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CONTENT / SADRŽAJ

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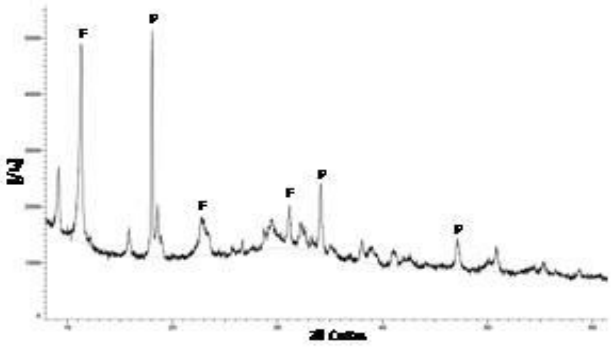
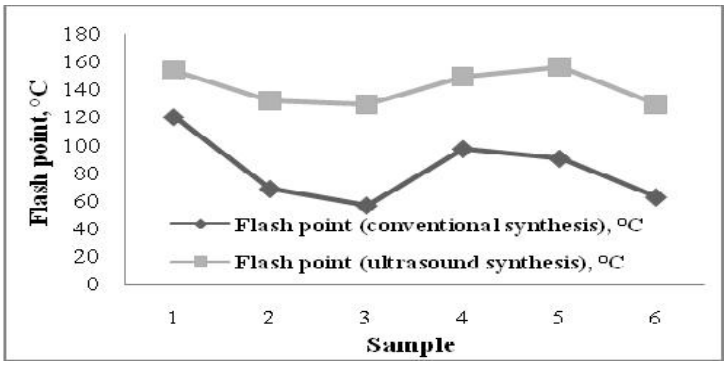
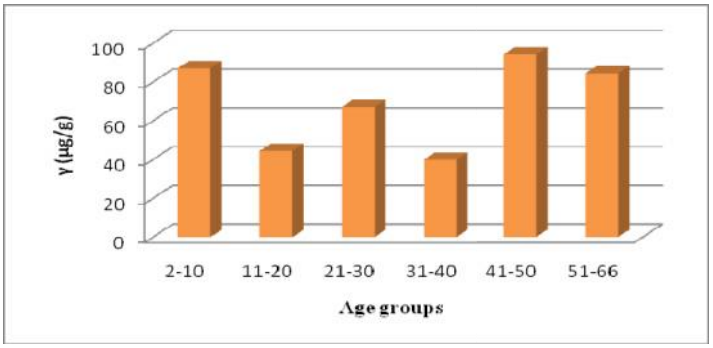
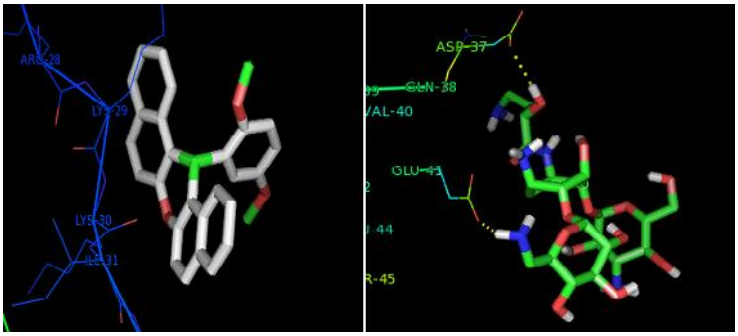
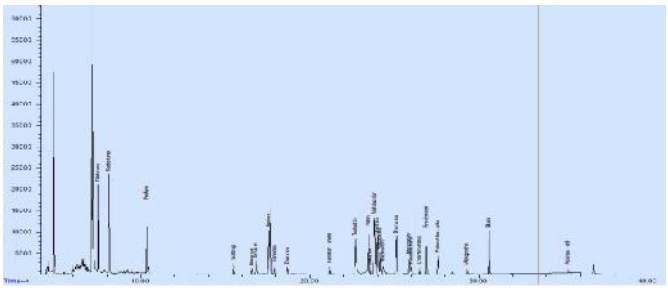
Humidity (%)	69,56	85,89	82,74	83,71	88,51
VTS (%)	74,53	65,89	67,12	65,90	60,59
Ash (%)	25,47	34,11	32,88	34,10	39,41
pH value	7,00	8,00	8,00	8,00	8,00
HPK (mg/g)	73,56	32,60	50,91	33,63	42,27
BPK$_5$ (mg/g)	19,84	-	-	13,00	27,23
VFA (mg/g)	30,01	14,36	19,96	3,59	4,31
Organic Nitrogen-N (mg/g)	9,10	-	-	0,96	1,06
Ammonia-NH$_3$ (mg/g)	2,74	-	-	6,00	8,50
Sulphides (mgS^{-2}/g)	0,740	0,240	0,400	0,470	0,460
Orthophosphate-P (mg/g)	0,150	0,118	0,122	0,116	0,118

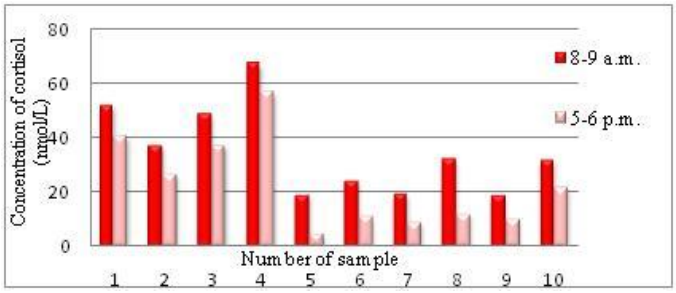


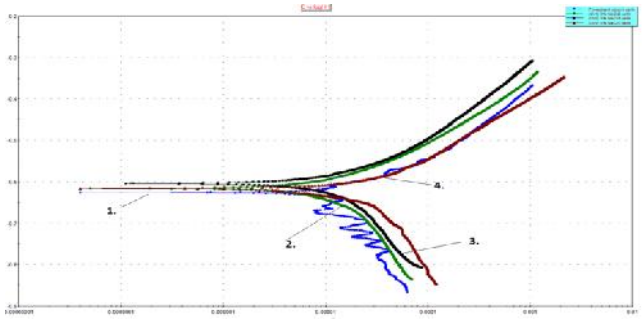


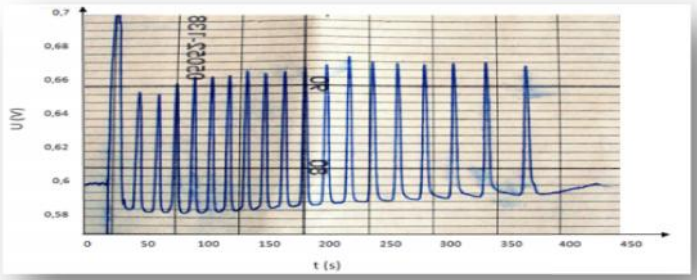












Editorial

Sneezing is not always the symptom of a cold. Sometimes, it is an allergic reaction to something in the air. An allergen is a substance that can cause an allergic reaction. In some people, the immune system recognizes allergens as foreign or dangerous. As a result, the immune system reacts by making a type of antibody called IgE to defend against the allergen. This reaction leads to allergy symptoms. These allergic reactions are most commonly caused by pollen and mold spores in the air, which start a chain reaction in our immune system. As a ritual, each spring summer and fall, tiny particles known as pollen are released from trees, grasses and weeds. Pollen is transported by air currents and enters human noses and throats, triggering an allergic reaction named allergic rhinitis, also known as Pollen Allergy.

The word pollen is derived from the Greek word meaning 'fine flour' and the role of the pollen grain is to fertilise the female flower to reproduce plant species. Pollen grains can be spread by birds, bees or wind. Pollination times vary with the plant variety and its location. For example, trees pollinate in late winter and early spring. Grasses flower next, and the weed 'Plantain' flowers from August through to May. Grass pollen numbers are also higher in inland areas, where there are no natural barriers to wind dispersal.

An allergic reaction can be caused by any form of direct contact with the allergen—eating or drinking a food you are sensitive to (ingestion), breathing in pollen, perfume or pet dander (inhalation), or brushing your body against an allergy-causing plant (direct contact, generally resulting in hives).

Almost any substance in the environment can be an allergen. The list of known allergens includes plant pollens, spores of mold, animal dander, house dust, foods, feathers, dyes, soaps, detergents, cosmetics, plastics, and drugs.

Editors

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Original Scientific Article

Biogas from Poultry Manure

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Abstract: It is estimated that around 2 billion of waste annually is formed in the European Union (EU), which is deposited-in sanitary and industrial dumps, and it is recorded continuous increasing in the production of organic waste. Biodegradability of organic substances enables the emission of CH_4 (biomethane) that has 25 times higher the greenhouse potential than CO_2 as a predominant greenhouse gas in the atmosphere. The treatment of organic waste through anaerobic digestion flourished in the 20th century, thus opening the way to the environmental remediation of manure, as one of the most influential source of methane emissions. This study research the production of methane in poultry manure, as one of the most usual animal fertilizers in Bosnia and Herzegovina. The research activities are comprised of waste characterization and testing the biodegradability of waste using Biomethan Potential Test BMP The temperature range of the study was 37 ± 1°C (mesophilic process). The research topic is manure of laying hens, without bed. The conducted research activities have shown the existence of possibilities for the production of biogas by using anaerobic biological treatment of poultry manure. The yield of biogas in the amount of 5752 mL was achieved with a methane content of 53.19% for the treatment of the substrate with 15% total solid (TS). By treating the substrate with 17% total solid (TS) less biogas is obtained, in the amount of 2337.50 mL of biogas but with a higher content of methane, in the amount of 56.36%. Physico-chemical analysis revealed a deviation ratio of C(COD):N:P:S from the optimal ratio for substrate and digestate, which caused disturbances in the performance of the anaerobic digestion process. Inadequate ratio is expressed with low carbon content and a high content of nitrogen and sulfur. The course of the study has proven to be extremely useful for testing the possibilities of biogas production in combination with other organic waste, which opens up opportunities for further research.

INTRODUCTION

Manure from all types of farms, is a potential polluter of the environment, due to the natural content and gas emission and with greenhouse effect. Also, manure is abundant with natural content of anaerobic microorganisms that produce methane (CH_4), which has 25 times higher the greenhouse effect than CO_2, which is the most abundant gas with greenhouse effect in the atmosphere. Among all of the manures, most intense emission of methane has bovine manure.

The utilization of the anaerobic digestion (AD) process has been recognized since ancient times, but it flourished in the 20th century. The treatment of organic waste, from which energy can be obtained in the form of methane, is carried out indoors at a temperature that corresponds to anaerobic microorganisms (mesophilic or thermophilic).
As the manure is naturally rich with anaerobic microorganisms and nutrients, it is suitable material for biological treatment, where as final products biogas and digestate are formed. It can be treated alone or in

combination with other organic waste (co-digestion), such as waste from the harvest, straw, corn silage, organic communal waste, etc., which are the most common. The energy value of biogas is chemically related to methane and increases according to the increasing content of methane in it. The average energy value of biogas is of 20 to 30 MJ/m^3 with a methane content of 55% to 70%. Biogas can be used for the simultaneous production of electricity and heat in cogeneration plants, it can be placed in the system of gas distribution network or used as a fuel for vehicles. The use of biogas depends on its quality.

Digest is the decomposition of anaerobic digestion, cleaned of pathogenic microorganisms and enriched with easily accessible and high-quality nutrients for plants. It is an excellent replacement for natural fertilizer. The amount of biogas and energy from manure depends on the type, weight and age of the animal. Organic waste of older animals has better biogas potential due to intensive metabolism and the formation of larger amounts of waste. Today, there are several million biogas plants for anaerobic digestion of various organic wastes, and in the European countries, the most of them are installed in Sweden, Germany, Finland, Latvia, etc. In 2013 the energy derived from biomass covered 10% of the global primary energy consumption in the amount of 56.6 EJ of energy.

MATERIALS AND METHODS

In the realization of experimental activities, as a substrate was used a manure from the poultry complex (meat industry) Brovis LLC, Donje Moštre, Visoko. Experimental activities are carried out in two parts: the physical and chemical analysis and using BMP test.

Physical and chemical analysis

Physical and chemical analysis were carried out on a sample of solid to the sludge consistency, so all the methods used for analysis are customized, in order to provide more representative and more accurate results. The analysis was conducted from a solution made of 10 g sample in a metering container of 1 L. In physico-chemical terms, the substrate and digestate (treated substrate) were analyzed. The analysis involved determining the following parameters: total solids (TS), Volatile solids (VTS), pH value, chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), volatile fatty acids (VFA), total nitrogen (N), total phosphorus (TP) in the form of orthophosphate and sulphide content (S^{2-}). Phosphorus was determined by spectrophotometry with ammonium molybdate and ascorbic acid (Čoha F., 1990). BPK_5 is analyzed in accordance with ISO 5815: 2003. The organic nitrogen (N) and ammonia (NH_3) were determined according to the Kjeldahl method (APHA, 2012). All other parameters were analyzed according to APHA, 1998.

