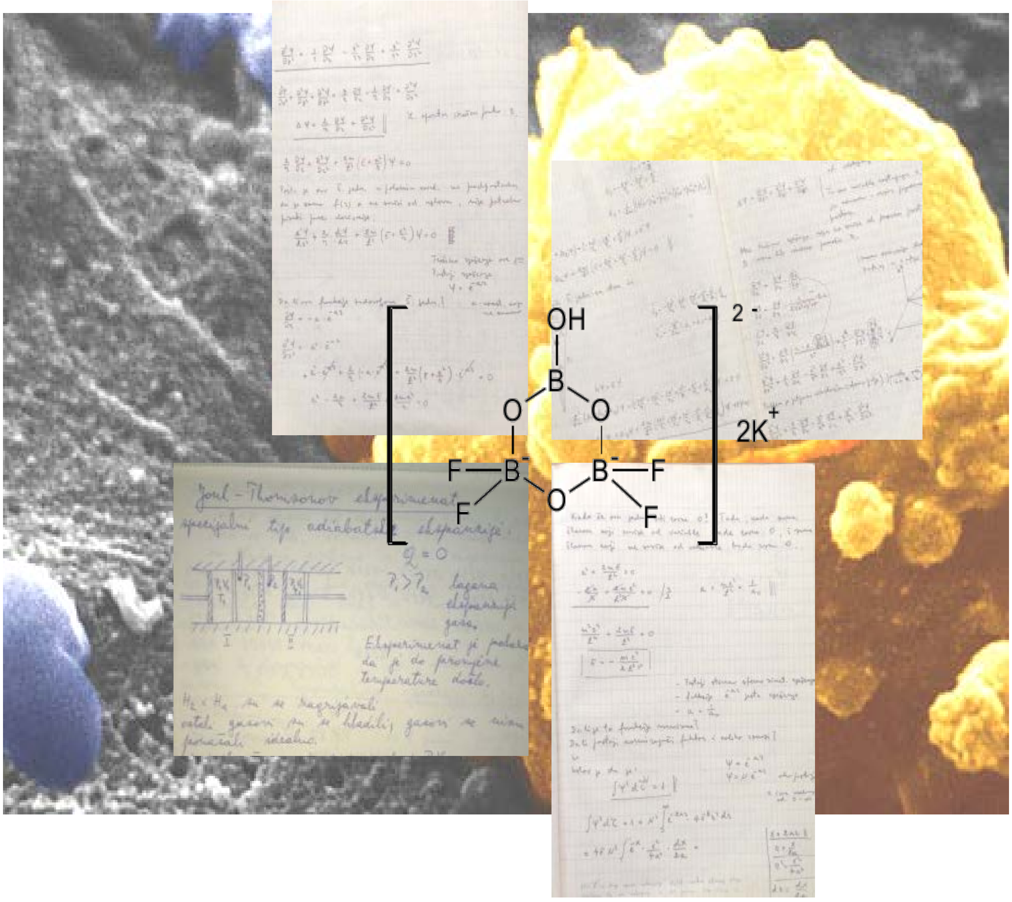
Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266



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Bosne i Hercegovine

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2017
Issue 49

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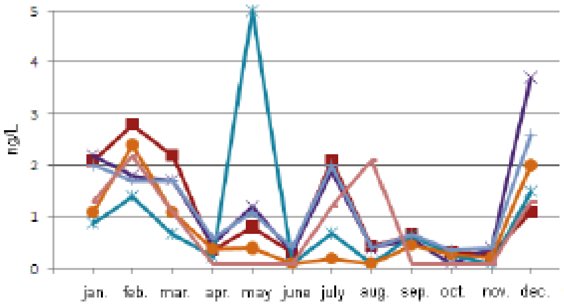
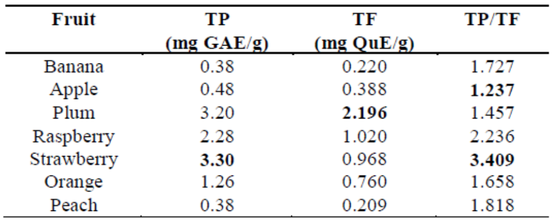
Coumarins	+	+	+
Emodins	+	+	+
Fatty acids	–	–	–
Flavonoids	+	+	+
Leucoanthocyanins	–	+	–





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Banana	0.38	0.220	1.727
Apple	0.48	0.388	**1.237**
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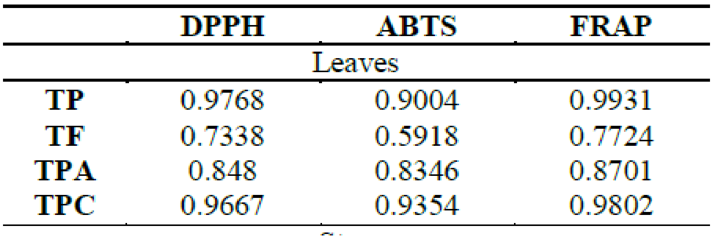

	DPPH	ABTS	FRAP
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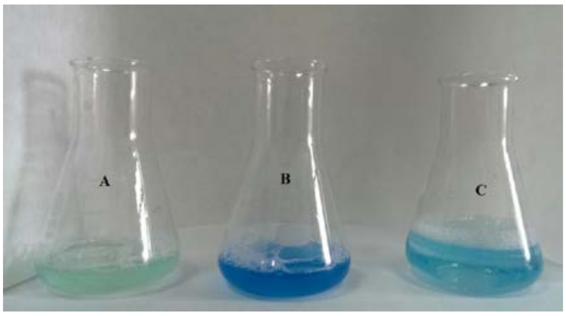

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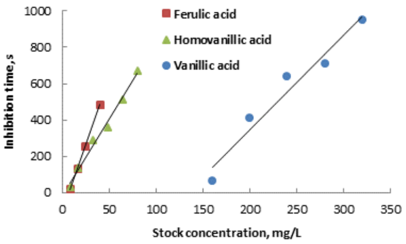

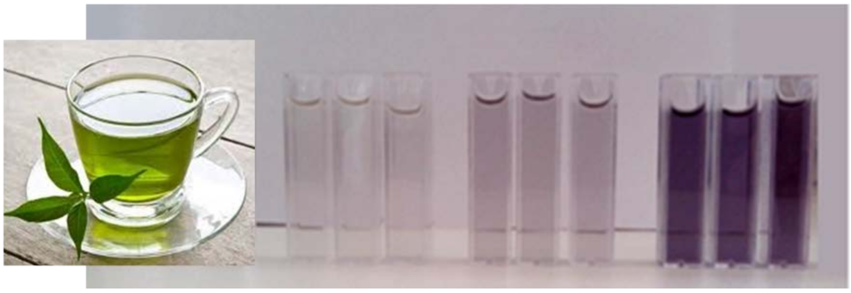

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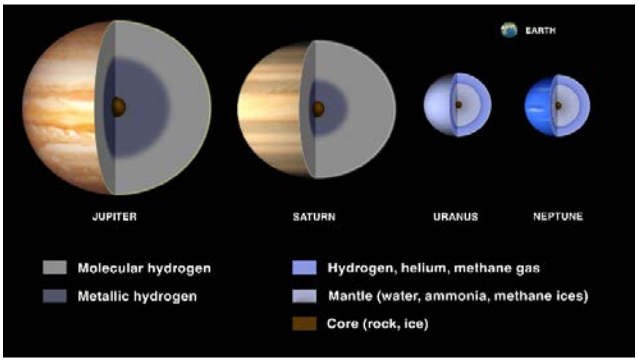

Editorial

Climate change is at the top of the list of global issues; not surprisingly, as the focal challenge to life and research which affects economy, policy, and the world at large. The cooperation and collaboration between nations is of vital importance in order to tackle these issues since it truly does change the way we look at protecting vulnerable populations.

The Kyoto Protocol (1997) is the first international treaty to cut greenhouse gas emissions, and it is linked to the United Nations Framework Conventions on Climate Change (1992). The Protocol commits parties by setting internationally binding emission reduction targets. Under the Protocol, countries must meet their targets primarily through national measures. The Protocol, since its adoption at the Third Conference of the Parties (COP3) in 1997, has become a beacon of climate action and an inspiring precursor to the Paris Climate Change Agreement (2015), because it demonstrated that international climate change agreements not only work but can significantly exceed expectations in meeting their objectives.[1]

Similarly, the UN Climate Conference (2017) in Bonn, Germany, gathered leaders of national governments, cities, states, business, investors, NGOs and civil society in order to stimulate climate action once again and to meet the goals of the Paris Climate Change Agreement.

The undertaken actions urge all nations to commit to a common cause – to confront climate change and to adapt to its effects with joint efforts and mutual understanding.

Editors

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[1] Celebrate the Kyoto Protocol's 20th Anniversary with the UN. (2017, Decembar 06). Retrieved from https://cop23.unfccc.int/news/celebrate-the-kyoto-protocol-s-20th-anniversary-with-the-un

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Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266

UDC: ________________________

Original Scientific Paper

2017
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1-8

Phytochemical screening, quantitative determination of phenolic compounds, and antioxidative activity of *Ostrya carpinifolia*

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Article info
Received: 20/07/2017
Accepted: 02/11/2017

Keywords:
Antimicrobial activity,
Balkans,
Betulaceae,
DPPH,
European hop-hornbeam,
Secondary metabolites

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Abstract: *Ostrya carpinifolia* is an interesting and suitable species for reforestation and landscaping. For the first time ever, phenolic profile, antioxidative and antimicrobial activity of *O. carpinifolia* was done in this study. Aqueous and methanol extracts of the aerial parts were analyzed using either fast screening methods of secondary metabolites, and UV/VIS spectrophotometry for determination of polyphenolic contents and antioxidant activity (DPPH). Antimicrobial activity of methanol extracts was investigated using the disc diffusion method against a selected nine microorganisms. Phytochemical tests confirm the presence of cardiac glycosides, coumarins, emodins, flavonoids, tannins, terpenes, terpenoids and steroids, while anthocyanins, fatty acids and saponins were absent in all aqueous extracts. Leucoanthocyanins were observed only in the stem extract. Methanol extracts of leaves contain the highest level of total phenolics and flavonoids (35.574 and 30.908 mg CE g^{-1} DW, respectively), while the inflorescences extracts were the richest with total proanthocyanidins and phenolic acids (19.165 mg CE and 9.342 mg CAE g^{-1} DW, respectively). All methanol extracts showed very strong antioxidative activity, where the lowest activity was recorded for inflorescences (IC$_{50}$: 0.242 mg mL^{-1}) and the highest for stem (IC$_{50}$: 0.107 mg mL^{-1}). Analyzed extracts showed no antimicrobial activity against the test organisms. ANOVA indicated the presence of significant differences between the total phenolics and flavonoids and DPPH ($p<0.05$). Duncan’s test confirmed the presence of statistically significant and very high positive correlation ($R=0.989$) between total phenolics and phenolic acids contents. Obtained results indicate the necessity of further research of European hop-hornbeam.

INTRODUCTION

Plants have an enormous ability to synthesize different bioactive organic compounds (i.e. secondary metabolites, SMs) that are not directly involved in their growth, development and reproduction but in absence they may be harmful or even fatal for plant's survivability. Secondary metabolites have essential roles in stabilizing cellular structure, plant adjustment and defense reacting under biotic and abiotic stresses. They are included in different physiology processes: pigmentation, pollination, seed germination, signaling, and resistance mechanisms under suboptimal conditions. The main characteristics of SMs are large chemical diversity, complexity and structure heterogeneity, as also the presence of diverse mechanisms and pathways of their biosynthesis (Bennet and Wallsgrove, 1994; Croteau, Kutchan and Lewis, 2000; Boudet, 2007; Edreva, Velikova, Tsonev, *et al*., 2008; Acquaviva, Menichini, Ragusa, *et al.*, 2012; Bartwal, Mall, Lohani, *et al*., 2013; Lattanzio, 2013; Murkovic, 2016).

According to available data, more than 200,000 different SMs have been reported, and this number is still continually increasing (Verpoorte, van der Heijden, ten Hoopen, *et al*., 1999; Hadacek, 2002; Edreva *et al.*, 2008; Springob and Kutchan, 2009; Bartwal *et al.*, 2013). Among them, the most abundant group of SMs with wide array of roles in plants are phenolic compounds including phenolic acids, tannins, lignins and most diverse flavonoids (Bartwal *et al*., 2013) with increasing biological interest of proanthocyanidins as the most widespread bioflavonoids (Škerget, Kotnik, Hadolin, *et al*., 2005). Namely, in recent years, phenolics and phenolic-related compounds gained huge importance and attention in medicine, pharmacology, food processing and cosmetics industry, due to their antioxidant, antimicrobial and anti-inflammatory properties (Oliviera, Sousa, Morais, *et al*., 2008; Abou-Zeid, Bidak and Gohar, 2014; Ashraf, Sarfraz, Rashid, *et al*., 2015; Hofmann, Nebehaj and Albert, 2016).

Ostrya carpinifolia Scop. (European hop-hornbeam), the only native species of genus in Europe and endemic to temperate West Eurasia, is a small to medium-sized broadleaved deciduous tree (Pasta, de Rigo and Caudullo, 2016). In terms of forestry, it is very interesting and suitable species for the reforestation of many degraded sites and landscaping (Weber, 2011; Ivetić, Devetaković, Davorija, *et al*., 2015; Pasta *et al*., 2016). Although, to date, chemical composition and biological activities of this species have been scarcely studied (Wollenweber, 1975), according to published data about other *Ostrya* species, *O. carpinifolia* can also be a source of natural phytochemicals with beneficial effects in broad arrays (Matsuki and Koike, 2006; Barbehenn, Weir and Salminen, 2008; Park, Kim, Ko, *et al*., 2010; Kim, Park and Lim, 2010).

Based on the lack of information and keeping the above facts in view, the aims of this study are: 1) qualitative and quantitative analysis of aqueous and methanol extracts of different aerial parts of *Ostrya carpinifolia* and 2) evaluation of antioxidant and antimicrobial activities of methanol extracts of European hop-hornbeam.

MATERIAL AND METHODS

Plant material

Plant material (leaves, stems, inflorescences) was collected from healthy trees of *O. carpinifolia* in its natural population near Sarajevo (43˚51’19’’ N, 18˚27’27’’ E, 595 m ASL, S-SW), in May 2015. Different plant parts were separated immediately and packed into paper bags. In the laboratory, the plant materials were washed with running tap water, rinsed with distilled water and naturally air dried during 10 days at room temperature in a dry, shaded and well-aerated place. Then, dried materials were grounded into powder using mixer and stored in plastic bags, at room temperature, until extraction processes.

Chemicals and reagents

Folin-Ciocalteu’s reagent, catechin, caffeic acid, DPPH (1,1-diphenyl-2-picryl-hydrazyl), sodium carbonate, absolute methanol, and aluminium chloride were purchased from Sigma-Aldrich (Steinheim, Germany), while Amoxicillin, Müeller Hinton and Sabouraud dextrose agars were purchased from HiMedia Chemicals Ltd. (Mumbai, India). All other used chemicals and solvents were of analytical grade.

Tested microorganisms

Antimicrobial activity was tested against a panel of microorganisms: Gram-positive bacteria (*Staphylococcus epidermididis* ATCC 8739TM, *Staphylococcus aureus* subsp. *aureus* ATCC 6538 TM, *Bacillus subtilis* subsp. *spizizenii* ATCC 6633™, *Enterococcus faecalis* ATCC 19433, and *Bacillus vulgatus* ATCC 8482), Gram-negative bacteria (*Salmonella abony* NCTC 6017TM, *Escherichia coli* ATCC 8739TM, and *Pseudomonas aeruginosa* ATCC 9027) and fungus *Candida albicans* (ATCC 10231). The bacterial strains were cultured in Müeller Hinton agar while fungus was cultured on Sabouraud dextrose agar.

Aqueous extracts preparation

Per 5 g of ground plant materials were placed in to the glass beakers and 50 mL of distilled water was added in each. After 30 min of incubation at 55°C in water bath the extracts were cooled, centrifuged for 20 min at 1800 rpm (Centric 322 B, Techtnica), and filtered through Whatman No.1 filter papers.

Preliminary phytochemicals screening

Preliminary fast phytochemical standard tests for the screening and qualitative identification of bioactive chemical constituents of plant aqueous extracts of *O. carpinifolia* were carried out by modified methods of Trease and Evans (2002), Savithramma, Linga Rao and Suhrulatha (2011), and Subhashini Devi, Satyanarayana and Tarakeswara Naidu (2014). Leaves, stem and inflorescences extracts were screened for 12 phytochemical constituents: anthocyanins, cardiac glycosides, coumarins, emodins, fatty acids, flavonoids, leucoanthocyanins, saponins, steroids, tannins, terpenes, and terpenoids.

Methanol extracts preparation

Grinded plant materials (0.5 g) were extracted with 25 mL of 80% methanol. The extraction process was carried out in two replicates (twice with 12 mL and adjusted to 25 mL) using both times an ultrasonic bath (Elma sonic S 60 H) for 30 min. Extracts were then centrifuged at 1800 rpm for 10 min. The obtained supernatants were stored in plastic tubes at 5°C for further analysis.

The Shimadzu UV-mini 1240 spectrophotometer was used for determination of polyphenolic contents and antioxidant activity of all methanol extracts. All samples were analyzed in triplicates.

Determination of total phenolic content

Total phenolic content of the methanol extracts was determined using modified Folin-Ciocalteu method (Wolfe, Wu and Liu, 2003). An aliquot of the methanol extract (20µL) was mixed with distilled water followed by addition of 100 µL of Folin-Ciocalteu’s reagent and 300 µL of freshly prepared sodium carbonate solution (7.5%). After mixing, the tubes were incubated for 30 min at 45°C in water bath until blue color development. The absorbance of the resulting solvents was measured at 765 nm. Total phenolic content was expressed as mg of catechin equivalents per gram of dry plant sample (mg CE g^{-1}).

Determination of total flavonoids

Total flavonoid content was determined by the modified method of Ordoñez, Gomez, Vattuone, *et al*. (2006). The methanol extract aliquots of both leaves (20 µL) and stem and inflorescences (60 µL) were mixed with 25 µL of 10% aluminum chloride water solution followed by the addition of 25 μL of 1M sodium acetate water solution. The mixture was left for homogenization and incubation at room temperature (24°C) for 20 min. The absorbance was measured at 415 nm. Total flavonoid content was expressed as mg of catechin equivalent per gram of dry sample (mg CE g^{-1}).

Determination of total proanthocyanidins

Total proanthocyanidins content was measured by modified method of Wettstein, Jende-Strid, Ahrenst-Larsen, *et al*. (1977). An aliquot of methanol extract (50 μL) was mixed with 750 μL of 4% vanillin-methanol solution and 375 μL of hydrochloric acid. The tubes were vortexed and the absorbance was immediately measured at 500 nm. Total proanthocyanidins content was expressed as mg of catechin equivalent per gram of dry weight (mg CE g^{-1}).

Determination of total phenolic acids

Quantification of total phenolic acids was carried out using slightly modified Arnow method (Haydrich and Goślińska, 2004). Namely, all the analyzed samples are diluted with methanol (1:1, v/v) because of reading of phenolic acids excessive values. The tubes were vividly shaken and after 20 min of incubation at room temperature, absorbance was measured at 490 nm. The results were expressed as caffeic acid equivalents per gram of dry sample (mg CAE g^{-1}).

DPPH Radical Scavenging Activity Assay

The antioxidant activity of the methanol extracts was assessed by DPPH free radical scavenging modified method by Meda, Lamien, Romito, *et al*. (2005). Aliquots of diluted methanol extracts (100, 80, 60, 40, and 20 μL) were mixed with freshly prepared ethanol DPPH solution and incubated for 30 min at room temperature in the dark. The absorbances of the resulting solutions were measured at 515 nm against 96% ethanol as a blank. The DPPH scavenging ability in percentage (AA %) was calculated as follows:

$$AA\% = \frac{(A_a - A_b) \times 100}{A_a}$$

where:

A_a– the absorbance of DPPH blank
A_b– the absorbance of DPPH with sample in different concentrations.

The antioxidant activity was expressed as half maximal inhibition concentration (IC_{50}) defined as the concentration of sample required for inhibition of 50% of initial amount of DPPH radicals. The IC_{50} values were calculated graphically based on the calibration curves for each sample. The lower IC_{50} values indicated the higher antioxidative activity in the methanol extracts.

Estimation of antimicrobial activity

Standard serial dilution assay with disc diffusion method (Bauer, Kirby, Sherris, *et al*., 1966) was used to test the antimicrobial activity of methanol extracts against selected microorganisms. Inoculum was prepared by diluting overnight grown microbial culture with 0.9% NaCl and adjusting the concentration of the medium to match the 0.5% McFarland standard. Then, 1 mL of bacterial suspension was spread on sterile Müeller Hinton agar plate, and Sabouraud dextrosa agar was inoculated with 1 mL of fungal suspension. Sterile discs impregnated with 25 μL of methanol extracts were placed on the inoculated agars. Standard disc of Amoxicillin (Amx10mcg) was used as control in bacterial plates, and 80% methanol was used as blank in bacterial and fungal plates. The inoculated plates with discs were incubated at 37°C for 24 hours to allow maximum growth of the organisms.

Statistical Analysis

Data was analyzed by Statistica 7 for Windows using a one-way analysis of variance (ANOVA). To determine the significant differences between groups, after analysis of variance, Duncan's *post-hoc* test was used at $p<0.05$.

RESULTS AND DISCUSSION

According to Boudet (2007) and Bartwal, *et al*. (2013), variations among plant species functionalities are result of their chemical features and content, where the key place takes the polyphenolic compounds, the most abundant and heterogeneous group of secondary metabolites. Besides important roles in the plant kingdom (color, taste, technical properties, mechanical support, protection, pollination, etc.) these compounds are primarily responsible for plant utilization in medicine, pharmacology, agriculture, cosmetic, aromatherapy and food industry, due to their positive effect on human health. With the respect to significance of natural products and concerns about unsafety and carcinogenic potential of widely used synthetic antioxidants and chemical preservatives, the science efforts are directed towards exploiting new antioxidants and antimicrobial compounds from natural sources and analyzing the phenols potential in these activities.

Scarcity of data on phenolic content, antioxidative and antimicrobial activity of *Ostrya carpinifolia* indicates a lack of exploration of this species. Exceptions are data of the Wollenweber (1975) who reported that *Ostrya* species possess polyphenols with dominant role of flavonoids. Therefore, this paper presents, for the first time, phenolic profile, antioxidative and antimicrobial activity of *Ostrya carpinifolia*.

Polyphenolic compounds of **Ostrya carpinifolia**

Phenolic compounds are generally present in many plants and can be concentrated in different plant parts. Among phenolic compounds, tannins, coumarins, phenolic acids and flavonoids, stand out with their antioxidant potential. Preliminary phytochemical tests confirm the presence of cardiac glycosides, coumarins, emodins, flavonoids, tannins, terpenes, terpenoids and steroids in all aqueous extracts of *Ostrya carpinifolia* (Table 1).

Table 1: Obtained results of phytochemical analysis of secondary metabolites presence in aqueous extracts of *Ostrya carpinifolia* by rapid screening methods ("–"indicates absence and "+" presence).

Secondary metabolites	Leaf	Stem	Inflorescences
Anthocyanins	–	–	–
Cardiac glycosides	+	+	+
Coumarins	+	+	+
Emodins	+	+	+
Fatty acids	–	–	–
Flavonoids	+	+	+
Leucoanthocyanins	–	+	–
Saponins	–	–	–
Steroids	+	+	+
Tannins	+	+	+
Terpenes	+	+	+
Terpenoids	+	+	+

Leucoanthocyanins were observed in the stem extract but not in the leaves and inflorescence extracts. Anthocyanins, fatty acids and saponins were absent in all studied extracts. Previous chemical investigation of *Ostrya japonica* Sarg. and *O. virginiana* (Mill.) K.Koch indicated the presence of polyphenolic compounds (Matsuki and Koike, 2006; Barbehenn, *et al.*, 2008; Kim, *et al.*, 2010; Park, *et al.*, 2010). In the family Betulaceae, for the genera *Alnus*, *Betula*, *Carpinus* and *Corylus* have been shown to produce wide variety of polyphenolic compounds (Wollenweber, 1975; Matsuki and Koike, 2006; Barbehenn, *et al.*, 2008; Kim, *et al.*, 2010; Park, *et al.*, 2010; Mushkina, Gurina, Konopleva, *et al.*, 2013; Costea, Vlase, Viorel, *et al.*, 2016; Hofmann, *et al.*, 2016; Orodan, Vodnar, Toiu, *et al.*, 2016; Riethmüller, Könczölb, Szakálc, *et al.*, 2016).

Although it is known that microsite conditions and the position of the leaves can influence phenolic content and its seasonal changes (Zhang, Gao, Zhang, *et al.*, 2010; Vagiri, Conner, Stewart, *et al.*, 2015; Hofmann, *et al.*, 2016), it is obvious that this family represents a valuable source of polyphenolic compounds and should be more investigated in the future, since our results indicate the presence of antraquinone emodin, coumarins and steroids in *O. carpinifolia*.

