. *R. crispus* dried roots are a gentle and safe laxative, useful for treatment of mild constipation, due to anthraquinone content which gives the roots yellow colored pigment [27].

The root of some species of the genus *Rumex* contains anthracene derivatives (0.2-1.7%) and catechin tannins (6-12%). Because the root does not contain toxic substances, it can be used as a safe natural remedy [16].

In some parts of Italy, the leaves of *R. crispus* L. are used for medicinal purposes – in the treatment of dermatitis, injuries, and in cosmetics [28]. Reuter et al. (2010) state that *R. crispus* L. is used in acne treatment [29]. *R. crispus* is also useful for treating a wide range of skin problems such as fungal disorders, spring eruption and scrofula [27]. Bussmann and Glenn (2010) mention that the whole plant *R. crispus* L. is used in medicinal purposes in northern Peru in the treatment of reproductive problems in women [30]. This plant is used fresh to treat uterus infections, inflammation of the female reproductive organs, and vaginal infections. In form of decoction or tincture *R. crispus* is used for the treatment of microbial infections, malaria, and sleeping sickness [31]. The infusion or decoction of *R. crispus* is commonly used for the treatment of helminths, wound, internal bleeding and vascular diseases in folk medicines of South Africa [32]. The mean phytochemical content of the root of *R. crispus* is higher than that of the leaf and this may also justify the frequent use of the root more than the leaves in South Africa traditional medicine for the cure of helminthic infections [33].

Šarić (1991) stated that the leaf and ripe fruit of *R. crispus* L. are used in area of former Yugoslavia in the treatment of diarrhea, hemorrhoids, anemia, as well as to improve digestion and appetite [11]. *R. obtusifolius* L. is used in treatments in the same way as *R. crispus*

L. It has a medicinal root [11]. *R. obtusifolius* has therapeutic indications, because its use cleanses toxins, has laxative, diuretic, calming effect, decrease the incidence of chronic skin diseases, liver disorders and anemia [19].

ANTIMICROBIAL POTENTIAL OF *RUMEX CRISPUS* L. AND *RUMEX OBTUSIFOLIUS* L.

Throughout human history, infectious diseases have been treated with herbal remedies [34]. Since numerous studies indicate a significant increase in bacterial resistance to several antibiotics [35], there is significant interest in medicinal plants, traditionally used in folk medicine, which are a potential source for the discovery of new and effective therapeutic agents. They are also interesting from the aspect of the use of plant extracts in fight against plant pathogens [36], [37], [38].

Studies examining the antibacterial activity of some *Rumex* species – such as *R. crispus* L. [39], *R. nervosus* L., *R. abyssinicus* L. [40], *R. japonicus* L. [2] and *R. alveollatus* L. [8] – showed significant inhibitory activity against majority of tested microorganisms. Extracts of these plants can be used as natural antibiotics in the treatment of some diseases, especially localized skin diseases.

The extracts of *R. crispus* have been declared to possess an antioxidant, antimicrobial and antifungal activities [27]. A study conducted by Yildirim et al. (2001) showed that extracts of *R. crispus* L. leaves in ether, ethanol and hot water are active against *Staphylococcus aureus* and *Bacillus subtilis*. The extracts do not show activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Clostridium albicans* [41].

Ulukanli et al. (2005) investigated the antibacterial activity of extracts of *R. crispus* L. root and aboveground parts [39]. According to them, *R. crispus* L. root extract in hexane shows *in vitro* activity against Gram-positive bacteria: *Micrococcus luteus*, *Mycobacterium smegmatis*, *Bacillus subtilis*, and *Bacillus subtilis* var. niger, as well as three species of Gram-negative bacteria: *Aeromonas hydrophila*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The extract in hexane shows no activity against *S. aureus*. The root extract in methanol does not inhibit the growth of the bacterial species *A. hydrophila* and *P. aeruginosa* only, while the acetone extract shows activity against all the aforementioned Gram-positive and Gram-negative bacteria. The effect of methanolic extracts of *R. crispus* L. root against the examined Gram-positive is more pronounced than against Gram-negative bacterial cultures. The extract

of the aboveground parts of *R. crispus* L. in hexane shows only antibacterial activity against *M. smegmatis*, *B. subtilis* and *B. subtilis* var. *niger*. Acetone extract of aboveground parts does not prevent the growth of *S. aureus*, *B. subtilis* var. *niger* and *P. aeruginosa*. Methanolic extract of aboveground parts of *R. crispus* L. does not show antibacterial effect against *P. aeruginosa* only. The acetone extract activity of *R. crispus* L. root has shown promising results against mostly Gram-positive pathogens. This indicates the presence of strong antimicrobial substances in the solid extract of this plant. Thus, its further purification and isolation is necessary in order to determine the types of substances responsible for the antimicrobial activity of this medicinal plant [39]. Research of antibacterial activity conducted by Coruh et al. (2008) revealed that the aqueous extract of the aboveground parts of *R. crispus* L. does not show antimicrobial activity, while the methanol extract shows an inhibitory effect on the growth of 15 tested microorganisms [3]. The extract has shown a very strong antibacterial effect against bacterial cultures: *Agrobacterium tumefaciens*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas corrugate*, *Pseudomonas syringae* pv. *tomato*, *Salmonella typhimurium*, *Serratia liquefaciens*, *Vibrio cholerae*, *Yersinia frederiksenii* and *Yersinia pseudotuberculosis*. *Xanthomonas compestris* was most sensitive to the extract activity. Its MIC (minimum inhibitory concentration) value was lower than the MIC value of the standard.

The study of Cowan (1999) showed that methanol extract is a strong antibacterial inhibitor, with high phenol content [42]. The antibacterial activity of the methanolic extract of *R. crispus* L. can be attributed to the presence of several compounds. Thus, the phenolic compound pyrogallol has a toxic effect on microorganisms – by inhibiting enzymes, and reacting with a sulfhydryl group [42].

Methanolic extract of *R. obtusifolius* L. shows antibacterial activity against *B. cereus*, *B. subtilis*, *E. coli*, *S. aureus* and *Salmonella tryphi* [43]. Aqueous fractions of *R. obtusifolius* L. herb extract showed weak inhibition against *S. epidermidis*, *S. aureus*, and MRSA, while aqueous fractions of *R. obtusifolius* L. root extract showed moderate inhibition against the same bacterial species [6]. The ethanolic extract of *R. obtusifolius* L. fruits possessed potential activity against the reference and clinical staphylococcal strains (MIC=500 µg/mL), also against *P. mirabilis* (MIC=500 µg/mL), lower inhibitory effect on the growth of Gram-negative bacteria – *E. coli* ATCC 3521, *P. vulgaris* and *P. aeruginosa* (MIC > 500 µg/mL), and higher antifungal activity against *T.*

menthagrophytes with MIC=250 µg/mL in agar dilution method [44].

It has been reported that three substances isolated from roots of *R. crispus* exhibit antifungal activity. They are identified as two anthraquinones – chrysophanol and parietin, and nepodin. These compounds in experiments *in vivo* screened for antifungal activity were effective in controlling of the disease development comparable to synthetic fungicide fenarimol, at similar concentrations [9].

ANTIOXIDATIVE POTENTIAL OF *RUMEX CRISPUS* L. AND *RUMEX OBTUSIFOLIUS* L.

Oxidative damage – a primary as a result of metabolism, or a secondary caused by environmental contaminants – leads to the formation of free radicals, which are thought to play a central role in the development of diseases such as cancer and atherosclerosis. Therefore, antioxidants, which can neutralize free radicals, can help prevent these diseases [45].

Although synthetic antioxidants – such as butylhydroxytoluene (BHT) and butylhydroxyanisole (BHA) – are used in food production, there are reports on their side effects [46]. Therefore, the substitution of synthetic antioxidants by natural ones, as well as the screening of new plant species with the aim of identifying new antioxidants, are of particular importance in recent years [47].

Reactive oxygen species (ROS) are formed in cells during normal aerobic metabolic processes. Endogenous sources of ROS are numerous membrane and cytosolic oxidoreductases, and the largest ROS production in the cell is related to mitochondria (respiratory chain), peroxisomes, and microsomes [48]. At low and moderate concentrations, ROS have numerous roles in the cell: defense against infectious agents and the functioning of regulatory mechanisms and intracellular signaling pathways. At low and moderate concentrations, ROS have numerous roles in the cell: defense against infectious agents as well as the functioning of regulatory mechanisms and intracellular signaling pathways.

ROS can also occur under the influence of various exogenous influences, such as various biotic and abiotic factors. Their activity can upset the balance between prooxidant and antioxidant reactions, resulting in increased production and accumulation of ROS. This condition is known as oxidative stress. Increased ROS production, along with decreased cell antioxidant protection, can lead to oxidative damage to cellular constituents such as: lipids, proteins, and DNA.

Phenolic components are considered to be the most important plant antioxidants, and thus the antioxidant activity of plant material is correlated with their content of phenolic components [2]. Phenols play a significant role in the fight against chronic diseases [4]. Variability in phenol content may be the result of: environmental factors, plant species, and collection periods [3].

Although the antimicrobial activity of some species of the genus *Rumex* has been investigated, the number of studies on the total phenols content, as well as the antioxidant and antibacterial activity of *R. crispus* L. is limited [3].

Research, conducted by Coruh et al. (2008), showed that the aboveground parts of the plant *R. crispus* L. are a rich source of phenol compounds. The difference in the antioxidant activity of the extracts in water, and methanol, is not statistically significant compared to BHA and BHT. The results indicate that the aboveground parts of the plant *R. crispus* L. have strong antioxidant activity, which can be significant for both human consumption and food safety [3].