BMP test

The BMP test was carried out using a volumetric method at the mesophilic temperature zone (37°C ± 1) for a period of 31 days of retention time. The experiments were performed in the so-called "batch" reactors fueled by the use of clogged glass bottles. BMP test apparatus used in the experimental section is shown in Figure 1.

Figure 1: BMP test apparatus

Analysis of biogas was performed by gas chromatography (SICK GMS 810).

RESULTS AND DISCUSSION

The efficiency of the anaerobic digestion depends on several key factors, and it is very important to ensure optimal conditions in the system of anaerobic microorganisms. BMP test was intended to examine the production of biogas with normal activity of anaerobic microorganisms in the substrate. The following samples were examined using BMP test:

- O1 - Substrate with 15% total solid
- O2 - Substrate with 17% total solid
- M1 - Inokulum1 (muddy consistency emerged AD settings O1) + O1
- M2 - Inokulum2 (muddy consistency emerged AD settings O2) + O2
- The content of examined mixture used in BMP test, was determined by calculating. For preparation the sample O1 was used 98.55 g substrate per100 mL water, and for the sample O2 was used 131.41 g per 100 mL water. Preparation the samples M1 and M2 was done the same like the samples O1 and O2 but on formed muddy consistency. With the realization of samples O1 and O2, a rich anaerobic environment is tried to be developed, to be used as inoculum for the realization of samples M1 and M2.

The results of physico-chemical analysis

Aims of physico-chemical analysis of substrate and digestate were monitoring of changes of the defined parameters during the anaerobic digestion process and their mutual impact. The results of physical-chemical analysis of substrate and digestate are listed in Table 1.

Analyzing the substrate, pH value of 7 was determined, which represents the optimal conditions for the operation and growth of microorganisms and correction of pH value with acid / base or the addition of buffer is not necessary. pH value of all digests did not exceed 8, which gives an

indication of weakly alkaline environment and partly a stable process that can stabilize over time. Variation of the pH environment was affected by the concentration of substances contained in the substrate, which are usually prone to change of the concentration of ammonia and VFA, and which have an inhibiting effect on the process in specific concentrations. Sulfur and heavy metals concentrations have inhibitory effect, in smaller cases. The inhibitory concentration of NH_3 is 600 mg/L, for the VFA it is 2000 mg/L (Al Seadi T. and al., 2008), while for the sulfur in the sulfide form it is 200 mg/L (Dodić J. and al., 2013).

The analysis revealed that contents of these substances in the substrate and digestate are not in the form of inhibitory concentrations.

Table 1. The results of physical-chemical analysis of substrate and digests

Parameter	Substrate	O1	O2	M1	M2
ST (%)	30,44	14,11	17,26	16,29	11,49
Humidity (%)	69,56	85,89	82,74	83,71	88,51
VTS (%)	74,53	65,89	67,12	65,90	60,59
Ash (%)	25,47	34,11	32,88	34,10	39,41
pH value	7,00	8,00	8,00	8,00	8,00
HPK (mg/g)	73,56	32,60	50,91	33,63	42,27
BPK_5 (mg/g)	19,84	-	-	13,00	27,23
VFA (mg/g)	30,01	14,36	19,96	3,59	4,31
Organic Nitrogen-N (mg/g)	9,10	-	-	0,96	1,06
Ammonia-NH_3 (mg/g)	2,74	-	-	6,00	8,50
Sulphides (mgS^{2-}/g)	0,740	0,240	0,400	0,470	0,460
Orthophosphate-P (mg/g)	0,150	0,118	0,122	0,116	0,118

It also established that substrate contained 27.60 mg/L of ammonia concentration, 300.11 mg/L of VFA, and 7.7 mg/L of sulfur in the form of the sulfide. Organic nitrogen in the amount of 92.61 mg/L (9.10 mg/g) came from the proteins present in the substrate. For digestate sample the following concentrations were determined: VFA in the amount of 143.60 mg/L and the sulfide in the amount of 2.42 mg/L. For digestate sample O2 the following concentrations were determined: VFA in the amount of 196.63 mg/L and the sulfide in the amount of 4.04 mg/L. Ammonia and organic nitrogen were not analyzed in samples O1 and O2. These samples were intended for the adaptation and development of micro-organisms, namely for the creation of anaerobic environments to be used as inoculum for M1 and M2. For digestate sample M1, the following concentrations were determined: ammonia in the amount of 62.72 mg/L, VFA in the amount of 35.95 mg/L and the sulfide in the amount of 4.73 mg/L. For digestate sample M2, the following concentrations were determined:ammonia in an amount up to 89.60 mg/L, VFA in the amount of 43.15 mg/L and the sulfide in the amount of 4.65 mg/L. Low phosphorus content is explained by the fact that it was determined in the form of orthophosphate, total phosphorus was not determined.

The most important assessment of the quality of the substrate and of the monitoring of anaerobic digestion is the ratio C(COD):N:P:S.

The optimal ratio of C(COD):N:P:S is 600:15:5:1 (Al Seadi T. and al., 2008, Imamović N., 2014). In this study through the physico-chemical analysis it is possible to determine the ratio of C(COD):N:P:S for M1 and M2 substrate and digestate samples. The analysis determined the relation C(COD):N:P:S -598.30:74.01:1.22:6.00 for substrates, 594.32:140.67:0.24:8.31 for digestate sample M1 and 598.23:135.30:0.20:6.51 for digestate sample M2. The deviation from the optimal ratio resulted because of the low carbon content in relation to the nitrogen and sulfur. According to the ratio, nitrogen deviates 4.9 and sulfur 6 times more in the substrate, nitrogen by 9.4 and sulfur 8.3 times more in digestate sample M1, while in the digestate sample M2 nitrogen deviates by 9.02 and sulfur 6.51 times more. Unbalanced ratio C(COD):N:P:S can lead to increased production of NH_3, H_2S, increasing the partial pressure of hydrogen (H_2) and reducing the production of VFA, which results in slowing or stopping the process.

Variations of those components cause changes in the pH environment and increase the sensitivity of bacteria in the methanogenic stage. The optimum ratio of the pH value

for the operation of microorganisms is 7-7.5, while the deviation of pH value leads to disturbances for the process of anaerobic digestion. Great amount of NH_3 observed in digestate M1 and M2 encourages the conversion of undesirable amino acids in NH_3 instead of in acetate. Although none of the digestates did not contain inhibitory concentration of NH_3 in the amount of 600 mg/L, the increased concentration of NH_3 had a braking effect on the implementation anaerobic digestion phase. The impact expected of undesirable proteins conversion is less production of biogas and methane content.

The impact of free NH_3 was sought to avoid with the dilution method of substrate in assay mixture, which reduced the total solid (ST) content and provided contact of anaerobic environment with substrate nutrients. However, due to the low content of organic carbon, anaerobic organisms spent more organic matter for the the formation of NH_3, H_2S, CO_2, NO_x and H_2, than for the formation of VFA. The formation of these gases led to a slowing of the process of anaerobic digestion. In phase of acetogenesis, homoacetogenic bacteria consume H_2 and CO_2 to form acetate to maintain a stable condition of low partial pressure of H_2 required for the process (Kukkonen T., 2014). The activity of these bacteria could not come to the fore due to the excessive accumulation of these gases and low-carbon content. The variation of TS and VTS results in digestate determined by physical and chemical analysis, originate from different activities of anaerobic microorganisms in phases of anaerobic digestion process.

The results of the analysis of digestate substrate inoculum in samples M1 and M2 revealed low amount of VFA which leads to the consumption of VFA in acetogenesis and methanogenesis processes. However due to a deviation of C(COD):N:P:S substrate ratio from the optimal, it is possible that there has been a brake in the formation of VFA influenced by unwanted gaseous products formed in phases of acidogenesis and of acetogenesis.

The aforementioned settings revealed increased ash content, which gives an indication that nutrients were not adequately spent in microbial food chain. Increased ash content came from the the formation of salts of heavy metals with sulfur and phosphorus.

A component which additionally affected breaking of the process is the partial pressure of hydrogen (H_2). This intermediator is a normal product of acidogenesisandacetogenesis phases of production, but due to insufficient carbon content, there was a non-conforming production of VFA in oxidation reactions and consumption of H_2 in respiratory reactions, where additionally,the production of VFA was slowed by NH_3 and H_2S. This is a normal occurrence in bioreactors, which becomes neutralized by adding more substrate rich in carbon, and by waiting for the system to return to a stable state. The lack of experimental activities is the interruption of the process after the 31st day.

The results of BMP test

In theory, the retention time of the mesophilic temperature is between 20 to 40 days (Ward A.J. and al., 2008). According to research of Kukić, S. and associates (2010) biogas production begins after the third day, and the maximum production is achieved on the 15th to the 20th day and reduces by the end of the retention time. The results of volumetric analysis of BMP test are shown in the diagram 1.

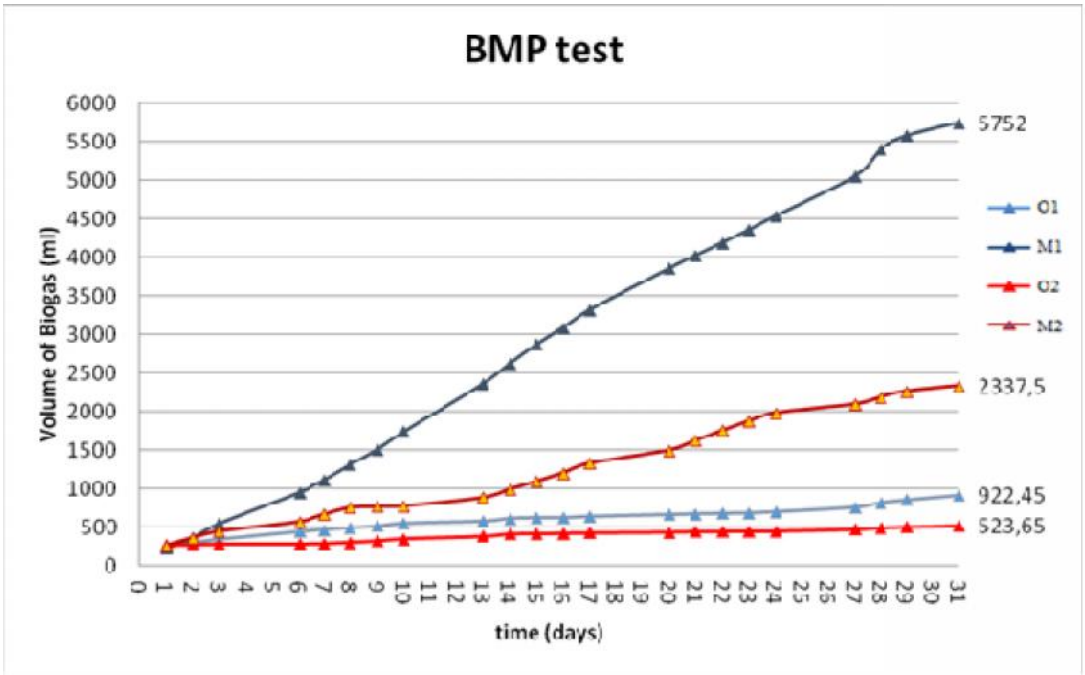

Figure 2. The results of BMP test

Using volumetric analysis of O1 and O2 samples, a slight inclination/slope is noticed to the end of the retention time. Although the daily volumes by the end of the analysis fluctuated, the process was terminated after thirty days. With the addition of new quantities of raw material of the substrate on the muddy consistency as inoculum in the settings of the M1 and M2, there was a remarkable activity of microorganisms and the production of biogas increases. According to physical-chemical analysis, allowable concentrations of influential substances in the substrate were established. However, their relationship which is expressed through optimal ratio C(COD):N:P:S is not satisfactory, because of the low content of organic carbon in relation to other substances. The deviation from the optimum relationship had an impact on the process of anaerobic digestion, where in the already formed biogas there was an increased extrication of NH_3, H_2S, CO_2 and H_2, which inhibited the methane separation. The disturbances in the quality of the production of biogas were already noticed in the settings of O1 and O2 samples, in which the conversion of cumulative production of methane from theoretical amounted only 36.35% for O1 and15.48% for O2. The low conversion was expected because of the sensitivity of anaerobic microorganisms since for their adaptation, a nutritious methanogens base wasn't used. The improvement in the quality of anaerobic digestion process was noticed in the settings of M1 sample, where the conversion of methane production amounted from cumulative to theoretical up to 95.18%, while for the M2 it was only 26.81%. Positive results in setting of the M1 can be seen in the diagram, where they grow without constant flow to the end of the retention time. In both M1 and M2 settings, the constant flow at the end of the retention time was not noticed, which means that AD was not completed until the end.The analysis of biogas on 31st day with gas chromatography revealed methane content in the amount of 53.19% for the sample of biogas settings M1 and 56.36% for the sample of biogas settings M2.

CONCLUSION

Animal manure is naturally abundant with anaerobic microorganisms which in the process of aerobic digestion consume nutrients from the manure and produce gases with greenhouse effect that accumulate in the atmosphere. The aim of the study was to examine the treatment of anaerobic digestion of one of the most usual aminal waste in Bosnia and Herzegovina, poultry manure, which is currently used as fertilizer for arable land. This study, testing the quality of the substrate determined deviation of C(COD):N:P:S ratio from optimal, but it did not prevent the process of anaerobic digestion to continue with a certain production of biogas and methane content.

