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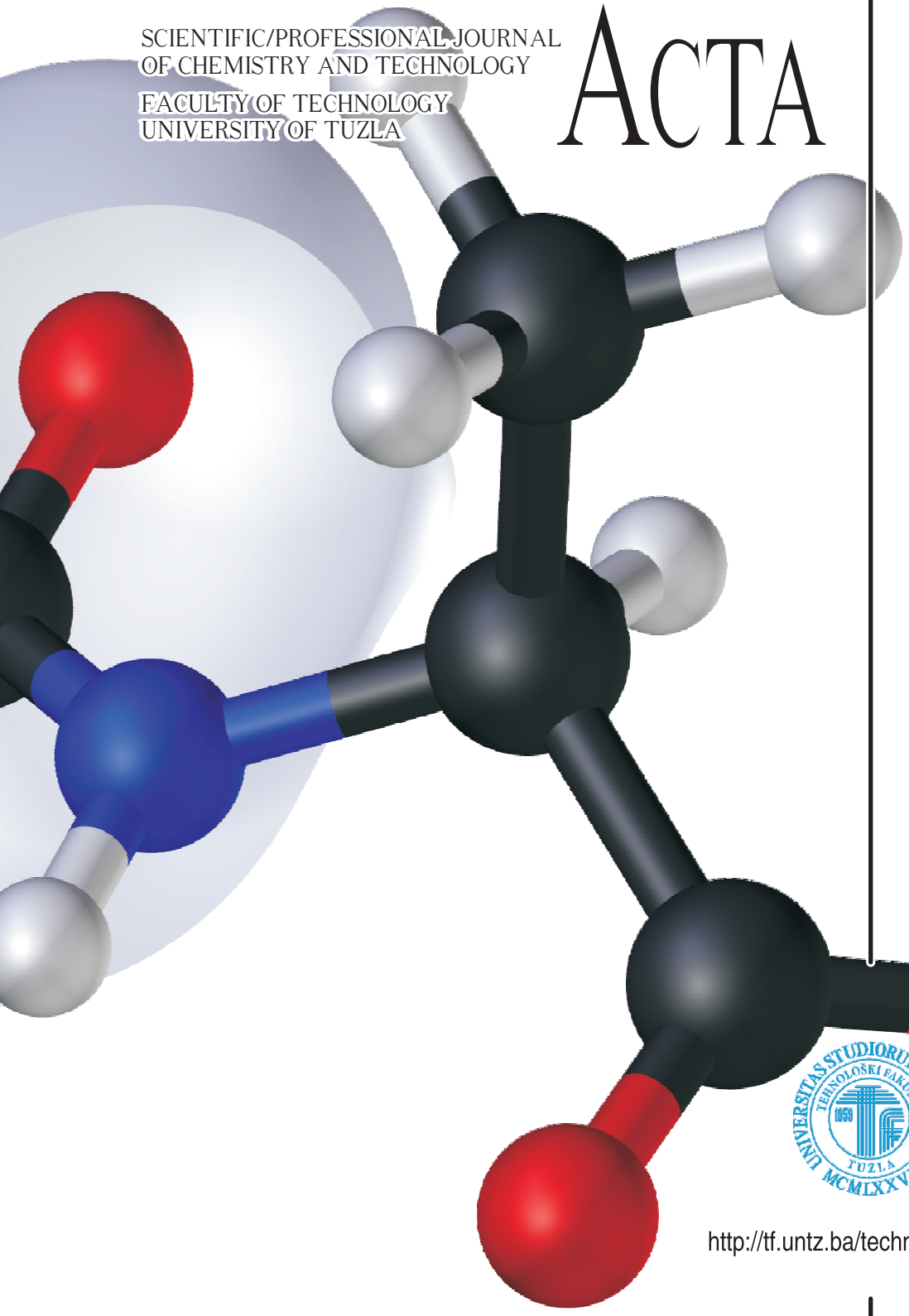

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CONTENT


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PROTONATION OF CITRACONIC AND GLUTACONIC ACID IN PERCHLORIC ACID MEDIA

ORIGINAL SCIENTIFIC PAPER

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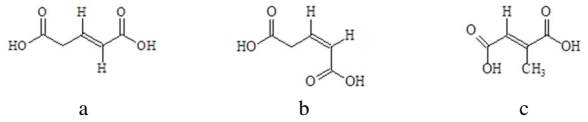
ABSTRACT: The protonation process of citraconic and glutaconic acid in perchloric acid media was followed using the method of UV spectroscopy. The observed changes in the UV spectra of investigated acids confirmed that the protonation process in perchloric acid with concentration from 5 to 10 mol/dm^3 occurred. Glutaconic acid behaved as weak organic base in perchloric acid media and existed in its monoprotonated form. On the other hand, citraconic acid existed in its protonated form and as protonated anhydride at higher perchloric acid concentration. Using the absorbance data the thermodynamic dissociation constants were calculated applying the methods of Yates and McClelland, Bunnett and Olsen, and the "excess acidity" function method. The solvatation parameters m, m^* and ϕ were evaluated, as well. In order to correct the medium effect the method of characteristic vector analysis was applied. The possible site where the protonation may take place was discussed using the partial atomic charge values determined according to AM1 and PM3 semiempirical methods.

KEYWORDS: citraconic acid, glutaconic acid, UV spectroscopy, thermodynamic dissociation constants, AM1 and PM3 semiempirical methods

INTRODUCTION

The structural formulas of citraconic (*cis*-2-methyl-1,2-ethylenedicarboxylic acid) and glutaconic (pent-2-enedioic acid) acid are shown in Scheme 1.



a b c

Scheme 1. Structural formulas of *trans*-glutaconic (a), *cis*-glutaconic (b) and citraconic acid (c)

Glutaconic acid exists in two forms: *cis*-glutaconic and *trans*-glutaconic acid. In the aqueous solutions *cis*-glutaconic acid undergoes izomerization and it is transformed into the *trans*-form which is more stable [1]. Glutaconic acid is important industrial substance because of their wide practical application. For instance, this acid can be used as monomer for production of biodegradable polyesters in chemical industry. Furthermore, glutaconic acid by condensation with suitable α,ω-diaminoalkanes participate in formation of polyamides [2]. Citraconic acid is used in the production of synthetic resins and polymers [1]. When the anhydride of citraconic acid reacts with primary amines an amide bond with a terminal carboxyl group can be formed. This reaction can be reversed in acidic condition *i.e.* pH from 3 to 4. It suggests that this anhydride can be used to block primary amine groups in alkaline conditions when pH value is between 7 and 9 [3].

The unsaturated dicarboxylic acids were studied by many authors, and some of them investigated the acid-base behavior of citraconic and glutaconic acid [4]-[7]. The protonation process of citraconic and glutaconic acid in sulfuric acid media was studied by several authors and the dissociation constants values (pK_{BH^+}) of protonated acids were determined, as well [5]-[8]. Knowing the pK_{BH^+} values of carboxylic acids is important in order to understand the mechanisms of the reactions in which they take part [9]. However, there is no literature data about thermodynamic dissociation constants values of citraconic and glutaconic acid determined in perchloric acid media. In order to investigate the influence of the strength of the acid and its anion on the protonation process it is important to use different mineral acids for protonation.

Taking into consideration that thermodynamic pK_{BH^+} values are important parameters for weak organic acids the purpose of this work was to follow the protonation process of citraconic and glutaconic acid in perchloric acid media by means of UV spectroscopy. The changes in the spectra can be used for determination of thermodynamic dissociation constant values of protonated acids. At the same time, using the semiempirical AM1 and PM3 quantum

chemical methods the possible protonation site in the molecules of the investigated acids can be predicted [10], [11].

EXPERIMENTAL

Chemicals and equipment: The stock solutions were prepared by dissolving a known amount of the investigated acids in redistilled water. The concentration of citraconic acid in the stock solutions was 9.8 10^{-5} mol/dm^3, while that of glutaconic acid was 8.3 10^{-5} mol/dm^3. Two series of test solutions with constant concentration of investigated organic acids were prepared. The concentration of perchloric acid in the test solutions was varied between 1 mol/dm^3 and 11 mol/dm^3. The UV spectra of the two series of solutions were recorded at room temperature in the wavelength region from 190 nm to 290 nm with 1 nm resolution, 24 hours after preparation of the test solutions. The absorbance data at the following wavelengths: 198, 206, 214 and 220 nm were used for calculation of the pK_{BH^+} values of glutaconic acid. The wavelengths were selected around the maximum of the absorption band. The molar absorption coefficient values of the unprotonated form of glutaconic acid were determined at perchloric acid concentration of 1 mol/dm^3 from the absorbance data at the same wavelengths. It was considered that at this mineral acid concentration glutaconic acid existed in its unprotonated form. The molar absorption coefficient values of protonated form of glutaconic acid were determined when the perchloric acid concentration was 11 mol/dm^3, because the protonation process of glutaconic acid at this concentration was completed. All used substances were of analytical grade p.a (Alkaloid and Merck). The concentration of perchloric acid was determined by titration with a standard solution of sodium hydroxide. The UV spectra were recorded on a Varian Cary spectrophotometer using quartz cell with 1 cm length, at room temperature. All calculations were performed by Microsoft Excel computer program.

Calculation of the pK_{BH^+} values: It is well known that the investigated carboxylic acids in water behave as proton donors, while in strong mineral acid media as proton acceptors. The protonation equilibria of these substances can be presented with the equation (1).

$$B + H_3O^+ \rightarrow BH^+ + H_2O \quad (1)$$

The pK_{BH^+} values in highly acidic media can be evaluated by Hammett [12] equation (2) in the following form:

$$pK_{BH^+} = H_0 + \log I \quad (2)$$

where, pK_{BH^+} is the dissociation constant of protonated form of the acid, H_0 is Hammett acidity function, I is a ratio between the concentration of the protonated and unprotonated form of the acid (ionization ratio).

When the absorbance values of protonated and unprotonated forms are measurable the ionization ratio can be calculated as $\log I = c(BH^+)/c(B)$. B is the set of primary nitroanilines that serve as reference bases which can be protonated in strongly mineral acid media. The Hammett acidity function (H_0) was no valid for all types of weak bases and it was upgraded by several authors. Bunnett and Olsen [13] calculated the thermodynamic dissociation constants according to the equation (3).

$$\log I + H_0 = \phi[H_0 + \log c(H^+)] + pK_{BH^+} \quad (3)$$

where, ϕ is a parameter which characterizes the changing activity coefficient behavior of bases with changing acidity.

Using the equation (3) necessity of different acidity functions to characterize the protonation process of different bases was reduced to one acidity function, *i.e.,* Hammett acidity function. The method of Yates and McClelland also can be used to determine the thermodynamic pK_{BH^+} values [14]. In this case, the calculations can be made by the equation (4).

$$\log I = -mH_0 + pK_{BH^+} \quad (4)$$

A plot of log$I$ vs. $-H_0$ gives a straight line with slope m, whose value is about 1. Actually, the pK_{BH^+} values should be calculated using the acidity functions which make possible the best slope of the dependence of log$I$ *vs.* H_0, *i.e.*, slope closest to unity. This suggest that the protonation process is favored compared to the solvatation process and the pK_{BH^+} values calculated by this method would be more accurate. Furthermore, the method of Cox and Yates [15], also known as the "excess acidity" function method (Eq. 5), was applied to determine the thermodynamic pK_{BH^+} values and the values of the solvatation parameter m^*.

$$\log I - \log c_{H^+} = m^* X + pK_{BH^+} \quad (5)$$

where, m^* expresses the hydrogen-bonding solvatation of the protonated base. X is excess acidity function which represents the difference between the observed acidity and that which the system would have if it was ideal.

The excess acidity function X is defined using the obtained data of the ionization ratio of 76 weak bases in 0-78% aqueous perchloric acid solutions [16]. These bases included compounds where the protonation centre is the atoms of N, C, O and S. Hence, using the acidity function X the values of the dissociation constants for different bases could be determined in aqueous perchloric acid solutions. The "excess acidity" function method is mostly used for determination of pK_{BH}^{+} values of organic bases in strong mineral acid media [16].

RESULTS AND DISCUSSION

UV spectra of citraconic and glutaconic acid: The UV spectra of citraconic and glutaconic acid were recorded at the wavelength region from 190 to 280 nm, as it is shown in the Figs. 1 and 2, respectively.

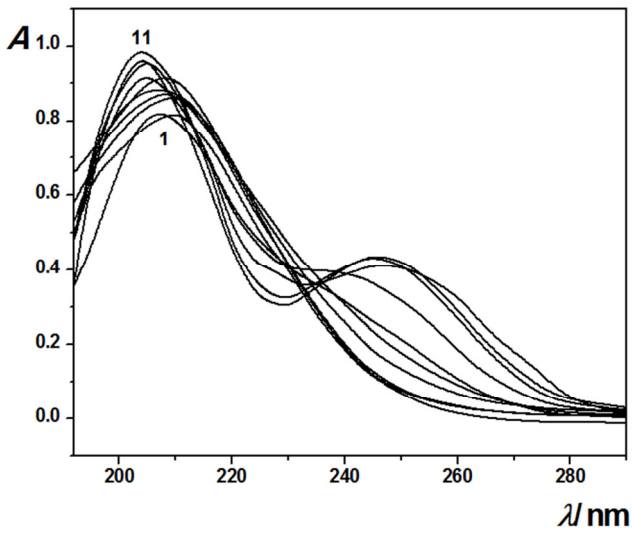


Fig. 1. Experimental UV spectra of citraconic acid(c = 9.8 10^{-5} mol/dm^3) in perchloric acid media (c($HClO_4$) = 1 mol/dm^3 (spectrum 1), c($HClO_4$) = 11 mol/dm^3 (spectrum 11))

At lower perchloric acid concentration (below 5 mol/dm^3) in the spectrum of citraconic acid a single band at 212 nm (marked with 1, in the Fig. 1) appeared (See Fig. 1). When the concentration of perchloric acid increased this band shifted monotonic to 204 nm (marked with 11 in the Fig. 1) and its intensity increased. At higher perchloric acid concentration a new band at 246 nm appeared. These two absorption bands are due to the $\pi \rightarrow \pi^*$ electron transitions. In accordance with the previous investigations [5]-[8] it was expected that the citraconic acid exists in the protonated form and protonated anhydride, as a result of partial dehydratation of the formed protonated acid [17].