The study of Idris et al. (2015) shows the presence of alkaloids, phenols (16.9 to 240.7 mg GAE/g), flavonoids (19.4 to 526.23 mg QE/g), saponins and proanthocyanidins in the solvents extract (methanol, ethanol, acetone, and water extract) of the root and leaf of *R. crispus* [33]. The root of the plant has higher content of phytochemicals than the leaf and also showed a slightly higher antioxidant activity compared with the leaf tested by ABTS, DPPH and FRAP, total antioxidant capacity, and nitric oxide scavenging assay. The study of Suh et al. (2011) revealed that curly dock seed extremely play an important role in reactive oxygen species (ROS) scavenging against oxidative stress [49].

The cyclohexane, dichloromethane, and methanol extracts of *R. crispus* L. aerial parts were rich in phenolic compounds (13.0 to 249.8 mg GAE/g of dry weight), and MeOH extract has presented remarkable activity in DPPH assay (IC₅₀ 6.2 µg/mL) [50].

A study on the antioxidant activity of ripe fruit extracts of *R. crispus* L., and their influence on lipid peroxidation in liposomes, conducted by Maksimović et al. (2011), revealed that methanol extracts of *Rumex crispus* L. fruit show antioxidant activity similar to BHT activity. Pre-treatment with the extract *in vivo* inhibits oxidative stress induced by carbon tetrachloride, bringing the level of antioxidant enzymes close to control values [51].

The aqueous extract of curly dock fruits evaluated for its antioxidant activity by *in vitro* assays for ferric-reducing antioxidant power (FRAP), NO•, OH• and 2,2-diphenyl-1-picrylhydrazyl (DPPH)-free radical

scavenging activities and the influence on lipid peroxidation in liposomes showed a potential antioxidant activity manifested in scavenging of free radicals as well as an ability to decrease lipid peroxidation in liposomes [27].

Methanolic extract of *R. obtusifolius* L. shows a high level of antioxidant activity [43]. In study of Sganzerla et al. (2019) phenolic, flavonoids, flavonols were quantified, and antioxidant activity by DPPH, ABTS and FRAP assays was tested in aqueous, ethanolic and hydroethanolic extracts. The best solvent tested in this study was the hydroethanolic extract to root and ethanolic extract to leaf. The hydroethanolic extract from root presents more antioxidant activity by ABTS (2695.3 mg TEAC 100 g⁻¹) and FRAP (13579.2 mg TEAC 100 g⁻¹) comparing to the other extracts. Compared to other examined extracts the TPC (981 mg GAE 100 g⁻¹), TFC (155.1 mg QE 100 g⁻¹) and TF (136.7 mg QE 100 g⁻¹) were more concentrated in hydroethanolic (70% ethanol) extract [19]. The antioxidant activity of the studied parts of plants *Rumex crispus* L. and *Rumex obtusifolius* L. by DPPH, ABTS and FRAP assay can be arranged in the following order: the generative part (flowers, seeds) > leaves > root > stem (for flowering and fruiting stages). In this study was found that parts of the root closer to the stem differed in higher activity [5].

CONCLUSION

Rumex crispus L. and *Rumex obtusifolius* L. are widespread weed plants. They have long been used in the diet and traditional medicine of people from different areas. Studies have shown that these plants have a rich nutritional composition and can be a good source of nutrients and bioactive compounds. The extracts of *R. crispus* and *R. obtusifolius* have been declared to possess antioxidant, antimicrobial, and antifungal activities, offering remarkable protection activity. The level of phenolic compounds and other phytochemicals identified in these plants contributed to their radical scavenging activity and medicinal properties. Research to date indicates that *R. crispus* and *R. obtusifolius* can be potential sources of biologically active molecules intended for use in the pharmaceutical and food industry. Further work needs to be established to identify other molecules responsible for the biological activity of these plants.

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THERAPEUTIC EFFECT OF OINTMENT FOR PSORIASIS BASED ON *ACHILLEA MILLEFOLIUM* L., *CALENDULA OFFICINALIS* L. AND *SALVIA OFFICINALIS* L.

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

This study investigates the efficacy of Dermavit cream for treatment of psoriasis, eczema and dermatitis made by Pharmamed d.o.o Travnik for treatment of patients diagnosed with psoriasis. Dermavit cream for treatment of psoriasis, eczema and dermatitis contains in composition *Achillea millefolium* extract, *Calendula officinalis* extract and *Salvia officinalis* essential oil. Due to the specific composition of extracts contained in Dermavit cream for treatment of psoriasis, eczema and dermatitis, it hydrates and softens the skin, helping it to regenerate and soothe the skin surface. For qualitative analysis of the extracts, UHPLC – MS (*Millefolium herba* and *Calendulae flos*) and GC-MS (*Salviae folium*) were used. According the study results conducted on subjects with diagnosis of psoriasis Dermavit cream for treatment of psoriasis, eczema and dermatitis can be determined to be successful in alleviating psoriasis symptoms. The results are obtained based on a study involving 30 patients with psoriasis, where each of them used the cream for a period of 60 days.

KEYWORDS: psoriasis, Dermavit cream for treatment of psoriasis, eczema and dermatitis, flavonoid components, UHPLC-MS, GC, GC-MS

INTRODUCTION

Psoriasis is a chronic, non-communicable, inflammatory, incurable skin disease characterized by hyperproliferation and abnormal keratinocyte differentiation, inflammatory cell infiltration, and vascular changes (angiogenesis). It is characterized by symmetrical skin lesions with crisp boundaries, red papules and plaques, and generally covered in white and silver scales. The disease's name derives from the Greek term $\psi\omicron\rho\iota\alpha\sigma\iota\varsigma$, which combines the words psora (itching) with the suffix -iasis, which means condition or activity [1]. Psoriasis affects around 2% to 3% of the world's population, or 125 million people in total. Therefore, this disease represents a significant public health issue [2].

A wide range of conventional medical therapies have been established for the treatment of psoriasis, from topical therapy (corticosteroids, vitamin D analogues, psoralen, 5-aminolevulinic acid, salicylates, fumaric acid esters, anthralin (dithranol), tacrolimus, retinoids - eg tazarotene) systemic drugs

(methotrexate, cyclosporine, retinoids, 6-thioguanine, mycophenolate mofetil, troglitazone, and new biologics, such as adalimumab, alefacept, efalizumab, etanercept, infliximab), to phototherapy, or a combination of all mentioned above [3].

However, most of these therapies cause a number of side effects and have limited effectiveness, leading to skin atrophy, organ toxicity (hepatotoxicity, nephrotoxicity, teratogenicity), carcinogenicity and immunosuppression, which limits their long-term use [4].

In turn, a short-term treatment of psoriasis causes its remission after finishing the treatment or only relieves the patient's condition. Therefore, the invention of new alternative treatments for psoriasis causing fewer side effects would be desirable. It seems that several herbal drugs can meet these requirements and have to be seen as promising new agents for psoriasis treatment. Herbal products are greatly accepted by patients because they are believed to be safer than conventional therapeutics. Moreover, herbal

products present a great structural diversity and multidirectional mechanisms of action, which is not commonly seen in synthetic compounds. Herbal drugs may become an effective treatment for psoriasis, causing lower costs and less side- or toxic effects in comparison to other therapies. Therefore, researchers are still looking for novel herbal products and/or their active constituents, which potentially could be used for the treatment of psoriasis instead of synthetic drugs [5].

However, most studies provide limited information on the effectiveness of topically used herbal products. Therefore, more scientific evidence and documentation is needed to promote the treatment of psoriasis with herbal products.

In this paper, Dermavit cream for psoriasis, eczema, and dermatitis, made by Pharmamed d.o.o. Travnik, further referred to as Dermavit cream, has been investigated. Dermavit cream is part of a line of specialized cosmetics. It's a cream for dry, cracked, dandruff-prone skin, as well as red and inflammatory skin. The line is available in Bosnia and Herzegovina. Phytochemical analysis of plant extract used for cream preparation was performed and the efficacy of Dermavit cream was investigated. A survey of patient with diagnosis of psoriasis was done to assess subjective improvement after using Dermavit cream.

MATERIALS AND METHODS

Extracts from *Millefolii herba* (*Achillea millefolium* L.) and *Calendulae flos* (*Calendula officinalis* L.), as well as *Salviae officinalis* essential oil (*Salvia officinalis* L.), provided by the Pharmamed laboratory, were used for qualitative investigation of potentially active compounds.

The extracts of *A. millefolium* and *C. officinalis* were prepared by maceration according to the Ph. Jug IV [11]. Single extraction of suitably crushed drug was performed at room temperature in a well-closed, light-protected container with 63 percent ethanol in a ratio of 1:5. Shaking and stirring was applied twice a day during maceration lasting 5 days. The macerate was then separated from the drug by squeezing, followed by pressing and left for 2 days in a cool, dark place and then filtered. The obtained extracts were analyzed qualitatively using UHPLC-MS method. The GC method and the GC-MS combination were used to undertake qualitative and quantitative analysis of *Salviae officinalis* essential oil. *Salviae officinalis* essential oil was prepared by steam distillation as shown in Figure 1.