The ratio C(COD):N:P:S for the substrate is 598.30:74.01:1.22:6.00, 594.32:140.67:0.24:8.31 for the digestate sample M1 598.23:135.30:0.20:6.51 for the digestate sample M2. Regardless of disturbances, the positive results were recorded in the settings of M1 and M2 samples, where the addition of a new quantity of nutrients has led to improvements in the quality of anaerobic digestion process. The poultry manure has a rather low methane potenthial with variation of substances necessary for the anaerobic digestion process. The analysis of biogas sample O1 found methane content in the amount of 33.80% and in the O2 biogas sample it amounted to 29.48%. To improve the quality of the substrate it must be combined with waste rich in oxygen. With the addition of new quantities of nutrients, an improvement in the production of biogas was visible, with an increase of a methane content by 1.5 times in M1 (53.19%) and 1.9 times in M2 (59.36%). The same substrate was used for co-digestion, but it would be useful to examine the co-digestion with another type of organic waste (sawdust, straw, agricultural waste, municipal organic waste). Combining different types of biomass should be tested in order to eliminate the negative impact on the efficiency of the entire process.

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Summary/Sažetak

U zemljama Europske Unije (EU) procjenjuje se da godišnje nastane oko 2 biliona otpada koji se odlaže na sanitarnim i industrijskim deponijama, a zabilježen je kontinuirani porast nastajanja organskog otpada. Biorazgradivost organskih tvari omogućava emisiju CH_4 (biometan) koji ima 25 puta veći stakleнički potencijal od CO_2 kao najzastupljenijeg stakleničkog plina u atmosferi. Tretman organskog otpada anaerobnom digestijom doživljava procvat u 20 vijeku čime otvara puteve ekološke sanacije stajskog gnojiva kao jednog od najuticajnijih izvora emisije metana. U radu ispitana je produkcija bioplina i sadržaja metana iz peradarskog đubriva, kao jednog od najzastupljenijeg animalnog otpada u Bosni i Hercegovini. Istraživačke aktivnosti su se sastojale od karakterizacije otpada i ispitivanja biorazgradivosti otpada putem BMP testa. Temperaturni opseg istraživanja je bio 37 ± 1°C (mezofilni proces) u trajanju od 31 dan. Predmet istraživanja je svjež gnoj nesilica, bez postelje. BMP testom ostvaren je prinos bioplina u iznosi od 5752 ml bioplina sa sadržajem metana od 53,19% za tretman supstrata sa 15% suhe tvari. Tretiranjem supstrata sa 17% suhe tvari dobiveno je manje bioplina, u iznosu od 2337,50 ml, ali sa većim sadržajem metana, u iznosu od 56,36 %. Fizičko-hemijskom analizom utvrđen je odstupanje omjera C(HPK):N:P:S od optimalnog, kod supstrata i digestata, što je prouzrokovalo smetnje u odvijanju procesa anaerobne digestije. Neadekvatan omjer izražen je niskim sadržajem ugljika, a visokim sadržajem azota i sumpora. Tok istraživanja pokazao se izuzetno korisnim za potrebe ispitivanja mogućnosti proizvodnje bioplina u kombinaciji sa drugim organskim otpadom, što otvara mogućnosti daljeg istraživanja.

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Original Scientific Paper

Investigation of the influence of magnesium chloride to sulfate corrosion of concrete

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Abstract: The paper research subject is to examine whether chlorides of magnesium chloride block the affect of sulphate of magnesium sulfate to the concrete, that is, whether they block the sulfate corrosion of concrete. The cylindrical samples of cement paste measuring 80 × 40 mm of varying water-cement ratio 0.5 and 0.7 (kg H_2O / kg of cement) were prepared for implementation of planned research. The samples were immersed and treated for nine months in following solutions: 1% $MgCl_2$, 1% $MgSO_4$, 1% $MgCl_2$ + 1% $MgSO_4$, 5% $MgCl_2$, 5% $MgSO_4$, 5% $MgCl_2$ + 5% $MgSO_4$. In order to study concrete corrosion, the samples were dried, grounded and analyzed by X-ray diffraction analysis (XRD), after being removed from the solutions. Treated cement paste samples in solutions of $MgSO_4$, the concentration of 1% $MgSO_4$ and 5% $MgSO_4$, at both water-cement ratios form ettringite formula $3CaO{\cdot}Al_2O_3{\cdot}3CaSO_4{\cdot}31H_2O$.Ettringite occurs in reactions of sulfate and aluminate hydrate and could lead concrete structure to a state of destruction. The reaction is also known as sulphate corrosion of concrete. In joint solutions of magnesium salt of concentration of 1% $MgCl_2$ + 1% $MgSO_4$ and 5% $MgCl_2$ + 5% $MgSO_4$, at both water-cement ratios, chlorides with aluminate hydrate form monochloroaluminate hydrate formula $3CaO{\cdot}Al_2O_3{\cdot}CaCl_2{\cdot}10H_2O$, while sulfates react with calcium hydroxide to gypsum without forming expansive ettringite on samples of the cement paste. Blocking the formation of expansive ettringite verifies the blocking of sulfate corrosion of concrete by chloride from magnesium chloride.

INTRODUCTION

A great threat towards concrete and reinforced concrete structures represent magnesium salts, mainly sulphates and chlorides. The basic processes that take place in cement stone in the presence of magnesium salt solution are based on their reaction with calcium hydroxide, known in the chemistry of cement as "portlandit" (Mladenović, 2008):

$$Ca(OH)_2+MgSO_4+2H_2O \rightarrow CaSO_4{\cdot}2H_2O+Mg(OH)_2 \quad (1)$$

$$Ca(OH)_2+MgCl_2 \rightarrow CaCl_2+Mg(OH)_2 \quad (2)$$

The product reaction of (1) and (2) is magnesium hydroxide, whose solubility is small, 18.2 $mgdm^{-3}$at room temperature, and as such precipitates from the solution. Binding of OH^- ions in the magnesium hydroxide is accompanied by reduction of pH value of the solution in the pores of the cement stone to pH = 10 (Mladenović, 2008). Reduction of pH of concrete leads to increased corrosion intensity of concrete and steel reinforcement. Otherwise, the pH of the solid concrete pores is in the interval from 12.5 to 13.5, thanks to the calcium hydroxide

generated through the process of cement hydration (Angst, 2011; Đureković, 1996). In addition to the basic reactions, reaction 1 and 2 in the concrete exposed to a solution of a magnesium salt lead to other reactions that occur between the mineral cement hydrate, chloride and sulfate, and magnesium ions. Among the most dominant reactions, as the research results show, are the reactions between the aluminate hydrate and chloride as well as sulphate of magnesium. Chlorides that penetrate to concrete from the environment enter into reaction with aluminate hydrate. The most common product of the reaction of chloride and aluminate hydrate is monochloroaluminate hydrate formula $3CaO{\cdot}Al_2O_3{\cdot}CaCl_2{\cdot}10H_2O$, known as the Friedel's salt. (Hirao et al. 2005; Sumranwanich et al. 2004; Hewlett, 1998; Bikić et al. 2009).

In the presence of magnesium sulfate in the surface layers of concrete, a magnesium hydroxide forms, while hydrated calcium aluminum sulfate hydroxide forms in the inner layers $3CaO{\cdot}Al_2O_3{\cdot}3CaSO_4{\cdot}31H_2O$, known in the chemistry of cement as "ettringite" (Mladenović, 2008; Đureković, 1996). Ettringite is the phase that occurs in concrete, in reactions of sulfate and aluminate hydrate and could lead concrete to a state of destruction. The reaction is known as sulphate corrosion of concrete. In some studies dealing with the test of concrete exposed to seawater, it was found that chlorides slow down the affect of sulphate ions to concrete. Without the presence of chloride, ettringite may form in the concrete in the presence of sulphate, while gypsum forms in the presence of chloride (Mladenović, 2008). However, a more detailed literature review about chloride role at the impact of sulfate on concrete point to the three scholar thoughts: (a) in the presence of chloride the sulfate intensity affect on the concrete increases, (b) in the presence of chloride the sulfate intensity affect on the concrete decreases, (c) there no significant impact of chloride at the affect of sulfates on concrete (Hossain et al. 2005). The paper research subject is to examine whether chlorides block the affect of sulphate on the concrete, that is, whether they block the sulfate corrosion of concrete.

EXPERIMENTAL

The cylindrical samples of cement paste measuring 80 × 40 mm were prepared for implementation of planned research. For sample preparation the Portland cement class PC 42.5 with following clinker mineralogical composition was used: alit, tricalcium silicate (C_3S) - 66.37%; belit, dicalcium silicate (C_2S) - 8.32%; tricalcium aluminate (C_3A) - 9.62%, celite, and tetracalcium alumoferit (C_4AF) - 10.77%. The samples were prepared in varying water-cement ratio 0.5 and 0.7 (kg H_2O / kg of cement). After preparation, the samples were placed in molds in a heated space with relative humidity of at least 90%. The molds were held in such state for 24 hours. After demolding, the samples were immersed in the following solutions: 1% $MgCl_2$, 1% $MgSO_4$, 1% $MgCl_2$ + 1% $MgSO_4$, 5% $MgCl_2$, 5% $MgSO_4$, 5% $MgCl_2$ + 5% $MgSO_4$. In the above mentioned solutions, the samples were treated at room temperature for the next 9 months. After 9 months, the cement paste samples were extracted from the above solution, dried in an oven at 105° C to constant weight, cooled in a desiccator and then grounded. In order to study the corrosion of concrete, the samples were analyzed after grinding using X-ray diffraction analysis (XRD). In addition, certain phases of importance were followed in assessing the corrosion of concrete. In this paper, three phases were followed, namely portlandite, ettringite and monochloroaluminate hydrate. Diffraction lines of highest intensity of portlandite are located at the following angles: 2Θ: 11,2˚ (I/I_0 = 100 %), 22,6˚ (I/I_0 =60 %), 30,9˚ (I/I_0 = 50 %). Diffraction lines of highest intensity of ettringite are located at the following angles: 2Θ: 9,65˚ (I/I_0 = 100 %), 5,58˚ (I/I_0 = 80 %), 3,21˚ (I/I_0 = 60 %). Diffraction lines of highest intensity of monochloroaluminate hydrate are located at the following angles: 2Θ: 11,2˚ (I/I_0 = 100 %), 22,6˚ (I/I_0 =60 %), 30,9˚ (I/I_0 = 50 %) (Bikić et al. 2009; Perkins, 2000; ARL X'TRA, 2008). Mineralogical analysis of cement clinker and x-ray diffraction analysis of the cement paste samples (XRD) were carried out on "X-Ray Diffractometer SIEMENS D 5000" device.

RESULTS AND DISCUSSION

Figures 1, 2, 3 and 4 show diffraction patterns of the cement paste treated in solutions of 1% $MgCl_2$ and 5% $MgCl_2$ of varying water-cement ratio.

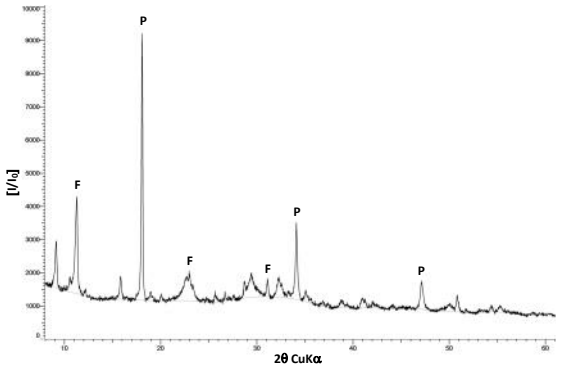

Figure 1. X-ray diffractogram of a sample treated in a solution of 1% $MgCl_2$ v/c=0,5

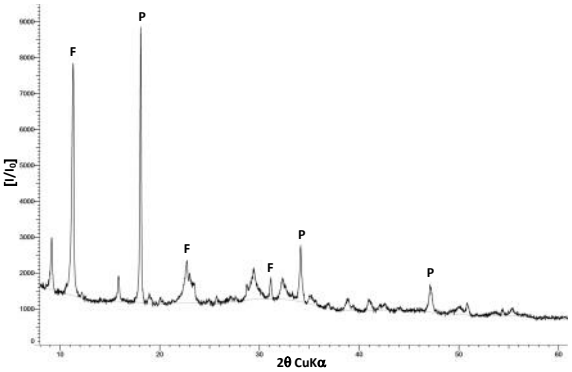

Figure 2. X-ray diffractogram of a sample treated in a solution of 1% $MgCl_2$ v/c=0,7

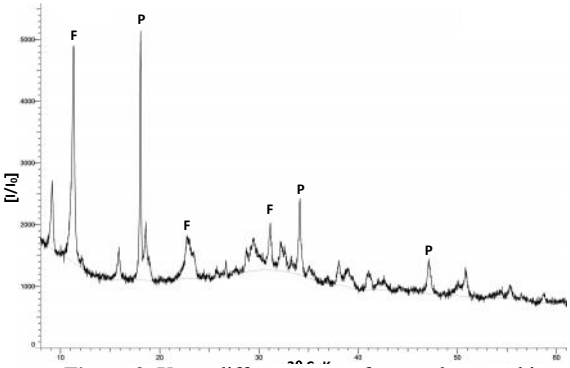

Figure 3. X-ray diffractogram of a sample treated in a solution of 5% $MgCl_2$ v/c=0,5

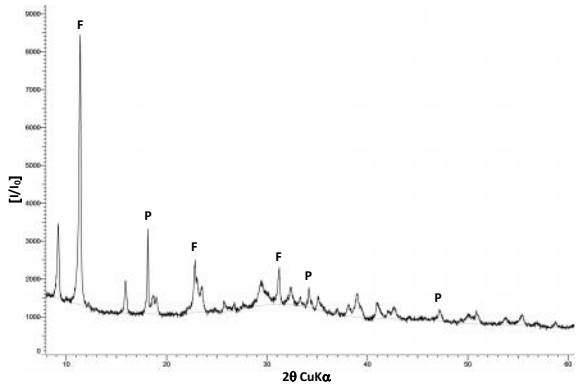

Figure 4. X-ray diffractogram of a sample treated in a solution of 5% $MgCl_2$ v/c=0,7

Figures 1, 2, 3 and 4 detect diffraction lines of portlandite, mark P, and monochloroaluminate hydrate, mark F. Diffraction lines of monochloroaluminate hydrate prove that there has been a reaction between chlorides that penetrate the concrete, the cement paste, and aluminate hydrate, at both concentrations of $MgCl_2$ and water-cement ratios. The formation of monochloroaluminate hydrates negatively impact concrete because it leads to the destruction of aluminate hydrate, one of the main constituents of concrete.

