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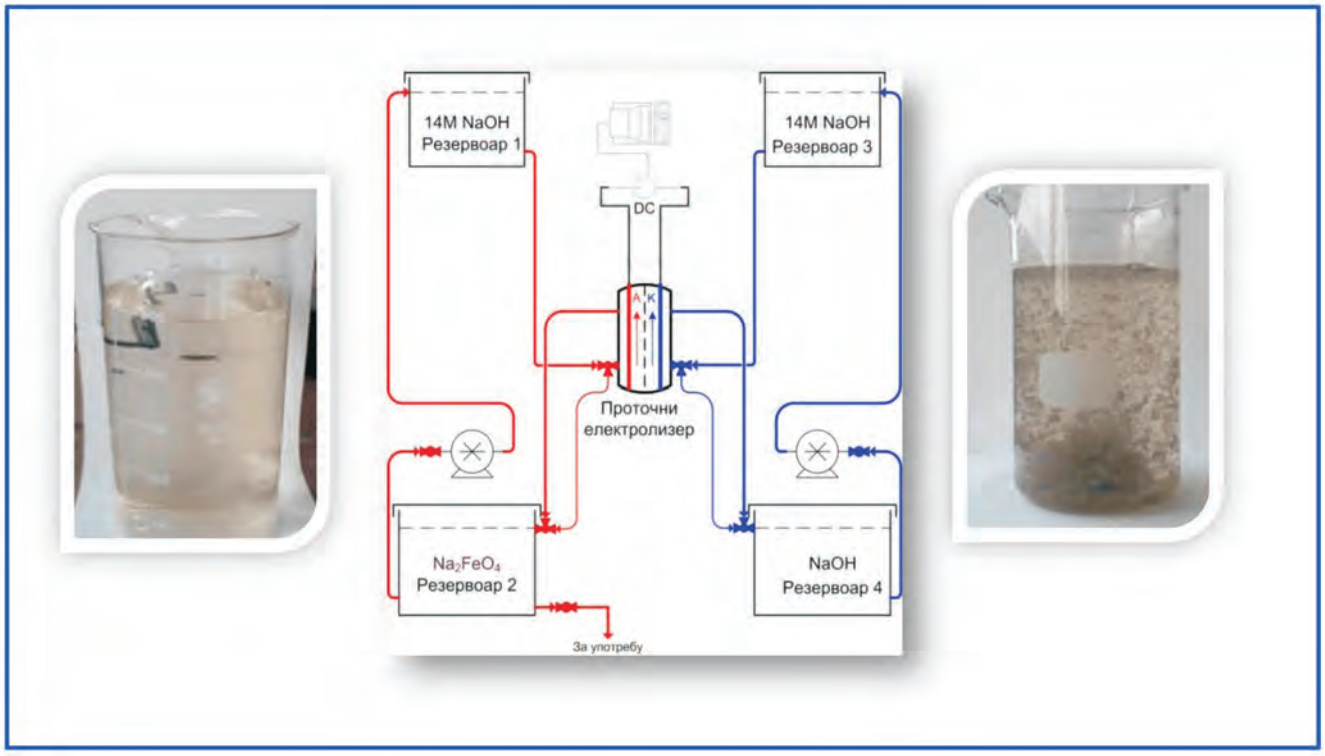

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The identification and quantification of bioactive compounds from the aqueous extract of comfrey root by UHPLC–DAD–HESI–MS method and its microbial activity

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Abstract

The qualitative, quantitative and microbial determination of the aqueous extract of comfrey root was done in this paper. The qualitative and quantitative analyses were done by the UHPLC–DAD–HESI–MS method. Allantoin, rosmarinic acid and ellagic acid were identified as major bioactive compounds and their quantification was also done. The obtained results showed a high content of allantoin, ellagic acid and rosmarinic acid (8.91, 7.4 and 12.8%, respectively) which indicated that the comfrey root can be used as a source for the isolation of these three compounds. The results obtained by the determination of the antimicrobial activity showed that *Escherichia coli* ATCC 8739 and *Salmonela typhimirium* ATCC 6538 were most sensitive to the aqueous extract of comfrey root. The results showed that allantoin did not express the antimicrobial activity on all the investigated bacteria species, and based on this it can be concluded that allantoin is not responsible for the antimicrobial activity of the aqueous extract of comfrey root.

Keywords: comfrey root, *Symphytum officinale* L., UHPLC–DAD–HESI–MS, microbial activity.

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Comfrey (*Symphytum officinale* L.) is a perennial herb which belongs to the genus *Symphytum* of the *Boraginaceae* family. It is a weed plant that is usually found in some parts of Asia, Europe and in North America [1,2]. Previous studies have shown that comfrey possesses good analgesic, anti-inflammatory, astringent, expectorant, antifungal and decongestant properties [3–5]. In folk medicine, the comfrey root has been used externally as a traditional medicinal plant (as an ointment, compress, or alcoholic fermentation) for treating fractures, strains, thrombophlebitis and haematoma, and internally (as tea, tincture or infusions) in treating gastro-intestinal and respiratory tract diseases [6]. On the other hand, the leaves and stems have also been used for treating the same disorders, as well as for treating rheumatism and gout [7].

Previous studies showed that these beneficial properties of comfrey are the result of the presence of numerous bioactive compounds [3,8]. It is known that comfrey contains allantoin, 18 amino acids, A, B and C vitamins, ellagic acid, auxin, triterpenoids, tannins, rosmarinic acid, steroidal saponins, inulin, pyrrolizidine alkaloids, calcium, potassium, iron, sulfur, copper, selenium. Hydrocolloid polysaccharides, which represent immunomodulatory substances, were also detected in comfrey [9,10].

Although comfrey consists of numerous different compounds, allantoin, ellagic acid and rosmarinic acid are probably the most important for its beneficial activities [11]. Allantoin stimulates the metabolic process in the subcutaneous tissue and stimulates the cell growth (proliferation), which results in epithelialization and a protective effect on the skin. It also strongly promotes the cell growth in bone cells (the bone fracture) and connective tissues (tendons, ligaments). Allantoin has a moisturizing and keratolytic effect, increases the water content of the extracellular matrix and improves desquamation of upper layers of the dead skin cells, increases the smoothness of the skin, promotes the cell proliferation and wound healing. It exhibits a mitigating, anti-irritating and protective effect on the skin by forming a complex with irritants and sensitizing agents [12]. On the other hand, rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, and it is consistent in *Boraginaceae* and *Lamiaceae* plant species. Rosmarinic acid possesses antioxidant activities [13,14], antiviral, anti-inflammatory effects and shows a very low toxicity [15] and antitumor potential. It is widely used for the preparation of many products in pharmaceutical, cosmetic and food

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industry. The rosemary essential oil has long been known for its antimicrobial and antivirus activities [16]. As well as the two previously mentioned compounds, ellagic acid also possesses many beneficial activities. It may occur in a free form in many fruits and vegetables and it has been investigated by researchers for a series of pharmacological properties such as antiviral, antioxidant, antimutagenic and anticancer activities [17,18]. In addition, ellagic acid has the effect to reduce a heart disease, birth defects, liver problems, the side effects of chemotherapy in men with advanced prostate cancer and it has also shown to be an effective inhibitor of *in vitro* lipid peroxidation [19,20].

Taking into account the multiple significance of comfrey, one of the aims of this study was to develop a new method, UHPLC–DAD–HESI–MS, for the qualitative analysis of the aqueous extracts of comfrey root and quantitative determination of allantoin, ellagic acid and rosmarinic acid as major bioactive compounds which are important pharmacodynamic properties of comfrey extract. Comfrey, as a source of bioactive compounds with potentially beneficial biological effects, is poorly investigated, but the results about its antimicrobial activity were not found in literature data [11]. Therefore, in this study, the microbial activity of the aqueous extracts of comfrey root was investigated and it was compared to the standard of allantoin that is the main pharmacological component in comfrey root.

MATERIALS AND METHODS

Reagents

Acetonitrile and water were purchased from Fisher Chemical (LC-MS and HPLC grade, respectively). Allantoin, ellagic acid and rosmarinic acid standards were purchased from Sigma-Aldrich. Formic acid was purchased from Carlo Erba (Italy).

The plant material

In this study, we used an air-dried comfrey root (*Symphyti* radix) which was obtained from the Institute for Medicinal Plant Research "Dr Josif Pančić". Dried root was milled in a laboratory disintegrator (the laboratory electric mill "Braun Aromatic KSM2").

Extract preparation

Chopped and homogenized plant material (30 g) was packed in the filter paper cocoon and placed in the Soxhlet extraction apparatus. 300 mL of water was measured in a reception vessel and the extraction was carried out at the boiling temperature of the solvent for 240 min. The solvent was removed by evaporation on a rotary vacuum evaporator at 50 °C. The resulting extract was dried in a laboratory oven at 50 °C to constant weight.

UHPLC–DAD–HESI–MS analysis

All the experiments were performed by using a Thermo Scientific liquid chromatography system (UHPLC) composed of a quaternary pump with a degasser, a thermostated column compartment, an autosampler, and a diode array detector connected to LCQ fleet ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with heated electrospray ionisation (HESI). Xcalibur (version 2.2 SP1.48) and LCQ Fleet (version 2.7.0.1103 SP1) software were used for the instrument control, data acquisition and data analysis. Separations were performed on a Hypersil gold C18 (50 mm×2.1 mm, 1.9 μm) from Thermo Fisher Scientific.

UHPLC–DAD–HESI–MS analysis was performed according to Kečkeš *et al*. (2013) with slight modifications [21]. The mobile phase consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. A linear gradient program at the flow rate of 0.350 mL/min was used for 0–2 min from 10 to 20% (B), 2–4.5 min from 20 to 90% (B), 4.5–4.8 min 90% (B), and 4.8––4.9 min from 90 to 10% (B), 4.9–12.0 min 10% (B). The injection volume was 10 μL, the column temperature was maintained at 25 °C, the separated compounds were detected at the wavelength of 272, 294, 308 and 360 nm, and each online spectrum was recorded within the range of 200–800 nm. The mass spectrometer was operated in a positive mode. HESI-source parameters were as follows: the source voltage 5.0 kV, the capillary voltage 46.00 V, the tube lens voltage 85.00 V, the capillary temperature 400 °C, the sheath and the auxiliary gas flow (N_2) 32 and 12 (arbitrary units). MS spectra were acquired by full range acquisition covering 150–700 *m*/*z*. For the fragmentation study, a data dependant scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the collision-induced dissociation (CID) cell was set at 25 eV.

Cultures of microorganisms used for the determination of microbial activity

To evaluate the *in vitro* microbial activity of the aqueous extract of comfrey root and allantoin standards, laboratory control microorganism strains that belong to the American Type Culture Collection (ATCC, Rockville, MD, USA) were used. *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538) were used as Gram-positive bacteria, *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27857) and *Salmonella typhimurium* (ATCC 14028) were used as Gram-negative bacteria, and *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231) were used as fungi.

The cultures of microorganisms were stored in the microbiology laboratory of the Faculty of Science in Niš

at –20 °C under appropriate conditions, subcultured twice immediately before the use in the microdilution test, when fresh suspensions of microorganisms were prepared. Standardized suspensions were prepared by flushing microorganisms with 0.9% NaCl solution with oblique and agar dilution to the appropriate number of cells, also saline. The number of microorganisms was determined with the turbitimer.

Determination of microbial activity

To determine the minimum inhibitory concentration (*MIC*) of the aqueous extract of comfrey root and allantoin, the microdilution method was applied and it was performed in microtiter plates with 96 conical holes [22,23]. The suspension was inoculated with bacteria that were prepared from the cultures of the appropriate microorganisms which were one day old, and the number of colonies in the suspension culture adjusted to 0.5 McFarland's turbitimer standards (bacterial colonies of microorganisms 2.0×10^{-6} CFU mL^{-1} and fungal spores 2.0×10^{5}). The initial concentration of the comfrey extract and allantoin were 40 and 10 mg/mL, respectively. After double dilution series *in situ*, the concentration of the samples was in the range of 2.2×10^{-6} to 10 mg/mL for the aqueous solution of allantoin and 1.96×10^{-2} to 40 mg/mL for the aqueous extract of comfrey root. Two columns in each plate were used as controls. One column was positive, and it included the antibiotic with a wide range of activities (doxycycline, a series of dilutions of 50–0.02 mg/mL). The second column contained the solvent. The growth of microorganisms was followed by the addition of 10 mL resasurine (BHD Laboratory Supplies) as an indicator (prepared by dissolving the tablets of 270 mg in 40 mL of sterile distilled water) into each indentation in the inoculated microtiter plate filled with the samples which were to be tested. The plates were incubated at 37 °C for 24 h for bacteria and at 28 °C for 48 h for fungi. After the incubation, the *MIC* values were determined based on the color change indicator. The color change from purple to pink or total bleaching was considered as a positive result. The lowest concentration where there was a change of color (bleaching) was adopted as the *MIC* value.

The minimum bactericidal/fungicidal concentration (*MBC*/*MFC*) was determined by streaking the contents of each microtiter plate recess in which the described change of the indicator color in the sterile surface of the nutrient agar prepared in Petri dishes was observed. Petri dishes were incubated at 37 °C for 24 h (bacteria) and at 28 °C for 48 h (fungi). After the incubation, the minimum bactericidal – fungicidal concentration was evaluated as one, where there was a lack of growth of 99.9% of the initial cell inoculum. All the tests were done in triplicate.

RESULTS AND DISCUSSION

UHPLC–DAD–HESI–MS analysis of the aqueous extract of comfrey root

Previous studies have shown that comfrey possesses numerous beneficial properties due to the presence of many compounds with biological activity. But unfortunately, the studies have also shown that this herb is also potentially toxic due to the presence of toxins in comfrey which include pyrrolizidine alkaloids (PAs) such as lycopsamine, echimidine and lasiocarpine, and their *N*-oxides [24,25]. Based on this, it can be concluded that the determination of the composition of the aqueous extract of comfrey root is of great importance. For the identification of the composition of the aqueous extract of comfrey root, a new UHPLC–DAD––HESI–MS method was developed and applied. All compounds were identified based on retention times, UV––Vis absorption spectrum and mass spectra by matching their molecular ions obtained by LC–HESI–MS and LC–MS/MS methods with theoretical molecular weights from literature data [6,21,25–27].

Figure 1 shows the UHPLC–DAD chromatogram of the aqueous extract of comfrey root at 272 nm. In Table 1, the identified compounds from the extract are shown, labeled as peaks 1–12 following the elution orders in the UHPLC–DAD chromatograms (mass spectra are not shown). In addition, structural formulas of the identified compounds in the extract are shown in Figure 2.

The first peak at retention time of 0.56 min was identified as allantoin. The presence of pseudomolecular ion $[M+H]^+$ in the positive ion mode at 159 *m*/*z* and its ion fragment at 116 *m*/*z* indicates that the first peak is allantoin. The presence of allantoin in the extract was also confirmed by standard reference.

Rosmarinic acid was identified as peak 2 at retention time of 5.31 min. MS Fragmentation of pseudomolecular ion $[M–H]^-$ in the negative mode at 359 *m*/*z* showed two ion fragments at 161 and 133 *m*/*z*. The presence of rosmarinic acid was also confirmed by standard reference. Peak 3 at retention time of 5.95 min was identified as lycopsamine. Lycopsamine contains the fragment ion at 120 *m*/*z*, which is a characteristic of the retronecine moiety, and represents the basic structure from which it is derived [25]. MS Fragmentation of lycopsamine showed two characteristic fragmenation ions at 120 and 138 *m*/*z*, which represent characteristic fragmentation ions of retronecine-type alkaloids. 5-*O*-Feruloylquinic acid was identified as peak 4 at retention time of 6.27 min. The presence of pseudomolecular ion $[M+H]^+$ in the positive mode at 369 *m*/*z* and ion fragments at 193 and 173 *m*/*z* after MS fragmentation indicates that 5-*O*-feruloylquinic acid is present in the aqueous extract of comfrey root. Peak 5

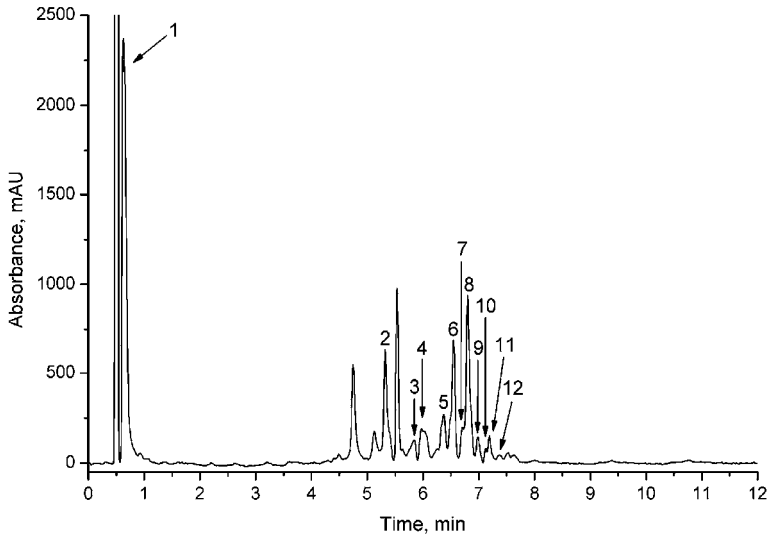

Figure 1. UHPLC Chromatogram of the aqueous extract of comfrey root (Symphytum officinale L.) detected at 272 nm.

Table 1. The identified compounds in the aqueous extract of comfrey root

Peak No.	t_R / min	Ionization mode	Pseudomolecular ion	MS/MS	Compound
1	0.56	+	159	116	Allantoin
2	5.31	–	359	161, 133	Rosmarinic acid
3	5.95	+	300	120, 138	Lycopsamine
4	6.27	+	369	193, 173	5-*O*-Feruloylquinic acid
5	6.48	+	273	255, 227, 153	Pinobanksin
6	6.62	+	291	245, 205, 179	Epicatechin
7	6.72	+	455	291, 163	*p*-Coumaroyl-hexoside-methylglutarate
8	6.79	+	539	455, 291, 163	Derivate of *p*-coumaroyl-hexoside--methylglutarate
9	6.97	–	315	151	Quercetin-3-methylether
10	7.10	–	301	257, 229	Ellagic acid
11	7.21	+	319	279, 149	Myricetin
12	7.51	+	412	394, 336, 238, 220, 120	Lasiocarpine

at retention time of 6.48 min with pseudomolecular ion $[M+H]^+$ at 273 *m*/*z* was identified as pinobanksin. After MS fragmentation, three ion fragments at 255, 227 and 153 *m*/*z* were present. Based on the obtained results from MS fragmentation it can be concluded that this compound is pinobanksin. Pseudomolecular ion $[M+H]^+$ in the positive mode at 291 *m*/*z* and its ion fragments at 245, 205 and 179 *m*/*z* indicated that peak 6 at retention time of 6.62 min was epicatechin. Peaks 7 and 8 were identified as *p*-coumaroyl-hexosidemethylglutarate and its derivate, respectively. After MS fragmentation, both compounds showed the same ion fragments at 291 and 163 *m*/*z* whereby peak 8 showed the additional ion fragment at 455 *m*/*z.*

Peak 9 at retention time of 6.97 min was identified as quercetin-*3*-methylether. Based on its pseudomolecular ion $[M–H]^–$ in the negative mode at 315 *m*/*z* and its ion fragment at 151 *m*/*z* it can be concluded that quercetin-*3*-methylether is present in the aqueous comfrey root extract. Ellagic acid was identified as peak **10** at retention time of 7.10 min. Ellagic acid and quercetin have a similar pseudomolecular ion $[M–H]^–$ in the negative mode at 301 *m*/*z*, but their MS fragmentation is different. After MS fragmentation, based on the presence of ion fragments at 257 and 229 *m*/*z* it can be concluded that peak 10 is ellagic acid. The presence of ellagic acid in the aqueous extract of comfrey root was confirmed by standard reference. Myricetin was identified as peak 11 at retention time of 7.21 min. In the positive mode its pseudomolecular ion $[M+H]^+$ at 319 *m*/*z*, and ion fragments at 279 and 149 *m*/*z* confirmed that myricetin was present in the aqueous extract of comfrey root. In the analysis of the aqueous extract of comfrey root, lasiocarpine (peak 12) was also detected. The presence of pseudomolecular ion $[M+H]^+$ in the positive mode at 412 *m*/*z*, and ion fragment at 120 *m*/*z*

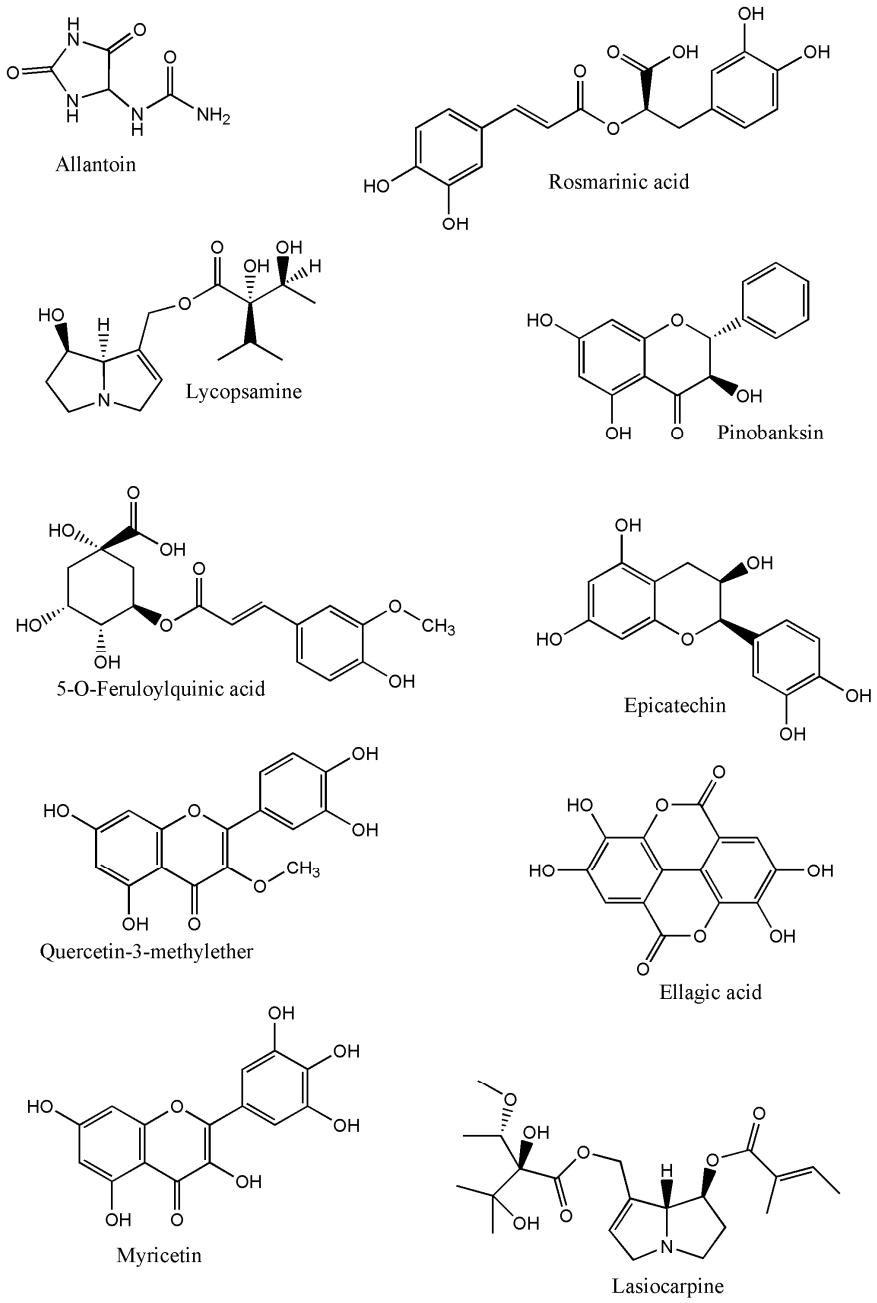

Figure 2. Structural formulas of the identified compounds in the aqueous extract of comfrey root.

indicates that peak 12 is lasiocarpine. Based on previous studies, it is known that lycopsamine and lasiocarpine belong to the group of pyrrolizidine alkaloids which are of special interest because several of them showed to cause toxic reactions in humans, primarily a veno-occlusive liver disease, when ingested as food or herbal medicines.

Quantification of bioactive compounds in the aqueous extract of comfrey root

The results of the qualitative analysis of the aqueous extract of comfrey root showed that allantoin and rosmarinic acid were present in the extract. Besides these two compounds, the presence of ellagic acid was also detected. Due to the biological activity of these three compounds and their role in the total beneficial activity of comfrey, quantitative contents were determined. In Table 2, the obtained results from the quantitative determination of allantoin, ellagic acid and rosmarinic acid are shown. The contents of these compounds were determined by UHPLC–DAD–HESI–MS method using the calibration curves which were constructed for the appropriate bioactive component at 360 nm.

Allantoin is one of the main components responsible for beneficial bioactive properties of comfrey.

Table 2. The yields of the total extractive matter and the content of allantoin, ellagic acid and rosmarinic acid in the aqueous extract of comfrey root

Compound	Regression equation[a]	R^2	g/100 g of dry extract	Total extractive matter (g/100 g of plant material)
Allantoin	$y = 12298.88x + 12.86$	0.9996	8.91	6.30
Ellagic acid	$y = 368210x + 110.15$	0.9969	7.4	
Rosmarinic acid	$y = 8514.7x + 1214.3$	0.9983	12.8	

[a]$y = ax+b$; where x is the concentration in mg/mL, and y is the area under the curve at the selected wavelength

From literature data, it is known that allantoin is present from 0.6–4.7% in comfrey [28]. The results of our study showed that the aqueous extract of comfrey root contains allantoin 8.91 g/100 g of the dry extract (*i.e*., 8.91%) [5]. Based on this it can be concluded that comfrey root represents a good source for the isolation of allantoin.

Literature data indicate that ellagic acid was mostly present in strawberry (162 mg/100 g dry matter) [29] and raspberry fruits (415 mg/100 g dry matter) [30]. In the aqueous extract of comfrey root, the content of ellagic acid is 7.4 g/100 g of the dry extract. The high content of ellagic acid in the aqueous extract of comfrey root can be responsible for its biological activity. Rosmarinic acid was also present in many plant materials, as are the leaves of Lemon balm (*Melissa officinalis*, Lamiaceae) in the concentration of 3.91% [31]. In *Rosmarinus officinalis* (Lamiaceae) rosmarinic acid was detected in leaves, flowers, stems and roots, but the highest amount of 2.5% was found during the first stages of the leaf growth [32]. In the crude extract of *Borago officinalis* (*Boraginaceae*) rosmarinic acid was present with 2.5% [33]. In these studies, the determined content was 12.8%, and to our knowledge this is the highest concentration of rosmarinic acid that has been found in all determined species so far.

Microbial activity of allantoin and the aqueous comfrey root extract

The presence of the identified compounds and the high content of allantoin, ellagic acid and rosmarinic acid in the aqueous extract of comfrey root, and the lack of the published results of the antimicrobial activity of comfrey, were a good motive for this study. The results of the microbial activity of the aqueous extract of comfrey root and allantoin are shown in Table 3.

Table 3. The microbial activity of allantoin and the aqueous extract of comfrey root

Microorganism	Tested sample				Positive control				Negative control	
	Allantoin (c_0 = 10 mg/mL)		Aqueous extract of comfrey (c_0 = 40 mg/mL)		Doxycycline µg/mL		Nystatin µg/mL		Sterilised aqua destilata	
	MIC	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC/MFC*
Bacterial strains										
B. subtilis (ATCC 6633)	–	–	–	–	1.56	1.56	nt[a]	nt[a]	na[b]	na[b]
E. coli (ATCC 8739)	–	–	10	40	0.78	0.78	nt[a]	nt[a]	na[b]	na[b]
P. aeruginosa (ATCC 27857)	–	–	–	–	12.5	12.5	nt[a]	nt[a]	na[b]	na[b]
S. typhimurium (ATCC 14028)	–	–	10	20	6.25	<50.0	nt[a]	nt[a]	na[b]	na[b]
S. aureus (ATCC 6538)	–	–	–	–	6.25	0.78	nt[a]	nt[a]	na[b]	na[b]
Fungi										
A. niger (ATCC 16404)	–	–	–	–	nt[1]	nt[a]	0.78	0.78	na[b]	na[b]
C. albicans (ATCC 10231)	–	–	–	–	nt[1]	nt[a]	6.25	6.25	na[b]	na[b]

[a]Standard reference of the sample that is not tested; [b]standard reference of the tested sample that did not show activity

Based on the obtained results, it can be concluded that *Escherichia coli* ATCC8739 and *Salmonela typhimirium* ATCC6538 were the most sensitive to the aqueous extract of comfrey root but, on the other hand, the aqueous extract of comfrey root did not show the activity on the investigated fungi strains. Doxycycline, a commercial antibiotic which was used as the control sample showed the antimicrobial activity on all the investigated bacteria species. The results of the microbial activity of allantoin showed that allantoin did not express the antimicrobial activity on all the investigated bacteria species. Based on this, it can be concluded that allantoin is not responsible for the antimicrobial activity of the aqueous extract of comfrey root.

CONCLUSIONS

By using a new UHPLC–DAD–HESI–MS method 12 compounds were separated and identified in the aqueous extract of comfrey root. The highest content of the identified components was determined for rosmarinic acid (12.8%), allantoin (8.91%) and ellagic acid (7.4%). The aqueous extract of comfrey root showed the best microbial activity against *Escherichia coli* ATCC8739 and *Salmonela typhimirium* ATCC6538, while the standard of allantoin did not show activity against any of the examined microbe strains. This indicates that allantoin is not responsible for the antimicrobial activity of the aqueous extract of comfrey root. The obtained results could be beneficial for the potential application of the aqueous extract of comfrey root as a source of bioactive components in pharmaceutical and cosmetic industry.

Acknowledgements

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IZVOD

IDENTIFIKACIJA I KVANTIFIKACIJA BIOAKTIVNIH JEDINJENJA IZ VODENOG EKSTRAKTA KORENA GAVEZA POMOĆU UHPLC–DAD–HESI–MS METODE I NJEGOVA MIKROBIOLOŠKA AKTIVNOST

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(Naučni rad)

U ovom radu ispitivan je kvalitativni i kvantitativni sastav vodenog ekstrakta iz korena gaveza (*Symphytum officinale* L.) dobijen metodom po Soxhletu. Pored kvalitativne i kvantitativne analize ispitivana je i upoređena antimikrobna aktivnost alantoina i vodenog ekstrakta korena gaveza. Primenom nove UHPLC–DAD–HESI–MS metode identifikovano je 12 jedinjenja. Visok sadržaj alantoina, elaginske kiseline i ruzmarinske kiseline (8.91, 7.4 i 12.8%, redom) u vodenom ekstraktu korena gaveza ukazuje na činjenicu da se koren gaveza može koristiti kao izvor za njihovo dobijanje. Prisustvo bioaktivnih jedinjenja u vodenom ekstraktu korena gaveza dalo je dobru osnovu za ispitivanje i mikrobiološke aktivnosti. Rezultati ispitivanja antimikrobne aktivnosti su pokazali da su *Escherichia coli* ATCC 8739 i *Salmonela typhimirium* ATCC 6538 najviše osetljive na vodeni ekstrakt iz korena gaveza. Međutim, s druge strane vodeni ekstrakt iz korena gaveza nije pokazao mirobiološku aktivnost na ispitivane sojeve gljivica. Pored vodenog ekstrakta korena gaveza ispitivana je i mikrobiološka aktivnost alantoina, kao važnog konstituenta korena gaveza. Iz dobijenih rezultata se vidi da alantoin nije pokazao aktivnost na ispitivane sojeve mikroba. Na osnovu dobijenih rezultata može se zaključiti da alantoin nije odgovoran za mikrobiološku aktivnost ekstrakta korena gaveza.

Ključne reči: Koren gaveza • *Symphytum officinale* L. • UHPLC–DAD–HESI–MS • Mikrobiološka aktivnost

The influence of Schiff base inclusion complexes with β-cyclodextrine on antibiotic production by *Streptomyces hygroscopicus* CH-7

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Abstract

A media consisting of isatin-Schiff bases and its inclusion complexes with β-cyclodextrine was developed to maximize the production of antibiotics Hexaene H-85 and azalomycine B by *Streptomyces hygroscopicus* CH-7. The media with β-cyclodextrine inclusion complex of isatin-3-thiosemicarbazone resulted in the maximum antibiotics concentration of 493 μg/cm^3 for Hexaene H-85 and 191 μg/cm^3 for azalomycine B. The production of hexaene H-85 and azalomycine is higher when β-cyclodextrine complex is added as a nitrogen source, comparing to pure isatin-Schiff base. The maximum concentration of hexaene H-85 in medium with inclusion complex of isatin-Schiff base is 1.4-2.3 times higher than the basal medium. The maximum production of azalomycine is 2.1-3.4 times higher in media with inclusion complex of Schiff base. During the fermentation process, the nutrient media with β-cyclodextrine inclusion complexes with isatin-Schiff bases affect the strain morphology, since it is in the form of compact pellets, which are formed from short and long, branched filaments.

Keywords: *Streptomyces hygroscopicus*, Schiff base, β-cyclodextrine, inclusion complex, antibiotic production.

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Actinomycetes, especially *Streptomyces* bacteria are extremely important group of microorganisms because of their ability to produce commercially important secondary metabolites [1,2]. Actinomycetes name comes from the Greek word "aktis" which means air and "mikes" which means mushroom. The smell of soil that occurs immediately after the rain just comes from actinomycetes, precisely from geosmin, streptomycetes metabolite [3].

Most antibacterial and antifungal agents, now in clinical use, were found during the "golden era" of antibiotics in forties and sixties of 20th century, by isolation and screening of soil fungi and actinomycetes. During this period, nearly 12,000 of bioactive secondary metabolites were discovered, of which 160 is used for clinical purposes such as natural, as well as semisynthetics products. 55% of discovered secondary metabolites were produced by the genus *Streptomyces*, 11% from other actinomycetes, 12% of non-filamentous bacteria and 22% of filamentous fungi [4].

Penicillins, cephalosporins, tetracyclines, aminoglycosides, glycopeptides, macrolides and polyenes discovered in time of the "golden era" still represent very important antibiotics against bacterial and fungal infections. Between sixties and eighties of 20th century, the pharmaceutical efforts have been directed towards improving existing chemo types, increasing potency, stability and pharmacokinetics.

However, despite significant advances in technology and the development of methods and instruments in the field of microbiological screening, the last twenty years have been without significant breakthroughs in terms of antibiotics. Actually, a small number of new drugs act selectively and often do not penetrate the microbial pathogen cell, or even deactivated *in vivo*. As a matter of fact, over the past few decades, antibacterial lipopeptides – daptomycins produced by *Streptomyces roseosporus* (active against Gram-positive bacteria that cause skin infections) and cyclic lipopeptides – echinocandins and pneumocandins (which inhibit the biosynthesis of fungal cell walls) can be considered significant [4].

Great ability of *Streptomyces* to produce antibiotics makes streptomycetes interesting for study, but old methods of screening these organisms should be modernized and some new should be introduced. Isolation must be made by, at least, unusual habitats, assuming that unknown types of endophytes, extremephiles or marine streptomycetes can produce new chemotypes.

Among them, abyssomicin C presents a new polycyclic and polyketide antibiotic produced by a marine strain *Verrucosispora* and it is very active against Gram-positive bacteria [5]. Diazepinomicin is a unique dibenzodiazepinon produced by a strain of *Micromonospora*

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[6] known for its antibacterial, antiinflammatory and antitumor activities. Salinosporamide A is a new β-lactone-γ-lactam isolated from the fermentation broth of marine actinomycetes *Salinispora tropical* [7].

It is also possible to reinvestigate antibiotics which activity is yet unknown. For example, early discovered antibiotic plantensimycin produced by *Streptomyces plantesis* is found to be an inhibitor of biosynthesis of certain fatty acids. Additional strategies in the discovery of new metabolites could be the study of genomes and detection of cryptic genes encoding secondary metabolism. Genome sequences of *Streptomyces coelicolor*, *S. avermitilis*, *S. griseus* and *Saccharopolyspora* showed that a large number of biosynthetic gene clusters contain all genes for the production, regulation, transportation and autorezistance of secondary metabolites [4].

Although this selective isolation method is so far allowed for the isolation of a large number of rare actinomycetes, the main problem still is difficult cultivation of rare actinomycetes and complicated genetic engineering [4].

The species *Streptomyces hygroscopicus* produces a various polyene antibiotics depending on environmental and nutritional conditions [8,9]. The production of antibiotics is usually affected by different parameters, such as chemically defined media. Species of the genus *Streptomyces* produce antibiotics and growth on substrates with different sources of carbon and nitrogen [10–12]. An increase of the yield of antibiotics can be achieved at an early stage of fermentation conditions and changes in the composition of culture medium [13,14]. In the present work, an extensive study has been made on the isatin-Schiff bases and their β-cyclodextrine inclusion complexes as a nitrogen source in chemically defined media for antibiotic production by *S. hygroscopicus*, as well as, on soil morphology.

MATERIALS AND METHODS

Bacterial strain, media and growth condition

A strain *Streptomyces hygroscopicus* CH-7 (NCAIM (P) B-001336) was gained from Microbial collection at Faculty of Chemistry and Institute of Chemistry, Technology and Metalurgy in Belgrade, Serbia [3,4]. *S. hygroscopicus* was maintained as spore and mycelia suspensions in sterile glycerol (20% *w*/*V*), which were prepared according to our previous work [13,14]. Suspensions were stored at –20 °C until required. The fermentation media were inoculated with 5% (*V*/*V*) of a preculture after 48 h growth and incubated at 30 °C for 240 h under the standard condition of aeration and agitation (200 rpm). The fermentation basal media has the following composition: glycerol 15 g/dm^3, $CaCO_3$ 3 g/dm^3, NaCl 3 g/dm^3, $MgSO_4$ 0.5 g/dm^3, $(NH_4)_2HPO_4$ 0.5 g/dm^3, K_2HPO_4 0.5 g/dm^3 and soya bean 10 g/dm^3. The fermentation modified media has the follow composition: glycerol 15 g/dm^3, $CaCO_3$ 3 g/dm^3, NaCl 3 g/dm^3, $MgSO_4$ 0.5 g/dm^3, $(NH_4)_2HPO_4$ 0.5 g/dm^3, K_2HPO_4 0.5 g/dm^3 and inclusion complex 10 g/dm^3.

Determination of antibiotics

Antibiotics were measured by Perkin-Elmer Lambda 15 UV/Vis spectrophotometer.

Determination of hexaene H-85

Hexaene H-85 was measured spectrophotometrically at λ = 364 nm. The mixture of 0.5 cm^3 fermentation broth and 2.0 cm^3 of 1-butanol was stirred and centrifugated. Absorbance (*A*) of 1-butanol extract was measured with 1-butanol as a control probe. The calculation was done by using the following equation:

$$\gamma(\mu g/cm^3) = 66.7A_{364}$$

where 66.7 is experimental calculated extinction coefficient for hexaene H-85 which is $E_{1cm}^{1\%} = 600$ [9].

Determination of azalomycine B

Azalomycine B was measured spectrophotometrically at λ = 252 nm. The mixture of 0.5 cm^3 fermentation broth and 2.0 cm^3 of ethyl acetate was stirred and centrifugated. Absorbance of ethyl acetate extract was measured with ethylacetate as a control probe. The calculation was done by using the following equation:

$$\gamma(\mu g/cm^3) = 25.3A_{252}$$

where 25.3 is experimental calculated extinction coefficient for azalomycine B which is $E_{1cm}^{1\%} = 790$ [15].

Growth was determined by measuring dry weights of cells. The broth was centrifuged at 4000 rpm for 15 min to separate the mycelial biomass. After that biomass was dried at 105 °C to constant weight and weighed.

General procedure for isatin-Schiff base

Equimolar amounts of isatin and thiosemicarbazide, semicarbazide and phenylhydrazine were dissolved in 95% ethanol. The solutions were heated under reflux for about 50 min [16,17].

General procedure for β-cyclodextrine inclusion complexes

Equimolar amounts of Schiff base and β-cyclodextrine (Merck, Dermstadt) were dissolved in 150 cm^3 distilled water. The solutions were stirred at room temperature for 24 h, concentrated at 50 °C to about 20 cm^3 and dried in vacuum over concentrated H_2SO_4.

Methods

The melting points were determined by using Thomas-Hoover melting point apparatus and are uncor-

rected. FTIR spectra were recorded using a Michaelson Bomen MB-series spectrophotometer, using KBr pellet (1 mg/100 mg) technique. The electronic spectra were recorded on a Perkin/Elmer Lambda 15 UV/Vis spectrophotometer using 10^{-3} mol/dm^3 solutions in DMF. The results of identification of Schiff bases are given in previous work [13].

RESULTS AND DISCUSSION

Schiff bases possess high biological activity, which is greater if their synthesis have been performed by using active component. Schiff bases have antibacterial, antifungal, antimicrobial, anti-HIV, anti-inflammatory and antitumor activity [16,17]. Some of them are natural analogues of amino acids, such as isatin-3-thiosemicarbazone analogue of tryptophan, so the positive effect of these compounds on the production of antibiotics is quite expected.

The identification and characterization of synthesized of β-cyclodextrine inclusion complexes with isatin-Schiff base were done by using standard analytical methods.

Inclusion complex of β-cyclodextrine and isatin-3-thiosemicarbazone (β-CD+ITC)

Yield, 85.9%, m.p. 134 °C, Color: pale yellow. IR (KBr, 1/cm): 1667 ν(C=O), 1585 ν(C=N), 1238 ν(C=S). UV/Vis (DMF, λ(nm/$\varepsilon\times10^3$ (dm^3·cm/mol): 200/3.784 π→π*, 250.0/1.187 π→π*, 348.4/0.813 π→π*.

Inclusion complex of β-cyclodextrine and isatin-3--semicarbazone (β-CD+ISC)

Yield, 80.9%, m.p. 131 °C, Color: pale yellow. IR (KBr, 1/cm): 1690, 1668 ν(C=O). UV/Vis (DMF, λ(nm/ /$\varepsilon\times10^3$ (dm^3·cm/mol): 195,0/4,286 π→π*, 220/4.776 π→π*, 265/3.791 π→π*, 330/2.988 π→π*.

Inclusion complex of β-cyclodextrin and isatin-3-phenylhydrazone (β-CD+IPH)

Yield, 45.54%, m.p. 136 °C, Color: pale yellow. IR (KBr, 1/cm): 1666 ν(C=O). UV/Vis (DMF, λ(nm/$\varepsilon\times10^3$ (dm^3·cm/mol):195/3.125 π→π*, 250/0.621 π→π*, 300/0.349 π→π*, 385/0.212 π→π*.

Schiff base as a source of nitrogen in a nutrition media has a significant impact on the production of antibiotics [13].

Isatin-3-thiosemicarbazone, isatin-3-semicarbazone, and isatin-3-phenylhydrazone significantly affect production of hexaene H-85 and azalomycine B using strains of *S.hygroscopicus* CH-7.

β-Cyclodextrine has a significant effect on the production of antibiotics by some microorganisms. Addition of this compound in the substrate has a stimulating effect on the production of lankacidin (macrocyclic antibiotic groups) using a microorganism *Streptomyces roche* var.*volubilis*, and does not increase microbial growth and is not used as a carbon source [18,19].

The production of hexaene H-85 and azalomycine B by *S. hygroscopicus* CH-7 was observed when the soya bean (10 g/dm^3) in basal medium was replaced with inclusion complexes of β-cyclodextrine with isatin Schiff bases (10 g/dm^3) as a nitrogen source. The maximum concentrations of hexaene H-85 and azalomycine B and dry biomass, achieved during the fermentation are given in Table 1.

Maximum microbial growth on all substrates is achieved from the second to the fourth day of fermentation (Figure 1b). Maximum concentrations of dry biomass on substrates with inclusion complexes of Schiff bases and pure β-cyclodextrine were lower than in the basic medium.

The pH values in all modified media achieved maximum at 3rd day of fermentation (Figure 1a). As it can be seen (Table 1), the maximum concentration of dry biomass (Figure 1b) is reached by 4th day of fermentation in all media. The best result among the tested β-cyclodextrine inclusion complexes is obtained for the medium with inclusion complex of ITC (8.7 g/dm^3). The maximum concentration of dry biomass in basal medium is reached by 3th day and its value is 8.9 g/dm^3.