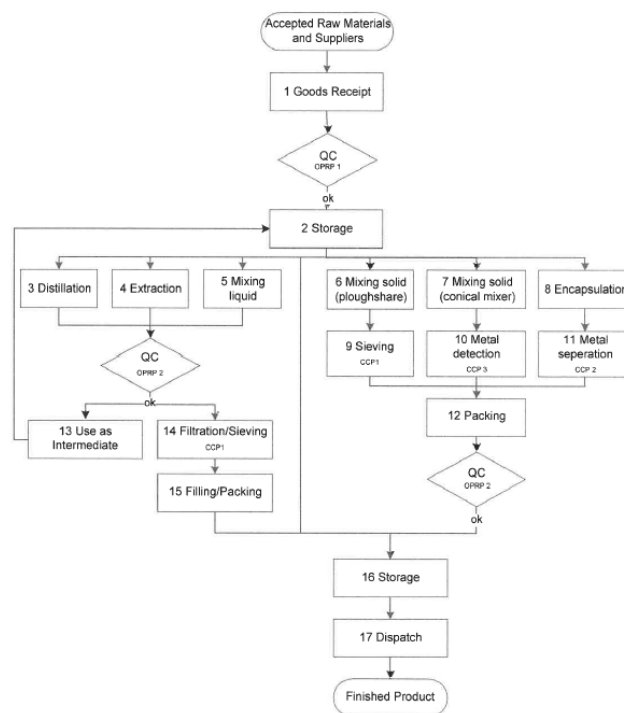


Figure 1. Flow chart production process of *Salviae officinalis* essential oil

ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – UHPLC

The Ultimate 3000 UHPLC system (Thermo Fisher Scientific - San Jose, CA) was equipped with a reversed phase C18 column (Thermo Acclaim; 2.1 mm × 15 cm, 3 μm, 120 Å). Mobile phase A (H₂O/FA, 100:0.01) and mobile phase B (ACN) were degassed prior to their usage. A 66 min binary gradient with flow rate set to 500 μL/min was applied as follows: 0–1 min, 5% mobile phase B; 2– 45 min, 5–95% mobile phase B; 45–55 min, 95% mobile phase B; 56–66 min re-equilibration with 5% mobile phase B). Five microliters of each sample (5mg/mL) were injected followed by a blank injection to ensure proper column washing and equilibration.

MASS SPECTROMETRY - MS

Mass spectrometric detection was performed with a LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific) using the HESI source (300°C heater temperature, 40/10/1 arb. units for the sheath, aux and sweep gases respectively and 3.5kV spray voltage at 275°C capillary temperature) to achieve negative/positive ion mode ionization. MS scans were performed with an m/z range from 150 to 2000. MS/MS scans of the three most abundant ions were achieved through collisional induced dissociation

(CID) with fragmentation at 30% normalized collision energy.

GAS CHROMATOGRAPHY - GC

A Shimadzu GC 2010 Plus gas chromatograph was equipped with Permabond SE54 quartz capillary column (10m x 0.25mm, 0.25m film thickness), and a flame ionizing detector (FID).

The GC apparatus was set to operate in the following manner: the injectors and detectors were kept at 270 °C, while chromatography was carried out in the linear temperature programming mode 50-125°C and at a heating rate of 3° C/min. It took 25 minutes to do the chromatography. Throughout the analysis, the carrier gas was nitrogen (N₂) 5.0 at a constant flow rate of 2.51 ml/min. In a 1:100 split, an essential oil solution in dichloromethane, CH₂Cl₂, was injected.

GAS CHROMATOGRAPHY - MASS SPECTROMETRY – GC-MS

GC-MS analysis of essential oil was performed on a GCMS-QP2010 Shimadzu device equipped with a MN Optima-5 capillary column, Macherey Nagel (50m x 0.25mm, film thickness 0.5µm) connected to a mass spectrometric selective detector (MSD).

The operating mode of the GC-MS apparatus was as follows: Analytical conditions identical to those for GC / FID were applied in the case of GC-MS analysis. Instead of nitrogen, helium (He) was used as the carrier gas at a constant flow rate of 1.14 ml/min throughout the analysis time. The sample was injected using a split/splitless injector at a 1:10 split ratio.

The conditions prevailing in MS are: the temperature of the ion source is 250°C, and the interface temperature is 270°C. Mass spectra were recorded for the mass range m/z of 40-500.

Identification was done in accordance with databases and literature.

OBSERVATIONAL CLINICAL STUDY CONDUCTED ON PATIENTS

The effects of Dermavit cream were studied in 30 patients for two months to investigate its efficacy. On a voluntary basis and with respect for privacy, patients were selected according to age, gender and disease's duration. Patients accepted to participate in the study by signing the informed consent form. The study was done in the MGM pharmacy, Zenica branch, at the address Dr. Abdulaziza Aska Borića 23. After 7 days, 15 days, 1 month, and 2 months of using Dermavit cream to treat psoriasis, the changes were

photographed and recorded. The parameter like skin flakes, redness and the thickened of the skin were followed and evaluated with scores from -1 to 2 with patient's dermatologist, who diagnosed the patient with psoriasis. The total withdrawal of symptoms was evaluated with a score of 2, partial withdrawal of symptoms with a score of 1, without effect with a score of 0 and deterioration with a score of -1. Classification of terms is determined in cooperation with dermatologist. For satisfactory improvement, the total number of grades from 4 to 6 was taken, for partial improvement of the total number of grades from 1 to 3, for lack of improvement the total score was 0, and for deterioration the score was less than zero. After 60 days of therapy with Dermavit cream, the patients were given a structured questionnaire (see Appendix).

The questionnaire consisted of two parts: the first part contained questions of the patient's demographics, lifestyle and habits, the second part questions about subjective improvement after using Dermavit cream. The answers to questions concerning the effects of the treatment were assessed on a 5-point Likert scale: Totally agree, Somewhat agree, Neither agree or disagree, Somewhat disagree, Totally disagree. The results were processed using google forms.

RESULTS AND DISCUSSION

The review of the literature shows that a great growth has taken place in the worldwide interest in the potential of herbal medicines for the treatment of psoriasis over the last 20 years. Parallel to various synthetic medicines used topically (corticosteroids, vitamin D analogues, retinoids) and systemic (methotrexate, retinoids, cyclosporin), or targeted (biological) therapies (e.g. alefacept, efalizumab, etanercept) also herbal products play an important role as therapeutic agents for psoriasis treatment [7].

There are many advantages of using natural drugs, including patient compliance, less side-effects, easy availability, low-costs, and more than one mode of biochemical action for psoriasis treatment. Therefore, researchers are searching for new herbal products, which have the potential to be an alternative for synthetic drugs in psoriasis therapy [8].

Herbal formulation of Dermavit cream is prepared from two different plant extracts, Calendulae flos extract and Millefolii herba extract and Salviae officinalis essential oil. Calendulae flos is marked by the European medicines Agency for use in the treatment of for skin inflammations, minor wounds and mouth or throat inflammation [9]. Moreover, the intended use does not require medical supervision.

Milefolii herba is recognised as traditional herbal medicinal product for the treatment of small superficial wounds [10]. *Salvia officinalis* is also made to be anti-inflammatory agent [11].

Based on our findings, no clinical studies have been reported with preparation that contain combination of plant extract and essential oil.

PHYTOCHEMICAL ANALYSIS

Flavonoid components present in *Calendula flos* extract (isoquercitrin, rutin, calendoflavoside, isorhamnetin, isorhamnetin-3-O-glucoside, narcissin)

and flavonoid components present in *Millefolii herba* extract (cinarozid, kosmosin, luteolin, apigenin, centaureidin, luteolin-7-O-glukozid, apigenin-7-O-glukozid, salvigenin, eupatilin) were identified by comparing the retention time, and by comparing m/z mass spectrum values with literature published data and databases and are in line with the manufacturer's specifications.

The spectrum of flavonoids rutin from *Calendula officinalis* extract and luteolin from *Achillea millefolli* extract are presented in Figure 2 and Figure 3.

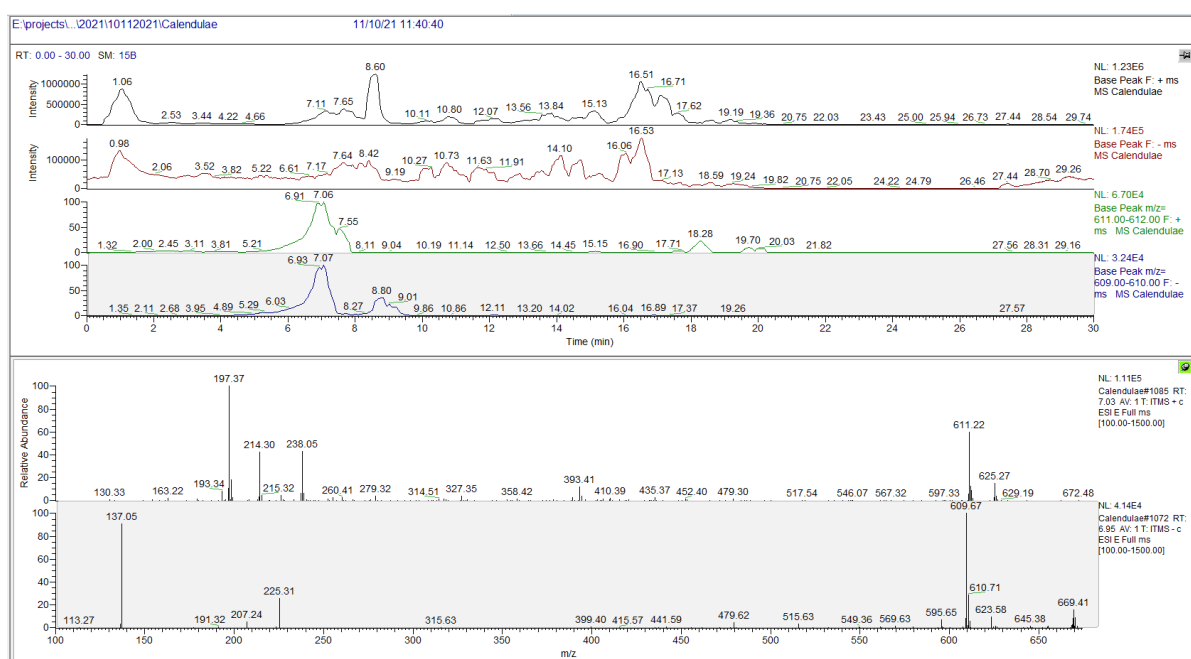


Figure 2. The spectrum of rutin

The observed mass spectrometer shows the dominant deprotonized ion $[M-H]^-$ at m/z 609.67. The spectrum of the base peak shows higher sensitivity in the negative ionization mode. The retention time of the

test analyte in the cause is 7.07, and deviates less than 1% from the retention time of the standard solution of 7.1.