Figures 5, 6, 7 and 8 show diffractograms of cement paste treated in solutions of 1% $MgSO_4$ and 5% $MgSO_4$ of varying water-cement ratio.

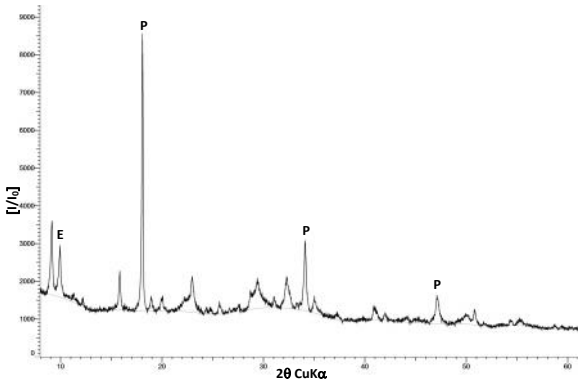

Figure 5. X-ray diffractogram of a sample treated in a Solution of 1% $MgSO_4$ v/c=0,5

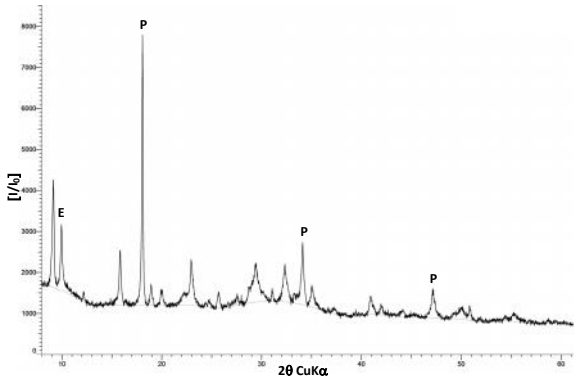

Figure 6. X-ray diffractogram of a sample treated in a solution of 1% $MgSO_4$ v/c=0,7

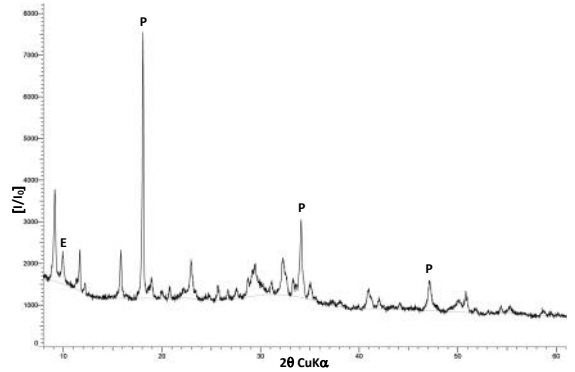

Figure 7. X-ray diffractogram of a sample treated in a solution of 5% $MgSO_4$ v/c=0,5

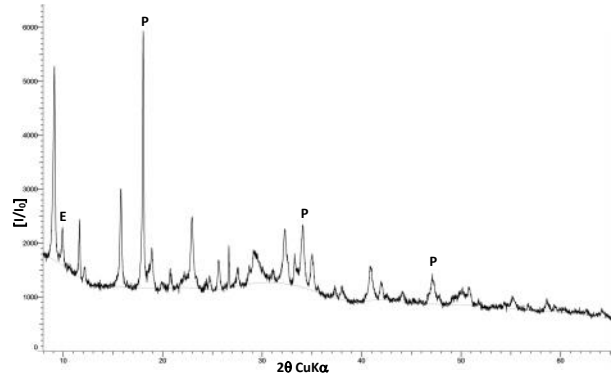

Figure 8. X-ray diffractogram of a sample treated in a 5% $MgSO_4$ v/c=0,7

Figures 5, 6, 7 and 8 detect diffraction lines of portlandite, mark P, and ettringite, mark E. Diffraction lines of ettringite prove that there has been a reaction between the sulfate of magnesium sulfate that penetrate the concrete and aluminate hydrate, at both concentrations of $MgSO_4$ and water-cement ratios. This also verifies that the samples of cement paste treated in solutions of $MgSO_4$ of both concentrations show sulphate corrosion of concrete. Key research carried out in this paper shown in Figures 9, 10, 11 and 12, represent the influence of magnesium chloride on the sulfate corrosion of concrete.

Figures 9, 10, 11 and 12 show comparative diffractograms of cement paste treated in joint solutions of magnesium salt 1% $MgCl_2$ + 1% $MgSO_4$, then 5% $MgCl_2$ + 5% $MgSO_4$, and concentration solutions of 1% $MgSO_4$ and 5% $MgSO_4$, while fluctuating water-cement ratio. The intensity of diffraction lines is proportional to the amount of certain mineral species in the testing sample (Petrovski, 2006).

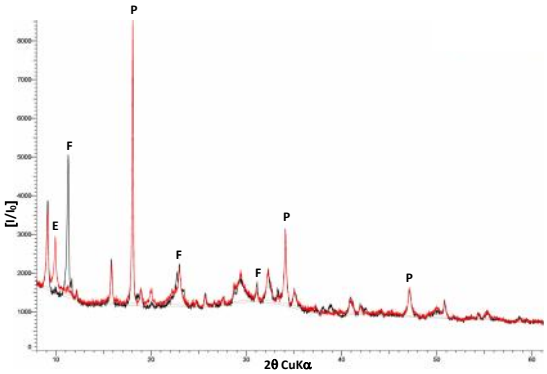

Figure 9. X-ray diffractograms of a samples treated in a solutions of: Black - 1% $MgSO_4$ + 1% $MgCl_2$ v/c=0,5; Red - 1% $MgSO_4$ v/c=0,5

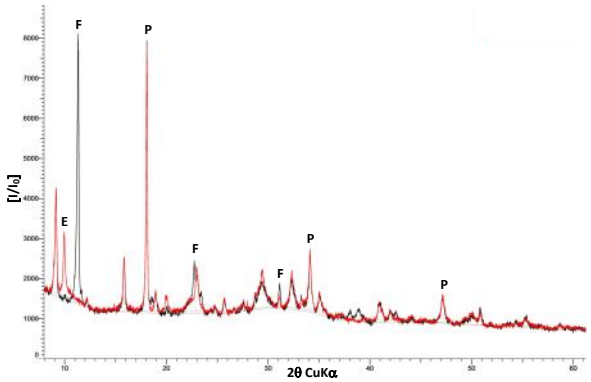

Figure 10. X-ray diffractograms of a samples treated in a solutions of: Black - 1% $MgSO_4$ + 1% $MgCl_2$ v/c=0,7; Red - 1% $MgSO_4$ v/c=0,7

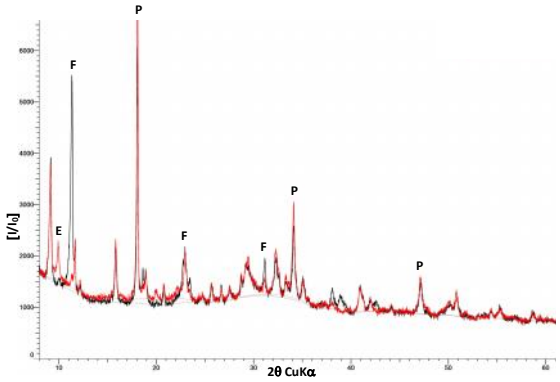

Figure 11. X-ray diffractograms of a samples treated in a solutions of: Black - 5% $MgSO_4$ + 5% $MgCl_2$ v/c=0,5; Red - 5% $MgSO_4$ v/c=0,5

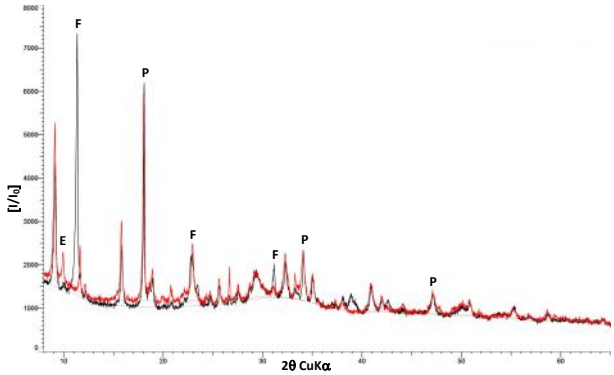

Figure 12. X-ray diffractograms of a samples treated in a solutions of: Black - 5% $MgSO_4$ + 5% $MgCl_2$ v/c=0,7; Red - 5% $MgSO_4$ v/c=0,7

Figures 9, 10, 11 and 12 depict that the addition of a solution of $MgCl_2$ to $MgSO_4$ block the formation of ettringite. As Figures 9, 10, 11 and 12 depict, diffraction lines of ettringite, mark E that appears on the samples of cement paste treated in solutions of $MgSO_4$, the concentration of 1% $MgSO_4$ and 5% $MgSO_4$, at both water-cement ratios, is disappearing in samples treated in joint solutions of magnesium salts. This proofs that chlorides of $MgCl_2$ block the formation of sulfate corrosion of concrete. That means that joint solutions of magnesium salts ($MgCl_2$ + $MgSO_4$) of higher and lower concentrations of chlorides of magnesium chloride form monochloroaluminate hydrate, while sulfates of magnesium sulfate react with portlandit to gypsum without forming expansive ettringite later on.

CONCLUSIONS

Researching the influence of magnesium chloride to sulfate corrosion of concrete, the following was verified:

- the XRD method verifies that treated cement paste samples in solutions of $MgCl_2$ of concentration of 1% $MgCl_2$ and 5% $MgCl_2$, in both water-cement ratios (v / c = 0.5 and v / c = 0.7), form a monochloroaluminate hydrate $3CaO{\cdot}Al_2O_3{\cdot}CaCl_2{\cdot}10H_2O$. The same occurs in reactions of chlorides and aluminate hydrate.
- the XRD method verifies that treated cement paste samples in solutions of $MgSO_4$ of concentration of 1% $MgSO_4$ and 5% $MgSO_4$, in both water-cement ratios, form ettringite $3CaO{\cdot}Al_2O_3{\cdot}3CaSO_4{\cdot}31H_2O$. The same occurs in reactions of sulfate and aluminate hydrate and could lead to the state of destruction of concrete structure. The reaction is also known as sulphate corrosion of concrete.
- the joint solutions of magnesium salt of concentration of 1% $MgCl_2$ + 1% $MgSO_4$ and 5% $MgCl_2$ + 5% $MgSO_4$, in both water-cement ratios, verifies that the chlorides of magnesium chloride block the formation of sulfate corrosion of concrete on cement paste samples. Chloride with aluminate hydrate form monochloroaluminate hydrate while sulfates of magnesium sulfate react with portlandit to gypsum

without forming expansive ettringite later on. Therefore, in the joint solutions of magnesium salts ($MgCl_2$ + $MgSO_4$), the chlorides are inhibitors of sulfate corrosion of concrete.