Production of hexaene H-85

The addition of β-cyclodextrine inclusion complexes with Schiff bases has the significant influence on the production of hexaene H-85. Maximum concentration of antibiotic is reached by 2th day in basal medium and by 2th (pure β-cyclodextrine and inclusion complex of

Table 1. The impact of inclusion complexes on maximum concentration of dry biomass (X_{max}) and maximum production (C_{max}) and yield of antibiotics (Y_{max}) during the fermentation of S. hygroscopicus CH-7

Nitrogen source	X_{max} g/dm^3	Hexaene H-85		Azalomycine B	
		C^H_{max} μg/cm^3	Y^H_{max} μg/g$_{s.b}$	C^A_{max} μg/cm^3	Y^A_{max} μg/g$_{s.b}$
Soya bean	8.9	212	23.82	56	6.29
ITC+β-CD complex	8.7	493	56.66	191	21.95
ISC β-CD complex	8.4	314	37.38	123	14.64
IPH β-CD (complex)	8.0	417	52.12	136	17.00
β-CD (1%)	8.2	272	33.17	102	12.43

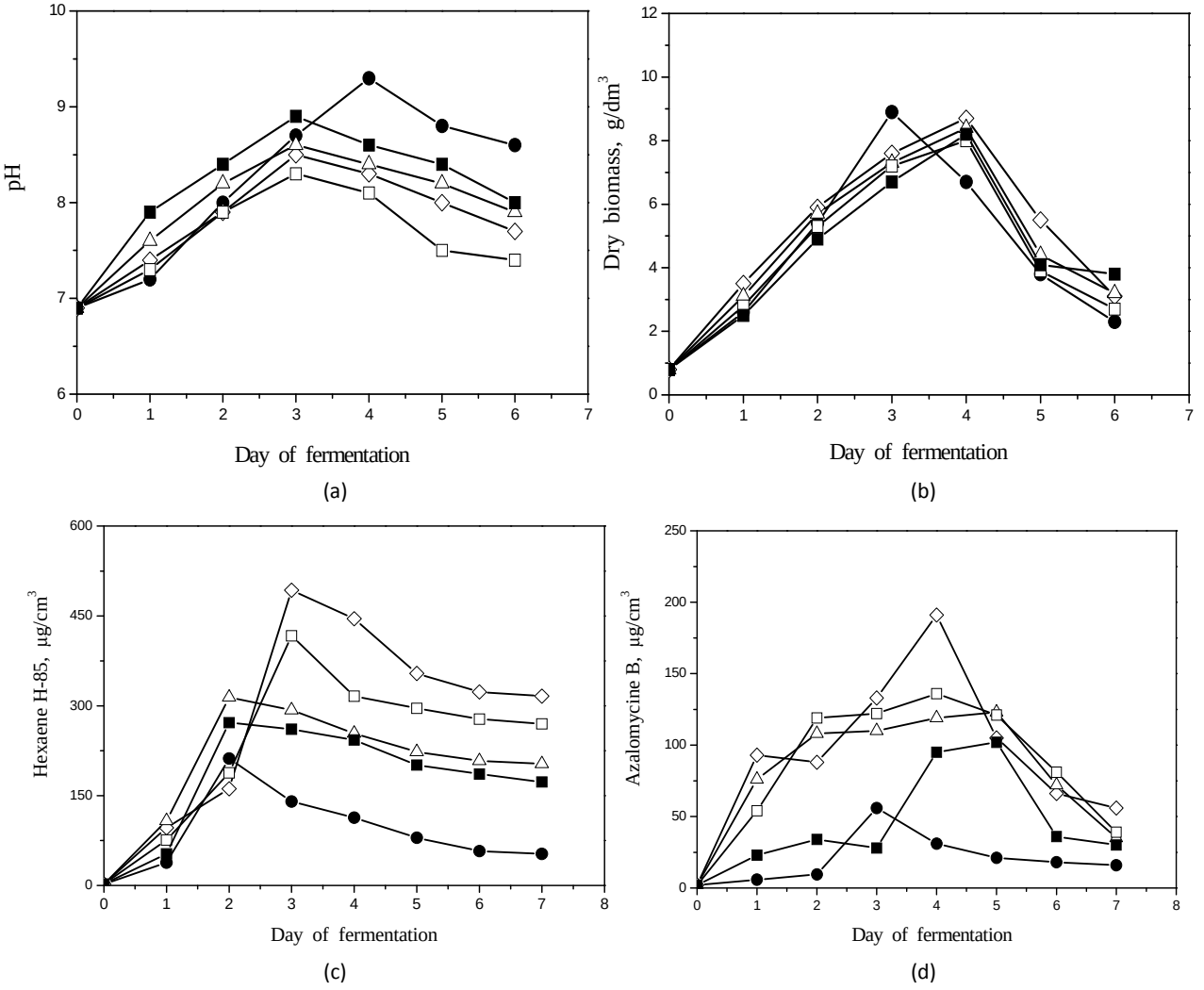


Figure 1. The change of pH (a), concentration of dry biomass (b), concentration of hexaene H-85 (c) and azalomycine B (d) in basal medium (-●-), in medium with pure β-cyclodextrine (-■-) and media with β-cyclodextrine inclusion complexes with: ITC (-◊-), ISC (-Δ-) and IPH (-□-).

ISC) and 3th (inclusion complex of ITC and IPH) day in modified media (Figure 1).

The maximum concentration of hexaene H-85 in medium with inclusion complex of ITC, ISC and IPH is 2.3, 1.4 and 1.9 times higher than the basal medium (212 μg/cm^3). It can be seen (Table 1), that the concentration of hexaene H-85 in medium with pure β-cyclodextrine is also 1.2 times higher than in the basal medium.

Production of azalomycine B

The production of Azalomycine B is also stimulated in media with β-cyclodextrine inclusion complexes with Schiff bases. Maximum concentration of antibiotic is reached by 3th day in basal medium and 4th (inclusion complex of ITC and IPH) and 5th (pure β-cyclodextrine and inclusion complex of ISC) day in modified media (Figure 1).

The maximum production of antibiotic is 56 μg/cm^3 in basal medium. The values are 3.4, 2.1, 2.4 and 1.8 times higher in media with inclusion complex of ITC, ISC, IPH and pure β-cyclodextrine, respectively (Table 1).

Comparing to the results with Schiff base [13] the production of hexaene H-85 and azalomycine B is higher when β-cyclodextrine complex with Schiff base is added as a nitrogen source (Table 2). The mechanism of action of tested compounds was not examined. Isatin-Schiff base was used as a nitrogen source due to their similarity with L-tryptophan. This amino acid was already used as a nitrogen source in a basal medium [13]. Since it has a structural similarity with used isatin-Schiff base (an indole moiety), it is obviously why this class of compounds was used for media modification. Those results are connected with fact that inclusion of Schiff bases with β-cyclodextrine increases their solubility, which has a positive effect on their assimilation by *S. hygroscopicus* CH-7 from media.

Table 2. Maximum production (C_{max}) and yield of antibiotics (Y_{max}) during the fermentation of S. hygroscopicus *CH-7 with Schiff base [13] and inclusion complexes with* β*-cyclodextrine*

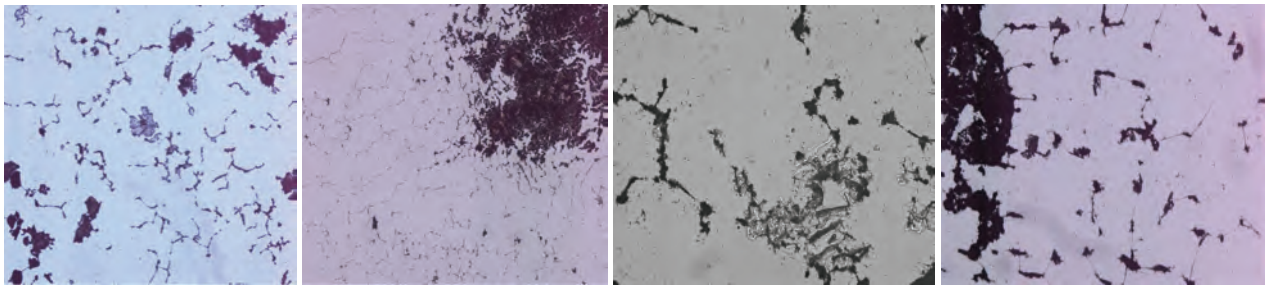
Nitrogen source	Hexaene H-85		Azalomycine B	
	C^H_{max} μg/cm^3	Y^H_{max} μg/g$_{s.b}$	C^A_{max} μg/cm^3	Y^A_{max} μg/g$_{s.b}$
ITC [13]	372	38.75	118	12.29
ITC+β-CD complex	493	56.66	191	21.95
ISC [13]	293	31.50	92	9.89
ISC +β-CD complex	314	37.38	123	14.64
IPH [13]	329	36.15	106	11.64
IPH+β-CD (complex)	417	52.12	136	17.00

The impact of β-cyclodextrine inclusion complexes with isatin-Schiff bases on *S. hygroscopicus* CH-7 morphology

Depending on fermentation conditions, *Streptomyces* can grow like single filaments, like a branched filaments or small pellets. Small and single pellets favourite the production of secondary metabolites [20]. The production of antibiotics by filamentous microorganisms sometimes depends upon shapes, size and cell branched filaments, which is similar to the pellets (Table 3). Comparing to Schiff bases [13] the morphology of strain has small differences, since they are in the form of pellets and single, free filaments. The highest yield of hexaene H-85 and azalomycine B was achieved in media with small, dispersive pellets and single filaments. The morphology of *Streptomyces hygroscopicus* CH-7 is shown in Figure 2.

Table 3. The impact of β-cyclodextrine inclusion complexes with isatin-Schiff bases on morphology S. hygroscopicus and production of antibiotics

Nitrogen source	The strain morphology	Yield of antibiotics	
		Y^H_{max} μg/g$_{s.b}$	Y^A_{max} μg/g$_{s.b}$
β-CD (1%)	Clear, compact pellets, small and short fillamnets	33.17	12.43
ITC+β-CD complex	Compact pellets, short, weakly branched fillaments	56.66	21.95
ISC β-CD complex	Large, compact pellets, long, branched fillaments	37.38	14.64
IPH β-CD (complex)	Pellets of short single and branched fillaments	52.12	17.00



(a) (b) (c) (d)

Figure 2. The morphology of S. hygroscopicus in medium with β-cyclodextrine inclusion complexes with isatin-Schiff bases. a) β-Cyclodextrine, b) β-cyclodextrine inclusion complexes with ITC, c) β-cyclodextrine inclusion complexes with ISC and d) β-cyclodextrine inclusion complexes with IPH.

aggregation.

During the fermentation, the nutrient media with β-cyclodextrine inclusion complexes with isatin-Schiff bases affects the growth of *S. hygroscopicus* CH-7. The strain in basal medium is in the form of large and single pellets [13]. The strain in modified media is in the form of compact pellets, which formed from short and long,

CONCLUSION

The antibiotic production by *Streptomyces hygroscopicus* CH-7 is improved by replacing the soya bean as a nitrogen source with β-cyclodextrine inclusion complexes with isatin-Schiff base. The media with β-cyclodextrine inclusion complex of isatin-3-thiosemi-

carbazone resulted in the maximum antibiotics concentration of 493 μg/cm^3 for hexaene H-85 and 191 μg/cm^3 for azalomycine B. The production of hexaene H-85 and azalomycine is higher when β-cyclodextrine complex is added as a nitrogen source, comparing to pure isatin-Schiff base. The maximum concentration of hexaene H-85 in medium with inclusion complex of isatin-Schiff base is 1.4–2.3 times higher than the basal medium. The maximum production of azalomycine is 2.1–3.4 times higher in media with inclusion complex of Schiff base. Those inclusion complexes also affect the growth of strain, which is in the form of compact pellets, which formed from short and long, branched filaments. Comparing to Schiff bases the morphology of strain has small differences, since they are in the form of pellets and single, free filaments. Thus, the future experiments will allow the replacement of commercial nitrogen sources with compounds such as inclusion complexes of different Schiff base.

Acknowledgments

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IZVOD

UTICAJ KOMPLEKSA ŠIFOVIH BAZA SA β-CIKLODEKSTRINOM NA PRODUKCIJU ANTIBIOTIKA POMOĆU *Streptomyces hygroscopicus* CH-7

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(Naučni rad)

Streptomyces hygroscopicus je soj poznat po produkciji različitih vrsta antibiotika i komercijalno važnih sekundarnih metabolita. Formiranje metabolita je rezultat mikrobioloških procesa u toku fermentacije i zavisi od nivoa biomase mikroorganizma, morfološkog profila kulture, kao i od ekoloških uslova. U cilju povećanja prinosa antibiotika, osnovna hranljiva podloga je redefinisana izmenom izvora azota, tako što su umesto sojinog brašna (10 g/dm^3) dodati kompleksi Šifovih baza sa β-ciklodekstrinom (10 g/dm^3). Dodatak kompleksa Šifovih baza sa β-ciklodekstrinom rezultirao je znatno povećanje koncentracije oba antibiotika. Maksimalna koncentracija heksaena H-85 je ostvarena na podlozi sa kompleksom izatin-3-tiosemikarbazona sa β-ciklodekstrinom i iznosila je 493 μg/cm^3, dok je za azalomicin B iznosila 191 μg/cm^3. Povećanje maksimalnih koncentracija antibiotika na podlogama sa kompleksima Schiff-ovih baza je iznosilo 1,4–2,3 puta za heksaen H-85, odnosno 2,1–3,4 puta za azalomicin B, u odnosu na osnovnu podlogu. U toku procesa fermentacije na hranljivim podlogama sa kompleksima Šifovih baza sa β-ciklodekstrinom, soj raste u obliku kompaktnih peleta, koji se formiraju od kratkih i dugih, razgranatih filamenata.

Ključne reči: *Streptomyces hygroscopicus* • Šifove baze • β-Ciklodekstrin • Inkluzioni kompleksi • Produkcija antibiotika

Fuzzy model for determination and assessment of groundwater quality in the city of Zrenjanin, Serbia

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Abstract

The application of the fuzzy logic for determination and assessment of the chemical quality of groundwater for drinking purposes in the city of Zrenjanin is presented in this paper. The degree of certainty and uncertainties is one of the problems in the most commonly used methods for assessing the water quality. Fuzzy logic can successfully handle these problems. Evaluation of fuzzy model was carried out on the samples from two representative wells that are located at depths of two aquifers from which water is taken to supply the population with drinking water. The samples were analyzed at 8 different chemical water quality parameters. In the research, the arsenic concentration (As^{3+} and As^{5+}) is considered as the dominant parameter due to its suspecting carcinogenic effects on human health. This type of research is for the first time conducted in the city of Zrenjanin, middle Banat region.

Keywords: groundwater quality, fuzzy logic, degree of certainty, arsenic.

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The availability and quality of groundwater as well as drinking water will be the main environmental and social issues in the future. Drinking water quality is currently defined only as the absence or presence of certain strictly limited undesirable substances. Water quality monitoring and quality decision-making based on the obtained data is a complex and multidimensional task for decision makers. The main reason of such heavy and chalanging work are uncertainties that occur in all steps, from sampling to analysis. The sets of the monitored data and limits should not be as crisp set, but as fuzzy sets [1].

In modeling complex of environmental problems, researchers often fail to define precise statements about input and outcomes of pollutants, but fuzzy logic could help to overcome these logical uncertainties. Fuzzy logic can be considered as a language that allows one to translate sophisticated statements from natural language into a mathematical formalism. Fuzzy logic can deal with highly variable, linguistic, vague and uncertain data or knowledge and therefore has the ability to allow a logical, reliable and transparent information stream from data collection to data usage in environmental application system. Fuzzy logic provides a framework to model uncertainty, the human way of thinking, reasoning and perception process [2]. The results on water quality obtained using the index developed on the basis of fuzzy set theory were found to be more useful than those derived from the Water Quality Index method that is currently used [3].

The assessment of the groundwater quality (GWQ) used as drinking water in the town of Zrenjanin, Vojvodina region, Serbia has been carried out by the flexibility of fuzzy set theory in decision-making through the environment assessment.

Problems with the water supply of the city of Zrenjanin are not solved through decades, therefore the population of the city of Zrenjanin has been long-term exposed to health risk and safering. In early 2004, Provincial Sanitary Inspection banned for drinking and cooking water from Zrenjanin due to multiple exceeded concentrations of maximum allowable concentration (MAC) of arsenic [4]. The ban is still in force. The groundwater is only chlorinated and without any purification process distributed to consumers. Drinking water is organoleptically not acceptable, which means that it has no appropriate phisical parameter as the colour, smell and taste. Application of fuzzy methodology to the selected chemical parameters of the quality of groundwater/drinking water is for the first time evaluated in Zrenjanin city and nearby surrounding.

MATERIALS AND METHODS

Fuzzy set theory

Fuzzy set theory is a generalization of classical set theory, since the elements membership to the fuzzy set may be characterized as a number in the interval [0,1].

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Fuzzy sets are the basic elements that describe the uncertainty. Fuzzy set theory allows the integration of information from different parameters in the processes of modeling and evaluation. A fuzzy set is defined in terms of a membership function (MF) which maps the domain of interest, *e.g.*, concentrations, onto the interval [0,1] [5]. The MF of the fuzzy set A defined over a domain *X* takes the form:[6]:

$$\mu_{\mathrm{A}} : \mathrm{X} \rightarrow [0,1] \tag{1}$$

Therefore, the fuzzy set A is defined by its MF:

$$\mu_A(x) = \{(\mu_A(x)), x \in X, \mu_A(x) \in [0,1]\} \tag{2}$$

In fuzzy water quality assessment the most often are used trapezoidal MFs. Trapezoidal MF achieves the highest classification accuracy in water quality assessment. Trapezoidal are preferable than triangular, due to certain water quality parameters cannot be determine, only one specific value with maximum MF as desirable is acceptable or not acceptable. These values should be included in certain intervals. In that way, trapezoidal MFs are characterizing the dynamic behavior of the water quality parameters. Changing the type of MF will affect the final output of fuzzy model, because the GWQ parameters would be assigned different MFs that were obtained by different formulas.

Fuzzy MFs are to be trapezoidal for all 8 analyzed parameters in this research due to nature of the parameters. Functions are created according to the allowable limits under current legislation and according to the studies and recommendations of the World Health Organization (WHO) and the perception of the authors (Table 1).

Equation (3) fuzzifies the data in terms of trapezoidal MF [6]:

$$\mu_A(x) = \begin{cases} 0, x < \alpha \\ \dfrac{x-\alpha}{\beta-\alpha}, \alpha \le x \le \beta \\ 1, \beta \le x \le \gamma \\ \dfrac{\delta-x}{\delta-\gamma}, \gamma \le x \le \delta \\ 0, x > \delta \end{cases} \tag{3}$$

For a trapezoidal MF, α is the minimum value, δ is the maximum value and β and γ are the two values which represent the interval of the most likely value.

Zadeh [9] proposed the following definitions for the operations on the fuzzy sets:

1. The union of two fuzzy sets A and B is fuzzy set C with its MF:

$$\mu_C(x) = \mu_A(x) \wedge \mu_B(x) = \max\{\mu_A(x), \mu_B(x)\} \tag{4}$$

2. The intersection of two fuzzy sets A and B is fuzzy set C with its MF:

$$\mu_C(x) = \mu_A(x) \vee \mu_B(x) = \min\{\mu_A(x), \mu_B(x)\} \tag{5}$$

3. The complement of a fuzzy set A can be defined by MF $\mu_C(x)$:

$$\mu_C(x) = 1 - \mu_A(x) \tag{6}$$

Fuzzy rule evaluation

Fuzzy rules are standard mathematical rules in the form: if X = A, then Y = B (A $\rightarrow$ B), where *x* and *y* are linguistic variables, while A and B are linguistic values defined on the universal sets X and Y. "X is A" is called the antecedent, assumption, premise, fact. "Y is B" is called a consequence, conclusion, implication.

In accordance with the fuzzy rules the classification of the groundwater is consequent to the system of fuzzy rules. Each rule consists of a set of antecedent statements, which are the names of properties (pH, $KMnO_4$ consumption, arsenic,...) and values of characteristics, *i.e.*, linguistic descriptions (desirable, acceptable and not acceptable). Linguistic descriptions are assigned according to the information about the health implications of each parameter on the human health.

Fuzzy logic is based on the expert's knowledge for developing a sopisticated system; the experts' knowledge is used together with the other parameters through the rules defined in a fuzzy inference system to create a system based on the knowledge captured [10].

Mamdani fuzzy reasoning

All of the parameters in the research will be connected with the operator "Λ" – AND operator. Applying

Table 1. The prescribed limits [4,7,8]

Parameter	Unit	Limits by current legislation	WHO Guideline values
pH	–	6.8–8.5	6.5–9.2
$KMnO_4$ consumption	mg/l	8	1–19
Ammonia (NH_4^+)	mg/l	1	1.5
Total iron (Fe^{2+},Fe^{3+})	mg/l	0.3	0.3–1
Arsenic (As^{3+},As^{5+})	mg/l	0.01	0.001–0.01
Calcium (Ca^{2+})	mg/l	200	75–200
Magnesium(Mg^{2+})	mg/l	50	50–100
Sodium (Na^+)	mg/l	150	200

a fuzzy "Λ" operation will yield a result that is the minimum of the fuzzy value of the number of input variables. The aggregation of the rule will be the truncation of the output fuzzy set. This method is applied to all rules to obtain the final result which gives the final shape of the output fuzzy membership function after aggregation of all the rules, respectively. Then the union operation is applied to all the output fuzzy sets to yield the final fuzzy set [11]. For example: "*IF ammonia is Unacceptable Λ iron is Desirable THEN the GWQ is Acceptable*". This type of fuzzy system reasoning is called Mamdani implication of max.min operator. Mamdani system assumes that the output of the process of reasoning is fuzzy set (Fig. 1). This fuzzy set requires aggregation in the process of defuzzification. The use of fuzzy numbers and aggregation of fuzzy sets are proposed as a suitable technique for handling the uncertainties in decision-making on environmental quality criteria [12].

Deffuzification

Defuzzification is the last step in the process of fuzzy reasoning. Defuzzification is the process of transforming the results obtained in the form of fuzzy set to the numeric value. In this study two defuzzification methods have been used: mean of maxima (MOM) and center of area (COA) or centroid method.

MOM method is given by the expression [13–15]:

$$z^* = \frac{a+b}{2} \tag{7}$$

where *a* and *b* are as defined in Fig. 2.

COA procedure is the most prevalent and physically appealing of all the defuzzification methods [13–15] and it is given by the algebraic expression:

$$z^* = \frac{\int \mu_C(x) z \mathrm{d}z}{\int \mu_C(x) \mathrm{d}z} \tag{8}$$

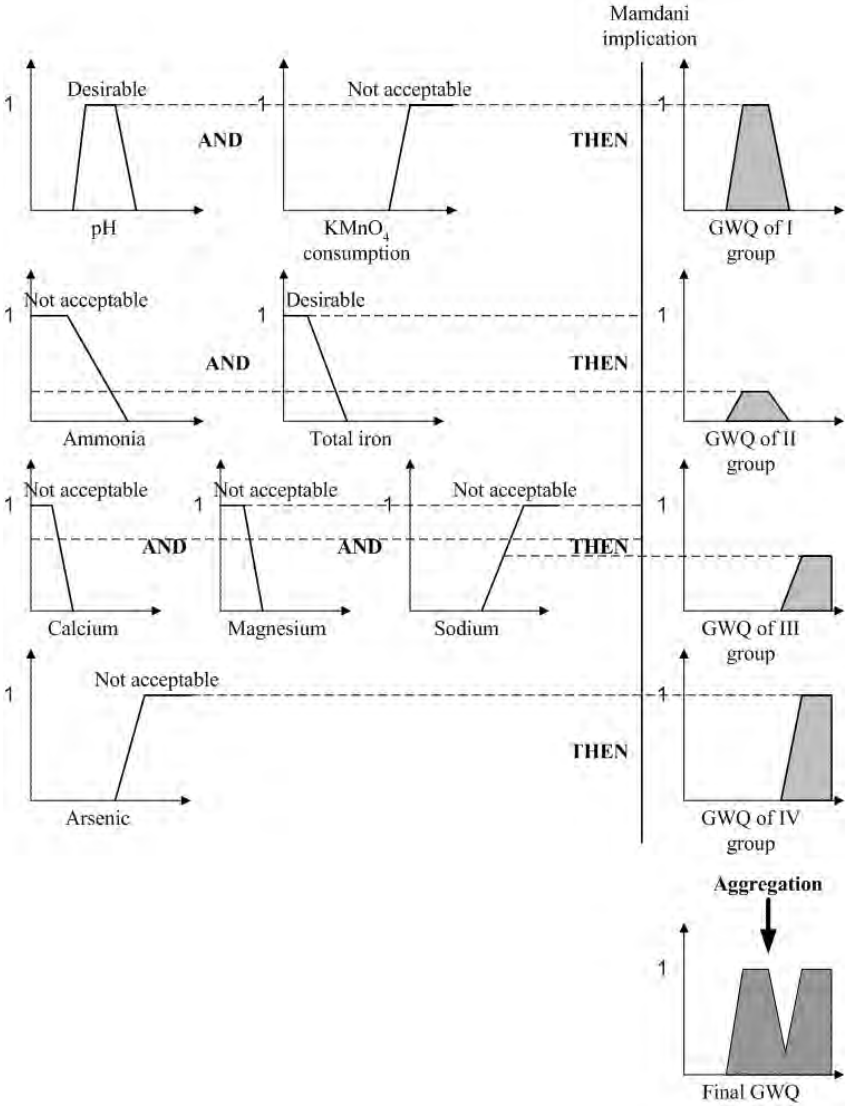

Figure 1. Graphical representation of Mamdani implication.

where $\int$ denotes an algebraic integration. This method is shown in Fig. 3.

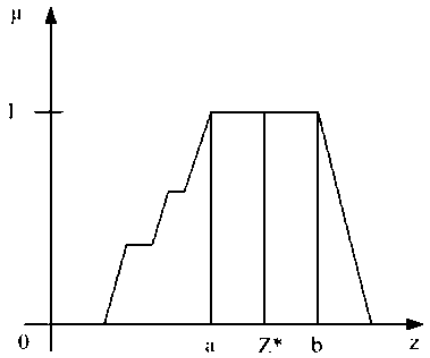

Figure 2. MOM defuzzification method.

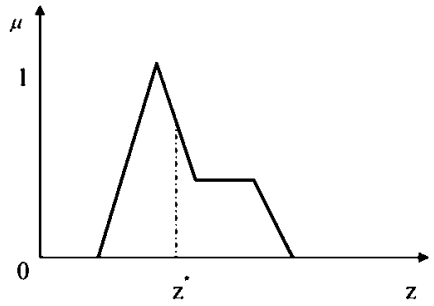

Figure 3. COA defuzzification method.

RESULTS AND DISCUSSION

Study area

City of Zrenjanin is situated on the western edge of the region Banat, in the province of Vojvodina, at 20°23' east longitude and 45°23' north latitude. Most of the population and the economy of the city is supplied with water from shallow (30–75 m) and deep (90––135 m) aquifers, which are characterized by high mineralization, a high content of iron, ammonia, manganese, sodium, organic matter, arsenic, unbalanced organoleptic properties. The water is oxygen-poor and loaded with dissolved sulfur hydrogen and methane. The main groundwater aquifers in Zrenjanin have extremely adverse physical characteristics according to the strategic development material focused inter alia on the quality of grounwater/drinking water [16].

Water supply of the City of Zrenjanin is based on utilization of groundwater from the two main aquifer with the wells located northwest of the city with a total of 35–40 wells. All wells were perforated to a depth of two aquifers and therefore this research was applied only on two representative wells from these depths. In Zrenjanin groundwater directly engages the pipeline system and it supplies the population as drinking water. The groundwater only passes through the microbiological treatment before enters the pipeline system.

The data of chemical parameters of groundwater used in this research are taken from the Institute of Public Health Zrenjanin (Table 2). The research was conducted from October 2010 to July 2011. The first well is located at a depth of 98–118 m while the second is at a depth of 36–61 m. Eight relevant parameters have been selected: pH, $KMnO_4$ consumption, ammonia, total iron, calcium, magnesium, sodium and arsenic on which the fuzzy logic approach would be applied. The observed parameters are selected on the basis of their concentrations which are above the permissible limits or significantly below the limit. In legislation on water quality, there are not considered extremely low concentrations of certain parameters, which can have adverse effects on human health.

For the selected GWQ parameters, fuzzy sets are assigned whose MFs are shown in the following Figures 4–11.

Fuzzy model of GWQ is created by dividing 8 selected parameters into 4 different categories, *i.e.*, groups according to predefined quality criteria. Groups were formed according to the main characteristics of the chemical composition of the groundwater. The first group consists of: pH and $KMnO_4$ consumption, which are the primary indicators of the chemical composition of the groundwater and the parameters to be tested in the initial survey of the water quality. The second group includes the total iron and ammonium cat ions which are micro components of the groundwater, and their presence does not affect the type of water, but

Table 2. Chemical parameters of groundwater from the city of Zrenjanin; concentrations are given in mg/l

Sample	pH	$KMnO_4$ consumption	NH_4^+	Fe^{2+}, Fe^{3+}	Ca^{2+}	Mg^{2+}	Na^+	As^{3+},As^{5+}
Well 1, October, 2010	7.97	37.16	1.46	0.22	14.3	15.6	315.8	0.187
Well 2, October, 2010	7.69	41.23	1.75	1.55	53.9	20.7	236.1	0.005
Well 1, January, 2011	8.2	38.37	1.04	0.19	13	15.4	225	0.17
Well 2, January, 2011	7.72	41.73	1.69	1.53	57.4	23.7	212.5	0.005
Well 1, April, 2011	8.12	32.62	1.17	0.01	14.9	12.1	160.8	0.155
Well 2, April, 2011	7.57	34.68	1.75	0.2	13.8	41.4	150	0.007
Well 1, July, 2011	7.44	36.79	1.58	0.23	23.6	4	250	0.102
Well 2, July, 2011	7.63	39.13	1.74	1.07	57.1	21.8	312.5	0.04

have a huge impact on the specific composition of the groundwater and often determine its ability to be used. The third group is structured by calcium, magnesium and sodium ions representing macro components which make up the basic composition of the groundwater and the type of water is determined on the basis of their content. The fourth group is composed of the ionic forms of arsenic. Arsenic compounds are considered separately because arsenic is one of the most important hazards, the key parameters of the groundwater quality of the examined area. Intoxication by arsenic through drinking water is accumulative nature. Long-term exposure with arsenic causes cancer of some organs, especially the skin, lung, bladder and kidney. Common signs of acute toxic effects include: vomiting, dry mouth and throat, muscle cramps, hallucinations [17]. Geological area in Zrenjanin and in the Banat region is well known of the content of the arsenic ions. Therefore the source of cat ions of arsenic is of geologically origin but it could be also of anthropogenic activities.

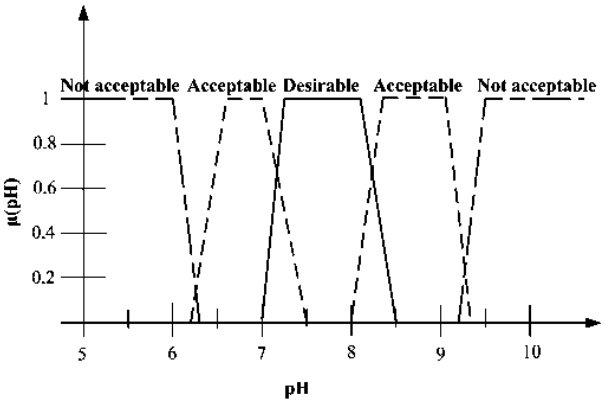

Figure 4. MF of the pH defined for the GWQ.

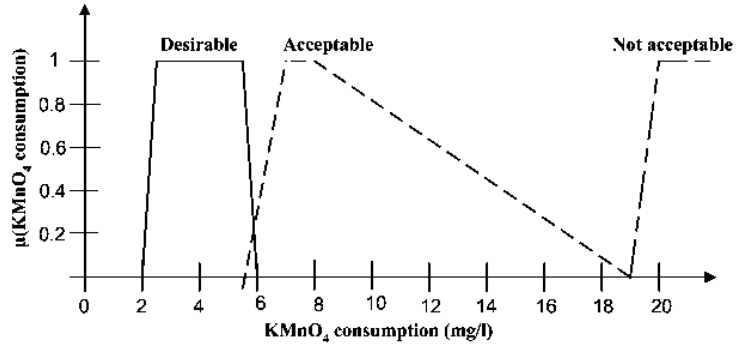

Figure 5. MF of the $KMnO_4$ consumption defined for the GWQ.

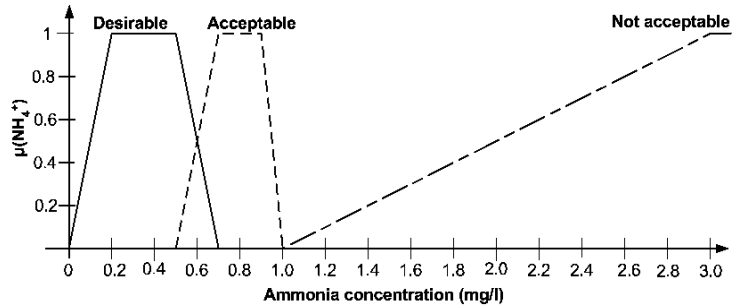

Figure 6. MF of the ammonia concentration defined for the GWQ.

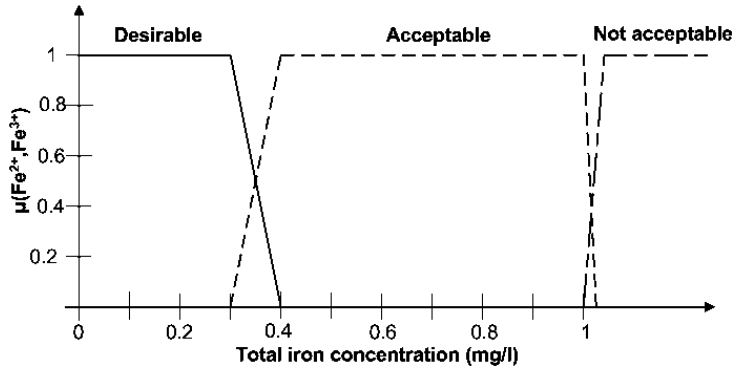

Figure 7. MF of the total iron concentration defined for the GWQ.

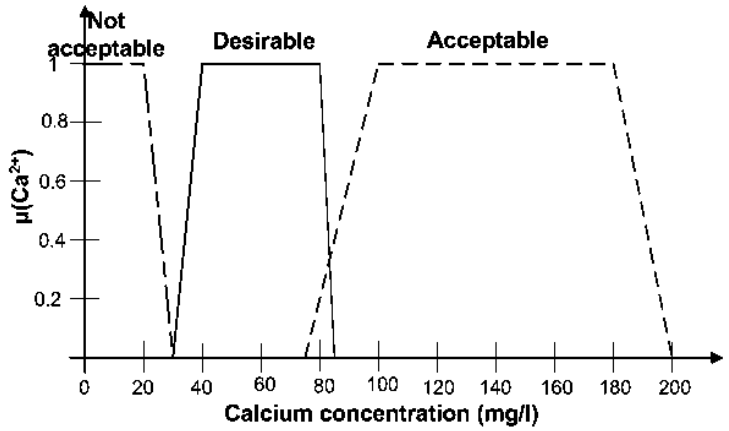

Figure 8. MF of the calcium concentration defined for the GWQ.

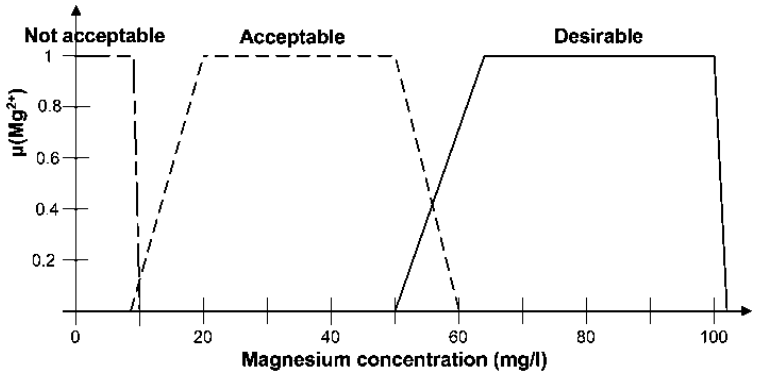

Figure 9. MF of the magnesium concentration defined for GWQ.

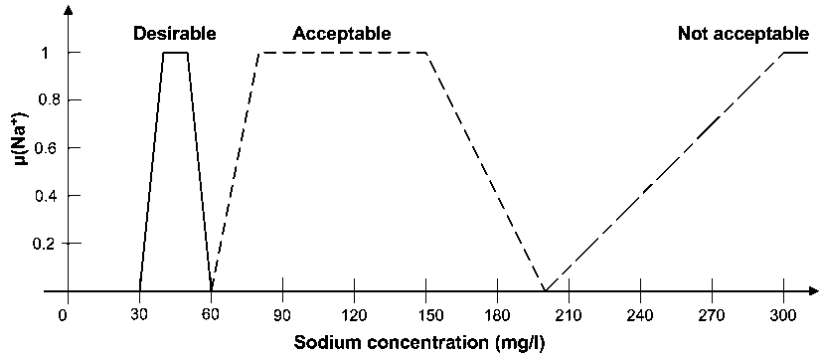

Figure 10. MF of the sodium concentration defined for the GWQ.

Under natural conditions, the greatest range and the highest concentrations of arsenic are found in groundwater as a result of the strong influence of the water––rock interactions and the favorable physical and geochemical conditions in aquifers for the mobilization and accumulation of arsenic. Arsenic is particularly mobile at pH values typically found in groundwater (pH 6.5–8.5) under both oxidizing and reducing conditions [18].

Inorganic arsenic compounds are classified by the International Agency for Research on Cancer (IARC) in Group 1 (carcinogenic to humans) on the basis of sufficient evidence for carcinogenicity in humans and limited evidence for carcinogenicity in animals [8]. There are numerous data on the relationship between the risk of cancer and drinking water with high content of arsenic, but has not yet assessed the risk caused by low concentrations of arsenic in water. Considering all the uncertainties related to the risk assessment, the World Health Organization in 1993 recommended the MAC of arsenic in drinking water of 10 μg/l. Recommendations from these have been adopted in the legislation of the Republic of Serbia in the Book of Regulations on the Hygienic Correctness of Drinking Water [7].

In the created fuzzy rules, all of the statements are coordinate with the intersection (AND) operator while other operators (OR and NOT operators) are not imple-

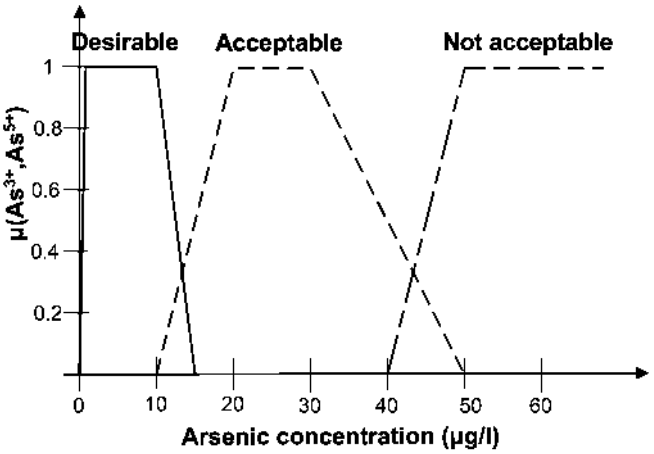

Figure 11. MF of the arsenic concentration defined for the GWQ.

mented. This is because, AND operator is more flexible and adaptive for such assessment. Operator is selected on the basis of previous experiences and on the perception of the authors.

Fuzzy rules defined for the first group of parameters: Rule 1: *If pH is Desirable AND $KMnO_4$ consumption is Not acceptable THEN GWQ of group 1 is Acceptable*. Rule 2: *If pH is Acceptable AND $KMnO_4$ consumption is Not acceptable THEN GWQ of group 1 is Acceptable*.

Acceptable pH indicates the significance of the aquatic environment existence of toxic cationic forms of arsenic, which is in accordance with the positive physical-chemical principles of oxidation-reduction process. The results of analyzed water samples are in excellent correspondence with the mathematical rules of fuzzy logic.

Fuzzy rules defined for the second group of parameters: Rule 3: *If ammonia is Not acceptable AND iron is Desirable THEN GWQ of group 1 is Acceptable*. Rule 4: *If ammonia is Not acceptable AND iron is Acceptable THEN GWQ of group 1 is Not Acceptable.* Rule 5: *If ammonia is Not acceptable AND iron is Not acceptable THEN GWQ of group 1 is Not Acceptable.*

The presence of ammonia in potable water supplies is often accompanied by the presence of iron and manganese cat ions in the concentrations above the permitted limits [19]. Simultaneous processes of microbial disintegration of sediment organic matter and reduction of some chemical species leads to the origin of unusual concentrations of ammonia (NH_4^+(aq)), dissolved organic carbon, iron (Fe^{2+}(aq), Fe^{3+} (aq)) [20].

Fuzzy rule defined for the third group of parameters: Rule 6: *If calcium is Not acceptable AND magnesium is Not acceptable AND sodium is Not acceptable THEN GWQ of group 2 is Not Acceptable.* Rule 7: *If calcium is Not acceptable AND magnesium is Acceptable AND sodium is Not acceptable THEN GWQ of group 2 is Acceptable.* Rule 8: *If calcium is Not acceptable AND magnesium is Acceptable AND sodium is Acceptable THEN GWQ of group 2 is Acceptable.* Rule 9: *If calcium is Desirable AND magnesium is Acceptable AND sodium is Not acceptable THEN GWQ of group 2 is Acceptable.*

The main components that determine the mineralization and the chemical type of groundwater are sodium, magnesium and calcium. They represent the macro components or the basic cat ions of groundwater.

Fuzzy rule defined for the fourth group of parameters: In this group there is only one parameter – arsenic. Results from the first three groups will be combined with a fourth group to obtain the final classification of the groundwater. The special rule has been created for this group which says: *If arsenic is Not acceptable then the GWQ is Not Acceptable, regardless of the other three groups. If arsenic is Desirable or Acceptable then the final GWQ is Acceptable*, but the degree of certainty is determined on the basis of all parameters.

Until recently, arsenic was often not on the list of constituents in drinking water routinely analyzed by national laboratories, water utilities and non-governmental organizations and so the body of information about the distribution of As in drinking water is not as well known as for many other drinking water constituents [21]. Indeed, As is now recognized as the most serious inorganic contaminant in drinking water on a worldwide basis. In the city of Zrenjanin groundwater arsenic is analyzed once a year.

A hierarchical structure for groundwater classification resulting in a set of fuzzy rules is constructed on the basis of knowledge from authors and experts in this scientific expertise. Experts, consulted in the creation of fuzzy rules, are from the field of physical chemistry and chemistry from the Institute of Public Health dealing with these issues as well as mathematicians focused on fuzzzy logic. The main condition for the support of hierarchy for created fuzzy rules is the impact of sel-

ected parameters on human health. This was carried out through dividing of parameters in different categories. The greatest importance is assign to a group 4, followed by group 2, 1 and 3.

Hierarchical structure for groundwater classification:

– IV group: Arsenic has with reason assigned the greatest emphasis due to carcinogenic effect on humans.

– II group: Iron in groundwater provides the typical well water "rust" taste. Health effects are not expected at levels normally found in natural waters. It can be indicator of some toxic elements in groundwater. Increased concentrations of iron are capable of releasing dangerous amounts of arsenic into the groundwater. The ability of arsenic to mobilize and enter into the aquifer is attributed to a process known as the reductive dissolution of iron. The process that is responsible for the reduction of insoluble ferric iron to soluble ferrous iron is known as reductive dissolution [22]. If the environment is such that a reduced iron form is produced, groundwater will contain higher concentrations of iron. The most frequent cause of reducing reactions is the presence of organic matter [23]. The ammonium is also interesting in regard to the high arsenic levels since infiltrating water from surface sources could contain high quantities of organic material which could decrease oxygen levels and redox conditions even more, which might increase the mobilization of arsenic. Ammonium does not pose a risk for people's health in concentrations that can be expected in groundwater. However, there is health implication with high concentrations of nitrate in drinking water, which ammonium can be oxidized to if exposed to oxygen. High ammonium concentrations are also a common sign that surface water influenced by anthropogenic activities are infiltrating to the groundwater [24].

– I group: Because we are dealing with high groundwater arsenic concentration, pH is the parameter which is also substantial for GWQ. The concentration of arsenic in groundwater in the first place depends on the pH of the water. Therefore, it is assigned a certain "weight" to this parameter. $KMnO_4$ consumption is a parameter indicating polluting organic compounds in the water. Organic matter in groundwater plays important roles in controlling geochemical processes by acting as proton donors/acceptors and as pH buffers, by affecting the transport and degradation of pollutants, and by participating in mineral dissolution/precipitation reactions. Dissolved and particulate organic matter may also influence the availability of nutrients and serve as a carbon substrate for microbially mediated reactions. Numerous studies have recognized the importance of natural organic matter in the mobilization of hydrophobic organic species, metals and radionuclides [25].

– III group: The least impact in assessment is assigned to the macro components of calcium, magnesium and sodium because they do not have severe implications on human health.

Fuzzy rules defined for the final GWQ are represented in the Table 3.

Changing in the fuzzy rules would not significantly affect final classification of GWQ. If within the group parameters would be changed certain statements this will affect the final class of groundwater. But the things that can most influence on the change in the final GWQ are concentrations of selected input parameters. Even small changes of concentration of one GWQ parameter can cause considerable changes of overall quality.

Final classification of GWQ from monitored locality has been divided into three categories: Desirable, Acceptable and Not acceptable. Inside those groups there are percentages which represent the degree of certainty. The degree of certainty shows on which level of safeness is drinking water reliable. MF of the final quality of the groundwater is shown in the following Fig. 12.

Table 4 shows the final classification of groundwater quality with its degree of certainty.

Advantage of this model is that it can be applied to any of locality with the proper selection of characteristic parameters for the site. Many authors have used fuzzy logic for assessing water quality [1–3,5,26–29]. Advantages and disadvantages of fuzzy models for assessing water quality are presented in the incoming Table 5.