It suggested that the reaction of protonation and dehydratation took place at the same time. Hence, the absorption band which appeared at 246 nm (See Fig. 1) was due to existence of the anhydride in the solution. The existence of two bands in the spectrum of citraconic acid was in agreement with the literature data which confirmed that for some organic acid at higher mineral acid concentration anhydrides are formed [17], [18]. In the spectrum of glutaconic acid a single absorption band around 214 nm (marked with 1 in the Fig. 2) appeared. When the perchloric acid concentration increased the hypsochromic effect was observed, similarly as it was case with the changes in the position of the spectral band of citraconic acid, and the intensity of this band insignificantly increased. When the concentration of perchloric acid was 11 mol/dm^3 the band was placed at 204 nm (marked with 11 in the Fig. 2).

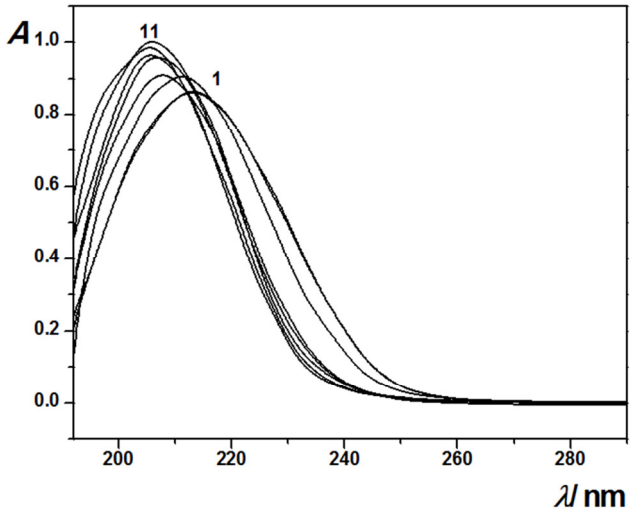


Fig. 2. Experimental UV spectra of glutaconic acid (c = 8.3 10^{-5} mol/dm^3) in perchloric acid media (c($HClO_4$) = 1 mol/dm^3 (spectrum 1), c($HClO_4$) = 11 mol/dm^3 (spectrum 11))

On the basis of these results and previous studies [4]-[8], the existence of one isosbestic point in the UV spectra of citraconic and glutaconic acid was expected. However, its position could not be established clearly, because of the influence of the solvent. In order to determine the precise position of the isosbestic points, and to separate the influence of the solvent the experimental spectra were reconstructed using the method of characteristic vector analysis (CVA) [19]. The obtained reconstructed spectra of citraconic and glutaconic acid are shown in the Figs. 3 and 4, respectively.

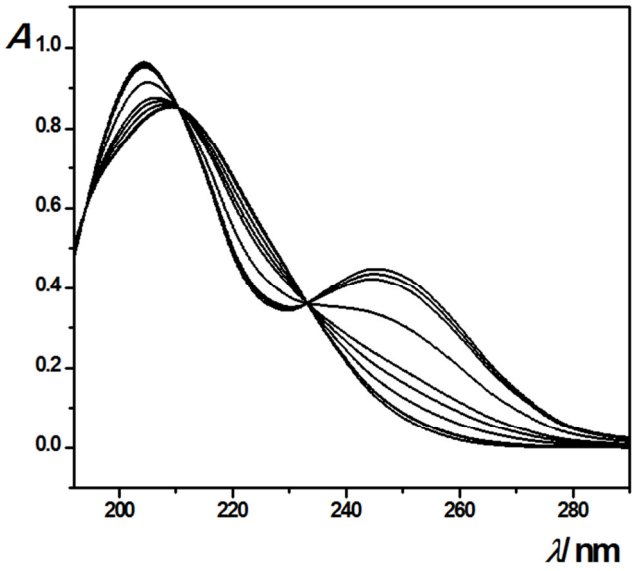


Fig. 3. Reconstructed UV spectra of citraconic acid (c = 9.8 10^{-5} mol/dm^3) in perchloric acid media

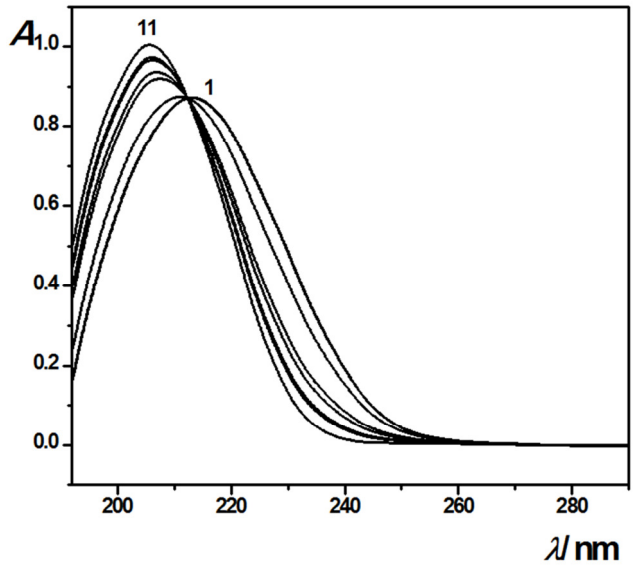


Fig. 4. Reconstructed UV spectra of glutaconic acid (c = 8.3 10^{-5} mol/dm^3) in perchloric acid media

As it can be seen from the Fig. 3, two isosbestic points existed in the spectrum of citraconic acid, the first one at 210 nm and the second one at 234 nm. It means that there were three forms able to absorb UV radiation in the system: the acid in its unprotonated form, the protonated form of the acid and the protonated anhydride. Because of that, it was complicated to determine the pK_{BH}^{+} values of citraconic acid. Hence, the protonation process of citraconic acid was characterized only qualitatively. The existence of one isosbestic point at 212 nm in the spectrum of glutaconic acid (See Fig. 4) suggested that in the reaction system two forms were present, *i.e.*, the unprotonated and protonated form of glutaconic acid. This result was in agreement with the investigations made for similar systems [4-8]. Furthermore, the one step of protonation of glutaconic acid was confirmed with the dependence of the absorbance at 220 nm on perchloric acid concentration. This dependence had a sigmoidal shape, *i.e.*, sigmoidal curve, S (See Fig. 5).

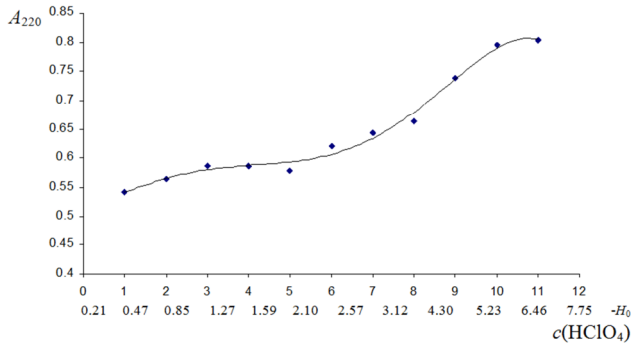


Fig. 5. The dependence of the absorbance (λ = 220 nm) of glutaconic acid (c = 8.3 10^{-5} mol/dm^3) on perchloric acid concentration

From the Fig. 5 it could be seen that the S curve had only one step. It confirmed that only one carboxylic group was protonated. When the concentration of perchloric acid was below 6 mol/dm^3 (H_0 = -2.10) the glutaconic acid existed in its unprotonated form, while at perchloric acid concentration up to 10 mol/dm^3 (H_0 = -5.23) the protonated form of the acid was formed. In agreement with previous investigations of similar organic acids obtained in mineral acid media and on the base of the results presented in this investigation, it was clear that in perchloric acid media the protonation process of citraconic and glutaconic acid took place [4]-[8].

Partial atomic charges: The exact place of protonation of the investigated organic acid is an important question. In order to establish the site of protonation of some organic acids (fumaric, maleic, aconitic, glutaconic, citraconic and mezaconic acid) many authors used different methods such as: ultraviolet, infrared, Raman, mass and NMR spectroscopy [20]-[23]. Most of them suggested that the protonation takes place on the oxygen of the carbonyl group [18], [24]-[26]. The possible protonation reaction of glutaconic and citraconic acid in perchloric acid media can be predicted according to the literature data and the calculated values of the partial charges using semiempirical methods [27]. In this work AM1 (Austin Model 1) and PM3 (Parametric Method 3) semiempirical methods were used for optimization of the geometry of investigated acids [10], [11]. For that

purpose HyperChem computer program was used [28]. The partial atomic charges of atoms which are possible protonation centers in the molecule of the investigated acids were determined by AM1 (Austin Model 1) and PM3 (Parameterized Model 3) methods (Tables 1-3).

Table 1. Atomic charges of *cis*-glutaconic acid calculated by AM1 and PM3 semiempirical methods

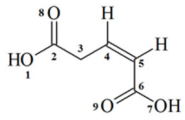
	protonation site	AM1	PM3
	1 O	-0.3114	-0.3045
	2 C	0.3089	0.3815
	3 C	-0.1560	-0.0869
	4 C	-0.0625	-0.0353
	5 C	-0.2031	-0.1940
	6 C	0.3359	0.4187
	7 O	-0.3081	-0.3011
	8 O	**-0.3640**	**-0.3971**
	9 O	**-0.3768**	**-0.4098**

Table 2. Atomic charges of *trans*-glutaconic acid calculated by AM1 and PM3 semiempirical methods

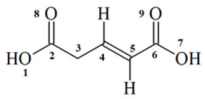
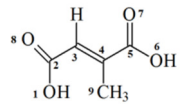
	protonation site	AM1	PM3
	1 O	-0.3178	-0.3110
	2 C	0.3064	0.3783
	3 C	-0.1455	-0.0769
	4 C	-0.0730	-0.0480
	5 C	-0.1972	-0.1909
	6 C	0.3383	0.4159
	7 O	-0.3145	-0.3052
	8 O	**-0.3475**	**-0.3826**
	9 O	**-0.3577**	**-0.3945**

Table 3. Atomic charges of citraconic acid calculated by AM1 and PM3 semiempirical methods

	protonation site	AM1	PM3
	1 O	-0.3060	-0.3003
	2 C	0.3342	0.4117
	3 C	-0.1400	-0.1287
	4 C	-0.0269	-0.0372
	5 C	0.3290	0.4045
	6 O	-0.3036	-0.2953
	7 O	**-0.3489**	**-0.3895**
	8 O	**-0.3549**	**-0.3917**
	9 C	-0.2030	-0.0918

According to the values of partial atomic charge (Tables 1 and 2) the protonation process of glutaconic acid occurred on the oxygen of the carbonyl group which is closer to the double bond (marked with 9) probably as a result of higher electronic density on that place in the molecule. The citraconic acid was protonated on the carbonyl oxygen of the carboxylic group marked with 8 (Table 3). The similar atomic charge values were obtained for the oxygen atom of the carbonyl group which is closer to the methyl group (marked with 7) probably because of its positive inductive effect.

Determination of the pK_{BH}^+ values: The protonation process of glutaconic acid was quantitatively characterized with the pK_{BH}^+ values calculated using the following methods: Bunnett and Olsen [13], Yates and McClelland [14], and Cox and Yates [15] (equations (3-5)). The pK_{BH}^+ values could be calculated using the data of the acidity functions H_0 and X which are known from the literature [12], [15], [29]-[31] and the ionization ratio between the concentration of protonated and unprotonated form of the acid, I. This ratio was determined from the absorbance data according to Beer's law [32], [33]. Actually, the concentrations of protonated and unprotonated form of the acids were determined from absorbance data (experimental and reconstructed) using an overdetermined system of four equations (absorbance values) with two unknown parameters (concentration of unprotonated and protonated form). The molar absorption coefficient values (needful for calculation of the ionization ratio) were obtained by measuring the absorbance values at the concentration of perchloric acid when glutaconic acid existed in its protonated and unprotonated form. The molar absorption coefficients, absorbance data, and the ionization ratio values at the selected wavelengths of glutaconic acid are given in Table 1.

There is no acidity functions determined when carboxylic acids were used as indicators. Thus, the pK_{BH}^+ values could be determined using the acidity functions obtained with compounds which are protonated at the same protonation center as the investigated acids [34]. When the method of Yates and McClelland [14] was used the calculations were made with the amide acidity function (H_A) instead of H_0. The pK_{BH}^+ obtained in this way was close to those calculated according to the "excess acidity" function method [15]. Because of that, the H_A acidity function was considered as more appropriate for calculation of the pK_{BH}^+ values. In this case it was expected that the slope parameter would have the value equal to unity.

The pK_{BH}^+ values of glutaconic acid also were determined graphically using the method of Davis and Geissman [35]. Namely, when log$I$ = 0, it is as-

sumed that the acid is half protonated, then $H_0 = (H_0)_{1/2}$. These values multiplied with the slope parameter m gave the graphically value of the pK_{BH^+}, *i.e* $pK_{BH^+} = m \cdot (H_A)_{1/2}$. The values of the solvatation parameters m, m^* and ϕ, the correlation coefficients, the standard deviation (SD) and relative standard deviation (RSD) were calculated, as well. The dependence of log$I$ on H_0 is linear with slope which depend on the method of calculation, and with an intercept equal to the graphic value of the thermodynamic dissociation constant. In the "excess acidity" function method the dependence of $\log I - \log c_{H^+}$ *vs*. X was linear. The dependence of log$I$ on H_A (Yates and McClelland method) and $\log I - \log c_{H^+}$ on X (Cox and Yates method) for glutaconic acid in perchloric acid media is shown in the Figs. 6 and 7, respectively.