ACKNOWLEDGEMENT

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Summary/Sažetak

Predmet istraživanja provedenih u ovom radu je ispitati da li hloridi iz magnezijum hlorida blokiraju djelovanje sulfata iz magnezijum sulfata na beton, odnosno da li blokiraju sulfatnu koroziju betona. Za realizaciju planiranih istraživanja pripremljeni su cilindrični uzorci cementne paste, dimenzija (80×40) mm, varirajući vodocementni faktor, 0,5 i 0,7 (kg H_2O/kg cementa). Uzorci su potapani i tretirani 9 mjeseci u sljedećim rastvorima: 1% $MgCl_2$, 1% $MgSO_4$, 1% $MgCl_2$ + 1% $MgSO_4$, 5% $MgCl_2$, 5% $MgSO_4$, 5% $MgCl_2$ + 5% $MgSO_4$. U cilju ispitivanja korozije betona, uzorci su nakon vađenja iz navedenih rastvora, sušenja i mljevenja analizirani koristeći rendgensko difrakcionu analizu (XRD). Na uzorcima cementne paste koji su tretirani u rastvorima $MgSO_4$ koncentracija 1% $MgSO_4$ i 5% $MgSO_4$, kod oba vodocementna faktora, formira se etringit formule $3CaO{\cdot}Al_2O_3{\cdot}3CaSO_4{\cdot}31H_2O$. Etringit nastaje u reakcijama sulfata i hidrata aluminata i zna dovesti betonsku konstrukciju do stanja destrukcije. Reakcija je poznata i kao sulfatna korozija betona. U zajedničkim rastvorima magnezijumovih soli koncentracija 1% $MgCl_2$ + 1% $MgSO_4$ kao i 5% $MgCl_2$ + 5% $MgSO_4$, kod oba vodocementna faktora, hloridi s hidratima aluminata formiraju monohloraluminathidrat, formule $3CaO{\cdot}Al_2O_3{\cdot}CaCl_2{\cdot}10H_2O$, dok sulfati reaguju s kalcijum hidroksidom do gipsa, ne formirajući kasnije ekspanzivni etringit na uzorcima cementne paste. Blokiranje formiranja ekspanzivnog etringita dokaz je blokiranja sulfatne korozije betona od strane hlorida iz magnezijum hlorida.

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Original Scientific Paper

Effect of Ultrasound on Biodiesel Synthesis from Plant Oil

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Abstract: The recent studies have clearly shown that the continuous exploitation of fossil fuels has adverse effects on the environment, while reserves of oils are sufficient for about next fifty years. As the need for energy rises, so do the energy policies tend to develop and research the renewable energy sources including biodiesel. The goal of this research is to examine the ultrasound effect on biodiesel synthesis process and to optimize synthesis conditions, examining the effect of several parameters in production process and biodiesel quality. Biodiesel synthesis was processed out of unused sunflower oil and sunflower oil used in fast-food (waist oil). Particularly, the ultrasound effect on production process-transesterification reaction was examined. After synthesis, examination of density, viscosity, flash point and yield were done using suitable apparatus and methods. The study results proved that the most optimal temperature with the use of ultrasound is 60°C, sonication time of 15 minutes and alcohol-oil molar ratio 3:1. Also, the results proved that using ultrasound during biodiesel synthesis, transesterification reaction can be processed on lower temperatures and still, biodiesel of good quality can be produced, contrary to conventional synthesis. Using the ultrasound generator for laboratory biodiesel synthesis largely shortens reaction time, increases rate of chemical reaction, decreases by-product amount, decreases alcohol amount, decreases waste water and, in the end, saves energy because the reaction is faster and takes less time at lower temperatures.

INTRODUCTION

In recent years, fatty acid methyl esters derived from vegetable oil, commonly known as biodiesel, have gained importance as alternate fuel for diesel engines. Due to closed carbon cycle, biodiesel does not contribute to global warming. An analysis of the life cycle of biodiesel shows that overall CO_2 emissions are reduced by 78% compared with petroleum-based diesel fuel(Gerpen*et al.,* 2005;Sheehan*et al.,*1998).

Biodiesel also has a relatively high flash point (150°C), which makes it less volatile and safer to transport or handle than petroleum diesel(Zhang *et al.,* 2003). Biodiesel can be synthesized by chemical, enzymatic and microbial methods. Alkali based esterification is the most often applied approach(Ranganathn*etal.,*2008). However, there are some drawbacks such as problems in the recovery of glycerol, the need to remove the catalyst, the

requirement to use oils without free fatty acids and high energy consumption. A high level of interest from industry initiated research concerning non-catalytic biodiesel synthesis from rapeseed oil in supercritical methanol, which has a conversion of 80% (350^{o}C, 45–65MPa, 240 s). Recently, the application of the ultrasound and hydrodynamic cavitation has also been reported to significantly intensify the synthesis of biodiesel (Gogate*et al.,* 2009; Kelkar*et al.,* 2008). It was demonstrated that hydrodynamic cavitation was about 40times more effective than acoustic methods (Gogate*et al.,* 2008; Wu *et al.,* 2007). The most important parameters that influence the transesterification reaction are: reaction temperature, ratio of alcohol to vegetable oil, type of acyldonor and acceptor, type and amount of catalyst, mixing intensity, quality (purity, free fatty acid content) of starting materials and water content(Nielsen *et al.,* 2008).

However, in this field, interest is increasingly moving in the direction of finding ways to minimize the cost of biodiesel production. The problem can be approached in two different ways: by finding a cheap source of raw material (Peterson, 1986; Alcantara*et al.,* 2000) or by intensifying the synthesis process by using novel reactors based on the use of enzymes, microwaves, supercritical fluids, ultrasound, or fluid energy (Kelkar*etal.*, 2008).

EXPERIMENTAL

Unused sunflower oil and used sunflower oil (used in fast food restaurants) were used for biodiesel production. KOH (p.a, Merck), methanol (p.a, Sigma Aldrich), ethanol (p.a, Sigma Aldrich), 2% phenolphthalein,ethanolic solution (Merck, p.a.) were used as received.

Used oil was purified by filtration to remove mechanical impurities (food residues). After that, oil was heated up to 105^{o}C during five to ten minutes, to remove possible water (Andričić, 2006).

Conventional synthesis was carried out at 65°C, with constant mixing (magnetic stirrer) during 45, 90 and 120 minutes. Methanol-oil molar ratio was 3:1. This ratio was chosen on the basis of experimental data from 12 synthesis samples (changing parameters: reaction time, catalyst type, oil type) (Čišija, 2013). Biodiesel synthesis using ultrasound generator (PHYWE, 800 kHz, 16 W/cm^2) was carried out on 25°C, 45°C and 60°C during 15,30 and 45 minutes, respectively. Methanol-oil molar ratios were 3:1, 6:1 and 9:1 (Gude*et al.,* 2013).

Product characterisation, after purification, was carried out by viscosity measurement (Ostwald), density measurement (pycnometer), flash point measurement (Marcuson), yield calculation (Vatrenjak-Velagić,1997).

RESULTS AND DISCUSSION

The goal of this research was to examine the ultrasound effect on the biodiesel synthesis process and to optimize synthesis conditions (shorten reaction time, to carry out reaction at lower temperatures, to get high quality biodiesel with economical benefit), contrary to conventional synthesis.

The results of biodiesel produced via conventional synthesis are given in Table 1. All reactions were carried out at 65°C during 45, 90 and 120 minutes with changing next parameters: reaction time, catalyst type, amount of catalyst and oil type. Alcohol-oil molar ratio for all reactions was 3:1 (Čišija, 2013).

Contrary to conventional synthesis, synthesis via ultrasound gave a product with much better characteristics (Lima *et al.,*2012),even at lower temperatures where conventional synthesis cannot be done, which is presented in this study.

Best quality biodiesel was produced from unused and used sunflower oil via conventional synthesis during 120 minutes with the magnetic stirrer.

Table1. Yield and biodiesel (conventional synthesis) characteristics, oil-alcohol molar ratio 3:1, temperature 65°C

Characteristic biodiesel produced out	Reaction time (min)	Unused sunflower oil	Used sunflower oil (kitchen)	Used sunflower oil (fast-food)
Density (g/mL)	45	0.8751	0.8749	0.684
	90	0.8898	0.8959	0.8801
	120	0.8983	0.8839	0.8698
Viscosity (mm^2/s) at 40°C	45	7.452	9.950	10.470
	90	7.112	9.807	9.870
	120	6.874	9.070	9.710
Flash point (°C)	45	48	44	51
	90	68	48	56
	120	97	51	62
Yield (%)	45	34.97	94.00	84.63
	90	93.00	95.41	89.23
	120	98.89	93.69	85.69

Reaction of transesterification (ultrasound synthesis) yields at 25°C were 90.56%, 93.08% and 95.75% with density 0.8691 g/mL, 0.8711 g/mL and 0.8771 g/mL. Viscosity value did not vary significantly: 6.11 mm^2/s, 5.93 mm^2/s and 5.71 mm^2/s. Also, flash point value did not vary significantly: 127°C, 129°C and 133°C.

Yields of reactions conducted at 45°C were 94.35%, 95.59% and 96.30%. Measured densities of biodiesel were 0.8832 g/mL, 0.8806 g/mL and 0.8873 g/mL. Viscosity values were 5.77mm^2/s, 5.89 mm^2/s and 5.92 mm^2/s. Flash point values were 131°C, 133°C and 135°C.

Yields of reactions conducted at 60°C were 97.50%, 98.57% and 96.40%. Measured densities of biodiesel were 0.8742 g/mL, 0.8790 g/mL and 0.8720 g/mL. Viscosity

values were pretty much close: 5.63 mm^2/s, 5.54 mm^2/s and 5.57 mm^2/s. In this case, flash point values were 138°C, 149°C and 153°C.

Colluci and co-workers (Colucci*et al.,* 2007), stated that yield of reaction which was administered by ultrasound mixing after 15 minutes at 25°C about 85%, at 40°C was around 87% and at 60°C yield was 88% (alcohol-oil molar ratio 3:1).

In the case of alcohol-oil molar ratio 6:1 at 25°C, yield was about 92%, at 40°C was 94.50%, and at 60°C yield was 97%. In the case of alcohol-oil molar ratio 9:1 at 60°C, after 15, 30 and 45 minutes, yields were just over 95%, with negligible variation.

Lima and co-workers(Lima *et al*., 2012) stated that at the 60°C (case with use of waste oil), alcohol-oil molar ratio 2.4:1, after 15 minutes yield was 98%, and after 30 and 45 minutes yields were very common99%. In the same conditions, but with conventional synthesis yield was lower, in fact, after 15 minutes was 88%, after 30 minutes was 92% and after 45 minutes was 96%.
Figure1 shows conventional reaction yield (Čišija, 2013) and ultrasound reaction yield depending onreaction time. It shows that ultrasound synthesis gives way much better yield in shorter time period contrary to conventional synthesis.

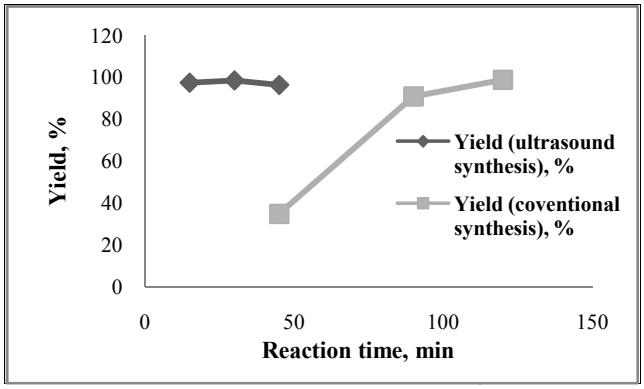

Figure1. Conventional reaction yield and ultrasound reaction yield depending on reaction time-used sunflower oil, alcohol-oil molar ratio 3:1, temperature 65°C

Figure 2.shows difference in viscosity values of biodiesel-conventional synthesis (Čišija,2013), and biodiesel-ultrasound synthesis.The flash point of a volatile material is the lowest temperature at which it can evaporate to form an ignitable mixture in air. At the flash point, the vapor may cease to burn when the ignition source is removed(Vatrenjak-Velagić, 1997).Biodiesel produced via ultrasound synthesis has much better values of viscosity: 5.49-5.72 mm^2/s. Biodiesel produced via conventional synthesis has higher values of viscosity: 9.71-10.47 mm^2/s(Čišija, 2013; Young *et al.,*1989).

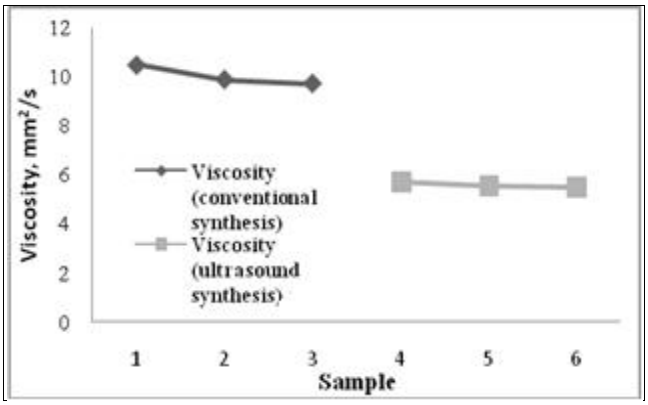

Figure 2. Dependence of biodiesel viscosity-ultrasound synthesis and conventional synthesis-used sunflower oil; alcohol-oil molar ratio 3:1

Flash point represents a major biodiesel quality factor. Biodiesels flash point is above 130°C to 180°C, which depends on raw material (Jurac, 2011). In this research, flash point values were all above 130°C (two exceptions-56°C and 120°C). The low flash point values were caused by impure chemicals (methanol) and bad mixing (magnetic stirrer)(Čišija, 2013).