From Figure 1 and Table 2 it is visible that leaves, in comparison to other plant parts, possessed very high concentrations of all analyzed phenolic compounds, except phenolic acids. In addition, in all plant parts the total proanthocyanidins had relatively high and more or less uniform values, whereas the total phenolic acids were at least present. The total flavonoids content was the highest in the leaves and the lowest in the stem.

Previous studies of phenolic compounds in Betulaceae members were, mainly, done on different type of leaves extracts. Analyses showed that these species were rich with phenolics, flavonoids and phenolic acids (Wollenweber, 1975; Barbehenn, *et al.*, 2008; Park, *et al.*, 2010; Mushkina, *et al.*, 2013; Costea, *et al.*, 2016; Hofmann, *et al.*, 2016; Orodan, *et al.*, 2016; Riethmüller, *et al.*, 2016). This data is in accordance with our results, except for phenolic acids which were present in the lower values in *O. carpinifolia* (Table 2). The presence of total proanthocyanidins in family Betulaceae is, for the first time, determined in this study. Namely, from obtained results is visible that all aerial parts of *O. carpinifolia* are relatively rich with proanthocyanidins.

It is known, among other things, that phenolic compounds are generally included in plant defense against pathogens and predators, in processes of pigmentation, UV protection and etc., and mostly included in plant-microbe interactions and initiation of mycorrhizal symbioses as signaling molecules (Winkel-Shirley, 2001; Crozier, Clifford and Ashihara, 2006; Mandal, Chakraborty and Dey, 2010; Khodammi, Wilkes and Roberts, 2013). Accordingly, it is possible to assume that the presence of relative high concentrations of phenolics, flavonoids and proanthocyanidins enable this species to survive on thermophilic habitats and to be used as a pioneer species in the reforestation and landscaping.

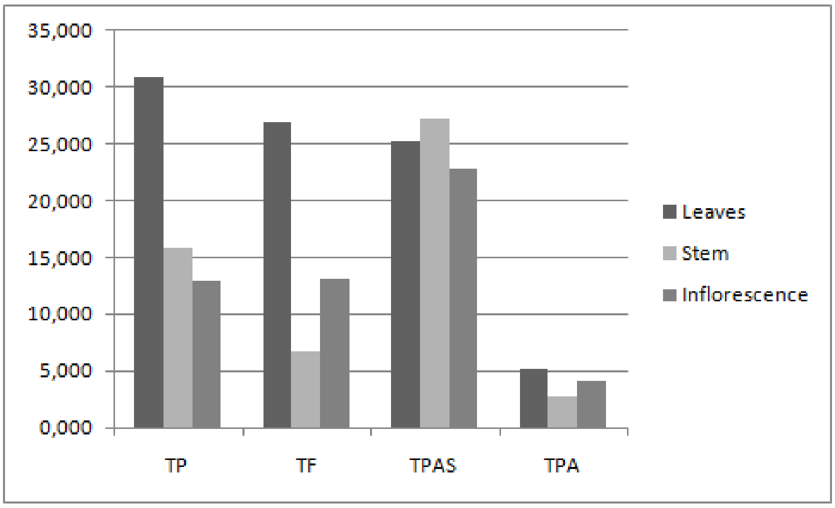

Figure 1: Representations of mean values of the analyzed polyphenolic compounds in different parts of *Ostrya carpinifolia.* TP – total phenolics, TF – total flavonoids, TPAS – total proanthocyanidins (all expressed in mg CE g^{-1} DW), TPA – total phenolic acids (mg CAE g^{-1} DW).

Table 2: Basic descriptive parameters of analyzed secondary metabolites and antioxidant activity of *Ostrya carpinifolia* (PP – plant part, *X* – average, SD – standard deviation, CV – coefficient variation, TP – total phenolics, TF – total flavonoids, TPAS – total proanthocyanidins, TPA – total phenolic acids, IC_{50} – half maximal inhibition concentration).

PP	**Parameter**	**TP (mg CE^a g^{-1})**	**TF (mg CE g^{-1})**	**TPAS (mg CE g^{-1})**	**TPA (mg CAE^b g^{-1})**	**IC_{50} (mg mL^{-1})**
Leaves	**Min**	27.982	21.975	17.422	3.467	0.164
	Max	35.574	30.908	32.469	6.860	0.213
	X	30.831	26.903	25.313	5.224	0.184
	SD	3.192	3.092	4.744	1.127	0.016
	CV (%)	10.353	11.494	18.743	21.580	8.459
Stem	**Min**	11.223	5.772	17.036	1.368	0.086
	Max	21.953	7.543	35.849	4.337	0.125
	X	15.895	6.628	27.299	2.666	0.107
	SD	4.121	0.753	6.651	1.176	0.013
	CV (%)	25.925	11.358	24.363	44.133	11.860
Inflorescence	**Min**	8.518	9.060	10.032	1.845	0.153
	Max	21.338	17.455	49.165	9.342	0.356
	X	12.908	14.171	22.861	4.050	0.242
	SD	5.739	4.486	18.263	3.574	0.084
	CV (%)	44.462	31.656	79.887	88.250	34.975

[a] – catechin equivalent
[b] – caffeic acid equivalent

Table 3: Duncan test ($p<0.05$) for the analyzed secondary metabolites (TP – total phenolics, TF – total flavonoids, TPAS – total proanthocyanidins, TPA – total phenolic acids) and antioxidant activity (IC_{50} – half maximal inhibition concentration) of methanol extracts of *O. carpinifolia*. Bold text indicates statistically significant correlations.

	TP	**TF**	**TPAS**	**TPA**	**IC_{50}**
TP	1.00000	-0.468653	0.884935	**0.988785**	-0.626422
TF	-0.468653	1.000000	-0.825824	-0.553269	0.703915
TPAS	0.884935	-0.825824	1.000000	0.924325	-0.781791
TP	**0.988785**	-0.553269	0.924325	1.000000	-0.735095
IC_{50}	-0.626422	0.703915	-0.781791	-0.735095	1.000000

Antioxidative and antimicrobial activity of **Ostrya carpinifolia**

Antioxidant potential of phenolic compounds, especially flavonoids, is connected to their ability of metal chelating and scavenging harmful free radicals and reactive oxygen species which attack lied to cell damages and various disorders and diseases (Michalak, 2006; Siatka and Kašparová, 2010; Bartwal, *et al.*, 2013; Radić, Vujčić, Glogoški, *et al.*, 2016; Rawat, Jugran, Bahukhandi, *et al.*, 2016).

The antioxidant activity of methanol extracts of *O. carpinifolia* was evaluated on the basis of their ability to scavenge free DPPH radicals. This is the first report about antioxidant activity of *O. carpinifolia* while the antioxidant activity of *O. japonica* was previously reported (Kim, *et al.*, 2010; Park, *et al.*, 2010).

All examined methanol extracts showed very high free radical scavenging activity (Table 2). Very low concentration of stem extracts exhibited the highest (IC_{50}: 0.107 mg mL^{-1}) activity, followed by leaves (IC50: 0.184 mg mL^{-1}), while inflorescences exhibited the smallest antioxidant activity (IC_{50}: 0.242 mg mL^{-1}).

According to available reports about family Betulaceae, strong antioxidant activity was obtained in *Carpinus betulus* L., *Betula megrelica* Sosn., *Corylus colurna* L. and *Alnus sibirica* (Spach) Turcz. ex Kom. (Kim, *et al.*, 2010; Zardiashvili, Jokhadze, Kuchukhidze, *et al.* 2014; Hofmann, *et al.*, 2016; Riethmüller, *et al.*, 2016), and mostly moderate to low activity in *Alnus glutinosa* (L.) Gaertn, *Alnus incana* (L.) Moench, *Betula pendula* Roth, and *Corylus avellana* L. (Acquaviva, *et al.*, 2012; Mushkina, *et al.*, 2013; Costea, *et al.*, 2016; Orodan, *et al.*, 2016; Riethmüller, *et al.*, 2016).

Very low concentrations of all samples showed strong antioxidant activity, where the lowest activity was recorded for inflorescences (IC_{50}: 0.242 mg mL^{-1}) and the highest was exhibited by stem's extracts (IC_{50}: 0.107 mg mL^{-1}), that had the lowest total flavonoids values in comparison to other samples.

Analysis of variance indicated the presence of significant differences between the DPPH radical scavenging and contents of total phenolics and flavonoids ($p<0.05$). Duncan's test (Table 3) confirmed the presence of statistically significant and very high positive correlation (R=0.989) between total phenolics and phenolic acids contents.

Considering no correlation was found between DPPH IC_{50} values and phenolic components it can be presumed that antioxidant activity is not simply related to the total phenolics and flavonoids content and that highest antioxidative activity of stem extracts is not the result of high concentration of any of analyzed phenolic compounds. This can be explained, as we assume, with: strong influence of unanalyzed constituents (e.g. tannins), presence of antioxidants with different chemical structures, and different antagonistic, synergistic and additive reaction among present components. Similar results were previously registered at *Carpinus betulus* and *Corylus* species (Hofmann, *et al*., 2016; Riethmüller, *et al*., 2016).

Since the methanol extracts of *O. carpinifolia* are potential natural antioxidants, it is necessary to analyze and compare *O. carpinifolia* extracts in different solvents in order to isolate and identify their chemical constituents and potential bioactive compounds.

Although the analyzed phenolic compounds in methanol extracts of *O. carpinifolia* were present in significant amounts and antioxidant activity was strong, no antibacterial and antifungal activities against the selected nine microorganisms were observed.

According to relevant literature, extracts of different parts of some related species to *O. carpinifolia* had shown antimicrobial activity, where Gram-positive bacteria (at the most *Staphylococcus aureus*, *Staphylococcus epidermididis*, *Bacillus subtilis*) were more sensitive than Gram-negative bacteria (Oliviera, *et al*., 2008; Acquaviva, *et al*., 2012; Orodan, *et al*., 2016). Antimicrobial activity of extracts of *Betula pendula* and *Corylus avellana* against *Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* was very low or not identified at all (Oliviera, *et al*., 2008; Orodan, *et al*., 2016), which coincides with our results.

Obviously, further investigation of antimicrobial activity of *O. carpinifolia* extracts on unanalyzed bacteria and fungi strains in this study should be considered.

CONCLUSIONS

Ostrya carpinifolia represents a valuable source of polyphenolic compounds and should be more investigated in the future, since our results indicate the presence of nine groups of secondary metabolites. This study, also, showed that the methanol extracts of *O. carpinifolia* are potential natural strong antioxidants, which can be explained with intense influence of unanalyzed compounds, presence of antioxidants with different chemical structures, and different antagonistic and synergistic reactions among present constituents. According to obtained results, it is possible to assume that the presence of relative high concentrations of phenolics, flavonoids and proanthocyanidins and a strong antioxidative activity enable this species to survive on thermophilic habitats and to be used as a pioneer species in the reforestation and landscaping. From all the above it can be suggested the necessity for further research of *Ostrya carpinifolia* extracts in different solvents in order to identify and isolate their chemical constituents and potential bioactive compounds as a potential natural source of antioxidant activities.

ACKNOWLEDGMENTS

The authors are grateful to N. Bašić for both help collecting plant material and in the statistical data treatment.

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Summary/Sažetak

Ostrya carpinifolia je zanimljiva i pogodna vrsta za pošumljavanje i uređenje okoliša. U ovoj studiji je, po prvi put, određen fenolni profil te antioksidativna i antimikrobna aktivnost *O. carpinifolia*. Vodeni i metanolni ekstrakti nadzemnih dijelova crnog graba analizirani su pomoću kvalitativnih metoda za brzo ispitivanje sekundarnih metabolita te UV/VIS spektrofotometrijom za određivanje polifenolnog sadržaja i antioksidativne aktivnosti (DPPH). Antimikrobna aktivnost metanolnih ekstrakata je analizirana disk difuzijskom metodom protiv devet odabranih mikroorganizama. Fitohemijski testovi su potvrdili prisutnost emodina, flavonoida, kardijačnih glikozida, kumarina, tanina, terpena, terpenoida i steroida. Prisustvo antocijanina, masnih kiselina i saponina nije uočeno niti u jednom vodenom ekstraktu, dok su leukoantocijanini uočeni samo u ekstraktu stabla. Metanolni ekstrakti listova su imali najveću količinu ukupnih fenola i flavonoida (35,574 i 30,908 mg CE g^{-1} SM), dok su ekstrakti cvjetova bili najbogatiji s ukupnim proantocijanidinima i fenolnim kiselinama (19,165 mg CE i 9,342 mg CAE g^{-1} SM). Svi metanolni ekstrakti pokazali su vrlo jaku antioksidativnu aktivnost, pri čemu je najniža aktivnost zabilježena za cvjetove (IC_{50}: 0,242 mg mL^{-1}) a najviša za stabljiku (IC_{50}: 0,107 mg mL^{-1}). Analizirani metanolni ekstrakti nisu pokazali antimikrobnu aktivnost protiv testnih mikroorganizama. ANOVA je pokazala prisutnost značajnih razlika između ukupnih fenola i flavonoida i DPPH ($p <0,05$). Duncanov test potvrdio je prisutnost statistički značajne i vrlo visoke pozitivne korelacije (R=0,989) između ukupnih sadržaja fenola i fenolnih kiselina. Dobiveni rezultati ukazuju na potrebu daljnjeg istraživanja *Ostrya carpinifolia*.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

Print ISSN: 0367-4444

UDC: ________________________

Online ISSN: 2232-7266

Original Scientific Paper

9-14

Spatial and seasonal variation of PAHs concentration in the Spreča river

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Article info
Received: 19/07/2017
Accepted: 02/11/2017

Keywords:
PAHs,
HPLC,
Spreča,
Modrac.

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Abstract: The largest part of the river Spreča flows through the heavily populated region of Tuzla canton, and it is also area of mining-industrial zone. This study monitors the PAHs content, in the part of Spreča's flow which is extremely exposed to these pollutants during the year of 2015. Content of PAHs in the river were analyzed on three locations: prior Modrac lake, in the lake and on the mouth of Spreča into river Bosna. Determination of PAHs in the water was performed by HPLC techniques - fluorescence detection, after liquid-liquid extraction. Eight PAHs, recommended by EU directive, have been analyzed: naphthalene, anthracene, fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene and benzo(ghi)perylene. The highest concentration was found for naphthalene. Except naphthalene, concentration of all other PAHs was significantly higher on the mouth of Spreča than on the two previous locations. Concentration of all PAHs significantly varies during seasons and it was highest during the winter period. High level of naphthalene and fluoranthene comes from coal-mining area which is prior to Modrac lake and also from the soda drinks chemical industry further away from the lake. Other six PAHs mainly come from soda drimks -chemical industry.

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are one of the most widely known pollutants in nature. They occur in all parts of the environment: air, soil, surface, underground, drinking and sea water, ice, food, sediment, plant, animal tissues etc. Depending on the chemical structure they can be poorly degradable with a long period of retention in the environment. They act toxically to the living world. Some of the PAHs show strong carcinogenic and mutagenic activity, leading to acute toxicity, developmental and reproductive toxicity, cytotoxicity and genotoxicity (Magi et al., 2002; WHO, 2000). PAHs, most commonly present in the environment, usually contain from two to seven benzene rings (Lundstedt, 2003). PAHs with the same number of condensed rings may be significantly different, depending on whether these rings have arranged linearly, angular or clustered (Lundstedt, 2003). In order to determine the good ecological status of water, the EU legislations have listed the priority substances whose concentrations have to be determined in surface waters. Among those substances, eight PAHs have been listed: naphthalene, anthracene, fluoranthene, benzo(b)-fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene and benzo(ghi)perylene (EU Directive, 2013).

PAHs arise mainly due to the incomplete combustion of fossil fuels, wood masses, waste materials or on natural way such as volcanic eruptions and forest fires (Maliszewska-Kordybach, 1999). However, a number of these compounds emerge as a product of the industry, primarily of chemical and mining. PAHs in surface waters mostly have come from air during atmospheric precipitation, then by discharging wastewater from settlements, waste water that comes from the area where coal has stored, wastewater after treatment of wood and wastewater of other types of industry (Achtena and Hofmann, 2009). The river Spreča, with its tributaries, is exposed to most of the mentioned PAHs emission sources, but the mining area and facilities of chemical industry in this region could be the main source of these pollutants. Coal from mining area is the important source of many PAHs, most naphthalene. These PAHs come in the river flows through the process of coal separation. The soda drinks chemical industry in Lukavac presents a potentially polluter of the river with many types of PAHs.

Purpose of this research was to monitor variation of PAHs content in the river Spreča, during different seasons of one calendar year. On the base of the results, it was done an assessment of the influence of industry, located in the river basin, on the content of PAHs in the aquatic environment. Seasonal monitoring of PAHs concentrations in the river can provide significant data on the impact of certain human activities on their content, which can be used to undertake the activity in order to reduce emissions or to prevent the harmful effects on the environment.

Methods of analysis, from sample preparation to instrumental analysis, must be highly specific and sensitive since that PAHs amounts in the samples are usually very low. The most widely used techniques in analysis of PAHs are high-performance liquid chromatography (HPLC) and gas chromatography (Khan et al., 2005). The most commonly used method for testing river water samples is HPLC technique with fluorescent detection, after liquid-liquid extraction. This method is also specified by ISO standard for the determination of 15 selected PAHs in surface waters, where a mass concentration above 0,010 μg/L has been expected for each individual component (ISO, 2002).

Liquid-liquid extraction of PAHs is quite complicated. However, this is one of the most reliable methods, especially due to the efficiency. Beside water, this method includes extraction of PAHs that absorbed on the suspended matter, which finally gives a better assessment of water pollution.

MATERIALS AND METHODS

Samples

In the middle flow, the river Spreča flows into Modrac lake and also flows out from the other side of the lake. A mining area is placed in part of flow prior the lake, while the chemical industry is situated near the Spreča, after lake. Related to this, the content of PAHs in the river has been analyzed on the three locations: prior the Modrac lake, in the lake and at the mouth of the Spreča into the river Bosna. Samples from locations prior the lake and at the mouth of the Spreča, have been collected and analyzed monthly, during the year of 2015. Samples from lake have been collected and analyzed four time in mentioned year.

Water was collected in dark-glass bottles, previously weighed. The bottles were filled to the top (approx. 1000 mL), stored at about + 4 ºC and protected from light until extraction was made. Extraction was performed within 24 h of the sampling, to avoid adsorption losses. If the complete analysis cannot be done within 24 h, the part of sample from the sampling bottle has to be removed until the volume of 1000 ± 10 mL. Volume of sample is determined by weighing of bottle. Then 25 mL of hexane is added and well-stirred. Sample treated on this way, protected from light, can be stored 72 h at + 4 ºC (ISO, 2014).

Selected PAHs

The analyses included 8 PAHs recommended by EU directive: naphthalene, anthracene, fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo-(a)pyrene, indeno(1,2,3-cd)pyrene and benzo(ghi)perylene.

Chemicals and equipment

The following chemicals and equipment were used for analysis. Chemicals: deionized water; hexane (HPLC grade, Fisher Scientific); N,N-dimetilformamid (>99,9 %, Roth), acetonitrile (HPLC grade, Baker); acetone (HPLC grade, Fisher Scientific); sodium sulfate (p.a.); nitrogen (99,999 %); PAH standards: naphthalene (99,7 %, Supelco), anthracene (99,0 %, Supelco), flouranthene (99,5 %, Supelco), benzo(b)flouranthene (97,3 %, Supelco), benzo(k)flouranthene (99,5 %, Supelco), benzo(a)pyrene (95 %, Supelco), indeno(1,2,3-cd)pyrene (99,7 %, Supelco) and benzo(ghi)perylene (99,4 %, Supelco). Equipment: magnetic stirrer (Agimatic-E, Selecta); rotavapor (Laborota 4011 digital, Heidolph); coulumn C18 (Zorbax Eclipse PAH, Analytical 4,6 x 250 mm; 5-Micron, Agilent); HPLC system with fluorescence detector (Solvent delivery module LC-20AT Prominence; On-line Degasser DGU-20A5; Auto sampler SIL-20A Prominence; Column oven CTO-20AC Prominence; Spectrofluorometric detector RF-10Axl; System controller CBM-20A Prominence, SHIMADZU).

The extraction of PAHs

Volume of the water sample in the bottle was adjusted on the 1000 ± 10 mL, by removing a part of sample. Exact volume of sample has been determined by weighing the bottle (difference between full and empty bottle).

Then, 25 mL of hexane was added and well-stirred. The sample was extracted by mixing it on a magnetic stirrer, set at 1000 rpm, about 60 min. After extraction, sample was transferred to a separating funnel and left until phase separation, for at least 5 minutes.

The hexane layer was transferred to a 100 mL conical bottle and the extract was dried with sodium sulfate for at least 30 min. The dried extract was decanted in the solvent evaporator flask. The conical bottle was washed with 5 mL of hexane, two times, and all added to the same bottle for solvent evaporation.

The hexane extract was lightly evaporated to volume of 2 mL approximately. It was done by rotary-evaporator, under pressure of 210 mbar and in the thermostatic bath at 30 ºC. It has been performed carefully to avoid evaporation to the dryness, because it can produce losses of PAHs with 2 and 3 benzene rings.

The concentrated 2 mL extract was transferred to the test tube. To this extract it was added 250 μL of N,N-dimethylformamide and 500 μL of acetone and mixed. Hexane and acetone were removed with a gentle stream of nitrogen, so that the volume of the extract was reduced to a volume between 200 µL and 250 µL, the remain was N,N-dimethylformamide with the analyte. The extract was diluted to 2 mL with acetonitrile (ISO, 2002).

Analysis

HPLC separation was performed with acetonitrile/water combination on the polymerically bonded C18 column optimized for the separation of PAHs. Elution was carried out with a solvent mixture in a gradient mode. Time and percentages of acetonitrile: 0-2 min. 60-60 %; 2-15 min. 60-100 %; 15-20 min. 100 %; 20-22 min. 100-60 %; 22-26 min. 60-60 %. Separation was carried out at constant temperature of 30 ºC. The injection volume was 10 µL. Fluorescence detector was used for detection. The appropriate wavelength for excitation and emission for respective individual PAHs has been adjusted with respect to their sensitivity and selectivity. The calibration curve was made from five different concentration standards, diluted in acetonitrile (ISO, 2002).

RESULTS AND DISCUSSION

The basin of the river Spreča is exposed by two major industrial sources of PAHs. The first are coal mines Đurđevik and Banovići, where inevitably comes the emission of PAHs into water during the coal separation process. The mines are bound to the river Spreča by the left tributary, prior to the Modrac lake. Another important source of PAH is a coke-chemical industry, which is potentially the most dangerous emitter of these substances. It is located in Lukavac, in the part of Spreča's flow down the lake. The habitants of heavily populated region surrounding the lake, commonly use coal and wood for the heating of their households. With rainfall and poor air circulation during the winter months, emission of PAHs from households may significantly increase of PAH concentration in the river, depending on weather conditions.

Concentration of all tested PAHs has been higher on the mouth of the river Spreča than on two previously locations, and for most of PAHs this difference in concentration was extremely high (Figure 1). This result was expected, with regard to the accumulation of PAHs from both significant sources during the flowing of the river, and the inability that PAHs to be degraded within a relatively short time of flow. The exception is naphthalene, which was on annual-average in the highest concentration at the mouth of Spreča, but in certain months naphthalene was in highest concentration on the location prior the lake. The reason for reduction of naphthalene at the mouth, in some months, could be relatively rapid degradation of naphthalene (Oleszczuk and Baran, 2003; ATSDR, 1995). Indication for degradation of naphthalene was also its content during the winter prior the lake and March's concentration in the lake (Figure 1b, Figure 1c). The concentration of naphthalene during the March in the lake was much lower than in the Spreča prior the lake during the previous months, that indicates the relatively rapid decomposition of naphthalene. It is true that lake also has been filled from several smaller streams, but it is not so significant for this decrease.