Table 4. The spectrophotometric data of glutaconic acid (c = 8.3 10^{-5} mol/dm^3) in perchloric acid media, and values of log$I$ (reconstructed spectra)

$c(HClO_4)$ [mol/dm^3]	log$I$	A_{198}	A_{205}	A_{214}	A_{220}
1		0.5018	0.7418	0.8683	0.7924
2		0.8815	0.9953	0.8174	0.5423
3		0.7547	0.9731	0.8013	0.5747
4		0.7832	0.9942	0.8212	0.5320
5	-1.185	0.8363	1.0108	0.8573	0.4580
6	-0.731	0.7207	0.9598	0.8542	0.5325
7	-0.219	0.6871	0.8931	0.8263	0.6259
8	0.284	0.5589	0.8871	0.8488	0.6827
9	0.791	0.5280	0.8409	0.8935	0.7599
10	1.602	0.5140	0.7534	0.8672	0.7913
11		0.5087	0.7218	0.8354	0.8047
	$^*\varepsilon_B$	95635	104791	89528	60968
	ε_{BH^+}	60234	90933	104653	95509

*The molar absorption coefficient values (ε [dm^3 mol^{-1} cm^{-1}]) of unprotonated and protonated form of glutaconic acid

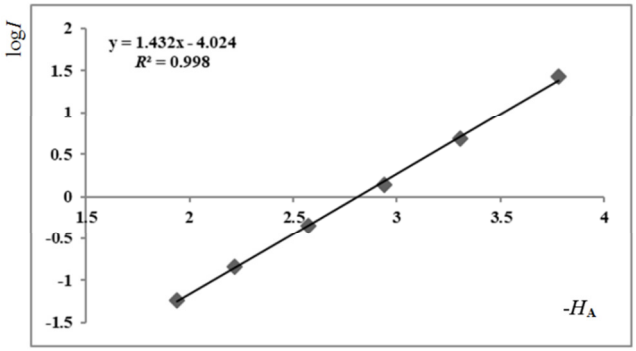


Fig. 6. The dependence of log$I$ on H_A for glutaconic acid

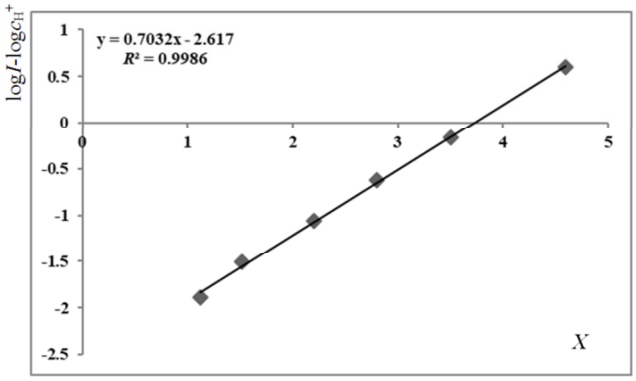


Fig. 7. The dependence of log$I$-logc_{H^+} on X for glutaconic acid

As it was mentioned before H_A is the most adequate one for determination of the pK_{BH^+} values for dicarboxylic acids, *i.e*., using this function the slope value closest to unity was obtained (See Fig. 6). The values of the numerically and graphically obtained thermodynamic dissociation constants (pK_{BH^+}) with their confidence interval at 95% confidence levels, the slope parameters (m, m^* and ϕ) and statistical data are presented in Table 2.

The thermodynamic dissociation constants (pK_{BH^+}) determined according to Bunnett and Olsen had more negative values compared to those calculated by other methods (see Table 5). As a result of different grade of solvatation of the used bases the slope parameter ϕ was not equal to unity. This parameter had positive value which means that the solvatation of glutaconic acid was more pronounced than that of Hamett's indicators used to set up the H_0 scale [12]. The values of the solvatation parameter m^* ("excess acidity" function method) for glutaconic acid were similar to those obtained for amides (m^* = 0.51 ± 0.07) which additionally confirmed that the amide acidity function was the most suitable one for calculation of the pK_{BH^+} for this acid [13]. Therefore,

in Yates and McClelland's equation (Eq. 3) H_A instead of H_0 was used [12]. As a result of that the values of the slope parameter m were close to unity, and the pK_{BH}^+ values can be considered as thermodynamic dissociation constants. The pK_{BH}^+ values calculated with this method were similar to those calculated according to the "excess acidity" function method [13]. It suggested that the method of Yates and McClelland was more reliable for determination of pK_{BH}^+ values than the method of Bunnett and Olsen. The pK_{BH}^+ values of glutaconic acid determined in perchloric acid media were more positive compared to pK_{BH}^+ values which we had reported in sulfuric acid media [8]. It was expected because perchloric acid is stronger than sulfuric acid as a result of the influence of the anion.

Table 5. Thermodynamic pK_{BH^+} values of glutaconic acid (experimental and reconstructed spectra)

experimental spectra			reconstructed spectra		
"excess acidity" function method					
pK_{BH}^+ (numerically)	pK_{BH}^+ (graphically)	m^*	pK_{BH}^+ (numerically)	pK_{BH}^+ (graphically)	m^*
-2.61±0.027 SD = 0.034 RSD = 1.31	-2.62	0.70	-2.66±0.017 s = 0.022 V = 0.81	-2.67	0.67
Bunnett and Olsen					
pK_{BH}^+ (numerically)	pK_{BH}^+ (graphically)	ϕ	pK_{BH}^+ (numerically)	pK_{BH}^+ (graphically)	ϕ
-3.81±0.035 s = 0.039 V = 1.03	-3.80	0.57	-3.92±0.031 s = 0.044 V = 1.12	-3.81	0.51
Yates and McClelland					
pK_{BH}^+ (numerically)	pK_{BH}^+ (graphically)	m	pK_{BH}^+ (numerically)	pK_{BH}^+ (graphically)	m
-2.83±0.043 s = 0.02 V = 1.22	-2.80	1.09	-2.75±0.032 s = 0.060 V = 1.11	-2.73	1.04

CONCLUSION

The protonation reaction of glutaconic acid took place in perchloric acid media, *i.e.*, this acid existed in its protonated form at perchloric acid concentration up to 10 mol/dm^3. On the other side, citraconic acid at higher perchloric acid concentration existed in its protonated form and as a protonated anhydride, as well. Thus, the thermodynamic dissociation constants were determined for glutaconic acid, while the protonation process of citraconic acid was characterized only qualitatively. There was no significant difference between the numerically and graphically calculated pK_{BH}^+ values which was statistically confirmed. The differences between the thermodynamic dissociation constants obtained from the experimental and the reconstructed spectra were also insignificant. It indicated that there was a little influence of the solvent on the appearance of the spectra compared to the effect of the protonation reaction. The correlation coefficient values of the dependence of log$I$ on H_A (amide acidity function) or X ("excess acidity" function method) used acidity function were between 0.995 and 0.998. This suggested a good correlation between the values used for calculation of the pK_{BH}^+ and the slope parameter values obtained using the different acidity functions.

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METHANOL EXTRACTS AND VOLATILES OF *TELEKIA SPECIOSA* (SCHREB.) BAUMG. FROM BOSNIA AND HERZEGOVINA

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: The aim of the present study was to analyse the methanol extracts from the aerial and the underground parts of *Telekia speciosa* (Schreb.) Baumg by HPLC and determine their antioxidant capacity. In addition, volatiles from both parts of the plant were analysed. The chlorogenic acid (CGA) and caffeic acid derivatives were present in the extracts. The CGA content was 977.8±85 mg per 100 g extract for underground and 272.0±18 mg per100 g extract for aerial parts of *T. speciosa*. The antioxidant capacity, determined by DPPH and FRAP method, was on average three and a half times higher for the extract of the underground parts in comparison with the extract of the aerial plant parts. The volatile constituents determined by GC-FID/MS were characterized by the presence of a high concentration of oxygenated sesquiterpenes. The obtained results contribute to better knowledge of phytochemical properties of *T. speciosa*, which is traditionally used in bronchial asthma therapy.

KEYWORDS: *Telekia speciosa*, chlorogenic acid (CGA), antioxidant capacity, volatiles

INTRODUCTION

The only representative of the genus *Telekia* in the flora of Bosnia and Herzegovina (BiH) is *Telekia speciosa* (Schreb.) Baumg. It inhabits wet and shady positions in mountain woodlands. It is a perennial herbaceous plant with alternating, wide, whole leaves and large heterogeneous heads which can be individual or in cluster blooms. It is widespread in Eastern and Central Europe and the Balkan Peninsula [1]. The root of *T. speciosa* is traditionally used as a remedy for bronchial asthma in Balkan countries. In BiH, root smoke of this plant is used in inhalations for bronchial asthma [2].

The root of *T. speciosa* contains essential oil, bitter compounds and inulin [3]. Phytochemical investigations have revealed *T. speciosa* as a rich source of sesquiterpene lactones, especially in its underground parts [4]. Isoalantolactone is almost exclusively contained in the essential oil of *T. speciosa* roots. It is a sesquiterpene lactone – an eudesmanolide with an antiproliferative and anti-inflammatory activity [5]. Sesquiterpene lactones have been found to be active bactericidal principles, as well [6].

The aerial part extracts have been found to contain fatty acids, namely palmitic, linoleic, oleic and caproic acids [7]. Reports concerning the sterols of *T. speciosa* extracts can also be found in the literature [8]. Pseudoguaianolide - 2,3-dihydroaromaticin and three thymol derivatives have been isolated as major secondary metabolites from the aerial parts of methanol extract of *T. speciosa*.

Phenol acids derivatives in earlier investigations were isolated from an extract of *T. speciosa* flowers. Those compounds were: one derivative of ferulic acid [(E)-ferulic acid 4-O-β-(6-O-2-hydroxy isovaleryl)-glucopyranoside] and five caffeic acid derivatives [(E)-caffeic acid 4-O-β-(6-O-2-hydroxyisovaleryl)-glucopyranoside, (E)-caffeic acid 4-O-β-(6-O-3-hydroxy-2-methylpropanoyl)-glucopyranoside, 6-O-(E)-caffeoyl-glucopyranose, (E)-caffeic acid 4-O-β-glucopyranoside] and 5-caffeoylquinic acid (chlorogenic acid) [9].

The aim of the present study was to identify and quantify phenol acids in the methanol extracts of *T. speciosa* aerial and underground parts and determine the antioxidant capacity of the extracts. To the best of our knowledge, the chemical composition of the extracts of underground parts as well as the antioxidant capacity of the plant extracts had never been studied before. In addition, we analyzed the volatiles of both, aerial and underground parts.

MATERIAL AND METHODS

PLANT MATERIAL

Aerial and underground parts of *T. speciosa* were collected at their specific location of Zlača, Banovići

municipality (BiH) during the flowering period in August 2015. Geographical coordinates of the location were N44°20'22.3" E18°33'39.6". The plant material was identified according to Flora Croatia by authors [10] and voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, the University of Tuzla. The plant material was cleaned, cut, and dried.

REAGENTS AND CHEMICALS

All the reagents used were of analytical grade. Folin-Ciocalteu´s phenol reagent, sodium carbonate, sodium acetate anhydrous, and ferric (III) chloride were obtained from Merck (Germany). HPLC-grade acetonitrile and formic acid were purchased from Merck. Water for HPLC was prepared by Milli-Q Water Purification System. Methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), ferrous (II) sulfate heptahydrate, hydrochloric acid, glacial acetic acid, HPLC-grade chlorogenic acid, caffeic acid, ellagic acid, gallic acid, and *o*-coumaric acid were purchased from Sigma-Aldrich (USA). Rutin and ferulic acid were obtained from Carl Roth (Germany).

PLANT EXTRACTS

The dried plant material was crushed in a grinder until powder formation. The samples were extracted with 98% methanol on a magnetic stirrer under reflux at 50 °C for 1 hour. The mixtures were filtered through a filter paper (Whatman No. 1). The methanol was removed by evaporation. The dried extracts were stored in the fridge at 4 °C, in glass bottles for further investigations.

HPLC ANALYSES

HPLC analyses of extracts (3 mg/mL in methanol) were carried out using an Agilent 1260 Infinity (Agilent Technologies, USA) system equipped with a Agilent 1260 Infinity Quaternary Pump, Agilent 1260 Infinity Standard Autosampler, Agilent 1260 Infinity Diode Array Detector, and Agilent 1260 Infinity Thermostatted Column Compartment. The separations were performed on a Merck LiChroCART$_R$250-4 C18 RP analytical column (250x4.6mm i.d., 5µm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was applied: 0-15 min, linear gradient from 10% to 20% B; 15-30 min, linear gradient from 20% to 30% B; 30-35 min, linear gradient from 30% to 40% B; 35-40 min, linear gradient from 40% to 90% B; 40-45 min, then returned to the initial conditions. The injection volume was 10 µL; the flow rate was 0.8 mL/min. The detection wavelength was 325 nm, and the column thermostat was set at 30 °C [11]. Component identification was performed comparing their retention times and UV spectra with those obtained from standards. The calibration curve for chlorogenic acid was obtained by the external standard method in the concentration range of 15.6-500 µg/mL (y=28.93x-220.2, R^2=0.9996).

TOTAL PHENOL CONTENT AND ANTIOXIDANT CAPACITY

The total phenol content was determined by the Folin Ciocalteu spectrophotometric method [12]. The *in vitro* antioxidant capacity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assays [13] and the ferric reducing antioxidant potential (FRAP) [14].

ISOLATION AND GC-FID/MS ANALYSES OF ESSENTIAL OILS

The dried aerial and underground parts of *T. speciosa* were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, according to the European Pharmacopoeia. The obtained oils were separated, dried over anhydrous sodium sulfate, and stored at -20º C until the analysis.

The volatile constituents were determined by the GC-FID/MS analyses using an Agilent 6890N GC system coupled with an Agilent 5975 MSD, FID, and equipped with a HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The oven temperature was programmed linearly rising from 60 to 280 ºC at 3 ° min^{-1} and then isothermal at 280 ºC for 5 min; injector 200 ºC; FID 300 ºC; transfer-line 250 ºC; carrier gas He (1.0 mL min^{-1}, constant flow mode); injection volume 1 µL of essential oil dissolved in ethanol; split ratio 10:1. EI Mass spectra (70 eV) were acquired over the m/z range of 35-550.

The identification of the individual compounds was based on the comparison of their retention times (t_R), retention indices (RIs), and mass spectra with those obtained from authentic samples and/or listed in the NIST, Wiley mass spectral libraries, and the literature [15]. For the quantification, the relative area percentages obtained by FID were used.

RESULTS AND DISCUSSION

The yields of the methanol extracts of *T. speciosa* were 4.31% and 11.52% for the aerial and underground parts, respectively.

The qualitative and quantitative analysis of the extracts was performed by the RP-HPLC method. The chlorogenic acid (CGA) and caffeic acid deriva-

tives (CA derivatives) were present in the extracts. In the aerial parts of *T. speciosa* except CGA, two CA derivatives (Rt - 22.464 min and Rt - 35.631min) were detected. In the underground parts, except CGA, four CA derivatives (Rt - 22.464 min, Rt - 25.433 min, Rt - 35.627 min and Rt - 37.694 min) were also present. Caffeic acid and other reference substances used in the analyses (gallic acid, o-coumaric acid, ferulic acid, ellagic acid and rutin) were not detected.

The content of chlorogenic acid was determined in the extracts (Table 1). In the extracts from the underground parts of *T. speciosa* the CGA content was on average three and a half times higher than the CGA content in the extracts from the aerial parts of *T. speciosa*.