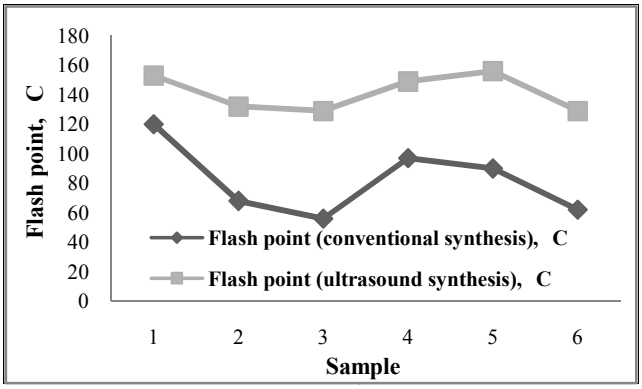

Figure 3. Dependence of biodiesel flash point, ultrasound synthesis, alcohol-oil molar ratio 9:1, and biodiesel flash point, conventional synthesis, alcohol-oil molar ratio 3:1- used sunflower oil

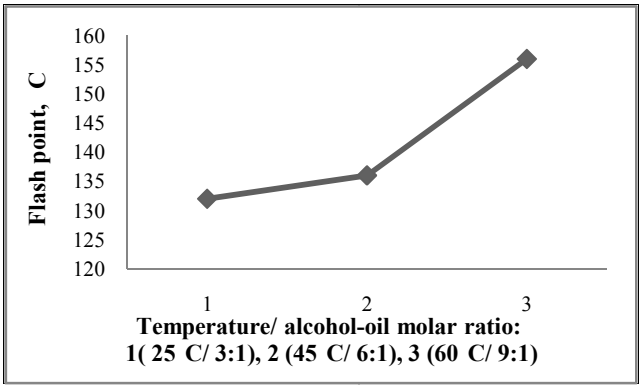

Figure 4. Flash point dependence (biodiesel produced via ultrasound synthesis) on alcohol-oil molar ratio and temperature

Comparing these results-Figures 1, 2, 3 and 4-one can conclude that using ultrasound gives much better quality of biodiesel contrary to conventional synthesis.

CONCLUSIONS

Synthesis conditions (temperature, reaction time, alcohol-oil molar ratio) have shown less effect on biodiesel produced out of unused sunflower oil, contrary to biodiesel produced out of used sunflower oil.

Considering the results, it is apparent that using the ultrasound generator, largely shortens reaction time and enhance yield, and improves biodiesel quality which, firstly, depends on raw material. Ultrasound field increasesrate of chemical reaction by intensifying mass transport and mixing. The outcome of ultrasound field is cavitation-bubble collapse-cavern which causes presence of local high temperatures, pressure and turbulence which, in the end results in speeding up chemical reaction. Also, better homogenization is a greatadvantage contrary to conventional synthesis.

Using the ultrasound generator for laboratory biodiesel synthesis largely shortens reaction time, increases rate of chemical reaction, decreases by-product amount, decreases alcohol amount, decreases waste water and, in the end, saves energy because reaction is faster and takes less time at lower temperatures.

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Summary/Sažetak

Iz dosadašnjih istraživanja vidljivo je da neprestano iscrpljivanje fosilnih goriva utiče na okoliš, a postojeće rezerve nafte dovoljne su za narednih pedesetak godina. Kako je potreba za energentima sve veća, tako se i energetska politika okreće razvoju i istraživanju obnovljivih izvora energije, među koje spada i biogorivo. Cilj ovog rada bio je istražiti postupak sinteze biodizela pomoću ultrazvuka iz biljnih ulja ispitujući uticaj odabranih parametara na proces sinteze biodizela i kvalitet istog. Sinteza biodizela rađena je iz nekorištenog suncokretovog ulja i suncokretovog ulja korištenog u fast-food-u (otpadno ulje). Posebna pažnja je posvećena djelovanju ultrazvučnog polja (kavitaciji) na proces sinteze biodizela, odnosno na hemijsku reakciju transesterifikacije. Nakon sinteza urađena su ispitivanja gustoće, viskoziteta, tačke plamišta i prinosa reakcije sinteze odgovarajućim metodama i aparaturama. Rezultati istraživanja su pokazali da je najoptimalnija temperatura reakcije transesterifikacije uz primjenu ultrazvuka na 60^0C, vrijeme djelovanja ultrazvuka od 15 minuta, te omjer alkohol-ulje 3:1. Također, rezultati su pokazali da upotrebom ultrazvuka u procesu sinteze biodizela, reakcija transesterifikacije može da se odvija i na znatno nižim temperaturama u odnosu na konvencionalnu sintezu biodizela, pri čemu se dobija biodizel zadovoljavajućih karakteristika.Primjenom ultrazvučnog generatora za laboratorijsku sintezu biodizela uveliko se skraćuje vrijeme trajanja sinteze, povećava se brzina reakcije, smanjuje se količina nusproizvoda - sapuna, može se koristiti manja količina alkohola i prilikom ispiranja smanjuje se problem otpadne vode i, naposlijetku, štedi se energija jer reakcija traje kraće i može da se odvija na nižim temperaturama.

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Analysis of Some Metals in Human Hair by the AAS Method

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Abstract: The essential and toxic elements are contained in drinking water, food and the air - in the entire general human surrounding. Considering the effects of these elements on human health, the recommended/allowed levels of their intake into the organism are defined by the national and international regulations. Those levels are an important indicator of the state an organism is, which is determined by different biological samples of human origin. In this work, the determination of the concentrations of metals was performed on human hair samples of the people living on the area of Kiseljak. The metal levels that were established in the hair were of those essential metals (copper, zinc, calcium, magnesium, iron) and of two toxic metals (chromium and cadmium). The human hair sampling was carried out during the period of November 2014. - February 2015. The group of responders was male and female donors of various ages, (2-66 years old). By the examination of the results, the specifications that were taken into consideration were the following: age and gender of the hair donor, chemical treatment of the hair, smoking habits of the donors. The technique that was applied for determining the concentration of the heavy metals in the samples was the atomic absorption spectrometry (AAS). The final results showed a normal and in some cases increased, concentration of essential metals. The content of Cr and Cd in all analyzed samples was below the limit of quantification of used technique.

INTRODUCTION

Heavy metals is a term that covers a group of elements with similar chemical properties. Some of them, including copper, iron, zinc, play an important role in human organism and are called essential metals, while others are not known as being useful for our health, or more precisely they are toxic. High concentrations of heavy metals may cause health problems (Puntaric et. al., 2012). People may commercial products of different purposes. Depending on their type and chemical properties, elements differ in their mobility in the environment and their toxic effect on plants, animals and humans. Although certain inorganic types also have different chemical properties, major differences are achieved by creation of metal-carbon links, or by creation of organometallic compounds (Bošnir, 2005). Hair, just like the fat tissue, is the organism's storage of toxic and other matters, and the longer the hair

is, the longer the period over which the analysis can determine the organism's status. Hair analysis is very important because it indicates the actual status of organism and the actual nutritional status, as well as the quantity of stored and accumulated toxins, all of which can be reliably determined only by the hair mineral analysis (Chojnacka et al., 2005). It is an analysis whose results do not vary day to day and are not subject to multiple changes like the blood count, or blood or urine tests (Chojnacka et al., 2005). Lack or increased concentration of essential trace elements in hair indicates serious problems in the physiology of human organism (Dombovari et al., 1998; (Dombovari et al., 1999). Thus, for example, a low concentration of bioelements such as Zn, Fe, Ca in human hair is a typical indicator of deficiency diseases, metabolic and physiological disorders (Katz et al., 1988). In addition to that, human hair is a very attractive biological material in terms of sampling, transporting and storage, and also because it provides information on the concentration of certain trace elements that are found in hair in much larger concentrations than in other biological samples which makes the analysis easier (Zhunk et al., 1995). For all the reasons described above, determination of contents (concentration) of trace elements in hair has been a continual activity in the field of biomedical and environmental studies over the past three decades (Arnold, Sachs, 1994), (Ciszewski et al., 1997).

EXPERIMENTAL

The experimental section of this paper is devoted to analyzing the contents of elements Cu, Zn, Ca, Mg, Fe, Cr and Cd in human hair using the method of atomic absorption spectrometry (AAS), flame technique. Hair samples were taken from 34 individuals, 14 male and 20 female. Determination of trace heavy metal concentration was performed on healthy population taking into consideration specifications such as gender, age, smoking habits and chemical treatment of hair.

Preparation of samples for analysis - Previously washed samples of human hair were cut into 0.5-1 cm long portions which were then weighed three times to within 1,0 ± 0,1 g. Acid digestion of samples was performed with concentrated HNO_3 adding 1 mL 30% H_2O_2. The solution was filtered and the contents quantitatively transferred to a 50 mL volumetric flask. Distilled water was added until the volumetric flasks were filled to the mark. These prepared solutions were used for determination of metals using the method of atomic absorption spectrometry, flame technique.

Determination of metal contents in hair samples - Contents of metals (essential and non-essential) were determined using the method of atomic absorption spectrometry - flame technique (FAAS, Spectra AA-10, Varian). The instrument was previously calibrated with standard solutions of the tested metals. Standard solutions of the metals, 1000 mg/L, are original standards of the Merck Company (Germany). Concentrations were determined using the calibration curve method.

Analytical quality control - All used reagents had the analytical grade of purity (Merck, Germany). Repeatability of results was checked by performing three tests for each sample and calculating the value of standard deviation. Given the lack of the certified reference material (CRM), analytical recovery was determined for the entire analytical procedure for all analyzed metals by adding standard metal solutions. Method accuracy was confirmed based on satisfactory recovery factor values (93.2 – 105.8%).

RESULTS AND DISCUSSION

In this paper the concentrations of copper, zinc, chrome, cadmium, calcium, magnesium and iron in 34 hair samples classified by gender, age, smoking habits and chemical treatment of hair, was determined. The respondent group consisted of male and female subjects of different age (2-66 years of age), but with the same place of residence, Kiseljak.

Table 1. Data on hair samples and abbreviated codes of samples used for analysis

Sample code	Sex	Age	Color	Smoker	Chemically treated
A	F	44	black	YES	NO
B	M	45	black	YES	YES
C	M	8	brown	NO	NO
Ć	M	14	brown	NO	NO
Č	M	60	gray	YES	NO
D	F	56	brown	YES	YES
Đ	F	33	brown	YES	YES
DŽ	M	15	brown	NO	NO
E	M	18	black	NO	NO
F	F	18	brown	NO	NO
G	F	60	gray	NO	YES
H	F	26	blond	NO	YES
I	M	2	blond	NO	NO
J	F	9	brown	NO	NO
K	M	47	black	NO	NO
L	M	25	black	NO	NO
LJ	M	41	brown	YES	NO
M	M	63	gray	YES	NO
N	M	11	brown	NO	NO
NJ	F	48	blond	YES	YES
O	F	23	brown	NO	YES
P	F	44	brown	YES	YES
R	F	41	red	YES	YES
S	F	35	blond	YES	YES
Š	F	45	blond	YES	YES
T	F	4	blond	NO	NO
U	F	27	brown	YES	NO
V	M	2	brown	NO	NO
Z	F	50	black	YES	YES
Ž	F	65	brown	YES	YES
X	M	66	gray	NO	NO
Y	F	60	brown	NO	YES
Q	F	14	brown	NO	NO
q	F	63	red	YES	YES

In all respondents the contents of toxic metals Cr and Cd was below the lower limit of quantification. Contents of the analyzed essential metals did not significantly deviate from normal values (Biolab Medical Unit, 2010) of the analyzed metals in healthy individuals and coincide with reference data from other international studies (Baranowska et al., 2004; Sokolowska et al., 2007).

Human hair is approximately 80 % protein and 15 % water, with smaller amounts of lipid and inorganic substances, and its composition also includes copper, zinc, iron and other elements (Wilson, 2007). Normal values, or intervals of concentration of some metals in hair, expressed in μg/g of hair are given in Table 2 (Biolab Medical Unit, 2010).

Table 2. Normal element concentrations in human hair (Biolab Medical Unit, 2010)

Element	Concentration (μg/g)	Element	Concentration (μg/g)
Ca	200 - 2800	Mn	0.2 – 2.00
Cr	0.10 – 1.50	Mg	60 – 160
Co	0.01 – 0.20	P	100 - 200
Cu	10 – 100	K	50 – 300
Fe	5.0 -30	Se	0.40 – 2.00
Na	50 - 1000	Zn	160 - 240
Al	< 50.0	As	< 1.00
Cd	< 0.10	Pb	< 2.00

In this work, mass concentration of seven metals was determined. Obtained results for the content of copper, zinc, calcium, magnesium and iron in hair samples will be presented in charts as mean values of concentrations. In addition to the presentation of total results, the results of analysis of these metals were compared based on gender, age, smoking habits and chemical treatment of hair. Contents of chromium and cadmium were below the limit of quantification of the used method in all samples.