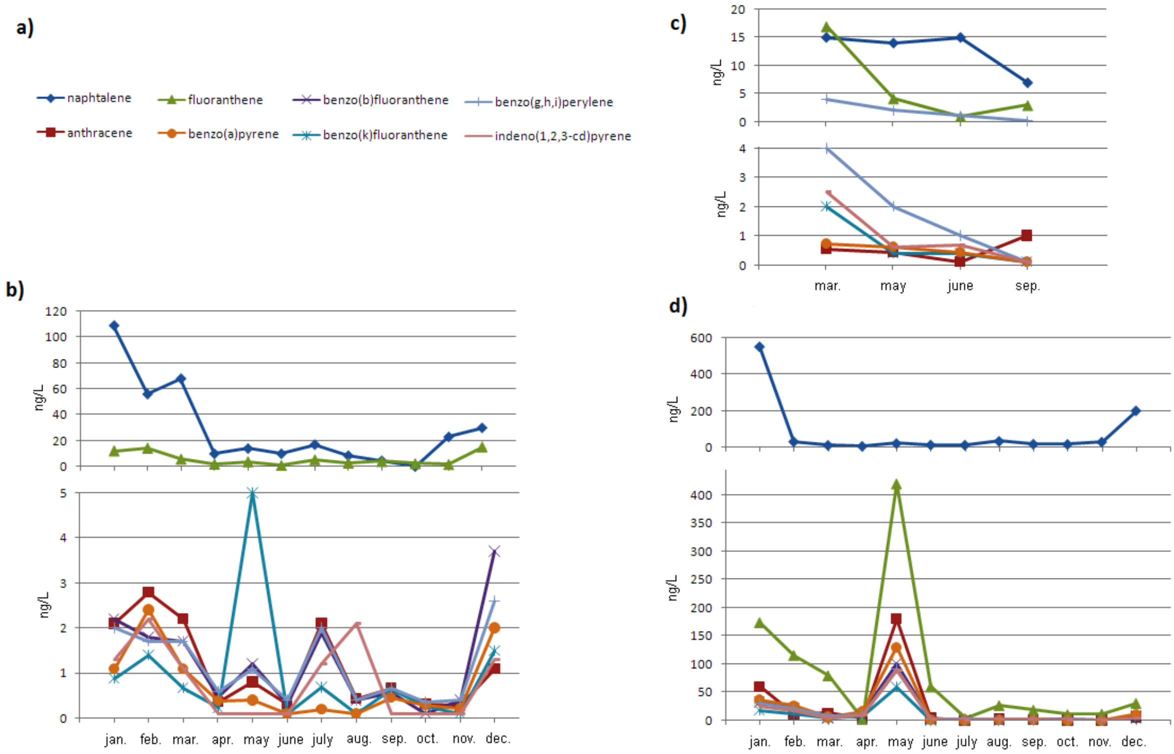

Figure 1: Concentration of PAHs: a) legend; b) in the Spreča river, prior the Modrac lake; c) in the Modrac lake; d) on the mouth of Spreča into Bosna river

The results, especially Figure 1d, indicate different sources of pollution for naphthalene compared to other PAHs, because concentration of naphthalene was not following variation in the content of other PAHs. Certainly, a great source of naphthalene in the river Spreča lies in the mining zone prior to the lake, but its increased concentration during winter months means that there is a significant emission during the heating season (Figure 1b). It means that naphthalene in the river comes from several sources, including non-industrial (coal and wood burning during the heating season), and not only from the coal separation process and the coke-chemical industry. Otherwise, among all tested PAHs, the greatest concentration was found for naphthalene. On the other side, that fact doesn’t present a problem because allowed concentration for naphthalene is high, so all values for this PAH were in the scope of acceptable ecological standards

Excluding naphthalene, other seven PAHs were in extremely higher concentration on the mouth of river than on the previous locations (Figure 1). It assumes that the main source of these PAHs is in the part of the river flow after the lake. Cause of this probably comes from soda drinks chemical industry located near the river, after the lake. Among these PAHs the highest concentration was found for fluoranthene. The reason could be in its emission from other sources - fluoranthene has been significantly contained in the river on the measure points prior to the lake and in the lake (Figure 1b, Figure 1c). Fluoranthene is contained in many types of coal and surely came into river by the process of coal separation (Chen at al., 2004; Willsch and Radke, 1995). As natural components of coal, from the group of analyzed PAHs, naphthalene is most common followed by fluoranthene, while other PAHs are significantly less presented (Achtena and Hofmann, 2009). Emission from process of coal separation, slow degradation in environment and emission from soda drinks chemical industry contributed to high content fluoranthene on the mouth of Spreča. However, obtained values of fluoranthene rarely exceed values that are environmentally hazardous, while the annual average level has been acceptable to the environment (EU Directive, 2013).

The winter-summer variations were the most expressed for the naphthalene and fluoranthene concentrations. Higher concentrations of naphthalene are usual in the environment during the heating season, what is also mentioned for fluoranthene in some studies (Bari et al., 2010). Other six PAHs: anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene, showed some variations of content during summer and winter season (Figure 1b, Figure 1c). For these six PAHs emission during coal separation was excluded because they didn’t show monthly extreme values. During the winter period they could be emitted in air by combusting biomass and coal, as they shown high content in that period. The densely populated region, with precipitation and poor air circulation contributes to this. Also, these six PAHs were in significantly higher concentration at the mouth of the river Spreča than on the two previous mentioned points. The reason for this is their very low presence in coal (Achtena and Hofmann, 2009). In the river, significant levels were detected in the river, down the lake presumably as a result of emission from the soda drinks chemical industry. In all measurements and calculations, monthly and yearly, the anthracene has been within the environmentally safe level. The other five PAHs, at the river mouth, often had value that a risky for the environment, as well as at the annual average, according to the environmental standards of EU.

The PAHs content in the lake showed the lower seasonal oscillations than in the river (Figure 1c). Of course, it is caused by long period of accumulation of water, mixing and homogenization of different monthly values for river. The concentration in March is a good indicator of the average value of the PAHs during the later winter period, and in September for the summer period. Ratio of PAHs concentration for March/September in the lake shows a clear seasonal variation, evident for most PAHs. There are several reasons for decreasing of PAHs content in the lake during the summer such as significant reduction of PAH emission in the air usually caused by coal using households, as well as stronger air circulation during that period. Also, more intense sunlight during the summer leads to faster degradation of PAHs (ATSDR, 1995).

The results from Figure 1d are the most important, because they are from the mouth of the river and sum up all the pollutants and consequences for the wider region. Regarding that, river Spreča has no good ecological status with regard to PAH concentration, which has been assumed.

CONCLUSIONS

Concentrations of PAHs often exceeded the maximum of allowed monthly value that specified in EU standards, related to good ecological status of the river (EU Directive, 2013).in The highest detected concentration was for naphthalene, but since is relatively quickly degradable, it is not serious threat for the environment. All PAHs showed seasonal variations and increased concentration during the winter. During the coal separation in mining area, the most emitted PAH is naphthalene, and slightly less fluoranthene. Among all tested PAHs, fluoranthene with its content and environmental impact presents the most serious ecological threat to the river Spreča. Naphthalene and fluoranthene come from multiple sources while the other PAHs mainly have the origin from the soda drinks chemical industrial zone, located in the part of the flow after the lake. Finally, it could be concluded that the river Spreča is contaminated with PAHs on the level that could be threat to living organisms in the river.

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Summary/Sažetak

Najvećim dijelom rijeka Spreča protiče kroz gusto naseljenu regiju Tuzlanskog kantona, a to je ujedno i područje rudarsko-industrijske zone. Ovaj rad prati sadržaj policikličnih aromatskih ugljikovodika (PAH) tokom kalendarske godine, u dijelu Spreče koji je izrazito izložen ovim polutantima. Sadržaj PAH-ova u rijeci je analiziran na tri lokacije: prije jezera Modrac, u jezeru i na ušću Spreče u rijeku Bosnu. Analiza PAH-ova obavljena je tehnikom HPLC uz fluorescentni detector, a nakon ekstrakcije tečno-tečno. Analizirano je osam PAH-ova, u skladu s preporukom direktive Evropske unije. Generalno, među analiziranim PAH-ovima, najveću koncentraciju je imao naftalen. Na ušću Spreče u Bosnu je nađena najveća koncentracija svih PAH-ova, osobito fluorantena. Izuzev naftalena, sadržaj svih ostalih PAH-ova je osjetno veći na ušću Spreče nego na dvije lokacije prije. Koncentracija svih PAH-ova značajno varira tokom sezona i najveća je u zimskom periodu. Visok nivo naftalena i fluorantena potiče iz zone rudnika uglja smještene prije jezera Modrac i koksno-hemijske industrije koja se nalazi u dijelu toka nakon Jezera. Ostali PAH-ovi najviše potiču iz koksno-hemijske industrije.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

15-18

Print ISSN: 0367-4444

Online ISSN: 2232-7266

UDC: ______________________

Original Scientific Paper

Antioxidant activity of fruits and vegetables commonly used in everyday diet in Bosnia and Herzegovina

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Article info
Received: 21/09/2017
Accepted: 10/11/2017

Keywords:
Fruits
Vegetables
Total Phenols
Total Flavonoids
Antioxidant Activity

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Abstract: Total phenols, flavonoids and their ratios, as well as antioxidant activity of selected fruits (banana, apple, plum, raspberry, strawberry, orange and peach) and vegetables (broccoli, onion, spinach, red cabbage, tomato, lettuce, leek and cauliflower) were determined by spectrophotometric methods. The antioxidant activity of the selected fruits and vegetables was evaluated by TEAC and FRAP methods. Results showed that total phenol content (mg GAE/g) in fruits was in the range from 0.38 to 3.30, while in vegetables it was in the range from 0.22 to 1.58. Total flavonoid content (mg GAE/g) in fruits was in the range from 0.21 to 2.20, while in vegetables it was in the range from 0.06 to 0.98. The highest ratio of total phenolic and flavonoid compounds was observed for strawberry and tomato, while the lowest one was for lettuce. Antioxidant activity by TEAC and FRAP methods showed the highest results for strawberries and red cabbage. Investigated fruits and vegetables consumed on a daily basis in the households in Bosnia and Herzegovina, can be considered as an exceptionally good source of natural phenols and flavonoids. Investigated fruits and vegetables are often used fresh in homemade dishes so that phenols and flavonoids are not destroyed by thermal processing and are therefore more available and more potent as antioxidants in vivo.

INTRODUCTION

Consumption of fruits and vegetables in large quantities has been considered crucial in human health prevention against numerous diseases - cancer, diabetes, neurodegenerative and cardiovascular diseases. Currently, the opinion is that protective properties of many foods come from low molecular antioxidants present in such foods which protect their structures from oxidative damage (Wang and Lin, 2000). Aforementioned risk-reducing effect for many diseases is not a result of the activity of an individual antioxidant like α-tocopherol, ascorbic acid or β-carotene, but could also be a result of antioxidant compounds not yet found, or of a synergy of several different antioxidants present in fruits and vegetables (Teow, Truong, McFeeters, et al., 2007). Research on macromolecules (DNA, nucleotides, proteins) being damaged by free radicals showed that diets enriched in additional amounts of fruits rich with β-carotene, tocopherols and ascorbic acid, had limited influence on inhibiting oxidation processes (Day, Seymour, Pitts, et al., 2009). However, research showed that consuming different sorts of fruits and vegetables contributes to lowering free radical processes, probably because they contain great amounts of non-vitamin antioxidants, such as polyphenols and anthocyanins (Zhishen, Mengcheng, Jianming, 1999; Lichtenthäler, Rodrigues, Maia, Papagiannopoulos, et al., 2005). Presence of phenolic compounds in food is of great importance because of the demonstrated antioxidant activity, prevention of cancerogenesis and mutagenesis (Re, Pellegrini, Proteggente, et al., 1999). Polyphenols have an important role in maintaining sensory properties of food during thermal processing. Knowledge of polyphenolic composition of the food and its change during storage and processing is essential in order to ensure a good food quality. Anthocyanins are responsible for red, blue and purple colors of many sorts of food, including fruits and vegetables, while flavonols and tannins are involved in the taste formation (astringent) and bitterness (Hangun-Balkir, McKenney, 2012). Everything aforementioned makes antioxidants an increasingly interesting research topic.

MATERIALS AND METHODS

Total phenols, flavonoids and their ratio, as well as the antioxidant activity of samples of the selected fruits (banana, apple, plum, raspberry, strawberry, orange and peach) and vegetables (broccoli, onion, spinach, red cabbage, tomato, lettuce, leek and cauliflower) were determined by spectrophotometric methods. The antioxidant activity of the selected fruits and vegetables was evaluated by TEAC and FRAP methods.

Sample preparation

Samples of the tested fruits and vegetables (500 mg each) were powdered in a mortar, homogenized by addition of ethanol (80% v/v), and then centrifuged (15000 rpm, 20 minutes) at temperature of 4 °C. Supernatants obtained by such processing were used for determination of phenols and flavonoids as well as of the antioxidant activity of the extracts.

Total phenolic content determination

Total phenols (TP) were determined spectrophotometrically using *Folin-Ciocalteu* reagent (Waterhouse, 2002). Total phenolic content in individual samples was determined by extrapolation from calibration curve obtained by measuring apsorption of gallic acid solutions of known concentrations (0 – 2.0 mg/mL) and was expressed in milligrams of gallic acid equivalent by gram of fresh weight of the sample (mg GAE/g).

Total flavonoid content determination

Total flavonoid content was determined by spectrophotometric method based on forming yellow flavonoid complex with aluminum (Willet, 2002). Absorbtion was measured at 420 nm. Quercetin was used as the standard. Total flavonoid content in the individual samples was determined by extrapolation from calibration curve obtained by measuring absorption of quercetin ethanolic solutions of known concentrations (0 – 30 mg/mL) and was expressed in milligrams of quercetin equivalent by gram of fresh weight of the sample (mg QuE/g).

Oxidation potential determination by TEAC method

TEAC test (Trolox Equivalent Antioxidant Capacity) is a method of the antioxidant activity determination which measures the ability of the sample to catch/neutralize $ABTS^{\cdot+}$ (2,2’-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid) radical action. $ABTS^{\cdot+}$ radical is generated directly into a stable form using potassium persulfate as an oxidation agent. Such formed radical absorbs at 732 nm, and after mixing with an antioxidant in the reactive medium, the absorbtion drop appears at that wavelength as a result of a reaction between the radical and the antioxidant. Trolox is usually used as the standard (watersoluble form of vitamin E) therefore the name of the method is TEAC (Trolox Equivalent Antioxidant Capacity), and the results are expressed as Trolox equivalents, or a Trolox solution concentration (mmol/mL) with the equivalent antioxidant activity as 1.0 mmol/mL solution of the tested compound (Prior and Schaich, 2005). The results of the samples testing are expressed by equation of calibration diagram as micromols of Trolox equivalent by gram of fresh weight of the sample (μmol Trolox/g).

Reduction potential determination by FRAP method

This method (Benzie and Strain, 1996) is based on reduction of colorless iron(III)-tripyridyltriazine (Fe^{3+} - TPTZ) complex into an intensively blue-colored fero form (Fe^{2+}). The antioxidant activity of the tested samples is determined by spectrophotometric measurement of absorption at 593 nm. The calibration curve for FRAP reagent is prepared by mixing acetate buffer (300 mmol/L, pH=3.6), TPTZ reagent (10 mmol/L in 40 mmol/L HCl) and $FeCl_3x6H_2O$ (20 mmol) in ratio 10:1:1. In each of the test tubes, 3 mL of FRAP reagent was measured and 0.1 mL of FeSO4x7H2O standard solution of 0.2-1 mmol/mL was added. Absorption was monitored at 593 nm compared to a blind test (3 mL of FRAP reagent and 0.1 mL of water). The concentration (mmol/mL) of Fe^{2+} in the sample was determined from the calibration curve equation and was converted to micromols of the exctract (μmol Fe^{2+}/g), representing the FRAP value.

RESULTS AND DISCUSSION

The total phenolic (TP) and total flavonoid (TF) contents, as well as their ratios in ethanolic extracts of the tested fruits and vegetables, were determined by spectrophotometry and the results are shown in Tables 1 and 2.

Table 1. The content of total phenols (TP) and flavonoids (TF) and their ratio (TP/TF) in fruit extracts

Fruit	TP (mg GAE/g)	TF (mg QuE/g)	TP/TF
Banana	0.38	0.21	1.727
Apple	0.48	0.39	1.237
Plum	3.20	**2.20**	1.457
Raspberry	2.28	1.02	2.236
Strawberry	**3.30**	0.97	**3.409**
Orange	1.26	0.76	1.658
Peach	0.38	0.21	1.818

Table 2. The content of total phenols (TP) and flavonoids (TF) and their ratio (TP/TF) in vegetable extracts

Vegetables	TP (mg GAE/g)	TF (mg QuE/g)	TP/TF
Broccoli	1.28	**0.98**	1.306
Onion	0.88	0.60	1.467
Spinach	0.72	0.32	2.250
Red cabbage	**1.58**	0.45	3.511
Tomato	0.30	0.06	**5.000**
Lettuce	0.14	0.36	0.389
Leek	0.22	0.75	0.293
Cauliflower	0.33	0.42	0.786

From the results shown in Tables 1 and 2, among the tested samples, strawberries and red cabbage are the richest source of total phenols, while plum and leek are the richest source of total flavonoids. Total phenolic content (mg GAE/g) in the fruits ranged from 0.38 to 3.30, while in the vegetables it ranged from 0.220 to 1.58 . Total flavonoid content (mg GAE/g) in the fruits ranged from 0.21 to 2.20, while in the vegetables it ranged from 0.06 to 0.98. The highest ratio of the total phenolic and flavonoid compounds was observed for strawberry and tomato, while the lowest was for lettuce.

TEAC and FRAP values of ethanolic extracts for the tested fruits and vegetables are given in Tables 3 and 4.

Table 3. TEAC and FRAP values of fruit extracts

Fruit	TEAC (µmol Trolox/g)	FRAP (µmol Fe^{2+}/g)
Banana	1.81	1.64
Apple	3.43	3.94
Plum	18.25	20.57
Raspberry	18.46	23.25
Strawberry	**25.90**	**33.50**
Orange	8.49	11.81
Peach	1.95	2.02

Table 4. TEAC and FRAP values of vegetable extracts.

Vegetables	TEAC (µmol Trolox/g)	FRAP (µmol Fe^{2+}/g)
Broccoli	6.48	8.33
Onion	5.32	3.69
Spinach	7.57	10.09
Red cabbage	**13.77**	**18.70**
Tomato	2.55	3.44
Lettuce	1.71	1.24
Leek	2.40	1.60
Cauliflower	2.95	2.59

According to the results presented in Table 3 (TEAC and FRAP for the ethanolic fruit extracts) the highest radical scavenging capacity by TEAC method was determined in the strawberry extract, while the lowest was in the banana extract. Also, Table 3 shows that the highest FRAP value, meaning also the best reduction ability, was observed in the strawberry extract, while the lowest was in the banana extract. Table 4 shows that the highest FRAP and TEAC values was observed in the red cabbage extract, while the lowest was in the lettuce extract.

The research has shown that many sorts of fruits contain higher amounts of phenolic compounds. The research on red fruits showed that an average phenolic content was in the range between 1.9 and 7.2 mg per 1 g of fresh fruit (Jakobek, Šeruga, Novak, et al., 2007). However, our research has shown that strawberry, plum and raspberry contain higher amounts of phenols and flavonoids than citrus fruits. Also, some research showed that different sorts of vegetables contain lower amounts of total phenols and flavonoids than citruses (Katalinic, Miloš, Kulisic et al., 2006). Our results support these findings.

The research revealed that TEAC and FRAP tests show differences in the antiradical effect which depends on the phenol content in fruits and vegetables; consequently, the higher total phenolic content, the stronger the antiradical effect it is (Katalinic, Miloš, Kulisic et al., 2006). Our results are in good accordance with other studies.

Different investigations have shown a relationship between the total phenolic contents and the antioxidative activity of the fruits, plants and vegetables (Deighton, Brennan, Finn, et al., 2000; Abdille, Singh, Jayaprakasha, et al., 2005; Vinson, Hao, Su, et al., 1998). The chemical composition and chemical components in plant extracts are important factors governing the efficacy of natural antioxidants. The antioxidant activity of an extract could not be explained on the basis of their phenolic content, which also requires their chemical characterization (Halliwell, Gutteridge, 2015).

CONCLUSIONS

For the purpose of the bioactive content determination in the tested fruits and vegetables, the total phenolic and flavonoid contents were determined using Folin-Ciocalteau reagent, and the antioxidant capacity was determined by FRAP and TEAC methods. Based on the obtained results, the conclusions can be drawn as follows.

Investigated fruits and vegetables, commonly used in everyday consumption in Bosnia and Herzegovina, can be considered as an exceptionally good source of natural phenols and flavonoids.

Investigated fruits and vegetables are often used fresh in homemade dishes; that is why phenols and flavonoids are not destroyed by thermal processing and are therefore more available and more potent as antioxidants *in vivo*.

Therefore, in order to ensure optimal nutrition, health, and well-being, consumers should obtain their nutrients, antioxidants, bioactive compounds, or phytochemicals from their balanced diet based on a wide variety of fruits, vegetables, and other plant foods.

Further research on the health benefits of the investigated plant material is necessary.

Acknowledgement

This work was supported by the Federal Ministry of Education and Science in Bosnia and Herzegovina and was carried out within the project „Investigation of the antioxidative status of different plants used in everyday nutrition and obtained from Bosnian markets“ (Grant no. 0101-7552-17/15, dated 14.12.2015).

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Summary/Sažetak

Ukupni fenoli, flavonoidi i njihov odnos, te antioksidativna aktivnost uzoraka odabranog voća (banana, jabuka, šljiva, malina, jagoda, naranča i breskva) i povrća (brokula, crveni luk, špinat, crveni kupus, paradajz, salata, prasa i karfiol) su određivani spektrofotometrijskim metodama. Antioksidativne aktivnosti odabranog voća i povrća određene su TEAC i FRAP metodama. Rezultati su pokazali da se sadržaj ukupnih fenola (mg GAE/g) u voću kreće u rasponu od 0.38 – 3.30, a u povrću u rasponu od: 0.22 – 1.58. Sadržaj ukupnih flavonoida (mg GAE/g) u voću kreće se u rasponu od: 0.21 – 2.20, a u povrću u rasponu od: 0.06 – 0.98. Najveći odnos ukupnih fenola i ukupnih flavonoida bio je za jagodu i paradajz, a najmanji za salatu. Za dio ispitivanja koji se odnosio na antioksidativnu aktivnost TEAC i FRAP metodom je utvrđeno da su najveću antioksidativnu aktivnost pokazale jagode i crveni kupus. Može se konstatovati da bi se ispitivane vrste voća i povrća, koje se najčešće konzumiraju u bosanskohercegovačkim domaćinstvima, mogu smatrati izuzetno dobrim izvorom prirodnih fenola i flavonoida.Ispitivano voće i povrće se često upotrebljava svježe u domaćim receptima i radi toga se fenoli i flavonoidi ne uništavaju termičkom obradom i stoga su dostupniji i potentniji kao antioksidanti in vivo.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

19-24

Print ISSN: 0367-4444

UDC: ___________________________

Online ISSN: 2232-7266

Original Scientific Article

Impact of the use of alternative fuels in cement production on emission of harmful pollutants

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Article info
Received: 03/11/2017
Accepted: 19/12/2017

Keywords:
Cement production
Alternative fuels
Pollutant emission

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Abstract: Cement industry is a huge energy consumer, and at the same time a huge greenhouse gases generator – approx. 7% seven percent of the total global CO_2 emission in 2010 (Deja, Uliasz-Bochenczyk and Mokrzycki, 2010). High fuel consumption weakens the economy of the process and the acceptance of cement’s market price, so many cement companies have turned to research to use alternative fuels in production in order to reduce both the production costs and greenhouse gas emissions. However, due to the fact that this process is basically a waste incineration, the cement industry is constantly under pressure from the general public and environmental associations, so the tests are constantly being carried out to determine emissions of harmful pollutants during the use of various alternative materials in the cement production process. This paper investigates the impact of the use of some alternative fuels (scrap tires, used oils, waste from the coal industry, waste from the tanneries and leather industry, waste from oil refining – filter cake and oily wipes & rags) in an amount of 10% (energy) on emission of harmful pollutants in cement production. The results obtained by these tests show that, although there are evident differences in the content of individual pollutants for different types of alternative fuels, total emissions stays within statutory limits.

INTRODUCTION

In today's world, cement factories are considered successful, if they have managed to get 70% energy needs from alternative sources. Some plants in the world in their plans already predict a one-hundred-percent replacement of fossil fuels by alternative fuels. FCL (Cement Factory Lukavac) has a relatively new production line that is designed so that it can use alternative fuels with continuous monitoring and control of all production and environmental parameters. The results of the previous measurements for selected pollutants, the emissions of which have been produced in the process of cement production and determined by strict EU standards, have shown values within the permitted limits and for some pollutants the obtained values have been lower than the prescribed ones. This kind of controlled combustion with continuous monitoring of conditions and product control can only be achieved with efficient plants – what's in the FCL embedded during the building a new furnace line and mills of raw meal, released in mid-2009.

Theory

The cement industry, due to alkaline environment, high temperature and long processing time, has the potential to adequately handle large amounts of fuels, including waste, by co-combustion (Rahman, Rasul, Khan and Sharma, 2013). Fuel incineration is carried out both in the primary air stream which carries fuel into the furnace and in the heated secondary air stream where the raw material is heated up to 1450°C, so the flue gas temperature reaches 2000°C. The gases are maintained at this temperature from5 to 7 seconds, that is enough to break all organic energy constituents while the inorganic residue of the incineration, or ashes, become part of the clinker. Along with the main components of ash (silicon oxide, aluminum, iron and calcium salts), heavy metals are also incorporated into clinker.

Thermal treatment of waste or its use as fuel in the Federation of Bosnia and Herzegovina must be carried out with full respect to emission limit values in accordance with the applicable ordinance (Pravilnik o uvjetima za rad postrojenja za spaljivanje otpada, 2005), that can be achieved by installation of equipment for control of the emission of flue gases. The use of alternative fuels in the cement industry began in the 1970s and since then, the number of cement factories in the world, using alternative fuels, is in constant growth.