Table 1. Chlorogenic acid content in the methanol extracts of *T. speciosa* aerial and underground parts

Extract	Chlorogenic acid (mg per 100 g extract)[a]
T. speciosa aerial parts summer Zlača	272.0±18
T. speciosa underground parts summer Zlača	977.8±85

[a] mean ± SD (n=3)

There were no available literature data about phenol acids in *T. speciosa* underground and aerial parts except some derivatives of phenol acids from a fraction of extract from *T. speciosa* flowers. Research studies have shown that chlorogenic acid demonstrates a wide range of pharmacological activities including antioxidant, antiobesity, anti-edematogenic and antinociceptive activities [16], [17].

Phenol compounds contribute to the overall antioxidant capacity of plants, which is why the extracts of *T. speciosa* were analyzed for their total phenol content by the Folin Ciocalteu method. DPPH and FRAP assays were used to evaluate the antioxidant capacity of those extracts (Table 2).

Table 2. Total phenol content, IC_{50} and FRAP values of *T. speciosa* methanol extracts

Sample	Total phenol content (mg GAE g^{-1} extract)[a]	IC_{50} (µg mL^{-1})[a]	FRAP µmol Fe^{2+} g^{-1} extract[a]
TSH	63.02±1.17	497.79±31.86	384.32±9.54
TSR	162.84±7.48	129.57±3.93	1337.48±48.03

[a] mean ± SD (n=3)
TSH = *T. speciosa* extract of the aerial parts
TSR = *T. speciosa* extract of the underground parts

To the best of our knowledge, the data about the antioxidant capacity of this plant have not been found by now. The extract of the underground parts of *T. speciosa* had on average three and a half times higher antioxidant capacity in comparison with the extract of the plant aerial parts. IC_{50} for rutin used as standard was 12.42 µg mL^{-1}. The content of chlorogenic acid indicates positive correlation existence between its content and antioxidant capacity in analysed extracts. The obtained results also confirm the well-known positive correlation between the total phenol content and antioxidant capacity.

The aerial parts of *T. speciosa* contained 0.04% (v/w) of yellow, liquid fragrant essential oil. The identified 69 constituents from the aerial parts of *T. speciosa* accounting for 87.9% of the oil are presented in Table 3. The oil of *T. speciosa* was characterized by the presence of a high concentration of oxygenated sesquiterpenes (51.8%). The major components were (*E*)-nerolidol (10.3%), caryophyllene oxide (8.2%), (*Z,E*)-farnesol (7.7%) and prenopsan-8-ol (4.9%). Sesquiterpene hydrocarbons constituted (5.8%) of the oil. Thymol derivative 10-isobutyryloxy-8,9-epoxythymyl isobutyrate (3.4%) was the major representative of oxygenated monoterpenes (12.0%). Non-terpene compounds presented an appreciable amount of essential oil (18.3%) with dominant (*E*)-phytol (4.9%) and hexadecanoic acid (3.3%).

Table 3. Chemical composition of essential oils from *T. speciosa* aerial and underground parts

No.	Compound	RIE[a]	Aerial parts (%)	Undergr. parts (%)
1	linalool	1101.4	**1.4**	0.2
2	nerol oxide	1155.3	0.4	-
3	*α*-terpineol	1192.5	0.3	-
4	*n*-decanal	1206.2	0.6	-
5	nerol	1229.5	0.7	-
6	geraniol	1255.1	0.3	-
7	dihydroedulan I	1294.1	**1.3**	-
8	silphiperfol-5-ene	1326.2	-	0.3
9	7-*epi*-silphiperfol-5-ene	1345.0	-	0.4
10	decanoic acid	1371.9	0.2	-
11	silphiperfol-6-ene	1377.3	0.3	0.2
12	modheph-2-ene	1381.0	-	0.2
13	*α*-isocomene	1387.8	-	0.2
14	*β*-elemene	1394.0	0.3	0.2
15	*β*-ionol	1406.2	0.7	-
16	*β*-isocomene	1407.2	-	0.2
17	(*E*)-*β*-caryophyllene	1421.4	**1.3**	0.5
18	*epi*-*β*-santalene	1448.2	-	0.2
19	geranyl acetone	1453.5	0.6	-
20	*α*-humulene	1455.7	0.2	-
21	4,5-di-*epi*-aristolochene	1472.9	0.2	-
22	*β*-chamigrene	1477.6	0.3	0.1

No.	Compound	RIE[a]	Aerial parts (%)	Undergr. parts (%)
23	thymol isobutyrate	1485.6	0.3	0.3
24	(*E*)-*β*-ionone	1487.8	0.7	-
25	neryl isobutanoate	1491.4	**1.7**	**1.3**
26	*α*-selinene	1498.3	0.7	0.2
27	*β*-bisabolene	1509.3	-	0.2
28	cameroonan-7*α*-ol	1512.1	0.7	-
29	modhephen-8*β*-ol	1515.5	0.3	0.4
30	(*E*)-dihydro-apofarnesal	1521.0	0.3	-
31	*δ*-cadinene	1525.8	**1.1**	-
32	*cis*-calamenene	1536.7	0.1	-
33	*α*-calacorene	1545.6	0.6	-
34	isocaryophyllene oxide	1556.5	**1.5**	-
35	*epi*-longipinanol	1562.1	0.2	-
36	(*E*)-nerolidol	1567.8	**10.3**	-
37	neryl (*S*)-2-methylbutyrate	1576.3	-	0.5
38	prenopsan-8-ol	1577.7	**4.9**	-
39	caryophyllene oxide	1587.6	**8.2**	**1.3**
40	humulene epoxide II	1612.4	0.9	0.2
41	*cis*-isolongifolanone	1614.3	0.5	-
42	muurola-4,10(14)-dien-1*β*-ol	1631.6	0.4	-
43	*α*-acorenol	1634.3	-	0.1
44	caryophylla-4(12),8(13)-dien-5- *α*-ol	1636.6	**1.1**	0.1
45	caryophylla-4(12),8(13)-dien-5- *β*-ol	1640.6	**2.6**	-
46	*β*-eudesmol	1645.8	**2.8**	**1.6**
47	*α*-eudesmol	1653.1	0.3	-
48	atractylone	1656.3	-	0.4
49	selin-11-en-4α-ol	1657.7	0.8	0.4
50	14-hydroxy-(*Z*)-caryophyllene	1667.9	0.7	0.2
51	*trans*-calamenen-10-ol	1670.2	0.3	-
52	14-hydroxy-9-*epi*-(*E*)-caryophyllene	1674.5	**2.0**	0.7
53	cadalene	1677.7	0.6	-
54	*epi*-*α*-bisabolol	1685.2	0.5	-
55	6-methoxythymyl isobutirate	1687.5	0.6	0.4
56	*n*-heptadecane	1698.4	0.7	0.2
57	*δ*-dodecalactone	1707.8	0.5	0.7
58	(*E,Z*)-farnesal	1716.3	0.7	-
59	3-methoxy-cuminyl isobutyrate	1719.7	-	0.3
60	6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphtalen-2-ol	1723.0	-	0.3
61	(*Z,E*)-farnesol	1725.0	**7.7**	-
62	(*E,E*)-farnesal	1743.8	**1.1**	-
63	fukinone	1749.1	0.4	-
64	cyclocolorenone	1753.8	0.4	-
65	tetradecanoic acid	1765.0	**1.1**	-
66	*α*-costol	1775.4	0.6	-
67	*n*-octadecane	1798.4	0.3	-
68	hexahydrofarnesyl acetone	1845.3	**1.9**	-
69	benzyl salicylate	1870.1	0.4	-
70	alantolactone	1902.1	-	**2.4**
71	10-isobutyryloxythymyl isobutyrate	1917.2	1.5	0.5
72	10-isobutyryloxy-8,9-dehydrothymyl isobutyrate	1921.5	0.7	0.4
73	isoalantolacton	1945.9	**1.9**	**77.2**
74	hexadecanoic acid	1969.5	**3.3**	-
75	10-isobutyryloxy-8,9-epoxythymyl isobutyrate	2034.6	**3.4**	**3.7**
76	(*E*)-phytol	2114.8	**4.9**	-
77	10-isovaleryloxy-8,9-epoxythymyl isobutyrate	2119.1	0.6	0.2
78	pentacosane	2498.1	0.5	-
79	heptacosane	2698.0	0.3	-
80	nonacosane	2899.9	0.2	-
Total			**87.9**	**96.9**
Oxygenated monoterpenes			12.0	8.1
Sesquiterpene hydrocarbons			5.8	3.2
Oxygenated sesquiterpenes			51.8	85.6
Others			18.3	0.0

RIE[a] – experimental retention indices
n/a – not available
„-„ - not detected

In the previous investigations, aerial parts collected during the flowering time of *T. speciosa* from Serbia contained 0.06% (v/w) of essential oil. Dominant compounds were sesquiterpenes, non-terpene components and oxigenated monoterpenes [4]. Furthermore, leaf, stem, flower and root essential oils were also studied. The qualitative composition of the essential oils from the examined plant material was similar, whereas the quantities of individual components of the oils varied widely depending on the kind of the plant material [18]. The results of essential oils components obtained from the literature were similar to our results.

The amount of the essential oil found in the underground parts of *T. speciosa* (0.29% (v/w)) was higher than in the aboveground parts. The oil was yellowish with needle crystals, and of aromatic odor. The identified 35 constituents from the underground parts of *T. speciosa* accounting for 96.9% of the oil are presented in Table 3. The oil was characterized by the presence of high concentration of oxygenated sesquiterpenes (85.6%) with isoalantolactone being the major component (77.2%). *β*-Eudesmol (1.6%) and caryophyllene oxide (1.3%) were also the representatives of oxygenated sesquiterpenes in the oil of the underground parts. Sesquiterpene hydrocarbons constituted only (3.2%) of the oil. Thymol derivatives [10-isobutyryloxy-8,9-epoxythymyl isobutyrate (3.7%), 9-isobutyryloxythymol isobutyrate (2.4%)] and neryl isobutanoate (1.3%) were the oxygenated

monoterpenes found in appreciable amounts. They constituted (8.1%) of the oil.

In the previous investigations, the underground parts collected during the flowering time of *T. speciosa* from Poland and Montenegro contained 0.4% - 1.7% of essential oils. The dominant compound of those oils was also isoalantolactone (62.3% - 95%) [1], [18].

CONCLUSION

To the best of our knowledge, the extracts from the aerial and underground parts of *Telekia speciosa* (Schreb.) Baumg were analysed on the presence of phenol acids by HPLC for the first time. The chlorogenic acid and caffeic acid derivatives were detected in the extract from the aerial and underground parts, with a higher amount of caffeic acid derivatives and three and a half times higher amount of the chlorogenic acid in the underground parts.

The presence of phenol compounds contributes to the antioxidant capacity, which was also evaluated in the extracts for the first time, as we know. On average the three and half times higher antioxidant capacity was determined for the underground parts than for the aerial parts of *T. speciosa* extracts. The results for the chemical composition of the essential oils of *T. speciosa* were similar to the previously published data.

The obtained results contribute to better knowledge of phytochemical properties of *T. speciosa*, which is traditionally used in bronchial asthma therapy. The recommendation for further investigations refers to the isolation of caffeic acid derivatives using the column chromatography and the determination of their structures as known or new compounds by comparison of their spectral data (1H NMR, UV) with those found in the literature.

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THERMAL PRETREATMENT OF CATTLE MANURE FOR ENHANCING BIOGAS PRODUCTION

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: The low degradability of waste materials containing lignocellulosic material is one of the factors that hinder the production of biogas. The increased need for advanced techniques in the anaerobic digestion process has led to the use of different pretreatment raw materials prior to the anaerobic digestion process in order to increase the yield of biogas. In order to maximize the yield of biogas from cattle manure in a mixture with waste sludge, the anaerobic digestion process was performed at mesophilic conditions for 54 days. As pretreatment, the thermal treatment of cattle manure at temperatures of 55 and 70 °C was applied. The highest biogas production was obtained in the sample of cattle manure, previously treated at 70 °C (M70), while the lowest was recorded in the control sample M. The specific biogas production in relation to the input quantity of volatile organic matter had the highest value in sample M70 and the lowest in the control sample. Overall results have shown that the thermal treatment of cattle manure before the anaerobic digestion process can increase both yield and biogas quality.

KEYWORDS: Anaerobic digestion, biogas, thermal pretreatment, cattle manure, waste sludge.

INTRODUCTION

One of the oldest and best researched processes by which the energy can be obtained from biomass is anaerobic digestion. Anaerobic digestion is a biological process of converting complex substrates of biogas and digestate by the action of various types of microorganisms without the presence of oxygen through four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis [1].

Today, biogas is commonly produced from raw materials that are easily degradable. Although there is a great potential for biogas production in organic raw materials, especially waste from agriculture, crop residues, animal waste, communal waste, food remains, these raw materials are characterized by a low degree of degradation and as such require longer retention time in the anaerobic digester [2].

Recently, the amount of manure produced on cattle farms has increased, most of which has been disposed in landfills or on agricultural land as a fertilizer without previous treatment. On the other hand, with application of the Nitrates Directive (91/676/EEC), the application of cattle manure for fertilization of agricultural land is limited[3]. In fact, anaerobic digestion provides an alternative solution for treatment, as well as for the recovery of energy from this type of waste [4].

In order to overcome the problem of slow degradation to ensure a shorter time of retention, with increased production of biogas and reduction of waste material that is being disposed, it is advisable to use certain methods for pretreatment of input raw material [5].

The optimal pretreatment method for use in the process of decomposing these raw materials should be economical, should increase the availability of raw materials to microorganisms, should not use or produce substances that inhibit biogas production, should not require high energy consumption and no environmentally harmful byproducts should be created.

MATERIAL AND METHODS

Fresh samples of cattle manure from the dairy farm "Spreča", which were thermally treated at temperatures of 55°C and 70°C and waste sludge from the municipal waste water treatment plant in Živinice, were used as substrates.

A laboratory reactor system for anaerobic digestion, consisting of six glass bottles of 500 ml volume, combined with eudiometric tubes which help to read the biogas production volume, was used for experimental studies. The constant temperature of 35°C was obtained by heating the water in the water bath (Figure 1). The pressure and the temperature of the ambient air were measured with the barothermohygrometer and the readings were used to convert the volume of obtained biogas into normal conditions.

Mixing of the substrate was performed mechanically using a magnetic stirrer.