Copper content in human hair samples - Concentration of copper in hair samples was in the range between 6.20 and 25.02 μg/g. Therefore, as the results show, most of the tested hair samples contain copper in concentrations which are, in most cases, slightly below the lower optimal concentration of copper, but within the range of normal values. Comparison of the results with the results of other published papers shows that the measured concentrations of copper are similar to those obtained in researches conducted by other authors in Bosnia and Herzegovina (Hajdar, 2014), as well as researches conducted in other European countries (Sokolowska et al., 2007; Biolab Medical Unit, 2010).
Copper content in the hair of female population ranged between 7.67 and 25.02 μg/g, while that in male population was between 6.20 and 21.12 μg/g. There are no pronounced gender-related differences in copper content.

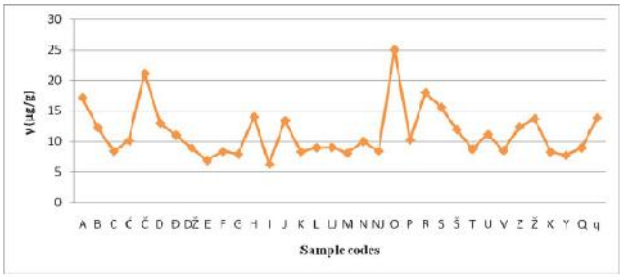

Figure 1. Mass concentration of copper in human hair samples

As can be seen from Figure 2, copper content increases with age until the age of 30 when it reaches its maximum value and then begins to slightly decrease with age.

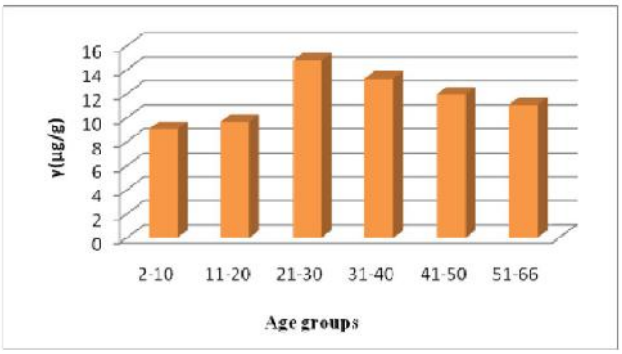

Figure 2. Comparison of age-related copper concentrations in hair

During the sampling, data related to smoking habits were collected from hair donors. Mean values of Cu concentrations in hair samples of smokers was 12.90 μg/g, and in non-smokers' hair 9.90 μg/g. However, it cannot be generally stated that copper content in all hair samples of smokers is higher than the content in non-smokers. As regards the copper content in chemically treated and untreated hair, content in chemically treated hair ranged between 7.67 and 17.98 μg/g, and in chemically untreated between 6.20 and 21.12 μg/g. A slightly higher mean value of copper concentration in chemically treated hair was obtained and it was 12.97 μg/g, while the content in untreated hair was 10.00 μg/g.

Zinc content in human hair samples - Obtained values of zinc concentration ranged between 30.40 and 214.55 μg/g and all of them fall within the normal range of zinc concentrations (Biolab Medical Unit, 2010) in hair samples and most are close to the optimum concentration values (The Agency for Toxic Substances Disease Registry, 2003). Similar results were also obtained in other publications (Mahmutović, 2012; Hajdar, 2014).
Lower content of zinc was found in nine samples. Those are samples taken from the youngest and the oldest individuals. There are numerous reasons for less-than-optimal zinc concentrations, such as diet, stress, menstrual

cycle, internal infections and numerous other factors (Freeman, 1999).

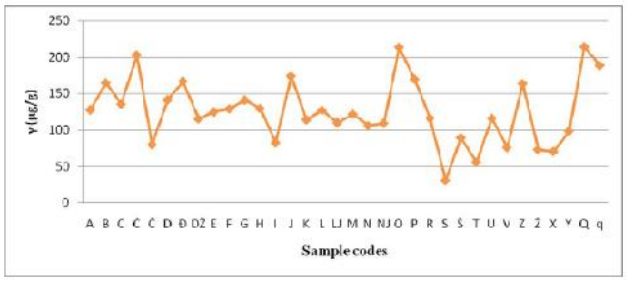

Figure 3. Mass concentration of zinc in human hair samples

Mean value of zinc concentration in male population is 111.32 µg/g, and in female population 138.58 µg/g, which indicates that zinc concentration in female hair is slightly higher.

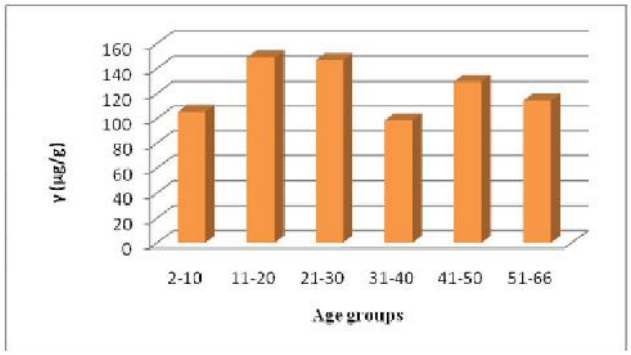

Figure 4. Comparison of age-related zinc concentrations in hair

As shown in Figure 4, obtained results do not indicate a regular age-related change in zinc concentration, more precisely, the data does not allow for reliable linking of zinc concentrations and respondents' age. Based on the mean values of Zn concentrations in samples of smokers, which was 123.0 µg/g, and in samples of non-smokers, which was 128 µg/g, it can be concluded that there are no pronounced differences in zinc content. A slightly higher zinc concentration of 133.11 µg/g was found in chemically treated hair in comparison to mean values of zinc concentration found in untreated hair which was 120.16 µg/g.

Calcium content in human hair samples - As shown in Figure 5, calcium concentrations in hair samples ranged between 99.04 and 14882.63 µg/g. It can be concluded that the values of calcium concentration obtained in most of the samples are within the range of normal values. Mean values obtained in several samples deviate from the normal concentration, but only in female hair samples, while in one sample the mean value of calcium concentration is below the lower limit of normal values and it is the sample taken from a male child aged 2 years. Increased calcium in blood can occur due to increased discharge of calcium from bones, increased absorption from the digestive system or due to decreased discharge in kidneys *(Phyllis, 2006*).

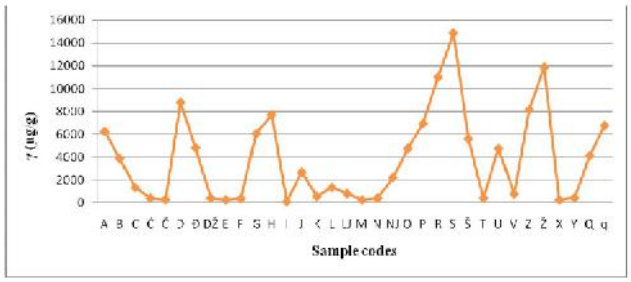

Figure 5. Mass concentration of calcium in hair samples

Mean value of Ca concentration in hair samples of smokers is 6080.48 µg/g, and the value in non-smokers' hair is 1798.33 µg/g. It can be concluded that higher calcium concentration was found in hair samples taken from smokers which can be explained by the fact that calcium is an integral component of tobacco (Rehak, 2013). Mean value of calcium concentration in chemically treated hair was 6928.93 µg/g, and mean value in chemically untreated hair was 1353.88 µg/g. Mean value of calcium concentration in male respondents is 795.05 µg/g, while mean value in female respondents is higher and above the limit of normal values and was 6224.83 µg/g. Results for age-related calcium content are shown in Figure 6.

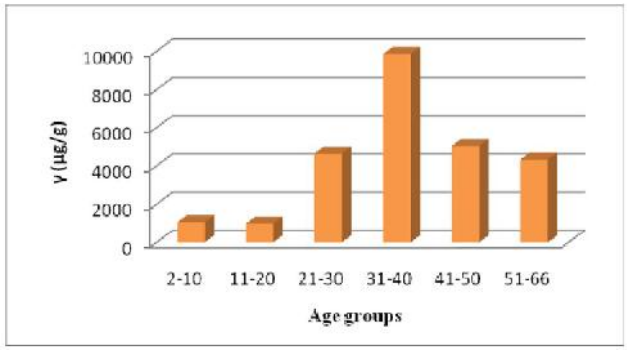

Figure 6. Comparison of age-related calcium concentration in hair

Calcium content changed with age, more precisely it reaches its peak at the age between 31 and 40 years, when it slowly begins to decrease, according to the literature (*Pereira, 2004*). The lowest calcium concentration in hair is found in age group 11 - 20 years because in this period our bones need a lot of calcium, which they obtain from the organism, in order to reach their maximum density before the age of 30 years, when the bones slowly start to lose calcium (Pereira, 2004), which is confirmed by the obtained results.

Magnesium content in human hair samples - Magnesium content in hair samples ranged between 2.11 and 382.34 μg/g. Obtained values of magnesium concentration are within the concentrations that are considered normal. Lower than normal magnesium values were found mainly in male respondents. Mean value of magnesium concentration in male hair was 47.43 μg/g, and in female hair was 135.44 μg/g. Mean values of magnesium concentration are shown in Figure 7.

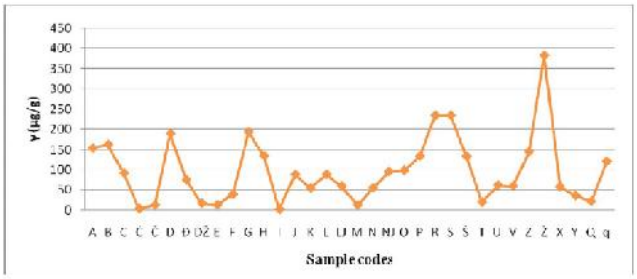

Figure 7. Mass concentration of magnesium in hair samples

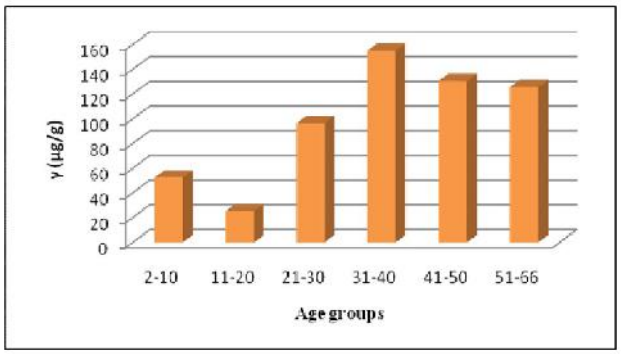

Figure 8. Comparison of age-related magnesium concentration in hair

Obtained results indicate a regular change in Mg concentration with age. Age group 11 - 20 years was the one with the lowest magnesium concentration, but this is explained by the fact that our bones need a lot of magnesium during childhood and at young age, which they then take from the organism to reach their maximum density at about the age of thirty years, when the bones slowly start to lose magnesium (Pereira et al., 2004), which is confirmed by the obtained results.

If we look at the mean value of Mg concentrations found in hair samples obtained from smokers, which was 138.03 μg/g, and those obtained from non-smokers, which was 59.81 μg/g, then we can conclude that higher magnesium concentration was found in hair samples taken from smokers, which can be explained by the fact that tobacco contains magnesium which participates in photosynthesis as an integral part of chlorophyll to a lower degree, and to a higher degree was found in the form of other compounds (Rehak, 2013).

Higher mean value of magnesium concentration of 158.06 μg/g was found in chemically treated hair when compared to the mean value of chemically untreated hair, which was 47.97 μg/g.

Iron content in human hair samples - Results of iron content in hair samples are shown in Figure 9. Iron concentrations in 34 analyzed samples range between 18.04 and 310.30 μg/g. If we compare the obtained results with the results of other experimental measurements, we can see that the found iron concentrations are similar to the values of concentrations obtained in other publications (Fleming, 2001).

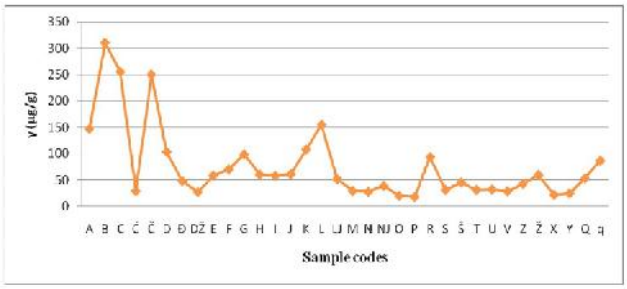

Figure 9. Mass concentration of iron in hair samples

Iron content in the hair of male population ranged between 22.17 and 310.30 μg/g, and the mean value of concentrations was 96.32 μg/g. Iron content in female population ranged between 18.04 and 147.53 μg/g, and the mean value of concentration was lower as expected and was 59.78 μg/g. As shown in Figure 10, it is not possible to link the iron content in hair with the age of respondents.