Table 3. Created fuzzy rules for definition of the final GWQ

Rule	IF I group is…	AND II group is…	AND III group is…	AND fourth group is…	THEN GWQ is…
1	Acceptable	Acceptable	Acceptable	Not acceptable	Not acceptable
2	Acceptable	Not acceptable	Acceptable	Acceptable	Acceptable
3	Acceptable	Acceptable	Acceptable	Not acceptable	Not acceptable
4	Acceptable	Not acceptable	Acceptable	Acceptable	Acceptable
5	Acceptable	Acceptable	Acceptable	Not acceptable	Not acceptable
6	Acceptable	Acceptable	Acceptable	Acceptable	Acceptable
7	Acceptable	Acceptable	Not acceptable	Not acceptable	Not acceptable
8	Acceptable	Not acceptable	Acceptable	Acceptable	Acceptable

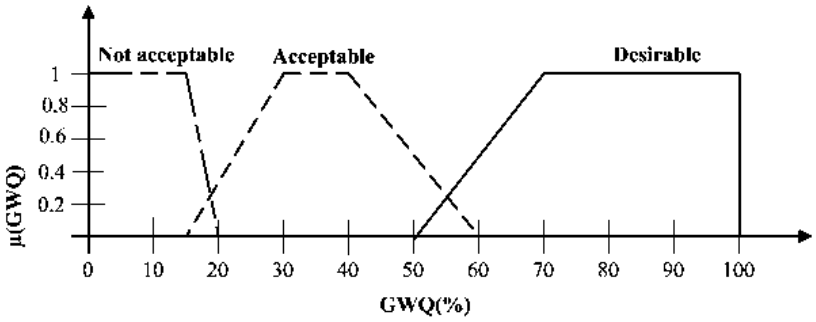

Figure 12. MF of the final GWQ.

Table 4. Final results of the GWQ

Sample	Groundwater quality	Degree of certainty	
		MOM / %	*COA* / %
Well 1, October, 2010	GWQ Not acceptable	22.5	27.05
Well 2, October, 2010	GWQ Acceptable	61.25	61.68
Well 1, January, 2011	GWQ Not acceptable	22.5	27.05
Well 2, January, 2011	GWQ Acceptable	61.25	61.68
Well 1, April, 2011	GWQ Not acceptable	22.5	27.05
Well 2, April, 2011	GWQ Acceptable	61.25	61.68
Well 1, July, 2011	GWQ Not acceptable	22.5	27.05
Well 2, July, 2011	GWQ Acceptable	22.5	27.05

Artificial intelligence technologies are becoming more and more important for water quality management in order to satisfy objectives of sophisticated water quality decision-making. The most important motive for choosing fuzzy logic for water quality assessment is flexible potential to combine different aspects. Fuzzy assessment of the water quality represents perspective for the future. In literature review are presented the latest researches in this scientific topic from 2013–2014.

An interval fuzzy credibility-constrained programming (IFCP) method is developed for river water quality management by Liu *et al*. [31]. A real-world case for water quality management planning of the Xiangxi River in the Three Gorges Reservoir Region is then conducted for demonstrating the applicability of the developed method. Priya [32] has been developed Fuzzy Inference System (FIS) to classify ground water for irrigation purposes. The fuzzy model was validated using the ground water quality data collected from Karunya watershed. Srivastava *et al.* [33] designed and deve-

Table 5. Advantages and disadvantages of fuzzy models for assessing water quality [30]

Advantages	Disadvantages
Easy interpretable by natural language	Not free of eclipsing but can be handled with trial and error process
Can handle complex and vague situation	Cannot incorporate guidelines values for water quality parameters
Can incorporate exports opinion with hard data	Suffer rigidity to some extent (careful selection of parameters can reduce it)
Can describe a large number of nonlinear relationships through simple rules	Easy to manipulate or can be biased due to human subjectivity
Provides a transparent mathematical model	
Able to account interconnection (interdependencies) among parameters	
Capable to handle missing data without influencing the final water quality index value	
Free of ambiguity and can represent different water quality usage if parameters are selected carefully	

loped soft computing system to assess the water quality of rivers Ganga and Yamuna during the Maha Kumbh 2013 in and around Sangam Zone, Allahabad, by making use of physicochemical parameters relationship. Nasr *et al*. [34] has offered the creation of a new fuzzy water quality index (FWQI) to assess the degree of drinking water resources in rural areas of Yazd province, Iran. Wang *et al*. [35] created a model for assessing the water quality status of the Meiliang Bay of the Taihu Lake in China. Results show that the proposed model can determine the water quality level and provide an acceptable alternative based on optimized objectivity in determining water quality level. Zhou *et al.* [36] examined 15 groundwater samples from SuoLuoShu water resource with fuzzy mathematics. On this basis, the membership degrees of the groundwater samples in the 5 grades were calculated and by using the maximum membership principle, the groundwater grade of each samples were determined. Aghaarabi et al. [37] presented the use of two multi-criteria decision-making frameworks based on hierarchical fuzzy inference engines for the purpose of assessing drinking water quality in distribution networks.

CONCLUSION

Classical methods for assessing the water quality are representing only the facts that the environmental data are in or out of the prescribed limits. Therefore, classical assessment of the water quality classified water as acceptable or not acceptable. The main disadvantage of such analyzes is that none of the tested parameter has assigned proper “weight”. But, applied fuzzy model of GWQ observed each parameter in terms of impact on human health and thus to each parameter is assigned a specific “weight”.

One of the main advantages of fuzzy approach is that the final water quality has assigned degree of certainty. This method is much more convincing and more accessible to the decision makers and public which should be informed about the state of drinking water quality. Fuzzy model simply represent a clearer and better view of decision-making.

GWQ in the city of Zrenjanin is assessed by taking account only the chemical indicator parameters. Chemical characteristics of groundwater involve: key indicators of the chemical composition (pH and $KMnO_4$ consumption), macro components (calcium, magnesium and sodium) and micro components (iron, ammonium and arsenic).

From all physical parameters from analysis, color and electrical conductivity values were slightly above the permissible limits but were not included in the calculations because they cannot greatly influence the change in the final quality of groundwater. Groundwater at the city of Zrenjanin is chlorinated before distributed for human consumption and the water is microbiologically correct. Therefore, the microbialogical parameters of groundwater quality are not implemented in the assessment.

The fuzzy analysis showed that samples from well 1 provide an unacceptable quality of groundwater while samples from well 2 have acceptable quality. All values of final GWQ are very closely despite of the method of defuzzification. In the shallow aquifer the arsenic concentrations are below the MAC and this groundwater can be used for drinking purpose. But there is not sufficient water in upper layers of soil to supply the entire population of the city of Zrenjanin for drinking usage. Groundwater from deeper aquifer are not acceptable as drinking water due to its highly elevated arsenic concentrations. This implies that the quality of drinking water in Zrenjanin only depends on the geological structure of the soil. This is the real state of the geochemical properties of the area on which Zrenjanin is situated.

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IZVOD

FAZI MODEL ZA ODREĐIVANJE I PROCENU KVALITETA PODZEMNE VODE U GRADU ZRENJANINU

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(Naučni rad)

U radu je predstavljena primena fazi logike za utvrđivanje i procenu hemijskog kvaliteta podzemnih voda za piće u gradu Zrenjaninu. Stepen pouzdanosti i neizvesnosti su neke od problema koje se sreću kod najčešće primenjivanih metoda za procenu kvaliteta vode. Fazi logika uspešno upravlja sa ovim poteškoćama. Evaluacija fazi modela je ostvarena na uzorcima iz dva reprezentativna bunara koji se nalaze na dubinima dva vodonosna sloja iz kojih se uzima voda za snabdevanje stanovništva grada Zrenjanina pijaćom vodom. U uzorcima je analizirano 8 različitih parametara hemijskog kvaliteta vode. U istraživanju, koncentracija arsena (As^{3+} i As^{5+}) je razmatrana kao ključni parametar zbog svojih kancerogenih efekata na ljudsko zdravlje. Ova vrsta istraživanja je po prvi put sprovedena u gradu Zrenjaninu.

Ključne reči: Kvalitet podzemne vode • Fazi logika • Stepen pouzdanosti • Arsen

Interpretacija rezultata kvaliteta površinskih voda primenom multivarijalne analize

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Izvod

Monitoring površinskih voda predstavlja veoma bitan faktor u kontroli kvaliteta voda, a samim tim i zdravlja živih bića. Kvalitet površinskih voda u velikoj meri određen je prirodnim i antropogenim procesima. Dobijeni rezultati monitoringa površinskih voda zbog prostornih i vremenskih varijacija kvaliteta su suviše obimni za njihovo pojedinačno tumačenje. Primenom multivarijalne statističke analize može se postići znatna redukcija obimnosti raspoloživih podataka i omogućiti interpretacija dobijenih rezultata o kvalitetu i ekološkom statusu/potencijalu voda. U ovom radu su primenom multivarijalne statističke analize (klaster analiza i analiza glavnih komponenti) obrađeni odabrani rezultati analiza površinskih voda na teritoriji AP Vojvodine u toku 2011. godine. Ove tehnike omogućavaju interpretaciju rezultata monitoring programa kvaliteta površinskih vodnih tela na teritoriji AP Vojvodine i istovremenu identifikaciju uticaja registrovanih i potencijalnih izvora zagađivanja na kvalitet datih vodnih tela.

Ključne reči: kvalitet voda, multivarijalna analiza, klaster analiza, analiza glavnih komponenti.

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U toku godišnjeg hidrološkog ciklusa, kvalitet površinskih voda zavisi od atmosferskih padavina, nanosa, odnosno erozije tla u slivu, naseljenosti i razvoja industrije u slivnom području. Pored toga, izmena temperature u toku godišnjih doba, kao i mešanje različitih vrsta voda takođe su činioci koji utiču na promenu hemijskog sastava površinskih voda [1].

Da bi bio postignut zadovoljavajući kvalitet površinskih voda neophodan je monitoring koji je izrazito bitan segment u upravljanju vodama. Monitoring programi površinskih voda uključuju analize vode, sedimenta i biote [2]. Krajnja informacija koja se dobija monitoringom površinskih voda ključna je za donošenje odluka u upravljanju vodama i zahteva odgovarajući način obrade podataka dobijenih merenjima u toku samog monitoringa. Kada su u pitanju monitoring programi koji se sprovode na godišnjem nivou, za obradu tako velikog skupa podataka najčešće se koriste statističke metode. Jedne od tih metoda su multivarijalne statističke metode kao što su faktor analiza, klaster analiza i analiza glavnih komponenti. Ove metode omogućavaju redukciju velikog broj podataka monitoringa i markiraju merne stanice sličnog kvaliteta, kao i problematične pokazatelje kvaliteta.

Multivarijalne statističke metode omogućavaju identifikaciju mogućih faktora/izvora koji su odgovorni za varijabilnost kvaliteta vode. Takođe, omogućavaju identifikaciju rasporeda izvora zagađenja i na taj način predstavljaju koristan alat za razvoj odgovarajuće strategije kako bi se ostvarilo efikasno upravljanje vodnim resursima [3–8].

Klaster analiza se koristi za redukciju obimnih podataka, kombinuju se objekti u grupe relativno homogenih sastava. Ona pomaže u grupisanju parametra (slučajeva) u klastere na osnovu sličnosti ili razlike između njih. Izazov mnogih istraživanja u kojima se radi sa velikim bojem podataka upravo je identifikovanje i grupisanje elemenata u manje grupe na osnovu neke povezanosti [9,10].

Analiza glavnih komponenti (*Principal Component Analysis*, PCA) ima sposobnost da prepoznaje i eliminiše suvišne podatke iz eksperimentalnih rezultata. Primenom analize glavnih komponenti redukuje se broj raspoloživih podataka, a kao rezultat se dobija različiti broj novih promenljivih tzv. glavne komponente (*principal components*, PC). Glavna komponenta, PC, je u stvari linearna kombinacija originalnih promenljivih. Koeficijent inverzne relacije od linearne kombinacije se zove komponenta opterećenja i predstavlja koeficijent korelacije između originalne promenljive i glavne komponente. U toku analize dobija se veći broj glavnih komponenti. Prva glavna komponenta, PC1, predstavlja maksimalni udeo ukupnih promenljivih. Druga glavna komponenta, PC2, ne korelira se sa PC1 a predstavlja maksimalni udeo od rezidualne promenljive. Na istom principu se formiraju i ostale glavne komponente, sve dok se ukupna varijansa ne izračuna. U praktičnom radu obično je dovoljno zadržati samo nekoliko glavnih

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komponenti, čiji zbir obuhvata veliki procenat ukupne promenljive [9–11].

Navedene statističke metode pružaju mogućnost lakšeg, bržeg i jasnijeg definisanja promenljivih koje imaju najveći uticaj na kvalitet površinskih voda. U ovom radu su primenom klaster analize i analize glavnih komponenti analizirani i interpretirani rezultati kvaliteta površinskih vodnih tela na teritoriji AP Vojvodine u cilju identifikacije mogućih izvora zagađenja.

EKSPERIMENTALNI DEO

Za potrebe ovog rada korišćeni su podaci o kvalitetu površinskih voda na teritoriji AP Vojvodine za mesec februar 2011. godine [12].

Pregled vodnih tela i mernih stanica na kojima je izvršeno uzorkovanje vode za analizu i čije su vrednosti pokazatelja kvaliteta obrađene u ovom radu dat je u Tabeli 1. Obrađeno je ukupno 28 mernih stanica na sledećim prirodnim, značajno izmenjenim vodnim telima: Dunav, Tisa i Tamiš i veštačkom vodnom telu Dunav–Tisa–Dunav.

Analizirano je 25 pokazatelja kvaliteta vode: suspendovane materije, UV ekstinkcija na 254 nm, HPK(Mn), BPK_5, pH, ukupna tvrdoća, alkalitet, ukupni alkalitet, bikarbonati, slobodni CO_2, Ca^{2+}, Mg^{2+}, K^+, Na^+, Cl^-, SO_4^{2-}, ukupne rastvorne soli, elektroprovodljivost, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, ukupni fosfor, rastvorni kiseonik i zasićenost vode kiseonikom.

Klaster analiza i analiza glavnih komponenti rađene su primenom softverskog programa Statistica 10.

REZULTATI I DISKUSIJA

Primenom klaster analize po Wards metodi dobijeni su dendrogrami prikazani na slikama 1 i 2.

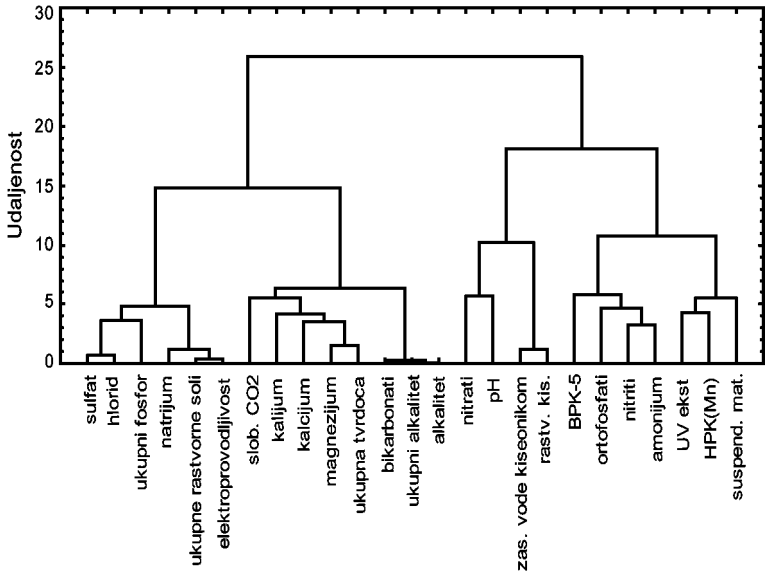
Na slici 1 prikazan je dobijen dendrogram ispitivanih parametara kvaliteta površinskih voda. Sa slike se vidi da se analizirani parametri grupišu u dva klastera u okviru kojih se može registrovati prisustvo podklastera. Prvi podklaster u okviru prvog klastera čine: Na^+, Cl^-, SO_4^{2-}, ukupne rastvorne soli, elektroprovodljivost i ukupni fosfor, dok se u drugom podklasteru nalaze: ukupna tvrdoća, alkalitet, ukupni alkalitet, bikarbonati, slobodni CO_2, Ca^{2+}, Mg^{2+} i K^+.

Ova dva podklastera, odnosno prvi klaster, obuhvataju hidrohemijske pokazatelje kvaliteta vode i pokazatelje geologije terena. Nitrati, pH, rastvorni kiseonik i zasićenost vode kiseonikom čine treći, dok se u četvrtom podklasteru nalaze: HPK(Mn), BPK_5, ortofosfati, nitrati, amonijum, UV ekstinkcija i suspendovane materije u okviru drugog klastera. Može se zaključiti da se u okviru njega grupišu faktori uticaja tačkastih i difuznih izvora zagađenja površinskih vodnih tela kao i ekološki faktori.

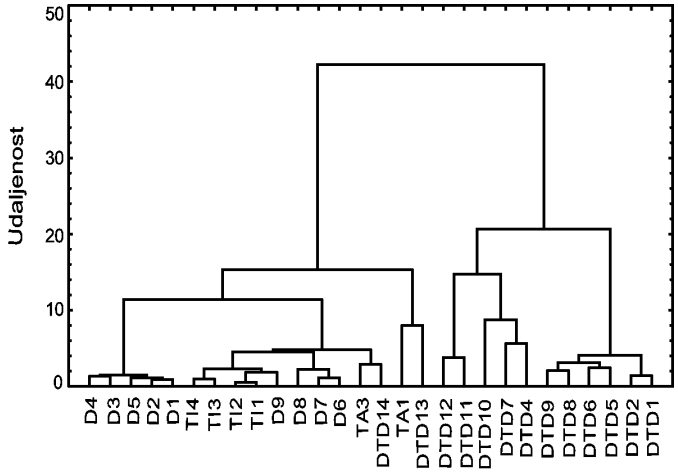
Rezultat klaster analize mernih stanica ispitivanih površinskih vodnih tela pokazuje razdvajanje prirodnih tj. značajno izmenjenih vodnih tela (Dunav, Tisa i Tamiš) i veštačkog vodnog tela (HsDTD) na dva osnovna klastera. Jednan klaster grupiše sve merne stanice na značajno izmenjenim vodnim telima, dok se u drugom nalaze merne stanice na HsDTD. U klasteru gde su grupisane sve merne stanice na značajno izmenjenim vodnim telima nalaze se i dve merne stanice veštačkog vodnog tela HsDTD (Vlajkovac i Kajtasovo). Razlog izdvajanja ove dve merne stanice HsDTD u klaster sa stanicama na prirodnim vodnim telima može se objasniti odstupajućim sadržajem kalijuma i natrijuma na mernim stanicama DTD13 i DTD14 (slika 3). Sadržaj ovih parametara u navedenim mernim stanicama je mnogo bliži vrednostima izmerenim na mernim stanicama TA1 i TA3 nego na ostalim stanicama u okviru HsDTD

Tabela 1. Pregled obrađenih vodnih tela – mernih stanica
Table 1. Summary of processed water bodies - monitoring stations

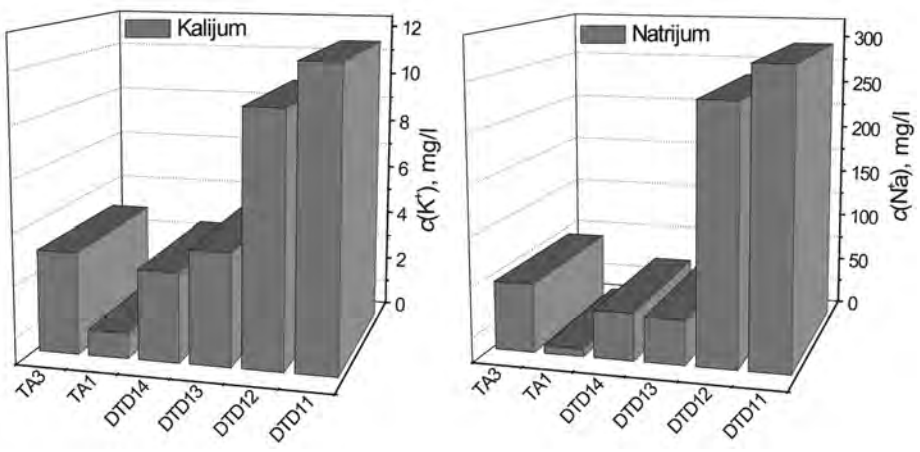
Oznaka	Naziv vodnog tela – merna stanica	Oznaka	Naziv vodnog tela – merna stanica
D1	Dunav – Bezdan	TA3	Tamiš – Pančevo
D2	Dunav – Apatin	DTD1	Kanal Dunav-Tisa-Dunav – Sombor
D3	Dunav – Bogojevo	DTD2	Kanal Dunav-Tisa-Dunav – Mali Stapar
D4	Dunav – Bačka Palanka	DTD4	Kanal Dunav-Tisa-Dunav – Vrbas 2
D5	Dunav – Novi Sad	DTD5	Kanal Dunav-Tisa-Dunav – S. Miletić
D6	Dunav – Slankamen	DTD6	Kanal Dunav-Tisa-Dunav – Savino Selo
D7	Dunav – Čenta	DTD7	Kanal Dunav-Tisa-Dunav – B. Gradište
D8	Dunav – Pančevo	DTD8	Kanal Dunav-Tisa-Dunav – Novi Sad
D9	Dunav – Banatska Palanka	DTD9	Kanal Dunav-Tisa-Dunav – Bač
TI1	Tisa – Maronoš	DTD10	Kanal Dunav-Tisa-Dunav – B. Petrovac
TI2	Tisa – Padej	DTD11	Kanal Dunav-Tisa-Dunav–Novo Miloševo
TI3	Tisa – Žabalj	DTD12	Kanal Dunav-Tisa–Dunav – Melenci
TI4	Tisa – Titel	DTD13	Kanal Dunav-Tisa–Dunav – Vlajkovac
TA1	Tamiš – Jaša Tomić (Graničeri)	DTD14	Kanal Dunav-Tisa–Dunav – Kajtasovo



Slika 1. Dendrogram pokazatelja kvaliteta ispitivanih površinskih vodnih tela.
Figure 1. Dendrogram of parameters of the quality of investigated surface water bodies.



Slika 2. Dendrogram ispitivanih vodnih tela.
Figure 2. Dendrogram of investigated water bodies.



Slika 3. Histogrami sadržaja kalijuma i natrijuma na odabranim lokalitetima.
Figure 3. Histograms of concentration of potassium and sodium in selected localities.

(npr. DTD11 i DTD12). Drugo objašnjenje odstupanja ove dve merne stanice može biti u njihovom geografskom položaju. Vlajkovac i Kajtasovo su merne stanice na banatskom delu HsDTD (kanal Banatska Palanka–Novi Bečej) u koji su uklopljeni Tisa i svi presečeni vodotoci (Nera, Tamiš, Karaš, Brzava, oba Begeja i Zlatica). Navedeni vodotoci, sa padina Karpata vode ka Tisi, odlikuju ih vrlo bujični režimi koji su sve nepovoljniji zbog nekontrolisane seče šuma na Karpatima i regulacionih radova u Rumuniji.

Takođe, posmatrano geografski, rezultati klaster analize, pokazuju raspodelu mernih stanica u okviru Banata i Bačke. Prvi podklaster (D1–D5) čine merne stanice u Bačkoj, drugi podklaster (D6–D9, TI1–TI4, TA1 i TA3, DTD13 i DTD14) formiraju merne stanice u Banatu. U drugom klasteru jedan podklaster (DTD11 i DTD 12) čine merne stanice u Banatu, a drugi podklaster (DTD1–DTD10) merne stanice u Bačkoj.

Analiza glavnih komponenati je statistička metoda koja se vrlo često koristi u cilju redukcije velikog broja podataka kako bi se omogućila lakša i pravilnija analiza rezultata. Obradom navedenih parametara, analiza glavnih komponeneti (PCA), sa četiri glavne komponenete opisuje ukupan udeo varijansi od 84,65%. Vrednosti pojedinih glavnih komponeneti, PC, prikazane su u tabeli 2.

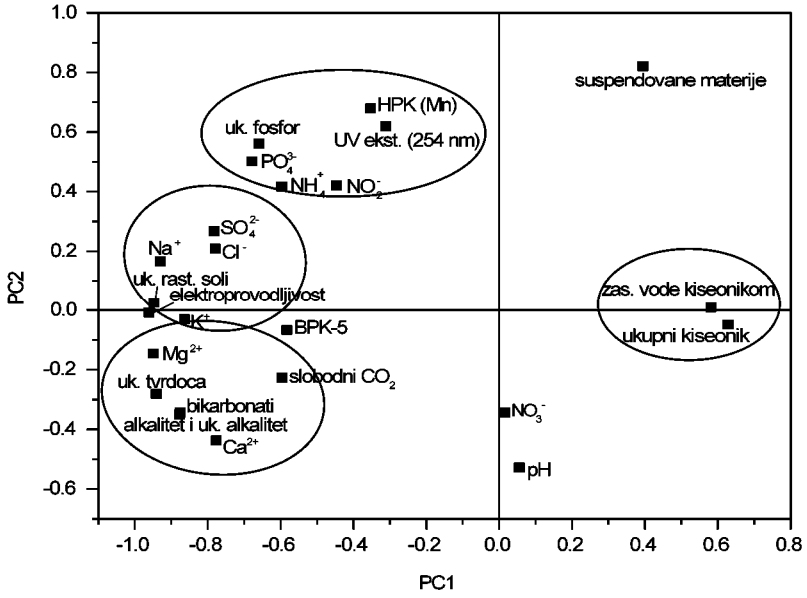
Vrednosti pojedinih PC pokazuju kakvo je slaganje novodobijenih glavnih komponenti sa početnim varijablama tj. analiziranim parametrima. Iz podataka prikazanih u tabeli 2 se uočava kao što je i očekivano da prva glavana komponeta, PC1, u najvećem broju slučajeva sadrži vrednosti veće od 0,7. Druga glavna komponeneta (PC2) samo u slučaju suspendovanih materija ima značajan udeo u opisivanju početnih varijabli, dok u trećoj i četvrtoj nema ni jedne dominatne vrednosti. Na osnovu toga može se zaključiti da su za dalju analizu rezultata najznačajnija analiza prve i druge glavne komponente.

Na slici 4 prikazane su korelacije dobijenih vrednosti prve dve glavne komponente. Uočava se skoro identično grupisanje analiziranih parametara kao što je dobijeno i primenom klaster analize (slika 1).

Slično kao u slučaju klaster analize može se potvrditi grupisanje hidrohemijskih faktora (alkalitet, tvrdoća vode i parametri koji utiču na elektroprovodljivost), tačkastih izvora zagađenja (fosforna i azotna jedinjenja,

Tabela 2. Rezultati analize glavnih komponenti za ispitivana vodna tela
Table 2. Results of the principal component analysis of the investigated water bodies

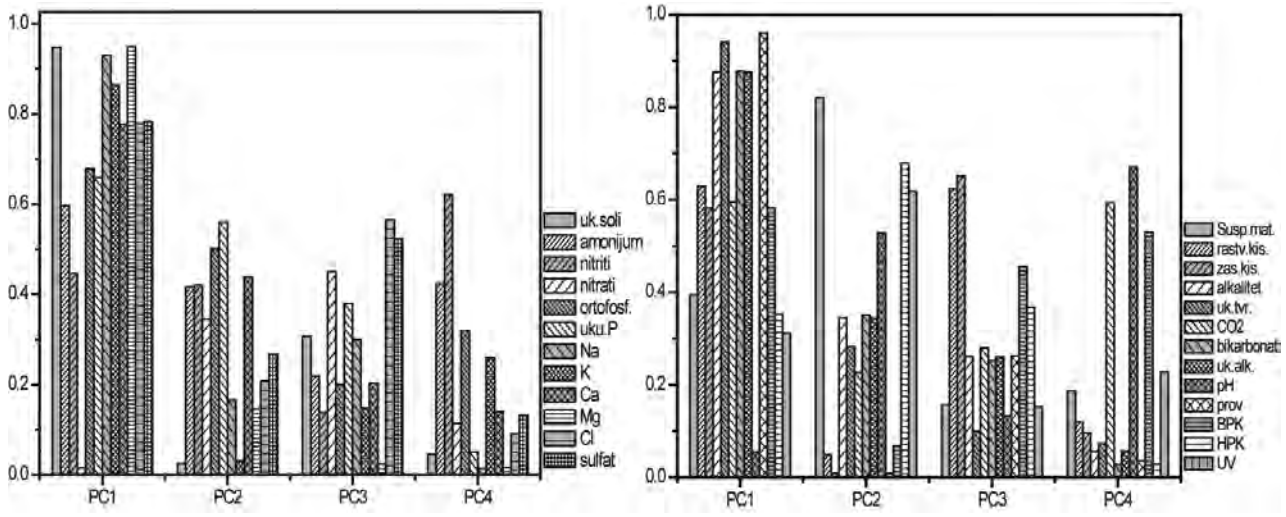
Parametar	PC1	PC2	PC3	PC4
Suspendovane materije	0,39422	**0,82048**	0,15689	–0,18676
Rastvorni kiseonik	0,62893	–0,04904	**–0,62391**	0,11943
Zasićenost vode kiseonikom	0,58203	0,00910	**–0,65101**	0,09661
Alkalitet	**–0,87537**	–0,34398	0,26093	0,05617
Ukupna tvrdoća	**–0,94060**	–0,28170	–0,09947	–0,07248
Slobodni CO_2	–0,59499	–0,22699	0,27985	–0,59365
Bikarbonati	**–0,87754**	–0,35110	0,25287	0,02751
Ukupni alkalitet	**–0,87580**	–0,34356	0,26049	0,05665
pH	0,05548	–0,52832	–0,13244	**0,67025**
Elektroprovodljivost	**–0,96037**	–0,00856	–0,26266	–0,03596
Ukupne rastvorne soli	**–0,94672**	0,02612	–0,30642	–0,04522
Amonijum joni	–0,59665	0,41636	0,21881	0,42420
Nitriti	–0,44686	0,42044	0,13770	0,62106
Nitrati	0,01497	–0,34450	–0,45038	0,11419
Ortofosfati	**–0,67818**	0,50192	–0,20014	0,32043
Ukupni fosfor	**–0,65883**	0,56011	–0,37961	0,05089
Na^+	**–0,92985**	0,16541	–0,29922	–0,01451
K^+	–0,08632	–0,03145	0,14746	–0,25984
Ca^{2+}	**–0,77654**	–0,43803	–0,20355	–0,13989
Mg^{2+}	**–0,94975**	–0,14648	–0,02377	–0,01634
Cl^-	**–0,77866**	0,20838	–0,56559	–0,09128
SO_4^{2-}	**–0,78266**	0,26709	–0,52300	–0,13183
BPK_5	–0,58203	–0,06741	0,45554	0,52988
HPK (mn)	–0,35263	**0,67909**	0,36766	–0,02884
UV ekstinkcija (254 nm)	–0,31104	**0,61900**	0,15333	–0,22807
Udeo u ukupnoj varijansi, %	50,39	14,74	11,49	8,03



Slika 4. Korelacija PC1/PC2 ispitivanih parametara.
Figure 4. Correlation of PC1/PC2 of investigated parameters.

HPK i UV ekstinkcija) i ekoloških faktora (zasićenost vode kiseonikom i ukupni kiseonik). Sa slike 4 je uočljivo da suspendovane materije nisu grupisane ni sa jednim od ostalih pokazatelja kvaliteta voda. Njihov sadržaj je u vodnim telima, prvenstveno u HsDTD, posledica upravljanja samim sistemom tj. dirigovanog režima protoka vode. Brane i ustave koje se nalaze na ovom veštačkom vodnom telu kao hidromorfološki pritisci, imaju značajan uticaj na dinamiku sedimenta i posredno na sadržaj suspendovanih materija. Sadržaj nitrata i pH vrednost vode su, predpostavlja se, posledica difuznih pritisaka na vodna tela. Pod ovim pritiscima se podrazumeva spiranje sa poljoprivrednih površina i neadekvatno upravljanje šumama tj. šumskim zemljištem. Nitratni jon u akvatičnim sistemima predstavlja „jak kiseli anjon" pa samim tim njegovo prisustvo u višim koncentracijama može da ima za posledicu povećanje kiselosti vode [13]. Ovakve promene u kvalitetu vode mogu biti izraženije u toku dužih i češćih, ali obilnih padavina kojima su vodna tela izložena u ranom prolećnom i kasnom jesenjem periodu godine. Treba napomenuti da nitrati mogu biti generisani mikrobiološkom transformacijom amonijaka. U područjima sa intenzivnim uzgojem stoke i lociranim farmama (slučaj i u pojedinim područjima na teritoriji Vojvodine), uticaj amonijaka na kvalitet površinskih voda može biti značajan [14].

Distribucija, tj. udeo pojedinih originalnih promenljivih u okviru svake glavne komponente predstavljeni su na slici 5. Sa slike se uočavava da u formiranju PC1



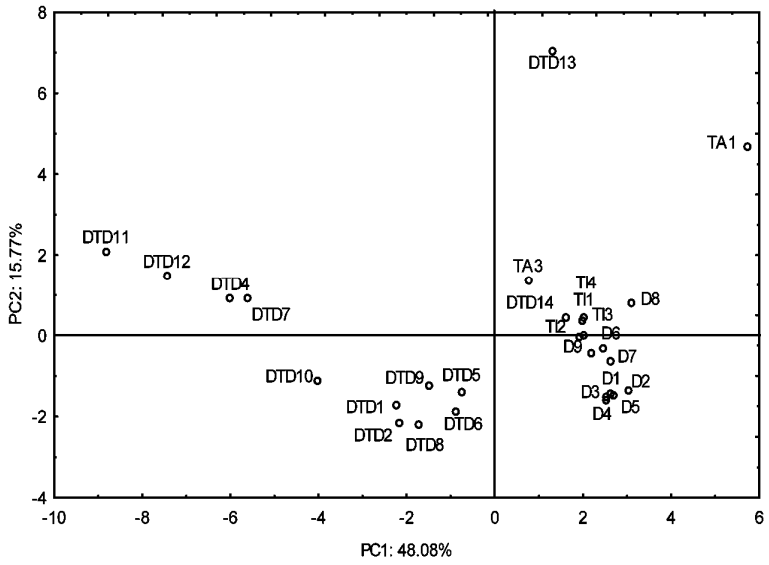
Slika 5. Raspodela apsolutnih vrednosti individualnih promenljivih u okviru četiri glavne komponente.
Figure 5. Distribution of the absolute values of the individual variables within the four principal components.

učestvuju sve analizirane varijable izuzev nitrata, pH, BPK_5 i suspendovanih materija što može biti ujedno i objašnjenje za njihovo odstupanje od ostalih parametra (slika 4).

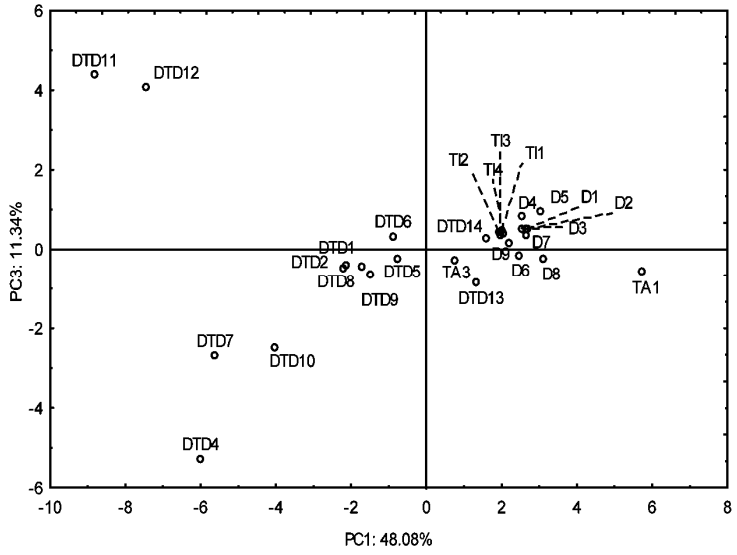
Na slikama 6 i 7 prikazane su korelacije prve glavne komponete sa drugom (slika 6) i trećom glavnom komponentom (slika 7) za analizirane merne stanice. Uočava se identična raspodela mernih stanica kao primenom klaster analize (slika 2). Javlja se odvajanje prirodnih i veštačkih vodenih tokova (odstupanje DTD13 i DTD14) i razdvajanje mernih stanica na osnovu geografskog pripadanja.

Sa slike 6 se vidi da dve merne stanice u okviru HsDTD koje se nalaze u Bačkoj (DTD4 i DTD7) odstupaju od ostalih mernih stanica u ovom regionu javljaju se bliže banatskim mernim stanicama DTD12 i DTD11. Razlog tome može biti prvenstveno zbog sadržaja ortofosfata i ukupnog fosfora izmerenih na ove četiri merne stanice koji su nešto viši u odnosu na izmerene vrednosti na ostalim mernim stanicama.

Fosfor je pored azota, i neorganskog ugljenika ključni element fotosinteze, ali i rasta algi i biljaka. Od velike važnosti je razumeti kompleksnost procesa eutrofikacije, ne samo u cilju procene ekološkog statusa vodnog tela, već i u cilju planiranja odgovarajućih mera za ublažavanje. Na ovim mernim stanicama je takođe bitno izvršiti analizu i klasifikaciju sedimenta, upravo iz tog razloga što je interakcija između vode i sedimenta u



Slika 6. Korelacija PC1/PC2 proučavanih vodnih tela.
Figure 6. Correlation of PC1/PC2 of investigated water bodies.



Slika 7. Korelacija PC1/PC3 proučavanih vodnih tela.
Figure 7. Correlation of PC1/PC3 of investigated water bodies.

rekama na prvom mestu važna zbog fosfora, koji se kao čestični može taložiti na rečnom dnu [15]. Ukoliko je koncentracija rastvorljivog reaktivnog fosfora u vodenom stubu veća nego ravnotežna koncentracija fosfora, fosfor će se adsorbovati na sedimentu [16].

ZAKLJUČAK

U ovom radu su primenom dve mulivarijalne statističke metode, klaster analize i analize glavnih komponenti obrađeni rezultati analize 28 mernih stanica na prirodnim vodnim telima (Dunav, Tisa i Tamiš) i na jednom veštačkom vodnom telu (HsDTD) prikupljenim u toku februara meseca 2011. godine na teritoriji AP Vojvodine. Obema primenjenim metodama izvršena je podela ispitivanih vodnih tela na sličan način, na osnovu porekla (prirodna i veštačka) i na osnovu teritorijalnog pripadanja mernih stanica (Banat i Bačka). Pojedinačna odstupanja, objašnjena su odgovarajućim razlikama na pojedinim mernim stanicama u odnosu na ostale. Na osnovu dobijenih rezultata potvrđuje se polazna pretpostavka, da se primenom raznih statističkih metoda mogu identifikovati glavni faktori koji imaju uticaj na ekološki status i ekološki potencijal vodnih tela kao i unaprediti postojeća mreža monitoringa.

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SUMMARY

INTERPRETATION OF THE RESULTS OF SURFACE WATER QUALITY APPLYING MULTIVARIATE ANALYSIS

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(Scientific paper)

Monitoring of surface water, through the analysis of physical-chemical and chemical parameters, is a very important factor in the control of water quality and the health of living beings. The surface water quality is largely determined by the nature (atmospherics) and anthropogenic processes (discharge of municipal and industrial wastewater). The results of monitoring of surface water are usually too expensive and difficult for correct interpretation, due to the spatial and temporal variations in water quality. The significant reductions of the ampleness of the available data and the better interpretation of the obtained results about the quality and ecological status/potential of water can be achieved by application of Multivariate Statistical Analysis. The selected results of the analysis of surface water in AP Vojvodina in 2011 were analyzed by using Multivariate Statistical Analysis (cluster analysis and principal components analysis) and represented in this paper. These techniques allow the interpretation of the results of the monitoring program of investigated surface water bodies and simultaneous identification of registered influence and potential sources of pollution on the quality of the given water bodies. The both methods are applied and the division of water bodies is tested in the same manner at the origin (natural and artificial) and on the basis of territorial belonging monitoring stations (Banat and Bačka). Individual variations are discussed in corresponding differences in individual measuring stations in relation to others. By application of the given method, a grouping of the examined indicators of water quality in the following factors: hydro-chemical factor, ecological factor, the factor point pollution and diffusion have been obtained. The obtained results confirm the initial hypothesis that the use of different statistical methods can identify the main factors that have an impact on the ecological status and ecological potential of water bodies and can improve the existing monitoring. In addition, the analysis of the extracted surface water bodies can be applied in cases where it is necessary to implement simultaneous monitoring of the biological quality elements to determine whether chemical parameters can ensure the functioning of ecosystems.

Keywords: Water quality • Multivarite analysis • Cluster analysis • Principal components analysis

Synthetic cinnamates as potential antimicrobial agents

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Abstract

This study deals with synthesis of methyl cinnamate, butyl cinnamate, and *p*-methoxy methyl cinnamate and testing of their *in vitro* antimicrobial activity. Antimicrobial activity was examined towards 29 microorganisms using microdilution method. It is shown that antimicrobial activity of methyl cinnamate and *p*-methoxy methyl cinnamate was better than that of butyl cinnamate. *Sarcina lutea*, *Bacillus subtilis* ATCC 6633, *B. subtilis* and *B. subtilis* IP 5832 (probiotic) were the most sensitive bacteria. It is established that *p*-methoxymethyl cinnamate can be a new, potential anti-*Staphylococcus aureus* agent with minimum inhibitory concentration of 62.5 μg/ml. Methyl cinnamate and *p*-methoxy methyl cinnamate inhibited the growth of *Aspergillus restrictus*, *A. flavus* and *A. fumigatus* in the concentration range from 62.5 to 250 μg/ml.

Keywords: methyl cinnamate, butyl cinnamate, *p*-methoxy methyl cinnamate, antimicrobial activity, bacteria, fungi.

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Cinnamates can be found in nature as secondary metabolites widely distributed in the plant kingdom. They are synthesized by plants and, actually, represent components of essential oils. Numerous studies have demonstrated that essential oils with methyl cinnamate as one of the main components, exhibit different kind of biological activity, such as antibacterial, antifungal, antithrombotic, anti-inflammatory and antioxidative activity [1–5].

Besides naturally occurring, the cinnamates can be synthesized using different methods: esterification of corresponding cinnamic acids with alcohols [6], Wittig reaction [7], Reformatsky reaction [8], Heck reaction [9], as well as other methods [10]. Due to the characteristic flavour and fragrance, as well as high boiling point and stability, synthetic methyl, ethyl- and butyl cinnamate are widely used in food industry, especially for beverages and baked goods. Council of Europe [11] included methyl cinnamate in the group of substances safe for use in foodstuffs. Application of cinnamic acid esters in cosmetic and pharmaceutical industry is also significant. Ethyl cinnamate is used especially in the manufacture of fine products, due to its sweet, fruity and strong cinnamon odour with hints of amber and vanilla. In the perfume industry it can be used as flavour fixative agent. Butyl cinnamate is used in the manufacturing of fine fragrances, decorative cosmetics, shampoos, toilet soaps and other toiletries, as well as in non-cosmetic products such as household cleaners and detergents [12,13].

The cinnamic acid derivatives showed different biological activities like antioxidant, antidiabetic, heaptoprotective, anti-inflammatory, cytotoxic, etc. [10]. According to *in vivo* observations, methyl cinnamate is not irritating to skin and mucous membrane [14]. Studies of mutagenicity and genotoxicity have shown that in rec assay with *Bacillus subtilis* strains H17 (rec^+) and M45 (rec^-), a dose of 20 μg/disk methyl cinnamate produced no genotoxic effects, while an *in vitro* assay in Chinese hamster ovary cells showed that methyl cinnamate, at concentrations below 100 μM, did not have any cytogenetic effect [14] .

Cinnamates have wide use in different areas of industry, especially in food industry. However, there is limited information about antimicrobial activity of cinnamates as synthesized compounds, therefore, the aims of this work were to synthesize and to estimate the antimicrobial effects of methyl cinnamate, butyl cinnamate, and *p*-methoxy methyl cinnamate on broad panel of bacteria and fungi.

MATERIALS AND METHODS

General

Methyl, butyl and *p*-methoxy methyl cinnamates were synthesized using the palladium-catalyzed phosphine-free Heck reaction. Different bases and ionic liquids [15,16] were used (Figure 1).

The Heck reaction products were analyzed with GC–MS chromatography and ^{1}H-NMR spectroscopy. GLC analyses were obtained with an Agilent 6890N (G 1530N) instrument (Serial # CN10702033), with capillary apolar column. The ^{1}H-NMR spectra were run in

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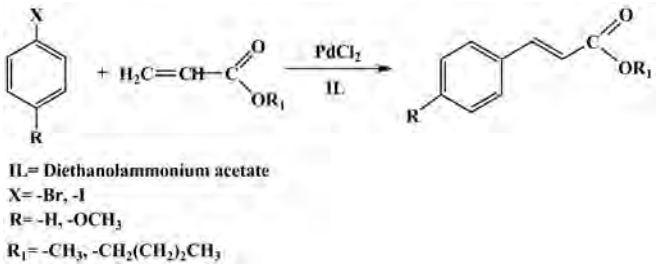

Figure 1. Synthesis of investigated cinnamates.