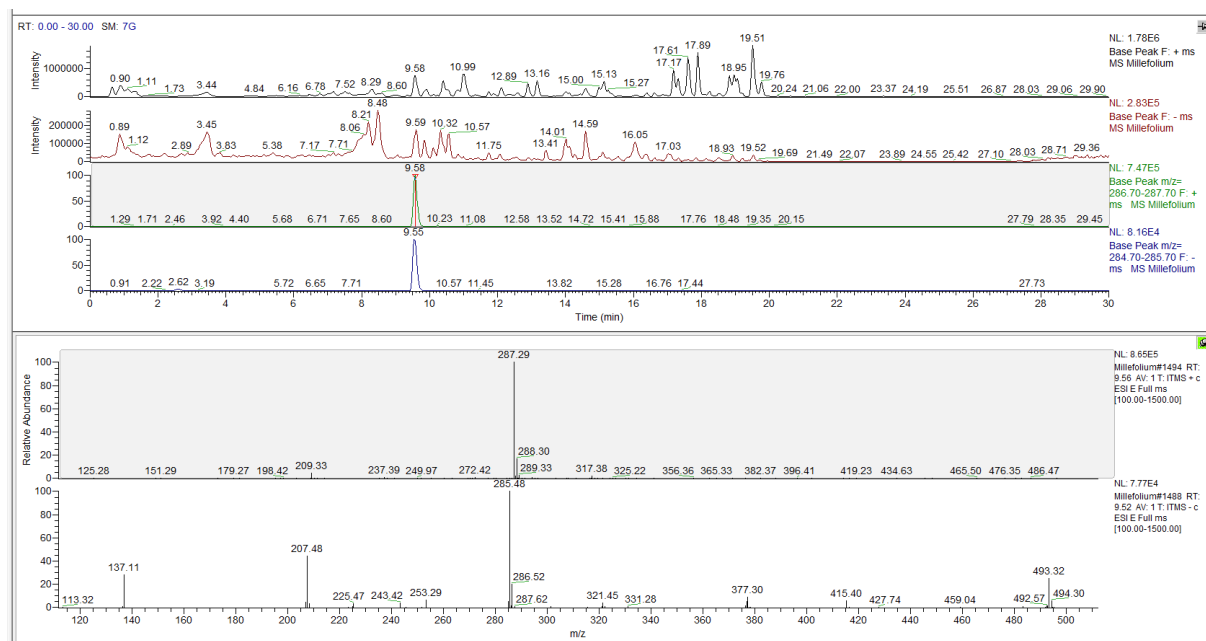


Figure 3. The spectrum of luteolin

The observed mass spectrometer shows a dominant ion at m/z 287.29. The spectrum of the base peak shows higher sensitivity in the positive ionization mode. The retention time of the test analyte in the cause is 9.58 and deviates less than 1% from the retention time of the standard solution which is 9.5. The chemical profile of *Salviae officinalis* essential oil obtained by GC analysis is given in Table 1 and the GC chromatogram is shown in Figure 4.

Table 1. Retention time and Area of *Salviae officinalis* essential oil

Peak	Retention time, tr	Area
1	3.349	123483
2	3.652	154900
3	4.299	59626
4	4.736	13063
5	5.028	1852
6	5.389	2125
7	5.631	27906
8	5.814	299695
9	6.117	1025
10	6.705	17634
11	7.034	1474

12	7.692	9237
13	8.302	675400
14	8.696	147160
15	9.654	485051
16	10.526	83203
17	11.005	.9419
18	15.451	32370
19	18,216	97853
20	18.294	55619
21	19.069	4144
22	19.253	9393
23	19.343	2653
24	19.421	2807
25	19.497	1176
26	19.537	1682
27	20.228	23548
28	20.665	92756
29	22.014	110868
30	22.751	76093
Total		2623213

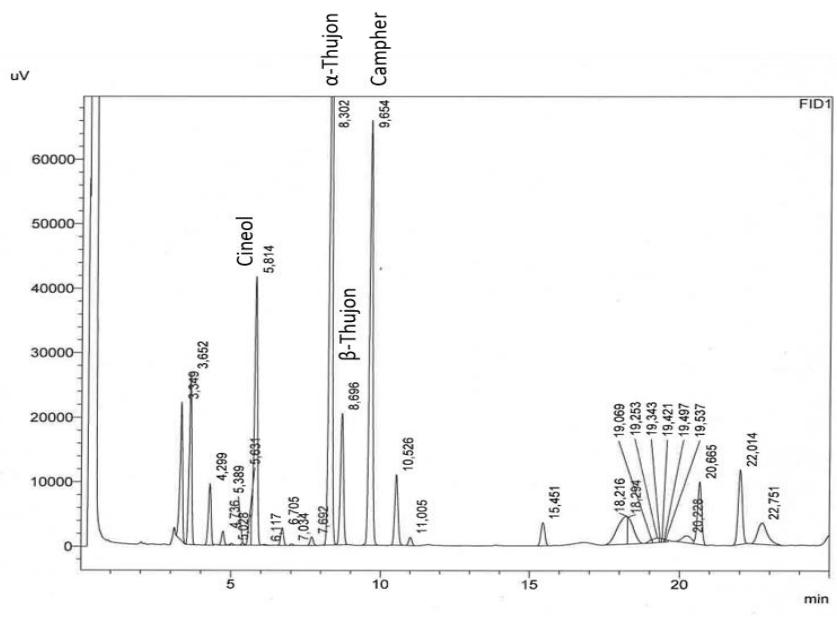


Figure 4. Chromatogram of *Salvia officinalis* essential oil

Salvia officinalis essential oil has been shown to have 30 different constituents, the highest percentage of α and β -thujone (31.36%), camphor (18.49%), and cineol (11.42%). Other compounds are present in a smaller percentage (Figure 4).

These results are in line with manufacturer's specifications.

OBSERVATIONAL CLINICAL STUDY CONDUCTED ON PATIENTS

A total of 38 patients who reported having symptoms related to psoriasis were assessed for eligibility criteria, of which 7 were excluded for various reasons (no objective signs of psoriasis, refuse to participate or not eligible for inclusion). A total of 31 patients were eligible for the study, and they randomized in four main groups, according to the disease's duration (Figure 5).