From the production costs of cement, 30-40% goes to the energy consumption (Radwan, 2012). With the aim of lowering these energy costs, alternative fuels were introduced in the cement industry, so today more than 200 cement factories worldwide use alternative fuels. In Germany, according to Verein Deutscher Zementwerke (2017), the share of fossil fuels (particularly lignite and coal) has continuously dropped from 74.3% in 2000 to currently 35.2%. Heat energy requirements are today primarily covered using alternative fuels such as treated commercial and municipal waste, scrap tires, used oil or sewage sludge (64.8% in 2016), while the high energy efficiency of the cement production process was not affected by this substitution.

It has been shown that combustion of waste as alternative fuels in cement plants preserves natural resources and reduces emissions. In comparison of the environmental impact of this technique and the environmental impact of the landfill, the landfill has a much larger emission and impact on the greenhouse effect due to the conversion of the organic part of the waste to the gases. Approximately half of degradable organic waste turns into CO_2, and the remainder into methane – CH_4, whose potential impact on global warming for the one hundred- year time horizon is 28 (IPCC, 2013). If the organic material from the waste is used in the cement industry, indirect decrease of potential methane emissions from the landfill is achieved in the incineration process.

Regarding scrap tires, or TDF (tire-derived fuel, which is composed of shredded scrap tires), numerous studies (e.g. Reisman, 1997; Baird, Horrocks, Kirton and Woodbridge, 2008; Cheema and Badshah, 2013 or The Pembina Institute and Environmental Defence, 2014) have shown that their use as an alternative fuels in cement kilns either reduces or does not significantly affect emission of most pollutants. For example, one extensive survey involving thirty-one cement plants (Richards, Goshaw, Speer and Holder, 2008) demonstrated that "Dioxin-furan emission test results indicated that kilns firing TDF had emissions approximately one-third of those kilns firing conventional fuels – this difference was statistically significant. Emissions of particulate matter (PM) from TDF-firing kilns were 35% less than the levels reported for kilns firing conventional fuels (not statistically significant due to the low PM emissions reported for essentially all cement kilns). Nitrogen oxides, most metals, and sulfur dioxide emissions from TDF-firing kilns also exhibited lower levels than those from conventional fuel kilns. The emission values for carbon monoxide and total hydrocarbons were slightly higher in TDF versus non-TDF firing kilns. However, none of the differences in the emission data sets between TDF versus non-TDF firing kilns for sulfur dioxide, nitrogen oxides, total hydrocarbons, carbon monoxide, and metals were statistically significant", which mostly confirms mentioned results of earlier research, however, it should be noted that the emissions are highly dependent on the technological process of a particular cement plant, which was a main motive for detailed research on a specific process: production of cement in FCL (Cement Factory Lukavac).

EXPERIMENTAL

In the FCL, for the production of heat and clinkering of meal various fuels are used: heavy fuel oil – SNS (with sulfur content up to 1%), domestic brown coal (with sulfur content of 2-3%) and brown coal imported from the Czech Republic (with sulfur content up to 1%). At present, a combination of domestic coal and imported coal is used in the ratio of 30:70 due to the reduction of SO_2 emissions during combustion in a rotating kiln. In the absence of imported brown coal, medium fuel oil with sulfur content below 1% (FCL, 2007) is used in the combination of fuels. The first attempts to use alternative fuels in the FCL started in 2010 when the first trial run was carried out, and since then several tests have been conducted alongside activities related to obtaining approval from relevant institutions for the use of alternative fuels in the cement production process. Therefore, it can be said that today FCL is capable of using various alternative fuels in the production process: scrap tires, used oils, waste from the leather and textile industry, filter cakes and oily waste. For conducting the testing of the impact of the use of alternative fuels on pollutants emissions in the period of 5 days, measurements of the pollutant concentration in flue gas from the rotary kiln in the Lukavac cement factory were performed.

During the first series of measurements, the rotary kiln worked with a normal working regime, i.e. with the only coal used as a fuel. Subsequently, in the rotary kiln, alternative fuels (scrap tires, used oils, waste from the leather industry, waste from the textile industry, waste from oil refinery – filter cake and oily wipes & rags) were used in a percentage of 10% (energy) of coal. Waste from the leather and textile industry was acquired from the company Prevent-Visoko, and used oil and oily wipes & rags was collected by the Kemokop from Tuzla, the company authorized to manage such types of waste, while the filter cake was purchased from the Oil Refinery Modriča.

The calorific values of liquid fuels were determined according to the ASTM D240-09, using the IKA C 5000 bomb calorimeter. For solid fuels, calorific values were determined according to the BAS ISO 1928:2010, using the same bomb calorimeter.

Since it was necessary to measure the mean concentration of flue gas, the sampling was carried out in several points per cross-section of the flue duct. Sampling was performed isokinetically, using Tecora G4 isokinetic automatic sampler, according to BAS ISO 9096, BAS EN 13284-

1:2006, BAS EN 1948-1 and BAS EN 1911:2011. The sampling site was on the chimney of the rotary kiln (Figure 1).

Figure 1: Sampling site at the chimney

Determination of dust concentration was performed according to BAS EN 13284-1:2006. Using the Horiba Model PG-250 multi-gas portable analyzer, the concentration of SO_2, CO and NOx were determined, according to BAS ISO 7935:2000, BAS EN 15058:2008 and BAS EN 14792:2007. Concentrations of HCl and HF were determined using Varian Cary 50 UV-Vis spectrophotometer, according to BAS EN 1911:2011 and BAS ISO 15713:2008. AA240FS fast sequential atomic absorption spectrometer was used for determination of Hg and other heavy metals, according to APHA Standard Methods 3111B and 3112 and also to BAS EN ISO 15586:2005. Procedure of determination of dioxins and furans was performed according to BAS EN 1948-2:2007 and BAS EN 1948-3:2007. Total organic carbon content was determined according to BS EN 12619:2013. The analysis were performed in cooperation with the laboratory of the company Dvokut from Sarajevo, accredited according to BAS EN ISO/IEC 17025:2006.

Results and Discussion

In order to determine the mixing ratio (90% of coal with 10% of alternative fuel, in an energetic sense), the calorific values of coal and alternative fuels were determined. These values are presented in Table 1.

Table 1: Calorific values of fuels

Fuel	**Calorific value [kJ/kg]**
Coal	23.330
Scrap tires	24.000
Waste from tanneries/leather industry	11.688
Waste from textile industry	22.000
Used oil	40.000
Waste from oil refinery – filter cake	23.000
Oily wipes & rags	22.000

Based on determined calorific values, the amounts of individual fuels were calculated and the operating modes, during the measurement is set, as shown in Table 2

Table 2: The amount of individual fuels in the mixture

Fuel	**15.07. 12:30-13:30**	**15.07. 14:00-15:00**	**18.07. 9:30-11:00**	**18.07. 12:00-13:30**	**18.07. 14:00-15:00**	**19.07. 9:00-11:00**	**19.07. 11:00-12:45**
				[kg/h]			
Coal	11500	10000	10000	10000	10000	10000	10000
Scrap tires		1050					
Waste from tanneries/leather industry				1950			
Waste from textile industry					900		
Used oil			585				
Waste from oil refinery – filter cake						1000	
Oily wipes & rags							950

The results of measurements given in the Table 3 are treated as the average values of all measured values of emissions in the period of measurement. All emission measurement results are presented in the form of measured values corrected to normal conditions and dry gas and reference content $O_{2\ REF}$ = 10%.

Comparing emission results regarding the use of coal fuels and a mixture of 90% coal and 10% (energy) of alternative fuels, it can be stated that concentrations of pollutants generated by the incineration of all alternative fuels used in these tests lie below the limit values prescribed by the applicable ordinance (Pravilnik o uvjetima za rad postrojenja za spaljivanje otpada, 2005 and 2012). NO_x concentration while waste is used as an alternative fuel is reduced, except in the case of waste from the leather industry. CO emission is increasing, with the largest increase of 300% seen when waste textile and oily wipes & rags are used as fuel, that could be attributable to the fact that this waste has a non-homogeneous composition and consists of different types of textiles (natural fibers, cotton, silk, etc., synthetic fibers, polyesters, viscose, etc.) and require different conditions for full combustion. The emission of dioxins and furans is rather low regardless of used fuel or fuel mixture, most probably due to high temperature and retention time of several seconds.

Table 3: The emission of pollutants and comparison with emission limit values (ELV)

Parameter	**ELV [mg/m^3]**	**The results normalized to dry gas, normal conditions and $O_{2,REF}$ [mg/Nm^3]**						
		Coal	**Coal + tires**	**Coal + used oil**	**Coal + leather waste**	**Coal + textile waste**	**Coal + filter cake**	**Coal + oily rags**
Dust, total	30*	3.2	6.1	7.0	5.8	6.6	10.8	10.9
SO_2**	50*	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CO	-***	60.2	71.6	74.8	82.4	106.5	106.5	212.6
NO_X	500*	420.0	408.1	394.3	464	344.6	361.7	377.6
HCl	10*	2.9	1.8	2.5	2.4	1.6	3.8	4.2
HF	1*	-	-	-	-			
Cd +Tl	0.05*	$12.6\cdot10^{-4}$	$11.9\cdot10^{-4}$	$6.9\cdot10^{-4}$	$10.3\cdot10^{-4}$	$7.8\cdot10^{-4}$	$21.5\cdot10^{-4}$	$36.0\cdot10^{-4}$
Hg	0.05*	$7.10\cdot10^{-5}$	$4.32\cdot10^{-5}$	$7.7\cdot10^{-5}$	$5.05\cdot10^{-5}$	$5.86\cdot10^{-5}$	$7.33\cdot10^{-5}$	$9.59\cdot10^{-5}$
Sb + As + Pb + Cr + Co + Cu + Mn + Ni + V	0.5*	$6.3\cdot10^{-4}$	$12.3\cdot10^{-4}$	$11.0\cdot10^{-4}$	$14.7\cdot10^{-4}$	$16.4\cdot10^{-4}$	$20.2\cdot10^{-4}$	$28.9\cdot10^{-4}$
Dioxins and furans	0.1*	0.019	0.011	0.017	0.0148	0.0628	0.0093	0.01
TOC	10*	4.81	5.71	4.79	6.60	4.68	9.37	8.14

* ELVs according to applicable ordinance (Pravilnik o uvjetima za rad postrojenja za spaljivanje otpada, 2005 and 2012)

** SO_2 was measured, and results were negative, which means that the SO_2 has been present in samples, but the lower calibration point of the instrument lied higher than measured value.

*** According to above mentioned Ordinance (Annex II, point 1.3), also according to the ☐Directive 2010/75/EU (Annex VI, Part II, point 2.4), "The competent authority may set emission limit values for CO", but still it wasn't.

Emissions are even lower when the mixtures of coal and alternative fuels are used, which is something that may be the subject of further research, but such a phenomenon could be the consequence of slight lowering the temperature during the combustion of the alternative fuels, as lower temperatures are believed to prevent the post combustion catalytic formation of dioxins and furans (Karstensen, 2006). An exception is the waste from the textile industry where increased concentration of dioxin (0.0628 mg/Nm^3) was recorded, which is attributable to the composition of the fabric and the fact that many of them contain plastic compounds.

The metal emission is increased but it is necessary to point out that all values lie below the emission limit value of 0.5 mg/Nm^3. Used alternative fuels contain metals in its composition so such an increase was expected. HCl and Cd + Tl emissions are reduced when alternative fuels are used. The exception is waste from the oil industry and the oily wipes & rags, which can be attributed to the character of this waste and the fact that it comes from the environment in which it could come into contact with the compounds (chlorine, heavy metals) which can cause the rise of emission of these compounds in the rotating kiln. Hg emission is reduced by the use of wastes from textile, leather and used oil while an increase has been recorded when scrap tires, waste from oil refining - filter cake and oily wipes & rags are used, what is expected with regard to the chemical composition of given alternative fuels.

The TOC concentration increases, except when scrap tires and waste from the textile industry are used as alternative fuels, where the emissions are approximately equal to emissions when using solely conventional fuel - coal. Total dust emissions are increasing

CONCLUSION

The basic requirement for co-combusting alternative fuels in the cement industry is that this process has no adverse impact on the environment or on the quality and properties of the main product.

The aim of the research described in this paper was to determine the emission level when burning alternative fuels in a rotary kiln in comparison to rotary kiln operation when only coal is used as fuel.

The results of the measurement showed that the values of all pollutants observed during the use of various alternative fuels in an amount of 10% (energy) were lower than the emission limit values, while the operator at the same time achieved economic benefits in terms of reducing the fuel costs.

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Summary/Sažetak

Cementna industrija je veliki potrošač energije i u isto vrijeme veliki generator stakleničkih plinova – oko 7% ukupne globalne CO_2 emisije u 2010 (Deja, Uliasz-Bochenczyk i Mokrzycki, 2010). Velika potrošnja goriva slabi ekonomiku procesa i cjenovnu prihvatljivost cementa na tržištu, tako da su se mnoge cementare okrenule istraživanjima sa ciljem upotrebe alternativnih goriva u proizvodnji kako bi se pojeftinio proizvodni proces i smanjila emisija stakleničkih plinova. Ipak, zbog činjenice da se u osnovi radi o spaljivanju otpada, cementare su stalno pod pritiskom javnosti i ekoloških udruga, stalno se rade ispitivanja sa ciljem određivanja emisije štetnih polutanata pri korištenju različitih alternativnih materijala u procesu proizvodnje cementa. U ovom radu je vršeno ispitivanje utjecaja korištenja nekih alternativnih goriva (auto gume, rabljena ulja, otpad iz kožarske industrije, otpad iz tekstilne industrije, otpad iz prerade nafte – filter kolač i zauljene krpe) u količini od 10% (energetski) na emisiju štetnih polutanata pri proizvodnji cementa. Rezultati dobijeni ovim ispitivanjima pokazuju da, iako postoje evidentne razlike u sadržaju pojedinih polutanata za različite vrste alternativnih goriva, ukupne emisije ostaju unutar zakonom dozvoljenih vrijednosti.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

25-30

Print ISSN: 0367-4444

Online ISSN: 2232-7266

UDC: ______________________

Original Scientific Paper

Determination of phenolic content and antioxidant properties of methanolic extracts from *Viscum album* ssp. *album* Beck.

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Article info
Received: 08/11/2017
Accepted: 13/12/2017

Keywords:
Viscum album
Phenolic
Antioxidant activity
DPPH
ABTS
FRAP

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Abstract: Content of phenolic compounds and antioxidant properties of methanolic extracts from *Viscum album* ssp. *album* Beck. leaves and stems was determined. Mistletoe was collected from four different hosts (*Betula* L., *Tilia cordata* Mill., *Robina pseudoacacia* L, and *Salix alba* L.). Folin-Ciocalteu method, $AlCl_3$ method, method with Arnow reagent, and acid-butanol assay were used for determination of total phenols, flavonoids, phenolic acids and proanthocyanidins respectively. Antioxidant properties of the extracts were investigated with DPPH, ABTS and FRAP method. Total phenols were in range 7.02-13.52 mg GAE/g DW, flavonoids 2.29-5.05 mg RE/g DW, phenolic acids 0.62–2.84 mg caffeic CAE/g DW and proanthocyanidins 0.63 -4.83 mg LCE/g DW. Content of flavonoids and proanthocyanidins was higher in leaves than the stems. The highest antioxidant activity in leaves (68.93-86.89 µmol Trolox equivalents/g DW) and in stems (67.28-81.72 µmol Trolox equivalents/g DW) with DPPH, ABTS and FRAP method had mistletoe collected from *Robina pseudoacacia* L. Also, high correlation was obtained between total phenols, phenolic acids and proanthocyanidins content and antioxidant activity.

INTRODUCTION

Viscum album L. (Loranthaceae) also known as European mistletoe or Common mistletoe is an evergreen semi-parasitic plants which grows on various host trees and shrubs. In Bosnia, the plant is represented by three subspecies: ssp. *album* Beck, ssp. *abietis* (Wiesb.) Abromeit. and ssp. *austriacum* (Wiesb.) Vollmann and can be found on approximately 46 different trees. The most abundant is *Viscum album* ssp. *album* Beck which has been identified on 42 leafy tree and shrubs in Bosnia (Treštić, 2015). Different pharmacological effects of *V. album* has been reported such as: antioxidant and antidiabetic (Orhan, Aslan, Sendogdu *et al.*, 2005; Gray and Flatt, 1999), antiepileptic, antipsychotic and sedative (Gupta, Kazmi, Afzal, *et al.*, 2012), vasodilator and antihypertensive properties (Tenorio-Lopez, del Valle, Gonzalez, *et al.*, 2005; Ofem, Eno, Imoru, *et al.*, 2007). Also, some studies cover immunostimulant, antimutagenic and anticancer effects of *V. album* samples (Yesilada, Deliorman, Ergun, *et al.*, 1998; Hong and Lyu, 2012; Sabova, Pilatova, Szilagy, *et al.*, 2010; Siegle, Fritz, MacClellan, 2001).The main bioactive compounds in *V. album* are polysaccharides, phenylpropanes, lecitins, viscotoxins, alkaloids, flavonoids, caffeic and other acids (Ergun and Deliorman, 1995). Phytochemical composition depends on host tree and as the main antioxidant flavonoids and phenolic acids are reported (Luczkiewich, Cisowski, Kaiser, *et al.*, 2001). In order to estimate antioxidant activity of plant extracts different methods were used: TEAC (Trolox equivalent capacity), FRAP (ferric-reducing ability), TRAP (total radical trapping capacity, ORAC (oxygen radical absorbance capacity) (Wu, *et al.*, 2004).

In this work, for the first time, we investigated total content of phenols, flavonoids, phenolic acids and proanthocyanidins in *V. album* ssp. *album* Beck. leaves and stems collected from several different hosts. Antioxidant activity of the extracts was determined with three methods: DPPH, ABTS and FRAP using Trolox as a standard. Correlations between investigated compounds and antioxidant activity in relation to their location (leaves and stems) are also reported.

EXPERIMENTAL

All chemicals used in this work were highest purity grade obtained from Sigma-Aldrich Chemical Company (Germany).

Plant material

Leaves and stems were collected from four different hosts in area of Sarajevo at localities Vraca and Miljevići in November 2015. According to the host tree, plant material was marked as follows: VAR *(Robina pseudoacacia*), VAT (*Tilia cordata*), VAB (*Betula* L.), and VAS (*Salix alba*). The plant material was dried in a ventilated place at room temperature and stored in paper bags until use. A voucher specimens of the plants were deposited at herbarium of Department of Forest Ecology at Faculty of Forestry.

Extraction

Powdered dry sample of leaves or stems (0.5 g) was extracted with 80% aqueous methanol (2 x 12 mL) during 30 minutes in an ultrasonic bath (Elmasonic S 60H). The mixture was centrifuged for 15 min at 3000 rpm (Centric 322 B, Techtnica). Obtained supernatants were combined, filtrated and volume of the extract was adjusted with extraction mixture up to 25 mL. Extracts were stored at -20ºC until analysis.

Determination of total phenols

Total phenolic content was determined with Folin-Ciocalteu method (Singelton, Orthofer, Lamuela-Raventos, 1974). Briefly, 0.1 mL of diluted sample was mixed with 7.9 ml of distillated water and 0.5 mL of Folin-Ciocalteu reagent was added. Approximately, after 5 minutes, 20% Na_2CO_3 (1.5 mL) was added to the reaction mixture, and it was left to stand for 30 minutes in a water bath at 40ºC. Blank was prepared by using distillated water instead extract. Absorbance of the colored product was measured at 765 nm and a calibration curve was prepared with gallic acid as a standard. Final results are expressed as mg gallic acid equivalents (GAE) per gram of dry sample. All spectrophotometric measurements were done with Shimadzu UV-mini 1240 spectrophotometer.

Determination of total flavonoids

Total flavonoids were determined with $AlCl_3$ method (Quettier, Gressier, Vasseur, *et al.*, 2000) using rutin as a standard. Equal volumes of extracts and reagent were mixed and left to stand at room temperature for 1 hour, and absorbance of the colored product was measured at 415 nm against the blank. Sample blank was also used in the analysis. Final results are expressed as mg rutin equivalents (RE) per gram of dry sample.

Determination of total phenolic acids

Quantification of total phenolic acid was done with Arnow reagent (Gawlic-Dziki, 2012). Briefly, 1 mL of diluted sample was mixed with 5 mL of water, 1 mL HCl (0.5 M), 1 mL of Arnow reagent and 1 mL of NaOH (1 M). Calibration curve with caffeic acid standards was established. Absorbance was measured at 490 nm, and the results are expressed as mg caffeic acid equivalents (CAE) per gram of dry sample.

Determination of total proanthocyanidins

Proanthocyanidins were determined by butanol-HCl assay (Hagerman, Harvey-Mueller, Makkar, 2000b). 0.5 mL of diluted extracts was mixed with 3.0 mL of the butanol-HCL reagent (butanol-HCl 95:5 *v/v)* and 0.1 ml of ferric reagent (2% ferric ammonium sulfate in 2 M HCl). Samples were heated at boiling water bath for 60 minutes. Absorbance was measure before and after heating at 550 nm against blank. Results are expressed as mg leucocyanidin equivalents (LCE) using specific absorbance of leucocyanidin 460.

Determination of antioxidant capacity

DPPH assay

Antioxidant activity of the extracts was measured by the method of Brand-Williams, Cuvelier, Berset, (1995) and Thaipong, Boonprakob, Crosby, *et al.* (2006). The extracts were dissolved in methanol while DPPH stock solution (0.094 M) was prepared in methanol on a daily basis and diluted to absorbance of 1.1±0.02 at 515 nm. Aliquots of extracts (0.1 mL) were mixed with 1.9 mL of DPPH solution and left at room temperate in the dark for 30 minutes. Standard solutions of Trolox were used for preparation of a calibration curve. Final results are expresses as µmol of Trolox equivalents (TE) per gram of dry sample.

ABTS assay

To determine ABTS assay, the method of Ree, Pellegrini, Proteggente, *et al.* (1999) modified by Thaipong, *et al.* (2006) was used. In brief, stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate were mixed in equal volumes and left to stand in the dark for 12-16 hours in order to obtain ABTS radical cation solution ($ABTS^{\bullet+}$). An aliquot of freshly prepared $ABTS^{\bullet+}$ solution was diluted with methanol to obtain absorbance of 1.1 ±0.02 units at 734 nm. Plant extracts (0.1 mL) were allowed to react with 1.9 mL of working $ABTS^{\bullet+}$ solution for 6 minutes after what the reduction in absorbance was measured. Standard solutions of Trolox were used to prepare a calibration curve, and the results are expressed as µmol of Trolox (TE) per gram of dry sample.

FRAP assay

Ferric reducing antioxidant power (FRAP) was determined by method of Benzie and Strain (1999). FRAP reagent was prepared by mixing 300 mM acetate buffer, pH= 3.6; 10 mM TPTZ (2,4,6-tripiridil-s-triazine) in 40 mM HCl acid and 20 mM $FeCl_3$ in the ratio 10:1:1.

Obtained solution was heated at 37ºC for 30 minutes in a water bath. Plant extracts (0.1 mL) were mixed with 1.9 mL of working FRAP solution and left in the dark for additional 30 minutes. Absorbance of the formed blue complex was measured at 593 nm against a blank. Standard solutions of Trolox were used to prepare a calibration curve, and the results are expressed as µmol of Trolox (TE) per gram of dry sample.

RESULTS AND DISCUSSION

Quantitative content of investigated bioactive compounds in leaves and stems of mistletoe samples hosted by different trees: *Robina pseudoacacia (VAR)*, *Tilia cordata (VAT)*, *Betula* L. (VAB), *Salix alba (*VAS) are presented in Table 1. In leaves, total phenols were in range 8.75-13.52 mg GAE/g DW, total flavonoids 3.34-5.05 mg RE/g DW, total phenolic acids 1.19-2.39 mg CAE/g DW, and total proanthocyanidins 2.29-4.83 mg LCE/g DW. In stems, total phenols were in range 7.02-13.51 mg GAE/g DW, total flavonoids 2.29-2.84 mg RE/g DW, total phenolic acids 0.62-2.84 mg CAE/g DW, and total proanthocyanidins 0.63-1.19 mg LCE/g DW. Generally, higher content of flavonoids and proanthocyanidins was found for leaves extracts compared with stems for all samples. Mistletoe leaves hosted by *Salix alba* (VAS) had higher content of all investigated compounds than the stems. However, stems of mistletoe from VAT and VAB were richer in the content of total phenols and total phenolic acids than their leaves. The highest content of total phenols (13.52 mg GAE/g DW), total flavonoids (5.05 mg RE/g DW) and total phenolic acids (2.23 mg CAE/g DW) was found in mistletoe leaves from VAR, while the lowest levels of phenols (8.75 mg GAE/g DW), flavonoids (3.34 mg RE/g DW) and phenolic acids (1.19 mg CAE/g DW) was determined in mistletoe leaves from VAS. The highest content of proanthocyanidins was found in mistletoe leaves fromVAB while the lowest was in mistletoe leaves from VAR.

The mistletoe stems extracts hosted by *Salix alba* (VAS) had the lowest content of all investigated compounds while the highest content was found in mistletoe stems hosted by VAR for phenols (13.51 mg GAE/g DW), phenolic acids (2.84 mg CAE/g DW) and proanthocyanidins (1.19 mg LCE/g DW). Content of flavonoids in mistletoe stems from VAR and VAT were very similar 2.82 and 2.84 mg RE/g DW respectively.