$CDCl_3$ on a Varian Gemini 200 MHz spectrometer. ^{1}H--NMR spectra are given in Supplementary material. The compounds $PdCl_2$, diethanolamine, aryliodide, olefins and amphotericin B were obtained from Aldrich Chemical Co, St. Louis, USA. Nutrient media, Mueller–Hinton broth was from Liofilchem, Roseto, Italy, while Sabouraud dextrose broth was from Torlak, Belgrade, Serbia. Doxycycline and fluconazole were from Galenika A.D., Belgrade, Serbia, and Pfizer Inc., New York, USA, respectively. Resazurin was from Alfa Aesar GmbH & Co, Karlsruhe, Germany.

In vitro antimicrobial assay

Test microorganisms

Antimicrobial activity was tested against 20 strains of bacteria and 9 strains of fungi. The list of tested microorganisms was presented in Tables 1 and 2. All clinical isolates of bacteria were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The other microorganisms (the ATCC strains and fungi) were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

Bacterial and yeast suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard. When adjusted to the turbidity of the 0.5 McFarland's standard, bacteria suspension contains about 10^8 colonies forming units (CFU)/mL and suspension of yeast contains 10^6 CFU/mL. The initial suspensions were additionally diluted in 1:100 ratio in sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spore from agar slants with growing aspergilli. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) using microdilution method with resazurin [17]. The tested compounds were first dissolved in dimethyl sulfoxide (DMSO) (10% of total volume) and then into nutrient liquid medium (up to 100% of total volume). The stock concentrations of tested compounds were 2000 μg/mL. Next, serial twofold dilutions were made in a concentration range from 7.81 to 1000 μg/mL in sterile 96-well microtiter plates containing Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi. After that, 10 μL of diluted bacterial, yeast suspensions and suspensions of spores was added to appropriate wells. Finally, 10 μL resazurin solution, as an indicator of microbial growth, was added to each well inoculated with bacteria and yeasts. Resazurin is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 24 h for bacteria, 28 °C for 48 h for yeasts and 28 °C for 72 h for moulds. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink. For moulds, MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelia growth. Minimum microbicidal concentration was determined by plating 10 μL of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration (MMC).

Doxycycline, fluconazole and amphotericin B, dissolved in nutrient liquid medium, were used as a positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. Also, in the experiment, the concentration of DMSO was subsequently decreased because of the twofold serial dilution assay, resulting in a working DMSO concentration of 5% or lower. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Statistical analysis

Data were analyzed using the Student's *t*-test and the one-way analysis of variance (ANOVA). In all cases p values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS package.

RESULTS

Synthesis of cinnamates

Methyl cinnamate (**MC**), butyl cinnamate (**BC**), and *p*-methoxy methyl cinnamate (**MMC**) were synthesized using the Heck reaction and subjected to investigation of their antimicrobial activity.

Antimicrobial activity

The results of *in vitro* testing of antibacterial and antifungal activities of cinnamates in relation to 29 species of microorganisms are shown in Tables 1 and 2. The tested compounds showed different degree of antimicrobial activity. *MIC* values were in range from 15.6 to >1000 μg/ml while MMC values were from 31.25 to >1000 μg/ml. It is found that **MC** and **MMC** exhibited better antimicrobial activity than BC ($p < 0.05$). Solvent control showed that 10% DMSO did not inhibit the growth of microorganism.

Regarding the tested bacteria, Gram-positive bacteria and probiotics were more sensitive than Gram negative bacteria ($p < 0.05$). The most sensitive bacteria were *Sarcina lutea*, *Bacillus subtilis* ATCC 6633, *B. subtilis* and *B. subtilis* IP 5832 (probiotic). In these cases, *MIC*s were 15.6 and 31.25 μg/ml, while *MMC*s were between 31.25 and 125 μg/ml. Moderate activity was shown against *S. lutea* ATCC 9341, *Staphylococcus aureus* and *Proteus mirabilis* (standard and clinical strain) with *MIC* at 62.5 and 125 μg/ml. The growth of *Enterococcus faecalis* ATCC 29212, *E. faecalis*, *Escherichia coli* ATCC 25922, *E. coli*, *Salmonella enterica*, *S. typhimurium*, *Lactobacillus rhamnosus* was not affected at least by one tested compounds.

Antifungal activity was a little bit better against moulds than yeasts, but not statistically significant ($p > 0.05$). *Aspergillus restrictus*, *A. fumigatus*, *A. flavus* have shown sensitivity at the lowest concentration (*MIC* = 250 μg/ml for **MC** and *MIC* = 62.5 and 125 μg/ml for **MMC**).

DISCUSSION

In vitro antibacterial and antifungal activity of cinnamates were tested against a panel of microorganisms including human pathogenic bacteria, yeasts, moulds, probiotics in order to evaluate broad-spectrum antimicrobial activity. Significant results were obtained for bacteria *S. lutea* and *B. subtilis.* These bacteria are important as contaminants because they are ubiquitous in the environment, particulary in soil and air. Therefore, they find their way easily into food products or colonize skin, gastrointestinal and respiratory tracts

*Table 1. Antibacterial activity (μg/ml) of methyl cinnamate (**MC**), butyl cinnamate (**BC**) and p-methoxy methyl cinnamate (**MMC**)*

Species	**MC**		**BC**		**MMC**		Doxycycline	
	MIC	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*
Gram-positive bacteria								
Sarcina lutea ATCC 9341	125	250	n.t.	n.t.	62.5	250	< 0.45	7.81
Sarcina lutea	31.25	125	>1000	>1000	15.6	62.5	< 0.45	3.75
Enterococcus faecalis ATCC 29212	1000	>1000	>1000	>1000	>1000	>1000	7.81	62.5
Enterococcus faecalis	>1000	>1000	>1000	>1000	>1000	>1000	7.81	62.5
Bacillus subtilis ATCC 6633	31.25	125	>1000	>1000	15.6	31.25	1.95	31.25
Bacillus subtilis	31.25	62.5	250	>1000	31.25	31.25	0.11	1.95
Staphylococcus aureus ATCC 25923	500	>1000	>1000	>1000	1000	>1000	0.22	3.75
Staphylococcus aureus	125	1000	>1000	>1000	62.5	1000	0.45	7.81
Gram-negative bacteria								
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	>1000	>1000	15.62	31.25
Escherichia coli	1000	>1000	>1000	>1000	>1000	>1000	7.81	15.63
P. aeruginosa ATCC 27853	1000	>1000	>1000	>1000	1000	>1000	62.5	125
Pseudomonas aeruginosa	1000	>1000	250	>1000	1000	>1000	250	> 250
Proteus mirabilis ATCC 12453	250	500	>1000	>1000	125	500	15.62	62.5
Proteus mirabilis	500	1000	>1000	>1000	125	500	250	> 250
Salmonella enterica	1000	>1000	>1000	>1000	>1000	>1000	15.62	31.25
Salmonella typhimurium	1000	>1000	>1000	>1000	>1000	>1000	15.62	125
Probiotics								
Lactobacillus rhamnosus	>1000	>1000	31.25	62.5	>1000	>1000	7.81	31.25
Lactobacillus plantarum	250	500	>1000	>1000	250	250	0.45	7.81
Bifidobacterium animalis subsp. *lactis*	500	500	31.25	62.5	1000	>1000	31.25	62.5
Bacillus subtilis IP 5832	125	500	250	500	31.25	125	1.95	15.63

*Table 2. Antifungal activity (μg/ml) of methyl cinnamate (**MC**), butyl cinnamate (**BC**) and p-methoxy methyl cinnamate (**MMC**)*

Species	**MC**		**BC**		**MMC**		Fluconazole		Amphotericin B	
	MIC	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*
Yeasts										
*Candida albicans*ATCC 10231	1000	> 1000	1000	> 1000	500	1000	31.25	1000	0.49	1.95
Candida albicans	1000	1000	1000	> 1000	500	1000	62.5	1000	0.98	1.95
Rhodotorula sp.	500	1000	500	1000	1000	1000	62.5	1000	3.9	3.9
Saccharomyces boulardii	250	500	n.t.	n.t.	250	500	31.25	1000	n.t.	n.t.
Moulds										
Aspergillus niger ATCC 16404	500	> 1000	> 1000	> 1000	500	> 1000	62.5	62.5	0.98	1.95
Aspergillus niger	500	> 1000	1000	> 1000	500	> 1000	500	1000	0.98	0.98
Aspergillus restrictus	250	1000	500	> 1000	125	250	500	1000	0.98	1.95
Aspergillus fumigatus	250	1000	1000	> 1000	125	250	500	1000	3.9	3.9
Aspergillus flavus	250	1000	1000	> 1000	62.5	1000	1000	1000	0.98	15.63

of people causing food poisoning or human infection. Also, *S. aureus* was inhibited by **MMC** at low concentration. This compound can be a new, potential anti-*S. aureus* agent since this bacterium has been developing resistance to common antibiotics. Majority of Gram-negative bacteria showed moderate sensitivity to tested cinnamates. It is known that Gram-negative bacteria, in contrast to Gram-positive bacteria, contain the outer membrane which serves as a permeability barrier and prevent the entry of noxious compounds including antibacterial compounds. With respect to activity of positive control, a broad-spectrum antibiotic doxycycline, the activity of tested compounds was lower. But, if we take into consideration the results of other studies about antibacterial activity of naturally occurring cinnamates or synthesized cinnamates, it can be concluded that *MIC* values below 1000 μg/ml indicate significant activity of cinnamates and their potential application as antibacterial agents [1–3,18,19]. Narasimhan *et al*. [18] synthesized and evaluated antimicrobial activity of series of esters, substituted derivatives and amides of cinnamic acid. The compounds, isobutyl cinnamate and dibromo cinnamic acid, were the most effective substances displaying growth inhibition of all the tested microorganisms. The authors observed that the removal of double bond in side chain of cinnamic acid was effected with –OH and –Br group. Also, the results have shown that addition of halogens to the side chain caused remarkable increase in growth inhibitory effect of cinnamic acid, whereas addition of hydroxy groups to the side chain double bond did not remarkably enhance the antimicrobial activity. Venkateswarlu *et al*. [19] tested derivatives of polyhydroxy cinnamic acid among which butyl hydroxy cinnamates showed high antibacterial and low antifungal activity. The most sensitive bacterium was *B. subtilis,* similarly with results in our study.

Moulds are ubiquitous microorganisms with a great capacity to colonize many kinds of substrates. Also, they produce mycotoxins that can be teratogenic, carcinogenic. In this study, tested compounds **MC** and **MMC** showed marked inhibitory activity of the growth of *A. restrictus*, *A. flavus* and *A*. *fumigates*. However, that activity was lower than positive control amphotericin B. Amphotericin B was used as a control since it is a drug of choice for infection caused by *Aspergillus* species. *MIC*s of amphotericin B for most *Aspergillus* species are clustered between 0.5 and 2 μg/ml, so for tested *A. fumigatus* strain with *MIC*s above 2 μg/ml, the activity of amphotericin B is associated with a high probability of therapeutic failure [20]. Among the tested compounds, **MMC** was the most active against *A. fumigatus*. The activity of tested cinnamates against the bacteria and fungi may open the possibility of their use as a preservative in food industry or as antimicrobial agents in pharmaceutical industry.

CONCLUSIONS

In this study synthetic cinnamates: methyl cinnamate, butyl cinnamate and *p*-methoxy methyl cinnamate were tested for their *in vitro* antibacterial and antifungal activity. It is found that methyl cinnamate and *p*-methoxy methyl cinnamate have shown better antimicrobial activity than butyl cinnamate. These synthetic cinnamates could be potential antimicrobial agents against food spoilage and human pathogenic bacteria and fungi.

Supplementary material

^{1}H-NMR spectra of investigated compounds are available from corresponding author on request.

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IZVOD

SINTETISANI CINAMATI KAO POTENCIJALNI ANTIMIKROBNI AGENSI

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(Naučni rad)

Cinamati, osim što su prisutni u prirodi kao produkti sekundarnog metabolizma biljaka, mogu se i sintetisati primenom više različitih metoda. Poznato je da ispoljavaju brojna biološka delovanja, a i da se primenjuju u industriji. Stoga, ciljevi ovog radu su sinteza cinamata, metil cinamat, butil cinamat i *p*-metoksi metil cinamat, i testiranje njihove antimikrobne aktivnosti. Antimikrobna aktivnost je ispitana mikrodilucionom metodom u odnosu na 29 vrsta mikroorganizama. Testirana jedinjenja, metil cinamat i *p*-metoksi metil cinamat, su pokazala bolju aktivnost nego jedinjenje butilcinamat. Bakterije *Sarcina lutea*, *Bacillus subtilis* ATCC 6633, *B*. *subtilis* i *B*. *subtilis* IP 5832 (probiotik) su najosetljiviji mikroorganizmi sa minimalnim inhibitornim koncentracijama (*MIK*) od 15,6 do 31,25 μg/ml, dok su minimalne mikrobiocidne koncentracije (*MMK*) bile od 31,25 do 125 μg/ml. Na osnovu prikazane aktivnosti (*MIK* = 62,5 μg/ml), uočeno je da *p*-metoksi metil cinamat može da predstavlja budući, potencijalni agens protiv infekcija izazvanih bakterijom *Staphylococcus aureus*. Testirana jedinjenja metil cinamat i *p*-metoksi metil cinamat su inhibirala rast gljiva, proizvođača mikotoksina, *Aspergillus restrictus*, *A. flavus* i *A. fumigates* u koncentracijama od 62,5 do 250 μg/ml. Prikazani rezultati potvrđuju antimikrobnu aktivnost testiranih cinamata i ukazuju na njihovu primenu u borbi protiv štetnih bakterija i gljiva.

Ključne reči: Metil cinamat • Butil cinamat • *p*-Metoksi metil cinamat • Antimikrobna aktivnost • Bakterije • Gljive

Mogućnost primene ferata(VI) u tretmanu efluenta industrijske otpadne vode u laboratorijskim uslovima

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Izvod

U ovom radu prikazani su efekti primene ferata(VI) u tretmanu efluenta industrijske otpadne vode (IOV) u laboratorijskim uslovima. Korišćeni uzorci su mešavina efluenata otpadne vode iz različitih industrijskih postrojenja čiji sastav je određen analizom uzoraka pre tretmana feratom(VI). Određivanjem fizičko–hemijskih karakteristika uzoraka, nađena je veoma visoka vrednost hemijske potrošnje kiseonika (HPK), 833,28 mg/l (uzorak 1) odnosno 26331 mg/l (uzorak 2), kao i koncentracija pojedinih polutanata viša od maksimalno dozvoljene. Primenjeni Na_2FeO_4 sintetisan je elektrohemijskom metodom i primenjivan *in situ*. Fizičko–hemijskim ispitivanjem uzoraka, posle tretmana različitom količinom (2, 5, 8 i 10 ml) Na_2FeO_4 koncentracije 8 g/l pokazano je da se ferat(VI) može koristiti kao višefunkcionalno sredstvo pri prečišćavanju industrijske otpadne vode, pri čemu se količina zagađujućih materija svodi ispod maksimalno dozvoljenih vrednosti. Pokazana je visoka efikasnost ferata(VI) kao snažnog osidansa pri uklanjanju ukupnog P i do 99,5%, kao i suspendovanog materijala. Takođe je ukazano na visoku sorpcionu moć nastalog gvožđe(III)-hidroksida koji svojom razvijenom površinom apsorbuje 95,5% F^- i uklanja ih iz rastvora u obliku mulja.

Ključne reči: industrijska otpadna voda, ferat(VI), uklanjanje P i F^-.

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NAUČNI RAD

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Industrijske otpadne vode (IOV) nastaju upotrebom vode u tehnološkim procesima kao i u procesima proizvodnje energije. Sadržaj zagađujućih materija u IOV, koje su danas najveći zagađivači vodnih resursa, potiče iz proizvodnih procesa i varira u zavisnosti od vrste industrije: metalska, industrija celuloze i papira, farmaceutska, industrija prerade kože, nafte, gasa, prehrambena industrija, duvanska industrija, itd. S obzirom na postojeće direktive i zakonske uredbe [1–4] o sastavu efluenta IOV pre ispuštanja u recipijent, svuda u svetu se izuzetna pažnja poklanja postrojenjima i metodama za prečišćavanje IOV. Kontrolom voda iz industrijskih postrojenja na ulazu u recipijent od strane referentnih laboratorija dobijaju se podaci o kvantitativnom i kvalitativnom prisustvu zagađujućih materija u IOV. Metode koje se koriste pri procesima prečišćavanja IOV ne daju uvek zadovoljavajuće rezultate u odnosu na maksimalno dozvoljene koncentracije polutanata, usled složenog sastava IOV kao i usled formiranja mnogih nusproizvoda tokom tretmana konvencionalnim metodama [5]. U tretmanu voda kao oksidaciona i dezinfekciona sredstva najčešće se primenjuju hlor, hipohlorit, hlor-dioksid, ozon, vodonik peroksid ili njihova kombinacija, kao i UV katalitički oksidacioni procesi. Međutim, problem ovih metoda je formiranje potencijalno štetnih bioprodukata kao trihalometana i bromida [6]. Osim što postoji potreba za ekološki povoljnim oksidansima, takođe postoji potreba i za ekološki povoljnim koagulantima jer se pokazalo da koagulanti na osnovi aluminijum-sulfata ili polialuminijum-hlorida značajno gube moć koagulacije u vodama bogatim organskim materijalom, što ih čini manje efikasnim, a posebno na temperaturama prerade voda nižim od 15 °C [7].

Primena ferata(VI) u tretmanu otpadnih voda (OV) predstavlja potencijalnu atraktivnu alternativu kao samostalni višenamenski proces tretmana ili integrisan sa konvencionalnim metodama. U procesu tretmana OV ferat(VI) je moguće koristiti kao snažno oksidaciono, koagulaciono i flokulaciono sredstvo i kao efikasan dezinfektant [8–10] koji u poređenju sa uobičajenim sredstvima tretmana ne daje toksične bioprodukte [11]. Jedna od bitnih prednosti ferata(VI) je netoksičnost $Fe(OH)_3$, snažnog koagulanta i flokulanta, koji nastaje redukcijom ferata(VI) pri oksidaciji polutanata prisutnih u OV različitog porekla i sastava [7,12,13]. Upotreba ferata(VI) kao višefunkcionalnih hemijskih reagenasa ima direktne aplikativne prednosti u jednostavnosti i ekonomskoj isplativosti postupka tretmana IOV, procednih voda deponija, pijaćih voda, sanitarnih otpadnih voda i otpadnih voda agrara. Primenom ferata(VI) u procesu tretmana voda postiže se izbegavanje formiranja toksičnih nusproizvoda, korišćenje jedne hemikalije, jednog sistema za doziranje i mešanje, manju dozu hemikalije nego kod konvencionalnih oksidanata i koagulanata, pa samim tim i manju proizvodnju mulja [14].

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Istraživanja [15] pokazuju visoku efikasnost ferata(VI) pri uklanjanju mikropolutanata u postupcima obrade OV. Ferat(VI) ima mogućnost uklanjanja polutanata prisutnih u koncentracijama reda μg/L do ng/L koji se konvencionalnim matodama tretmana OV ne mogu ukloniti ili je preostala koncentracija polutanata viša od maksimalno dozvoljene [16].

Visok oksido–redukcioni potencijal ferata(VI) u odnosu na konvencionalna sredstva dezinfekcije vode, oslobađanje nascentnog kiseonika pri oksidaciji vode, kao i izostanak formiranja hlornih i drugih toksičnih nusproizvoda, daje izuzetnu prednost feratu(VI) kao dezinfektantu. Poređenja su pokazala [17,18] veću efikasnost ferata(VI) primenjenog za prečišćavanje vode u odnosu na aluminijum sulfat i gvožđe sulfat, i to za 50% kod smanjenja zamućenja, 30% kod smanjenja ukupne hemijske potrošnje kiseonika i 10% veću efikasnost uništavanja bakterija.

Reakcija redukcije ferata(VI) u kiseloj sredini prikazana je jednačinom (1) [19]:

$$FeO_4^{2-} + 8H^+ + 3e^- = Fe^{3+} + 4H_2O,\ E_0 = 2{,}20\ V \quad (1)$$

a u alkalnoj sredini reakcijom (2) [19]:

$$FeO_4^{2-} + 4H_2O + 3e^- = Fe(OH)_3 + 5OH^-,\ E_0 = 0{,}72\ V \quad (2)$$

dok se pri oksidaciji vode feratom(VI) oslobađa nascentni kiseonik, snažni oksidacioni agens [20]:

$$FeO_4^{2-} + 5H_2O + 3e^- = Fe^{3+} + O_2 + 10OH^- \quad (3)$$

Feratom(VI) se mogu efikasno oksidovati različite vrste organskih i neorganskih jedinjenja i kompatibilan je sa drugim metodama prerade OV pa ih je moguće kombinovati [21]. Prema istraživanjima [22], maksimalan opseg procenta oksidacije faratom(VI), pri pH 8 je bio 18–47%, 23–47%, 85–100% i 32–55% za benzen, hlorobenzen, alilbenzen i 1-heksen-4-ol, redom, dok se fenoli kao frekventne zagađujuće materije u otpadnim i rečnim vodama mogu ukloniti od 99 do 100% tretmanom feratom(VI) koncentracije 0,1–2,0 mg/dm^3 tokom delovanja u trajanju od 30 min pri pH > 8 na temperaturi 25 °C [23]. Proučavana je i oksidacija nekoliko neorganskih polutanata, kao što su cijanidi [24,25]:

$$2HFeO_4^- + 3HCN + OH^- \rightarrow$$
$$\rightarrow 2Fe(OH)_3 + 3CNO,\ [Fe(VI)]:[CN]=1:1{,}1 \quad (4)$$

amonijak [24,26]:

$$2K_2FeO_4 + 2NH_3 + (n–1)\ H_2O \rightarrow$$
$$\rightarrow N_2 + Fe_2O_3{\cdot}nH_2O + 4KOH,\ [Fe(VI)]:[NH_3] = 1:1 \quad (5)$$

$$2K_2FeO_4 + NH_3 + nH_2O \rightarrow$$
$$\rightarrow KNO_2 + Fe_2O_3{\cdot}nH_2O + 3KOH \quad (6)$$

$$8K_2FeO_4 + 3NH_3 + (n+2)\ H_2O \rightarrow$$
$$\rightarrow 3KNO_3 + 4Fe_2O_3{\cdot}nH_2O + 13KOH \quad (7)$$

i vodonik-sulfid [27,28]:

$$8HFeO^- + 3H_2S + 6H_2O \rightarrow$$
$$\rightarrow 8Fe(OH)_3 + 3SO_4^{2-} + 2OH,\ [Fe(VI)]:[H_2S] = 2:5 \quad (8)$$

Istovremeno odigravanje procesa oksidacije i koagulacije primenom ferata(VI) omogućava uklanjanje niza metala i teških metala iz rastvora čak i arsena(III) koji se efikasno oksidiše feratom(VI) prema jednačini (9), a zatim koagulacijom i flokulacijom nastalim $Fe(OH)_3$ uklanja u vidu mulja [29,30]:

$$2HFeO_4^- + 3H_3AsO_3 \rightarrow 2Fe_3^+ + 3HAsO_4^- \quad (9)$$

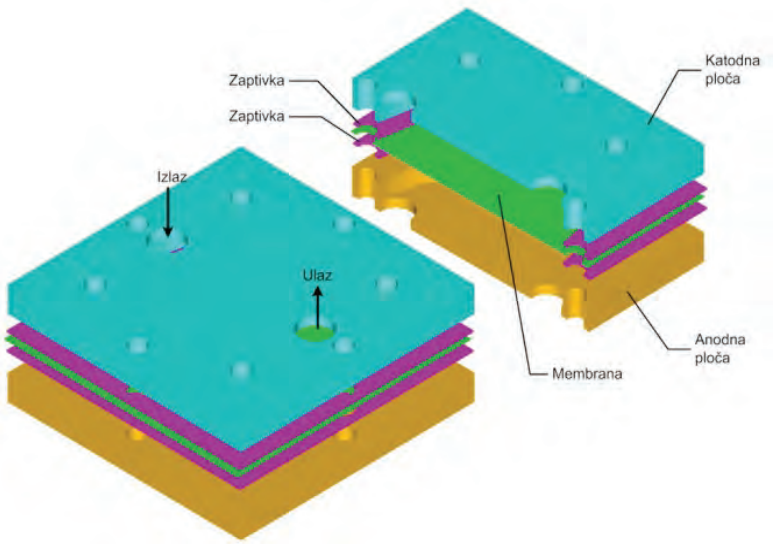
Ferat(VI) se može dobiti hemijskom sintezom: oksidacijom feri jedinjenja hipohloritima u jako alkalnim vodenim rastvorima i u rastopima oksidacijom feri jedinjenja alkalnim peroksidima, ili elektrohemijskom sintezom, anodnim ratvaranjem gvožđa u jako alkalnim rastvorima (pH > 10) u transpasivnoj oblasti [31,32]. Najprihvatljiviji metod sinteze ferata(VI) pri kontinualnoj primeni je elektrohemijska sinteza pri čemu se ferat(VI) *in situ* primenjuje pri prečišćavanju OV. Višegodišnjim naučno istraživačkim radom, prvi put kod nas, uspešno je elektrohemijskom metodom sintetisan ferat(VI), a uz optimizaciju i objašnjenje anodnih procesa [32–34].

Ovaj rad prikazuje rezultate laboratorijskog eksperimenta primene elektrohemijski sintetisanog ferata(VI) u tretmanu efluenta IOV. Cilj eksperimenta je bio da se pokaže mogućnost i efikasnost primene ferata(VI) pri procesu uklanjanju polutanata iz efluenta IOV.

S obzirom na to da je tretman rađen samo na laboratorijskom nivou neophodno je dalje optimizovati količinu primenjenog ferata(VI) za pojedinačne polutante kao i obezbediti ponovljivost dobijenih rezultata.

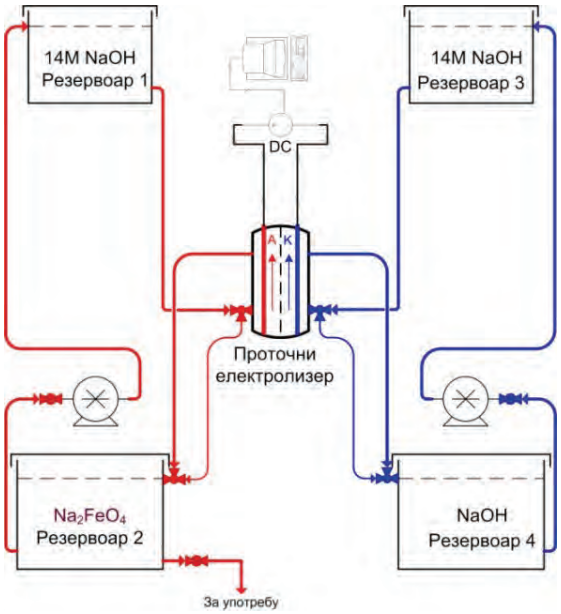
EKSPERIMENTALNI RAD

Korišćeni rastvor 10 M NaOH pripreman je od NaOH p.a. kvaliteta, proizvođača Centrohem, Stara Pazova. Za pripremu rastvora korišćena je demineralizovana voda. Rastvor Na_2FeO_4 koncentracije 8 g/l korišćen za tretman uzoraka efluenta IOV sintetisan je elektrohemijskim postupkom. Postupak elektrohemijske sinteze alkalnog rastvora ferata(VI) vršen je u laboratorijskom postrojenju za elektrohemijsku sintezu ferata(VI) sa protočnom dvodelnom elektrohemijskom ćelijom [34], slike 1 i 2, i zasnovan na transpasivnom anodnom rastvaranju legura gvožđa u rastvoru 10 M NaOH, u skladu sa prethodnim istraživanjima [32,33]. Anoda je od legure gvožđa sa sadržajem: silicijuma između 1,6 i 6%, ugljenika do 0,1%, mangana do 0,1%, bakra, sumpora i aluminijuma na nivou nečistoća. Katoda je od nerđajućeg čelika u obliku lima. Postupak anodnog rastvaranja vršen je u vremenu od 3 časa jačinom struje od 2,5 A (j = 85 mA cm^{-2}) i pri temperaturi od 25 °C, pri



Slika 1. Šema protočne elektrohemijske ćelije za sintezu ferata(VI).
Figure 1. Scheme of the flow electrochemical cell for the synthesis of ferrate(VI).

čemu je dobijen rastvor Na_2FeO_4 koncentracije 8 g/l. Koncentracija sintetisanog ferata(VI) kontrolisana je titrimetrijskom hromitnom metodom [35] pri temperaturi od 25 °C. Za tretman uzoraka efluenta IOV korišćen je sveže sintetisan ferat(VI).



Slika 2. Šema pilot postrojenja za sintezu ferata(VI).
Figure 2. Scheme of the pilot plant for the synthesis of ferrate(VI).

Uzorci IOV korišćeni u eksperimentu uzorkovani su iz rezervoara smeše efluenata više vrsta IOV koji potiču iz različitih industrija: gumarske, prehrambene, proizvodnje toplotne energije, farmi. Korišćena IOV prethodno je tretirana nekom od osnovnih metoda tretmana kao što su taloženje prisutnog čvrstog materijala, filtracija i neutralizacija. Rezultati kvalitativne i kvantitativne analize uzoraka smeše efluenata IOV dati su u tabeli 1.

Korišćena su dva uzorka zapremine od po V = 1 l, fizičko–hemijskih karakteristika datih u tabeli 1. Oba ispitivana uzorka, najpre su tretirana sa 10 ml 10 M NaOH i posle taloženja od 24 h dekantovana. Posle tretmana NaOH izmerena je vrednost pH=12 kod oba uzorka i 1 M sumpornom kiselinom podešena na vrednost pH 9. U dekantovane uzorke rastvora od 250 ml dodato je po 2, 5, 8, i 10 ml Na_2FeO_4 koncentracije 8 g/l. Posle taloženja od 24 h uzorci su dekantovani i 1 M sumpornom kiselinom pH vrednost oba dekantovana rastvora je podešena do pH 7.

U tabeli 1 dati su rezultati kvalitativne i kvantitativne analize uzoraka IOV posle tretmana feratom(VI).

REZULTATI I DISKUSIJA

Analiza fizičko–hemijskih karakteristika ispitivanih uzoraka efluenta IOV posle tretmana Na_2FO_4, tabela 1, pokazala je izuzetnu efikasnost ferata(VI) u uklanjanju organskih i neorganskih zagađujućih materija. U ispitivanim uzorcima, HPK je bio 833,28 mg/l (uzorak 1) odnosno 26331 mg/l (uzorak 2) što predstavlja 18 odnosno 580 puta veću vrednost od dozvoljene (dozvoljeno 45–150 mg/l) u zavisnosti od vrste tehnološke otpadne vode [4], dok su koncentracije pojedinih metala bile nekoliko puta veće od dozvoljenih (Cu dozvoljeno 0,1 mg/l, Zn dozvoljeno 0,2 mg/l) [4]. Takođe, nađena je visoka koncentracija ukupnog fosfora (dozvoljeno 2 mg/l) i fluorida (dozvoljeno 20 mg/l), 2–3 puta veća od dozvoljene [4]. Zabeležena je i izuzetno visoka kiselost ispitivane IOV pH < 1 koja je nastala zakišeljavanjem uzoraka sumpornom kiselinom u cilju

Tabela 1. Fizičko–hemijske karakteristike efluenta industriske otpadne vode – uzorci 1 i 2, pre i posle tretmana sa Na_2FO_4
Table 1. Physicochemical characteristics of industrial wastewater effluent – samples 1 and 2, before and after the treatment

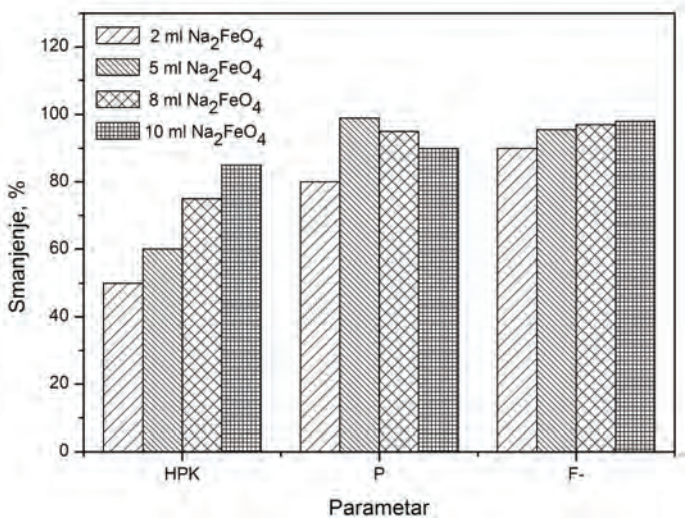
Parametar	Jedinica	Rezultati analize OV uzorak 1		Rezultati analize OV uzorak 2	
		Pre tretmana	Posle tretmana	Pre tretmana	Posle tretmana
Suspendovane materije	–	0,074	290	0,194	878
Taložne materije	mg/l	< 0,3	< 0,1	< 1	< 0,1
HPK	mg/l	833,28	333	26331	2 916
Amonijak	mg/l	7,9615	8,70	1,0839	44,75
Nitriti	mg/l	0,2902	3,12	0,2829	0,560
Nitrati	mg/l	44,64	100	42,66	128,4
Ukupan fosfor	mg/l	5,06	0,025	4,16	0,92
Fluoridi	mg/l	49	2,22	51,6	2,61
Sulfati	mg/l	2197	13917	9578	13898
Olovo	mg/l	< 0,030	< 0,03	0,072	< 0,03
Cink	mg/l	0,116	0,02	0,498	0,021
Bakar	mg/l	2,945	0,174	9,205	0,0848
Kadmijum	mg/l	< 0,005	0,005	< 0,005	< 0,005
Nikl	mg/l	0,016	0,002	0,048	0,002
pH	–	< 1	6,61	< 1	7,43

konzervacije. Iz tog razloga su najpre uzorci tretirani čistim 10 M NaOH što je povećalo pH vrednost, ali i uklonilo deo prisutnih jona metala koji su prešli u nerastvorno stanje i kao takvi uklonjeni iz rastvora. U tabeli 1 prikazani su rezultati fizičko-hemijske analize uzoraka od 250 ml IOV, pre i posle tretmana sa 5 ml Na_2FeO_4 koncentracije 8 g/l. Analiza uzoraka koji su tretirani sa 2, 8 i 10 ml Na_2FeO_4 rađena je samo za pojedinačne parametre (HPK, P i F^-) i uporedni rezultati su prikazani na histogramima, slike 3 i 4.

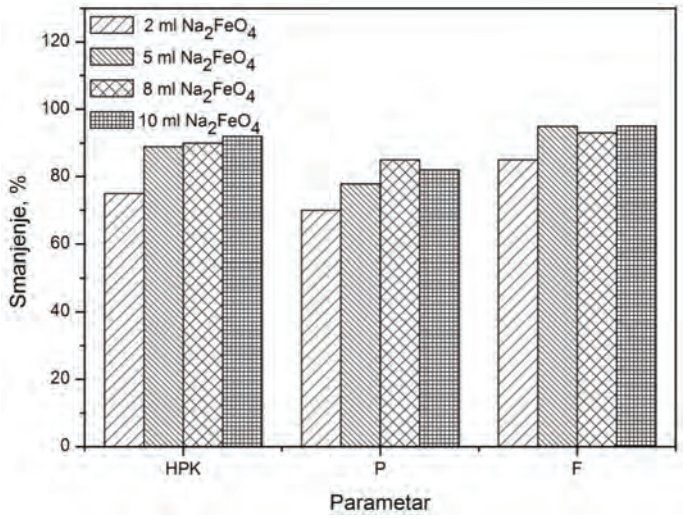
Predtretmanom uzoraka IOV NaOH eliminisan je iz rastvora deo polutanata, jona metala. Sa jedne strane predtretman NaOH umanjuje količinu potrebnog ferata(VI) za oksidaciju prisutnih polutanata, jer se ferat(VI) neće trošiti na uklanjanje metala. Istovremeno, NaOH rastvoru podešava vrednost od pH 9 pri kome je ferat(VI) najstabilniji [7,36].

Na slikama 5 i 6 jasno se uočava promena u rastvoru nakon dodatka ferata(VI), koagulacija i flokulacija prisutnih zagađujućih materija fero(III)hidrokisidom koji nastaje kao produkt redukcije ferata(VI). Gvožđe(III)-hidroksid ima veoma razvijenu površinu što mu omogućava adsorpciju nastalih produkata u reakciji oksidacije prisutnih polutanata feratom(VI), njihovu koagulaciju, flokulaciju i uklanjanje u obliku mulja.

Prema fizičko–hemijskim karakteristikama ispitanih uzoraka pre i posle tretmana efluenta IOV, koncentracija većine polutanata smanjena je za oko 60–99% u



Slika 3. Uporedni rezultati hemijske analize odabranih parametara uzorka 1 otpadne vode pre i posle tretmana.
Figure 3. Comparative results of the chemical analysis of selected parameters sample 1 wastewater before and after treatment.



Slika 4. Uporedni rezultati hemijske analize odabranih parametara uzorka 2 otpadne vode pre i posle tretmana.
Figure 4. Comparative results of the chemical analysis of selected parameters sample 2 wastewater before and after treatment.

Slika 5. Izgled uzorka otpadne vode pre tretmana feratom(VI).
Figure 5. Physical samples of wastewater before treatment by ferrate(VI).

Slika 6. Izgled uzorka otpadne vode po dodatku ferata(VI), koagulacija i obrazovanje flokula.
Figure 6. Physical samples of wastewater by addition of ferrate (VI), coagulation and formation of flocs.

odnosu na početno stanje. S obzirom na to da su metali uglavnom istaloženi i uklonjeni natrijum(I)hidroksidom značajno je uporediti procenat smanjenja HPK, P i F^- u zavisnosti od koilčine dodatog ferata(VI). Prema rezultatima prikazanim na histogramima, slike 3 i 4, značajno je smanjenje koncentracije ukupnog P, 7099,5%, čijem se uklanjanju u tretmanu voda posvećuje velika pažnja [37,38] jer je direktan uzročnik eutrofikacije (prekomernog rasta algi), i fluorida od 89-95,5% u odnosu na početnu vrednost. Međutim, s obzirom na to da je ferat(VI) neselektivno oksidaciono sredstvo veliki deo primenjenog ferata(VI) troši se na oksidaciju organskog materijala. Kako je nivo HPK tretiranih uzoraka IOV bio visok naročito u uzorku 2 i efikasnost uklanjanja P je nešto smanjena. Snažna koagulaciona svojstva $Fe(OH)_3$ dolaze do izražaja upravo u uklanjanju F^- koji se mogu uklanjati upravo kompleksiranjem i koagulacijom sa Fe^{3+} [39]. Sa povećanjem količine dodatog ferata(VI) dolazi do nešto većeg procenta eliminisanja P. Međutim, istovremeno se povećava i količina nastalog $Fe(OH)_3$ u reakciji oksidacije polutanata, koji svojim snažnim sorpcionim svojstvima značajno povećava količinu uklonjenih fluorida. Formiranje veće količine $Fe(OH)_3$, usled katalitičkog efekta $Fe(OH)_3$ na proces raspadanja ferata(VI), prouzrokuje slabije iskorišćenje ferata(VI) u procesu oksidacije polutanata. Takođe treba ukazati na činjenicu da se usled veoma pozitivnog oksido-redukcionog potencijala deo ferata(VI) utroši na oksidaciju vode prema jednačini (3).

Rezultati analize IOV, tabela 1, posle tretmana feratom(VI) pokazuju i znatno povećanje prisutnog amonijaka, nitrata i nitrita, što je posledica prisustva velike kontaminiranosti organskim i biološkim materijalom koje sadrže azotna jedinjenja, a koje ferat(VI) oksidiše i razgrađuje do amonijaka, nitrata i nitrita. Uklanjanje azotnih jedinjenja iz rastvora može se vršiti procesima nitrifikacije odnosno oksidacije NH_3 do NO_3 pod dejstvom nitrifikacionih bakterija u aerobnim uslovima i denitrifikacije, redukcije NO_3 bez prisustva O_2 do elementarnog azota [40]. Međutim, oksidacija NH_3 se

takođe može uspešno vršiti feratom(VI) [26] prema jednačinama (6)–(8) pri čemu se pod određenim uslovima (Fe:NH_3 = 1:1 i pH 9–10) 83% NH_3 oksidiše do elementarnog azota, jednačina (6). Kako je u ranijim istraživanjima [10] pokazano ponovnim tretmanom ispitivanih uzoraka IOV feratom(VI), prisustvo amonijaka, nitrata i nitrita bilo bi značajno smanjeno jer bi se usled jako male konecentracije drugih polutanata ferat(VI) trošio na oksidaciju amonijaka, nitrata i nitrita. Proces denitrifikacije se time može izbeći.

Povećana koncetracija sulfata u ispitivanim uzorcima, posledica je dodavanja sumporne kiseline rastvoru zbog podešavanja pH vrednosti za optimalno delovanje ferata(VI). Posle tretmana IOV feratom(VI) prisutne sulfate je moguće lako neutralizovati rastvorom $Ca(OH)_2$ i ukloniti ih iz rastvora kao talog $CaSO_4$.

ZAKLJUČAK

Primerom tretmana IOV u laboratorijskim uslovima pokazano je da se elektrohemijski sintetisan ferat(VI) može uspešno koristiti u procesu obrade IOV kao snažno oksidaciono, koagulaciono i flokulaciono sredstvo uz ekološki provoljne produkte, svođenjem količine zagađujućih materija na maksimalno dozvoljene vrednosti. Utvrđena je visoka efikasnost ferata(VI) pri uklanjanju ukupnog P i F^-, ali u zavisnosti od visine HPK odnosno prisustva ukupnih organskih materija. Pri visokim vrednostima HPK veći deo ferata(VI) se troši na oksidaciju organskog materijala što doprinosi manjoj efikasnosti pri uklanjanju P i F^-. Snažno sorpciono svojstvo i velika razvijena površina $Fe(OH)_3$, kao produkta redukcije ferata(VI) dolazi do izražaja posebno kod uklanjanja F^- koji se samo kompleksiranjem sa Fe^{3+} mogu ukloniti iz rastvora. Istovremeno sa većom količinom dodatog ferata(VI) dolazi do izražaja katalitički efekat $Fe(OH)_3$ na proces raspadanja ferata(VI) što prouzrokuje slabije iskorišćenje ferata(VI) u procesu oksidacije polutanata.

Međutim, sa stanovišta efikasnosti i što većeg iskorišćenja ferata(VI) neophodna je dalja optimizacija procesa tretmana feratom(VI) za pojedinačne zagađujuće materije.

Zahvalnica

Autori zahvaljuju Ministarstvu prosvete, nauke i tehnološkog razvoja u okviru čijih projekata, TR 34025 i 31080, projektnog perioda 2011–2014, je ovaj rad urađen. Takođe zahvaljujemo Institutu "Vatrogas", Novi Sad, na pruženoj tehničkoj podršci pri eksperimentalnom radu.

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SUMMARY

POSSIBLE APPLICATIONS OF FERRATE(VI) IN THE TREATMENT OF INDUSTRIAL WASTEWATER EFFLUENT IN THE LABORATORY

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(Scientific paper)

This paper shows the effects of ferrate(VI) application in the treatment of industrial wastewater effluent in laboratory conditions. The used samples are mixture of wastewater effluent from various industrial plants whose composition was determined by analyzing samples before the ferrate(VI) treatment. Determining physical-chemical characteristics of the samples showed very high chemical oxygen demand (COD) and the concentrations of individual pollutants are higher than the allowed maximum. In the tested samples, the COD was from 18 (sample 1) to 580 times (sample 2) greater than allowed (allowed 45–150 mg/l), while the concentrations of certain metals were several times higher than allowed (Cu allowed 0,1 mg/l, Zn allowed 0,2 mg/l). Also, a high concentration of total phosphorus content (allowed 2 mg/l) and fluoride was found (allowed 20 mg/l), 2–3 times higher than permissible. The applied Na_2FeO_4 was synthesized by electrochemical method and applied *in situ*. Physical–chemical testing of samples, after treatment with different amounts (2, 5, 8, and 10 ml) of Na_2FeO_4 in concentration of 8 g/l showed that ferrate(VI) can be used as a multifunctional agent in the purification of industrial wastewater, where in the amount of contaminating matter is reduced below the maximum permitted level. It was demonstrated the high efficiency of ferrate(VI) as a strong oxidant in the removal of total P and suspended materials. Also pointed out was the high sorption power of the generated ferric(III)hydroxide, which with its developed surface absorbs 95,5% of the F^- and removes it from the solution in the form of sludge. The shown high efficiency of ferrate(VI) in the total removal of P (70 to 99.5%) and F^- (89 to 95.5%), depending on the presence of the total COD value or the presence of the total organic substances. At high values of the COD the major part of ferrate(VI) is consumed in the oxidation of organic material and the formation of $Fe(OH)_3$, which accelerate the process of decomposition of ferrate(VI), which contributes to reduced efficiency of the removal of P and F^-.