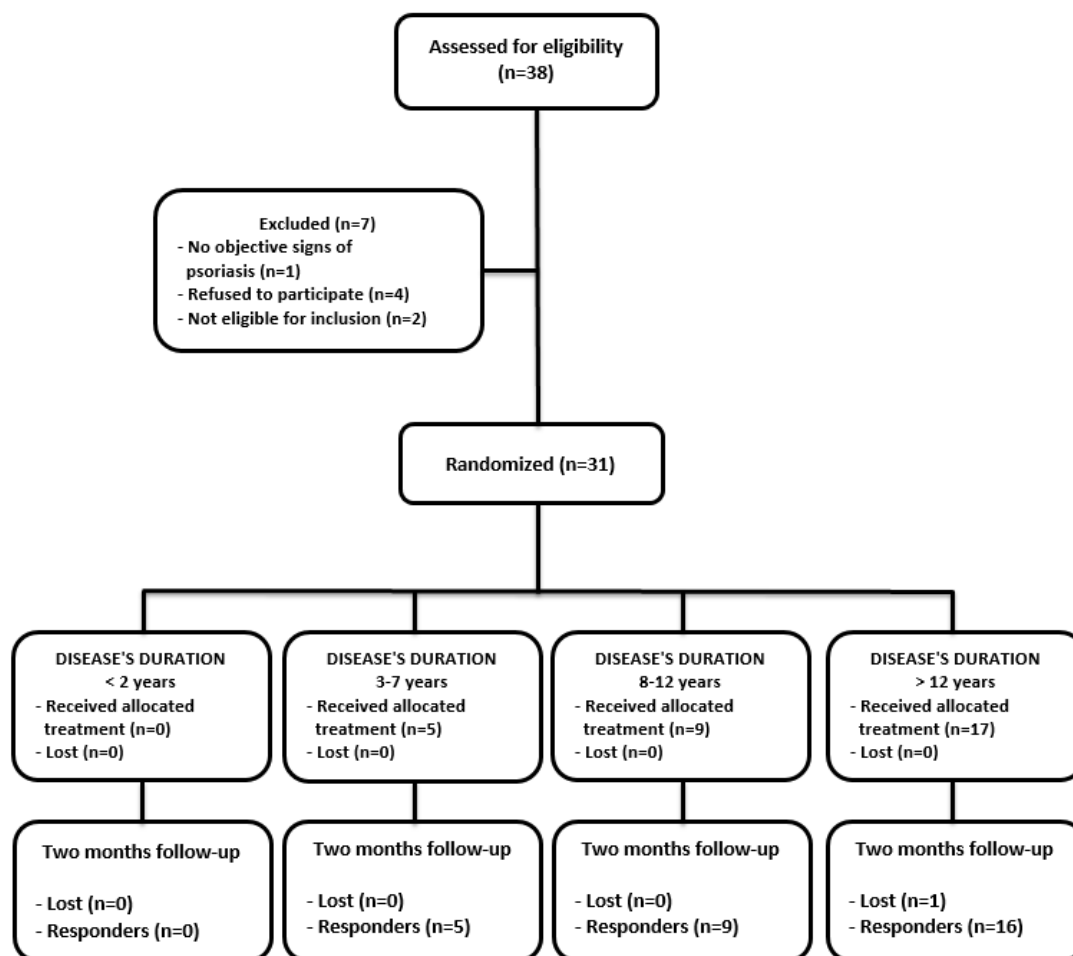


Figure 5. Flow chart of the study

After applying Dermavit cream for sixty days, satisfactory improvement was achieved to the biggest number of patients participating in the study a total of 19 (63.3%), respectively. Partial improvement was achieved on a limited number patients participating in the study, a total of 10 (33.3%), respectively. In the

smallest number of patients participating in the study, 1 patient (3.3%), there was no change. There were no cases of psoriasis worsening, after using Dermavit cream. Efficacy of Dermavit cream by age, gender and disease's duration is shown in Table 2.

Table 2. Efficacy of Dermavit cream by age, gender and disease's duration

		EFFICACY OF DERMAVIT CREAM			
		Satisfactory improvement n (%)	Partial improvement n (%)	Lack of improvement n (%)	Deterioration n (%)
GENDER	F	9 (30.0)	4 (13.33)	1 (3.33)	0 (0)
	M	10 (33.3)	6 (20.00)	0 (0)	0 (0)
AGE	< 30 years	3 (10.00)	1 (3.33)	0 (0)	0 (0)
	30–39 years	4 (13.33)	0 (0)	0 (0)	0 (0)
	40-49 years	1 (3.33)	4 (10.00)	1 (3.33)	0 (0)
	50-64 years	7 (23.33)	3 (10.00)	0 (0)	0 (0)
	> 65 years	3 (10.00)	3 (10.00)	0 (0)	0 (0)
DISEASE'S DURATION	< 2 years	0 (0)	0 (0)	0 (0)	0 (0)
	3-7 years	3 (10.00)	1 (3.33)	1 (3.33)	0 (0)
	8-12 years	9 (30.00)	0 (0)	0 (0)	0 (0)
	> 12 years	7 (23.33)	9 (30.00)	0 (0)	0 (0)

Based on the obtained results, no relationship between gender, age, duration of the disease and the effectiveness of the Dermavit cream can be established.

Figure 6 and Figure 7 show examples of satisfactory improvement and partial improvement, respectively.

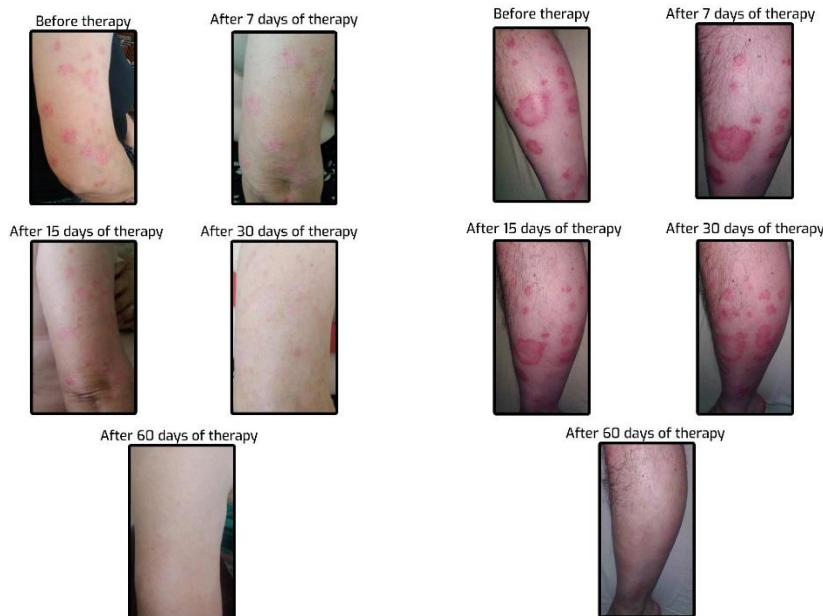


Figure 6. Examples of satisfactory improvement after applying Dermavit cream



Figure 7. Examples of partial improvement after applying Dermavit cream

According to the answers from the questionnaire, the majority of patients are frequently disturbed by itchy and/or irritated skin or pain, and are frequently concerned about their skin's look. For most patients participating in the study, skin redness, skin thickening, itching and skin peeling were reduced after using Dermavit cream.

CONCLUSION

Herbal products are being increasingly used in the treatment of skin diseases like psoriasis. Unfortunately, most studies provide only limited information about the efficacy and safety of topically used herbal products in the treatment of psoriasis. Therefore, more scientific evidence and

- 5. How often do you experience itchy skin?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 6. How often are you bothered by the feeling of irritated skin or pain?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 7. How often are you worried about the appearance of your skin?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 8. Does the condition of your skin affect the interaction with other people?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 9. Does the condition of your skin limit the type of clothing you wear?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 10. Does the condition of your skin affect the choice of social activities?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 11. Does your skin condition cause problems at school / work?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 12. Does your skin condition cause relationship / marriage problems?
 - never
 - rarely
 - sometimes
 - often
 - very often
- 13. Have you ever used herbal products in the treatment of diseases? YES NO
- 14. Have you ever used Dermavit cream for treatment of psoriasis, eczema and dermatitis? YES NO
- 15. How did you start using Dermavit cream for treatment of psoriasis, eczema and dermatitis:
 - on the recommendation of family / friends
 - on the recommendation of a physician
 - on the recommendation of a pharmacist
 - based on information from the media
 - other, _____
- 16. I use Dermavit cream for treatment of psoriasis, eczema and dermatitis regularly and in the way recommended by my doctor / pharmacist: YES NO

17. What are your expectations from the application of Dermavit cream for treatment of psoriasis, eczema and dermatitis:

- alleviation of symptoms
- treatment of diseases
- something else, _____

18. Since i have started to use Dermavit cream for treatment of psoriasis, eczema and dermatitis, my skin redness has decreased:

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

19. Since i have started to use Dermavit cream for treatment of psoriasis, eczema and dermatitis I have no thickening on the skin :

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

20. Since i have started to use Dermavit cream for treatment of psoriasis, eczema and dermatitis I have no scales on the skin:

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

21. Since i have started to use Dermavit cream for treatment of psoriasis, eczema and dermatitis I have no itchy skin :

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

22. I generally consider Dermavit cream for treatment of psoriasis, eczema and dermatitis very effective in treatment of psoriasis:

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

23. I would recommend Dermavit cream for treatment of psoriasis, eczema and dermatitis to other patients who have not used it before:

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

24. I have not noticed any side effects while using Dermavit cream for treatment of psoriasis, eczema and dermatitis:

- totally agree
- somewhat agree
- neither agree, or disagree
- somewhat disagree
- totally disagree

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DEFINITION OF INITIAL PHOSPHORUS SOURCE CONCENTRATION IN WASTE GLYCEROL-BASED MEDIUM FOR XANTHAN BIOSYNTHESIS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Phosphorus nutrition appears to be very important in the xanthan production, next to carbon and nitrogen. Various studies indicated that xanthan production is controlled by phosphorus concentration in cultivation medium and that phosphorus is generally added in the form of phosphate salts. This paper presents an analysis of the effect of various K_2HPO_4 concentrations (0-3.00 g/L) in waste glycerol-based medium on xanthan production using *Xanthomonas* strain PL 4 isolated from pepper leaves. The bioprocess success was assessed based on the values of xanthan concentration in media at the end of biosynthesis, average molecular weight of the polymer, degree of glycerol conversion into xanthan and degree of total phosphorus conversion. The obtained results show that the increase of K_2HPO_4 concentration in medium from 0 g/L to 2.50 g/L significantly contributes to the rise in xanthan amount and its molecular weight, while further increase of phosphorus source concentration has a statistically insignificant effect on the values of these parameters. The greatest bioprocess success is achieved when the concentration of K_2HPO_4 in medium was 2.50 g/L. Cultivation of producing strain on medium with optimal K_2HPO_4 concentration resulted in high production of xanthan (13.26 ± 0.20 g/L) of good quality ($3.38 \pm 0.14 \cdot 10^5$ g/mol) along with a relatively high degree of phosphorus conversion ($73.62 \pm 0.02\%$).