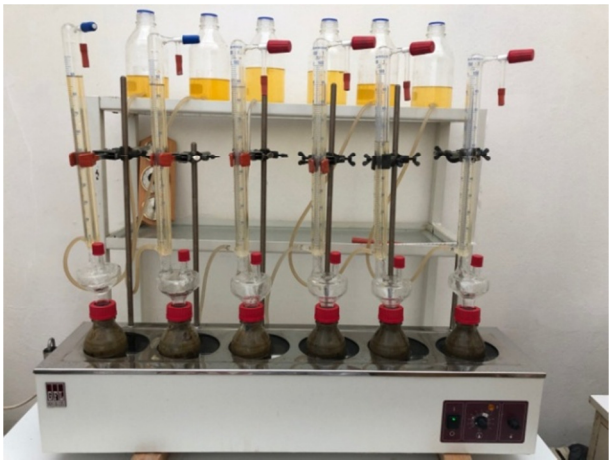

Figure 1. Laboratory reactor system for anaerobic digestion

An incubator with a thermo regulator for thermal pretreatment of cattle manurewas used (Figure 2). The samples were tempered for two hours in the incubator, with certain amounts of manure being subjected to temperatures of 55°C and 70°C.

Figure 2. Incubator with thermo regulator

The characterization of both raw materials, cattle manure and waste sludge, especially in the mixture, was performed before and after the anaerobic digestion by measuring the pH value and determining total dry matter (TS), volatile organic matter (VS), total Kjeldahl nitrogen (TKN) and chemical oxygen demand (COD). The methods used in the analysis of physico-chemical characteristics are standard methods and modified standard methods for wastewater testing (ISO and Standard Methods for Examination of Water and Wastewater (APHA)).

The pH value was measured by a digital measuring device with direct immersion of the electrodes in samples, using pH meter Mettler Toledo FE20/EL20. Prior to each measurement, the control of the measuring device was performed using standard buffer pH 4.01, 7.01, 10.01.

Determination of dry and volatile organic matter was carried out according to the standard Method *Method 2540-Solid B i 2540-Solid E. Standard Methods for the Examination of Water and Wastewater 21st edition* APHA, Washington, DC (2005) [6].

For the determination of the content of total Kjeldahl nitrogen, *Method 4500-N_{org} B. Standard Methods for the Examination of Water and Wastewater 20nd edition* APHA, Washington was used. The method consists of three parts: digestion at a temperature of 340 °C in the presence of concentrated sulfuric acid and Kjeldahl catalyst, distillation in the presence of NaOH where the distillate is absorbed into a 2% boric acid solution and titration with 0.1 M HCl in the presence of indicator bromocresol green [7].

Chemical oxygen demand was determined according to the modified standard method BAS ISO 6060:2000 [8].

The duration of the experiment was 54 days. The amount of biogas produced was read on a daily basis and the gas composition was determined on the gas chromatograph "PERKIN ELMER", equipped with the software package "Arnel".

RESULTS AND DISCUSSION

The experiment of anaerobic digestion of cattle manure and waste sludge mixtures was conducted at mesophilic conditions (35 ± 2 °C). Prior to the experiment, physico-chemical analysis of substrate, cattle manure (CM) and waste sludge (WS) was performed and the formed mixture was used as control sample (M). After the thermal processing of cattle manure at temperatures of 55 °C and 70 °C, two more mixtures (M55 and M70) were formed, which were subjected to the process of anaerobic digestion together with the control mixture.

In order to ensure the optimal value of dry matter content (about 8% mass) for the anaerobic digestion process, with respect to the results of the physico-chemical analysis, mixtures with equal ratio (by mass) of untreated or treated cattle manure and waste sludge (1:1) were formed. The physico-chemical characteristics of cattle manure and sludge, and the mixture of untreated and thermally treated cattle manure and sludge are shown in Table 1.

Table 1. Physico-chemical characteristics of cattle manure, sludge and mixture of CM:WS

parameter	Unit	CM	WS	M	M55	M70
pH	-	7.7	6.87	6.61	6.97	6.87
TS	%	14.99	3.82	9.47	10.09	9.56
VS	%	13.50	2.31	7.93	8.52	7.92
VS/TS	-	0.9	0.6	0.83	0.84	0.82
TKN	g/kg	3.68	2.85	3.20	3.07	3.26
COD	g/kg	178.90	46.39	70.98	89.06	93.97

TS-total solids VS-volatile solids; TKN-total Kjeldahl nitrogen; COD- chemical oxygen demand

The pH value measured in all three formed mixtures ranged from 6.61 to 6.97, while according to literature, the optimum pH value for an undisturbed process of anaerobic digestion ranged from 6.5 to 7.6 [9]. The content of dry matter in the control sample was slightly higher than the recommended value. The VS/TS ratio was approximately 0.8 in all formed mixtures, so the requirement for a sufficient amount of organic matter in reactors was satisfied [10].

The ratio of available organic matter and nitrogen, expressed through COD:N, ranged from 22:1 in control sample, 29:1 in sample M55, and 29:1 in sample M70, while the optimal value of this ratio was 30:1.

The differences in regard of pH value, dry matter content, VS/TS and COD:N ratios between M, M55 and M70 samples are mainly caused by the thermal treatment of the part of the M55 and M70 samples, although the inability to fully homogenize such mixtures should be considered, because it certainly has an influence on the parameters of the samples, but significantly lower than the influence of thermal treatment has.

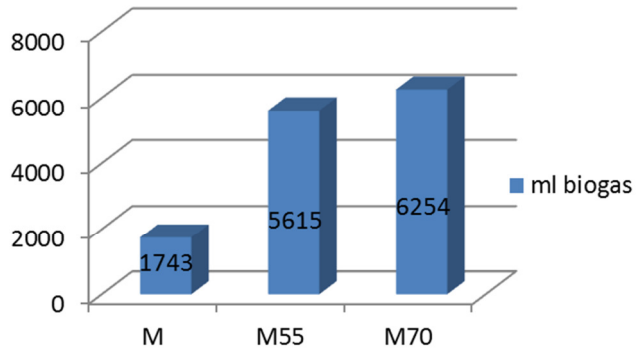

Diagram 1. Quantity of biogas obtained from untreated and thermally treated cattle manure and sludge in a period of 54 days

Diagram 1 shows the total amount of biogas produced during the time of substrate retention in reactors for 54 days. During the study, the amount of biogas produced in the reactor with a thermally treated cattle manure at 70°C (M70) was 6254 ml, a slightly smaller amount of biogas was recorded at 55°C (M55) (5615 ml), while the smallest biogas production in the amount of 1743 ml was recorded in the control sample (M). Also, the specific production of biogas based on the input VS expressed in [ml/g VSi] had the highest value for sample M70 (263.22), slightly smaller for sample M55 (219.68), while the lowest (73.27)for control sample (M) (Diagram 2).

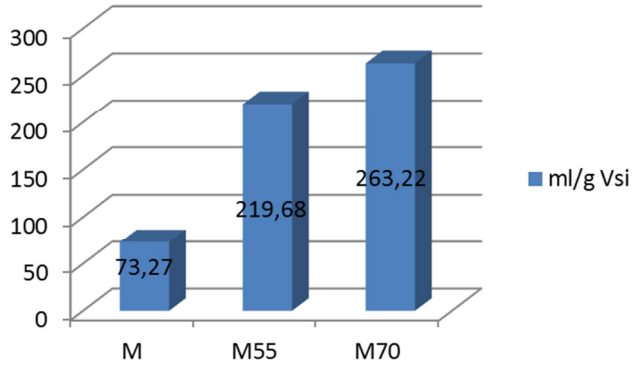

Diagram 2. Specific biogas production per g VSi

Table 2 shows the characteristics of the digestate remaining after the anaerobic degradation process, the amount of produced biogas, the specific biogas production in relation to the input volume of VSi, and the proportion of methane in the biogas. It is apparent that the values for certain parameters in the digestate were significantly lower than those prior to the digestion process, except for the pH value in samples M (7.03) and M70 (7.53), where the value was increased while slight reduction occurred in sample M55 (6.92), indicating that during the process there was no inhibition of the process with volatile fatty acids that represent an anaerobic degradation intermediate and the values remained within the range optimal for anaerobic digestion.

Reduction of dry and volatile organic matter suggests that part of organic matter was transformed into gas products. Also, significantly lower values of COD in the digestate (reduced by about 80%) were observed, in relation to the sample values prior to the start of the process. The TKN content in the samples prior to the start of the process was significantly

higher than the TKN values in the digestate, due to the fact that nitrogen is a macronutrient necessary for anaerobic microorganisms for growth and development during degradation of organic matter.

The highest value for the proportion of methane in biogas was in sample M70 (49.44%), while the lowest was in control sample (M) in the amount of 45.14%, and in sample M55 was 45.16%.

Table 2. Physico-chemical characteristics of cattle manure/waste sludge mixture after the experiment

Parameter	unit	M	M55	M70
pH	-	7.03	6.92	7.53
TS	%	8.15	6.61	6.83
VS	%	5.50	4.95	4.81
TKN	g/kg	2.27	2.84	2.95
COD	g/kg	13.48	13.35	10.88
V biogas	ml	1743	5615	6254
W methane	%	45.14	45.16	49.44
V methane	ml	768.79	2535.73	3091.97
Specific production of biogas	ml/g Vsi	73.27	219.68	263.22

TS-total solids VS-volatile solids; TKN-total Kjeldahl nitrogen; COD- chemical oxygen demand; V biogas – volume of biogas; W methane – share of methane in biogas; V methane – volume of methane;

CONCLUSION

Comparing the values obtained by physico-chemical analysis of the formed mixtures before and after the anaerobic digestion process, it could be concluded as follows:

- The pH value slightly increased in M and M70 samples.
- The reduction of volatile organic matter by 42% in sample M55 and 39.3% in M70 and about 30% for control sample (M), leads to the conclusion that a certain amount of organic matter was translated into gas products.
- The largest biogas production was recorded in sample M70 (6254 ml), while the smallest was obtained in the control sample M (1743 ml); in the M55 sample, 5615 ml of biogas was produced.
- The largest proportion of methane in biogas was in M70 (49.44%), while the values of this parameter for M and M55 samples were close to 45%.
- The specific production of biogas in relation to the input volume of volatile organic matter had the highest value for sample M70 (263.22 ml/g VSi), while for control sample (M) was the lowest and was 73.27 ml/g VSi.

This experiment has shown that the thermal treatment of cattle manure at temperatures of 55°C and 70°C in a mixture with waste sludge, increases biogas production by 3.22 and 3.59 times (respectively) compared to the control sample with untreated cattle manure. The increase in methane share in produced biogas of almost 9% compared to samples M and M55 was obtained in sample M70.

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INFLUENCE OF PARAMETERS ON SENSORY PROPERTIES AND CONSUMER ACCEPTANCE OF PROBIOTIC FRESH CHEESE

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: Probiotics, because of their beneficial effects on human health, are used in the treatment of digestive disorders, the prevention of colon cancer, restoration of the intestinal microflora balance and stimulate the immune system. New discoveries of soy enter the list of functional foods, i.e. found between food and medicine. The aim of the work was to examine the effect of soy drink, thermalization (60°C/3 min), combinations of microbial starter cultures and addition of prebiotics on sensory properties, and acceptability of probiotic fresh cheese. Fresh cheese samples were produced from skimmed milk (0.9% milk fat) and combination of cow's milk and soy drink. Milk fermentation was carried out at 25ºC and 40ºC, with addition of Lyofast EF1 and combination of microbial starter cultures Lyofast MWO 030 and Lyofast EF1 in a ratio of 1:1. Half of the obtained fresh cheese was subjected to a thermalisation process (60ºC/3 min). All the samples produced had a creamy consistency. The sensory properties of probiotic fresh cheese were evaluated by a group of 15 assessors. The acceptability of probiotic fresh cheese was evaluated by a group of 40 consumers. The samples were evaluated after 1, 7 and 14 days of storage. The addition of soy drink and the combination of microbial starter cultures had an influence on the improvement of the sensory properties of probiotic fresh cheese, which was confirmed by statistical analysis of the results.

KEYWORDS: probiotic fresh cheese, Lyofast EF1, Lyofast MWO 030, soy drink, thermalization, sensory properties, acceptability

INTRODUCTION

The development of cheese production through history is the result of the importance and role that milk and dairy products have taken in the nutrition of population, as well as the aspiration of people to ensure adequate storage of the most important foods for them. Fresh cheeses are usually not, or are minimally aged, have high moisture content, do not have rind, and got very mild flavor and a soft and smooth texture. In this category, milk coagulation is due to rennet and/or acid produced from a bacterial culture or other sources such as lemon juice.

When bacteria are involved in their manufacture, they also contribute to the development of typical flavors, quality improvement, and/or promote health benefits if they display probiotic properties [1]. The use of soy as a food ingredient is gaining importance in the food industry and for consumers due to its role as functional food [2].

Soymilk is an inexpensive, nutritive dairy substitute that is used to make cheese and cheese analogs by people worldwide [3]. Soy prebiotics such as oligofructose and inulin have a great application potential in the food industry due to their functional properties. The use of alternative ingredients such as soy drink reduces lactose content in yogurt [4]. According to a study carried out by Martinez – Villaluenga et al. [5], it is possible to use *Enterococcus faecium*, isolated from raw soy drink, in production of fermented soy drink, which could be a promising strategy in the preventative therapy against cardiovascular diseases. Because of their role in mating and the development of cheese flavor, enterococci have been proposed to be used as a starter culture in the production of European cheeses due to the preferred technological and metabolic properties [6,7,8,9].

Bedani et al. [10] examined the effect of inulin and okara flour on textural and sensory properties of probiotic soy yoghurt (SY) throughout 28 days of storage at 4 °C. According to them, inulin has influnce on higher scores for sensory acceptability. Bibiana et al. [11] compared reduced-fat fresh cow's milk cheese with inulin (3%) with both full-fat and reduced-fat cheeses without prebiotic. The results showed that the reduced-fat cheese with inulin was more acceptable than its counterpart without inulin. Kinik et al. [12] investigate the effects of probiotics, and inulin on aromatic compounds, and on the textural and sensory properties of symbiotic goat

cheese during its ripening period. They study showed that the most favoured cheeses were found to contain *E. faecium* and oligofructose.