Keywords: Industrial wastewater effluent • Ferrate(VI)• P and F^- removal

Emission of SO_2 and SO_4^{2-} from copper smelter and its influence on the level of total S in soil and moss in Bor, Serbia, and the surroundings

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Abstract

The city of Bor and the surroundings (Eastern Serbia) have been known for exploitation and processing of sulphide copper ores for more than 100 years. Emissions of waste gases and particulate matter rich in heavy metals are characteristic for pyrometallurgical production of copper. Long-term measurement results (2005–2008) indicate an increased sulphur dioxide level in the urban-industrial zone of Bor since it is closest to the copper smelter which is a dominant source of air pollution in the studied area. Average annual sulphur dioxide concentrations at four measuring sites in the urban-industrial zone exceeded the maximum allowable value of 50 μg/m^3. However, the maximum allowable value of the total atmospheric depositions (200 mg/m^2 per day on an annual basis) exceeded only at two of 15 measuring sites in the urban-industrial and rural zone. The highest annual deposition rate of sulphates from deposition was detected in the urban-industrial zone. Since the maximum permitted value for sulphates is not defined by the Serbian regulations, the extent of the pollution cannot be discussed. Since the environment can continuously be polluted through the wet and dry depositions, the biomonitoring by moss was conducted, which revealed significantly higher concentrations of total sulphur in moss in the urban-industrial zone, compared to the background zone. The obtained results confirm the reliability of moss as a bioindicator of ambient pollution. Higher total S concentration in soil samples was noted at the rural site (Ostrelj) located in the close vicinity of two tailing ponds.

Keywords: air pollution, copper smelter, sulphur dioxide, atmospheric depositions.

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The air quality depends mostly on anthropogenic pollution sources, such as transport and industry. Metallurgical production of non-ferrous metals is a significant source of waste gases and particulate matter emitted in the environment. By melting ores with a high content of sulphur, the smelter emits sulphur dioxide (SO_2) in excessive concentrations, which poses threat to the environment and the humans [1,2]. Open pits and tailing ponds from which the coarser particles are distributed by wind represent additional sources of environmental pollution in the vicinity of the metallurgical complexes, consequently they can be rich in hazardous materials [3].

The basic components of biosphere are affected by atmospheric deposition of various pollutants. Atmospheric particles, depending on a size, can be settled by gravity close to the source of pollution, or can be carried by wind to various distances from the source. The most direct consequences of atmospheric deposition, both dry and wet, are acidification of soil and water, and accumulation of heavy metals in the biosphere [3,4].

Biomonitoring is used to monitor a number of pollutants, since it is a reliable way to determine connection between the air pollution in large areas and the temporal and spatial concepts at an affordable price. Information on atmospheric trace elements can be obtained by modelling their atmospheric dispersion and deposition, based on a-priori known emission sources, and by measuring the actual atmospheric occurrences and/or deposition [5]. Moss monitoring technique is an efficient method for the quantitative determination of atmospheric deposition of various pollutants and pollution sources [6–9].

Sulphur is an important nutrient. Plants compensate their need for S, absorbing it from soil in the form of sulphate ions, and partly by foliar absorption from the air. Discarded parts of plants (*e.g.*, leaves and fruits) increase the concentration of S in soil because they are subjected to decomposition and oxidation on the surface of soil. During that process, S is transformed to a form acceptable to plants [10]. However, Sun *et al.* [11] argue that the absorption of S from soil is well regulated and does not cause increased concentrations in plants. When the concentration of S in the soil is sufficient, the ambient air with excessive SO_2 concentration can have a toxic effect on vegetation

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[12]. The natural level of SO_2 in the air (about 5 μg/m^3) is not toxic to plants. However, concentrations of SO_2 which are below the maximum allowable concentrations safe for humans can cause serious damage to plants [10]. According to Jablanović *et al.* [10], the concentrations of SO_2 in the air below 50 μg/m^3 may be useful as a source of the necessary S, while concentrations above 100 μg/m^3 cause adverse effects, even to tolerant plant species.

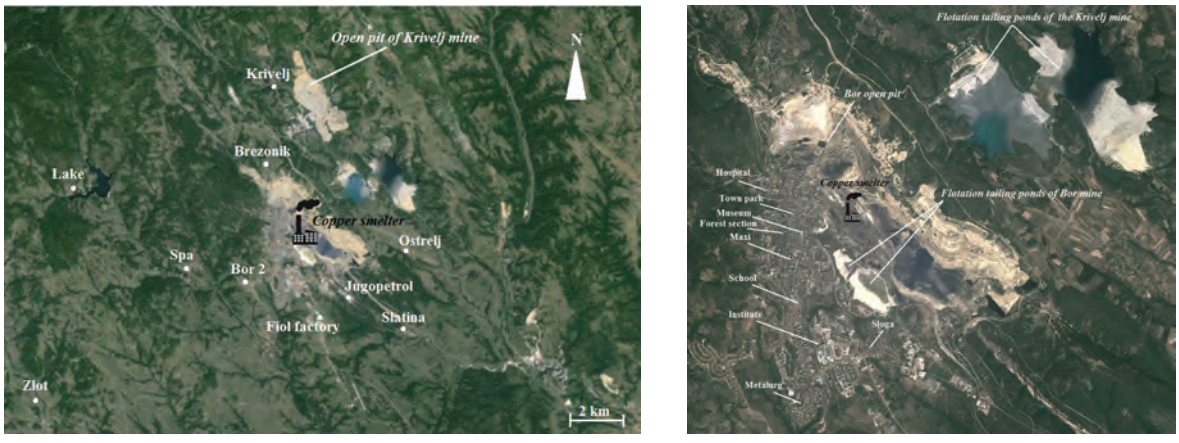
The primary sources of environmental pollution in Bor (Eastern Serbia) are mining and metallurgical processes (pyrometallurgical copper production from the sulphide ores $CuFeS_2$, Cu_2S and CuS). Besides primary emissions of waste gases from the copper smelter, secondary sources of pollution are open pits and tailing ponds situated in close vicinity of the town of Bor and surrounding villages (Figure 1), from which particles rich in sulphates are emitted. The Bor copper mine has started to work in 1903 after the discovery of abundant reserves of copper ores, which were exploited until 1993. In 1906, the first copper smelter started its operations. In the period 1961–1968, a new smelter was built, where sulphide copper ores are melted even today. Smelter waste gases containing on average 3––7% SO_2 are converted to H_2SO_4 in the sulphuric acid plant located within the Mining-Metallurgical Complex Bor. Since the plant provides accepting and processing less than 60% of the gases from the smelter, the rest of the gases are emitted untreated into the atmosphere [13].

The aim of this paper is to present air pollution data on ambient SO_2 concentrations, deposition rate of sulphates (SO_4^{2-}) from atmospheric depositions and total atmospheric depositions in the period of 2005–2008, as well as total S concentrations in soil and moss sampled in the vicinity of the Mining–Metallurgical Complex Bor during 2009.

EXPERIMENTAL

The measuring and sampling sites were selected depending on the dominant source of pollution (copper smelter), prevailing wind directions and the topography of the selected terrain. The studied area (Figure 1) includes four zones, two of which are situated in the close vicinity of the Complex (urban-industrial and rural zone) and the other two are not (background and tourist zone). Ambient SO_2 concentrations were measured at four sites in the urban-industrial zone: Town park, Jugopetrol, Institute and Brezonik. Total atmospheric deposition (TAD) and SO_4^{2-} were determined at 15 sites distributed in the urban-industrial zone (Hospital, Forest section, School, Institute, Sloga, Brezonik, Jugopetrol, Metalurg, Bor 2 and Foil factory), in the rural zone (Slatina, Krivelj and Ostrelj) and the tourist zone (Spa and Lake). Soil sampling sites were distributed in the urban-industrial (Town park and Hospital), the rural (Ostrelj and Slatina) and the background zone (Zlot), while moss was sampled in the urban-industrial (Town park, Institute, Museum and Maxi) and the background zone (Zlot).

Chemical analysis of the samples collected during the air quality monitoring program in the Bor area was performed in the Mining and Metallurgy Institute [14]. Ambient SO_2 concentrations were measured with stationary automatic and mobile measuring stations. At the sites, Town park and Jugopetrol, SO_2 concentrations were measured automatically by the UV fluorescent sulphur dioxide analyser (Model AF22M) according to the ISO standard (ISO 10498:2004). The method is based on detecting the characteristic fluorescence radiation emitted by SO_2 molecules. In the presence of a specific wavelength of UV light (214 nm), the SO_2 molecules reach a temporary excited electronic state. The subsequent relaxation produces fluorescence radiation, which is measured by a non-cooled photomultiplier tube. The analyser provides 15-min concentra-



(a) (b)

Figure 1. The map of the studied area: a) Eastern Serbia; b) the town of Bor.

tions of SO_2. The measurement is in the range 0–10000 μg/m^3. At the measuring sites, Institute and Brezonik, SO_2 concentrations were measured by the hydrogen peroxide method, based on the absorption of SO_2 from the air in the reagent containing 0.03 mol/dm^3 H_2O_2 at pH 5. The obtained solution is titrated with a standard solution (0.002 mol/dm^3 NaOH). The lower limit of detection for this method is 25 μg/m^3 [13].

Determination of total atmospheric deposition was conducted by the sedimentation method [15,16]. The method enables determination of liquid and solid fraction from depositions. During the period of 30±2 days, the particles were collected according to their ability to settle under their own weight. The sedimentator, mounted on 1.5 m-high tripods to avoid the collection of dust picked up by wind, consists of a funnel and a dry polyethylene cylindrical container. In the summer period, solution of copper(II) sulphate is added into the sedimentator in order to prevent algae growth. In the laboratory, solid fraction is separated from liquid by filtration. From the liquid fraction SO_4^{2-} concentrations are determined by the turbidimetric method. Total atmospheric depositions represent a sum of soluble and insoluble fraction given in milligrams per square metre per day (mg/m^2 per day).

Soil and moss samples were air dried first for 10 days at room temperature. In order to obtain concentrations on a dry matter basis, the samples were further dried in a dryer for a minimum of 24 h at 50 °C. The dried moss samples were ground in a laboratory mill into fine powder (average particle diameter less than 100 μm). To avoid contamination, the mill was thoroughly cleaned after each grinding. Soil samples were collected from the A-horizon (top soil 10–20 cm in depth) and stored in clean polyethylene bags. The soil samples were sieved through steel sieve, and then ground in the same way as the moss samples. After preparation, soil and moss material were digested according to the U.S. EPA method 3050B as in Piczak *et al.* [17]. Determination of total S concentration in the samples of soil and moss by ICP-AES and the quality control was performed at the Mining and Metallurgy Institute in Bor [18].

RESULTS AND DISCUSSION

Air pollution data

Taking into account the location of the Mining Metallurgical Complex and dominant wind directions, pollutants are spread over the town of Bor and the surrounding area [13] according to patterns given by the wind rose diagrams (Figure 2). From the shown 4-year period, it could be concluded that dominant winds in the studied area are in the West, East and South directions.

The average monthly SO_2 concentrations obtained at the four measuring sites in the urban-industrial zone of Bor in the period from 2005 to 2008 are given in Figure 3.

It can be seen that the highest monthly SO_2 concentration reached 450 μg/m^3 at the measuring site Jugopetrol in February 2005 (Figure 3a). At all the measuring sites during a 4-year period the lowest average SO_2 concentrations were detected at the sites

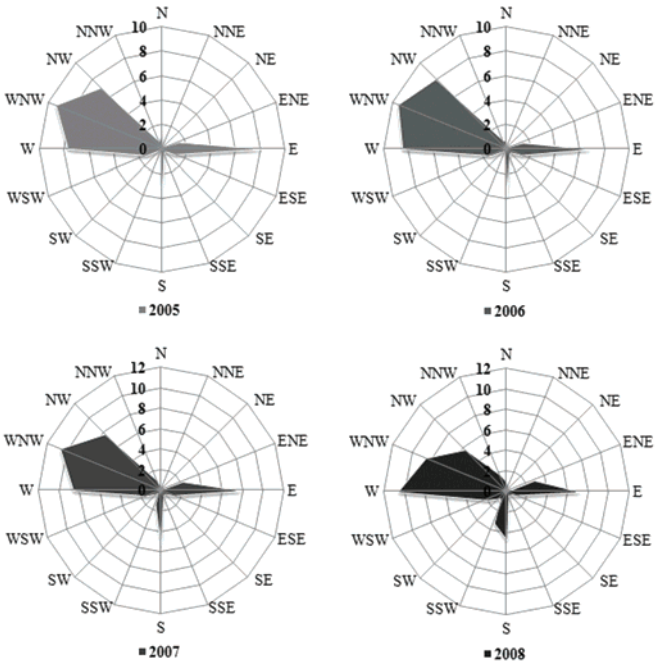


Figure 2. Wind rose diagram (%) for the study area in the period 2005–2008.

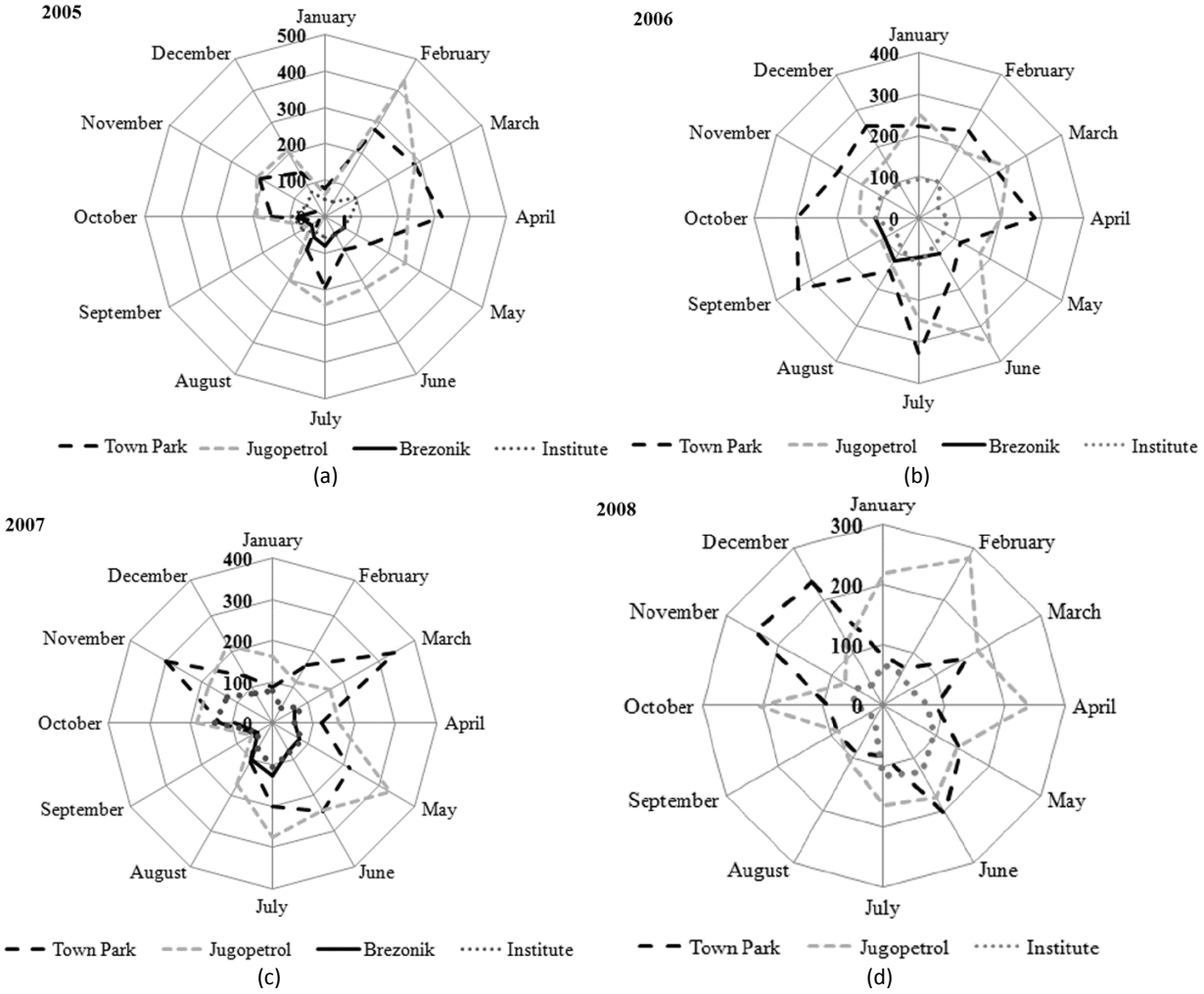


Figure 3. The average monthly SO_2 concentrations ($\mu g/m^3$) at the measuring sites Town park, Jugopetrol, Brezonik and Institute for a) 2005; b) 2006; c) 2007; d) 2008.

Institute and Brezonik, and the highest were at the sites Town park and Jugopetrol. The episodes of extremely high concentrations were probably a consequence of irregularities during the operation of the sulphuric acid plant when all the waste gases from the smelter were discharged without treatment into the atmosphere of Bor, as well as unfavourable winds (Figure 2). By comparing the average annual SO_2 concentrations (Table 1) with the current air pollution standard in Serbia [19], it can be said that the inhabitants of Bor live in the endangered environment, since only one annual SO_2 concentration (Institute, 2005) of all the measuring sites was within the maximum allowable value. Šerbula *et al.* [13] showed that ambient SO_2 concentrations mostly followed the anode copper production of the Complex. However, in some cases, the trend was not present and increasing copper production was accompanied by decreasing SO_2 concentrations and vice versa, which was a result of frequent stoppages of the copper smelter and limited capacities of the sulphuric acid plant.

Table 1. The average annual SO_2 concentrations ($\mu g/m^3$) in the urban-industrial zone of Bor in the period 2005–2008; maximum allowable concentration for residential areas according to the Serbian Regulation No. RS 75/10 [19]; 50 $\mu g/m^3$

Measuring site	2005	2006	2007	2008[a]
Town park	169	238	175	112
Jugopetrol	215	199	189	177
Institute	49	86	82	71
Brezonik	58	104	91	–

[a]Measurements for the period January–September

The Table 2 shows the average annual deposition rates of total atmospheric depositions and SO_4^{2-}. Based on the annual deposition rates of the TADs, it can be said that the maximum allowable value (MAV) given by the Serbian Regulation [19] exceeded only at the measuring sites Slatina (during 2007 and 2008) and Forest section (during 2007). Also, at the site School, during the course of 2008, deposition rate was 199.1 mg/m^2 per day, which is close to the MAV. In general, the

Table 2. The average annual deposition rates (mg/m^2 per day) of total atmospheric depositions (TAD) and sulphates from soluble fraction at 15 measuring sites in Bor and the surroundings for period 2005–2008; values above the maximum allowable value (MAV) shown in bold

Measuring site	2005		2006		2007		2008[a]	
	TAD	SO_4^{2-}	TAD	SO_4^{2-}	TAD	SO_4^{2-}	TAD	SO_4^{2-}
Institute	38.1	6.2	113.4	21.5	117.1	14.6	81.7	12.8
Jugopetrol	49.3	10.1	106.7	33.7	137.8	7.8	102.7	11.3
Brezonik	57.3	5.9	116.7	19.9	178.3	15.8	173.8	13.6
Hospital	64.1	7.4	171.0	49.0	187.6	16.9	193.3	39.9
Forest section	70.0	7.2	171.0	38.6	**212.5**	15.0	171.9	22.8
School	48.0	6.5	124.1	27.0	188.5	21.9	199.1	20.6
Metalurg	30.4	3.2	99.9	24.7	116.8	12.2	89.4	13.6
Spa	28.9	2.6	78.6	15.1	74.8	5.6	119.1	17.7
Foil factory	39.7	6.1	117.8	30.3	97.0	4.6	89.8	13.5
Bor 2	35.2	3.3	95.2	22.2	153.1	42.1	105.4	13.5
Lake	35.2	3.1	91.2	20.8	153.4	18.4	117.7	9.5
Slatina	49.6	6.5	141.1	30.3	**240.6**	17.2	**211.3**	18.0
Krivelj	50.6	5.6	123.2	16.1	125.0	12.3	100.1	7.5
Ostrelj	55.5	10.2	139.2	23.9	110.1	3.6	162.6	12.2
Sloga	44.7	8.9	145.7	28.3	144.3	9.4	171.4	16.5
MAV	200	–	200	–	200	–	200	–

[a]Measurements for the period January–September

lowest deposition rates at all the measuring sites were observed in 2005, compared to the rest of the studied period.

The highest deposition rate of sulphates from atmospheric deposition was noted at the measuring sites in the close vicinity of the Complex and in the prevailing wind directions. Also, in 2006, the average SO_4^{2-} deposition rate was higher compared to the values in 2005 and 2007. Considering that the copper smelter is a dominant source of air pollution in the studied area, increased anode copper production could cause a higher level of ambient pollution. During 2005, 2006, 2007 and 2008 the average anode copper production in the Mining–Metallurgy Complex was 3539.5, 3897.5, 3257.5 and 3346.8 t, respectively. However, the maximum allowable value for sulphates from atmospheric deposition is not defined by the Serbian Regulation [19] or by the European Environmental Agency.

In the study of Moreno-Grau *et al.* [15] where the sources of air pollution are mining and metallurgical activities, the deposition rates of the TADs on an annual base were: 787.5 (detected in the urban-industrial zone), 331.3 (urban zone), 386.4 mg/m^2 per day (intermediate zone). Compared to these values, the TADs deposition rates in the Bor area were a few times lower.

In Figure 4, box plots summarize the average

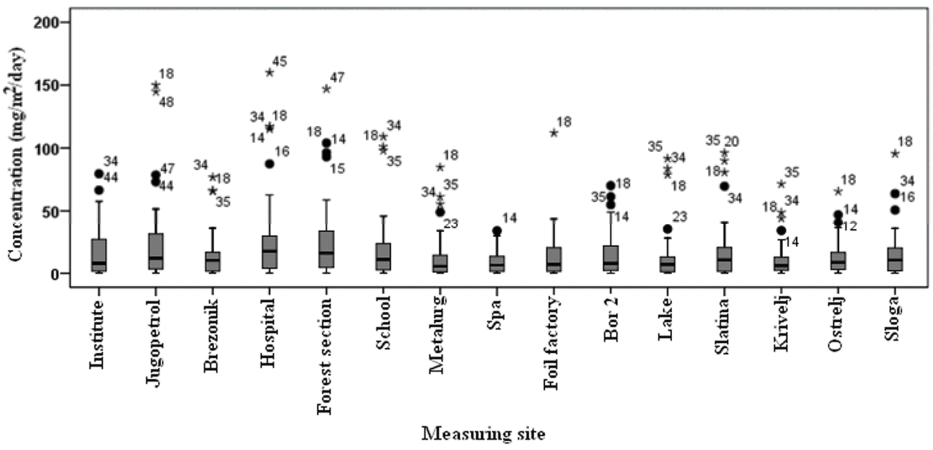


Figure 4. Box plots based on the average monthly sulphate deposition rates at 15 measuring sites in Bor and surroundings in the period 2005–2008 (numbers represent months of 4-year period, N = 48).

monthly SO_4^{2-} deposition rates at 15 measuring sites in the studied area. The upper and lower lines of the "boxes" represent lower and upper quartiles (25 and 75% of the data set, interquartile range), while middle line is called median and it represents the middle of the data set (50%). The T-bars that extend from the boxes are called whiskers and represent minimum and maximum values of the data set, excluding outliers. Outliers are the values that are ≥1.5 times greater than the interquartile range (marked with a dot) or ≥3 times greater than the interquartile range (marked with asterisks – extreme values). A few extreme values were detected at the sites Hospital (during September 2008), Forest section (November 2008) and Jugopetrol (June 2006 and December 2008). The measuring sites Hospital and Forest section are located in less than 1 km from the copper smelter, while the Jugopetrol site is situated in the dominant West wind direction. At the measuring site Spa the lowest level of pollution with sulphates is observed in the presented 4-year period.

Biomonitoring data

Concentrations of total S in soil and moss at the sampling sites in the urban-industrial, rural and background zone are given in Tables 3 and 4, respectively. The maximum concentration of S in soil was detected in the rural area at the sampling site Ostrelj (>1200 μg/g), which is probably a consequence of proximity of Bor and Krivelj tailing ponds and unfavourable winds.

Table 3. Average total S concentrations (μg/g of dry weight) in soil samples at the sampling sites in three zones

Urban-industrial		Rural		Background
Town park	Hospital	Ostrelj	Slatina	Zlot
848.7	687.5	1230.3	817.0	561.5

Table 4. Average total S concentrations (μg/g of dry weight) in moss samples at the sampling sites in two zones

Urban-industrial				Background
Town park	Museum	Maxi	Institute	Zlot
7363.0	7009.0	7196.0	3674.0	1482.0

The concentration of total S in moss, sampled in the urban-industrial zone is significantly higher than the concentrations in the background zone and at the Institute site which is not in the direction of the dominant winds. According to Sucharova and Suchara [6], a typical S content in mosses is in the range of 1200–1400 μg/g, which is far lower than the presented concentrations in the urban-industrial zone of Bor. Only the concentration obtained at the sampling site Zlot (the background zone) is within the typical range for sulphur concentrations [6]. The other obtained results are in agreement with Sucharova and Suchara [6], showing that the moss is an efficient bioindicator of ambient pollution by sulphur dioxide. Availability of moss was reduced in the studied area, but obviously certain types of mosses can survive a high level of air pollution, contrary to conclusions of Reimann *et al.* [20].

CONCLUSION

The main air pollution problems in the studied area of Bor and its surroundings originate from copper smelter, open pits and tailing ponds. Waste gases (which contain 3–7% SO_2) and atmospheric depositions (rich in sulphates and heavy metals) are continuously emitted in the atmosphere from these sources. The results presented in the paper are an outcome of a 4-year program (from 2005 to 2008) of air quality monitoring in Bor, which was conducted by the Mining and Metallurgy Institute, combined with biomonitoring data. The measuring sites of ambient SO_2 concentration are distributed in urban-industrial zone, while total atmospheric deposition and SO_4^{2-} sites are distributed in three zones: urban-industrial, rural and tourist, with the emphasis on the urban-industrial zone since it is closest to the copper smelter. Biomonitoring by moss was conducted at four sampling sites in the urban-industrial zone and in the background, while soil samples were taken from three zones. Although the measuring and sampling sites do not overlap completely due to different monitoring networks, measuring methods and availability of samples, some conclusions can be made from the obtained data sets. According to the average 4-year concentrations of SO_2 and deposition rates of the TADs, the level of pollution is decreasing from the Town park (*i.e.*, Forest section for TADs is the closest to the SO_2 site Town park) and Jugopetrol, towards Brezonik and Institute, which can be an evidence of the same pollution source of gaseous and particulate pollution. Higher level of pollution at these sites had an obvious influence on the total S concentrations in moss samples. Moss sampling sites Museum and Maxi are close to the measuring site Town park, where total S concentrations exceeded 7000 μg/g. Similar to the air pollution data, at the sampling site Institute total S concentration was lower. Significantly higher concentrations of total S in mosses from the urban-industrial zone compared to the background indicate its anthropogenic origin and a good response of site-available moss to the airborne pollution. The maximum concentration of total S in soil was detected in the rural area (Ostrelj), which is probably a consequence of proximity of Bor and Krivelj tailing ponds and unfavourable winds. In general, higher concentrations of the monitored polluting substances were noted at the sites closer to the copper smelter and/or at the prevailing wind directions.

It should be noted that during 2015 a new smelter, based on the autogenous flash smelting technology, should be operational within the Mining– Metallurgy Complex. The obtained data provides vital documentation of the current state of the environment in order to assess the efficiency of the planned technical measures to reduce pollutant emissions in the future in the studied area.

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IZVOD

EMISIJA SO_2 I SO_4^{2-} IZ TOPIONICE BAKRA I NJIHOV UTICAJ NA NIVO UKUPNOG S U ZEMLJIŠTU I MAHOVINI U BORU I OKOLINI

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(Naučni rad)

Područje Bora i okoline poznati su po eksploataciji i preradi sulfidnih ruda bakra više od jednog veka. Tokom dugogodišnje proizvodnje bakra emitovane su u atmosferu velike količine otpadnih gasova i čvrstih čestica sa visokim sadržajem teških metala (karakteristično za pirometaluršku proizvodnju bakra) što ima negativan uticaj na celokupnu životnu sredinu. U radu su predstavljeni podaci o zagađenju vazduha za period 2005–2008. godina, i to: ambijentalne koncentracije sumpor-dioksida (SO_2) na četiri merna mesta u urbano-industrijskoj zoni, brzine taloženja ukupnih taložnih materija (UTM) i sulfata (SO_4^{2-}) iz rastvornih materija na 15 mernih mesta u urbano-industrijskoj, ruralnoj i turističkoj zoni. Takođe, prikazane su koncentracije ukupnog sumpora (S) u uzorcima zemljišta i mahovine prikupljenih u septembru 2009. godine na mestima u urbano-industrijskoj, ruralnoj i kontrolnoj zoni ispitivanog područja. Izabrana merna mesta i mesta uzorkovanja nalaze se na pravcima dominantnih vetrova i na različitim udaljenostima od glavnog izvora zagađenja – topionice bakra. Rezultati dugoročnih merenja zagađenja vazduha ukazuju na povećani nivo sumpor-dioksida u Boru u odnosu na vrednost maksimalno dozvoljene koncentracije SO_2 u naseljenim područjima definisane pravilnikom. Prosečne godišnje koncentracije SO_2, izuzev na mernom mestu Institut, tokom ispitivanog perioda višestruko su premašivale maksimalno dozvoljenu vrednost (50 μg/m^3), što ukazuje na visok stepen zagađenja životne sredine. Maksimalno dozvoljene vrednosti za brzinu taloženja ukupnih taložnih materija na godišnjem nivou bile su prekoračene samo na dva od 15 mernih mesta (Šumska sekcija u urbano-industrijskoj zoni i Slatina u ruralnoj). Najviša prosečna godišnja brzina taloženja sulfata iz ukupnih taložnih materija detektovana je u urbano-industrijskoj zoni. Maksimalno dozvoljena vrednost za ove materije nije definisana pravilnikom, pa se ne može sa sigurnošću tvrditi o zagađenju životne sredine sulfatima, ali je očigledno povećano taloženje na mestima u blizini rudarsko–metalurškog kompleksa. Veće koncentracije ukupnog sumpora u uzorcima zemljišta detektovane su na mestu uzorkovanja Oštrelj u ruralnoj zoni, što je najverovatnije posledica blizine flotacijskih jalovišta borskog i kriveljskog rudnika. Značajno veće koncentracije ukupnog sumpora u uzorcima mahovine u urbano-industrijskoj zoni (preko 7000 mg/kg) u poređenju sa kontrolnom zonom (1482 mg/kg) potvrđuju da je mahovina dobar bioindikator zagađenja vazduha sumpor-dioksidom.

Ključne reči: Zagađenje vazduha • Topionica bakra • Sumpor-dioksid • Atmosferska depozicija

Quality of spelt pasta enriched with eggs and identification of eggs using ^{13}C MAS NMR spectroscopy

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Abstract

This paper deals with the characteristics of spelt pasta enriched with eggs. Eggs were added to spelt farina in the quantity of 0, 124 or 248 g/kg (equivalent to 0, 3 or 6 eggs, respectively). Post-hoc Tukey's HSD test at 95% confidence limit showed significant differences between various samples. Relatively low coefficients of variation have been obtained for each applied assay (1.25–12.42%), which confirmed the high accuracy measurements and statistically significant results. Standard score analysis is applied for accessing the contribution of eggs' content to spelt pasta quality. Maximum scores regarding quality (0.89) and chemical characteristics (0.70) have been obtained for 6 eggs spelt pasta formulation. It is also shown that the presence of eggs in pasta can be clearly confirmed by ^{13}C MAS NMR spectroscopy. Simultaneous increase in area of peak positioned at 29.5 and 176 ppm is directly associated with the increase in the content of added eggs in the corresponding samples. Pertinent data showed positive contribution of eggs to the spelt pasta and also that NMR spectrum can be used in the egg quantity control.

Keywords: eggs, ^{13}C MAS NMR, spelt pasta, quality.

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The dynamic way of life has the tendency to simplify and decrease in preparing healthy, quick, cheap, safe organic meals, and spelt pasta is meeting these goals. Pasta can be stored a long time without deterioration in flavor, odor and usability, and it doesn't lose quality like bread. Pasta is also characterized by good digestion. It is a significant source of proteins with relatively satisfactory composition of essential amino acids [1]. Eggs added to pasta contribute to better mechanical properties and quality of the product and also increase the nutritive and biological value of the product, which is reflected in the increase of lysine and ω-3 fatty acids and natural sources of lecithin. By consumption of products enriched with eggs human meets recommended needs [2–5].

Spelt wheat has shown potential in various food applications, including bread, pasta, breakfast cereal and other products of altered nutritional characteristics compared to conventional wheat products. It has very high protein content and even 30 to 60% higher concentration of mineral elements Fe, Zn, Cu, Mg and P compared to *Triticum aestivum* [6–9]. Spelt pasta is produced without additives, food colours and it doesn't contain genetically modified material, thus meets the requirements of an organic product. Organic food can be rated from satisfactory to good, because it contains much higher levels of nutrients [1,10].

Cross-polarization (CP) has been established as a method which provides significant enhancement in sensitivity of ^{13}C MAS NMR spectrum of solid samples, allowing characterization of molecular structures present in the food samples in their native forms [11]. The change in ^{13}C CP/MAS NMR peak intensity has been related to a change in molecular mobility, with higher ^{13}C CP/MAS NMR peak intensities being due to a decrease in segmental mobility resulting in more efficient cross-polarization [12]. On the other hand, for molecular segments with long aliphatic chains (such as lipids) undergoing fast and isotropic reorientations, dipolar interactions are averaged to zero and therefore CP MAS is incapable to observe such kind of structures. Application of direct polarization (DP) ^{13}C MAS NMR spectroscopy provides opportunity to detect both mobile and rigid structures together, also representing more quantitatively reliable method.

The aim of this study is to investigate the influence of eggs quantity on spelt pasta quality as well as the posibility of the identification of eggs by ^{13}C MAS NMR spectroscopy *via* variation of quantity of lipid and protein component originated from added eggs in pasta samples.

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EXPERIMENTAL

Material

In this experimental investigation the following materials were used for pasta production:

– whole meal spelt flour grown in the year 2012 in Serbia is characterized by protein, starch, sugar, lipids and cellulose content of: 15.6, 56.6, 2.20, 2.5 and 2.4% d.m (Table 1).

– liquid eggs concerning hygiene and microbiology were correct and were taken from a local food market.

Methods

Preparation of pasta

Pasta was made using the device "La Parmigiana D45" MAC 60. In a paddle mixer the whole meal moisture was adjusted to 31.5% by adding water or water and liquid eggs. Liquid eggs were first mixed into a homogeneous mass. Based on whole meal flour, applied quantity of eggs was 0, 124 or 248 g/kg (equivalent to 0, 3 or 6 eggs, respectively). Hydrated whole meal entered an extrusion worm which moved the loose dough forward and simultaneously compresses it into a homogeneous plastic mass prior to extrusion through a die with 1.4 mm diameter used for spaghetti. Mixing time was 15 min [13]. Raw pasta was dried in a cabinet drier about 12 h at controlled temperature that did not exceed 45 °C and humidity round 70% until pasta reached the moisture around 12.5%, followed by cooling to 25 °C, for 4 h and then stored at room temperature in sealed containers. Drying conditions and air flow were stringently controlled to avoid the creating of a discontinuity in the moisture gradient between the interior and exterior of spaghetti.

Pasta quality

Quality of pasta was evaluated in terms of cooking characteristics (water uptake, volume increase and cooking loss). Stickiness was sensory evaluated by numeric scores 0–10 by a panel of trained panellists. High scores were allocated to pasta with smooth, non-sticky surface [13].

Pasta texture – Texture analysis

Textural properties of cooked pasta were measured with Texture analyzer TA.HD plus (Stable Micro System, U.K.) equipped with a 5-kg load cell. The preparation procedure was the same for all tested samples (duration of cooking, time period between cooking and testing). Hardness and adhesiveness of cooked pasta were measured using a 36 mm cylinder probe (P/36R) (instrument settings were as follows, mode: measure force in compression, pre-test speed: 2.0 mm s^{-1}; test-speed: 1 mm s^{-1}; post-test speed: 10 mm s^{-1}; strain: 75%; trigger force: 5 g). The maximum force correlates to the hardness of the sample. Adhesiveness is calculated from the negative areas of the plots. Toughness (area under the force/time curve) was measured using the Warner–Bratzler shear blade (type HDP/BS) and the following settings: pre-test speed: 2 mm s^{-1}; test-speed: 2 mm s^{-1}; post-test speed: 10 mm s^{-1}; distance: 15.00 mm; trigger force: 10 g. The tests were performed on 10 replicates per batch. The two spaghetti strands were held close together and positioned centrally under the probe during testing.

Pasta colour

Pasta colour (L^*- lightness, b^*- share of yellow colour if positive value and blue colour if negative value, C^*-differences in colouration) was measured by objectively colourimeter Chroma meter (CR-400, Konica, Minolta, Japan) and was determined according to the procedure described by Filipović *et al*. [5].

Chemical analyses

Basic chemical analyses: protein, starch, sugars, lipids, cellulose and sugars of pasta spelt were defined according to approved AOAC method cited by Kaluđerski and Filipović [13]. Nitrogen was determined by Kjeldahl method (979.09) [14] and converted to protein using factor of 5.75.

^{13}C MAS NMR analyses

The ^{13}C MAS NMR spectrum was recorded at 100.627 MHz using a Bruker MSL 400 NMR spectrometer Tecmag console upgraded (Houston, TX, USA), operating at room temperature. Samples of spelt pasta

Table 1. Chemical characteristics of whole meal spelt flour and pasta with eggs, component content, % d.m.; values with the same letter are not statistically different at the p < 0.05 level (Turkey's HSD test)

Component	Whole meal spelt flour	Quantity of eggs with pasta			Polarity
		0	3	6	
Protein	15.6±1.8^a	15.80±0.37^a	17.14±0.10^b	18.12±0.02^c	+
Starch	56.6±2.3^a	56.44±3.08^a	55.86±4.93^a	51.40±0.51^b	+
Cellulose	2.4±0.1^a	2.00±0.46^a	1.96±0.74^a	2.13±0.24^a	+
Sugars	2.2±0.8^a	2.38±0.18^a	2.50±0.13^a	2.44±0.11^a	+
Lipids	2.5±0.2^a	2.70±0.07^a	3.54±0.19^b	4.69±0.09^c	+
SS	–	0.24	0.58	0.70	–

were packed into a 7 mm zirconium rotor and spun at 4 kHz. The ^{13}C DP MAS NMR spectrum was obtained with high power decoupling during acquisition and repetition time of 30 s. The accumulation of 2048 scans was done to obtain a satisfactory signal-to-noise ratio. The Kel-F rotor end caps background signal was removed by subtracting of each of DP spectrum from spectrum of empty rotor recorded under same condition. All chemical shifts are expressed relative to tetramethylsilane (TMS) using adamantane peak observed at 29.5 ppm.

Statistical analyses

Descriptive statistical analyses for all the obtained results were expressed as the mean ± standard deviation (*SD*). Analysis of variance (ANOVA) has been utilized to show relations between applied assays. Principal component analysis (PCA), used as pattern recognition technique, has been applied within assay descriptors to characterize and differentiate various analysed samples. The evaluation of one-way ANOVA and PCA analyses of the obtained results were performed using StatSoft Statistica 10.0® software. Applied methodology was the same as described before [5].

Determination of normalized standard scores (SS)

Standard scores is one of the most widely used technique to compare various characteristics of complex food samples determined using multiple measurements, where samples are ranked based on the ratio of raw data and extreme values of the measurement used. Since the units and the scale of the data from various nutritive, textural and colour characteristics measuring methods are different, the data in each data set should be transformed into normalized scores, dimensionless quantity derived by subtracting the minimum value from the raw data, and divided by the subtract of maximum and minimum value, according to following equations:

$$\overline{x}_i = 1 - \frac{\max\limits_i x_i - x_i}{\max\limits_i x_i - \min\limits_i x_i}, \quad \forall i$$

in case of "the higher, the better" criteria, or

$$\overline{x}_i = \frac{\max\limits_i x_i - x_i}{\max\limits_i x_i - \min\limits_i x_i}, \quad \forall i$$

in case of "the lower, the better" criteria, where x_i represents the raw data. The normalized scores of a sample for different measurements when averaged give a single unitless value termed as *SS*, which is a specific combination of data from different measuring methods with no unit limitation. This approach also enables the ease of employing some others set of pasta formulations to this elaboration.

Standard scores for different samples investigated in this article were calculated and the result has been shown in Tables 1 and 2.

RESULTS AND DISCUSSION

Quality of Spelt pasta with eggs

ANOVA test shows statistically significant differences ($p < 0.05$ level, 95% confidence limit) in protein content among the values of samples with 0 eggs and pastas with 3 and 6 eggs, Table 1. Egg proteins in pasta are increasing its biological value and quantity of essential amino acids of the product. Addition of eggs in pasta also results in statisically significant difference in starch content, while there are no statistically significant differences in the values of cellulose and sugar content. As expected, analysis of variance indicates the statistically significant differences in lipid content in

Table 2. Quality of pasta with eggs; values with the same letter are not statistically different at the p < 0.05 level (Turkey's HSD test)

Parameter	Qantity of eggs			Polarity
	0	3	6	
Quality of cooking pasta				
Water uptake, g	4.10±0.15^{a}	4.01±0.21^{a}	5.00±0.24^{b}	+
Volume increase, α / %	2.50±0.15^{a}	2.40±0.30^{a}	2.40±0.27^{a}	-
Cooking loss, *R* / % d.m.	3.90±0.15^{b}	3.62±0.24^{ab}	3.42±0.27^{a}	-
Pasta texture				
Hardness, g	3117.48±119.8^{a}	3253.28±280.4^{a}	4087.15±336.6^{b}	+
Adhesiveness, g s	2.02±0.6^{a}	0.37±0.22^{b}	0.12±0.07^{b}	-
Work of shear – toughness, g s	13.18±0.62^{a}	19.31±3.31^{b}	14.29±2.10^{ab}	+
Color of pasta				
*L** Brightness	70.15±1.19^{a}	70.77±0.30^{ab}	72.05±0.46^{b}	+
Difference in tone (colors)	15.46±0.07^{a}	16.25±0.19^{b}	16.29±0.16^{b}	+
Share of yellow color, *b**	14.96±0.16^{a}	15.77±0.19^{b}	15.94±0.15^{b}	+
SS	0.01	0.63	0.89	

samples of spelt pasta with 0, 3 and 6 eggs. This indicates that the addition of eggs, significantly affects the lipid content in the pasta and is highly appreciated as a source of monounsaturated fatty acids and ω-3 fatty acids that are necessary for normal functioning of the human organism [3].

In the of prevention of noninfectious, chronic diseases such as obesity, diabetes and cardiovascular disease, the nutrition experts recommend normal and balanced fat intake through diet. Total fat should be from 15 to 30%, saturated fatty acids (SFA) < 10%, polyunsaturated fatty acids (PUFAs) from 6 to 10% and trans fatty acids *MK* < 1%. The human body needs very small amounts of essential fatty acids that must enter by food and requirements for ω-3 fatty acids are even smaller. Consummation of pasta with eggs, introduces to the body ω-3 fatty acids which are necessary for the normal functioning of the human organism. According to Hayes [15], one egg contains 0.68% of ω-3 fatty acids necessary for daily human needs. Thus consumer satisfies 0.2 and 0.4% of daily needs of ω-3 fatty acids, by consummation of 100 g spelt pasta with 3 and 6 eggs, respectively.

Related to pasta quality, the addition of 6 eggs statistically significantly increases pasta water absorption in comparison to the values of the samples with 0 and 3 eggs (Table 2). Volume increase is ability of starch to swell and this parameter indicates that there were no statistically significant differences between pastas with eggs (3 and 6) and pasta without eggs. Cooking loss is one of parameter of the cooked pasta quality. Cooking loss has been decreased with addition of eggs, which indicates that protein and fatty substances from eggs reinforce and strengthen the gluten structure of the product.

The texture and colour of pasta are important quality characteristics of product and all contribute to the sensor properties of product, which are very important for consumers. Based on the analysis of the quality of the spelt pasta with 0, 3 and 6 eggs, the ANOVA test showed that the addition of 6 eggs statistically significantly increased the hardness of the pasta with 0 or 3 eggs (Table 2). Hardness of pastas is increased with the quantity of added eggs due to the positive influence of egg proteins and lipids on the gluten matrix. Similary to sensory evaluation, analysis of variance showed that the addition of eggs (3 and 6) also statistically significantly reduces the adhesiveness of pasta and have a positive impact on the properties of the chewing. ANOVA test showed that the addition of 3 eggs statistically significantly increased the toughness of the pasta with 0 eggs (Table 2). Eggs lipids and phospholipids stabilize the three-dimensional structure of gluten. Our results are similar to results which were obtained by Raina *et al*. [16], who claimed that the mean value for toughness of raw pasta increased with increasing the protein level.

Eggs are also improving yellow color and taste of pasta while whole meal flour causes darker color of the finished product. Adding 6 eggs improves the brightness of whole meal spelt pasta (Table 2). Addition of eggs (3 to 6) contributes to statistically significant differences in share of yellow color and increases difference in tone. In all pasta samples the positive values of yellow color (b^*) are registered. As expected, share of yellow color (b^*) increases with increasing content of eggs. This parameter indicates the presence of natural pigment in egg, but it can't quantify the egg quantity. These pigments are also recognized as natural antioxidants that positively contribute to free radicals elimination and are also anticarcinogenic agents [3].