KEYWORDS: biotechnological production; xanthan; *Xanthomonas* isolate; cultivation media; waste glycerol, phosphorus source

INTRODUCTION

Xanthan is a microbial extracellular heteropolysaccharide that is produced by bacteria of the genus *Xanthomonas* [1]. Due to its outstanding rheological characteristics and its ability to modify the flow behavior of solutions, it is widely used in the food, pharmaceutical, petrochemical, chemical and textile industries [2]. Although many types of bacteria from the genus *Xanthomonas* biosynthesizes xanthan, it is industrially most widely produced by aerobic submerged batch cultivation of reference strain *Xanthomonas campestris* ATCC 13951 on the appropriate medium under optimal conditions [3]. According to Allied Market Research it is estimated that xanthan market value in 2023 will reach amount to 1184 billion US dollars. Commercially available xanthan is relatively expensive due to usage of glucose or sucrose as carbon sources in the xanthan production medium. However, the results of several studies show that waste glycerol, which is generated in huge amounts during the production of biodiesel, is a cheap and available alternative substrate, suitable for use in biotechnological production of xanthan [4-6]. Research related to the xanthan biosynthesis on waste glycerol-based media is still in initial stages and there

is a need for further research and optimization of biotechnological production of xanthan on this complex cultivation medium in order to achieve high efficiency.

Cultivation medium composition, producing strains and bioprocess parameters have significant effect on the yield and the properties of xanthan [7]. Besides carbon and nitrogen sources, cultivation medium for xanthan production contains other important nutrients such as phosphorus, sulfur, potassium, calcium and magnesium. These nutrients are most commonly added to cultivation media in the form of phosphoric and/or sulfuric acid salts [8]. According to the data from available literature, phosphorus is usually added to cultivation medium in the form of K_2HPO_4 , in an amount from 1.00 g/L to 3.00 g/L [4, 7]. Although recent studies have been focused on optimization of waste glycerol-based medium in order to ensure maximal biosynthesis of xanthan of great quality, there are no available data from scientific literature related to the examination of the effect of phosphorus concentration and phosphorus-containing compounds in cultivation medium on the success of xanthan biosynthesis.

The aim of this study is to examine the effect of the different K_2HPO_4 concentrations in waste glycerol-based cultivation medium on xanthan production using strain *Xanthomonas* PL 4 isolated from pepper leave. Process efficacy was estimated based on the quantity and quality of biosynthesized xanthan and conversion of phosphorus.

EXPERIMENTAL

PRODUCING MICROORGANISMS

The strain *Xanthomonas* PL 4 isolated from infected pepper leave was used as the producing microorganism in these experiments. Producing strain was stored at 4°C on agar slant (YMA[®], HiMedia, India) and subcultured every four weeks.

CULTIVATION MEDIA

The commercial medium (YMB[®], HiMedia, India) and glycerol-based growth medium (2 g/L glucose, 3 g/L glycerol from waste glycerol and 5 g/L malt extract) were used for inoculum preparation (inoculum I and inoculum II, respectively), while xanthan production was performed on medium with waste glycerol in a quantity of 16.0 g/L. Cultivation medium for xanthan production also contained $(NH_4)_2SO_4$ in a quantity of 3.0 g/L. The pH value of both glycerol-based media was adjusted to 7.0 ± 0.2 . All media were sterilized by autoclaving (121°C, 2.1 bar, 20 min) and stored at 4°C until use.

INOCULUM PREPARATION

Producing strain was subcultured on agar slant and incubated at 25°C for 48 h. Inoculum preparation procedure was included the incubation of refreshed producing microorganism in liquid media in two steps: preparation of inoculum I and preparation of inoculum II. First step (inoculum I preparation) considered the suspending of producing microorganism cells in YMB[®] and incubation for 48 h. Inoculum II preparation considered addition of 10% (v/v) of inoculum I to glycerol-based growth medium and incubation for 36 h. Both suspensions were incubated in aerobic conditions at 25°C and 150 rpm (laboratory shaker KS 4000i control, Ika[®] Werke, Germany).

XANTHAN PRODUCTION

The xanthan production was carried out in 300 mL Erlenmeyer flasks with 100 mL of the waste glycerol-based medium. Inoculation was performed by adding 5% (v/v) of inoculum II prepared as previously described.

The biosynthesis was performed under aerobic conditions at 30°C and 150 rpm (laboratory shaker KS 4000i control, Ika[®] Werke, Germany) for 168 h.

XANTHAN SEPARATION

At the end of biosynthesis, the xanthan was separated from the supernatant of cultivation medium obtained using an ultracentrifuge (Hettich Rotina 380 R, Germany) at 10,000 rpm for 10 min, by precipitation with cold 96% (v/v) ethanol in the presence of the potassium-chloride as electrolyte as described in previous research [9]. The concentration of the produced biopolymer was evaluated by determining the weight of the dry product per litre of cultivation medium.

DETERMINATION OF CULTIVATION MEDIUM RHEOLOGICAL PROPERTIES

The rheological behavior of cultivation medium samples taken at the end of the bioprocess were evaluated using rotational viscometer (REOTEST 2 VEB MLV Prüfgerate-Verk, Mendingen, SitzFreitel) with double gap coaxial cylinder sensor system, spindle N. Based on deflection of measuring instrument (α , Skt), shear stress (τ , Pa) was calculated under defined values of shear rates (D , 1/s) using the Eq. (1):

$$\tau = 0.1 \cdot z \cdot \alpha \dots\dots\dots (1)$$

where z is the constant with the value 3.08 $\text{dyn/cm}^2 \cdot \text{Skt}$. The pseudoplastic behavior of the cultivation medium was confirmed by fitting the experimental data to the Ostwald-de-Waele model using the power regression. The values of the consistency factor (K , $\text{Pa} \cdot \text{s}^n$), flow behavior index (n) and determination coefficient (R^2) were determined by Excel software 2013 and used for calculation of medium apparent viscosity (η_a , $\text{mPa} \cdot \text{s}$) from Eq. (2):

$$\eta_a = K \cdot D^{n-1} \dots\dots\dots (2)$$

where D is shear rate with the value of 100 1/s.

DETERMINATION OF XANTHAN MOLECULAR WEIGHT

The average molecular weight of the separated xanthan was estimated based on the intrinsic viscosity of its solution in 0.1 M sodium chloride using the Mark-Houwink type equation [10].

DETERMINATION OF GLYCEROL AND PHOSPHOROUS

CONTENT

The samples of cell-free cultivation media taken after inoculation and 168 h of cultivation, obtained by centrifugation at 10,000 rpm for 15 min (Rotina 380 R, Hettich Lab Technology, Germany), were analyzed for glycerol and total phosphorus contents. Glycerol content was determined by high performance liquid chromatography (HPLC). The samples were filtered through a 0.45 μm nylon membrane (Agilent Technologies Inc, Germany) and then analysed. The HPLC instrument (Thermo Scientific Dionex UltiMate 3000 series) was equipped with a HPG-3200SD/RS pump, WPS-3000(T)SL autosampler (10 μL injection loop), Zorbax NH2 column (250 mm \times 4.6 mm, 5 μm) and RefractoMax520 detector. 70% (v/v) acetonitrile was used as eluent with a flow rate of 1 mL/min and elution time of 10 min at a column temperature of 30 $^{\circ}\text{C}$. The glycerol content results after inoculation and xanthan concentration in media at the end of biosynthesis were used to calculate the degree of conversion of glycerol to xanthan ($K_{S/P}$, %) using equation:

$$K_{P/S} = \frac{P}{S_0} \cdot 100 \dots \dots \dots (3)$$

where S_0 is the initial glycerol content (g/L), whereas P is xanthan concentration in media at the end of biosynthesis (g/L).

The content of total phosphorus was determined using the spectrophotometric method proposed by Gales et al., 1966 [11]. The phosphorus content results after inoculation and 168 h of cultivation were used to calculate the degree of total phosphorus conversion (K_{P_t} , %) using equation:

$$K_{P_t} = \frac{P_{i0} - P_t}{P_{i0}} \cdot 100 \dots \dots \dots (4)$$

where P_{i0} is the initial phosphorus content (g/L) and P_t is the residual phosphorus content (g/L).

DATA ANALYSIS

All experiments were carried out in triplicate and the results were averaged. The experimental data were processed by one-way analysis of variance (One-Way ANOVA). Significant differences between the means were determined by Duncan's multiple range test at the significance level of $\alpha=0.05$ using Statistica 13.2 software (Dell Inc., USA).

RESULTS AND DISCUSSION

In accordance with the defined aim of this research, xanthan biosynthesis was performed by the strain *Xanthomonas* PL 4 isolated from pepper leaves on waste glycerol-based cultivation media containing K_2HPO_4 in concentrations from 0 g/L to 3.00 g/L.

Considering the fact that the xanthan solutions and xanthan cultivation broths at the end of biosynthesis are highly viscous and show pseudoplastic behavior [8], the possibility of xanthan production on waste glycerol-based cultivation media with addition of K_2HPO_4 in different concentrations were evaluated comparing the rheological properties of cultivation media after biosynthesis. The rheological parameters were determined from the relationship between shear rate and shear stress, and obtained values are presented in Table 1.

Table 1. Rheological properties of waste glycerol-based cultivation media containing K_2HPO_4 in different concentrations

K_2HPO_4 (g/l)	K [$\text{Pa}\cdot\text{s}^n$]	n [1]	R^2	η_a [$\text{mPa}\cdot\text{s}$]
0.0	0.2998	0.5238	0.916	29.98 \pm 4.02
0.5	0.2421	0.5653	0.954	31.04 \pm 2.47
1.0	0.3366	0.5120	0.943	34.36 \pm 0.38
1.5	0.3275	0.5383	0.928	36.77 \pm 0.77
2.0	0.4286	0.4870	0.9487	37.52 \pm 2.62
2.5	0.4614	0.4838	0.944	38.77 \pm 3.53
3.0	0.4612	0.5027	0.947	40.93 \pm 4.52

K - consistency factor; n - flow behavior index;
 R^2 - determination coefficient; η_a - apparent viscosity.