MATERIAL AND METHODS

MATERIALS

Production prototypes of milk based products with fresh cheese and fruits were conducted in the laboratory of Food Technology, the Faculty of Technology, University of Tuzla. For the production of fresh cheese, commercial UHT cow milk (Meggle) with 0.9% milk fat and commercial soy drink (Alpro) were used. For direct inoculation of milk, FD-DVS cultures were used:

1. Lyofast EF1 (*Enterococcus faecium)*
2. Lyofast MWO 030 : Lyofast EF1 in a ratio of 1:1

Lyofast MWO 030 (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris)*

As a prebiotic supplement, Orafti®Synergy1, powder of inulin, was used.

The parameters of the production of fresh probiotic cheese were as follows:

- Thermalization: 60°C/3 min
- 2 volumes of the soy drink content: 10% and 15% in relation to the total amount of milk
- 2 different starter cultures: Lyofast EF1 and Lyofast MWO 030
- Orafti®Synergy1 supplement: 2.5g/ 100g cheese.

The milk was thermally processed at inoculation temperature (25°C and 40°C) and primed with the microbial starter cultures. The fermentation lasted between 19 and 20 hours (samples A and B) and between 10 and 11 hours (samples C and D) until a pH value of 4.6 was reached. Subsequently, curd was cut (samples A1-3, B1-3, C1-3 and D1-3 were thermalized and then cooled at temperature 37°C), squeezed through cotton fabrics for about 6 to 11 hours at 20°C. In samples B1-6 and D1-6, a prebiotic supplement was added. 24 samples were produced. The obtained products were stored in a refrigerator in a plastic container for 14 days at + 4°C. The sample markings are shown in Table 1. The sensory properties of probiotic fresh cheese samples were evaluated by a group of 15 assessors using a scoring method, after 1, 7 and 14 days of storage [13], and they are trained. The acceptance of the samples of produced fresh cheese was tested by 40 consumers using the hedonic scale according to Peryam. They expressed their general impression by rating the tested products on a scale of 1 to 9 [14]. The sample markings are shown in Table 1.

Samples	Thermalization 60°C/3 min	% soy Drink	Starter culture	**Orafti® Synergy1**
A1	-	-	Lyofast EF1	-
A2	-	10 %		-
A3	-	15 %		-
A4	+	-		-
A5	+	10 %		-
A6	+	15 %		-
B1	-	-		+
B2	-	10 %		+
B3	-	15 %		+
B4	+	-		+
B5	+	10 %		+
B6	+	15 %		+
C1	-	-	Lyofast MWO 030:Lyofast EF1 =1:1	-
C2	-	10 %		-
C3	-	15 %		-
C4	+	-		-
C5	+	10 %		-
C6	+	15 %		-
D1	-	-		+
D2	-	10 %		+
D3	-	15 %		+
D4	+	-		+
D5	+	10 %		+
D6	+	15 %		+

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was carried out using SPSS software (version 22). Duncan test was used to determinate which samples were statistically different by all the sensory properties and acceptance of product ($P<0.05$).

RESULTS AND DISCUSSION

The average values of sensory properties of fresh probiotic cheese are shown in Tables 2, 3 and 4.

During storage, the samples had a relatively good external appearance. The addition of soy drink had a positive effect on the appearance and consistency of fresh cheese samples. The combination of Lyofast MWO 030 starter

culture: Lyofast EF1 = 1:1 influenced better sample consistency (Tables 2, 3 and 4). Observing the sensory sensitivity of the fragrance, it is evident that the addition of Lyofast MWO 030 and soy drink contributed to better grades, which was also reflected in the taste of the product (Tables 2, 3 and 4). The cheese samples produced by inoculation of milk with Lyofast EF1 only had slightly bitter but still pleasing taste. The addition of the soy drink neutralized bitter taste, giving the sweet taste and smell of probiotic cheese, a better feeling in the mouth when consumed, and the creamy consistency.

Table 2. Average values of sensory properties of probiotic fresh cheese after 1 day of storage

Sample	External appearance (2,0)	Consistency (4,0)	Smell (3,0)	Taste (9,0)	Color (2,0)	Σ (20,0)
A1	1.73(±0.45)^{1}	3.25(±0.84)^{1}	2.38(±0.87)^{1}	7.69(±1.09)^{1}	1.98(±0,07)	17.01
A2	1.94(±0.17)^{2,3}	3.88(±0.35)^{3}	3.00(±0.00)^{3}	8.31(±0.79)^{1,2,3,4,5}	2.00(±0.00)	19.13
A3	2.00(±0.00)^{3}	3.88(±0.35)^{3}	2.91(±0.18)^{3}	8.16(±0.90)^{1,2,3,4,5}	2.00(±0.00)	18.95
B1	1.94(±0.17)^{2,3}	3.41(±0.71)^{1,2}	2.68(±0.50)^{3}	8.08(±0.91)^{1,2,3,4}	2.00(±0.00)	18.10
B2	1.96(±0.07)^{2}	3.61(±0.43)^{1,2,3}	2.88(±0.35)^{3}	8.81(±0.37)^{4,5}	2.00(±0.00)	19.26
B3	1.86(±0.09)^{1,2,3}	3.80(±0.36)^{2,3}	2.98(±0.07)^{3}	8.73(±0.45)$^{3.4.5}$	2.00(±0.00)	19.36
C1	2.00(±0.00)^{3}	3.99(±0.03)^{3}	2.95(±0.09)^{3}	8.56(±0.49)^{2,3,4,5}	1.98(±0.07)	19.48
C2	2.00(±0.00)^{3}	4.00(±0.00)^{3}	3.00(±0.00)^{3}	8.88(±0.35)^{5}	2.00(±0.00)	19.88
C3	2.00(±0.00)^{3}	4.00(±0.00)^{3}	3.00(±0.00)^{3}	8.75(±0.46)^{3,4,5}	2.00(±0.00)	19.75
D1	2.00(±0.00)^{3}	3.88(±0.35)^{3}	3.00(±0.00)^{3}	7.88(±0.58)^{1,2}	2.00(±0.00)	18.75
D2	2.00(±0.00)^{3}	3.88(±0.35)^{3}	2.98(±0.07)^{3}	8.25(±0.65)^{1,2,3,4,5}	2.00(±0.00)	19.10
D3	2.00(±0.00)^{3}	4.00(±0.00)^{3}	2.94(±0.17)^{3}	8.56(±0.03)^{2,3,4,5}	2.00(±0.00)	19.50
A4	1.79(±0.36)^{1,2}	3.56(±0.72)^{1,2,3}	2.53(±0.71)^{1,2}	8.01(±1.05)^{1,2,3}	1.97(±0.737)	17.99
A5	1.94(±0.17)^{2,3}	3.94(±0.17)^{3}	2.93(±0.21)^{3}	8.75(±0.46)^{3,4,5}	2.00(±0.00)	19.55
A6	1.94(±0.17)^{2,3}	3.88(±0.35)^{3}	3.00(±0.00)^{3}	8.56(±0.82)^{2,3,4,5}	1.97(±0.70)	19.35
B4	1.98(±0.07)^{3}	3.88(±0.23)^{3}	2.85(±0.22)^{3}	8.59(±0.41)^{2,3,4,5}	1.93(±0.17)	19.23
B5	2.00(±0.00)^{3}	3.81(±0.37)^{2,3}	3.00(±0.00)^{3}	8.88(±0.23)^{5}	2.00(±0.00)	19.69
B6	2.00(±0.00)^{3}	3.81(±0.37)^{2,3}	2.75(±0.46)^{2,3}	8.19(±0.88)^{1,2,3,4,5}	2.00(±0.00)	18.75
C4	1.94(±0.17)^{2,3}	4.00(±0.00)^{3}	2.81(±0.37)^{2,3}	8.44(±0.56)^{1,2,3,4,5}	1.93(±0.17)	19.13
C5	2.00(±0.00)^{3}	3.98(±0.07)^{3}	3.00(±0.00)^{3}	8.75(±0.46)^{3,4,5}	2.00(±0.00)	19.73
C6	2.00(±0.00)^{3}	3.98(±0.07)^{3}	3.00(±0.00)^{3}	8.81(±0.37)^{4,5}	2.00(±0.00)	19.79
D4	2.00(±0.00)^{3}	4.00(±0.00)^{3}	3.00(±0.00)^{3}	8.44(±0.53)^{1,2,3,4,5}	2.00(±0.00)	19.44
D5	2.00(±0.00)^{3}	3.75(±0.46)^{2,3}	2.94(±0.17)^{3}	8.75(±0.46)^{3,4,5}	2.00(±0.00)	19.44
D6	2.00(±0.00)^{3}	3.88(±0.35)^{3}	3.00(±0.00)^{3}	8.31(±0.53)^{1,2,3,4,5}	2.00(±0.00)	19.19

^{1,2,3,4,5,6} *- Mean values in the same column, with different superscript are significantly different (P<0.05)*

Table 3. Average values of sensory properties of probiotic fresh cheese after 7 days of storage

Sample	External appearance (2,0)	Consistency (4,0)	Smell (3,0)	Taste (9,0)	Color (2,0)	Σ (20,0)
A1	1.63(±0.58)^{1}	2.71(±1.00)^{1}	2.50(±0.92)^{1,2,3,4}	6.38(±1.59)^{1}	1.98(±0.07)	15.19
A2	1.79(±0.36)^{1,2}	3.59(±0.49)^{2,3,4,5}	2.86(±0.35)^{3,4}	7.50(±1.06)^{1,2,3,4,5}	1.98(±0.07)	17.71
A3	1.85(±0.35)^{1,2}	3.29(±0.92) ^{2,3}	2.60(±0.68) ^{2,3,4}	7.50(±1.30)^{1,2,3,4,5}	1.91(±0.18)	17.15
B1	1.81(±0.37)^{1,2}	3.01(±0.87) ^{1,2}	1.94(±0.62) ^{1}	6.63(±1.30)^{1,2}	1.88(±0.35)	15.26
B2	1.85(±0.22)^{1,2}	3.35(±0.55)^{2,3,4}	2.33(±0.70)^{1,2,3}	7.56(±0.82)^{1,2,3,4,5}	2.00(±0.00)	17.09
B3	1.71(±0.18)^{1,2}	3.44(±0.41)^{2,3,4,5}	2.29(±0.56)^{1,2,3}	7.73(±1.55)^{1,2,3,4,5}	1.88(±0.35)	17.04
C1	1.88(±0.23)^{1,2}	3.94(±0.17)^{4,5}	2.60(±0.50)^{2,3,4}	6.81(±1,06)^{1,2}	1.98(±0.07)	17.20
C2	1.95(±0.10)^{1,2}	4.00(±0.00)^{5}	2.81(±0.37)^{2,3,4}	7.63(±1.48)^{1,2,3,4,5}	2.00(±0.00)	18.39
C3	1.90(±0.19)^{1,2}	3.81(±0.37)^{3,4,5}	2.75(±0.46)^{2,3,4}	7.38(±0.80)^{1,2,3,4}	2.00(±0.00)	18.46
D1	1.81(±0.25)^{1,2}	3.38(±0.44)^{2,3,4,5}	2.69(±0.44)^{2,3,4}	7.44(±0.51)^{1,2,3,4}	2.00(±0.00)	17.31
D2	1.94(±0.17)^{1,2}	3.63(±0.44)^{3,4,5}	2.81(±0.25)^{2,3,4}	7.88(±0.64)^{2,3,4,5}	2.00(±0.00)	18.25
D3	2.00(±0.00)^{2}	4.00(±0.00)^{5}	3.00(±0.00)^{4}	8.44(±0.41)^{4,5}	2.00(±0.00)	19.44
A4	1.79(±0.36)^{1,2}	3.31(±1.16)^{2,3,4}	2.19(±0.63)^{1,2}	6.88(±1.55)^{1,2,3}	1.87(±0.23)	16.04

Sample	External appearance (2,0)	Consistency (4,0)	Smell (3,0)	Taste (9,0)	Color (2,0)	Σ (20,0)
A5	1.63(±0.44) [1]	3.75(±0.37)[3,4,5]	2.4(±0.60)[1,2,3,4]	7.25(±0.92) [1,2,3,4]	1.75(±0.46)	16.79
A6	1.80(±0.22)[1,2]	3.80(±0.35)[3,4,5]	2.63(±0.51)[2,3,4]	7.81(±0.70)[2,3,4,5]	1.85(±0.22)	17.89
B4	1.79(±0.36)[1,2]	3.50(±0.70)[2,3,4,5]	2.44(±0.49)[1,2,3,4]	7.31(±1.38)[1,2,3,4]	1.81(±0.37)	16.85
B5	1.85(±0.22)[1,2]	3.87(±0.41)[3,4,5]	2.50(±0.44)[1,2,3,4]	7.56(±0.45)[1,2,3,4,5]	1.93(±0.23)	18.64
B6	2(±0.00)[2]	3.78(±0.23)[3,4,5]	2.91(±0.22)[3,4]	8.31(±0.83)[4,5]	2.00(±0.00)	18.56
C4	1.88(±0.23)[1,2]	3.88(±0.23)[3,4,5]	2.50(±0.92)[1,2,3,4]	7.56(±1.14)[1,2,3,4,5]	1.94(±0.17)	17.75
C5	1.94(±0.17)[1,2]	3.79(±0.24)[3,4,5]	2.91(±0.18)[3,4]	8.31(±0.88)[4,5]	1.88(±0.35)	18.83
C6	1.88(±0.23)[1,2]	3.76(±0.253)[3,4,5]	2.66(±0.35)[2,3,4]	7.73(±1.33)[1,2,3,4,5]	1.99(±0.03)	18.01
D4	2.00(±0.00)[2]	4.00(±0.00)[5]	3.00(±0.00)[4]	8.44(±0.53)[4,5]	2.00(±0.00)	19.44
D5	2.00(±0.00)[2]	3.88(±0.35)[3,4,5]	3.00(±0.00)[4]	8.88(±0.35)[5]	2.00(±0.00)	19.75
D6	2.00(±0.00)[2]	4.00(±0.00)[5]	2.88(±0.35)[3,4]	8.25(±0.37)[3,4,5]	2.00(±0.00)	19.13

[1,2,3,4,5,6] *- Mean values in the same column, with different superscript are significantly different (P<0.05)*

Table 4. Average values of sensory properties of probiotic fresh cheese after 14 days of storage