SS is a numerical, unitless scale value, which has not consistent agreement with any measuring methods. Although it is a relative index and may not represent a specific property of different samples, previous experience with other samples [5] proved that *SS* provides a reasonably accurate rank of spelt pasta. In this article, standard scores are calculated for chemical properties and overall obtained data were presented in Tables 1 and 2. Standard score above 0.5 stands for the high standard in observed characteristics. Spelt pasta properties with *SS* above 0.5 showed more competitive qualities compared to other pastas.

Using the standard score analysis and revealing the standard scores of different spelt pasta formulations cannot only realize their position regarding other formulations, but also have a reference for developing strategies for improving their characteristics. The best scores for chemical properties and overall quality are obtained for pasta with more eggs added.

Analyzing of pasta samples by ^{13}C MAS NMR spectroscopy

In Fig. 1 is presented the spectrum of pasta samples with different number of eggs added (0, 3 and 6), affecting incensement of protein and lipid content in the pasta samples. Line assignments in the spectrum could be divided in two region: first one belonging to starch component (60–105 ppm), and second one associated to lipid and protein component (14–40 ppm aliphatic carbons, ~130 ppm aromatic and unsaturated carbons, and ~175 ppm carbonyl carbons) [17,18]. The whole meal spelt flour (Table 1) used in this experiment contained about 56.6% d.m. of starch and 15.6% d.m. of protein. Other ingredients include 2.5% of lipids, 2.2% of sugars and 2.4% of cellulose. According to the chemical analysis of pasta samples given in Table 1, protein component dominated over lipids (average 17.1% of proteins compared to 3.64% of lipids). Presence of lipids significantly complicated NMR peak resolving in obtained spectrum. However, good resolved

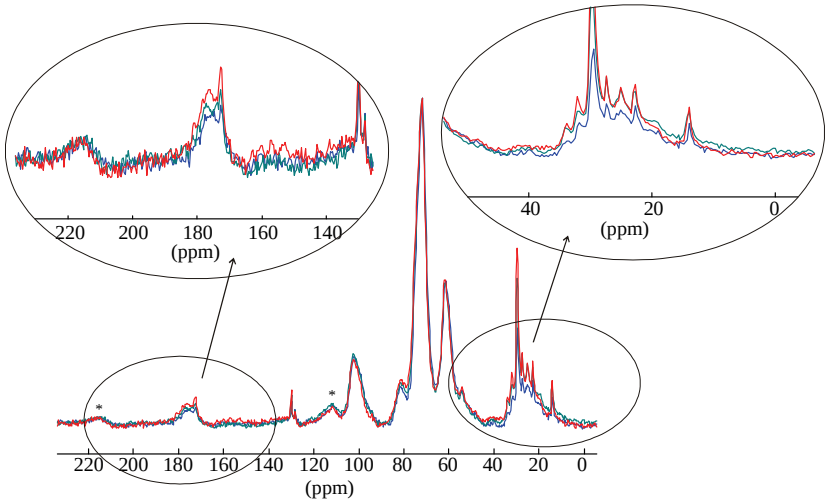

Figure 1. ^{13}C MAS NMR spectrum of pasta samples with 0, 3 and 6 eggs given one over another. Aliphatic (0-40 ppm) and carbonyl carbons peaks (130–230 ppm) regions are given enlarged separately. Asterisks mark position of spinning side bands (SSB).

sharp peaks (line width at half-height is less than 100 Hz) that are embedded on the relative broad resonance belonging to protein component represent strong evidence of the lipid component presence into the pasta samples. According to ^{13}C NMR chemical shift assignation for lipids previously reported [19,20], peaks between 14–40 ppm represent saturated carbons in all fatty acid chains [18], whereas shifts observed around 130 ppm are characteristic for unsaturated carbons. The carbonyl carbons (ester and fatty acids) appeared at high field around 173 and 180 ppm, respectively. The glycerol external $C_{1',3'}$ and central $C_{2'}$ carbons regularly are observed at 62 and 69.5 ppm, respectively, but in the case of our pasta samples, they are completely overlapped by polysaccharide starch component. Therefore, as the most sensitive for this analysis, was selected peaks at 29.5 ppm in the chemical shift region of aliphatic carbons, and peak positioned around 176 ppm for the chemical shift of carbonyl carbons representing mutual contribution from fatty acids and amino acids as the result of egg addition (Fig. 1).

The values of areas for selected peaks determined from ^{13}C MAS NMR spectrum of pasta samples with 0, 3 and 6 added eggs are represented in Table 3. Increase in peak area of signal positioned at 176 and 29.5 ppm (approximately 0.2 to 0.3 % for each egg added in the pasta sample), could be directly correlated with cumulative contribution of protein and lipid component originated from eggs addition in pasta samples. Although, discrimination between lipid and protein part could not be clearly determined from NMR spectrum presented in this work, based on the increase of peak area at selected position in the ^{13}C MAS NMR spectrum of pasta samples could clearly confirm presence of egg component in given amount.

By increasing the eggs quantity from 0 to 6, the peak areas at 176 and 29.5 ppm are also increased, and the protein and lipid content could be calculated as follows:

Protein content = (8.54±1.28) + (1.69±0.25)×Area of peak 176 ppm (r^2 = 0.990, p < 0.05)

Lipid content = (-3.71±0.22) + (1.47±0.04)×Area of peak 176 ppm (r^2 = 0.999, p < 0.05)

Table 3. Results of NMR analysis of pasta with eggs samples presented in the Figure 1 (peak area, %); peak areas are expressed as percentage of the total spectrum area

Line position, ppm	Quantity of eggs		
	0	3	6
176	4.36	4.97	5.72
29.5	3.96	4.87	5.84

CONCLUSIONS

Based on data of investigation of quantity eggs influence on spelt pasta quality and the possibility of egg identification by ^{13}C MAS NMR spectrum it can be concluded:

– Applied standard score analysis revealed the rank of each sample in comparison to other samples, regarding its chemical characteristics and overall quality. The best scores are calculated for pasta with more eggs added, and the best standard scores for chemical properties and overall quality (0.70 and 0.89, respectively) is experienced with 6 eggs, thus pointing at the strategies for improving spelt pasta characteristics.

– Egg lipids positively affect the pasta texture, and increase hardness (for 4 and 31%), reduce adhesiveness (for 82 and 94%) and increase toughness (for 46 and 8%) of the cooked pastas.

– Spelt pasta with eggs (3 and 6) contibutes to the positive balance of essential amino acids and ω-3 fatty

acids in human organism, with modified biologically and nutritive properties.

– Besides significant overlapping between peaks originated from lipid and protein component originated from addition of egg component inside spelt pasta samples, ^{13}C MAS NMR spectrum enables their mutual confirmation in order to correlate amount of egg component present in particular sample.

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IZVOD

KVALITET TESTENINE OD SPELTE SA JAJIMA I IDENTIFIKACIJA JAJA ^{13}C MAS NMR SPEKTROSKOPIJOM

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(Naučni rad)

Dinamičan način življenja ima za tendenciju da se pojednostavi i vremenski skrati način pripremanja zdravog i nutritivno vrednog obroka. Dobra osobina testenine je što se može čuvati dugo vremena bez pogoršanja ukusa, mirisa i upotrebne vrednosti i ne stari kao hleb. U ovom radu je ispitana mogućnost dokazivanja jaja u testenini od spelte. Jajni melanž je dodavan u količini od 0, 124 i 248 g/kg krupice što odgovara količini od 0, 3 i 6 jaja. *Post-hoc* Tukijev (Tukey's) HSD testom, pri 95% granici poverenja pokazano je da postoji statistički značajna razlika između različitih uzoraka. Relativno niski koeficijenti varijacije, koji su dobijeni za svako od posmatranih merenja (1,25–12,42%) ukazuju na visoku tačnost merenja i na statističku značajnost rezultata. Primenjena je analiza standardne ocene („standard score analysis"), radi potpunijeg sagledavanja uticaja količine jaja na kvalitet testenine od spelte. Najveće ocene („*score*") za kvalitet (0,89) i za hemijske karakteristike testenine (0,70), dobijene su za formulacije testenine od spelte sa 6 jaja. Lipidi jaja pozitivno utiču na teksturu testenine, povećavaju tvrdoću (od 4 do 31%), smanjuju lepljivost (za 82 i 94%) i povećavaju žilavost (za 46 i 8%) kuvane testenine. Jaja poboljšavaju senzorne osobine integralne kuvane testenine od spelte, doprinose povećanju svetloće i udela žutog tona. Takođe je pokazano da se prisustvo jaja u testenini može jasno potvrditi pomoću ^{13}C MAS NMR spektroskopije. Istovremeni porast površine pikova na 29 i 176 ppm dobijenih na osnovu polaznih spektara se direktno povezuju sa povećanjem sadržaja jaja u odgovarajućim uzorcima. Rezultati pokazuju da testenina sa jajima od spelte ima dobar tehnološki kvalitet, a da se u kontroli kvaliteta može koristiti NMR analiza za dokazivanje prisustva jaja.

Ključne reči: Jaja • ^{13}C MAS NMR • Testenina • Kvalitet

Flow cytometric determination of osmotic behaviour of animal erythrocytes toward their engineering for drug delivery

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Abstract

Despite the fact that the methods based on the osmotic properties of the cells are the most widely used for loading of drugs in human and animal erythrocytes, data related to the osmotic properties of erythrocytes derived from animal blood are scarce. This work was performed with an aim to investigate the possibility of use the flow cytometry as a tool for determination the osmotic behaviour of porcine and bovine erythrocytes, and thus facilitates the engineering of erythrocytes from animal blood to be drug carriers. The method of flow cytometry successfully provided the information about bovine and porcine erythrocyte osmotic fragility, and made the initial steps in assessment of erythrocyte shape in a large number of erythrocytes. Although this method is not able to confirm the swelling of porcine erythrocytes, it indicated the differences in porcine erythrocytes that had basic hematological parameters inside and outside the reference values. In order to apply/use the porcine and bovine erythrocytes as drug carriers, the method of flow cytometry, confirming the presence of osmotically different fractions of red blood cells, indicated that various amounts of the encapsulated drug in porcine and bovine erythrocytes can be expected.

Keywords: flow cytometry, osmotic swelling, osmotic fragility, mechanical fragility, microcytic anaemia, erythrocytes.

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Erythrocytes are the most abundant cellular components (>99%) of blood in humans. Besides their well known physiological functions, they serve as a natural blood compartment participating in biodistribution, metabolism and action of certain drugs [1]. On the other hand, the erythrocytes possess potential carrier capabilities and can be used for the controlled and targeted delivery of various bioactive compounds, including peptides and genetic materials [2]. Erythrocytes feature some unique advantages compared to other delivery systems, such as high biocompatibility, biodegradability, possibility of targeted drug delivery to the RES (reticuloendothelial system) organs, modification of the pharmacokinetic and pharmacodynamic parameters of the drug, etc. [3]. There are numerous reports that describe successful fabrications of engineered erythrocyte as novel carriers of various types of conventional and non-conventional drugs [4–8]. Various types of mammalian erythrocytes have been used for drug delivery, including erythrocytes of human, mice, cattle, pigs, dogs, sheep, goats, monkeys, rats and rabbits [2,3]. Methods based on the osmotic properties of the cells, such are hypotonic haemolysis, hypotonic dilution, hypotonic preswelling, hypotonic dialysis, etc., are the most widely used for loading of drugs in human and animal erythrocytes [2]. However, data related to the osmotic properties of erythrocytes derived from animal blood are scarce.

Classic tests for determination the osmotic behaviour of erythrocytes, including osmotic fragility test for determination of osmotic resistance [9] and microhematocrit method for determination of osmotic swelling [10], were found to be labour intensive and time consuming. The modern sophisticated method of flow cytometry emerges as a promising method for analysis of osmotic behaviour parameters for large number of cells in short time [11], although this method is rarely used for such studies in animals. This work was performed with an aim to investigate the possibility of use the flow cytometry as a tool for determination the osmotic behaviour of porcine and bovine erythrocytes, and thus facilitate the engineering of erythrocytes from animal blood to be drug carriers.

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EXPERIMENTAL

Chemicals

The salts used in the preparation of buffers were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA).

Blood samples and preparation of packed erythrocytes

This study was performed by using bovine and porcine blood, acquired from slaughterhouse "PKB Imes" in Belgrade, Serbia, and collected during slaughtering. Transport and treatment of the animals in the slaughterhouse was in obedience to the National Regulation on Animal Welfare, and all studies were performed in compliance with institutional animal care and use policies.

Blood of Holstein–Friesian calves and Swedish Landrace swine was taken from jugular vein and collected in a sterile glass bottle containing 3.8% sodium-citrate as an anticoagulant agent. Blood samples were transported at ambient temperature and processing started two hours after the collection. Centrifugation of whole blood at 2450*g* for 20 min at 4 °C was performed in Megafuge 1.0R, Heraus centrifuge (Langenselbold, Germany). Plasma and leucocytes (buffy coat) were carefully discarded by vacuum aspiration. The remaining pelleted erythrocytes were resuspended in isotonic (0.9%, *w*/*V*) saline solution, washed twice via centrifugation, and finally resuspended in an isotonic phosphate buffered saline, pH 7.2–7.4 (PBS, 0.8% saline buffered with 10 mM sodium phosphate).

The cyanmethaemoglobin method [12] was used for determination of haemoglobin concentration in erythrocyte suspension. Haematocrit (Hct expressed as %) was measured by the microhaematocrit method [13], while the erythrocyte were stained with Hayeam solution and counted using a Spenser haemocytometer. Haematological parameters, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to the formulas proposed by Wintrobe [14].

Osmotic fragility test

The osmotic resistance of erythrocytes was determined by the method of Beutler [9]. The extent of haemolysis (EH) in each sample was expressed as a percentage of the control, where the optical density (OD_{540}) of a distilled water-lysed sample of erythrocytes was set as 100%. The cumulative osmotic fragility curve was plotted from the *EH* values obtained in serial dilution of saline by Boltzmann sigmoidal function.

Determination of osmotic swelling by microhaematocrit method

The osmotic swelling index and extent of haemolysis were determined according to the methodology described by Vitvitsky *et al*. [10] and modified by Stojanović *et al*. [15].

Analysis of osmotic behaviour by flow cytometry

To analyse the osmotic behaviour of bovine and porcine erythrocytes by flow cytometry, a protocol based on determination of relative size of erythrocytes, after their incubation in series of buffer solutions with decreasing concentrations, was introduced.

In series of tubes each containing 2 mL of 5 mM sodium phosphate-buffered saline with NaCl solution of different concentrations (range from 155 to 80 mM), 5 µL of packed erythrocytes were added per tube. The obtained suspension was incubated at room temperature for 20 min. The erythrocytes' size was assessed by forward scatter (FSC) analysis on CyFlow® SL flow cytometer (Partec, Münster, Germany) using FlowMax 2.4 software (Partec, Münster, Germany). At least 60000 up to maximum 100000 events per sample were analysed using 0.1 µL/s flow rate. Under this condition acquisition speed was approximately 7000 events/s. The intensity of forward scatter (FSC) from each individual event was detected and assigned to one of 256, 512 or 1024 quantity classes, and presented in FSC/SSC dot plots as relative values in 3 decades logarithmic scale or in FSC/Events counts histograms in linear scale.

In order to evaluate the erythrocyte osmotic behaviour, the technique described by Piagnerelli *et al.* [16] was modified and expanded using the FSC signal in isotonic, as well as in hypotonic conditions.

Mechanical fragility test

Suspension of packed erythrocytes was prepared in PBS to approximate value of haematocrit 40%. Packed erythrocytes' suspension was added in each of five marked test tubes in volume of 5 mL. Three of them contained nothing but erythrocytes, and two of them additionally contained glass balls. One of the test tubes with and one without glass balls were placed in horizontal shaker with 320 rpm, and erythrocytes were exposed to mechanical force during 90 min. After the exposure to mechanical force (rocking), erythrocytes were centrifuged (20 min, 2465*g*), and the obtained supernatant was used for measuring the OD_{540}. Erythrocytes' pellet from the test tube which contained erythrocytes without balls and without exposing to mechanical force was overwhelmed with distilled water. After lysis (few minutes later) and centrifugation the obtained haemolysate was used for measuring the OD_{540}. The mechanical fragility index (*MFI*) in percentage was obtained according to the pattern described by Kameneva and Antaki [17]:

$$MFI(\%) = \frac{OD_{540\text{ tube with mechanical stress}} - OD_{540\text{ tube without mechanical stress}}}{OD_{540\text{ tube with hemolysate in water}} - OD_{540\text{ tube without mechanical stress}}}$$

RESULTS AND DISCUSSION

Red blood cells from mammals differ in many properties (cell diameter, volume and shape, membrane lipid composition, composition and organization of transmembrane and cytoskeleton protein, enzyme activity, ionic composition, ATP and other metabolite contents,…) [18,19], and each of them has less or more impact on osmotic behaviour. In this work, osmotic resistance of bovine and porcine erythrocytes was analysed by standard osmotic fragility test. All examined samples had basic erythrocytes indices in the reference range: *MCV* 40–55 and 53-54×10^{-15} L; *MCH* 11–17 and 17–21 pg; and *MCHC* 300–322 and 300–322 g/L, for bovine and porcine erythrocytes, respectively. The osmotic fragility curves obtained by plotting the extent of haemolysis against NaCl concentration were of a sigmoidal shape (Figure 1). Porcine erythrocytes were more osmotically fragile then bovine ones. The literature data regarding osmotic properties of animal erythrocytes are limited and differ, even opposite reports have been published. Cardoso and Camargos [20] claimed that porcine and human erythrocyte membranes possessed similar rheological properties. On the other hand, the articles published by Matsuzawa and Ikarashi [21], Brzezińska-Slebodzińska [22] and Johnstone and co-workers [23] are in agreement with our findings which show that bovine erythrocytes exhibit higher resistance to osmotic lysis than porcine erythrocytes.

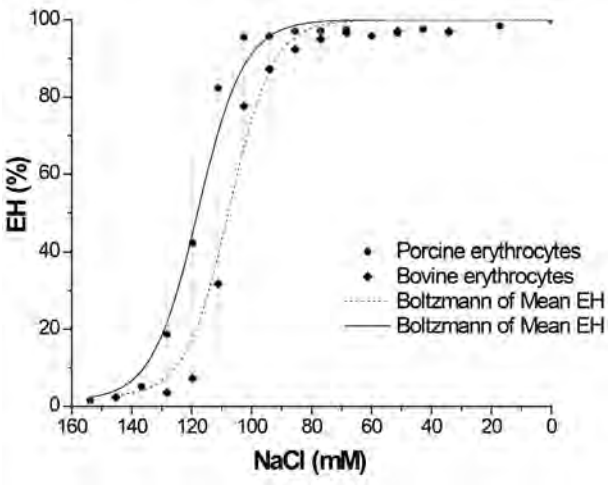

Figure 1. Mean cumulative osmotic fragility curves of bovine and porcine erythrocytes. Points – mean values of four samples; vertical bar – standard deviation; lines – fitting curves.

Parallel with the classical osmotic fragility test it was performed the mechanical fragility (MF) test, as a reliable and reproducible method of applying shear stress to erythrocytes. The output of the MF test is the Mechanical Fragility Index (*MFI*). Higher *MFI* values indicate that erythrocytes are more predisposed to lysis when exposed to shear stress. Both sets of the examined erythrocytes revealed similar low extent of mechanically induced haemolysis, as shown in Figure 2.

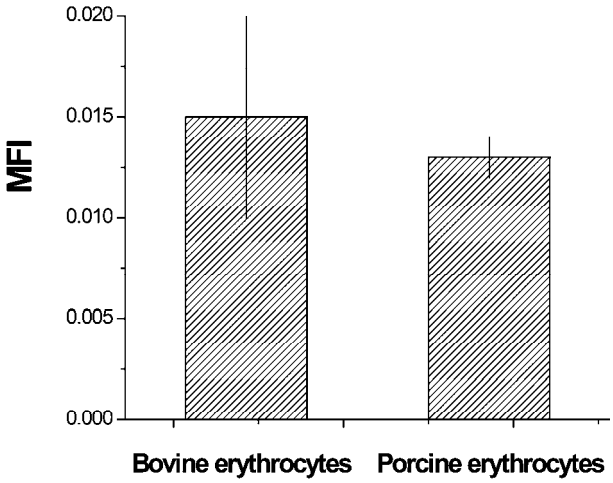

Figure 2. Mechanical fragility indexes (MFI) of bovine and porcine erythrocytes. Results are presented as mean ± SD values of three experiments.

Flow cytometric analyses were performed on erythrocytes incubated in the same buffer solutions which had been used for classic test of osmotic fragility. As depicted in FSC/SSC diagram in Figure 3 (a1 and b1), flow cytometer viewed the flow of both kinds of the examined erythrocytes in isotonic buffer solution as essentially two populations of cells. That phenomenon occurs due to biconcave shape of erythrocytes, and has been reported for human and mice erythrocytes [16,24,25], thus we fixed two gates of interest for these diagrams: R1 and R2. As presented in Figure 3 (a-2, b-2, a-3 and b-3), with decrease of buffer concentration from 155 to 65 mM one other population of small "events" (population R5) became visible. Most probably, the population of small vesicles was developed due to fragmentation of mechanically impaired swelled cells caused by shear flow in flow cytometer. This population of small vesicles enlarged with the decrease of buffer concentration down from 94 mM, and in the case of porcine erythrocytes at buffer concentration of 80 mM became predominant. In the case of bovine erythrocytes, FSC/SSC diagram obtained in buffer concentration of 65 mM (Figure 3, a-3) revealed solely the presence of those small vesicles. This result was in accordance with the previous finding obtained by osmo-

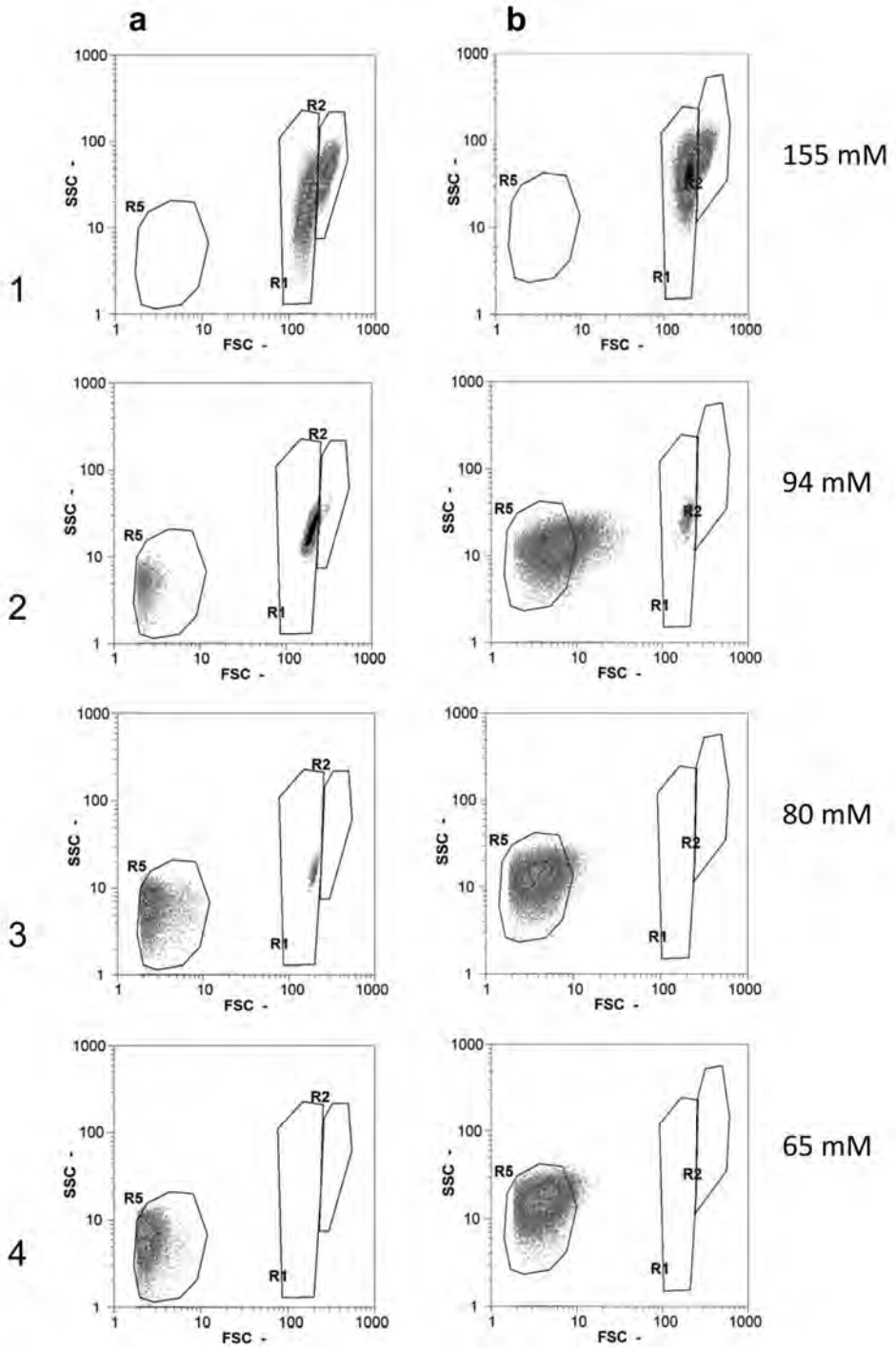

Figure 3. Flow cytometric analysis (FSC/SSC diagrams) of bovine (a) and porcine (b) erythrocytes exposed to hypotonic sodium-phosphate/NaCl buffers of decreasing concentrations. R1, R2 – erythrocyte populations, R5 – small vesicles.

tic fragility test that porcine erythrocytes were more osmotically fragile compared to bovine ones. Although the mechanical stress caused by shear flow in flow cytometer could not be neglected, the demonstrated equal MFI for bovine and porcine erythrocytes indicated that the obtained differences in FSC/SSC diagrams of erythrocytes incubated in same hypotonic buffer really reflected their different osmotic properties.

Regardless of the pronounced vesiculation, it was possible to analyse erythrocytes swelling by flow cytometry in 139 mM buffer, and buffers with lower concentration. *FSC*/counts histograms obtained for 2 samples of each bovine and porcine erythrocytes (Figures 4 and 5) in isotonic buffer solution (155 mM), because of their biconcave shape, showed bimodal size distribution (as represented by gates R1 and R2 in Fig-

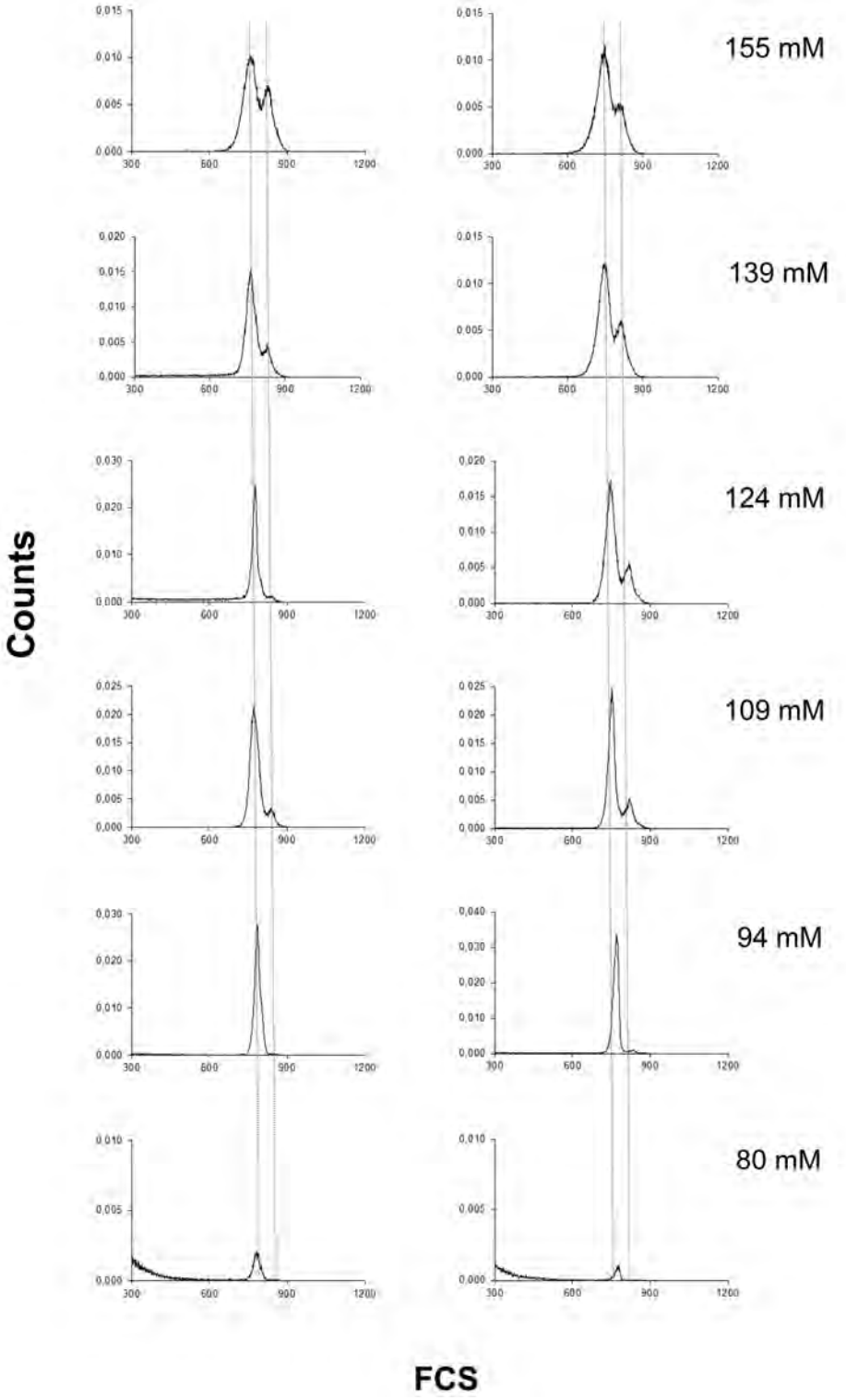

Figure 4. Changes in FSC values of bovine erythrocytes with decreasing concentration of sodium-phosphate/NaCl buffers.

ure 3). It is evident that bovine erythrocytes are smaller than porcine cells (reflected in minor *FSC* values), as also reported by other authors [26]. The hypotonic buffers were used with an aim to promote swelling and gave spherical shape of erythrocytes, thus eliminating variations in *FSC* caused by biconcave shape, as proposed by van den Bos [25]). As depicted in Figure 4, two representative samples of bovine erythrocytes with basic parameters within reference range revealed uniform pattern after exposing to hypotonic solutions. In the case of bovine erythrocytes, alterations in shape of the resulting *FSC*/counts histogram in corresponding

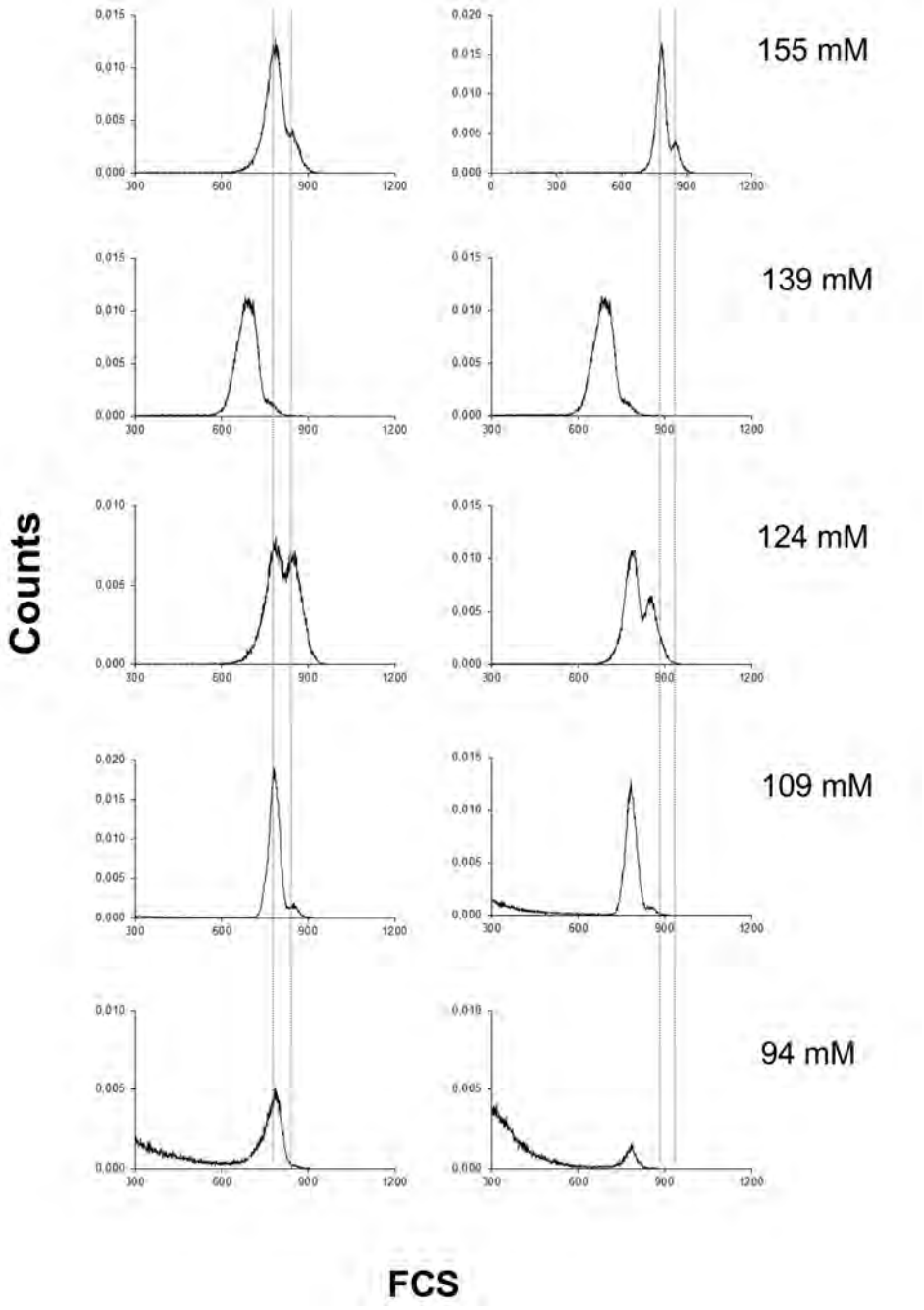

Figure 5. Changes in FSC values of porcine erythrocytes with decreasing concentration of sodium-phosphate/NaCl buffers.

hypotonic buffer reflected changes in erythrocyte osmotic properties, from swelling to fragmentation to small vesicles in 80 mM.

In the case of porcine erythrocytes, two samples from apparently healthy pigs with erythrocytes indices within reference range (Figure 5) did not reveal uniform *FSC*/counts pattern. Moreover, the resulting *FSC*/ /counts histograms in corresponding hypotonic buffers did not reveal swelling. The fragmentation of porcine erythrocytes has already been detected in 109 mM buffer, confirming once more their increased osmotic fragility compared to bovine erythrocytes. This finding about more osmotically fragile porcine erythrocytes was opposite to the reports of Jain [26], who declared that there was a linear relationship between cell size and osmotic resistance. Thus, smaller bovine erythrocytes (with minor *FSC* values) are expected to be less osmotic resistant than larger porcine ones (with higher *FSC* values), but results obtained by flow cytometry showed the opposite.

Furthermore, from this study we postulated the existence of osmotically different fractions of erythrocytes derived from bovine and porcine blood. Obviously, the fractions were different in size (as bimodal

size distribution in hypotonic buffer was confirmed by flow cytometry) and in swelling ability. As far as we know, bimodal distribution of size detected by flow-cytometry is typical for biconcave cells, such are the erythrocytes, as long as they are in the isotonic suspension [25], while this is not a case for cells which suffered from shape distortion to spheres upon treatment with a hypotonic solution. Thus, different loaded amount of drug in individual erythrocyte derived from both porcine and bovine blood could be expected, as it was reported for human erythrocyte [27].

Besides samples which had normal erythrocytes indices, one of the examined samples of porcine erythrocytes had *MCV* (42×10^{-15} L) and *MCH* (15 pg) values lower than the reference ones, indicating to a potential disorder of microcytic hypochromic anaemia [28]. *FSC*/ /counts histograms of that sample of porcine erythrocytes incubated in isotonic and hypotonic buffers (Figure 6) exposed *FSC*/counts patterns different from

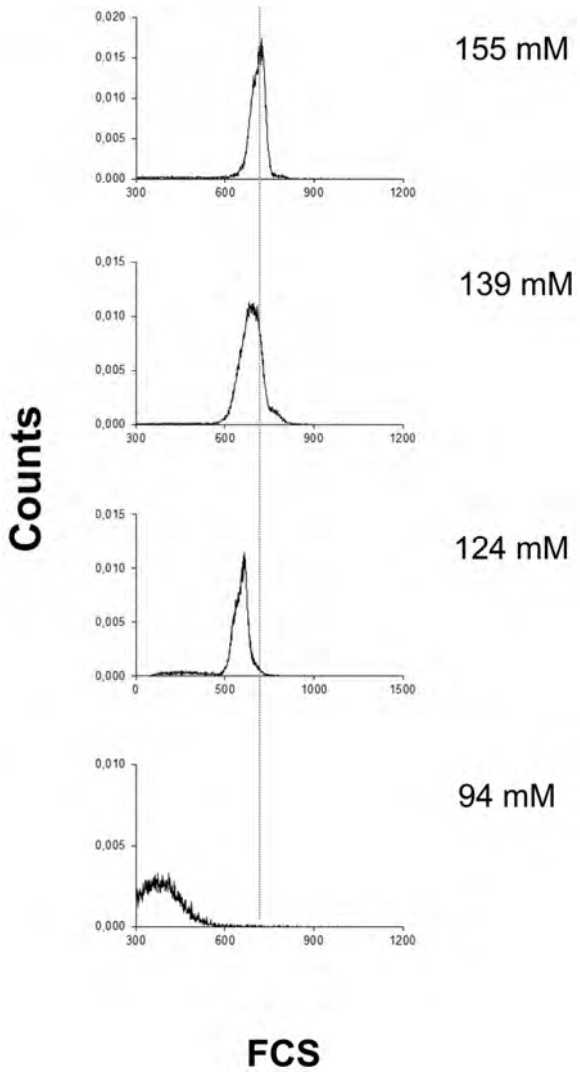

Figure 6. Changes in FSC values of "microcytic" porcine erythrocytes with decreasing concentration of sodium-phosphate/NaCl buffers.

those of apparently healthy samples of porcine erythrocytes, with decreased *FCS* values and changed shape of the *FCS*/counts curve (Figure 5). Since the flow cytometry technique has already been successfully described for assessment of erythrocytes shape in isotonic conditions in different pathologies [16], we compared the samples of apparently healthy bovine and porcine erythrocytes, as well as sample of porcine erythrocytes having basic erythrocyte parameters without reference range. As described by Piagnerelli *et al*. [16], the calculated spherical indexes amounted to 1.58±0.08 and 1.67±0.08, respectively, for the samples of porcine and bovine erythrocytes, while the potentially pathological sample of porcine erythrocytes gave the index of 1.45. The obtained minor index in the case of porcine erythrocyte sample with potential microcytic anaemia was in accordance with reports of human spherocytes which possess increased osmotic fragility [29].

Furthermore, we extended the procedure for shape assessment described by Piagnerelli *et al*. [16], and compared the mentioned indexes of the determined gates R1 and R2, obtained in hypotonic solutions, with results of osmotic swelling obtained from more routinely used microhematocrit test. In the mentioned comparison, the index obtained in isotonic solution was taken as a unit value. The correlations of the results are shown in Figure 7a and b, for bovine and porcine erythrocytes, respectively. The swelling index obtained by microhematocrit method was strongly correlated with the indexes obtained by flow cytometry in the case of bovine erythrocytes, Hct: R2/R1 = 0.84, p = 0.036, Hct: R1/R1 = 0.95, p = 0.0034, Hct: R2/R2 = 0.83, p = 0.039 ($p < 0.05$), as shown in Figure 7a. In the sample of porcine erythrocytes, there was no significant correlation between the swelling index calculated by microhematocrit method and indexes obtained by flow cytometry (Figure 7b).

CONCLUSION

The flow cytometry technique seemed to be a rapid technique, easier than the conventional methods for determination of osmotic behaviour of erythrocytes. By the use of this technique we successfully provided the information about bovine and porcine erythrocyte osmotic fragility, and made initial steps in assessment of erythrocyte shape in a large number of erythrocytes. Nevertheless, this technique could not provide confirmation of swelling of porcine erythrocytes, but indicated the difference in samples of porcine erythrocytes within and without range of basic haematological parameters. Toward use of bovine and porcine erythrocytes as drug carriers, the demonstrated existence of osmotically different fractions of erythrocytes indicated that different loaded amount of drug in individual erythrocyte derived from both porcine and bovine blood can

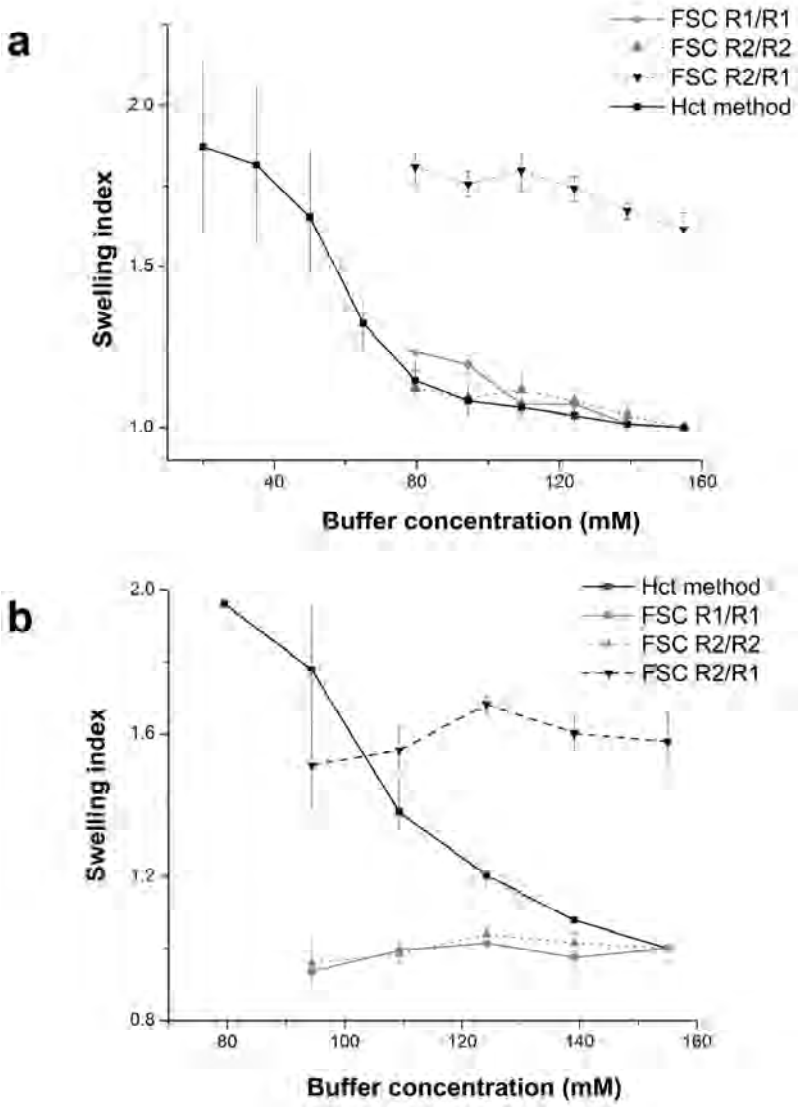

Figure 7. The swelling index of bovine (a) and porcine (b) erythrocytes determined by microhematocrit method, as described by Stojanović et al. (2012) (results are presented as mean ± SD values of five experiments) and by flow cytometry (results are presented as mean ± SD values of three experiments).

be expected. With an aim to define more in depth the osmotic and related rheological properties of different mammalian erythrocytes, application of flow cytometry technique merits further investigation.

Acknowledgement

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IZVOD

ODREĐIVANJE OSMOTSKIH OSOBINA ŽIVOTINJSKIH ERITROCITA PROTOČNOM CITOMETRIJOM U CILJU NJIHOVOG INŽENJERINGA KAO NOSAČA LEKOVA

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(Naučni rad)

Uprkos činjenici da su metode koje se baziraju na osmotskim osobinama ćelija najčešće korišćene metode za inkapsulaciju lekova u humane i životinjske eritrocite, podaci o osmotskim osobinama eritrocita životinjskog porekla su vrlo oskudni. Cilj ovog rada bio je ispitivanje mogućnosti korišćenja metode protočne citometrije za određivanje osmotskih osobina svinjskih i goveđih eritrocita, čime bi se olakšao inženjering pomenutih životinjskih eritrocita za otpuštanje lekova. Metodom protočne citometrije uspešno su dobijene informacije o osmotskoj fragilnosti svinjskih i goveđih eritrocita i načinjeni su početni koraci u proceni oblika velikog broja eritrocita. Iako ova metoda nije uspela da potvrdi bubrenje svinjskih eritrocita, ukazala je na razliku u uzorcima svinjskih eritrocita koji su imali osnovne hematološke parametre izvan i unutar referentnih vrednosti. U cilju primene svinjskih i goveđih eritrocita kao nosača lekova, metoda protočne citometrije je, potvrdivši prisustvo osmotski različitih frakcija eritrocita, ukazala na to da se različite količine inkapsuliranog leka u pojedinačnim, kako svinjskim, tako i goveđim eritrocitima mogu očekivati.