Vicaş, Rugina, Leopold, *et al.* (2011) pointed that influence of the host tree may have a key role in the phenolic composition of mistletoe leaves or stems. Orhan, Senol, Hosbas, *et al.* (2014) investigated *V. album* spp *album* hosted by different trees and found that *V. album* from *Robina pseudoacacia* L. had higher content of flavonoids (5.53 ±0.74 mg QE/g extract) than *V.album* from *Salix sp.* (4.66 ±0.73 mg QE/g extract).

Table 1: Total phenols (TP), total flavonoids (TF), total phenolic acids (TPA), total proanthocyanidins (TPC) in leaves (l) and stems (s) of mistletoe

Samples	**TP** (mgGAE/g)	**TF** (mgRE/g)	**TPA** (mgCAE/g)	**TPC** (mgLCE/g)
		Leaves		
VAR(l)	13.52±0.01	5.05±0.02	2.39±0.1	2.29±0.2
VAT(l)	10.34±0.01	4.56±0.05	1.31±0.19	4.11±0.23
VAB(l)	9.28±0.01	3.44±0.02	1.62±0.01	4.83±0.16
VAS(l)	8.75±0.006	3.34±0.01	1.19±0.02	4.78±0.07
		Stems		
VAR(s)	13.51±0.02	2.82±0.02	2.84±0.002	1.19±0.2
VAT(s)	11.32±0.01	2.84±0.02	1.59±0.003	1±0.32
VAB(s)	11.11±0.01	2.6±0.003	1.96±0.001	0.83±0.13
VAS(s)	7.02±0.007	2.29±0.02	0.62±0.005	0.63±0.03

Mistletoe from VAR-*Robina pseudoacacia*; VAT- *Tilia cordata*; VAB-*Betula* L.; VAS- *Salix alba*

On the other hand, the content of phenols was higher in plant hosted by *Salix sp.* (29.41 ±1.45 mg GAE/g extract) than *R. pseudoacacia* (27.08 ±1.87 mg GAE/ g extract). As it was observed by Vicaş, Rugina, Socaciu (2011) differences in phenolic contents highly depends on harvesting seasons and generally they are higher for samples harvested in spring than in autumn. The same authors found that leaves of mistletoe are richer in phenolic contents (total phenols and phenolic acids) than the stems which is similar to our results. (Vicaş, Rugina, Leopold, *et al.*, 2011).

Antioxidant activity

Antioxidant activity of mistletoe leaves and stems extracts were investigated by three methods: DPPH, ABTS and FRAP method. The results are presented in Table 2.

Table 2. Antioxidant activity in mistletoe leaves (l) and stems (s) determined with DPPH, ABTS and FRAP method.

	DPPH (µmolTE/g)	**ABTS** (µmolTE/g)	**FRAP** (µmolTE/g)
		Leaves	
VAR(l)	68.93±0.12	72.21±0.10	86.89±0.60
VAT(l)	34.01±0.86	43.39±0.11	54.39±0.51
VAB(l)	32.12±0.27	41.20±0.14	48.13±0.18
VAS(l)	31.25±0.19	43.79±0.31	42.78±0.24
		Stems	
VAR(s)	67.28±0.12	67.28±0.42	81.72±0.11
VAT(s)	42.41±0.29	51.59±0.41	67.71±0.44
VAB(s)	45.00±0.20	46.92±0.46	59.12±0.15
VAS(s)	30.13±0.21	36.86±0.26	38.04±0.34

Mistletoe from VAR-*Robina pseudoacacia*; VAT- *Tilia cordata*; VAB-*Betula* L.; VAS- *Salix alba*

The comparative antioxidant activity of *V. album* leaves is presented in Figure 1 and for stems in Figure 2. In mistletoe leaves, antioxidant activity varied from 31.25 to 68.93 µmol Trolox/g DW for DPPH, from 41.20 to 72.21 µmol Trolox/g DW for ABTS, and from 42.78 to 86.89 µmol Trolox/g DW for FRAP method (Table 2).

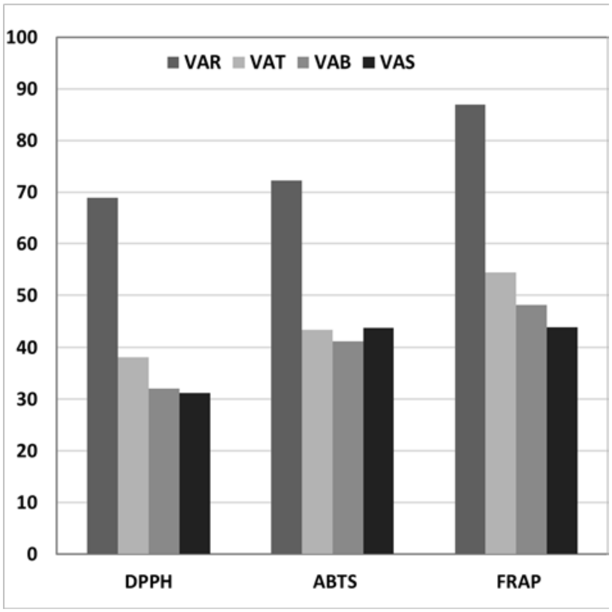
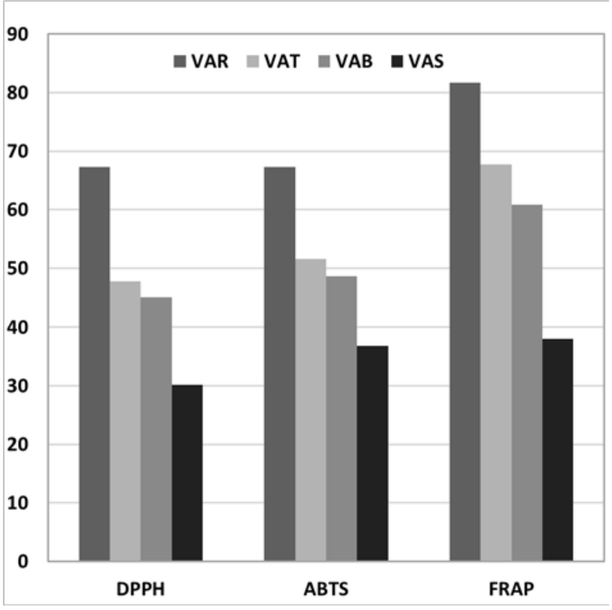


Figure 1: Antioxidant activity (µmol TE/g DW) of mistletoe leaves determined by DPHH, ABTS and FRAP method.

Generally, antioxidant activity decreased in the order: FRAP>ABTS>DPPH. (Figure 1). This can be explained by the fact that methanolic extracts of *V. album* harvested in autumn were richer in phenolic as antioxidant with ferric reducing ability (Vicaş,Prokisch, Rugina, *et al.*, 2009). Antioxidant activity of mistletoe leaves extracts from VAR and VAS were higher than the corresponding stems extracts. Interestingly, antioxidant activity of mistletoe leaves extracts collected from VAT and VAB were lower than corresponding stems extracts. This can be explained by higher contents of phenols and phenolic acids found in mistletoe stems from VAT and VAB than in leaves which may contribute to higher values of antioxidant activity in stems. (Figure 2).

Figure 2: Antioxidant activity (µmol TE/g DW) of mistletoe stems determined by DPPH, ABTS and FRAP method.



The highest antioxidant activity was determined in mistletoe leaves VAR while the lowest values were found for mistletoe leaves from VAS (DPPH and FRAP) and VAB (ABTS). These results are in agreement with other investigators who found that antioxidant capacity differs depending on the host trees (Onay-Ucar, Karagoz, Arda, 2006; Oluwaseun and Ganiyu, 2007; Vicaş *et al.*, 2009).

Antioxidant activity for stems varied from 30.13 to 67.28 µmol Trolox/g DW for DPPH, from 36.86 to 67.28 µmol Trolox/g DW for ABTS and from 38.04 to 81.72 µmol Trolox/g DW for FRAP (Table 2). Antioxidant activity for stems decreased in the following order: VAR >VAT> VAB >VAS for all three methods (Figure 2).

The highest values of antioxidant activity were determined for mistletoe stems from VAR and the lowest for stems from VAS.

Correlations investigated between antioxidant activity and bioactive compounds in leaves and stems are given in Table 3.

Table 3: Correlations between antioxidant activity and phenolic compounds in mistletoe leaves and stems

	DPPH	ABTS	FRAP
	Leaves		
TP	0.9768	0.9004	0.9931
TF	0.7338	0.5918	0.7724
TPA	0.848	0.8346	0.8701
TPC	0.9667	0.9354	0.9802
	Stems		
TP	0.9146	0.9116	0.9812
TF	0.688	0.6999	0.873
TPA	0.9316	0.9199	0.8912
TPC	0.9399	0.9495	0.965

TP- total phenols, TF-total flavonoids, TPA – total phenolic acids, TPC- total proanthocyanidins

We found strong correlation between phenols, phenolic acids, proanthocyanidins and antioxidant activity. In case of leaves, correlation coefficients were in range r^2= 0.9004-0.9768 for phenols; r^2= 0.8346-0.8701 for phenolic acids and for proanthocyanidins r^2= 0.9354-0.9802. In case of stems, they were in range 0.9116-0.9812 for phenols, 0.8912-0.9316 for phenolic acids and 0.9399-0.965 for proanthocyanidins. The lowest correlation coefficients were found for flavonoids r^2= 0.5918–0.7724 in leaves and for stems r^2= 0.688-0.873 which suggest that these compounds also contribute to the antioxidant activity of the extracts but in some less extend. Other investigators found that phenolics and flavonoids may contribute mostly to antioxidant activity of mistletoe extracts (Papuc, Crivineanu, Goran, *et al.*, 2010; Pietrzak, Nowak, Olech, 2014; Orhan *et al.*, 2014).

CONCLUSION

In all investigated samples, flavonoids and proanthocyanidins were present in higher amounts in leaves than the stems. Mistletoe from VAR was the richest in the content of phenols and phenolic acids. Mistletoe from VAS had the lowest content of phenols, flavonoids and phenolic acids in leaves and lower content of all investigated compounds in stems compering with other samples.

Mistletoe from VAT and VAB had higher content of total flavonoids and proanthocyanidins in leaves while stems were richer in content of total phenols and phenolic acids.

Antioxidant activity of *V.album* spp *album* leaves and stems generally were similar and decreased in the order: FRAP>ABTS>DPPH for leaves and stems. The highest antioxidant activity was found for mistletoe leaves and stems from VAR. This indicate that host three is important parameter in assessment of the mistletoe as a row material in medical and pharmaceutical application.

Antioxidant activity was highly correlated with content of total phenols, proanthocyanidins and phenolic acids. Moderate correlation was noticed between antioxidant capacity and total flavonoid content.

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Summary/Sažetak

Određivan je sadržaj fenolskih jedinjenja i antioksidacijska svojstva metanolnih ekstrakata listova i grančica imele *Viscum album* ssp. *album* Beck. Imela je prikupljana sa četiri različita domaćina (*Betula* L., *Tilia cordata* Mill., *Robina pseudoacacia* L, and *Salix alba* L.). Folin –Ciocalteu metoda, $AlCl_3$ metoda, metoda sa Arnow reagensom i kiselinsko-butanolna metoda korištene su u određivanju ukupnih fenola, flavonoida, fenolskih kiselina i proantocijanidina respektivno. Antioksidacijska svojstva ekstrakata ispitivana su upotrebom DPPH, ABTS i FRAP metode. Ukupni fenoli kretali su se u granicama 7,02-13,52 mg GAE/g s.u., flavonoidi 2,29-5,05 mg RE/ g s.u., fenolske kiseline 0,61-2,84 mg CAE/g s.u. i proantocijanidini 0,63-4,83 mg LCE/g s.u. Dobiveni sadržaj flavonoida i proantocijanidina je bio veći u listovima u odnosu na grančice. Najveća antioksidacijska aktivnost (68,93-86,89 µmol ekvivalenata Troloxa/g s.u. za listove i 67,28-81,72 µmol ekvivalenata Troloxa/g s.u. za grančice) za DPPH, ABTS i FRAP metodu određena je za imelu prikupljenu sa *Robina pseudoacacea* L. Takođe, dobivena je visoka korelacija između sadržaja ukupnih fenola, fenolskih kiselina, proantocijanidina i antioksidacijske aktivnosti.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

31-34

Print ISSN: 0367-4444
Online ISSN: 2232-7266

UDC: ________________________

Original Scientific Paper

Optimization of the spectroscopic method using potassium peroxymonosulfate for determination of antioxidant capacity

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Article info
Received: 22/11/2017
Accepted: 13/12/2017

Keywords:
antioxidants
potassium peroxymonosulfate
EAPK
FRAP

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Abstract: In this study, we were testing possible usage of commercial tablets for dental prosthesis, containing potassium peroxymonosulfate as a reagent for determination of antioxidant capacity *in vitro*. Our aim was to develop fast, simple and cheap method for determination of antioxidant capacity that will be suitable for laboratories with modest resources. This method was previously proposed, but we have chosen somewhat different approach. Because of the quite narrow linear range, when ascorbic acid was used as standard, for the preparation of the calibration curve, we have used a catechin in the concentration range from 0.125 mg/mL to 12.5 mg/mL. Obtained calibration curve was linear with correlation coefficient of $R^2=0.992$ and it was used for further determination of antioxidant capacity of selected samples. In order to test the possibility of this method for determination of antioxidant capacity of real samples, we have used five samples which antioxidant capacity was proven in previous testing with different *in vitro* and *in vivo* methods.

INTRODUCTION

Reactive oxygen species (ROS) are byproducts of normal cellular metabolism. Uncontrolled ROS production leads to their accumulation in cells, where they can cause oxidative damage, due to oxidative stress. Endogenic antioxidant repair systems are not enough to prevent disruption of normal cellular homeostasis, which requires substitution with exogenic antioxidants (Kunwar and Priyadarsin, 2011). For epidemiologic purposes it would be useful to determine total antioxidant capacity (TAC) of different classes and types of foods. So far many *in vitro* and *in vivo* methods for determination of TAC have been developed. In this study we were testing possible usage of commercial tablets for dental prosthesis, containing potassium peroxymonosulfate as a reagent for determination of antioxidant capacity *in vitro*. Our aim was to develop fast, simple and cheap method for determination of antioxidant capacity that will be suitable for laboratories with modest resources. In order to test our method, antioxidant capacity of selected samples was determined and compared with the result obtained using classical FRAP method.

EXPERIMENTAL

For determination of antioxidant capacity of selected food products, effervescent tablets for cleaning dental prosthesis, containing potassium peroxymonosulfate were used. This method was originally proposed by Al-Shahrani, Zaman and Amanullah (2013), but in this study method was modified and improved. In order to test this spectrophotometric method five samples available at the market in Bosnia and Herzegovina were analyzed: the Noni juice (*Morinda citrifolia L., Rubiaceae*), semi sweet red wine from Herzegovina, dry white wine from Herzegovina, 100% natural tomato juice (*Lycopersicum esculentum Mill., Solanaceae*) and Goji berries (*Lycium barbarum L., Solanaceae*).

Goji berries (5 g of homogenized sample) were extracted with 25 mL of boiled water. After 10 minutes, sample was filtered, and obtained infusion was used for further analysis. All the other samples were analyzed without prior preparation

For each sample 1 mL was mixed with 10 mL of reagent solution. All the spectroscopic measurements (Spectronic Genesis 2 UV/VIS spectrometer) were performed at 616 nm, with reagent solution as blank.

Reagent solution was prepared according to the previously described method by dissolving one effervescent tablet in 80 mL of saline solution. After adding a sample, the change of colour or the change in the blue color intensity indicates the presence of antioxidants. After 25 minutes this change can be quantified by measuring the absorbance at 616 nm. If the antioxidants are not present in the samples, blue colour wanish, while in the case of positive reaction (presence of antioxidant) intensity of blue colour is increased (Al-Shahrani, *et al.*, 2013). The most imortant ingredients of effervescent tablets for cleaning dental prosthesis (available at the market in BiH) are potassium peroxymonosulfate ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$), tetraacetylethylenediamine (TAED), sodium perborate monohydrate ($BNaO_3xH_2O$), and indicators indigotin and brilliant blue.

Potassium peroxymonosulfate is a component of a triple salt that acts as oxidant. Sodium perborate from the tablet is soluble in water and releases hydrogen peroxide in the solution.

In order to investigate the influence of oxidants and reductants on spectral characteristics of indicator present in reagent solution, H_2O_2 and sodium dithionite were used. For each probe one effervescent tablet was dissolved in 80 mL of saline solution and one was used as blank, while in the other two 1 mL of H_2O_2 or 0.1 g of solid sodium dithionite were added, respectively (Figure 1). After 25 minutes, the spectra of these solutions were recorded in the 200-1100 nm wavelength range.

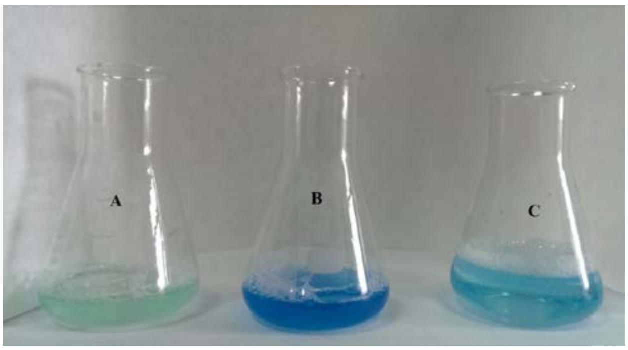

Figure 1: Solution of effervesent tablet after adding sodium dithionite (A), native solution (B), after adding H_2O_2 (C)

In order to quantify amount of antioxidant capacity of the selected samples calibration curve of methanolic catechin solution was used. The concentration range was from 0.125 mg/mL to 12.5 mg/mL. Antioxidant capacity of 10 mg of catechin was used as one EAPK (equivalent of antioxidative potential of catechin) unit. After measuring the absobance for each selected sample antioxidant capacity was expressed in EAPK units.

Ferric Ion Reducing Antioxidant Power Assay (FRAP assay)

This method was described by Benzie and Straine (1996) and has been used since then with small adjustments (Gorjanović, Alvarez-Suarez, Novaković, *et al.*, 2013). Complex Fe(III)-TPTZ (2,4,6-tri(2-pyridil)-s-triazine) is reduced to Fe(II)-TPTZ (intense blue complex) in the presence of antioxidant and its absorbance can be measured at 593 nm. Results are expressed in FRAP (mmol/L Fe^{+2}) and relatively to ascorbic acid as pure antioxidant (Gorjanović, *et al.*, 2013).

To each investigated sample (50 µL) before measurement (at 593 nm) 1.5 mL of FRAP reagents is added. Abosrbance is measured in 0 and 4 minute, and mean value was used for further calculation. In order to prepare calibration curve, solution of Fe(II) sulphate $x7H_2O$ was dilute in concentration range of 100 - 4000 µM. Results are expressed as FRAP (mmol/L Fe^{2+}).

FRAP reagent was prepared briefly before measurement by mixing in 10:1:1 ratio of 300 mM acetate buffer (pH 3.6), 10 mM solution of TPTZ (in 40 mM HCl) and 20 mM solution of $FeCl_3 \times 6\ H_2O$, respectively.

RESULTS AND DISCUSSION

According to Apak, Gorinstein, Böhm, *et al.*, (2013) the basis of most of the antioxidant based assays is either electron transfer or hydrogen atom transfer. In the first type of assays measuring the capacity of an antioxidant is based on reduction of an oxidant, which further changes colour of inidicator. The degree of colour change correlates with the concentration of antioxidant present in sample. On the other hand, reactions based on hydrogen atom transfer apply different reaction scheme, in which antioxidant quench free radicals by hydrogen donation (Apak, *et al.*, 2013).

TAED from the tablet reacts with hydrogen peroxide, and partly removes it, and the presumption is that a dominant mechanism of reaction is based on electron transfer, where antioxidants pottentialy present in samples react with potassium peroxymonosulfate which acts as oxidant.

So the possible mechanism of this reaction can be presented with equation 1.

$$HSO_5^- + 2H^+ + 2e^- \longrightarrow HSO_4^- + H_2O \qquad (1)$$

The change of colour of indigotin present in tablet during the reaction can be measured spectrophotometrically. Indigotin, (2-(1,3-dihydro-3-oxo-2H-indol-2-ylidene)-1,2-dihydro-3H-indol-3-one) is a dark blue crystalline powder, incorporated in effervescent tablet along with other indicator, brilliant blue. In oxidized form, indigotin is insoluble in water, so it has to be reduced to its leuco form, which is water soluble. During this reduction dye changes colour from blue to yellow-green. On the other hand, oxidation of indigotin leads to decreasing the intensity of colour, which vanishes in the end, because of the total oxidative degradation of indigotin (dehydroindigo) (Polette – Niewold, Manciu, Torres, *et al.*, 2007).

These colour changes are additionally proven, by investigation of spectral characteristics of indigotin, which is present is effervescent tablet. It is observed that change from indigotin to reduced leuco form does not lead to notable shift of absorption maximum, and the effects of colour change are obvious through absorption intensity change. During measurement of absorbance, spectra for solution with reducing agent (sodium dithionite), native solution and with added oxidant (hydrogen peroxide) are recorded (Figures 2-4).

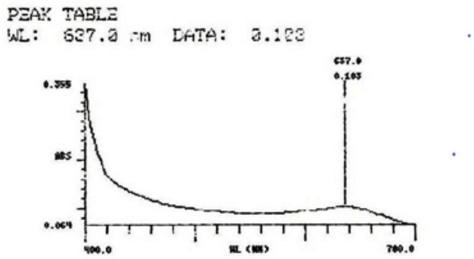

Figure 2: Spectrum of reduced indigotin from effervescent tablet after adding sodium dithionite

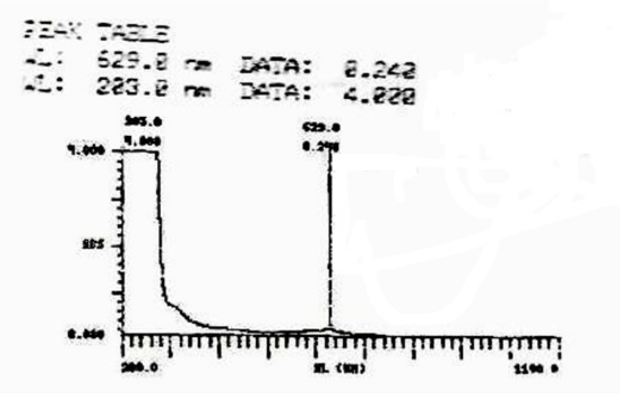

Figure 3: Spectrum of indigotin from effervescent tablet

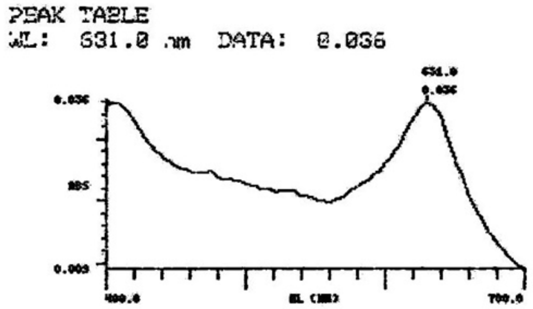

Figure 4: Spectrum of oxidized indigotin from effervescent tablet after adding H_2O_2

In this work, somewhat different approach was chosen comparing to originally proposed method (Al-Shahrani, *et al.*, 2013). For determination of antioxidant capacity calibration curve was made with the standard solution of ascorbic acid, within the 10 mg/80 mL (0.125 mg/mL) to 1000 mg/80 mL (12.5 mg/mL) concentration range. Obtained calibration curve was linear for concentrations from 10 mg/80 mL to 100 mg/80 mL, following the decrease of absorbances (for the concentration range from 100 mg/80 mL to 500 mg/80 mL), with no further change of the intensity of the absorption for the concentration range from 500 mg/80 mL to 1000 mg/80 mL. Because of this quite narrow linear range, we have investigated other option, which was to use catechin standard instead of ascorbic acid for preparation of the calibration curve. The same concentration range was chosen (0.125 mg/mL to 12.5 mg/mL). In this case obtained calibration curve was linear with correlation coefficient of R^2=0.992. This curve was used for further determination of antioxidant capacity of selected samples.

In order to test the possibility of this method in determination of antioxidant capacity of real samples, we have used five samples which antioxidant capacity was proven in previous testing with different *in vitro* and *in vivo* methods (Wang, Lutfiyya, Weidenbacher-Hoper, *et al*., 2009; Bramorski, Cherem, Marmentini, *et al*., 2010; West, Deng and Jensen, 2011; Iloki Assanga, Lewis Luján, Rivera-Castañeda, *et al.*, 2013; Ionica, Nour and Trandafir, 2012; Tarko, Duda-Chodak, Satora, *et al*., 2013; Katalinić, Milos, Modun, *et al*., 2004).

Selected samples were also tested using FRAP assay. Results of measurement are expressed as FRAP units (mmol/L Fe^{+2}) and were calculated from the calibration curve that was previously constructed (R^2=0,998).

Results of testing antioxidant capacity of selected samples in EAPK and FRAP units are presented in Table 1.