Sample	External appearance (2,0)	Consistency (4,0)	Smell (3,0)	Taste (9,0)	Color (2,0)	Σ (20,0)
A1	1.75(±0.35)[1,2,3]	2.81(±0.56)[1]	2.08(±0.61)[3,4]	4.85(±1.47)[1]	1.98(±0.07)[2,3]	13.40
A2	1.86(±0.22)[2,3]	3.49(±0.44)[2]	2.41(±0.60)[3,4,5,6]	5.88(±1.80)[1,2,3,4]	1.98(±0.07)[2,3]	15.61
A3	1.81(±0.37)[2,3]	3.38(±0.87)[2]	2.19(±0.84)[3,4,5]	6.44(±2.19)[1,2,3,4,5]	1.85(±0.35)[2,3]	15.66
B1	1.44(±0.49)[1]	2.31(±0.45)[2]	1.25(±0.37)[1]	5.50(±0.75)[1,2,3]	1.88(±0.35)[2,3]	12.38
B2	1.85(±0.22)[2,3]	3.56(±0.56)[2]	2.25(±0.65)[3,4,5]	7.25(±1.03)[4,5,6]	2.00(±0.00)[3]	16.91
B3	1.76(±0.23)[1,2,3]	3.44(±0.41)[2]	2.25(±0.26)[3,4,5]	7.50(±1.36)[4,5,6]	1.88(±0.35)[2,3]	16.83
C1	1.75(±0.21)[1,2,3]	3.44(±0.49)[2]	2.26(±0.54)[3,4,5]	5.94(±1.01)[1,2,3,4]	1.98(±0.07)[2,3]	15.36
C2	1.88(±0.23)[2,3]	3.81(±0.37)[2]	2.31(±0.59)[3,4,5]	6.38(±1.30)[1,2,3,4,5]	2.00(±0.00)[3]	16.38
C3	1.88(±0.23)[2,3]	3.88(±0.23)[2]	2.19(±0.75)[3,4,5]	7.31(±1.33)[4,5,6]	2.00(±0.00)[3]	17.25
D1	1.76(±0.33)[1,2,3]	3.63(±0.44)[2]	2.13(±0.64)[3,4,5]	5.25(±1.90)[1,2]	2.00(±0.00)[3]	14.76
D2	1.89(±0.18)[2,3]	3.88(±0.35)[2]	2.33(±0.70)[3,4,5]	5.50(±2.20)[1,2,3]	2.00(±0.00)[3]	15.59
D3	2.00(±0.00)[3]	4.00(±0.00)[2]	3.00(±0.00)[6]	8.50(±0.46)[6]	2.00(±0.00)[3]	19.50
A4	1.56(±0.49)[1,2]	2.75(±1.25)[1]	1.44(±0.67)[1,2]	4.88(±2.41)[1]	1.88(±0.22)[2,3]	12.50
A5	1.66(±0.44)[1,2,3]	3.50(±0.70)[2]	1.89(±0.62)[2,3]	6.56(±0.11)[2,3,4,5]	1.63(±0.51)[1]	15.24
A6	1.81(±0.25)[2,3]	3.68(±0.69)[2]	2.35(±0.40)[3,4,5,6]	6.94(±0.77)[3,4,5,6]	1.73(±0.36)[1,2]	16.50
B4	1.81(±0.37)[2,3]	3.44(±0.72)[2]	2.56(±0.49)[3,4,5,6]	7.00(±1.07)[3,4,5,6]	1.81(±0.37)[1,2]	16.63
B5	1.91(±0.18)[2,3]	3.63(±0.44)[2]	2.69(±0.45)[4,5,6]	7.88(±0.64)[5,6]	1.94(±0.17)[2,3]	18.04
B6	2.00(±0.00)[3]	3.69(±0.37)[2]	2.81(±0.25)[5,6]	7.31(±0.75)[5,4,6]	1.94(±0.17)[2,3]	17.75
C4	1.75(±0.37)[1,2,3]	3.63(±0.74)[2]	2.19(±1.06)[3,4,5]	6.38(±1.99)[1,2,3,4,5]	1.94(±0.17)[2,3]	15.88
C5	1.85(±0.22)[2,3]	3.73(±0.36)[2]	2.73(±0.45)[4,5,6]	7.35(±1.58)[4,5,6]	2.00(±0.00)[3]	17.65
C6	1.75(±0.26)[1,2,3]	3.75(±0.37)[2]	2.54(±0.39)[3,4,5,6]	7.06(±1.08)[3,4,5,6]	2.00(±0.00)[3]	17.10
D4	2.00(±0.00)[3]	4.00(±0.00)[2]	3.00(±0.00)[6]	8.50(±0.65)[6]	2.00(±0.00)[3]	19.50
D5	2.00(±0.00)[3]	3.88(±0.35)[2]	2.19(±0.37)[3,4,5]	7.75(±0.46)[5,6]	2.00(±0.00)[3]	17.81
D6	2.00(±0.00)[3]	4.00(±0.00)[2]	2.50(±0.53)[3,4,5,6]	7.00(±0.00)[3,4,5,6]	2.00(±0.00)[3]	17.50

[1,2,3,4,5,6] *- Mean values in the same column, with different superscript are significantly different (P<0.05)*

The combination of starter cultures Lyofast MWO 030 with Lyofast EF1 in the ratio of 1:1 improved the taste of cheese, which is also evident from the ratings given by the sensory assessors, and which was shown by statistical analysis (Tables 2, 3 and 4). In the research carried out by Rasouli Pirouzian et al. [15], samples of Iranian white UF cheeses containing *Enterococcus faecium* showed lower scores of cheeses that were diluted with milking with a combination of mesophilic (*Lactococcus cremoris* and *L. lactic*) and thermophilic (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) cultures. The panelists commented that the cheeses produced with *E. faecium* lacked a good taste and had a less pronounced aroma after two months of storage. Some bacteria produce additional cellular proteolytic and lipolytic enzymes

that can cause unwanted texture and flaws [16]. There are contradictory statements about the influence of enterococci on the sensory characteristics of cheese. High concentrations of enterococci are considered to cause deterioration in organoleptic properties in parmesan [17, 18]. In contrast, many authors claim that enterococci can play a positive role in the cheese production process [19]-[22]. The color, as sensory property, of all samples was the most stabile during storage. The consistency of thermalized samples was enhanced during storage, indicating that thermalisation had a positive impact on consistency.

Table 5. Average values of acceptance of non thermalized probiotic fresh cheese after 14 days of storage

Sample	X	D (%)	ND (%)
A1	5.188(±1.06)^{1,2,3}	87.50	12.50
A2	6.438(±1.267)^{1,2,3,4}	87.50	12.50
A3	6.588(±2.01)^{2,3,4}	75.00	25.00
B1	5.750(±0.70)^{1,2,3}	100.00	0
B2	7.125(±0.83)^{3,4}	100.00	0
B3	7.250(±1.16)^{3,4,5}	100.00	0
C1	6.225(±1.12)^{1,2,3,4}	100.00	0
C2	6.750(±1.38)^{2,3,4}	100.00	0
C3	7.438(±1.23)^{4,5}	100.00	0
D1	5.000(±1.30)^{1}	75.00	25.00
D2	5.500(±1.92)^{1,2}	75.00	25.00
D3	8.625(±0.35)^{5}	100.00	0

x- Average values; D – desirability; ND – non desirability
^{1,2,3,4,5}- Mean values in the same column, with different superscript are significantly different (P<0.05)

Table 6. Average values of acceptance of thermalized probiotic fresh cheese after 7 days of storage

Sample	X	D (%)	ND (%)
A4	6.938(±1.26)^{1}	100.00	0
A5	7.93(±0.41)^{2,3}	100.00	0
A6	8.125(±1.64)^{3}	100.00	0
B4	7.063(±1.65)^{1,2}	100.00	0
B5	8.313(±0.37)^{3}	100.00	0
B6	8.000(±0.91)^{2,3}	100.00	0
C4	8.000(±0.92)^{2,3}	100.00	0
C5	8.438(±0.72)^{3}	100.00	0
C6	8.000(±1.02)^{2,3}	100.00	0
D4	8.500(±0.41)^{3}	100.00	0
D5	8.875(±0.35)^{3}	100.00	0
D6	8.475(±0.35)^{3}	100.00	0

x- Average values; D – desirability; ND – non desirability
^{1,2,3}- Mean values in the same column, with different superscript are significantly different (P<0.05)

Table 7. Average values of acceptance of thermalized probiotic fresh cheese after 14 days of storage

Sample	X	D (%)	ND (%)
A4	5.188(±2.06)^{1}	62.50	37.50
A5	7.313(±0.94)^{2}	100.00	0
A6	7.313(±1.16)^{2,3}	100.00	0
B4	6.875(±1.45)^{2}	100.00	12.50
B5	7.938(±0.41)^{2,3}	100.00	0
B6	7.375(±0.69)^{2,3}	100.00	0
C4	6.938(±1.74)^{2}	100.00	0
C5	7.375(±1.57)^{2,3}	100.00	0
C6	7.313(±1.03)^{2,3}	100.00	0
D4	8.563(±0.49)^{3}	100.00	0
D5	7.875(±0.35)^{2,3}	100.00	0
D6	7.375(±0.51)^{2,3}	100.00	0

x- Average values; D – desirability; ND – non desirability
^{1,2,3}- Mean values in the same column, with different superscript are significantly different (P<0.05)

The analysis of the variance (Table 5, 6 and 7) showed that there was a statistically significant difference ($p < 0.05$) between the sensory properties of the samples of probiotic fresh cheese during storage, for appearance, consistency, smell and taste for all days of storage. According to the results (Table 4), the color, as a sensory property, was statistically significant ($P < 0.05$) only after 14 days of storage. Considering the statistically significant difference ($P<0.05$), the Duncan test was conducted by which it was determined which samples were the best by certain sensory properties also during the storage. The samples which were produced with the combination of starter cultures and addition of soy drink had a better external appearance, consistency and smell than other samples. Also, addition of inulin had a positive influence on consistency and smell. The samples with soy drink C2 and B5 had the best taste. The worst smell and taste had the sample without soy milk and inulin A1. There was a change of sensory properties during storage. Based on the statistical analysis of the results, it is evident that most D group samples were the most stable during storage. The results of the consumer acceptance for the produced fresh cream cheese samples are shown in Tables 5, 6 and 7. The preferred product is considered to be the one whose average grade (x) is at least 7.5 when applying a scale with nine possible grades [14]. This criterion was satisfied by all samples of probiotic fresh cheese after 1 day of storage. All samples were

100% preferred, indicating that no assessor rated the fresh cheese samples with a grade lower than 5. Soy drink influenced the stable acceptability during the first 7 days of storage. After 14 days of storage, only the samples with added soy drink and inulin D3, B5, D4 and D5 might be considered desirable. Considering the statistically significant difference ($P<0.05$), the Duncan test was conducted.

CONCLUSION

Sensory properties of probiotic fresh cheese were variable during storage for 14 days. During storage, the samples had a relatively good outer appearance.

Soy drink and the combination of microbial starter cultures had a positive influence on the external appearance, consistency, smell and taste of the samples.

Observing the results for the color of the probiotic fresh cheese, this sensory characteristic was found to be the most stable during storage. The thermalisation of the curd affected a slightly firmer consistency of the product, and during storage, the surface of the product did not extract whey.

The samples inoculated with the combination of microorganisms Lyofast MWO 030 and Lyofast EF1 with inulin addition were the most stable during storage for all sensory properties. All the investigated samples showed a high percentage of 100% preference for the first day of storage. During storage, the percentage of desirability decreased in each sample (up to 62.50%).

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SYNTHESIS, SPECTRAL CHARACTERIZATION, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF COPPER(II)-PABA COMPLEX

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: pABA (p-aminobenzoic acid or 4-aminobenzoic acid) is a chemical component of the folate molecule produced by plants and bacteria, and found in many foods. It is best known as a UV-blocking sunscreen applied to the skin, and is sometimes taken orally for certain medical conditions. Today it is known that many organic molecules in the human body can react with biometals such as copper, cobalt, manganese, iron and others. This study was performed to investigate the interaction of Cu(II) ions with p-aminobenzoic acid. Spectroscopic methods (FTIR and UV/Vis spectroscopy) were used to characterize the product obtained. The antimicrobial activity of the synthesized complex was tested by diffusion techniques. The results of spectroscopic analysis indicate the interaction of Cu(II) ions with pABA. Interaction is realized through oxygen donor atom of ligand. It was found that the Cu(II) complex has significant antimicrobial activity compared to the pABA ligand.

KEYWORDS: p-aminobenzoic acid, copper, FTIR, UV/Vis, antimicrobial analysis

INTRODUCTION

Para-aminobenzoic acid (pABA), (Figure 1), is a precursor for the synthesis of folic acid (also known as vitamin B9 or folacin). Folic acid is an enzyme cofactor, and it is involved in some basic biological reactions, as nucleotide biosynthesis, DNA repair and DNA methylation [1].

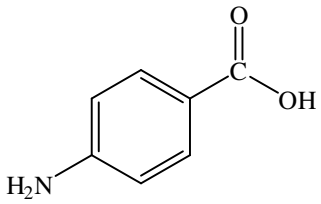

Figure 1. Structure of pABA

pABA is a chemical compound which has been of medical interest during the past decade because of its effects on bacterial metabolism and pigment metabolism [2]. It is also used as a protective drug against solar insolation and in diagnostic tests for the state of the gastrointestinal tract in medicine [3]. In normal man, pABA is rapidly absorbed and excreted, in small amount as free pABA or as acetylated pABA. Most of this drug is excreted either as a conjugation product with glycine (p-aminohippuric acid) or as the glucuronate [4]. pABA have shown a stronger antimicrobial activity at lower pH values. It is supposed that this acid reacts in at least two mechanisms of antibacterial activity: one mechanism in common with other organic acids and the other mechanism by interfering with the synthesis of the peptidoglycan layer by an action on the dihydrofolate reductase enzyme [5].

Complexing of biologically important molecules as well as molecules introduced into the body by drugs and supplements can significantly influence their biological activity [6,7]. The analysis of the interaction of biogenic metal M(II) cations with O, N, S-donor atoms of ligands often used in the treatment of a wide spectrum of diseases is important for monitoring of distribution, pharmacokinetics, excretion, drug efficacy and adverse effects [8].

MATERIAL AND METHODS

CHEMICALS

All reagents used were p.a. purity and were purchased from Sinex (Bosnia and Herzegovina), Euro-Lab (Bosnia and Herzegovina), Sigma Aldrich (United States) and Fisher Scientific (United States).