Ključne reči: Protočna citometrija • Osmotsko bubrenje • Osmotska fragilnost • Mehanička fragilnost • Mikrocitna anemija • Eritrociti

Optimization of frozen wild blueberry vacuum drying process

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Abstract

The objective of this research was to optimize the vacuum drying of frozen blueberries in order to preserve health benefits of phytochemicals using response surface methodology. The drying was performed in a new design of vacuum dryer equipment. Investigated range of temperature was 46–74 °C and of pressure 38–464 mbar. Total solids, total phenolics, vitamin C, anthocyanin content and total color change were used as quality indicators of dried blueberries. Within the experimental range of studied variables, the optimum conditions of 60 °C and 100 mbar were established for vacuum drying of blueberries. Separate validation experiments were conducted at optimum conditions to verify predictions and adequacy of the second-order polynomial models. Under these optimal conditions, the predicted amount of total phenolics was 3.70 g CAE/100_{dw}, vitamin C 59.79 mg/$100g_{dw}$, anthocyanin content 2746.33 mg/100 g_{dw}, total solids 89.50% and total color change 88.83.

Keywords: blueberry, vacuum drying, response surface methodology, product quality.

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Blueberries (*Vaccinium myrtillus* L.) are a valuable fruit worldwide, due to their improved nutritive value and many health benefits. The use of blueberries as a medicine dates since the 16th century. It has been included in many pharmacopoeias, and used in medicine and pharmacy [1]. In addition, berry fruits and their extracts can nowadays be used as a component of functional foods, dietary foods or dietary supplements [2]. Blueberries are also known for their high anthocyanin and flavonoid content. Their high antioxidant capacity has been attributed to high anthocyanin pigment content; their health-promoting features are attributed to phenolic acids and flavonoids. Many scientific papers report protective effect of berries and berry extracts such as antioxidant [2–6], anti-inflammatory, hepatoprotective and anticarcinogenic [1,7]. Reported values can vary depending on the variety. Moreover, total phenolic, total anthocyanin content, and antioxidant activities were significantly higher in wild berries than in cultivated ones [8].

As there are no many data about wild blueberries, the aim of this research was to determine the main chemical composition, vitamin C, anthocyanin, phenolics of wild blueberry fruits from Kopaonik mountain region in Serbia. In the present study, we applied vacuum drying to produce dried blueberries. To preserve the main health benefits of phytochemicals of dried blueberries, the influence of temperature and pressure on the quality of the final blueberry dried product under the applied experimental range using response surface methodology (RSM) was investigated.

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MATERIAL AND METHODS

Chemicals

Vitamin C, produced by J.T. Baker (Holland) was used as a standard. Standard substance and samples were dissolved/extracted in the solution of 3% *m*-phosphoric acid (Riedel-de Haën, Germany) in 8% acetic acid (J.T. Baker, Holland). Ammonia-acetate solution was used as a mobile phase (0.1 M; pH 5.1). Solutions were prepared in redistilled water with appropriate quality for HPLC analysis. Folin-Ciocalteu reagent was purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Chlorogenic acid was purchased from Sigma (Sigma, St. Luis, MO, USA). All other chemicals and reagents were of analytical reagent grade.

Material

Fruits of wild blueberry were grown in the region of Kopaonik mountain, Serbia, and hand-harvested at commercial maturity stage in season 2011. The collected fruits were washed, frozen and stored at –35 °C until analysis.

Drying procedure

Drying was performed in a vacuum dryer prototype (Figure 1) constructed and installed at the Department of Food Preservation, Faculty of Technology, Novi Sad (Serbia). Experimental drying facility consists of a cylindrical vacuum chamber made of steel sheet with a volume of about 70 L. The vacuum pump provides pressure in the chamber of 2 mbar. The chamber is equip-

ped with the condensate collector. The aluminum tray is fixed in a special frame and connected to a balance.

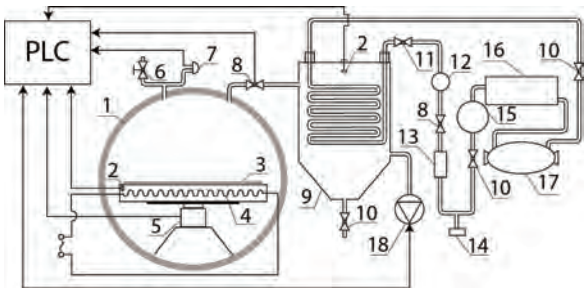

Figure 1. Schematic diagram of the vacuum drying equipment. 1: drying chamber; 2: temperature sensor; 3: sample holder; 4: heater; 5: load cell; 6: vacuum break-up valve; 7: pressure sensor; 8: solenoid valve; 9: condensate collector; 10: valves; 11: expansion valve; 12: flow indicator; 13: evaporator; 14: receiver; 15: pressure control; 16: condenser; 17: compressor; 18: vacuum pump.

The drying procedure control system (PLC) registers all working parameters (pressure in vacuum chamber, temperature on the heater surface and the change in product weight) during the drying process and the system controls the level of electric power supplied to the heaters to provide a product temperature not more than 75 °C.

The most relevant technical features relating for device are the following: 25–75 °C working temperature range; sensor sensitivity ±0.3 °C; 1.000 g balance maximum load; 0.03% balance sensitivity; 0.1 g balance resolution; 2–1000 mbar working pressure range, sensor sensitivity ±0.5.

The samples were uniformly arranged on the tray as a thin layer. Sample size was kept constant (about 400 g) for each experiment. Weight loss was recorded in 5 minute intervals and drying was continued until no mass change was detected (final moisture content in equilibrium). Drying runs were performed at pressures of 38 to 462 mbar and temperatures of 46 to 74 °C, according to the experimental plan given in Table 1. Drying times ranged from 5.5 to 29.1 h, depending on working conditions (temperature and pressure).

Table 1. The uncoded and coded levels of independent variables used in the RSM design

Independent variable	Symbol	Level				
		–1.414	–1	0	+1	1.414
Drying temperature, °C	X_1	46	50	60	70	74
Vacuum pressure, mbar	X_2	38	100	250	400	464

Experimental design

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. RSM is a critical technology in developing new processes and optimizing their performance. The objectives of quality improvement, including reduction of variability and improved process and product performance, can often be accomplished directly using RSM [9]. The central composite rotatable design (CCRD) was used for determining optimal drying temperature and vacuum pressure for drying process of frozen blueberries [10]. Drying air temperature (X_1) and vacuum pressure (X_2) were independent variables studied to optimize the drying process in terms of getting better final product quality (y). Investigated factors and levels tested were reported in Table 1. Experimental data were fitted with second order response surface model with the following form:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \quad (1)$$

where y are investigated responses (total solids, total phenolics, vitamin C, anthocyanin content and total colour change), β_0, β_j, β_{jj} and β_{ij} are constant coefficients of intercept, linear, quadratic, and interaction terms, respectively; X_i and X_j are coded independent variables (drying temperature and vacuum pressure).

Statistical analysis

Statistical analysis was performed using RSM software Design-Expert® v.7 (Stat-Ease, MN, USA). The results were statistically tested by the analysis of variance (ANOVA) at the significance level of p = 0.05. The adequacy of the model was evaluated by the coefficient of determination (R^2) and model p-value. A mathematical model was established to describe the influence of single process parameter and/or interaction of multiple parameters on each investigated response. Response surface plots were generated with the same software and drawn by using the function of two factors, and keeping the other constant.

Total solids

Total solids were determined by drying the samples at 105 °C until constant weight. Experiments were replicated three times for statistical purpose.

Total phenols

Dried blueberry samples were ground in a blender before the extraction. 10.0 g of this way prepared sample was transferred to volumetric flask and 50 ml of methanol, as extraction solvent, was added. Extraction was carried out for 24 h at the room temperature, after obtained extract was filtered. Prepared blueberry extracts were placed into a glass bottles and stored to prevent oxidative damage until analysis. The content of total phenolic compounds in blueberry extracts was

determined by Folin–Ciocalteu procedure [5,11] using chlorogenic acid as a standard. Absorbance was measured at 765 nm. Content of total phenolic compounds has been expressed as g of chlorogenic acid equivalent per 100 g of dried blueberries (g CAE/100 g_{dw}). Experiments were replicated three times for statistical purpose.

Vitamin C

2.5 g of ground dried blueberries was transferred to 25 ml volumetric flask, 3% m-phosphoric acid in 8% acetic acid was added and the mass was mixed for 5 min. The flask was filled up to the volume and filtered. Activated carbon was added to the filtered solution to remove the colour and filtered through filter paper (blue label) and membrane syringe filter with diameter pore of 0.45 μm. The filtrate was used for HPLC analysis of vitamin C at the HPLC system (Agilent 1100, USA) equipped with C-8 column and DAD detector. Mobile phase (0.1 M ammonia-acetate) flow rate was 0.4 ml/min and column temperature 37 °C. All analyses were performed in triplicate.

Anthocyanin content

Anthocyanin content (total monomeric anthocyanin content, TMA) in dried blueberries was determined as described by Giusti and Wrolstad [12], based on the pH-differential method previously described by Fuleki and Francis [13]. Blueberry extract absorbances were measured at 510 and 700 nm in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). Anthocyanin content was expressed as mg of cyanidine 3-glucoside equivalents per 100 g of dry weight (total solids) of blueberries. Experiments were replicated three times for statistical purpose.

Surface colour

The CIE *L*a*b** colour coordinates were measured using MINOLTA Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan). The apparent (surface) colour of samples was measured in terms of *L* (degree of darkness), *a* (degree of redness and greenness) and *b* (degree of yellowness and blueness). Finally, the total color change between blank white (L_0^*, a_0^* and b_0^*) and dried blueberry samples (L^*, a^* and b^*) was determined according to:

$$\Delta E = \sqrt{\left[\left(L_0 - L\right)^2 + \left(a_0 - a\right)^2 + \left(b_0 - b\right)^2 \right]} \quad (2)$$

Samples were placed on the measure head of Chroma Meter and measurements of color were performed for all prepared samples. A standard white color was used for calibration. Experiments were replicated five times for statistical purpose.

RESULTS AND DISCUSSION

Response surface methodology (RSM) was used in order to optimize vacuum drying process of blueberries. In this study, effects of drying temperature (46–74 °C) and pressure (38–462 mbar) on the total solids, total phenolics content, vitamin C, antioxidant activity, total anthocyanin content and total colour change of the blueberries dried in vacuum drier were investigated (Table 2). Table 3 shows the corresponding *p*-values for selected response variables of blueberries for each obtained coefficients and interactions. The effect of the linear, quadratic or interaction coefficients on the response was tested for significance by analysis of variance (ANOVA). Analysis of variance (Table 4) shows that the regression models for all investigated responses were statistically relevant with a significance

Table 2. The experimental design and data for the response surface analysis

Run	Temp. °C	Vacuum pressure mbar	Total solids %	Total phenolics content g CAE/100 g_{dw}	Vitamin C mg/100 g_{dw}	Anthocyanin content mg/100 g_{dw}	Total color change
1	64	250	89.11	3.6395	52.622	2455.10	89.89
2	60	400	87.98	2.9025	47.699	1986.40	89.68
3	60	100	90.26	3.5061	67.593	2753.45	88.33
4	50	462	76.41	2.1529	36.760	1984.02	90.21
5	50	250	79.41	2.9782	36.991	2067.01	91.05
6	50	250	81.53	2.9891	37.649	1975.59	91.21
7	50	250	82.68	2.8828	39.859	1927.40	90.88
8	50	250	83.82	3.1804	36.814	1955.22	91.24
9	50	250	74.84	2.7565	34.482	1920.42	90.63
10	50	38	83.84	3.3039	35.699	2455.74	89.58
11	40	400	80.45	2.8211	32.703	1828.70	91.88
12	40	100	76.99	2.5993	28.154	1938.93	91.57
13	36	250	76.86	2.7003	34.805	1771.99	90.60

Table 3. Corresponding p-values for selected response variable of dried blueberries for each obtained coefficients; X_1: drying temperature; X_2: vacuum pressure; $p < 0.01$ highly significant; $0.01 \leq p < 0.05$ significant; $p \geq 0.05$ not significant

Response	Term				
	X_1	X_2	X_1^2	X_2^2	X_1X_2
Total solids	0.0009	0.3774	0.1533	0.6419	0.2777
Total phenolics	0.0058	0.0114	0.2141	0.1987	0.0889
Vitamin C	0.0010	0.3790	0.0664	0.7882	0.0515
Anthocyanin content	< 0.0001	< 0.0001	0.0234	0.0011	0.0007
Total color change	0.0096	0.1834	0.2439	0.1013	0.4465

Table 4. Analysis of variance (ANOVA) of the modelled responses; the recovery

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
		Total solids			
Model	213.37	5	42.67	7.18	0.0111
Residual	41.63	7	5.95	–	–
Lack of fit	27.74	3	9.25	2.66	0.1838
Pure error	13.89	4	3.47	–	–
Total	255.00	12	–	–	–
		$R^2 = 0.9217$			
		Total phenolics			
Model	1.54	5	0.31	7.06	0.0117
Residual	0.31	7	0.044	–	–
Lack of fit	0.21	3	0.070	2.87	0.1677
Pure error	0.097	4	0.024	–	–
Total	1.85	12	–	–	–
		$R^2 = 0.8344$			
		Vitamin C			
Model	1094.23	5	218.85	8.05	0.0081
Residual	190.23	7	27.18	–	–
Lack of fit	175.39	3	58.46	15.75	0.0111
Pure error	14.84	4	58.46	–	–
Total	1284.46	12	–	–	–
		$R^2 = 0.8519$			
		Anthocyanin content			
Model	984000	5	196800	60.88	< 0.0001
Residual	22630.64	7	3232.95	–	–
Lack of fit	8700.87	3	2900.29	0.83	0.5415
Pure error	13929.77	4	3482.44	–	–
Total	1007000	12	–	–	–
		$R^2 = 0.9775$			
		Total color change			
Model	8.29	5	1.66	3.99	0.0495
Residual	2.91	7	0.42	–	–
Lack of fit	2.65	3	0.88	13.85	0.0140
Pure error	0.26	4	0.064	–	–
Total	11.20	12	–	–	–
		$R^2 = 0.8368$			

level ranging from $p < 0.0001$ (for anthocyanin content) to $p = 0.0495$ (for total colour change). The fitted model represent the experimental data well with high correlation coefficients, R^2, varying from 0.8344 to 0.9775, depending on investigated responses. The second order polynomial models used to express the investigated responses (y) as a function of independent variables (in terms of coded values) are shown in Table 5.

Total phenolic content of dried blueberries

Blueberries are a very valuable fruit due to their high concentration of phenolics and anthocyanins [6]. Total phenolic content in different varieties of blueberry can be from 251 [8], 300 to 384 [14], but can range up to 929 mgGAE/100 g [15]. The data about individual phenolics are different in the literature. You and others [14] identified seven phenolics in blueberries: caffeic acid, chlorogenic acid, *p*-coumaric acid, 4-*O*-feruloulquinic acid, 5-*O*-feruloylquinic acid, trans--ferulic acid and quercetin, with chlorogenic acid being the dominant. They did not found gallic acid, or catechin, which were dominant in the research of Sellapan and others [15]. Generally, depending on the cultivar, growing season and location, the content and profile of phenolic compounds vary [8].

In this study, the total phenolic content of dried blueberries varied from 2.15 to 3.64 g CAE/100 g_{dw} according to different investigated parameter levels. Total phenolic content was significantly influenced by linear term of drying temperature and pressure (Table 3). Furthermore, the interaction between temperature and pressure (X_1X_2) didn't have a significant effect on total phenolic content ($p = 0.0889$) as well as quadratic term of temperature ($p = 0.2141$) and quadratic term of pressure ($p = 0.1987$). The second order polynomial model used to express the total phenolic content (y_3) as a function of independent variables (in terms of coded values) are shown in Table 5. Figure 2 shows that total phenolic content in dried blueberries increased with increasing of drying temperature. It can be seen also that the total phenolic content increased slightly with increasing pressure until 260 mbar, while further increase did not show any significant change on total phenolic content.

Table 5. The second order polynomial models used to express the investigated responses (y) as a function of independent variables (in terms of coded values); X_1: drying temperature; X_2: vacuum pressure

Response	Second order polynomial model	Eq.
Total solids	$y_1 = 81.46 + 4.77X_1 - 0.81X_2 + 1.48X_1^2 - 0.45X_2^2 - 1.43X_1X_2$	(3)
Total phenolics	$y_2 = 2.96 + 0.29X_1 - 0.25X_2 + 0.11X_1^2 - 0.11X_2^2 - 0.21X_1X_2$	(4)
Vitamin C	$y_3 = 37.16 + 9.95X_1 - 1.73X_2 + 4.29X_1^2 + 0.55X_2^2 - 6.11X_1X_2$	(5)
Anthocyanin content	$y_4 = 1969.13 + 242.29X_1 - 193.05X_2 + 62.25X_1^2 + 115.4X_2^2 - 164.20X_1X_2$	(6)
Total color change	$y_5 = 91.00 - 0.81X_1 + 0.34X_2 - 0.31X_1^2 - 0.46X_2^2 + 0.26X_1X_2$	(7)

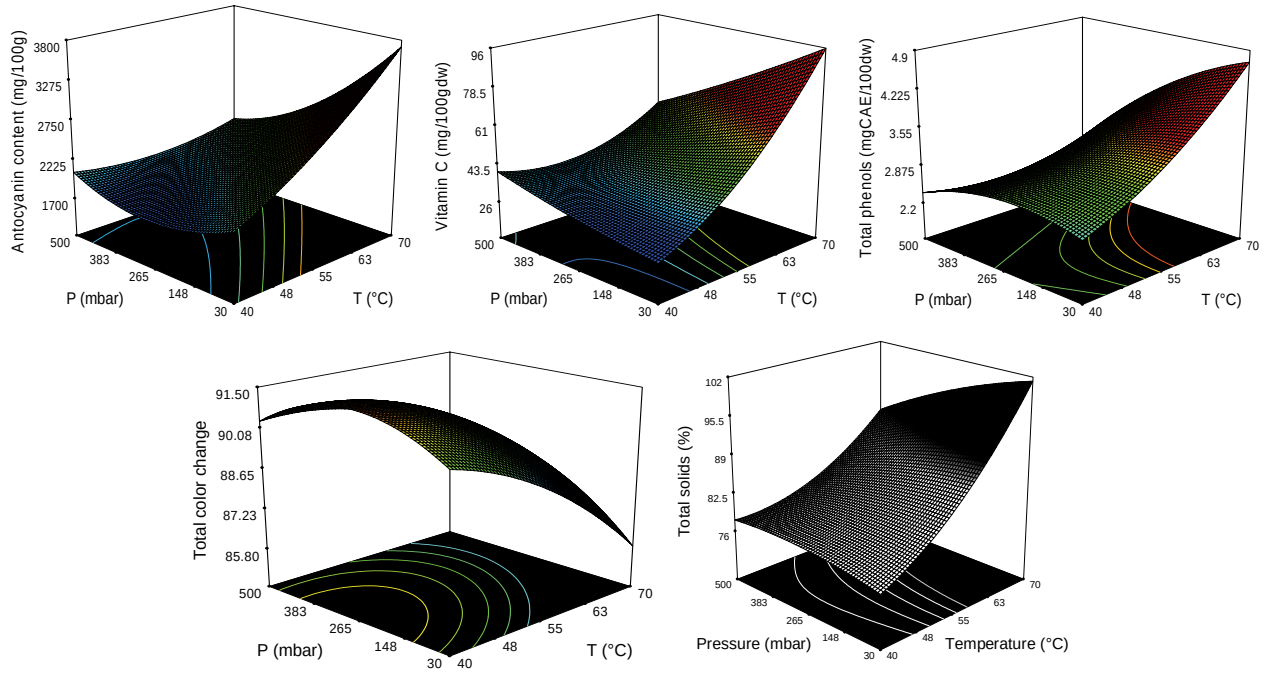

Figure 2. Surface plot for obtained responses as a function of vacuum pressure and drying temperature.

Anthocyanin content of dried blueberries

Besides their role as colour substances, anthocyanins are group of compounds which can provide many health benefits to humans. In general, total phenolics and total anthocyanins are higher in wild in comparison to cultivated fruits [8]. Sellapan and others [15] reported that blueberries had 87.4–197 mg anthocyanins per 100 g of fruit. Results obtained by You and others [14] showed that total anthocyanin contents in different cultivars was in the range from 116 to 224 mg/100 g, and there was even no significant difference between samples grown in organic and conventional conditions. Five main groups of anthocyanidins were identified in blueberries: cyanidin, delphinidin, peonidin, malvidin and petunidin [14,16]. They concluded that HPLC–MS method for individual anthocyanins and spectrophotometric method for total anthocyanins were reliable.

In this study, anthocyanin content was in the range from 1771.99 to 2753.45 mg/100 g_{dw}. According to the data from the Table 3, it can be seen that linear term of drying temperature had statistically significant influence on anthocyanin content of dried blueberries (p < < 0.0001). Furthermore, the quadratic terms of vacuum pressure and drying temperature also show significant effect on anthocyanin content of dried blueberries as well as the interaction between these two parameters (p = 0.0007). Effects of drying temperature and vacuum pressure on anthocyanin content of dried blueberries can be described by equation (Eq. (7)) and is presented in Figure 2. From this figure it can be seen that by increasing of drying temperature anthocyanin content significantly increase. With the increase of pressure to about 300 mbar, anthocyanin content slightly decreases. Further increase of pressure led to increase of antocyanin content in dried blueberries.

Vitamin C

Vitamin C is a compound that has many important biological functions in human body. When speaking about drying of fruit, the vitamin C content can serve as an indicator of severity of the drying process. In fresh blueberries, vitamin C content can range from 1.3 to 16.4 mg/100 g [6,17]. Also, depending on the cultivar, there can be a significant variation in the vitamin C content.

In this study, the vitamin C content of dried blueberries varied from 28.15 to 67.59 mg/100 g_{dw}. It is evident from Table 3 that linear term of drying temperature was the most predominant factor influencing vitamin C content (p = 0.0010) . According to the Figure 2, it can be seen that by increasing of drying temperature vitamin C content increases significantly. The vitamin C content also increases with the increasing of vacuum pressure.

Total solids

Total solids in fresh blueberries can range from 13.4 to 15.6 g/100 g [6] up to 16.8 [18]. In vacuum dried sour cherries [19], the total solids were in the range from 32.52 to 86.47% (*i.e.*, 67.48–13.53% of moisture), depending on the drying conditions used.

Figure 2 shows that temperature had significant influence on total solids of dried blueberries. With the increase of temperature, the total solids increase also. This can be also confirmed by p-value (Table 3), where linear term of drying temperature (p = 0.0009) significantly influenced total solids, while other variables didn't show significant influence on total solids.

Total colour change

According to Mascan [20], total colour difference, ΔE, "which is a combination of parameters L-, a- and b--values, is a colorimetric parameter extensively used to characterise the variation of colors in foods during processing". If the difference between the samples is less than 1.0, it is assumed that difference would not be sensitively perceptible [21].

Total colour change (ΔE) of the vacuum dried blueberries varied from 88.33 to 91.88 which showed small difference in total colour change of dried samples. Liner terms of temperature (p = 0.0096) significantly affect the colour of the samples, while linear term of pressure, quadratic terms of investigated parameters and interaction between temperature and pressure didn't have a significant effect on total colour change (Table 3). Figure 2 shows surface plot for total colour change value as a function of temperature and pressure for the vacuum drying of blueberries. The plots indicate that ΔE values increased with increasing of drying temperature. It can be seen also that ΔE increased with increasing pressure up to about 300 mbar, while further increase show decrease of ΔE the dried blueberries.

Optimization of frozen blueberry vacuum drying process

The first goal for Response Surface Methodology is to find the optimum response. When there is more than one response then it is important to find the compromise optimum that does not optimize only one response [22]. The second goal is to understand how the response changes in a given direction by adjusting the design variables. The investigated response surface variables determine the quality of the product. The main goal of this research was to find the best settings for drying parameters, temperature and vacuum pressure. Desirability function was developed for the following criteria: maximum content of total phenols, vitamin C and antocyanin in dried blueberries and minimum total color change. By applying desirability function method, the optimum conditions of 60 °C and 100

mbar were obtained for vacuum drying of blueberries. At this optimum point, the calculated investigated responses were as follows: total phenolics 3.70 g CAE/ /100 g_{dw}, vitamin C 59.79 mg/100 g_{dw}, anthocyanin content 2746.33 mg/100 g_{dw}, total solids 89.50% and total colour change 88.83.

CONCLUSIONS

Response surface methodology was used for determining optimal drying temperature and vacuum pressure for drying process of frozen blueberries in terms of getting better final product quality. The total solids, total phenolic, vitamin C, anthocyanin content and total colour change were used as quality indicators of dried blueberries. The analysis of variance (ANOVA) showed that the regression models were statistically good with a significance level of $p < 0.05$ for all investigated responses. Considering the maximum amount of total phenolic content, vitamin C, antocyanin in dried blueberries as well as the minimum total colour change of the samples, the following optimum drying conditions were obtained: temperature of 60 °C and vacuum pressure of 100 mbar. The calculated responses were as follows: total phenols 3.70 g CAE/100 g_{dw}, vitamin C 59.79 mg/100 g_{dw}, anthocyanin content 2746.33 mg/ /100 g_{dw}, total solids 89.50%, and total colour change 88.83. Separate validation experiments were conducted at optimum conditions to verify predictions and adequacy of the second-order polynomial models. The experimental values agreed with those predicted, thus indicating the success of response surface methodology in optimizing the investigated drying conditions.

Acknowledgment

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IZVOD

OPTIMIZACIJA VAKUUM SUŠENJA ZAMRZNUTE DIVLJE BOROVNICE

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(Naučni rad)

Cilj ovog istraživanja bila je optimizacija vakuum sušenja zamrznute borovnice primenom metode odzivne površine, u cilju očuvanja fitohemikalija. Sušenje je izvedeno u vakuum sušnici nove konstrukcije. Ispitani opseg temperatura i pritiska bio je 46–74 °C i 38–464 mbar. Kao pokazatelji kvaliteta sušene borovnice korišćeni su suva materija, ukupni fenoli, vitamin C, antocijani i ukupna promena boje. U okviru ispitanih parametara u eksperimentalnom opsegu, utvrđeni su optimalni uslovi vakuum sušenja borovnice na 60 °C i 100 mbar. Sprovedeni su odvojeni eksperimenti validacije u optimalnim uslovima u cilju verifikacije predviđanja i pogodnosti polinomskih modela drugog reda. U ovim optimalnim uslovima predviđena količina ukupnih fenola bila je 3,70 g CAE/100 g_{SM}, vitamina C 59,79 mg/100 g_{SM}, antocijana 2746,33 mg/100 g_{SM}, ukupna suva materija 89,50% i ukupna promena boje 88,83.

Ključne reči: Borovnica • Vakuum sušenje • Metoda odzivne površine • Kvalitet proizvoda

Effect of plant extracts of *Kitaibelia vitifolia* on antioxidant activity, chemical characteristics, microbiological status and sensory properties of Pirotski Kachkaval cheese

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Abstract

The aim of our study was to evaluate the impact of cheese (Pirotski Kachkaval) fortification by polyphenols attributed to *Kitaibelia vitifolia* ethanol herb extract, applied in two different manners (added to the cheese curd after texturizing or sprayed on surface of cheese). Investigation of the used antioxidant effects of polyphenols, physic-chemical composition, microbiological quality and sensory properties of Pirotski Kachkaval was undertaken. Antioxidant activity of conventional and fortified cheese was evaluated by five contemporary and compatible methods, and revealed a slight emphasis on phenol-linked antioxidant activity of fortified samples of cheese in comparison to samples of the control group. Fortified Pirotski Kachkaval had higher sensory evaluation scores than the controls. Statistically significant ($P < 0.05$) changes were observed in moisture content and total solids of control and modified series of cheese, but other parameters did not differ significantly ($P > 0.05$).

Keywords: Pirotski Kachkaval, *Kitaibelia vitifolia*, polyphenols, antioxidant activity, microbiological quality, sensory properties.

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Nowadays, there is a strong trend of investing into research and testing of natural antioxidants (preservatives) and their biological activity for use in the food industry, because many of the substances of synthetic origin have proven to be carcinogens [1]. Application of polyphenols from natural sources through diet may prevent oxidative stress and its deleterious effects and thereby improve the quality and nutritional value of food [2]. Polyphenols are complex group of secondary metabolites, widely distributed in plants and food of plant origin, divided into several classes [3]. The most represented classes of phenol compounds in human diet are phenolic acids and flavonoids [4]. There are numerous other biological processes affected by polyphenols, such as antioxidant activity, protection against cancer [5], cardiovascular diseases [6], inflammatory, allergic, diarrheic and ulcerous disorders [7] and antihypertensive effects [8]. Polyphenols may be detrimental when taken in larger doses and found in dietary supplements and fortified foods, despite the numerous health benefits [9]. In an applied work of the Phenol-Explorer database, which contained information of 502 polyphenols in 452 foods, the richest sources of polyphenols were identified as various spices and dried herbs [10]. The fat in cheese can be degraded by lipolysis due to lipase activity (somatic cells and microorganisms) or oxidation. The extent of lipolysis in Cheddar cheeses has a major impact on its sensory characteristics and excessive lipolysis is associated with downgrading due to oxidative rancidity [11]. One of the studies shows a potential use of rosemary oleoresin as an antioxidant to increase the shelf life of aged Cheddar [12]. Lipolysis and proteolysis are higher in ripened than in fresh cheese for all cheese varieties [13]. The use of the wild garlic herb (*Allium* sp.) in herby pickled cheese production revealed a significant increase in total free fatty acids (TFFAs) contents due to an increase in the herb level [14]. The increasing of mendi (*Chaerophyllum* sp.) amount in cheese samples had a significant ($P < 0.05$) effect on the lipolysis level at 2 and 90 days [15]. In one recent review authors confirmed the benefits of using of antimicrobial herb and spice compounds in food [16].

Design of our study is supported by the results obtained by HPLC/DAD analysis on the phenol component of *Kitaibelia vitifolia* ethanol herb extract [17]. Rosmarinic acid (2.937 mg/g of extract) was determined as a dominant compound. Total phenolics, flavo-

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noids, condensed tannins, and gallotannins were 85.25±0.69 mg of GA/g, 45.32±0.55 mg of RU/g, 54.25±0.75 mg of GA/g, and 41.74±0.55 mg of GA/g, respectively. Extract of *K. vitifolia* possesses total antioxidant capacity of 75.45±0.68 μg of AA/g. Antimicrobial activity of the *K. vitifolia* extract was determined by the dilution method, with Minimal Inhibitory Concentrations from 15.62 to 62.50 μg/mL [17].

The main aim of fortification of Pirotski Kachkaval using identified and quantified polyphenolic compounds which contain ethanol extract of the *K. vitifolia* was to get healthier and more sustainable delicacies. The purpose of the research within this study was to investigate the antioxidant effects of polyphenols in the complex food matrix such as the cheese, physico-chemical composition, microbiological quality, sensory properties of Pirotski Kachkaval, using the herb extract applied in two different manners.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl hydrate (DPPH), Folin–Ciocalteu, ascorbic acid and butylated hydroxytoluene (BHT) were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Hydrochloric acid, formaldehyde, anhydrous sodium carbonate, methanol, ethanol, acetone and petroleum ether were purchased from Centrohem (Centrohem, Stara Pazova, Serbia). All of the other chemicals and reagents were of analytical reagent grade.

Plant material

Kitaibelia vitifolia is a member of the *Malvaceae* family. The above-ground part of the test plant was collected in Central Serbia, at the flowering stage. The species was identified and the voucher specimen was deposited at the Department of Botany, Faculty of Biology, University of Belgrade (16350 BEOU).

Preparation of herb extract

Samples prepared from over ground part of the plant *K. vitifolia* (10.0 g) were extracted by 96% ethanol (100.0 mL) as a solvent. The extraction process was carried out using an ultrasonic bath (Branson and Smith-Kline Company, model B-220, Danbury, CN, USA) at room temperature for 1 h. The goal was the highest extraction yield of phenol acids. After filtration, 5 mL of the liquid extract was used for extraction yield determination. The solvent was removed by a rotary evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) under vacuum, and was dried at 60 °C to constant weight. The dried extracts were stored in glass bottles at 4 °C to prevent oxidative damage until analysis. Spectrophotometric measurements were performed using a UV–Vis spectrophotometer MA9523-SPEKOL 211 (ISKRA, Horjul, Slovenia).

Cheese formulation and processing

Pirotski Kachkaval (one of the cheese variety with unique technological process which included texturizing of ripe curd, categorized in the group of Pasta-Filata-Cheese), produced in the laboratory of "Obren Pejić" Dairy School in Pirot, Republic of Serbia by followed procedure: mixture of cow, sheep and goat raw milk (82:16:6 by volume) was heated in cheese vat at the temperature of 32 °C. Heated milk blend was coagulated with rennet for 40–45 min. Processing of cheese curd consists of cutting and stirring the curd in order to achieve forming of grain and it is done using special cheese making harps. Attrition of curd lasts 10 min, subsequently the process of grain formation is stopped for 10 min in order for grains to settle and squeeze whey as much as possible. Second heating-scalding was performed at the temperature of 38 to 42 °C. The dynamics and intensity of heat required to raise the temperature of curd for 1 to 2 °C every 2–3 min. Drying takes 30 to 40 min with constant stirring. Pressing was carried out at a pressure of 5 to 10 kg of cheese per kg of weight in a time of 30 to 45 min, to separate whey and then cut. The resulting fresh curd is cut into pieces the size of 5 to 10 kg and transferred to the ripening chamber and left on the draining table until the next day for acidification (cheddaring). Maturation of the fresh curd, so-called "baskija", aims to change its proteins. Ripe, acidified curd, shows ability for stretching and kneading by soaking in hot water. Maturation of the "baskija" performed by the lactic bacteria acquired in milk during milking and cooling in the chambers at a temperature of 30 °C and it lasts from 4 to 12 h. After completing the ripening, "baskija" is cut into slices with a thickness of 0.5 cm using a special macerator. "Baskija" is afterwards thinly placed into weaved baskets of hazel brushwood on texturizing, in the quantity needed for a one ball of Kachkaval, and immersed in hot water at a temperature of 75 °C. Water for texturizing consisted of one third of used water that was used the day before and two thirds of fresh water. Texturizing in a basket takes 5 to 8 min. On a separate cheese-making table, the textured curd is streched, twisted and dry salted. Obtained texturized curd was divided into three experimental groups and 3 balls for each group (nine balls in total). The extract of *K. vitifolia*, in the active concentration of 3.0 mL/100 g of cheese was added to the curd of the three balls during their formation (experimental group I – EG I). In addition to the 3 balls that belong to EG I, six more cheese extracts were formed into balls, and all nine balls were placed in molds where remains until the next day. Ripening of Kachkaval performed in the room for cheese ripening at the temperature of 15–18 °C.

Once the cheese is removed from the mold, we separated three cheese samples belonging to the EG I. Surface of the next three balls of cheese was treated with a spray solution (10 mL per ball) of the *Kitaibelia vitifolia* extract and formed experimentally group II – EG II. The remaining 3 samples of cheese were the control group C, produced in conventional manner, without any modifications. Samples of EG II were packaged after a 20-min period of herb extract spray solution exposure. At the same time, samples of control and EG I of ripened Kachkaval was packaged in the same manner (under vacuum in polyethylene bags), labeled and declared as Pirotski Kachkaval.

Preparation of the cheese aqueous extract for antioxidant activity testing

Kachkaval cheese was chopped and then homogenized in the blender. Ten grams of homogenized cheese sample was taken and dissolved in 10 mL of distilled water to obtain a concentration of 1 mg per 1 mL of distilled water.

Determination of total antioxidant capacity

The total antioxidant activity was evaluated by the phosphor-molybdenum method [18]. The assay is based on the reduction of Mo (VI)–Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A total of 0.3 mL of cheese sample was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then, the absorbance of the solution was measured at 695 nm using spectrophotometer against the blank after cooling to room temperature. Methanol (0.3 mL) was used as the blank. Ascorbic acid was used as the standard and total antioxidant capacity was expressed as mg of ascorbic acid per g of prepared cheese extract.

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The described method used by group of authors [19] was adopted with suitable modifications [20]. DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 μg/mL. Serial dilutions were carried out with the stock solution (1 mg/mL) of the cheese extract. Solutions (2 mL each) were then mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid, gallic acid and butylated hydroxytoluene were used as referential standards and dissolved in methanol to make the stock solution with the same concentration (1 mg/mL). Control sample was prepared containing the same volume without test compounds or reference antioxidants. Ninety-five % of methanol cheese extract was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\text{Inhibition} = 100\frac{A_c - A_s}{A_c} \quad (1)$$

The percentage inhibition values were calculated from the absorbance of the control (A_c) and of the sample (A_s), where the controls contained all the reaction reagents except the extract or positive control substance (Eqs. (1)–(3)). The IC_{50} value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated as μg/mL through a sigmoid dose-response curve.

Determination of inhibitory activity against lipid peroxidation

Antioxidant activity was determined by the thiocyanate method [21]. Serial dilutions were carried out with the stock solution (1 mg/mL) of the cheese extract, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 mL 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 mL with 40 mM phosphate buffer, pH 7.0. After incubation at 37 °C in the dark for 72 h, a 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL $FeCl_2$ (20 mM) and 0.1 mL ammonium thiocyanate (30%). The absorbance of the mixture was measured at 500 nm and the mixture was stirred for 3 min. Ascorbic acid, gallic acid, α-tocopherol and butylated hydroxytoluene were used as reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition percent of linoleic acid peroxidation was calculated using Eq. (1).

Measurement of ferrous ion chelating ability

The ferrous ion chelating ability was measured by the decrease in absorbance at 562 nm of the iron (II)––ferrozine complex [22,23]. One milliliter of 0.125 mM $FeSO_4$ was added to 1.0 mL sample (with different dilutions), followed by 1.0 mL 0.3125 mM ferrozine. The mixture was allowed to equilibrate for 10 min before the absorbance was measured. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the expression of the right-hand side of Eq. (1).

Determination of hydroxyl radical scavenging activity

The ability of *K. vitifolia* to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out

according to the described method [24]. The reaction mixture contained 100 μL of cheese extract dissolved in water, 500 μL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4–NaOH buffer (50 mM, pH 7.4), 200 μL of premixed 100 μM $FeCl_3$ and 104 mM EDTA (1:1, *V*/*V*) solution, 100 μL of 1.0 mM H_2O_2 and 100 μL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50 °C for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0 % TBA were added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The values are presented as the means of triplicate analyses.

Proximate composition of Pirotski Kachkaval

Moisture content, total solids, fat content, fat in total solids and proteins were determined by standard methods [25].

Determination of NaCl content

Sodium chloride content was determined by a modified Volhard method [26]. After acidification, the sample was added to the solution of silver nitrate in excess, and this excess was titrated standard volumetric solution of ammonium thiocyanate, to the emergence of a stable pink-brown coloration.

Determination of pH values

The pH values of the samples were measured using a laboratory pH-meter, model Cyber Scan 510 pH Meter, EUTECH Instruments, Landsmeer, The Netherlands. pH is measured in the homogenized Kachkaval sample by the procedure that corresponds to the used pH-meter. When a constant value is reached, the pH is read directly from the instrument with an accuracy of 0.01 pH units [27].

Determination of water activity (a_w value)

a_w Value was obtained according to the manufacturer's instructions, FA-st/1 User Manuel (GBX Scientific Instruments, Bourg de Peage, France). Container unit of device FA-st/1 is filled with prepared Kachkaval sample to the ¾ and read off a_w value. The device is calibrated by adjusting the values of a_w corresponding value for the selected reference salts.

Determination of total free fatty acids (TFFA) content

The total free fatty acids (TFFA) content of cheese samples was determined by the extraction titration method [28]. Three grams of grated cheese (W) were made into a paste with 5 mL of distilled water. Ten mL of extraction mixture containing isopropanol, petroleum ether and 4 N sulphuric acid in the proportion of 40:10:1 by volume was added to the paste. Six mL of petroleum ether was then added thorough mixing. The test tubes were kept at 40 °C for 10 min. The contents were vigorously shaken for 20 s. The two layers were allowed to separate (5–10 min) and an aliquot of the upper layer (V = 5 mL) was withdrawn and transferred to a 50 mL conical flask. After adding six drops of 1% methanolic phenolphthalein indicator, the contents were titrated with 0.02 N methanolic potassium hydroxide solution (V). Designation N in Eq. (2) is used for normality of methanolic potassium hydroxide solution, and its value is constant (0.02 N). The reagent blank was used to obtain background titration. The TFFA content of cheese was calculated by the following formula:

$$TFFA = 1000\frac{V\mathrm{N}}{VW}\ [\mu g/g \text{ of fat}] \quad (2)$$

Microbiological analysis

Processed cheese samples were analyzed for colony count of aerobic bacteria according to ISO 6610:2002 [29], coagulase-positive staphylococci according to ISO 6888-1:2005+A1:2005 [30] and anaerobic sulfite-reducing bacteria according to ISO 15213:2003 [31] at the first day of the storage.

Sensory analysis

Sensory analyses of Pirotski Kachkaval samples were performed by five members of the expert committee applying scoring system including the following characteristics: appearance (max. 2 points), colour (max. 1 points), cross section (max. 3 points), odour (max. 2 points) and taste (max. 10 points). The total number of points was a maximum of 18.

Statistical analysis

The results of examinations are presented as mean ± standard deviations of three determinations. Statistical analyses were performed using analysis of variance. Multiple comparisons of means were done by least significant difference (LSD) test. All computations were made by employing the SPSS software, version 15.0 (SPSS, Chicago, IL, USA). IC_{50} values were calculated by nonlinear regression analysis from the sigmoid dose-response inhibition curve.

RESULTS AND DISCUSSION

Antioxidant effects

Results of antioxidant activity testing obtained are shown in Table 1.

The levels of phenolic-linked antioxidant activity revealed a slight emphasis of phenol-linked antioxidant activity of fortified samples of cheese in comparison to samples of the control group. Samples of cheese from EG II (treated with a spray solution of the *Kitaibelia vitifolia* extract – 10 mL per ball) shown the best antioxidant activity. Plant extracts were added to balls of

Table 1. The antioxidant activities of Pirotski Kachkaval samples after 45 d of ripening; mean values ± standard error of three trials; EG I – experimental group I; EG II – experimental group II; C – control group

Sample	IC_{50}[a] / μg mL^{-1}				Total antioxidant capacity μg AA/g
	DPPH scavenging activity	Inhibitory activity against lipid peroxidation	Metal chelating activity	Hydroxyl radical scavenging activity	
EG I	67.45±1.45	94.46±1.23	55.01±0.18	109.41±0.32	58.85±0.80
EG II	63.48±1.44	92.90±0.91	53.15±0.95	101.25±0.43	59.34±0.47
C	66.20±0.89	93.23±1.22	55.89±0.86	108.47±0.52	59.15±0.65
Gallic acid (GA)	3.79±0.69	255.43±11.68	–	59.14±1.10	–
Ascorbic acid (AA)	6.05±0.34	> 1000	–	160.55±2.31	–
Butylated hydroxytoluene (BHT)	15.61±1.26	1.00±0.23	–	33.92±0.79	–
α-Tocopherol	–	0.48±0.05	–	–	–

[a]Values were determined by nonlinear regression analysis

EG I before the heat treatment, but in EG II after heat treatment, on the surface like spray. Our results showed that the temperature of 75 °C during heat treatment inhibited the activity of used plant extracts and thereby reduce the antioxidant activity of the same samples in EG I. Lower IC_{50} value indicates stronger antioxidant activity, and the first four test methods demonstrated the strongest antioxidant activity in samples of EG II. Total antioxidant capacity method revealed the highest capacity (expressed as– μg ascorbic acid (AA)/g) in samples of cheese from the EG II. Herbal extract (applied in different manners) as expected emphasized the antioxidant activity in comparison to samples of the control group. Direct interactions between polyphenols and food proteins and polysaccharides may affect their absorption. The presence of phenol hydroxyl groups of phenol acids is partly responsible for the antioxidant potency of modified cheese. Unsignificantly different level of total antioxidant capacity was found in comparison of the samples of Pirotski Kachkaval from the experimental group EG I to the experimental group EG II. Comparing the level of activity was obtained by addition of phenolic and flavonoid substances identified from above-ground parts of the *K. vitifolia* and activity of BHT, it was observed that the synthetic oxidant with a significantly stronger effect. From the Table 1, the antioxidant activity of modified EG I and EG II experimentally groups of Pirotski Kachkaval cheese was lower than synthetic antioxidant BHT which could be due to the chemical composition of the cheese (fat and protein contents) and the heat treatment of cheese (75 °C) which makes the cheese more sensitive to the oxidation. DPPH is a steady radical, often used to estimate the antioxidant activity of certain natural products and tracklements as well. The degree of the decolorization of purple color of DDPH radicals and reduction of absorbance to 517 nm imply to "scavenger" potential of the samples used in the research of Pirotski Kachkaval. The results of the DPPH scavenging activity with IC_{50} values from 67.45 (EG I), 63.48 (EG II) and 66.20 μg/mL (C) show an average high antioxidant potential in the examined samples. In comparison with the antioxidants that we used as a control group for the level of antioxidant activity, the samples of Pirotski Kachkaval from the control groups EG I, EG II and C have higher IC_{50} values, thus resulting in a lower antioxidant activity.