Table 1: Antioxidant capacity of selected samples

Sample	**Antioxidant capacity (EAPK)**	**Antioxidant capacity FRAP** (mmol/L Fe^{+2})
Dry white wine	0,81±0,017	7,96±0,01
Semi sweet red wine	1,60±0,046	11,9±0,005
Noni juice	1,50±0,046	11,2±1,79
Goji berries	9,19±0,04	33,8±1,20
Tomato juice	2,30±0,08	13,2±2,01

Since proposed mechanism of action for the method tested in this study is electron transfer, results are compared with those obtained with FRAP assay that has similar mechanism. There is a strong, positive correlation ($p<0.05$) with correlation coefficient of R^2=0,997 (Figure 5).

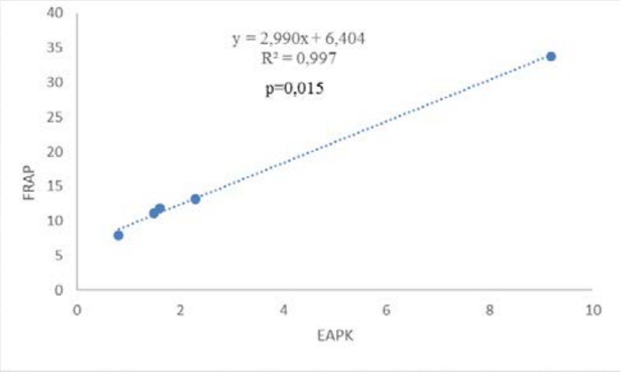

Figure 5: Correlation of results of our method and FRAP assay

CONCLUSION

Although very few samples were analysed with this method, obtained results show that potassium peroxymonosulfate can be used as a reagent for determination of antioxidant capacity *in vitro* in routine analysis. Advantages of this proposed method are cheap and available reagents that are used for the performing the analysis, and also a fact that there is no need for expensive equipement, which makes it suitable for laboratories with modest resources.

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Summary/Sažetak

U našem radu, ispitivana je mogućnost upotrebe komercijalnih tableta za čišćenje zubnih proteza, koje sadrže kalij peroksimonosulfat kao reagens za određivanje antioksidativnog kapaciteta *in vitro*. Cilj je bio razviti brzu, jednostavnu i jeftinu metodu za određivanje antioksidativnog kapaciteta, koja će se moći koristiti i u skromnije opremljenim laboratorijama. Ovaj metod je prethodno objavljen, međutim u našem radu korišten je različiti pristup uz modifikaciju originalne metode. Korištenjem askorbinske kiseline kao standardne supstance za izradu kalibracione krive dobivao se uzak raspon linearnosti, pa je iz tog razloga kao standardna supstanca korišten katehin u rasponu koncentracija od 0,125 mg/ml do 12,5 mg/ml. Dobivena kalibraciona kriva bila je linearna sa koeficijentom korelacije R^2=0,992 i ona je dalje korištena za određivanje antioksidativnog kapaciteta odabranih uzoraka. Kako bi se testirao predloženi metod u realnim uslovima, ispitano je pet uzoraka čiji je antioksidativni kapacitet potvrđen prethodnim testiranjima različitim *in vitro* i *in vivo* metodama.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

35-38

Print ISSN: 0367-4444

Online ISSN: 2232-7266

UDC: ________________________

Original Scientific Paper

Evaluation of the antioxidant activity of ferulic, homovanillic and vanillic acids using the Briggs-Rauscher oscillating reaction method

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Article info
Received: 06/12/2017
Accepted: 19/12/2017

Keywords:
Antioxidants
Phenolic acids
Briggs-Rauscher reaction
Inhibition time
Oscillations

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Abstract: In this study, the antioxidant activity of aqueous and ethanolic solutions of ferulic, homovanillic and vanillic acids were evaluated using Briggs-Rauscher oscillating reaction method. This method is based on the inhibitory effects of antioxidants on the oscillations of the Briggs-Rauscher reaction mixture. The inhibitory effect consists of an immediate quenching of oscillations, an inhibition time that depends on the amount and type of the antioxidant added, and a subsequent regeneration of oscillations. Flow oscillations in the Briggs-Rauscher reaction mixture were followed potentiometrically. In all samples, the inhibition times increased with increasing concentration and linearity was found in a wide concentration range of phenolic acid added. The antioxidant activity decreased in following order: ferulic acid > homovanillic acid > vanillic acid. It was also investigated the antioxidant activity for two-component and three-component mixtures of aqueous solutions examined phenolic acids.

INTRODUCTION

Phenolic acids are widely distributed in the plant world and have prominent antioxidant activity. They are ubiquitous in all plant organs and are therefore an integral part of the human diet (Ota *et al.*, 2011). It is estimated that humans consumed from 25 mg to 1 g phenolic acids a day depending on diet (Robbins, 2003). According to the basic structure, phenolic acids are divided into two classes: derivates of benzoic acid such as vanillic acid and derivates of cinnamic acid as ferulic acid (Rice-Evans *et al.*, 1996).
Ferulic acid (FA) is a ubiquitous plant constituent that occurs primarily in seeds. Ferulic acid is found in wheat, maize, rye, barley, oats, spinach, sugar beet, and water chestnuts, generally esterified, and rarely as free form, such as in barley. Due to its phenolic nucleus and an extended side chain conjugation, it readily forms a resonance stabilized phenoxy radical that accounts for its potent antioxidant activity. Ferulic acid has been shown to be protective against oxidative stress *in vitro*. It is absorbed and excreted by humans. The low lipophilicity impairs its *in vivo* efficiency, bioavailability and stability (Fraga, 2010).
Homovanillic acid (HVA) is a major catecholamine metabolite and is produced by the consecutive action of monoamine oxidase and catechol-O-methyl transferase on dopamine. It is used as reagent for the fluorimetric determination of glucose oxidase and other oxidative enzymes (Curzon *et al.*, 1970). In psychiatry and neuroscience, brain and cerebrospinal fluid levels of homovanillic acid are measured as a marker of metabolic stress caused by 2-deoxy-D-glucose (Marcelis *et al.*, 2006). Homovanillic acid presence supports a diagnosis of neuroblastomas and malignant pheochromocytoma (Candido *et al.*, 2002).
Vanillic acid (VA) is an oxidized form of vanillin produced during the conversion of vanillin to ferulic acid. The highest quantity of vanillic acid in plants has been found in the roots of *Angelica sinensis*. Vanillic acid is used as a flavoring agent. Various studies have provided evidence of the effectiveness of vanillic acid in the management of immune or inflammatory responses (Kim *et al.*, 2010). Vanillic acid showed antigenic and genotoxic effects depending on the dose on human lymphocytes (Bival Štefan, 2015).

Many analytical methods have been developed for the determination the activity of antioxidants (Shalaby and Shanab, 2013). The Briggs-Rauscher oscillating reaction method is very useful and applicable method as a test for the activity of antioxidants because it is works at pH about 2 and partially mimics the physiological conditions similar to those of the fluids in the human stomach. When antioxidants are added to an active oscillating Briggs-Rauscher reaction mixture, there is an immediate quenching of the oscillations for a certain time, denominated as inhibition time. An inhibition time is linearly dependant on the concentration of the antioxidant added to the reaction mixture (Cervellati *et al*., 2001; Cervellati *et al*., 2002). The efficiency of the corresponding antioxidant is expressed as inhibition time before oscillations restart. A better antioxidant as well as higher concentration of it leads to a prolonged inhibiton time. Relative antioxidant activity with respect to a substance chosen as standard can then be determined on the basis of inhibition time (Hönor and Cervellati, 2002; Hönor *et al*., 2002). The Briggs-Rauscher oscillating reaction method can give useful *in vitro* information on the antioxidant activity at low pH values and has many advantages. Milos and Makota (2012) demonstrated some new possibilities of this method for determine the synergistic and antagonistic effects in mixture of compounds, which often poses a problem when using standard methods.

Oscillatory behaviour in the Briggs-Rauscher reaction system can be easily followed potenciometrically using a bright platinum electrode and a suitable reference electrode under thermostated and stirred conditions.

The aim of the present study is to evaluate the antioxidant activity of aqueous and ethanolic solutions of ferulic acid, homovanillic acid and vanillic acid using the Briggs-Rauscher oscillating reaction method.

EXPERIMENTAL

Reagents

All used reagents were of analytical grade and were used without further purification. Potassium iodate, sulfuric acid, hydrogen peroxide and ethanol were obtained from Semikem (Sarajevo, BiH), malonic acid, manganese(II) sulfate monohydrate and starch were obtained from Merck (Darmastadt, Germany), ferulic acid, homovanillic acid and vanillic acid were obtained from Sigma (St. Louis, USA).

Preparation of the solutions for the Briggs-Rauscher reaction

Three colourless stock solutions were prepared daily. Solution A: 43 g potassium iodate and 4.5 mL 96% sulfuric acid were dissolved in distilled water and diluted to 1 L; Solution B: 15.6 g malonic acid, 3.4 g manganese(II) sulfate monohydrate and 3.0 g starch were dissolved in distilled water and diluted to 1 L; Solution C: 500 mL of 30% hydrogen peroxide was diluted to 1 L.

Mixture of equal volumes of stock solutions (A, B and C) represents the Briggs-Rauscher reaction mixture, which is used for evaluation of antioxidant activity (Džomba and Gojak-Salimović, 2017).

Preparation of the solutions of phenolic acids

Solutions of acids where in the concentration range of 8-40 mg/L, 8-80 mg/L and 160-320 mg/L for ferulic, homovanillic and vanillic acids, respectively; all of phenolic acids are ethanol and water soluble.

Apparatus

The oscillating behavior of the Briggs-Rauscher reaction was followed visually and potentiometrically by recording the potential of the reaction mixture using a platinum wire electrode and $Ag/AgCl/KCl_{(sat)}$ reference electrode (+197 mV vs. SHE). The electrodes were connected to a pH multimeter (Phywe, Model 13702.93). The accuracy of the multimeter was ±1 mV. All measurements were conducted at temperature (25±0.5°C) using a suitable thermostating system. The reaction mixture was stirred by a magnetic stirrer (600 rpm).

The Briggs-Rauscher oscillating reaction method for the determination of antioxidant activity

The Briggs-Rauscher reaction mixture were prepared by mixing the appropriate amounts of stock solutions of reagents of A, B and C. For each measurement 10 mL of each solution A and B were mixed into the double-wall thermostated beaker equipped with a magnetic stir bar and placed on a stirring plate. The 10 mL of solution C was used to initiate the oscillations. After the third oscillation, 1 mL solution of phenolic acid at corresponding concentration was added to 30 mL of an active Briggs-Rauscher reaction mixture. The inhibition times were then measured from the recordings. Typical potentiometric recordings for a non-inhibited and an inhibited Briggs-Rauscher reaction mixture were shown in our previous works (Dacić and Gojak-Salimović, 2016; Džomba and Gojak-Salimović, 2017).

The inhibition time defined as the time elapsed between the end addition of the phenolic acid and first regenerated oscillation. The addition of 1 mL of ethanol or water, without phenolic acid does not interrupt the oscillations. The pH value of a non-inhibited-Briggs-Raucher reaction mixture was 1.56.

RESULTS AND DISCUSSION

Our previous works showed the ability of some selected phenolic acids (gallic, caffeic, chlorogenic, rosmarinic and *p*-coumaric acids) to inhibited oscillations of the Briggs-Rauscher reaction mixture (Džomba and Gojak-Salimović, 2017). In this study, the inhibitory effect of various concentrations of the ethanolic and aqueous solutions of ferulic acid, homovanillic acid and vanillic acid on the oscillatory system Briggs-Rauscher reaction were evaluted at 25°C.

Addition of a solution of ferulic, homovanillic or vanillic acids in the active Briggs-Rauscher reaction mixture causes an immediate effect of quenching of oscillations and after period inhibition start again because the reaction produces hydroperoxyl radicals that are quenched by antioxidants. The total antioxidant activity is measured as an inhibition time of Briggs-Rauscher oscillating reaction. The obtained values of inhibition time of aqueous and ethanolic solutions of ferulic, homovanillic and vanillic acids are presented in Table 1.

Table 1: Inhibitory effects of individual ferulic acid, homovanillic acid and vanillic acid at different concentrations on total antioxidant activity measured

Ferulic acid		
Concentration (mg/L)	**t_{inhib} (s)**	
	Water	**Ethanol**
8	20	16
16	177	133
24	319	251
40	550	483
Homovanillic acid		
Concentration (mg/L)	**t_{inhib} (s)**	
	Water	**Ethanol**
8	100	30
16	163	135
32	235	290
48	406	360
64	800	510
80	1144	670
Vanillic acid		
Concentration (mg/L)	**t_{inhib} (s)**	
	Water	**Ethanol**
160	20	64
200	314	410
240	660	640
280	829	710
320	1250	950

The results collected in Table 1 shown that the majority of the ethanolic solutions of phenolic acids have much less antioxidant activity than the corresponding aqueous solutions.

In all samples, the inhibition times increased with increasing concentration and linearity was found in a wide concentration range of phenolic acid added. Therefore, the requirement for the possibility of using Briggs-Rauscher reaction as a test for antioxidant activity was fulfilled. The linear behavior of the inhibition time versus concentration of ethanolic solutions of ferulic, homovanillic and vanillic acids added shown in Figure 1.

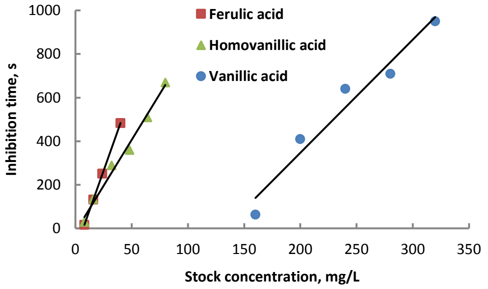

Figure 1: Straight lines of inhibition time versus concentration for the ethanolic solutions of phenolic acids studied

As shown in Figure 1, the slopes of the straight lines are different. The parameters of the straight lines and *R*-squared values are presented in Table 2.

Table 2: Parameters of straight-lines equations ($t_{inhib} = m(\text{antioxidant}) + q$) and R^2 values

Water solution	***m* (s L/mg)**	***q* (s)**	**R^2**
ferulic acid	16.42	−94.77	0.994
homovanillic acid	14.23	113.7	0.924
vanillic acid	7.482	−1170	0.987
Ethanol solution	***m* (s L/mg)**	***q* (s)**	**R^2**
ferulic acid	14.59	−100.4	1.00
homovanillic acid	8.409	−15.08	0.989
vanillic acid	5.195	−692.6	0.955

The antioxidant activity decreased in the following order: ferulic acid > homovanillic acid > vanillic acid, similar to the results previously shown in studies of other authors using Briggs-Rauscher oscillating reaction, TEAC and DPPH methods (Cervellati *et al*., 2001; Cervellati *et al*., 2002; Rice-Evans *et al*., 1996; Karamać *et al*., 2005).

The synergism between the antioxidants in the mixture makes the antioxidant activity not only dependent on the concentration, but also on the structure and the interaction between the antioxidants. In order to evaluate the impact of interactions among examined phenolic acids on their antioxidant activity, the inhibition time of ferulic acid, homovanillic acid and vanillic acid was compared with values obtained by mixing them in different combinations. The results obtained experimentally for nine two-component mixtures and one three-component mixture of aqueous solutions of phenolic acids were compared with the theoretical values by adding up the effects of two or three individual phenolic acids analyzed separately. The obtained results are presented in Table 3.

Table 3: Inhibitory effects of ferulic acid (16 mg/L), homovanillic acid (32 mg/L) and vanillic acid (200 mg/L) with various combinations on total antioxidant activity measured

Combination of compounds	**t_{inhib} (s)**
homovanillic acid + vanillic acid (50%:50%)	498 (275)
homovanillic acid + ferulic acid (50%:50%)	101 (206)
vanillic acid + ferulic acid (50%:50%)	300 (246)
homovanillic acid + vanillic acid (75%:25%)	560 (255)
homovanillic acid + ferulic acid (75%:25%)	504 (220)
vanillic acid + ferulic acid (75%:25%)	1502 (279)
vanillic acid + homovanillic acid (75%:25%)	1335 (294)
ferulic acid + vanillic acid (75%:25%)	1250 (255)
ferulic acid + homovanillic acid (75%:25)	580 (192)
ferulic acid + homovanillic acid + vanillic acid (33.3%:33.3%:33.3%)	320 (242)

*The values in parentheses are the summations of antioxidant activities of individual compounds at corresponding concentrations

Our results indicate that all combinations of phenolic acids demonstrated some level of discrepancy in antioxidant activity when compared to individual values of their constituents. Eight combinations of two and one combination of three phenolic acids showed more or less synergistic effect. The mixture of homovanillic acid and ferulic acid (50%:50%) showed a loss of antioxidant activity when compared to their individual values.

CONCLUSIONS

The Briggs-Rauscher oscillating reaction is suitable as an analytical method to determine antioxidant activity of the ethanolic and aqueous solutions of ferulic, homovanillic and vanillic acids. In all samples, the inhibition time of Briggs-Rauscher oscillating reaction increased with increasing concentration and linearity was found in a wide concentration range of phenolic acid added. The ethanolic solutions of examined phenolic acids had much less antioxidant activity than the corresponding aqueous solutions. The antioxidant activity decreased in following order: ferulic acid > homovanillic acid > vanillic acid. All combinations of phenolic acids demonstrated some level of discrepancy in antioxidant activity when compared to individual values of their constituents.

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Summary/Sažetak

U ovom radu ispitivana je antioksidacijska aktivnost vodenih i etanolnih rastvora ferulinske, homovanilinske i vanilinske kiseline primjenom metode Briggs-Rauscher oscilirajuće reakcije. Ova metoda se bazira na inhibitorskom efektu antioksidanasa na oscilacije Briggs-Rauscher reakcijske smjese. Inhibitorski efekat se sastoji od trenutnog gašenja oscilacija, vremena inhibicije koje zavisi od količine i vrste dodanog antioksidansa, i ponovne regeneracije oscilacija. Tok oscilacija Briggs-Rauscher reakcijske smjese praćen je potenciometrijskom metodom. Sa porastom koncentracije fenolskih kiselina linearno se povećavalo vrijeme inhibicije u širokom opsegu koncentracija. Antioksidacijska aktivnost je opadala prema sljedećem nizu: ferulinska kiselina > homovanilinska kiselina > vanilinska kiselina. Također je ispitana i antioksidacijska aktivnost dvokomponentnih i trokomponentnih smjesa vodenih rastvora fenolskih kiselina.

Glasnik hemičara i
tehnologa
Bosne i Hercegovine

Bulletin of the Chemists and Technologists
of Bosnia and Herzegovina

2017

49

39-42

Print ISSN: 0367-4444
Online ISSN: 2232-7266

UDC: ________________________

Original Scientific Paper

Spectrophotometric determination of Fe ions using green tea extract

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Article info
Received: 20/07/2017
Accepted: 20/12/2017

Keywords:
Natural reagents,
Green tea extract
Fe ion
Spectrophotometry
Green analytical chemistry

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Abstract: A batch spectrophotometric method for determination of Fe (II) and Fe (III) using green tea extract as reagent is proposed. The method is based on complex formation reaction between Fe and polyphenol compounds from green tea in buffered medium (pH = 4.8). Absorbance of Fe-polyphenol complex formed during the reaction was measured at 570 nm wavelength. The linear dynamic ranges are obtained from 1.0×10^{-5} to 5.0×10^{-4} mol/L. The applicability of the proposed method was demonstrated by determination of Fe ions in pharmaceutical dosage forms, orange juice, degassed mineral water and candy.

INTRODUCTION

This paper describes the possibility of using green tea extract as a natural reagent for spectrophotometric determination of Fe ions in different samples.

The plant for preparation of green tea (*Camellia sinensis*) is widely known. The popularity of this plant is based on its healing properties related to the polyphenol content. Green tea and its extracts are used to enrich energy drinks, juices and other types of foods (Balentine *et al.*, 1997).

One of the interesting properties of polyphenols of green tea is their ability to affect the absorption of iron in physiological system. Polyphenol compounds, particularly catechins, have the ability to react with iron ions and form a Fe-polyphenol complex (Ryan and Hynes, 2007). Various methods of using different natural reagents in order to efficiently develop analytical methods have been described in a literature very often. Plant extracts that can be used as acid-base indicators are the most commonly known. As natural reagents, enzymes can be isolated from tissues and bacteria (Grudpan, *et al.*, 2010, 2011).

One of the examples is a method for spectrophotometric determination of iron ions using a guava leaf extract as a natural reagent that has been +optimized (Settheeworrarit *et. al.*, 2005). However, Pinyou and co-workers (2010) have developed a new analytical method for the determination of iron ions using flow injections and natural reagents extracted from green tea. Recently, our research group was optimized the system for sequential analysis by injection based on the same reaction (Martinović Bevanda *et al.*, 2015).

Analytical methods that use natural reagents meet basic principles defined by green chemistry framework. Therefore, we consider it worthwhile to adapt the application of green tea for the batch spectrophotometric determination of Fe ions.

The basic theoretical and practical principles of green chemistry are defined by well-known twelve principles (Anastas and Warner, 1998). The most important among them is the principle in regards to sustainable development concerns as follows: avoid or reduce the use of toxic solvents, reagents, and the production of harmful waste. Furthermore, the benefits of applying green chemistry in industry or education are: faster and cheaper methodology, cleaner and healthier environment. We are witnessing that costs and other issues affecting the sustainability of chemistry education are growing concern nowadays (Kradtap Hartwel, 2012). Therefore, the proposed method can be used in analytical laboratories that do not have the possibility of flow analysis. Also, green principle on which this method is based offers a simple, interesting and innovative approach to modern education for sustainable development. The proposed method was tested in the analysis of real samples. Analytical use and the influence of different matrices were tested on orange juice sample, mineral water, candy and pharmaceuticals.

EXPERIMENTAL

Reagents and chemicals

All chemicals were of analytical-reagent grade and all solutions were prepared in deionized (DI) water.

A stock solutions, 50.0 mL each of 0.1 mol/L Fe(II) and Fe(III) were prepared by dissolving 1.3504 g $FeCl_3 \times 6H_2O$ (Sigma-Aldrich Chemie) and 1.3900 g $FeSO_4 \times 7H_2O$ (Sigma-Aldrich Chemie) respectively, in DI water containing 1.0 % (v/v) concentrated H_2SO_4. To the stock solution of Fe (II) was added 5.0 mL of H_2O_2 due to complete oxidation to Fe (III).

Acetate buffer, pH = 4.8, was prepared by mixing 14.44 g of dissolved sodium acetate in proper volume of deionized water with 5.5 mL of concentrated acetic acid. The obtained mixture was diluted to 1000.0 mL with DI water. If necessary, the pH value of the buffer was adjusted by adding small volumes of NaOH or HCl (4.0 mol/L)

Green tea extract (GTE) suspension was prepared with 8.0 g green tea in 150 mL acetate buffer (0.2 mol/L, pH = 4.8). The suspension was stirred for 10 minutes using magnetic stirrer (300 rpm). Then, the suspension was filtered and diluted with acetate buffer (pH= 4.8) to volume ratio 1:1. The extract was prepared daily. Green tea is purchased from local stores.

Sample solutions preparation

Twenty tablets of pharmaceutical preparations were accurately weighed and ground. A portion of powder was weighted and dissolved in mixture of 5.0 mL concentrated HCl and DI water. The mixture can be filtered, if necessary. The required aliquot of the filtrate was transferred to 50.0 mL volumetric flask. Hydrogen peroxide (5.0 mL) was added, to ensure the complete oxidation of Fe (II) to Fe (III).

Samples of degassed mineral water, juices and candy are spiked with Fe (III) and Fe (II) to verify the applicability of the method on samples with different matrices. An aliquot of 20.0 mL of liquid samples was mixed with required volume of standard solution of Fe ions.

Ten pieces of candies were accurately weighed and ground. A portion of powder was weighted and dissolved in deionized water. The working solution of this sample was also spiked with required volume of standard solution of Fe ions.

Apparatus

The absorbance was measured with a double-beam UV-Vis spectrophotometer, UV-1800 Shimadzu, with 1.0 cm width paired quartz cuvettes.

Measurements of pH were performed using a pH meter (Mettler Toledo, Schwerzenbach, Switzerland) equipped with a combined glass electrode.

Procedure

To determine both, Fe (III) and Fe (II), reaction solutions were prepared as follows. The volume of 3.0 mL of working solution of green tea extract (GTE) and 5.0 mL of standard solution of Fe ions or sample was precisely pipetted in laboratory tube with stopper. The formation of stable complex between polyphenols from green tea and the Fe ion is practically instantaneous. Depending on the concentration of the Fe ion, the color of the reaction solution is in different shades of deep blue. The absorbances of these obtained solutions against reagent blank were measured at 570 nm.

RESULTS AND DISCUSSION

Optimization of process of preparing a natural reagent and reaction condition

The effect of water, acetate buffer and temperature on the stability of the tea extract was tested. Also, both Fe (II) and Fe (III) could form color complexes with green tea extract and Fe (III)-green tea complex has higher molar absorptivity (Pinyou *et al.* 2010) and therefore for the optimization experiment, Fe (III) was used.