Flow behavior index (n) shows a level of deviation from Newtonian flow behavior. For a Newtonian fluid, flow behavior index is equal to 1, greater than 1 for a dilatant and less than 1 for a pseudoplastic fluid [12]. According to the results presented in Table 1, values of flow behavior index for cultivation media obtained after biosynthesis in applied experimental conditions were in the range from 0.4838 to 0.5653. The obtained values of flow behaviour index confirm the pseudoplastic characteristics of the cultivation media and this indicates that xanthan biosynthesis is confirmed in all media.

Values of the coefficient of determination (R^2) higher than 0.910 indicate a very good corresponding of the measured values with the Ostwald-de-Waele model, which further confirms pseudoplastic behavior of all tested samples.

The viscosity of xanthan solution is greatly effected with the concentration of the biopolymer, but also on its structure [8]. Taking into account that the consistency factor (K) is proportional to the viscosity,

the different values of this parameter shown in Table 1 indicate the difference in the quantity and quality of xanthan produced by the strain *Xanthomonas* PL 4 on waste glycerol-based cultivation media containing K_2HPO_4 in concentrations from 0 g/L to 3.0 g/L. The obtained results presented in Table 1 show that values of the apparent viscosity (η_a) of cultivation media at the end of bioprocess were in the range from 29.98 ± 4.02 mPa·s to 40.93 ± 4.52 mPa·s.

The values of apparent viscosity of cultivation medium obtained in this study are in accordance with result from previous research when different *Xanthomonas campestris* strains were cultivated on waste glycerol-based medium containing 3.00 g/L K_2HPO_4 [5]. The highest value of viscosity of cultivation medium (40.93 ± 4.52 mPa·s) was obtained when strain *Xanthomonas* PL 4 was cultivated on waste glycerol-based cultivation medium containing 3.00 g/L K_2HPO_4 and the lowest value of this

parameter (29.98 ± 4.02 mPa·s) was achieved by cultivation of the same producing strain on waste glycerol-based medium without any addition of K_2HPO_4 .

In order to examine the effect of the different K_2HPO_4 concentrations (0-3.00 g/L) in waste glycerol-based medium on xanthan production using strain *Xanthomonas* PL 4, statistical analysis of experimental data was carried out. The results of One-Way ANOVA analysis, as well as results of post hoc analysis by Duncan's multiple range test for xanthan concentration in media, its average molecular weight and degree of total phosphorus conversion are further discussed.

The results of statistical analysis of the effect of the different K_2HPO_4 concentrations in cultivation medium on xanthan concentration (P, g/L), its average molecular weight (M_w , 10^5 g/mol) and degree of total phosphorus conversion (K_p , %) are shown in Table 2.

Table 2. Analysis of variance (One-Way ANOVA) of the different K_2HPO_4 concentrations in cultivation medium on xanthan concentration, its average molecular weight and degree of phosphorus conversion

Parameters	Variability	SS	DF	MS	F-odnos	p-vrednost
P (g/L)	K_2HPO_4 (g/l)	27.155	6	4.526	39.280	< 0.000001
	Error	1.613	14	0.115	-	-
M_w (10^5 g/mol)	K_2HPO_4 (g/l)	11.509	6	1.918	61.632	< 0.000001
	Error	0.436	14	0.031	-	-
$(K_p, \%)$	K_2HPO_4 (g/l)	1600.500	5	320.100	79453.000	< 0.000001
	Error	0	12	0	-	-

SS – sum of squares; DF – degrees of freedom; MS – mean square; P-xanthan concentration in media; M_w -average molecular weight of xanthan; K_p -degree of total phosphorus conversion.

From the results presented in Table 2 it can be noticed that the p-values are much lower than 0.05 for all three observed parameters, which indicates that K_2HPO_4 concentration has a statistically significant effect on xanthan concentration in cultivation media, its average molecular weight and degree of total phosphorus conversion.

The obtained results of statistical analysis of the effect of the different K_2HPO_4 concentrations in cultivation medium on xanthan concentration, its average molecular weight and degree of total phosphorus conversion are presented graphically by Box & Whisker Plots in Figure 1-3.

Observing the results presented in Figure 1 it can be noticed that there is statistically significant difference in xanthan concentration in media when K_2HPO_4 was added to waste glycerol-based cultivation medium in different concentrations.

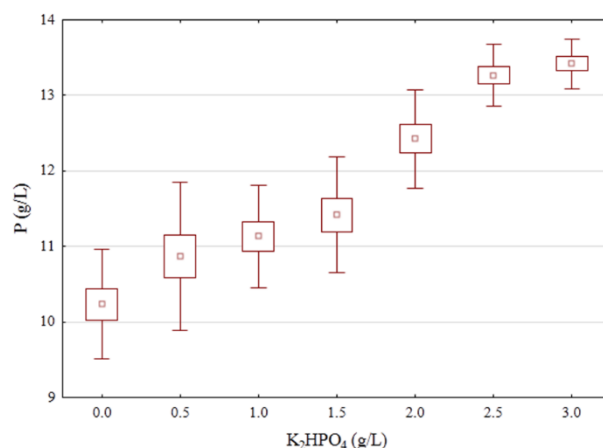


Figure 1. Effect of the different K_2HPO_4 concentrations in waste glycerol-based cultivation medium on xanthan concentration (P)

According to the results presented in Figure 1, concentration of xanthan produced in applied experimental conditions was in the range from around 9.50 g/L to 14.00 g/L. It is evident that the highest xanthan concentration was obtained when K_2HPO_4 concentration in cultivation medium was 3.00 g/L.

Results presented in Figure 1 suggest that there is no statistically significant difference in the xanthan concentration in media when K_2HPO_4 concentration in cultivation medium was 2.50 g/L and 3.00 g/L. The lowest xanthan concentration was obtained when biosynthesis was performed on medium without addition of K_2HPO_4 .

The graphically presented results in Figure 2 show that there is statistically significant difference in average molecular weight of xanthan obtained when K_2HPO_4 was added to cultivation medium in different concentrations. Considering the results presented in Figure 2 it can be noticed that average molecular weight of xanthan obtained on media with different concentration of K_2HPO_4 ranged from about $1 \cdot 10^5$ g/mol to $4 \cdot 10^5$ g/mol. The obtained results show that xanthan with the highest average molecular weight was achieved when K_2HPO_4 was added to cultivation medium in maximal concentration (3.00 g/L). As it can be seen from the Figure 2, there is no statistically significant difference in the average molecular weight of xanthan when K_2HPO_4 concentration in cultivation medium was 2.50 g/L and 3.00 g/L. Xanthan with the lowest average molecular weight was obtained when biosynthesis was performed on medium without addition of K_2HPO_4 .

From graphically presented results (Figure 3) it can be observed that there is statistically significant difference in degree of total phosphorus conversion obtained when K_2HPO_4 was added to waste glycerol-based cultivation medium in different concentrations. The results presented in Figure 3 show that a value of degree of total phosphorus conversion achieved after cultivation of producing strain on media with different concentration of K_2HPO_4 ranged from about 60% to 90%. The highest degree of total phosphorus conversion was achieved when K_2HPO_4 was added to cultivation medium in minimal concentration (0.50 g/L). High value of degree of total phosphorus conversion was also achieved when K_2HPO_4 concentration in cultivation medium was 1.00 g/L, 1.50 g/L and 2.00 g/L. The obtained results show that

the lowest conversion of total phosphorus was achieved when K_2HPO_4 was added to cultivation medium in maximal concentration (3.00 g/L).

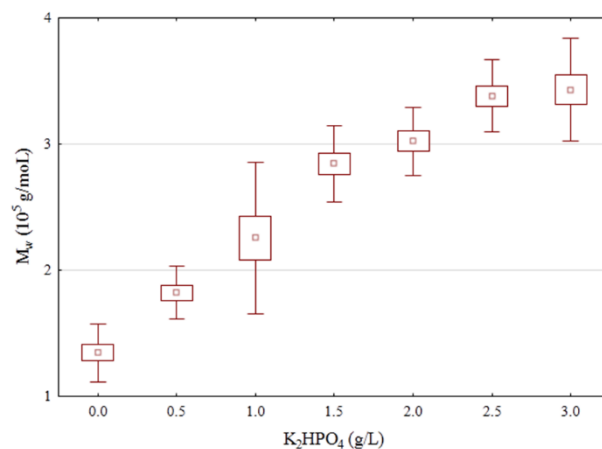


Figure 2. Effect of the different K_2HPO_4 concentrations in waste glycerol-based cultivation medium on xanthan average molecular weight (M_w)

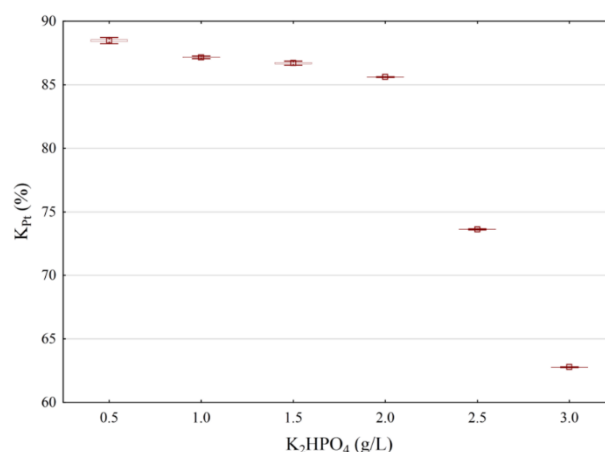


Figure 3. Effect of the different K_2HPO_4 concentrations in waste glycerol-based cultivation medium on degree of total phosphorus conversion (K_{Pt})

Homogeneous groups of experiments were established based on statistical significance of differences in the obtained xanthan concentration, its quality, the degree of conversion of glycerol to xanthan and the degree of total phosphorus conversion by using Duncan's multiple range test comparisons. The obtained results are shown in Table 3.