SYNTHESIS OF CU(II)-PABA COMPLEX

The complex synthesis was performed according to a previously published procedure [6, 8]. A mixture

of ethanol and water in a volume ratio 1:1 was used as a solvent for metal salt and pABA. For the synthesis of complex, solutions of $CuCl_2$ x $2H_2O$ (0.005 mol L^{-1}) and pABA (0.01 mol L^{-1}) were prepared. The solutions were mixed in an equimolar volume ratio and mixed on a magnetic stirrer for 30 minutes at room temperature, with an adjustment of pH value at 5.6. After stirring, the solution was left in dark space for three days to separate the solid complex. The resulting dark green product was filtered, washed with ethanol and water and then dried in a drying oven at 50 °C. The dry product is stored in a dessicator until analysis.

FTIR CHARACTERIZATION

In order to determine structure of the complex, samples were recorded on Nicolet iS10 FT-IR spectrophotometer - Thermo Fisher Scientific. The ATR technique was used for sample analysis. Samples were recorded in the range of 4000-650 cm^{-1}.

UV CHARACTERIZATION

The aqueous solutions of pABA (0.12 x 10^{-3} mol L^{-1}) and Cu(II) salt (0.06 x 10^{-3} mol L^{-1}) were used for recording the UV spectra. The solutions were mixed in equimolar volume ratio, stirred for 2 hours at 300 rpm, and then the UV spectra were recorded. Absorption spectra were recorded on a UV/Vis spectrophotometer Perkin Elmer λ25, in the range of wavelengths of 200-400 nm. Based on the position of the absorption maximum (λmax) in the tested model system, the value of the energy splitting of the central ion was calculated.

MORPHOLOGICAL CHARACTERIZATION

Before morphological characterization, solid complexes were treated with DMSO. The color, size and shape of Cu(II) crystal complex were determined by microscopic analysis. Shots were performed on the binocular microscope, the Leica DM 2500P mark.

ANTIMICROBIAL ACTIVITY *IN VITRO*

Antimicrobial activities were investigated by diffusion method on reference bacterial strains *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*. Antifungal activity of the complex was tested on *Candida albicans*. From the microorganisms strains of overnight cultures, suspensions of 0.5 McFarland turbidity were prepared (density 10^7-10^8 CFU/mL, depending on soy).

The strains were then placed on the surface of the nutrient substrate-Mueller-Hinton agar (MH), dispersed in sterile Petri dishes. Substrate thickness was 4 mm. In the agar sterile drill-shaped holes were made ("wells"), into which 80 μL of pABA and Cu(II) complex solutions in concentration of 5 mg mL^{-1} were added. After the plates were left at room temperature for 15 minutes, the substance was diffused into agar, incubated at 37^0C/24 h. After the incubation period, the size of the inhibitory zone was measured and the sensitivity of the microorganisms was expressed as follows: if the inhibitory zone of the microorganism growth was greater than 20 mm, it was marked with three pluses (+++), which is the highest sensitivity of microorganisms. If the inhibitory zone was in the range of 16-20 mm it was marked with two pluses (++). Very low sensitivity is indicated with one plus (+), if the inhibitory zone is 10-15 mm in diameter. The minus (-) mark is used for an inhibitory zone of less than 10 mm or if it's absolutely absent [9].

RESULTS AND DISCUSSION

STRUCTURE OF THE COMPLEX

Figure 2 shows the reaction scheme and the proposed structure of the Cu(II)-pABA complex. Metal and ligand react in a 1:2 molar ratio (M:L). The oxygen atom from the carboxyl group is involved in the formation of a bond with metal. The assumption is that the metal ion can bind two molecules of water (from the metal salt).

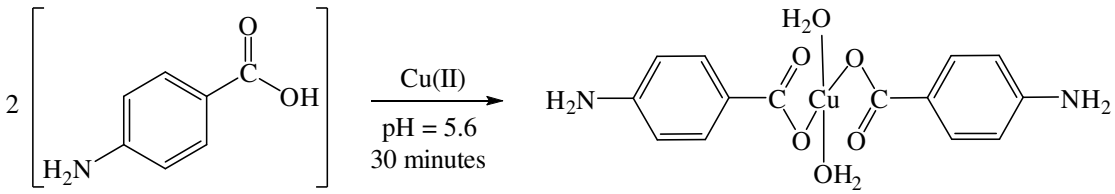

Figure 2. Reaction scheme and proposed structure of the complex

SPECTRAL CHARACTERIZATION

On the FTIR spectrum of the ligand, a strip characteristic of the O-H stretch vibration is seen at 3458 cm^{-1}, which is not visible in the spectrum of the complex. This indicates the interaction of copper(II) ions with the ligand over the oxygen atom of the carboxyl group. C=O stretch vibration is recorded as a high intensity strip at 1657 cm^{-1}. For the carboxylate ion in the spectrum of the complex the characteristic band is 1554 cm^{-1} (ν_{as}) (slight shift to smaller wave numbers compared to the ligand spectrum) and 1380 cm^{-1} (ν_s). The tape characteristic of the N-H stretch vibration was recorded at 3360 cm^{-1} in the ligand spectrum, or 3248 cm^{-1} at the complex spectrum. Insights into the electronic spectra of the ligand, peaks at 279, 217 and 202 nm were determined. In the spectrum of the complex, two absorption peaks were recorded, with hypochromic shifts. The difference in these two spectra is that the absorbance peak at 202 nm visible in the pABA spectrum is not visible in the spectrum of complex. Based on the absorption peak for the Cu(II)-pABA model system, splitting energy of d-orbitales was determined and it was 592 kJ mol^{-1}. Spectral data for pABA and $Cu(pABA)_2(H_2O)_2$ are shown in Table 1.

Table 1. Spectral data for pABA and $Cu(pABA)_2(H_2O)_2$

Infra-red spectral bands (cm^{-1})			
Sample	Functional group		
	N-H	O-H	C=O
pABA	3360	3458	1657
$Cu(pABA)_2(H_2O)_2$	3248	-	1554
Electronic spectral bands (nm)			
pABA	279, 217, 202		
$Cu(pABA)_2(H_2O)_2$	277, 217		

MORPHOLOGICAL CHARACTERIZATION

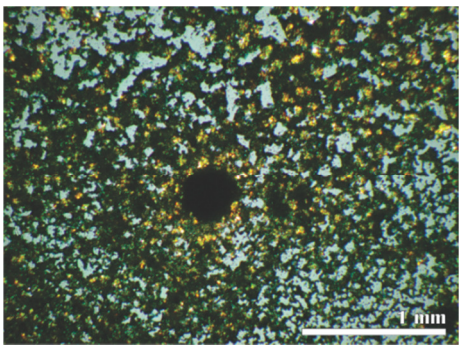

Figure 3. Morphology of $Cu(pABA)_2(H_2O)_2$ crystals

The morphologies of $Cu(pABA)_2(H_2O)_2$ crystals are presented in Figure 3. The images show the developed crystals, but irregular amorphous bodies. Severe interference colors are present due to the sample thickness. The size (diameter) is up to 0.25 mm.

ANTIMICROBIAL ACTIVITY *IN VITRO*

Table 2 shows the results of antimicrobial testing of pABA and Cu(II) complex. Antimicrobial screening revealed the effect of Cu(II) complexes on all tested gram positive bacteria. The largest inhibition zone was recorded with *Enterococcus faecalis* (16 mm) and the smallest in *Staphylococcus aureus* (11 mm). The synthesized complex does not act against gram negative bacteria and *Candida albicans*. In the parent ligand, antimicrobial activity is completely lacking in all bacterial strains and *Candida albicans* at a concentration of 5 mg mL^{-1}. In comparison with the control antibiotic Ciprofloxacin (conc. 1 mg mL^{-1}), significantly less antimicrobial activity of $Cu(pABA)_2(H_2O)_2$ is observed.

Table 2. Antimicrobial activities of pABA and $Cu(pABA)_2(H_2O)_2$

Microorganism	Inhibition Zone [mm]	
	1	2
E. coli	-	-
E. faecalis	-	16 (++)
S. aureus	-	11 (+)
B. subtilis	-	13 (+)
L. monocytogenes	-	13 (+)
P. aeruginosa	-	-
C. albicans	-	-

*Legend: (1) - pABA; (2) - $Cu(pABA)_2(H_2O)_2$

CONCLUSION

$Cu(pABA)_2(H_2O)_2$ complex is formed by interaction of Cu(II) ion with pABA in 1:2 (M:L) molar ratio. In the formation of the bond, oxygen atom of the carboxyl group are involved. There is a difference in the spectral and morphological properties of the complex and the ligand. Complexation of pABA with Cu(II) ion forms a compound that has significant antimicrobial activity on gram positive bacteria as opposed to pABA.

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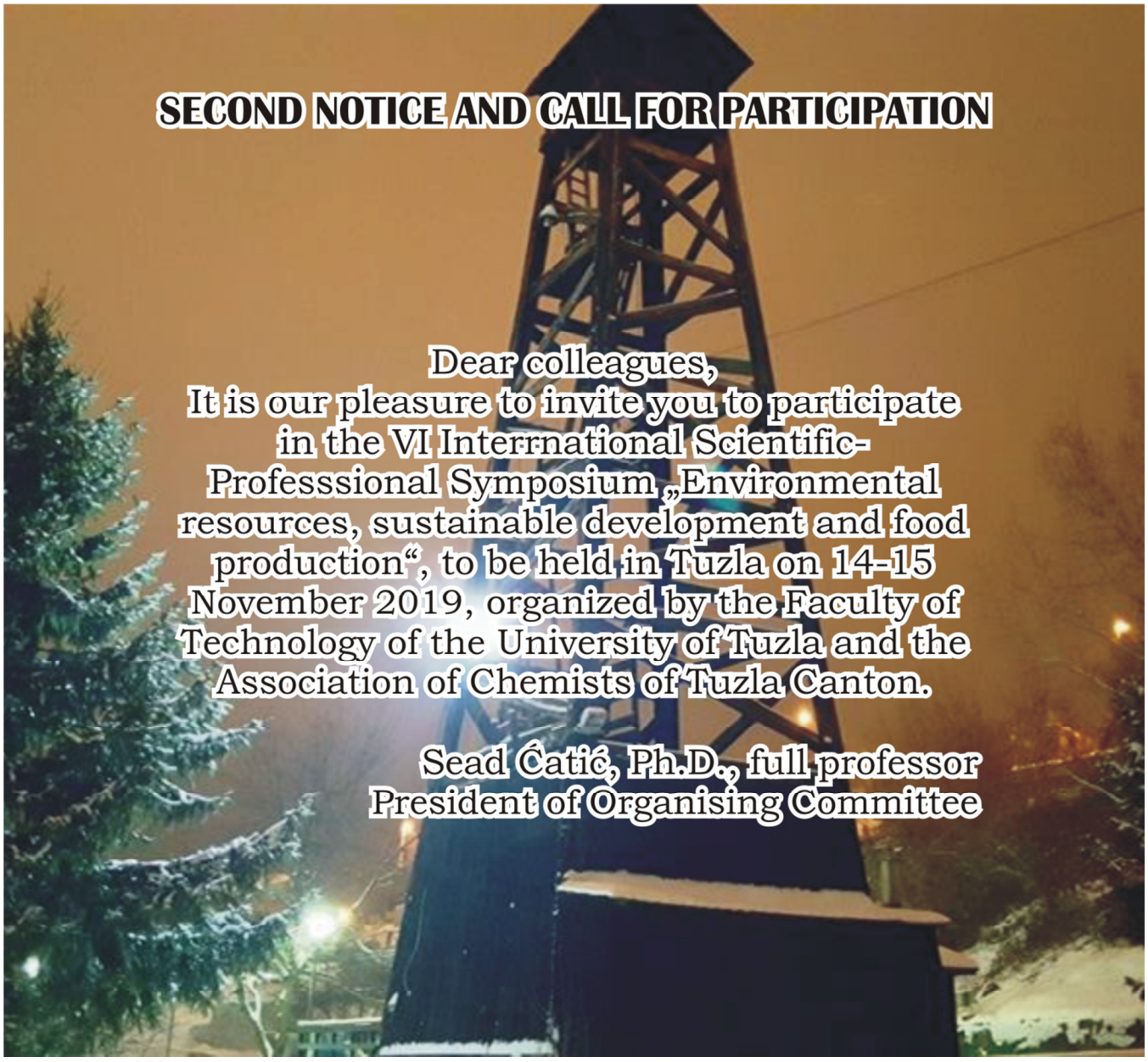
VI INTERNATIONAL SCIENTIFIC-PROFESSIONAL SYMPOSIUM »ENVIRONMENTAL RESOURCES, SUSTAINABLE DEVELOPMENT AND FOOD PRODUCTION - OPORPH 2019«

14-15 November 2019, Tuzla, Bosna i Hercegovina



Dear Colleagues,

It is our great honour to invite you to participate in the VI International Scientific-Professional Symposium „Environmental resources, sustainable development, and food production - OPORPH 2019“, to be held in Tuzla on 14 - 15 November 2019, organized by the Faculty of Technology of the University of Tuzla and the Association of Chemists of Tuzla Canton. The holding of the symposium "OPORPH" becomes a tradition of the Faculty of Technology of the University of Tuzla, and this year our Faculty is proud to celebrate 60 years of successful work, therefore, as part of the conference, a formal academy will be held.

As in previous years, our goal is to promote and popularize excellence in scientific and professional research of scientists and experts in the fields of chemistry, chemical engineering and technology, food technology, biotechnology, environmental protection and agronomy, and this year under the common motto "Sustainable development today is a change you want to see tomorrow."

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SECTIONS

- Chemical analysis, control and monitoring
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- Food technology and biotechnology
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FORMS OF PARTICIPATION

- Plenary presentations (at the invitation of the organizer)
- Oral presentations
- Poster presentations
- Students section (authors i co-authors of papers are exclusively students) NEW!

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*Abstract (up to 250 words in an officially available template) is to be sent in English and in one of the official languages of BiH to the e-mail address: sabina.begic@untz.ba

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Papers that pass the review process will be published in one of the upcoming issues of Technologica Acta.

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Journal article	[4] J. U. Duncombe, "Infrared navigation - Part I: An assessment of feasability," *IEEE Trans. Electron. Devices*, vol. ED-11, pp. 34-39, Jan. 1959.
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Technical report	[10] J. H. Davis and J. R. Cogdell, "Calibration program for the 16-foot antenna," Elect. Eng. Res. Lab., Univ. Texas, Austin, Tech. Memo. NGL-006-69-3, Nov. 15, 1987.
Patent	[11] J. P. Wilkinson, "Nonlinear resonant circuit devices," U.S. Patent 3 624 125, July 16, 1990.
Standard	[12] *IEEE Criteria for Class IE Electric Systems*, IEEE Standard 308, 1969.
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