The optimization of the preparation of the green tea reagent was made as follows. Volumes of 2.0, 4.0 and 8.0 g of green tea were added to the 150.0 mL of acetate buffer (pH 4.8) or water heated to 40 ° C. The extraction time was 10, 20 and 30 minute at mixing conditions (300 rpm). Mixture with water is thermostated at 40°C during extraction. Then the plant material was separated by filtration and the extracts were diluted with acetic buffer (pH= 4.8) or ultra-pure water in a 1:1 ratio.

Obtained reagents were used in optimization experiments for reaction condition for determination Fe ion. A volume of 5.0 mL green tea reagent and 5.0 mL working solution of Fe (III) were mixed. In resulting solutions concentration of Fe (III) was 1.0×10^{-3} mol/L and 1.0×10^{-4} mol/L. The absorbance was measured at 570 nm.

The obtained results show that absorbance was increased with increasing green tea weight mass, but it also slight increase of absorbance with higher extraction time, in both acetic buffer and DI water was observed. In order to obtain method with good sensitivity, stability and reasonable time of reagents preparation, 8.0 g of green tea and 10 minute extraction time in acetate buffer (pH = 4.8) were chosen as optimal for green tea reagents.

It has been observed that the absorbance of the reaction solution made with older reagents (24 h after preparation) is decreased by 33%, which indicate that tea extracts should be made daily.

In the next experiment, it was necessary to determine the optimal method for preparing the reaction solution, the volume of extract and the standard / sample solution which were mixed.

A green tea extract was prepared as described above. Different volumes of green tea reagents were mixed with 5.0 mL of standard Fe (III) solution, $c = 1.0 \times 10^{-4}$ mol/L in the following ratios: 1:1 (5 mL GTE + 5 mL Fe (III)); 3:5 (3 mL GTE + 5 mL Fe (III)) and 1:5 (1 mL GTE + 5 mL Fe (III)).

In this experiment one concentration of Fe (III) 1.0×10^{-4} mol/L was used and the highest absorbance was obtained for the reaction solution prepared by mixing 1.0 mL GTE with 5.0 mL of Fe (III) ion standard solution.

Also, reaction solutions were prepared at the same ratios, but this time with all five concentrations in the tested concentrations range. Measured and compared absorbance indicate that volume ratio (GTE:Fe) 3:5 give better regression of calibration line.

Table1. Equations of calibration lines for Fe (III) and Fe (II), linear regression coefficient, R^2 and detection limits

Concentration range, mol/L 1.0×10^{-5} - 5.0×10^{-4}	Equation	R^2	Detection limit, mol/L
Fe (III)	$A = 1.105 \cdot c + 0,0188$	0.9997	8.08×10^{-6}
Fe (II) + H_2O_2	$A = 1.524 \cdot c + 0.0195$	0.9980	9.52×10^{-6}

Analytical application

Under the optimized conditions given above, rectilinear calibration graphs were obtained in the concentration ranges from 1.0×10^{-5} to 5.0×10^{-4} mol/L. The limits of detection ($3s_b/m$, three of the standard deviation blank divided by slope of the calibration curve) and regression equations are given in Table 1.

In order to obtain good sensitivity and the same condition in reaction solution with sample and standard, calibration lines were prepared for both, Fe (III) and Fe (II). For determination of Fe (II) in standard or in real samples H_2O_2 was added because of oxidation of Fe (II) to Fe (III).

In order to evaluate the potential of the proposed method to analysis of real sample, the method was applied for analysis of commercially available pharmaceutical samples, Table 2. The measurements with spiked samples of degasses mineral water, juice and candy, (Table 3.) were assessed to verify the possible interference of different matrices. As shown in Table 2. the proposed method was successfully applied for the determination of Fe (II) in pharmaceutical preparation.

As it could be assumed, measurements obtained for spiked samples of juice, candy and mineral water showed poor recovery.

In the candy sample, there was very high concentration of sugars (w/w 95 %) that was more then 1000 –fold sugars excess then in spiked samples. The sugars in candy can be interference, and main reason for poor recovery.

It was found that the system could tolerate some cations ($Ca^{2+,}$ Mg^{2+},Co^{2+}, Ni^{2+},Cr^{3+} , Cu^{2+} and Mn^{2+}) at least up to 1:1 concentration ratio (Pinyou *et al.* 2010). In the spiked sample of mineral water concentration ratio was much higher. Tested aliquot (20 mL) of spiked sample of mineral water contains 0.1972 mg/L (from labeled value) of cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}). The amount of Fe added is 0.038 mg/L, which significantly exceeds the ratio of 1:1. We can conclude that cations present in the sample interfere determination of Fe ion, which is the main reason for poor recovery.

Table 2. Results for the determination of Fe (II) in pharmaceuticals

Sample	Taken, mol/L	Found ± SD, mol/L (n = 3)	Recovery
Heferol, Alkaloid AD Skopje	1.0×10^{-4}	$9.7\times10^{-5} \pm 0.029$	97.0 %
Retafer, Krka Farma doo	1.0×10^{-4}	$9.6\times10^{-5} \pm 0.003$	96.0 %

Table 3. Results for the determination of Fe (III) and Fe (II) ions in spiked samples

Sample	Fe (III), mol/L			Fe(II), mol/L		
	Added	Found* ± SD	Recovery	Added	Found* ± SD	Recovery
Orange juice	5.0×10^{-4}	$3.6\times10^{-4} \pm 0.024$	72.0 %	5.0×10^{-4}	$3.8\times10^{-4} \pm 0.029$	76.0 %
Degasses mineral water	7.0×10^{-5}	$2.4\times10^{-5} \pm 0.014$	34.2 %	7.0×10^{-5}	$3.3\times10^{-5} \pm 0.008$	47.0 %
Candy	1.0×10^{-4}	$3.2\times10^{-5} \pm 0.011$	32.0 %	1.0×10^{-4}	$3.6\times10^{-5} \pm 0.005$	36.0 %

*Values are mean of three replicates (n=3)

CONCLUSIONS

The proposed spectrophotometric method can be a good “green” alternative for routine analysis of the Fe ions in pharmaceuticals. The main advantages of the proposed methodology are the simple “green reagents” approach with detection limit of Fe ions at 8.08×10^{-6} mol/L and low cost.

Good linearity between measured signal and concentration was achieved in the range from 1.0×10^{-5} to 5.0×10^{-4} mol/L for both, Fe (II) and Fe (III). Considering that the proposed method meets some principles of green analytical chemistry it is very suitable as “Green Chemistry for life” (PhosAgro /UNESCO/ IUPAC Partnership) approach in undergraduate and graduate analytical chemistry laboratory.

Acknowledgement

This work was supported by the Federal Ministry of Education and Science of Federation of Bosnia and Herzegovina**.**

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Summary/Sažetak

U ovom radu je opisana mogućnost primjene ekstrakta zelenog čaja kao prirodnog reagensa za spektrofotometrijsko određivanje iona Fe u realnim i laboratorijskim uzorcima. Reakcijski sustav se temelji na formiranju kompleksa između iona Fe i polifenola iz ekstrakta zelenog čaja u puferiranom mediju pri pH = 4,8. Absorbancija formiranog tamnoplavog kompleksa mjeri se pri valnoj duljini od 570 nm. Linearna ovisnost postignuta je u području koncentracija od $1{,}0\times10^{-5}$ do $5{,}0\times10^{-4}$ mol/L. Utjecaj različitih matrica testiran je na uzorcima voćnog napitka, negazirane mineralne vode, i bombone u koje je dodana određena koncentracija iona Fe (III) i Fe (II), a mogućnost analitičke primjene testirana je na farmaceutskim pripravcima. Predložena metoda je jednostavna, jeftina i primjenjiva u analitičkim laboratorijima.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2017

49

43-48

Print ISSN: 0367-4444

Online ISSN: 2232-7266

UDC: ________________________

Original Scientific Paper

Chemical Characterisation of the Spring Waters used for Health Care, Guber, Srebrenica, Bosnia and Herzegovina

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Article info
Received: 01/08/2017
Accepted: 20/12/2017

Keywords:
Spring water
Heavy metals
Guber
Srebrenica

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Abstract: The Guber spring waters in Srebrenica (Bosnia and Herzegovina) were used for centuries, and continue to be used for health-care purposes. The experts noted 48 mineral springs of different discharges and chemical composition. In this study, the physico-chemical properties, content of heavy metals and anions were determined in four selected spring waters (Mali Guber, Očna voda, Sinus voda and Ljepotica). Very low pH and very high concentrations of iron and sulphate are found in all springs. The highest concentrations of iron (2069 µg/mL) and sulphate (2486 µg/mL) and the lowest pH (1.67) were measured at spring Očna voda. The concentration of other metals and anions varied between different springs. Correlations between physico-chemical parameters and concentration of metals and anions in the water samples were also evaluated. Results for the spring water Sinus voda were evaluated for the first time.

INTRODUCTION

Water is essential for life on earth and its quality is of great importance to human health. It is an important source of trace elements for the proper functioning of the human organism (Ristić, Popović, Pocajt, *et al.*, 2011). Mineral water always contains various minerals and trace elements and can be defined as water containing minerals, which are natural compounds formed through geological processes, or other dissolved substances that alter its taste or give it therapeutic properties (Rezaee, Hassanzadeh-Khayyat, Mehri, *et al.*, 2012; Albertini, Dacha, Teodori, *et al.*, 2007). The World Health Organization (WHO) has been concerned with health aspects of the management of water resources for many years and publishes various documents (WHO, 1997; WHO, 1996) concerning the safety of water environment and its importance for health. In the European Community (EC), natural mineral water is strictly defined in the EC Directive 80/777 that it must be groundwater and clearly distinguishable from ordinary drinking water by its nature (Lau and Luk, 2002). Mineral water include groundwater, due to the general mineralization, ion composition, gas content, the presence of therapeutic active ingredients (mineral and organic), radioactive elements, alkalinity or acidity and elevated temperatures, have beneficial physiological effects on the human organism (Dragišić, 1997). The healing mineral water of Srebrenica is known from the time of the ancient Romans who called its spring *Domavia*, and the whole area in this beautiful corner of Eastern Bosnia - *Argentaria*. During the reign of the Turks the fame of the healing water from the Javor Mountain spring has spread, and it is interesting that today's name of Srebrenica spa dates from these times. Turkish soldiers came to Srebrenica to be treated for leprosy hence the name Guber (Operta and Hyseni, 2013).

The Srebrenica area is characterised by numerous Pb-Zn sulphide ore bodies and several iron-sulphate mineral water springs. Srebrenica is also well known for its long mining history (Dangić and Dangić, 2007). Recent sediment analysis of these water springs (Dangić and Dangić, 2001), showed that they are very rich in arsenic, lead and iron. These waters are rare and have effective physico-chemical components and are significant due to the high content of iron, arsenic and trace elements; they are similar to the well-known mineral resources Levico in South Tyrol (Italy) (Hasić, Lončarević, Marić, *et al.*, 1976). Due to its chemical composition spa Guber is unique in Europe. Inside the spa Guber there are 48 springs, most notably: Veliki/Crni Guber (Big/Black Guber), Mali Guber (Small Guber), Kožna voda (Skin water), Očna voda (Eye water), Sinus voda (Sinus water) and Ljepotica (Beauty - water for face).The most important and known healing water is Crni Guber. It is potable in its natural state and experts verify it as curable for many diseases. This spring contains bivalent iron, copper, cobalt, nickel, manganese and a lot of other elements. It cures hypocrome anomalies, essential hypocrome anomalies, weak appetite, general weakness of the organism, weight loss, fatigue, exhaustion, asteny, neurovegetative defects, chronically skin diseases, rheumatism, multiple-sclerosis and many others health problems. Every-day consumption of the healing water from Crni Guber has to be under medical control (Pašagić, 2008). Few other mineral water springs appear near the Crni Guber spring. This spring is the most analyzed in many studies during previous years. Because of that, our research was based on the analysis of other important and often used springs. Among them, the spring Ljepotica has some similarities with the Crni Guber spring in the water composition and spring sediment formation.

The spring Ljepoticais the most favorable among users, because only washing with it makes prettier someone complexion and skin of the face and by sinking and rinsing help to cure all kinds of skin diseases (Dangić and Dangić, 2001). One of the most-visited healing spring is called Očna voda. It has low abundance but it is highly mineralized. Washing helps to cure mucous membrane of eyes particularly conjunctivitis and it considerably improves sight (Hasić,*et al.*, 1976). Mali Guber contains twice more iron than Crni Guber, higher quantities of calcium and manganese but it has lower abundance. Because of high mineralization it is possible to use this water for specific diseases. In the vicinity of Mali Guber is the spring Kožna voda that is used for the treatment of psoriasis, dermatitis and warts. Sinus voda heals and cures inflammation and painful sinus. This treatment is performed by sniffing. With the constant use of Sinus water it is possible to neutralize pain in sinus (Pašagić, 2008). The composition and properties of all springs and the river water (after all inputs) resemble of an acid mine drainage (Lenoble, Omanovic, Garnier, *et al.*, 2013).

There are many parameters that determine the quality of water. This study include 20 parameters such as: temperature, pH, conductivity, total solid after evaporation at 105°C, chemical oxygen demand (COD), chlorides, phosphates, sulphates and heavy metals (Ag, Au, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb and Zn).

EXPERIMENTAL

Study area

Srebrenica is located in the mountainous area of north-eastern Bosnia and Herzegovina. The urban part of Srebrenica is situated in the valley of the Krljevica River at 448 m above sea level and is surrounded by the hills of the Sušica Mountain. The town is about 160 km away from Sarajevo, and 120 km from Tuzla. Near Srebrenica, at 560 m above sea level is Guber, Srebrenica's most renowned spa resort. The path to the Guber resort is beautified with gorgeous coniferous and deciduous forests.

The climate is temperate continental sub-mountainous under the influence of no wind and fog (Bećirović, 2004).

Sampling

The ISO standard method was used for sampling (ISO, 1992). Water samples were collected in polyethylene bottles. All glass and plastic ware used for sampling and analysis were washed with 10% HNO_3 and rinsed with Milli-Q water. Sampling was carried out in April 2012 at four mineral springs (Mali Guber, Očna voda, Ljepotica and Sinus voda). The sampling locations are presented in Figure 1.

Figure 1. Sampling sites at Guber, Srebrenica
Source: https://www.openstreetmap.org/copyright.

Instruments and Reagents

Four spring water samples in triplicates were analysed by 20 parameters: temperature, pH, conductivity, COD, total solids after evaporation at 105°C, metal concentrations (Ag, Au, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb and Zn) as well as anion mass concentration (chlorides, sulphates, phosphates).

Atomic absorption spectrometer (AA240FS, Varian, Australia) was used for the determination of metals except for Fe. UV/Vis spectrophotometer (Carry 50, Varian, Australia) was used for phosphates analysis. Conductivity meter (HANNA Instruments, Model HI 8733, Sigma, Singapore) and pH meter (CG841, Schott, Germany) were used for conductivity and pH measurements.

Content of sulphate was determined by gravimetric method (precipitation with barium chloride). The content of total solid after evaporation at 105°C was determined by gravimetric method. Iron and chloride content was determined by volumetric method, dichromometric and mercurimetric titration, respectively.

All used reagents were analytical grade. Milli-Q water was used throughout the complete experimental work. All samples were analysed in triplicate. Comparison of the triplicate results shows significant agreement, confirming the quality of analytical data.

The detection limits (LOD) were calculated on the basis of three times the standard deviation of the blank signal. The obtained values of LOD for the investigated metals are: Mn (0.002 µg/mL), Zn (0.001 µg/mL), Cu (0.003 µg/mL), Cr (0.006 µg/mL), Pb (0.01 g/mL), Cd (0.002 µg/mL), Ni (0.01 µg/mL), Co (0.005 µg/mL), Sb (0.02 µg/mL), Au (0.002 µg/mL)and Ag (0.01 µg/mL).

RESULTS AND DISSCUSION

Srebrenica area is well known for its long mining history, as well as for its spring waters of specific composition (Dangić and Dangić, 2007). The healing springs from spa Guber in Srebrenica are the only springs of this kind in Bosnia and Herzegovina. Due to the great interest in the healing waters of the spa Guber in Srebrenica, and just a few available previously published papers which are mainly related to the spring Crni Guber (Lenoble, *et al.*, 2013; Blagojević, Lazić, Škundrić, *et al.*, 2008; Pašagić, Pašagić, Jatić, *et al.*, 2006; Miholić, 1954) the aim of this work was to determine the 20 selected parameters that characterise the spring waters and determine their invaluable quality. Ernst Ludwig, a Viennese chemist published the first chemical analysis of Srebrenica area mineral waters in 1890 in "Die Mineralquellen Bosniens — Die arsenhaltigen Eisenquellen von Srebrenica" ("Bosnian mineral sources — iron sources from Srebrenica containing arsenic") (Lenoble, *et al.*, 2013). The yield of springs is in most cases low (Q < 0.1 L/s – Očna voda) (Miholić, 1954).

Results of analysis of spring waters Mali Guber, Očna voda, Ljepotica and Sinus voda are shown in Table 1. The content of anions and metals is presented as the average values ± standard deviation of triplicate measurements.

Physico-chemical parameters and content of anions

All springs were of very acidic pH, and the lowest pH is determined at Očna voda (1.67), where also the highest sulphate content was found (2486 µg/mL). High sulphate concentration, originating from sulphide oxidation, results in low pH of these spring waters. That low pH leads to the dissolution of minerals, which then enriches the water with different elements and can lead to excessive concentrations (Lenoble *et al.,* 2013; Casiot, Lebrun, Morin, *et al.*, 2005). This is the common scenario in active or abandoned mines with acid mine drainage waters, which are considered to have such harmful effects on aquatic life that only adapted microorganisms can encountered (Bruneel, Duran, Casiot,*et al.*, 2006). High values of Pearson´s correlation coefficient obtained between pH and content of sulphate (r = 0.9646), pH and content of phosphate (r = 0.9915), conductivity and total solid after evaporation at 105°C (r = 0.9759) and conductivity and content of sulphate (r = 0.9832) confirms the strong chemical relationship between these parameters. There was no significant correlation between pH and chloride (r = 0.1086). The chloride content in all samples was significantly lower than the limit values prescribed by legislation (Službeni glasnik BiH, 26/10).

According to the Ordinance on natural mineral waters and natural spring waters (Službeni glasnik BiH, 26/10) limit value for COD is 5.00 µg/mL. Significantly higher values were obtained for all analysed springs. The lowest COD value was determined in Očna voda (111 µg/mL), while the highest value of 323 µg/mLwas found in Sinus voda. This can be probably explained by the presence of fulvic acid completely complexed by trace/major metals, humic acids are not expected at so low pH (Mounier, Zhao, Garnier, *et al.*, 2011).

Table 1. Summary results of spring waters (Guber, Srebrenica) analysis

Parameters	Mali Guber (Small Guber)	Očna voda (Eye water)	Sinus voda (Sinus water)	Ljepotica (Beauty)	Limit value*
Temperature (°C)	12.0	7.0	13.5	11.5	-
pH	4.91	1.67	2.82	2.36	6.5-9.5
Conductivity (mS/cm)	0.7	7.9	2.70	2.00	up to 2.5
Total solid after evaporation at 105 °C (mg/L)	907	7153	3413	1279	-
COD (µg/mL)	139	111	323	315	5.00
SO_4^{2-} (µg/mL)	595±7	2486±38	1422±22	930±16	250.00
PO_4^{3-} (µg/mL)	0.05±0.03	0.08±0.00	0.06±0.01	0.07±0.01	-
Cl^- (µg/mL)	2.98±0.20	3.42±0.15	2.63±0.12	2.43±0.16	250.00
Fe (µg/mL)	148±2	2069±26	1011±2	313±2	0.20
Mn (µg/mL)	5.80±0.50	1.42±0.03	14.06±0.90	< LOD	0.50
Zn (µg/mL)	13.20±1.20	4.30±0.09	10.57±0.80	2.29±0.04	3.00
Cu (µg/mL)	2.81±0.01	22.39±0.06	0.24±0.01	0.90±0.03	1.00
Cr (µg/mL)	< LOD	0.05±0.01	0.07±0.008	< LOD	0.05
Pb (µg/mL)	0.26±0.02	0.95±0.02	1.42±0.10	0.57±0.01	0.01
Cd (µg/mL)	0.06±0.00	0.07±0.00	0.17±0.002	< LOD	0.003
Ni (µg/mL)	0.09±0.00	0.26±0.01	0.20±0.01	0.09±0.00	0.02
Co (µg/mL)	0.05±0.01	0.34±0.01	0.18±0.00	0.07±0.00	-
Sb (µg/mL)	0.02±0.00	0.41±0.00	0.23±0.01	0.16±0.00	0.005
Au (µg/mL)	< LOD	< LOD	< LOD	< LOD	-
Ag (µg/mL)	< LOD	< LOD	< LOD	0.11±0.00	-

*Limit value according to the Ordinance on natural mineral and natural spring waters (Službeni glasnik BiH, 26/10); < LOD– below detection limit

Content of heavy metals

Following heavy metals Mn, Zn, Cu, Cr, Pb, Cd, Ni, Co, Sb, Au and Ag were analysed by flame atomic absorption spectrometry (FAAS) in spring waters from spa Guber Srebrenica. The content of iron was very high and was determined by volumetric method. Iron content ranged from 148 mg/mL (Mali Guber) to 2069 mg/mL (Očna voda). The obtained results shows that the concentrations of iron in samples of Guber spring waters are far above the limit value. Such a high concentration of iron is one of the main features of healing springs. According to the amount of iron, Očna voda is ranked on third place of the European mineral springs (Hasić,*et al.*, 1976).

Srebrenica mineral waters which contain $FeSO_4$ and sulphuric acid are unstable in contact with atmospheric oxygen. The water quickly turns yellow and slurred, causing brown precipitate of $Fe(OH)_3$. Additionally, while it is consumed during the oxidation of $FeSO_4$ to $Fe_2(SO_4)_3$, the amount of free sulphuric acid increases during the precipitation of $Fe(OH)_3$ (Miholić, 1954). Since $Fe(OH)_3$ already starts to precipitate from a weak acid solution (pH = 3), it is a characteristic of all Guber spring waters that after a short-time exposure to air, they begin to precipitate iron (III) hydroxide and are, thus, necessary to be conserved (e.g. by vitamin C). Guber waters lose almost all of their medicinal qualities for health-care purposes after a longer period of time, with the exception of Očna voda which has a lower pH amounting to 1.67. The content of metals except iron was presented in Figure 2.

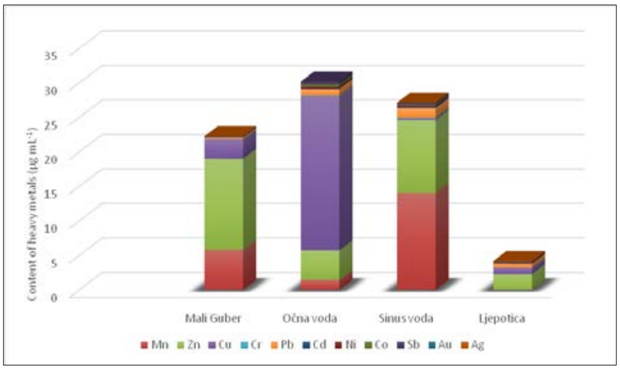

Figure 2. Content of heavy metals in the water springs Guber, Srebrenica

The concentration of heavy metals varied between different springs (Figure 2). By concentrations in different water springs, the elements were arranged as the following descending series:

Mali Guber: Fe>Zn>Mn>Cu>Pb>Ni>Cd>Co>Sb
Očna voda: Fe>Cu>Zn>Mn>Pb>Sb>Co>Ni>Cd>Cr
Sinus voda: Fe> Mn>Zn>Pb >Cu>Sb>Ni>Co>Cd
Ljepotica: Fe>Zn>Cu>Pb>Sb>Ag>Ni>Co

Enrichments of microelements are significant (Mn = 14.06 µg/mL and Pb = 1.42 µg/mL in Sinus voda, Zn = 13.20 µg/mLin Mali Guber, Cu = 22.39 µg/mL in Očna voda).

Concentrations of some heavy metals in the spring waters were compared with the limit values prescribed by the Ordinance on natural mineral and natural spring waters (Službeni glasnik BiH, 26/10). Obtained concentration for most of metals were above these limit values.

The resulting concentrations of most analysed parameters were the highest in the spring Očna voda.

Although Srebrenica has always been known for its mineral deposits of silver, the content of this metal in the concentration of 0.11 µg/mLwas only determined in the Ljepotica spring. The Ljepotica spring is particularly popular among women, because the washing water results in more beautiful and healthier complexion, and cleaner facial skin. Moreover, many users of this water healed and removed facial acne and blackheads (Pašagić, 2008). Scientific studies have shown that colloidal silver quickly "kills bacteria". Perhaps the presence of silver is one of the reasons for the effective purposes of Ljepotica on skin diseases.

The results were compared with the results given in the previous published paper (Lenoble, *et al.*, 2013). Generally, the obtained results in this study are in agreement with the previous published data. There are no available previously published papers relating to Sinus voda. First results for Sinus voda are presented in this paper.