The examined samples of Pirotski Kachkaval from the experimental series EG I and EG II, as well as those from the control group C show an incredibly high level of inhibitory activity against lipid peroxidation compared to gallic acid and ascorbic acid as the control antioxidants, while the same groups show a rather low level of inhibitory activity against lipid peroxidation when compared to the other two synthetic antioxidants (BHT and α-tocopherol). This result is probably the consequence of the forming of oxidant products and the creation of evaporable substances. Malondialdehyde and the other short chain products are not steady and they are reduced to alcohols and acids which cannot be defined using the method of inhibitory activity against lipid peroxidation.

Hydroxyl radical is a highly aggressive oxidant, capable of oxidizing the majority of biomolecules at a very high speed. Hydroxyl radicals can oxidize polyunsaturated fatty acids and start the natural lipid peroxidation. IC_{50} value of the samples of Pirotski Kachkaval of the control groups EG I, EG II and the control group C, using the method hydroxyl radical scavenging activity show a high level of activity compared to ascorbic acid, and a significantly lower degree of activity compared to gallic acid and BHT. Moreover, since all the antioxidants act synergistically against the noxious effects of oxidative stress [32], the assessment of the total amount of electron-donating antioxidants in foods including cheese may be an interesting approach as a supplement to the measurement of individual dietary antioxidant contents [33].

Physicochemical analyses of Pirotski Kachkaval

Statistically significant ($P < 0.05$) changes were recorded in moisture content and total solids of control (C) and experimental series (EG I and EG II) of cheeses. Fat content, milk fat in dry matter, protein content, pH and a_w values did not differ significantly ($P > 0.05$) between samples of control and experimental groups (Table 2). Lipolysis results in the formation of free fatty acids (FFAs), and statistically significant ($P < 0.05$) changes were recorded between control and cheese samples belonging EG I, where herb extract added in cheese body. Two groups of authors reported very similar results for 20 traditional Turkish ripened herby cheese [13,34]. By comparing the results of our testing with the results that are obtained in specific characteristics of Pirotski Kachkaval study [35,36] minor differences were observed in mean values of chemical parameters (lower total solids and fat content and higher content of fat in total solids), and protein content is very similar. These differences were probably caused by the use of different raw materials compared to those that were used in the previous studies as well as due to application of herb extract and minor differences in the cheese manufacturing process.

The results of microbiological tests

Microbiological analysis revealed that values for colony count of aerobic bacteria, *Coagulase-positive staphylococci* and *Staphylococcus aureus* and anaerobic sulfite-reducing bacteria did not differ significantly ($P > 0.05$) between samples of control and experimental groups (Table 3). The samples from the control and experimental groups of Pirotski Kachkaval were of a high microbiological quality immediately after production, due to the steaming heath treatment of cheese balls on pasteurization temperatures (75 °C for 5 to 8 min). That is very important because it is known that the total aerobic bacteria counts and moulds yeasts were higher in dry-salted and raw milk cheeses [37].

The results of sensory analysis

The sensory evaluation of the fortified Pirotski Kachkaval cheese of EG I and EG II and conventionally produced cheese originating from control group (C) was conducted using 5-point hedonic scale and the mean scores obtained are summarised in Fig. 1. The panellist could not find any difference in appearance, color, cheese body and cross section up to 45 days of ripening for both control and fortified groups; Assesors gave slightly higher scores for the odour of Pirotski Kachkaval samples from EG I compared with C and EG II samples, up to 45 days of ripening. The quality of the cheese body was high and uniformed (in all 3 groups ranked from 1.5 out of a maximum of 2 points). Pirotski Kachkaval was slightly softer consistency as expected, because the assessed after 45 days of ripening, so that the period of ripening after vacuuming was relatively short, it can take up to 3 months). The cross section of samples was very high and uniformed (2.5 out of a maximum of 3 points), with a note that the cheese was of a closed structure with a small number of technological tiny holes.

The odour of the Pirotski Kachkaval cheese was typical, pleasant, stronger in the EG I, under-expressed

Table 2. Physicochemical properties of Pirotski Kachkaval samples after 45 d of ripening; mean values ± standard error of three trials; EG I – experimental group I; EG II – experimental group II; C – control group; a and b indicate differences (P < 0.05) between rows, LSD test

Physicochemical property	C	EG I	EG II
Moisture content, %	47.85±0.06[a]	47.07±0.04[b]	47.20±0.05[b]
Total solids, %	52.15±0.06[b]	52.93±0.04[a]	52.80±0.05[a]
Fat content, %	26.6±0.33[a]	26.67±0.33[a]	26.50±0.29[a]
Fat in total solids, %	51.14±0.66[a]	50.38±0.65[a]	50.19±0.51[a]
Proteins, %	21.39±0.10[a]	21.62±0.69[a]	21.71±0.05[a]
NaCl, %	1.99±0.03[a]	2.03±0.02[a]	2.06±0.01[a]
pH	4.88±0.00[a]	4.90±0.00[a]	4.88±0.00[a]
Water activity (a_w value)	0.94±0.00[a]	0.95±0.00[a]	0.94±0.00[a]
Total free fatty acids (TFFA) content, μg/g	2.18±0.00[a]	2.00±0.00[b]	2.18±0.00[a]

Table 3. Microbiological quality of Pirotski Kachkaval samples; EG I – experimental group I; EG II – experimental group II; C – control group

Experimental group	Aerobic colony count, cfu/g	Coagulase-positive *Staphylococci* and *Staphylococcus aureus*, cfu/g	Anaerobic sulfite-reducing bacteria, cfu/g
C	< 3.000.000	< 100	< 100
EG I	< 3.000.000	< 100	< 100
EG II	< 3.000.000	< 100	< 100

in samples from the control group and the samples collected from the EG II, so that the maximum number of points (2). Commission awarded evaluated samples from the EG I, and the samples evaluated for the samples from the control and EG II at 1.5 points (high score). In judging the taste, predominant flavor was the one of unmaturated cheese, not enough for the type of cheese to be expressed as the salty taste, and the experimental group specific and more prominent than in EG II. The best taste was found in the samples from the EG I (8.5 point out of a maximum 10 points). Generally, the highest sensory quality of the cheese was noted in the evaluation of EG I (17.5 out of a maximum 18 points), followed by samples of the EG II (16.5 points) and control group (16.0 points). Modification of the cheese by treatment with the extract of *K. vitifolia* has beneficial effects on the sensory quality regardless on the way of application, where the effects were more noticeable when adding the extract to the cheese body after texturizing of ripened curd in relation to the spraying of the cheese surface with herb extract.

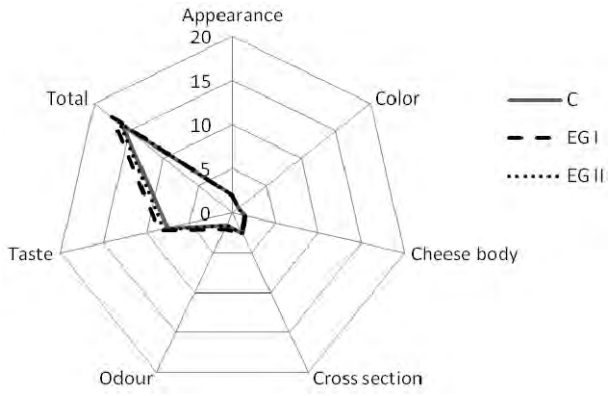

Figure 1. Sensory properties of cheese samples after 45 d of ripening.

CONCLUSIONS

The Pirotski Kachkaval samples were produced in a mixture of cow, sheep and goat raw milk in traditional technological process under industrial conditions, applying the appropriate extract or a spray solution of ethanol extract of the *Kitaibelia vitifolia*. Significant differences were found in moisture content and total solids of control (C) and experimental series (EG I and EG II) of cheeses. The application of the sprayed ethanol extract of the *K. vitifolia* on the surface of the cheese led to a stronger level of antioxidant activity and the highest total antioxidant capacity in the samples of EG II. Plant extract (applied in different manners) as expected fortified the antioxidant activity in comparison to samples of the control cheese. The fortification of Pirotski Kachkaval with *K. vitifolia* plant extract didn't interfere with the sensory perception of traditional cheese. The modified cheese samples (EG I and EG II) had higher sensory evaluation scores than the control group samples.

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IZVOD

EFEKAT EKSTRAKTA BILJKE *Kitaibelia vitifolia* NA ANTIOKSIDATIVNU AKTIVNOST, HEMIJSKI SASTAV, MIKROBIOLOŠKI STATUS I SENZORNA SVOJSTVA PIROTSKOG KAČKAVALJA

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(Naučni rad)

Cilj ispitivanja je da se odredi uticaj fortifikovanja sira (Pirotski kačkavalj) polifenolima poreklom iz etanolnog ekstrakta biljke *Kitaibelia vitifolia*, primenjenog na dva različita načina (dodavanjem u grudu pre uobličavanja ili prskanjem površine sira). Sproveli smo ispitivanja antioksidativnog efekta polifenola, fizičko–hemijskog sastava, mikrobiološkog kvaliteta i senzornih svojstava Pirotskog kačkavalja. Antioksidativna aktivnost konvencionalno proizvedenih i fortifikovanih sireva je ocenjivana korišćenjem pet savremenih i kompatibilnih metoda, i utvrđen je umereni rast antioksidativne aktivnosti poreklom od fenola u uzorcima fortifikovanog sira u odnosu na uzorke kontrolne grupe. Fortifikovani Pirotski kačkavalj je imao više ocene pri senzornom ispitivanju u odnosu na uzorke kontrolne grupe. Statistički značajne razlike ($P < 0.05$) su zapažene u sadržaju vlage i suve materije između uzoraka kontrolne grupe i uzoraka modifikovanih oglednih grupa sira, a ostali parametri se nisu značajno razlikovali ($P > 0.05$).

Ključne reči: Pirotski kačkavalj • *Kitaibelia vitifolia* • Polifenoli • Antioksidativna aktivnost • Mikrobiološki kvalitet • Senzorna svojstva

Discrete element modelling of screw conveyor-mixers

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Abstract

Screw conveyors are used extensively in food, plastics, mineral processing, agriculture and processing industries for elevating and/or transporting bulk materials over short to medium distances. Despite their apparent simplicity in design, the transportation action is very complex for design and constructors have tended to rely heavily on empirical performance data. Screw conveyor performance is affected by its operating conditions (such as: the rotational speed of the screw, the inclination of the screw conveyor and its volumetric fill level). In this paper, horizontal, several single-pitch screw conveyors with some geometry variations in screw blade were investigated for mixing action during transport, using Discrete Element Method (DEM). The influence of geometry modifications on the performance of screw conveyor was examined, different screw designs were compared, and the effects of geometrical variations on mixing performances during transport were explored. During the transport, the particle tumbles down from the top of the helix to the next free surface and that segment of the path was used for auxiliary mixing action. The particle path is dramatically increased with the addition of three complementary helices oriented in the same direction as screw blades (1458.2 mm compared to 397.6 mm in case of single flight screw conveyor). Transport route enlarges to 1764.4 mm, when installing helices oriented in the opposite direction from screw blades. By addition of straight line blade to single flight screw conveyor, the longest particle path is being reached: 2061.6 mm.

Keywords: DEM, modified screw conveyor, premixing, optimization.

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Screw conveyors are widely used for transporting and/or elevating particulates at controlled and steady rates. They are used in many bulk materials' applications in industries ranging from industrial minerals, agriculture (grains), pharmaceuticals, chemicals, pigments, plastics, cement, sand, salt and food processing. Screw conveyors can be designed with the same or variable pitch, cone-shaped screw conveyors are also used, with constant or variable pitch. If not designed properly for the transported material, the experienced problems include: surging and unsteady flow rates, inaccurate metering and dosing, inhomogeneity of the product, product degradation, excessive power draw, high start-up torques, high equipment wear and variable residence time and segregation. In case of hygroscopic material transport, it is possible that material is being pasted to the screw blade and/or to the casing, reducing the gap size between them. This affects the screw transporting capacity, increasing energy consumption.

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The segregation of powder materials should be attributed to the specific shape of particles and the differences in weights, but also to the length of the screw transporter. It is very difficult to design and manufacture screw transporter with increased length, particularly due to deformation of long screw shaft, in which case additional support(s) are required between the initial and final bearings case. A summary of current design methods and problems experienced for screw conveyors can be found in Bortolamasi and Fottner (2001) [1]. The description of the theoretical behavior of screw conveyors can be found in articles by Yu and Arnold (1997) [2] and Roberts (1999) [3].

Discrete element modeling (DEM), of particulate flow in a screw conveyor was first reported by Shimizu and Cundall (2001) [4]. They examined the performance of horizontal and vertical screw conveyors and compared their results with previous work and empirical equations. Owen *et al*. (2003) [5], introduced the use of a periodic slice model to explore the performance of a long screw conveyor. Cleary (2004) [6] used DEM to study draw down patterns from a hopper by a 45° inclined screw conveyor. This work was extended by Cleary (2007) [7] to examine the effect of particle shape on the draw down flow from the hopper and on the transport characteristics of the screw conveyor.

Screw conveyors are also used for metering (measuring the flow rate) from storage bins and adding small controlled amounts of trace materials (dosing) such as pigments to granular materials or powders [8––10]. Dosing feeders are often constructed by adding frequency converters for speed change and fine dosing to the desired value. In this case it is very important to properly choose the geometry of the screw transporter. Changes in screw geometry, with several additional elements welded on screw blade can significantly increase the homogeneity and reduce the segregation of materials by particle size.

Screw transporters are frequently used to remove powder or grain material from silos, and transport it to the mixer. It is very important to mix thoroughly all individual components (for instance in animal food industry), in order to obtain the homogeneous product. Before the mixing process is performed, it is often practice that some premixing action is done, using some type of the auxiliary mixer. There are many mixer types, used for this action, mostly counter-screw types, and more recently specially profiled blade mixers, utilized in the industry.

The leading idea in this article was to analyze transport action of screw conveyor and to utilize the screw blade with changed geometry as continuous premixing agent, before material enters the mixer. The volumetric fill level of the screw transporter depends on many processing parameters, but is never equal to one, and the possibility of welding additional elements to screw coil geometry exists. It was intended to improve mixing by inserting additional helix or helical strips, in the same or opposite direction of material flow, on the periphery of the spiral screw trajectory. The transport of specific particles depend on the rotational speed of screw conveyor, but also on the geometry of the helix, and the transporting path can be greatly prolonged by inserting these element to the screw coil. In this case, particle velocity is significantly increased, and the probability of mixing is also enhanced, in respect to distance traveled is much longer.

The main aim of this paper is to consider the possibility of prolonging particle transport path from the moment of entering to the moment of leaving the screw conveyor, with addition of new elements welded on the helix of screw conveyor, in order to increase the effect of auxiliary mixing along with the transport of particles. In this way a screw transporter could be considered as transporter and also the continous premixer. Discrete Element Method (DEM) was used to explore the modifications in screw geometry and the influence on transport path, during the transport of just one particle, with the intention to keep the material flow unspoiled.

MATERIALS AND METHODS

DEM simulation involves following the motion of every particle involved in the model definition, and modeling of each collision: inter-particle and between the particles and their environment (*e.g.*, the internal surface of the screw casing and the surface of the rotating screw). The boundary geometry is built using a CAD package and imported as a small sized triangular surfaces mesh into the DEM package. This provides unlimited flexibility in specifying the three dimensional geometries with which the particles interact. Here the particles are modeled as spheres (also imported as small sized triangular surfaces).

The modeling technique is based on the assumption that the particle is soft (soft particle method), and that particles are allowed to overlap. The amount of overlap is labeled as Δx, and the normal and tangential relative velocities determine the collisional forces (F_n and F_t). Figure 1 illustrates the collisional force as the result of normal and tangential forces. The normal force F_n is considered as the repulsive force that pushes the particles apart (or particle from bounding geometry), depicted as the action of the spring, and also dissipation action, resulting in an effective coefficient of restitution, shown as dashpot action. Tangential component is considered as an incrementing spring action and dashpot action that is subjected to frictional limit.

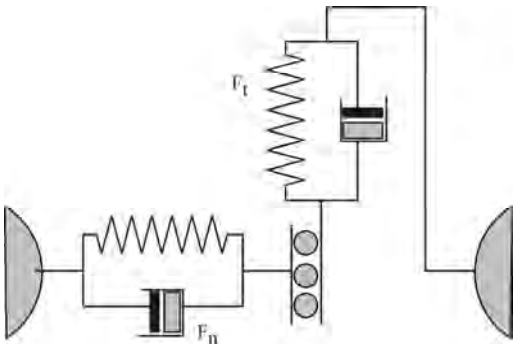

Figure 1. The contact force model.

In this article, DEM analysis was used to investigate the path of single particle, during transport, considering the differences in helix of the screw conveyor. Here applied DEM analysis can be summarized as follows: neighboring interaction list is based on the used grid (defined by used triangular surface mesh), and the boundary objects (also defined by triangular surface mesh), which are treated as virtual, non-moving particles. The collisional forces on the specific particle and boundaries are efficiently evaluated using the neighboring list and the spring-dashpot interaction model (Fig. 1) [12]. All the forces on the boundary objects and specific particles are summed and the resulting equations of motion are integrated using DEM

package. The particle velocities and their axial and tangential (swirl) components were invariant to changes of particle–wall friction.

RESULTS AND DISCUSSION

In this work the influence of helix geometry on single spherical particle trajectory is investigated. Applied DEM analysis, the mutual influence of different configuration of helix geometry and observed particle, is focused to inspect the possibility of prolonging single particle path during transport. The analysis of particle trajectory was intended to start in the moment when spherical particle enters the screw conveyor and stop in the moment when particle leaves the transporter. However, it was noticed that the segment of particle trajectory repeats each time the helix makes one revolution, concluding that it is possible to draw conclusions on prolonging the path, during transport, by observing only one segment of the particle trajectory. This conclusion was employed to shorten the process simulation, and also to reduce computer's central processing unit (CPU) calculation time. One of the key factors that the modeler should be aware in DEM analysis is shortening the CPU time and also the reduction of other computer resources (such as amount of RAM memory, motherboard performances, multiprocessors support, etc.), by making these basic assumptions.

The basic screw conveyor used in this study was a standard pitch, single flight screw conveyor with no additional helices, which is commonly used in processing industry. The pitch of the screw is defined as the length, along the drive shaft, of one turn of the helical blade, as shown in Figure 2. A standard pitch screw has its pitch equal to the outer diameter of the helical blade. The DEM model was simplified (and the CPU time is significantly reduced) by applying periodic boundary conditions to a single pitch of the screw as shown in Figure 2. The pitch of the screw was 50 mm, the diameter of the screw shaft was 15 mm, and the blade thickness was approximately 1 mm. The internal diameter for tubular case was 47 mm, giving a gap of about 1.5 mm between the outer edge of screw blade and the internal surface of the casing. Screw conveyor length was 400 mm. All simulations used the same rotational speed of 20 rpm.

DEM particles are modeled as spheres in three dimensions. Small-sized triangular surfaces mesh was used for geometrical modeling of seed, (grain that is very close to spherical shape), and for the DEM calculation, and the size of the particles used was 4.0 mm, with a density of 500 kg/m^3. The particle–boundary frictions used for the DEM (base case) simulations were 0.3, and particle–boundary coefficients of restitution were 0.3. The maximum overlap between particle and boundary is determined by the normal spring stiffness. Typically, average overlaps of 0.1–0.5% are desirable, requiring a spring constant of 1000 N/m for this type of simulation.

A series of DEM simulations was performed for various screw conveyor-mixer geometry. Standard pitch, single flight screw conveyor's transporting action was used in comparison to other modified screw conveyors with additional flights welded on the periphery of the spiral helix in order to improve mixing. Examined modified screw conveyor-mixers used in this article were:

1. Screw conveyor-mixer with three additional helices oriented in the same direction as screw transporting helix, welded on the periphery of the helix, Fig. 3a,

2. Screw conveyor-mixer with three additional helices oriented in the opposite direction from transporting helix, welded on the periphery of the helix, Fig. 3b,

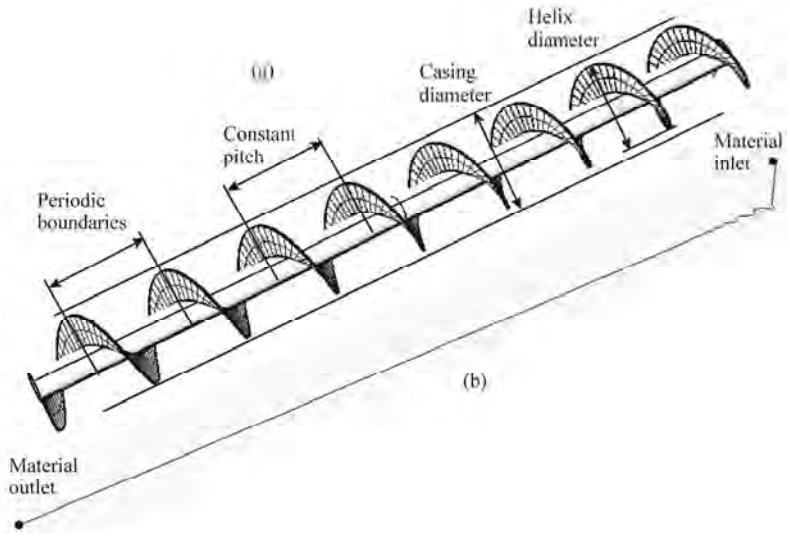

Figure 2. a) Standard pitch, single flight screw conveyor; b) particle path.

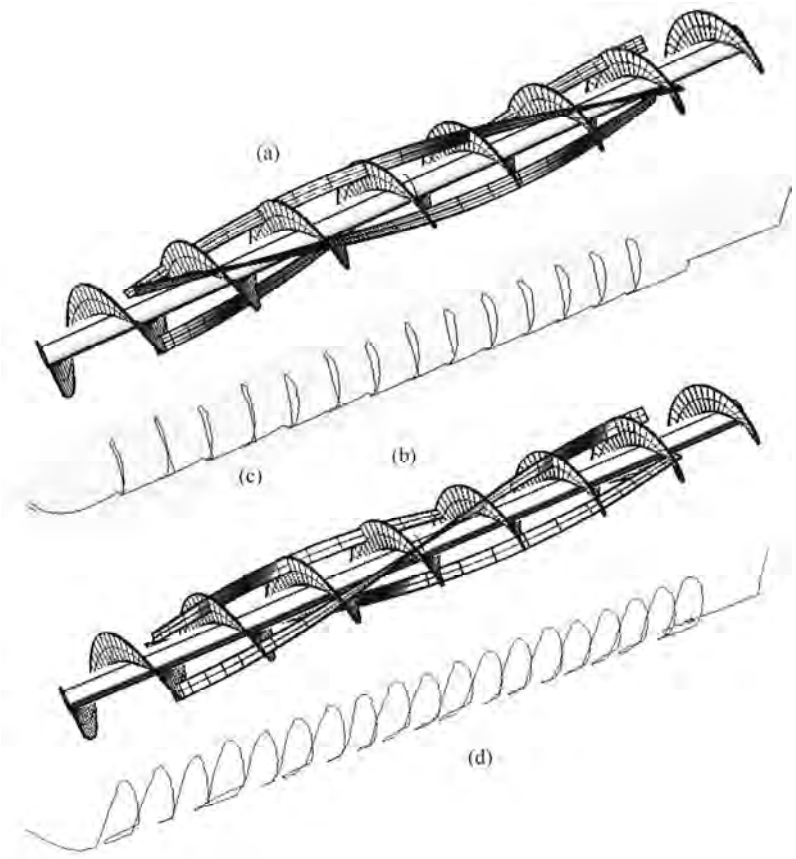

Figure 3. a) Screw conveyor-mixer with three additional helices oriented in the same direction as screw blades, b) screw conveyor-mixer with three additional helices oriented in the opposite direction from screw blades, c) particle path and d) particle path.

3. Screw conveyor-mixer with three truncated additional helices oriented in the opposite direction as transporting helix, welded on the periphery of the helix, Fig. 4a and

4. Screw conveyor-mixer with additional straight line blades, welded on the periphery of the helix, Fig. 4b.

The movement of granular particles, modeled as spheres in DEM simulation, was observed from the initial moment, the entering in screw transporter-mixer to the moment of leaving the external tube, and the motion path was analyzed. The full length of obtained path, during the simulation, and also the retention time were recorded.

The purpose of this analysis is to improve the geometry of the standard screw transporter with additional elements, welded on the perifery of the helix that enables prolonging of particle path within the screw conveyer. Also, the velocity increase of the single particle could be expected.

It is well known, that the screw conveyor fill level should be less than 50%, *i.e*., much of the volume above the helix blade is empty during transport, and this volume can be used for additional mixing action during transport. During the transport in classical screw transporter, the particles generally travel in the straight line, along the transporter length.

Using DEM simulation of the particle trajectories, single particle coordinates $x = f(t)$, $y = f(t)$ and $z = f(t)$ have been obtained, and the spatial curve showing the trajectory of that particle is plotted (Figs. 2b, 3c and d and 4c and d), from the moment of entering until the moment of leaving the screw conveyor.

The effect of single path prolonging leads to enhance the interferences between observed particles and increase probability of particles being mixed during the transport (in case that screw conveyor is transporting several different components and/or different particle sizes). The movement of just one single particle has been observed in order to show possible solutions that would increase the particle path by adding elements to the screw conveyor, not changing the basic dimensions of the screw conveyor.

In the first case, when the screw transporter works only as a conveyor, the particle path is almost a straight line (Fig. 2b), while in all other cases the particle is moving on a much longer path which was particularly evident in the case of screw conveyor-mixer with additional straight line blade (Fig. 4d).

In case of single flight screw conveyor (Fig. 2a), the total particle path is only 397.6 mm, according to DEM

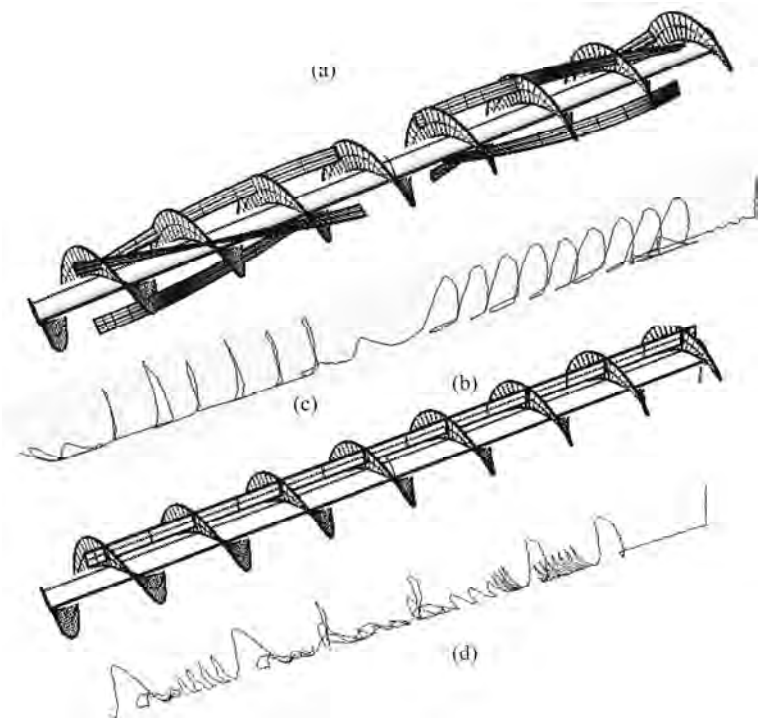

Figure 4. a) Screw conveyor-mixer with three truncated additional helices oriented in the opposite direction as screw blades, b) Screw conveyor-mixer with additional straight line blade, c) particle path and d) particle path.

simulation. Initial small perturbation was observed, and afterward straight lined path, caused by screw conveyor transporting action.

Screw conveyor-mixer with three additional helices oriented in the same direction as screw blades (Fig. 3a), strongly enlarges the total particle path, calculating more than a three times longer trajectory of 1458.2 mm, for equal transport time of 23.5 s. After reaching the top of the screw the particle tumbles down from the top of the helix. The particle tumbling down to the next free surface on the heap and that segment of path can be used for auxiliary mixing action.

When using screw conveyor-mixer with three additional helices oriented in the opposite direction from screw blades (Fig. 3b) for transporting and auxiliary mixing action, transporting path enlarges even more, to 1764.4 mm, which was expected, because opposite oriented helices return the single particle a bit backward, as can be seen from Fig. 3d.

By truncated additional helices oriented in the same direction as screw blades (Fig. 4a), particle path is being shortened (due to broken helices at the middle of screw conveyor). In this case, total path is 1728.8 mm.

Screw conveyor-mixer with additional straight line blade exerts the longest single particle path in this simulation (Fig. 4b): 2061.6 mm, which is a less more than five times compared with single flight screw conveyor.

CONCLUSION

Modified geometry screw conveyor and its utilization in mixing action were analyzed. The main idea was to improve mixing action by inserting additional helix or helical strips, on the periphery of the helix, in the same or opposite direction of material flow. The transport action of single particle depends on the geometry of the helix, and the transporting path can be significantly prolonged by inserting these elements to the helix of screw transporter. Particle retention time remains constant, but the velocity is significantly increased, and the probability of mixing of two or more particles is also enhanced, in respect to traveled distance is much longer.

Discrete Element Method (DEM) was used for an investigation of the effects of differences in screw geometry and the influence on transport path, during the transport of just one particle, with an intention to use a screw conveyor as transporter, but also as the continous pre-mixer.

The particle path is being extended by addition of complementary helices oriented in the same direction as screw blades (particle path is enlarged more then three times), or in the opposite direction of screw blades (when particle path is endured extended more then four times).

The longest path result obtained in DEM simulation was with the screw conveyor with additional straight

line blade, which is a less more than five times compared with single flight screw conveyor.

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IZVOD

KORIŠĆENJE METODE DISKRETNIH ELEMENATA NA MODELOVANJE PUŽNIH TRANSPORTERA-MIKSERA

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Pužni transporteri se veoma intenzivno koriste u industriji za proizvodnju i preradu hrane, plastike, mineralnih sirovina, u poljoprivrednoj proizvodnji kao i u prerađivačkoj industriji za podizanje i/ili transport rasutih materijala na kratkim i srednjim rastojanjima. Uprkos njihovoj očiglednoj konstrukcionoj jednostavnosti, sam čin transporta je veoma složen za razumevanje i konstruktori se često oslanjaju na iskustvene podatke pri konstruisanju i izradi. Osobine pužnih transportera su određene radnim uslovima (kao što su: brzinu rotacije vratila puža, ugao pod kojim je nagnut pužni transporter, nivo zapreminskog punjenja puža, itd.). U ovom radu je opisano nekoliko horizontalnih puževa, konstantne dužine koraka, pri čemu su geometrije pužnih spirala neznatno izmenjene radi ispitivanja procesa mešanja tokom transporta, korišćenjem metode diskretnih elemenata (*Discrete Element Method* – DEM). Ispitivani su uticaji geometrijskih izmena na osobine pužnog transportera, različita konstrukciona rešenja pužne spirale su međusobno poređena, kao i efekti geometrijskih izmena na mešanje u toku transporta. Tokom transporta u pužnom transporteru, čestice padaju sa vrha pužne spirale na prvu sledeću slobodnu površinu pužne spirale i taj segment putanje čestice može da bude iskorišćen za dopunsko mešanje materijala tokom transporta. Putanja čestice se drastično povećava ugradnjom tri dodatne zavojne površine usmerene u istom pravcu kao i pužna spirala (1458,2 mm u poređenju sa 397,6 mm u slučaju pužnog transportera sa jednom spiralom). Skraćivanjem dodatnih zavojnica, koje su usmerene u istom smeru kao i pužna spirala, unekoliko se smanjuje putanja čestice, na dužinu od 1728,8 mm (usled prekidanja zavojnice na sredini pužnog transportera). Putanja čestice se produžava na 1764.4 mm, kada se ugrade dodatne zavojne površine koje su usmerene u suprotnom pravcu od pravca pužne spirale. Ugradnjom tri dodatna pravolinijske letve, dobijena je najduža putanja čestice: 2061,6 mm.

Ključne reči: DEM • Modifikovani pužni transporter • Predmešanje • Optimizacija

DOKTORSKE DISERTACIJE I MAGISTARSKE TEZE HEMIJSKO–TEHNOLOŠKE STRUKE ODBRANJENE NA UNIVERZITETIMA U SRBIJI U 2014. GODINI

TEHNOLOŠKO–METALURŠKI FAKULTET, UNIVERZITET U BEOGRADU

Ime i prezime	Tema	Mentor
	Doktorske disertacije	
BOLTIĆ ZORANA	Obezbeđenje kvaliteta i uvođenje čistije proizvodnje u generičkoj farmaceutskoj industriji	Dr Mića Jovanović
SERATLIĆ SANJA	Uticaj pulsirajućih električnih polja na rast i aktivnost autohtonog soja *Lactobacillus plantarum* 564	Dr Branko Bugarski
SLEEM FARG ASHWEN HMUDA	Sinteza, struktura i solvatohromizam potencijalno formakološki aktivnih 3-(4-supstituisanih benzil)-5-alkil-5-fenilhidantoina	Dr Gordana Ušćumlić
ĆOSIĆ MILENA	Korelacija parametara Rheocasting procesa – strukture i svojstva nadeutektičkih aluminijum-silicijum legura	Dr Zagorka Aćimović
ABDUALNASER MOFTAH ALMAGRBI	Matematičko modelovanje višefaznih reakcionih procesa u proizvodnji obnovljivih i mineralnih dizel goriva	Dr Aleksandar Orlović
DAPČEVIĆ ALEKSANDRA	Sinteza i karakterizacija dopiranih oksida bizmuta sa silenitskom i defektnom fluoritskom strukturom	Dr Dejan Poleti
VASIĆ MILOŠ	Modelovanje i optimizacija procesa sušenja opekarskih proizvoda	Dr Željko Grbavčić
ALKOASH ALHKEM ABED	Silicijumski i silicijum-karbidni unipolarni tranzistori specifičnih geometrija	Dr Rajko Šašić
SOMAJA AHMED BEN HASAN	Struktura i fizičko-mehanička svojstva stomatoloških hibridnih kompozitnih materijala	Dr Radoslav Aleksić
STOJANOVIĆ ZORAN	Proučavanje procesa sinteze i svojstava višefaznih oksidnih prahova dobijenih hidrotermalnim procesiranjem	Dr Radoslav Aleksić
OMAR ALI SAIDED MOFTAH	Proizvodnja proteaza i lipaza fermentacijom sporednih proizvoda i otpadnih tokova industrije maslinovog ulja	Dr Zorica Knežević
MIRKOVIĆ MARIJA	Sinteza novih alifatičnih diimino-dioksima i diamino-dioksima i njihovih helatnih kompleksa sa prelaznim i radiaktivnim materijalima: potencijalna primena u medicini	Dr Dušan Mijin
RADOMAN TIJANA	Uticaj površinski modifikovanih nanočestica titan-dioksida na svojstva alkidnih i epoksidnih smola	Dr Enis Džunuzović
SPASOJEVIĆ VUK	Termodinamička analiza i modelovanje procesa uklanjanja ugljen-dioksida iz dimnih gasova	Dr Slobodan Šerbanović
TOMIĆ SLAVICA	Primena prirodnih zeolita u disperznim sistemima za pripremu vode za piće	Dr Dragan Povrenović
ČOLOVIĆ MIRJANA	Bioanalitičke metode za detekciju i evaluaciju toksičnosti organo-tiofosfatnih insekticida i proizvoda njihove degradacije	Dr Gordana Ušćumlić Dr Vesna Vasić
GRBAVČIĆ SANJA	Proizvodnja mikrobnih lipaza i proteaza kao aditiva u formulacijama detergenata	Dr Zorica Knežević-Jugović
LUKOVIĆ NEVENA	Razvoj enzimskog postupka za sintezu metil estara masnih kiselina	Dr Zorica Knežević-Jugović
STAMENIĆ DRAGANA	Sinteza, struktura i svojstva biodegradabilnih alifatskih poli(estar etara)	Dr Jasna Đonlagić
RADOJKOVIĆ ALEKSANDAR	Svojstva keramike na bazi barijum-cerijum-itrijum-oksida kao elektrolita za čvrste gorivne ćelije	Dr Jelena Miladinović Dr Milan Žunić
STIJEPOVIĆ VLADIMIR	Nova metoda za energetsku integraciju procesnih postrojenja u industrijskim kompleksima	Dr Mirjana Kijevčanin
RADISAVLJEVIĆ IGOR	Uticaj parametara zavarivanja na svojstva zavarenih spojeva aluminijumskih legura dobijenih postupkom zavarivanja trenjem alatom	Dr Nenad Radović

ANTANASIJEVIĆ DAVOR	Modelovanje indikatora kvaliteta životne sredine primenom veštačkih neuronskih mreža	Dr Viktor Pocajt
ĐURIŠ MIHAL	Ispitivanje fluidizacionih karakteristika polidisperznih smeša nesferičnih čestica	Dr Željko Grbavčić
POPOVIĆ DANIELA	Koeficijenti aktivnosti u trokomponentnim vodenim rastvorima elektrolita sa zajedničkim kalijum jonom na T=298,15 K	Dr Jelena Miladinović
RUŽIĆ JOVANA	Sinteza i karakterizacija kompozita sa osnovom bakra ojačanog nano- i mikro česticama ZrB2	Dr Karlo Raić
MARKOVIĆ JELENA	Proučavanje strukture i svojstava tečnih kristala oblika banane sa piridinom kao centralnim prstenom	Dr Aleksandar Marinković
MARKOVSKI JASMINA	Uklanjanje arsena primenom prirodnog i solvotermalno sintetisanog kalcita modifikovanog oksidima metala	Dr Aleksandar Marinković
	Magistarske teze	
MILIĆ-DEYAB JELENA	Uticaj mehaničke aktivacije liskuna na svojstva vatrostalnih premaza za nove tehnologije livenja	Dr Zagorka Aćimović Pavlović
POTKONJAK ŽELJKO	Uticaj unete količine toplote na mehanička svojstva zavarenog spoja debelozidnih ploča čelika za statičke offshore konstrukcije	Dr Nenad Radović

TEHNOLOŠKI FAKULTET, UNIVERZITET U NOVOM SADU

Ime i prezime	Tema	Mentor
	Doktorske disertacije	
DRAGAN ŽIVANČEV	Analiza uticaja genetskih, mikroklimatskih i ekoloških faktora na sastav glutena i tehnološki kvalitet sorti pšenice	Prof. dr Eva Lončar
TAMARA PREMOVIĆ	Uticaj vremena skladištenja, sadržaja nečistoće i ljuske semena na senzorni kvalitet, bioaktivne komponente i oksidativnu stabilnost hladno presovanog ulja suncokreta	Prof. dr Etelka Dimić
SNEŽANA FILIP	Ekstrakcija bosiljka (Ocimum basilicum, Lamiaceae) ugljendioksidom u superkritičnom stanju i modelovanje ekstrakcionog sistema	Prof. dr Zoran Zeković
MILAN NIKOLIĆ	Sinteza i karakterizacija nanokompozitnih čestica sa strukturom jezgro-omotač	Prof. dr Vladimir Srdić
DUŠICA ČOLOVIĆ	Ispitivanje uticaja procesa ekstrudiranja na dobijanje i stabilnost funkcionalnog hraniva za životinje na bazi lanenog semena	Prof. dr Ljubinko Lević
JELENA ŽIVANČEV	Napredne spregnute tehnike u analizi ksenobiotika	Prof. dr Biljana Škrbić
GORDANA LUDAJIĆ	Uticaj blizine frekventnih saobraćajnica na sadržaj toksičnih elemenata u zemljištu i pšenici	Prof. dr Nada Filipović
BOJANA LANTÉ	Sinteza nanoprahova i dobijanje kompozitne keramike sa magnetnom i dielektričnom fazom za primenu u mikroelektronici	Prof. dr Vladimir Srdić
SANJA PANIĆ	Fizičko-hemijske i katalitičke osobine ugljeničnih nanocevi sintetisanih metodom katalitičke hemijske depozicije iz gasne faze-korelacija sa osobinama primenjenih katalizatora na bazi prelaznih metala (Fe,Co,Ni)	Prof. dr Goran Bošković
ŠUMIĆ ZDRAVKO	Optimizacija sušenja voća u vakuumu	Prof. dr Aleksandra Tepić
ŠKALJAC SNEŽANA	Uticaj različitih tehnoloških parametara na formiranje boje tradicionalne fermentisane kobasice (Petrovačka kobasica) tokom standardizacije bezbednosti i kvaliteta	Prof. dr Ljiljana Petrović
CVETKOVIĆ BILJANA	Primena tehnoloških postupaka spontane fermentacije i osmotske dehidratacije za unapređenje nutritivnog profila senzornih svojstava i održivosti kupusa	Prof. dr Ljubinko Lević Žarko Kevrešan
KANURIĆ KATARINA	Promene komponenata i strukture mleka tokom fermentacije dodatkom nekonvencionalnog startera	Prof. dr Spasenija Milanović
	Magistarske teze	
MOŠIĆ IVANA	Tehnološka svojstva sorte vinove loze Prokupac u proizvodnji roze vina	prof. dr Vladimir Puškaš

TEHNOLOŠKI FAKULTET U LESKOVCU, UNIVERZITET U NIŠU

Ime i prezime	Tema	Mentor
	Doktorske disertacije	
СВЕТИСЛАВ ЦВЕТКОВИЋ	Анализа хемијских и енергетских постројења у функцији унапређења концептуалне фазе пројектовања	Предраг Рашковић
ВЕСНА НОВКОВИЋ	Добијање дигоксина из смеше секундарних гликозида *Digitalis lanata* Ehrh различитим техникама екстракције течност-течност	Влада Вељковић
СЛОБОДАН ПЕТРОВИЋ	Утицај различитих техника екстракције и дестилације на хемиски састав, етарског уља и екстраката из биљних врста рода *Thimus* l.	Миодраг Лазић
МИЛИЋ ПЕТАР	Оптимизација и моделовање кинетике екстракције резиноида и минералних материја из белог ивањског цвећа (*Galium mollugo* L.)	Влада Вељковић
ИВАН САВИЋ	Оптимизација технолошког поступка изолације амигдалина и кверцетина из биљног материјала и њихова фармаколошка активност	Весна Николић
СУЗАНА ЂОРЂЕВИЋ	Синтеза деривата на бази скроба и њихова примена у процесима скробљења прађе	Љубиша Николић
ИВИЦА СТАМЕНКОВИЋ	Континуална хомогена базно катализована алкохолиза биљних уља у реактору са вибрационом мешалицом	Миодраг Лазић
	Magistarske teze	
НОВИЦА ЂОРЂЕВИЋ	Модификација памучних отпадних влакана и примена у поступку обезбојавања ефлуента памучне текстилне индустрије	Драган Ђорђевић
ВЛАДИМИР МИЛОЈЕВИЋ	Оптимизација поступка синтезе поли(акрилне киселине) и њена примена у технологији детерџената	Љубиша Николић
ДРАГАН ЈОВАНОВИЋ	Модификација својстава поли(винил хлорида) калемљењем винилних мономера	Љубиша Николић
РАДИША КИТАНОВИЋ	Микробиолошке, физичко-хемијске и сензорне промене дувана типа Berlej сосираног у Ridraingu	Драгиша Савић
ДАНИЈЕЛА МАНЧИЋ	Оптимизација екстракције и карактеризација екстраката плода биљне врсте *Solanum retroflexum* Dun	Миодраг Лазић

TEHNIČKI FAKULTET U BORU, UNIVERZITET U BEOGRADU

Ime i prezime	Tema	Mentor
	Doktorske disertacije	
АНА СИМОНОВИЋ	Електрохемијско понашање бакра у киселом раствору натријум-сулфата у присуству органских инхибитора	др Милан Антонијевић, ред. професор
Мр СИЛВАНА ДИМИТРИЈЕВИЋ	Синтеза и карактеризација електролитичког купатила за позлату на бази комплекса злата са меркаптотриазолом	др Мирјана Рајчић Вујасиновић, ред. професор
Мр РАДМИЛА МАРКОВИЋ	Третман отпадних раствора из процеса електролитичке рафинације бакра коришћењем бакарних анода нестандардног хемијског састава	др Јасмина Стевановић, научни саветник
Мр ИВАНА МАРКОВИЋ	Истраживање ефекта ојачавања жарењем код синтерованих и ливених легура система бакар-злато	др Светлана Несторовић, ред. професор
Мр МИРОСЛАВА МАРИЋ	Могућности коришћења неких дивљих и култивисаних биљака за ремедијацију земљишта	др Милан Антонијевић, ред. професор
Мр РАДИША ПЕРИЋ	Испитивање ојачавања старењем легура система Au-Ag-Cu за производњу накита	др Драгослав Гусковић, ред. професор
ИВАНА ИЛИЋ	Примена ГИС-а у контролној стратегији мониторинга укупне емисије загађујућих материја у друмском саобраћају	др Милован Вуковић, ред. професор

АЛЕКСАНДРА ИВАНОВИЋ	Моделовање процеса производње паладијумских катализатора у циљу дефинисања оптималних механичких карактеристика	др Милован Вуковић, ван. професор
	Magistarske teze	
ГРАЦИЈАН СТРАИНОВИЋ	Флотација минерала бакра из нископроцентних руда применом колектора типа тионокарбамата	др Зоран Марковић, ред. професор