Table 3. Duncan's multiple range test: mean \pm standard deviation for the xanthan concentration, average molecular weight of xanthan, degree of conversion of glycerol into xanthan and degree of total phosphorus conversion

K_2HPO_4 (g/L)	P (g/L)	$K_{S/P}$ (%)	M_w (10^5 g/mol)	K_{Pt} (%)
0	10.24 \pm 0.36 ^a	63.38 \pm 2.23 ^a	1.35 \pm 0.11 ^a	-
0.5	10.87 \pm 0.49 ^b	67.32 \pm 3.03 ^b	1.82 \pm 0.10 ^b	88.48 \pm 0.12 ^f
1.0	11.13 \pm 0.34 ^b	68.93 \pm 2.10 ^b	2.26 \pm 0.30 ^c	87.15 \pm 0.05 ^e
1.5	11.42 \pm 0.38 ^b	70.70 \pm 2.36 ^b	2.84 \pm 0.15 ^d	86.70 \pm 0.08 ^d
2.0	12.42 \pm 0.33 ^c	76.91 \pm 2.02 ^c	3.02 \pm 0.13 ^d	85.60 \pm 0.02 ^c
2.5	13.26 \pm 0.20 ^d	82.13 \pm 1.25 ^d	3.38 \pm 0.14 ^e	73.62 \pm 0.02 ^b
3.0	13.42 \pm 0.17 ^d	83.09 \pm 1.02 ^d	3.43 \pm 0.21 ^e	62.78 \pm 0.01 ^a

*Values in the same column marked with the same letter are not significantly different at $\alpha=0.05$.

As it can be noticed from the Table 3, xanthan concentration in medium at the end of biosynthesis ranged from 10.24 \pm 0.36 g/L to 13.42 \pm 0.17 g/L. The highest xanthan concentration was obtained when K_2HPO_4 was added to waste glycerol-based cultivation medium in maximal concentration (3.00 g/L). According to the results presented in Table 3, the value of degree of conversion of glycerol into xanthan was in range from 63.38 \pm 2.23% to 83.09 \pm 1.02% and the highest value of this parameter was also achieved when K_2HPO_4 was added to medium in maximal concentration. The obtained results suggest that there is no statistically significant difference in xanthan concentration (13.26 \pm 0.20 g/L and 13.42 \pm 0.17 g/L) and degree of glycerol conversion into xanthan (82.13 \pm 1.25% and 83.09 \pm 1.02%) when K_2HPO_4 was added to cultivation medium in concentration of 2.50 g/L and 3.00 g/L, which is confirmed by the p -values of 0.584152 and 0.584152, respectively. Statistically significant lower xanthan concentration was obtained when cultivation of producing strain was performed on medium containing K_2HPO_4 in concentrations from 0.50 g/L to 2.00 g/L, which is confirmed by p -values lower than 0.05. However, the results of xanthan concentration obtained when using 1.0 g/L K_2HPO_4 (11.13 \pm 0.34 g/L) show greater success comparing to the results obtained in previous study where 7.23 g/L of xanthan was achieved by cultivation of the production strain *X. campestris mangiferaeindicae* 2103 on waste glycerol-based medium with the addition of 1.0 g/L K_2HPO_4 [4].

Based on the results presented in Table 3 it can be noticed that the lowest xanthan concentration was achieved when waste glycerol-based cultivation medium did not contain K_2HPO_4 . Conversion of carbon source into product was also the lowest when cultivation of producing strain was performed on medium with the same composition. Considering the results presented in Table 3 it is evident that increasing the concentration of K_2HPO_4 in waste glycerol-based

medium for xanthan biosynthesis up to 2.50 g/L, in the applied experimental conditions, has a positive effect on the xanthan concentration and degree of glycerol conversion into xanthan. The values of both parameters obtained in this research are higher comparing to the values achieved in previous research when cultivation of reference strain *Xanthomonas campestris* ATCC 13951 on waste glycerol-based medium containing 3.00 g/L K_2HPO_4 resulted in production of xanthan in concentration of 6.68 g/L where is 66.46% of glycerol converted to xanthan [13]. Taking into account that in industrial conditions, the degree of conversion of carbon sources into xanthan ranges from 50-85% [14], it can be concluded that within this research, a very high efficiency of bioprocess has been achieved.

Analysing the values of average molecular weight of xanthan produced by cultivation of producing strain on waste glycerol-based medium with different concentrations of K_2HPO_4 (Table 3) it can be observed that the values of this indicator of biopolymer quality were in the range from 1.35 \pm 0.11 \cdot 10⁵ g/mol to 3.43 \pm 0.21 \cdot 10⁵ g/mol. The obtained results suggest that the highest average molecular weight of xanthan was obtained when the cultivation of producing strain was performed on medium containing 3.00 g/L K_2HPO_4 . Xanthan of statistically insignificant lower average molecular weight (3.38 \pm 0.14 \cdot 10⁵ g/mol) was obtained when bioprocess was performed on medium containing 2.50 g/L K_2HPO_4 ($p=0.737000$). From the results shown in Table 3 it is evident that xanthan of the lowest average molecular weight was achieved when cultivation was performed on medium with no K_2HPO_4 . Considering the results discussed above, it can be concluded that increasing the concentration of K_2HPO_4 in cultivation medium up to 2.50 g/L has a positive effect on the average molecular weight of xanthan produced in applied experimental conditions.

The results of Duncan's multiple range test for the degree of total phosphorus conversion achieved after

cultivation of producing strain on waste glycerol-based medium with addition of K_2HPO_4 in concentration from 0.50 g/L to 3.00 g/L are presented in Table 3. Experiment in which cultivation was performed on medium without any addition of K_2HPO_4 was excluded from consideration. According to the results shown in Table 3 it can be noticed that the degree of total phosphorus conversion in waste glycerol-based medium after cultivation of producing strain in applied experimental conditions ranged from $62.78 \pm 0.01\%$ to $88.48 \pm 0.12\%$. The highest degree of total phosphorus conversion was achieved when medium containing 0.50 g/L K_2HPO_4 was applied. Very high conversion of total phosphorus was also achieved in experiments where the concentration of K_2HPO_4 in the medium for xanthan production was 1.0 g/L ($87.15 \pm 0.05\%$), 1.50 g/L ($86.70 \pm 0.08\%$) and 2.00 g/L ($85.60 \pm 0.02\%$). The lowest degree of total phosphorus conversion was achieved when K_2HPO_4 was added in cultivation medium in the concentration of 3.00 g/L. The values of degree of total phosphorus conversion accomplished in this research are much higher comparing to the results obtained in previous research when degree of total phosphorus conversion during cultivation of strains of the genus *Xanthomonas* isolated from pepper leaves on waste glycerol-based media containing 3.00 g/L K_2HPO_4 was in the range from 25.70% to 31.03% [6].

The results obtained within this research show that the increase of K_2HPO_4 concentration in medium from 0 g/L to 2.50 g/L significantly contributes to the rise in xanthan concentration in media, its average molecular weight and degree of conversion of glycerol into xanthan, while further increase of phosphorus source concentration has a statistically insignificant effect on the values of these parameters. Taking into account previously discussed results it can be concluded that the greatest bioprocess efficacy is achieved when the concentration of K_2HPO_4 in medium was 2.50 g/L. Waste glycerol-based medium with the initial K_2HPO_4 concentration of 2.50 g/L was suggested as the most appropriate for further research related to xanthan production.

CONCLUSION

In accordance with the defined aim, in this study the effect of various K_2HPO_4 concentrations (0-3.00 g/L) in waste glycerol-based medium on xanthan production using *Xanthomonas* strain PL 4 isolated from infected pepper leaf was examined. The obtained results suggest that increasing the concentration of used phosphate salt in the cultivation medium has a positive effect on the xanthan concentration, degree of glycerol conversion into

xanthan and its average molecular weight. The highest values of aforementioned parameters were achieved when bioprocess was performed on medium containing 3.00 g/L K_2HPO_4 . The obtained results also indicate that increasing the concentration of K_2HPO_4 in the cultivation medium from 2.50 g/L to 3.00 g/L does not lead to a statistically significant change in the values of analyzed parameters. On the other hand, increasing the concentration of K_2HPO_4 in the cultivation medium in the range from 0.50 g/L to 3.00 g/L has a negative effect on the degree of total phosphorus conversion and that the highest degree of phosphorus conversion was achieved when the cultivation of producing strain was performed on medium containing 0.50 g/L K_2HPO_4 .

According to the results achieved in this study it can be concluded that the optimal concentration of K_2HPO_4 in waste glycerol-based medium for high efficiency xanthan production by selected producing strain is 2.50 g/L. Results obtained in this study represent valuable information for future research related to development of biotechnological production of xanthan on waste glycerol-based medium.

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