CONCLUSION

The physico-chemical properties (temperature, pH, conductivity, chemical oxygen demand (COD) and total solid after evaporation at 105°C), the content of metals (Fe, Mn, Zn, Cu, Cr, Pb, Cd, Co, Sb, Au and Ag) and content of anions (Cl^-, SO_4^{2-}, PO_4^{3-}) were determined in four spring waters (Mali Guber, Očna voda, Sinus voda and Ljepotica) near Srebrenica town (Bosnia and Herzegovina). Correlations between physical-chemical analyzed parametres and concentration of metals and anions of the water samples were also evaluated.

Chemical analysis showed that spring waters contain very high concentrations of elements, particularly iron, and also of many other heavy metals and sulphate (causing low pH).

The highest concentrations of iron (2069 µg/mL) and sulphate (2486 µg/mL) and the lowest pH (1.67) were measured at spring water Očna voda. The concentration of other metals and anions varied between different springs. High sulphate concentration results in the low pH of these spring waters. A good correlation was found between pH-sulphate, pH-phosphate, conductivity-sulphate and conductivity-total solid after evaporation at 105°C.

Bearing in mind that people still use these spring waters for health-care purpose; that there is a plan for restarting the bottling of these mineral waters; that some plans for reactivation of the spa Guber Srebrenica are under consideration, further evaluation of chemical analysis is necessary.

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Summary/Sažetak

Izvorske vode Guber u Srebrenici (Bosna i Hercegovina) koristile su se stoljećima i koriste se u zdravstvene svrhe. Istraživači navode 48 mineralnih izvorarazličitih hemijskih sastava. U ovom radu određene su fizikalno-hemijske osobine, sadržaj teških metala i aniona u četiri odabrane izvorske vode (Mali Guber, Očna voda, Sinus voda i Ljepotica). Jako nizak pH i veoma visoka koncentracija željeza i sulfata nađena je u svim izvorima. Najviša koncentracija željeza (2069 µg/mL) i sulfata (2486 µg/mL) i najniži pH (1.67) izmjereni su za izvor Očna voda. Koncentracije ostalih metala i aniona varirale su između različitih izvora. Izračunate su korelacije između fizikalno-hemijskih parametara i koncentracija metala i aniona uzoraka vode. Rezultati za izvor Sinus voda predstavljeni su po prvi put u ovom radu.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266

UDC: ________________________

Technical Paper

2017

49

49-52

Incorporation of astronomy topics in the chemistry curriculum at Gymnasiums in Canton Sarajevo

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Article info
Received: 14/10/2017
Accepted: 12/12/2017

Keywords:
Gymnasium
Astronomy topics
Education in chemistry
Curriculum of chemistry
Astrochemistry

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Abstract: Astronomy, like no other scientific discipline, combines elements from almost the whole spectrum of research, from high energy physics to philosophy and psychology. It is expected that chemistry, as one of the fundamental sciences, finds significant place in this ever increasing field of frontier research. Astronomy topics in gymnasiums in Canton Sarajevo are, at present time, part of physics and geography programs. This paper explains how study of astronomy can be progressed by its incorporation in gymnasium subject such as chemistry. Topics can be chemical composition of celestial bodies, organic molecules present in gas clouds and exotic types of matter not found on Earth. The benefit of this incorporation does not hold only at purely educational level but expands on the goal of bringing somewhat abstract and fascinating ideas of reality beyond the tangible borders of Earth with the aim of increasing the interest of students in the subject of chemistry.

INTRODUCTION

Probably one of the earliest memories of any individual are those of the landscapes and night sky. The celestial phenomena have fascinated humanity from the inception of human civilization, regardless of age. Indeed, the prospects of great beyond had marked our lives in ways we can hardly quantify. It was in no small part to these that science and civilization have progressed side by side to reach the heights of today.

Research in astronomy is conducted observation and experimentation as is the case with all natural sciences. The observation comes from studying light sources and their interaction with outer spare materials and objects and experimentation by analyzing samples obtained by the manned mission and robotic probes. This has lead to vast application of chemistry principles and methods, such as the analysis of emission and absorption spectra of light originating from objects in space (Sun, other stars, nebulae…), content determination of samples of soil from Mars, asteroids and comets by robotic laboratories located on unmanned probes and so forth. This has also lead to the rise of the field of astrochemistry and cosmochemistry. (Muminovic, 2014)

In educational systems of Bosnia and Herzegovina gymnasiums represent the continuation of general education after elementary schools without clear vocational direction, with the exception of a mild increase in certain groups of subjects in higher years depending on students choice. As astronomy is in general one of the most fascinating subjects for students in general, this study aims to explain ways how it can be incorporated in chemistry curriculum with the aim of increasing the student interest in chemistry as well as natural sciences in general.

The current chemistry curriculum for gymnasiums contains a wide range of topics with the aim of familiarising students with basic facts from all areas of chemistry. As chemistry is significantly interconnected with other natural sciences it is possible to make connections with them in certain lessons and same goes for other natural sciences. This movement has gained ground in recent years and is now commonly referred to as STEM (science, technology, engineering and mathematics). This paper aims to show how that integration can be achieved in case of chemistry and astronomy on level of gymnasiums.

INCORPORATION SEGMENTS

This part aims to elaborate on the specific astronomy themes that can be incorporated at different class years in accordance with present curriculum:

First year

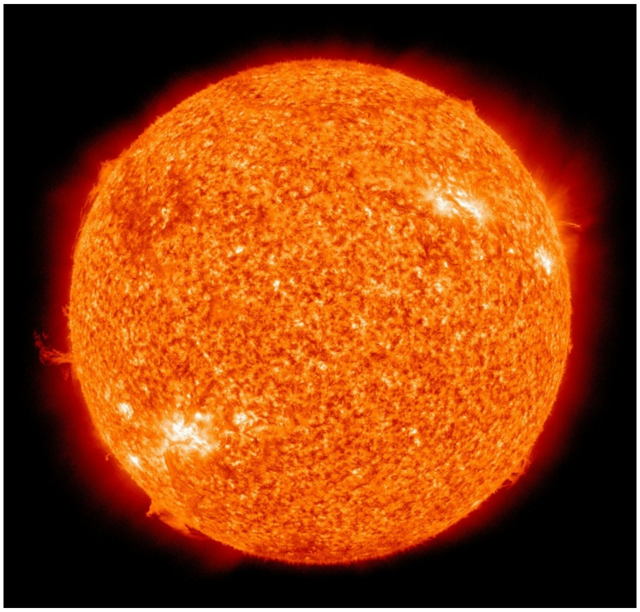

Figure 1: The Sun

The first year chemistry gives the main emphasis on the basic chemistry laws and principles and gives an introduction to chemical elements and their basic properties.While discussing gases and their characteristics added segments would be the composition of atmospheres on different planets and interplanetary bodies as opposed to Earth and gas cloud called nebulae. In addition to basic properties and aggregate states, this paper suggests incorporation of exotic state of hydrogen called “metallic hydrogen” that exists in systems of high pressure where hydrogen is compressed enough to become a solid electrical conductor.

With the discussion of quantum physics and work of Max Planck, the example of black body radiation can be based on the spectrum of Sun and how that has allowed astronomers to determine its composition and temperature. It would also include the nucleosynthesis of elements in the Big Bang explosion and in subsequent synthesis during bombardments of atoms with cosmic rays, fusion in stars and supernova explosions as part of studying the periodic table of elements. This also enables establishing astrochemistry and cosmochemistry distinct disciplines that bridge chemistry and astronomy and cosmology respectively (Muminovic, 2014).

Second year

The second year chemistry deals with organic and biochemistry topics. Here it is suggested that topics would include the presence of basic organic molecules in atmospheres of different objects and gas clouds with a comparison between different compositions, as well as examples of biomolecules such as amino acids. As many astronomical photographs are made in IR spectra this could be correlated with the tendency of organic molecules to be excitable while subjected to IR light.
(Muminovic, 2014; Cameron, P.J. (1968)

Figure 2: Orion nebula imaged in IR spectrum; credit: ESO/H. Drass et al.

Third year

The third-year curriculum includes discussion of inorganic chemistry that would be an opportunity to include information about diversification of elements in Earth's crust in comparison to other objects in space, as well as universe in general, with the emphasis on rare elements on Earth that are abundant in space with example of these being presence of hydrogen and helium u Earth’s atmosphere in comparison to the Sun. Space probes can be discussed in the topics of analytical chemistry as small robotic laboratories applying instrumental analytical methods. (Muminovic, 2014; Richter, N.B., 1963; Bizony, P. 1998)

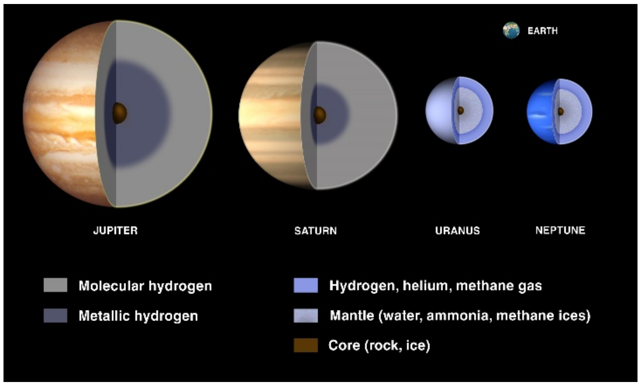

Figure 3: Comparison of chemical composition of Jovian planets; source: Lunar and Planetary Institute

Fourth year

The fourth year includes the student projects in ecochemistry and it is suggested to add a project that explores the influence of solar cycles and space debris on Earth. (Muminovic, 2014)

TEACHING TOOLS

As the aim of incorporation of astronomy is in opening new horizons to students it is suggested to make significant use of multimedia, simulations and digital photographs from different past and ongoing missions from space as well from Earth-based observatories. These include European Southern Observatory, Hubble Space Telescope, different National Aeronautics and Space Administration (NASA) and European Space Agency (ESA) missions and so forth. The multimedia and simulations provide a new dimension to teaching in addition to benefits provided by astronomy subjects in terms of providing teaching benefits. Also with the rising popularity of citizen science undertakings, students could be actively engaged with the aim of encouraging scientific initiative. This can include interactive web application Galaxy Zoo that enables participants to classify galaxies from image library and therefore engage in scientific astronomy firsthand. Another interactive experience would be the use of online simulation such as http://astro.unl.edu/.

CONCLUSION

By analyzing the current chemistry curriculum it has been determined that suggested additions can be made without requiring significant restructuring of lessons and textbooks, as these can be incorporated as additional materials presented within currently planned lessons. With the addition of multimedia and interactive materials, these could also serve a purpose as giving new insights and view on chemistry from angles of another natural science.

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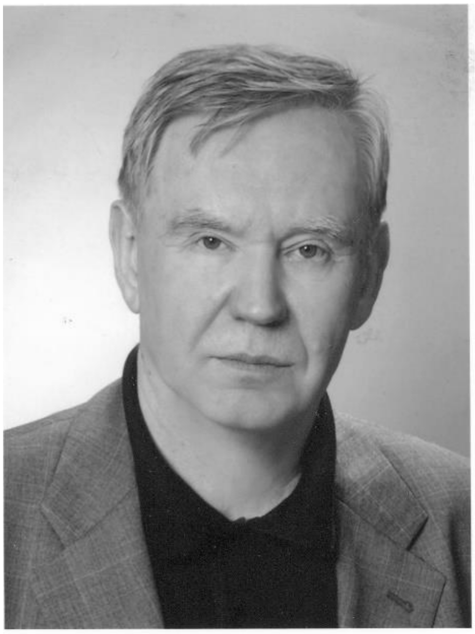
Summary/Sažetak

Astronomija, kao nijedna druga naučna disciplina, kombinuje elemente iz skoro cijelog spektra načnih istraživanja, od fizike visokih energija do filozofije i psihologije. Za očekivati je da hemija, kao jedna od fundamentalnih prirodnih nauka, nalazi značajno mjesto u ovom polju vrhunskih istraživanja koje se konstantno širi. Tematika astronomije u gimnazijama u Kantonu Sarajevo je, u ovom trenutku, dio programa fizike i geografije. Ovaj rad objašnjava kako učenje o astronomiji može napredovati uz njegovu inkorporaciju u okviru gimnazijskih predmeta poput hemije. Teme mogu biti hemijski sastav nebeskih tijela, organske molekule pristune u oblacima gasa i egzotični tipovi materije koji se ne mogu naći na Zemlji. Prednost ove inkorporacije se ne ogleda samo na čisto edukacijskom nivou, već što radi dalje na cilju donošenja donekle apstraktnih i fascinantnih ideja realnosti izvan dodirnih tačaka Zemlje sa ciljem povećanja interesa učenika za predmet hemije.

IN MEMORIAM



Prof. dr. Borivoj Galić

(1938–2017)

S velikom tugom smo primili vijest o smrti našeg dragog profesora i prijatelja Prof. dr. Borivoja Galića koji nas je napustio 20. novembra 2017. godine, u 80. godini života.

Profesor Galić je rođen 1938. godine u Sarajevu. Završio je studij hemije na Prirodno-matematičkom fakultetu 1964. godine. Godine 1966. zaposlio se na Prirodno-matematičkom fakultetu Univerziteta u Sarajevu kao asistent na Katedri za fizikalnu hemiju. Magistrirao je 1971. godine na Katedri za fizikalnu hemiju, a doktorirao 1975. godine na Prirodno-matematičkom fakultetu Univerziteta u Sarajevu.

Na Katedri za fizikalnu hemiju ostao je zaposlen sve do odlaska u penziju 2008. godine. U penziju je otišao kao vanredni profesor. Njegov odlazak u penziju nije značio prestanak rada, čak naprotiv. Baš tada je imao najviše ideja i elana da radi. Može se reći da je njegov rad u tom periodu bio najplodniji, što ga je održavalo sve do zadnjih dana.

Njegova veza sa hemijom je duža nego što postoji PMF. On je jedan od ljudi koji su podizali PMF od samih početaka. Bio je jedan od pokretača osnivanja Hemijskog instituta i Instituta za genetičko inženjerstvo i biotehnologiju.

Na PMF-u je bio u funkciji šefa Katedre za fizikalnu hemiju, šefa Odsjeka za hemiju, voditelj postdiplomskog studija u Odsjeku za hemiju, predsjednik Savjeta PMF-a, prodekan za opšte poslove i finansije, kao i mnoge druge funkcije.

Profesor Galić je bio priznati građanin Sarajeva, pogotovo među ljudima iz privrednog sektora. Njegova veza sa privredom je prepoznata 1984. godine kada je dobio 27-julsku nagradu za primjenu naučnih znanja u privredi, najveće priznanje koje se moglo dobiti u SR Bosni i Hercegovini.

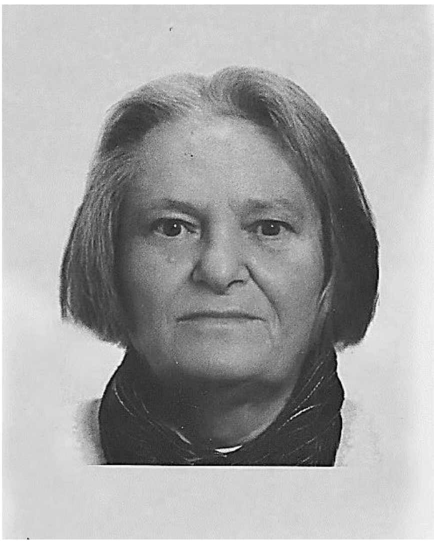
Pored značajnog doprinosa nauci, prof. Galić je bio svestrana osoba. Naime, bio je veoma uspješan sportista. Bio je reprezentativac Jugoslavije u velikom i malom rukometu. Profesor Galić bio je veoma pozitivna ličnost, njegova najveća osobina je pomaganje ljudima u svakom smislu. On je jednostavno pronalazio zadovoljstvo u pomaganju.

Kao njegovi studenti, kolege i saradnici drago nam je što smo imali priliku upoznati takvog čovjeka, te od njega naučiti mnogo toga iz nauke kao i života. Ostavio je veliki trag u našim srcima i uvijek će ostati dio nas i Katedre za fizikalnu hemiju.

Dragi profesore, rekli bismo Vam da počivate u miru, ali to se kaže ljudima koji su umrli. Fizikohemičari ne umiru, oni postižu termodinamičku ravnotežu.

Katedra za Fizikalnu hemiju

IN MEMORIAM



Doc. dr. Vjeročka Šišlov

(1936-2017)

Vjeročka Šišlov rođena je 1936. godine u Daruvaru, Hrvatska. Osnovno obrazovanje stekla je u Daruvaru i Zavidovićima, a srednjoškolsko u Visokom i Sarajevu. Studij hemije upisala je u Sarajevu 1956. godine, gdje je po odbrani diplomskog rada 1961. godine stekla diplomu Diplomirani hemičar.

U martu 1963. godina je izabrana za asistenta na Katedri za fizikalnu hemiju Odsjeka za hemiju Prirodno-matematičkog fakulteta u Univerziteta u Sarajevu. U periodu od 1966. do 1970. dodine boravila je u Engleskoj i to godinu dana na St. Andrews univerzitetu u Škotskoj i dvije godine na Imperial College-u u Londonu gdje je odbranila doktorsku tezu iz oblasti fizikalne organske hemije.

Godine 1972. je izabrana u zvanje docenta na Katedri za fizikalnu hemiju gdje je izvodila nastavu iz predmeta Fizikalna hemija II što obuhvata oblasti principa građe atoma, hemijske veze, strukturu molekula, jonske provodnike i termodinamiku elektrohemijskih procesa. Nekoliko godina izvodila je nastavu iz predmeta Fizikalna hemija I, što obuhvata hemijsku termodinamiku, statističku termodinamiku i kinetičku teoriju gasova. Deset godina izvodila je nastavu iz predmeta Fizikalna hemija za studente Poljoprivrednog fakulteta Univerziteta u Sarajevu. Preko deset godina izvodila je nastavu iz predmeta Instrumentalne metode za studente nastavničkog smjera hemije.

I kao asistent a i kao nastavnik, vodila je računske i eksperimentalne vježbe iz osnovnih predmeta fizikalne hemije opšteg smjera, te za studente Farmaceutskog fakulteta Univerziteta u Sarajevu. Na postdiplomskom studiju u Odsjeku za hemiju je izvodila nastavu iz oblasti realnih gasnih smjesa i tečnih dvokomponentnih mješovitih rastvarača. Bila je mentor u oko 40 diplomskih radova, te nekoliko magistarskih radova.

Naučna oblast iz koje su publikovani radovi je homogena kataliza, te hemijske ravnoteže u mješovitim rastvaračima sa posebnim aspektom na međumolekularnu interakciju komponenata rastvarača.

Na Prirodno-matematičkom fakultetu bila je u funkciji šefa Katedre za fizikalnu hemiju, te šefa Odsjeka za hemiju. Na Katedri za fizikalnu hemiju ostala je zaposlena sve do odlaska u penziju 2005. godine. I kao penzioner bila je aktivna i nastavila je obrazovanje u oblasti homeopatije o čemu nam je pričala prilikom svojih posjeta.

Vjeročka Šišlov, kao osoba sa duboko usađenim osjećajem odgovornosti, pristupala je svakom poslu, pa tako i profesorskom, veoma savjesno. Bila je samozatajna, skromna, čestita i uvijek spremna za druženje i razgovor i poneku šalu, pa makar i na vlastiti račun.

Ostaje nam zadatak da se sa puno zahvalnosti sjećamo drage profesorice, naše šefe, kako smo je od milja zvali.

Vesna Marić
Sabina Gojak-Salimović

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4. type of contribution,
5. a statement that the article is original and is currently not under consideration by any other journal or any other medium, including preprints, electronic journals and computer databases in the public domain, and
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    - ***Experimental*** section should give the purity and source of all employed materials, as well as details of the instruments used. The employed methods should be described in sufficient detail to enable experienced persons to repeat them. Standard procedures should be referenced and only modifications described in detail.
    - ***Results and Discussion***should include concisely presented results and their significance discussed and compared to relevant literature data. The results and discussion may be combined or kept separate.
    - The inclusion of a ***Conclusion***section, which briefly summarizes the principal conclusions, is highly recommended.
    - ***Acknowledgement*** (optional).
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e) Patents:
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f) Chemical Abstracts:
Habeger, C. F., Linhart, R. V., Adair, J. H. (1995). Adhesion to model surfaces in a flow through system. *Chemical Abstracts,* CA 124:25135.

g) Standards:
ISO 4790:1992. (2008). *Glass-to-glass sealings - Determination of stresses*.

h) Websites:
Chemical Abstract Service, www.cas.org, (18/12/2010).

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The following is the recommended style for analytical and spectral data presentation:

1. ***Melting and boiling points***:
   mp 163–165°C (lit. 166°C)
   mp 180°C dec.
   bp 98°C
   Abbreviations: mp, melting point; bp, boiling point; lit., literature value; dec, decomposition.
2. ***Specific Rotation***:
   $[a]^{23}_{D}$ –222 (*c* 0.35, MeOH).
   Abbreviations: a, specific rotation; D, the sodium D line or wavelength of light used for determination; the superscript number, temperature (°C) at which the determination was made; In parentheses: *c* stands for concentration; the number following *c* is the concentration in grams per 100 mL; followed by the solvent name or formula.

3. ***NMR Spectroscopy***:

   ^{1}H NMR (500 MHz, DMSO-d_6) d 0.85 (s, 3H, CH_3), 1.28–1.65 (m, 8H, 4´CH_2), 4.36–4.55 (m, 2H, H-1 and H-2), 7.41 (d, *J* 8.2 Hz, 1H, ArH), 7.76 (dd, *J* 6.0, 8.2 Hz, 1H, H-1'), 8.09 (br s, 1H, NH).

   ^{13}C NMR (125 MHz, $CDCl_3$) d 12.0, 14.4, 23.7, 26.0, 30.2, 32.5, 40.6 (C-3), 47.4 (C-2'), 79.9, 82.1, 120.0 (C-7), 123.7 (C-5), 126.2 (C-4).

   Abbreviations: d, chemical shift in parts per million (ppm) downfield from the standard; *J*, coupling constant in hertz; multiplicities s, singlet; d, doublet; t, triplet; q, quartet; and br, broadened. Detailed peak assignments should not be made unless these are supported by definitive experiments such as isotopic labelling, DEPT, or two-dimensional NMR experiments.
4. ***IR Spectroscopy***:

   IR (KBr) n 3236, 2957, 2924, 1666, 1528, 1348, 1097, 743 cm^{-1}.

   Abbreviation: n, wavenumber of maximum absorption peaks in reciprocal centimetres.
5. ***Mass Spectrometry***:

   MS *m/z* (relative intensity): 305 (M^+H, 100), 128 (25).

   HRMS–FAB (*m/z*): $[M+H]^+$calcd for $C_{21}H_{38}N_4O_6$, 442.2791; found, 442.2782.

   Abbreviations: *m/z*, mass-to-charge ratio; M, molecular weight of the molecule itself; M^+, molecular ion; HRMS, high-resolution mass spectrometry; FAB, fast atom bombardment.
6. ***UV–Visible Spectroscopy***:

   UV (CH_3OH) l_{max} (log e) 220 (3.10), 425 nm (3.26).

   Abbreviations: l_{max}, wavelength of maximum absorption in nanometres; e, extinction coefficient.
7. ***Quantitative analysis***:

   Anal.calcd for $C_{17}H_{24}N_2O_3$: C 67.08, H 7.95, N 9.20. Found: C 66.82, H 7.83, N 9.16.All values are given in percentages.
8. ***Enzymes and catalytic proteins relevant data:***

   Papers reporting enzymes and catalytic proteins relevant data should include the identity of the enzymes/proteins, preparation and criteria of purity, assay conditions, methodology, activity, and any other information relevant to judging the reproducibility of the results[1]. For more details check Beilstein Institut/STRENDA (standards for reporting enzymology data) commission Web site (http://www.strenda.org/documents.html).

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[1] For all other data presentation not mentioned above please contact Editor for instructions.

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- All tables (including title, description, footnotes),
- Manuscript has been "spellchecked" and "grammar-checked",
- References are in the correct format for the journal,
- All references mentioned in the Reference list are cited in the text, and *vice versa*.

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Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266

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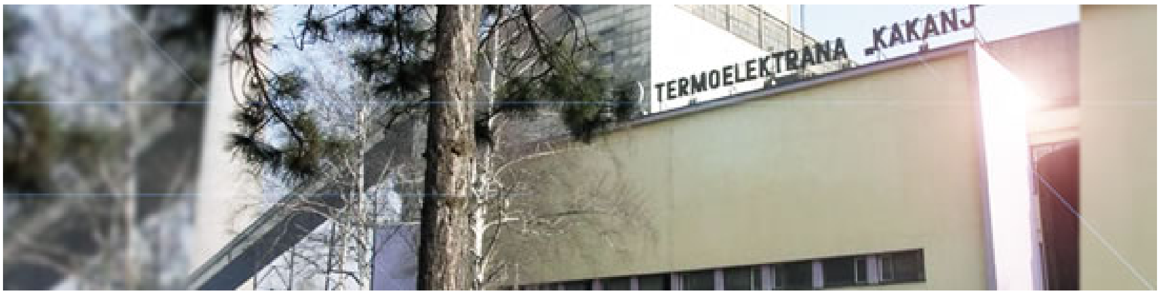

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