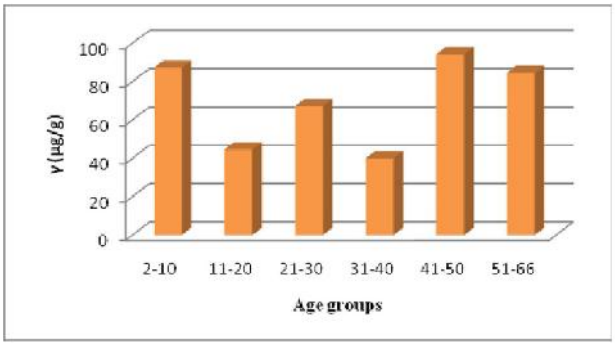

Figure 10. Comparison of age-related iron concentration in hair

Mean Fe concentration in hair samples obtained from smokers were slightly higher and was 86.87 μg/g, while in non-smokers were 66.18 μg/g. Thus, in both cases we have close values. Somewhat higher mean value of iron concentration at 78.88 μg/g was found in chemically untreated hair compared with the mean value of chemically treated hair which was at 72.17 μg/g.

Analysis of concentration of some metals in hair indicated age and gender-related differences in concentrations. In a

Polish research conducted on large groups of respondents (600 to 2,500 respondents out of the total of 3,349 tested individuals), analysis of biometals and toxic metals (Ca, Mg, Zn, Cu, Fe, Pb, Cd) was performed in several groups of different age, or different gender (men and women). Results of the research have indicated differences in the concentration of some of the mentioned metals, or their fluctuations in different periods of life, especially in female respondents (same as in our work) (Baranowska et al., 2004; Sokolowska et al., 2007). The mentioned results almost entirely coincide with the results obtained in this paper, in terms of the stated tested parameters.

CONCLUSIONS

Content of copper, zinc, calcium, magnesium and iron, chrome and cadmium in human hair samples was determined using the method of atomic absorption spectrometry, flame technique. In most of the cases, obtained concentrations were within the range of values that are considered normal. Chromium and cadmium contents were below the limit of quantification of the used method in all samples.

There are no age-related differences in copper content in hair. Higher concentrations of zinc, calcium and magnesium in hair were found in female individuals, and as expected, mean values of iron concentrations in hair were found in male respondents.

Copper content increases with age until the age of 30, when it reaches its maximum value and then begins to slightly decrease with age. Obtained results indicate a regular age-related change in calcium and magnesium content. Age group 11 - 20 years is the one with the lowest magnesium concentration, but this is explained by the fact that our bones need a lot of magnesium during childhood and at young age, which they then take from the organism to reach their maximum density at about the age of thirty years, when the bones slowly start to lose magnesium, which is confirmed by the obtained results. It is not possible to link the zinc and magnesium content in hair with the age of respondents.

Higher mean concentrations of copper, calcium, magnesium and iron were found in smokers. However, it cannot be generally stated that content of these metals in hair samples of smokers is higher than the content in non-smokers. Higher content of calcium and magnesium in hair could be explained by the fact that calcium and magnesium are contained in tobacco leaves. Zinc concentration in smokers' hair is slightly lower than that in non-smokers' hair.

Mean values of copper, zinc, calcium and magnesium concentrations are higher in individuals whose hair has been chemically treated that in those with untreated hair. Slightly higher mean value of iron concentration was found in chemically untreated hair.

The mass concentration of most of analyzed metals are over the normal concentration of metals in human hair (Biolab MedicalUnit, 2010).

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Summary/Sažetak

Esencijalni i toksični elementi se nalaze u hrani, vodi za piće i zraku - u cjelokupnoj ljudskoj, općoj i radnoj okolini. Preporučene ili dopuštene razine njihovog unosa u organizam, vezano za efekte na zdravlje, regulisane su nacionalnim i međunarodnim propisima i pokazateljima. Sadržaj teških metala je važan indikator stanja organizma i određuje se u različitim biološkim uzorcima humanog porijekla. U ovom radu određivane su koncentracije metala u ljudskoj kosi osoba koje žive na području općine Kiseljak. Određivana je koncentracija esencijalnih metala (Cu, Zn, Caj, Mg i Fe) i dva toksična metala (Cd i Cr). Uzorkovanje kose vršeno je u periodu od novembra 2014. godine do februara 2015. godine sa ciljem utvrđivanja razlike u koncentracijama tragova elemenata u ljudskoj kosi kod donatora. Grupa ispitanika sačinjena je od osoba muškog i ženskog spola različitih starosnih skupina (2-66 godina), a u obzir je uzeta starosna dob, spol, da li je kosa hemijski tretirana ili ne, da li je donator pušač ili ne. Za određivanje koncentracije ispitivanih teških metala primijenjena je instrumentalna metoda analize - atomska apsorpciona spektrometrija (AAS), plamena tehnika. Sadržaj esencijalnih metala je u granicama normalnih vrijednosti za većinu ispitivanih uzoraka, uz izvjesna odstupanja koja su bliska području normalnih koncentracija. Sadržaj Cd i Cr u svim uzorcima je bio ispod donje granice kvantifikacije korištene metode.

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DFT Structural Analysis of Chamazulene

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Abstract: A combined theoretical and experimental study on the structure, infrared,UV-Vis, ^{1}H and ^{13}C NMR data of chamazulene is presented. Theoretical geometry optimizations and some additional properties of chamazulene and their IR, UV-Vis, NMR spectra were calculated using the DFT B3LYP /6-31G(d) level. Calculations were done using software Spartan 10. Experimental data showed that chamazulene have absorption maximum at 340 nm to 530 nm. The position of max did not much differ from the theoretically calculated value. The calculated density of states showed excellent agreement with UV/Vis diffuse reflectance spectra predicting the absorption maximum at 310 nm (calculated 332 nm) to 530 nm (calculated 516 nm). The IR normal modes were assigned for the two very small sp^2 CH valence bands and strong sp^3 CH vibrations. The aromatic overtone vibrations can hardly be detected and also the C=C vibration is very weak.^{1}H NMR spectroscopy, showed resonances of the ring protons between 7 and 8 ppm. The methyl groups and the methylene group appear rather deshielded at 2.7 and 2.9 ppm. The calculations yielded reliable results that were in good correlation with experimental data. This study is a good basis forcollaboration between experimentalists and quantum chemists.

INTRODUCTION

Chamazulene (1,4-dimethyl-7-ethylazulene) is an aromatic chemical compound obtained by steam distillation of a variety of plants including chamomile (*Matricaria chamomilla*), wormwood (*Artemisia absinthium*), and yarrow (*Achillea millefolium*). It is a blue-violet derivative of azulene which is synthesized from the sesquiterpene matricin (Meisels and Weizmann 1993; Özgür, Cemal, Senyel2009). For the quantitative determination of essential oil, total azulenes and chamazulene in chamomile, variousmethods are used, such as gravimetry, spectrophotometry in the visible region and gas chromatography (Peters, Lanzilotta, Lemon, et al., 1998).Density Functional Theory (DFT) has been accepted by the quantum chemistry community as reliable and effective approach for the computation of molecular structure, vibration frequencies and energies of chemical reactions (Beyramabadi and Morsali 2011; Kadhum, Al-Amiery, Shikara, et al., 2011). DFT calculations provide an excellent agreement with experimental vibrational frequencies of investigated compounds. These calculations have been used extensively for calculating a wide variety of molecular properties such as equilibrium structure, charge distributionUltraviolet–visible spectroscopy(UV/Vis), Infrared frequencies (IR) and Nuclear Magnetic Resonance (NMR) spectra, and provide reliable results which are in agreement with experimental data (Becke, 1993). The purpose of this work is to determine the chamazulene of essential oil from Bosnia and Herzegovina chamomile samples by Thin Layer Chromatography (TLC) and UV/Vis method. Furthermore, chamazulene wascharacterized theoretically and experimentally by IR, UV-Vis and NMR

spectroscopy. The Beck´s three-parameter exchange functional with the Lee, Yang and Parr correlation functional (B3LYP),developed by Truhlar et al. were used to perform theoretical calculations of the investigated compound (Lee, Yang, Parr, 1988; Zhao, Y., Truhlar, 2005).

MATERIAL AND METHODS

Materials

Chamomile tea commercially available from Bosnian markets was used in this study. All the reagents and chemicals were purchased from Sigma-Aldrich Co. LLC.

Investigated compounds

The compound chamazulene was studied for their experimental and theoretical properties. Structure of investigated compound is presented in Figure 1.

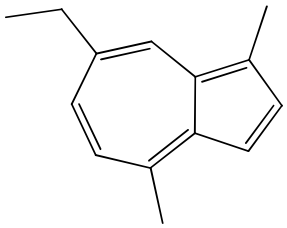


Figure 1: Structure of chamazulene

UV-Vis and TLC analysis

The methods for chamazulene determination in chamomile essential oil was developed based on silica gel G TLC and UV-spectrophotometry.
The chamazulene was isolated from chamomile by steam distillation and extracted with methyl tert-butyl ether (3 × 100 mL). The combined extracts are dried over $MgSO_4$, filtered and the solvent is removed in vacuo.The sample was dissolved in ethanol and applied to precoated TLC. The chromatographic separations were done on the silica gel F254. TLC plates developed with dichloromethane: ethylacetate (9:2 v/v). Detection was performed under UV lamp at 254 nm and the evaluation of the chromatographic plate was based on processing of chromatographic images. Then Rf value was calculated and compared with literature data (Padula, Rondina and Coussio 1976; Roth and Rupp 1995).
Ultraviolet Spectra were recorded using LAMBDA 25, PerkinElmer UV-Vis Spectrometer, and ethanol was used as solvent for the dilution of sample as well as blank.

Theoretical calculations

All the calculations were carried out with the Spartan 10 software. The geometries were optimized using the method: B3LYP basis set: 6-31G(d) as shown in Figure 2. The harmonic vibration frequencies were calculated by this method and the results were compared with experimental spectra. This method was used for calculating IR, UV-Vis and NMR spectra at the B3LYP/6-31G (d) level for chamazulene.

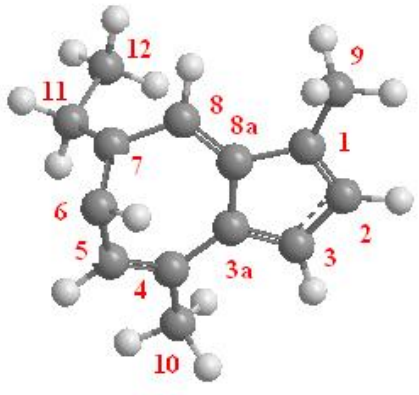


Figure 2. The optimized geometry of the chamazulene

RESULTS AND DISCUSSION

TLC and UV/Vis data of the prepared chamazulene

TLC study of the isolated compound was found (Rf=0.80) almost similar to that of the literature (Rf=0.78) data so this study concludes that the isolated compound may be chamazulene. Chamazulene was determined directly by measuring absorbance at 310 nm to 530 nm.

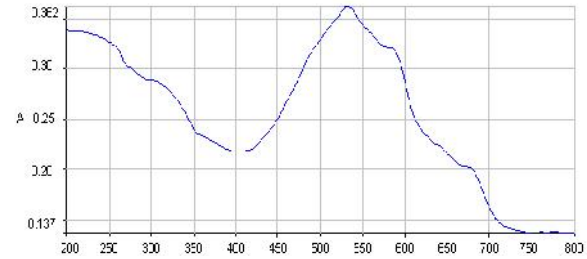
Experimental and theoretical UV/Vis spectral data

The electronic spectra of the chamazulene exhibit two characteristic broad bands at 340 nm and 530 nm (Table 1).

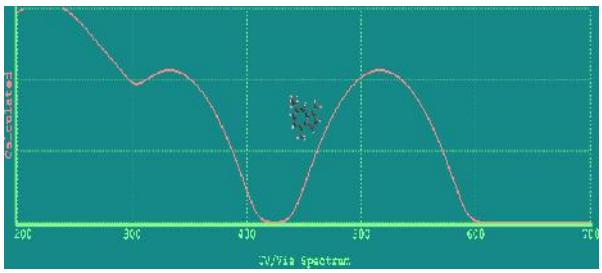
Table 1. Experimental and theoretical UV/Vis spectral data for chamazulene

Compound	λmax [nm] (Exp.)	λmax [nm] (Calcd.)	Intensity
Chamazulene	225	228	1.1687
	266	264	0.0529
	340	332	0.0184
	530	516	0.0183

The chamazulene exhibits the absorption maximum at 340 nm to 530 nm (Figure 3a). The position of λmax did not much different with theoretical calculation (Figure 3b). The calculated density of states showed excellent agreement with UV–Vis diffuse reflectance spectra predicting the absorption maximum at 310 nm (calculated 332 nm) to 530 nm (calculated 516 nm). The calculated values are lower than the experimental absorption maximun (Table 1). The reason for the discrepancies between the theory and experiment can be the vibrational effects, which are not taken into account and hydrogen bonding with the solvent molecules.The correlation factor from the linear regression was 0.9997.



(a)



(b)

Figure 3: Experimental (a) and theoretical (b) calculation of UV/Vis absorption spectra of chamazulene

Experimental and theoretical IR spectral data

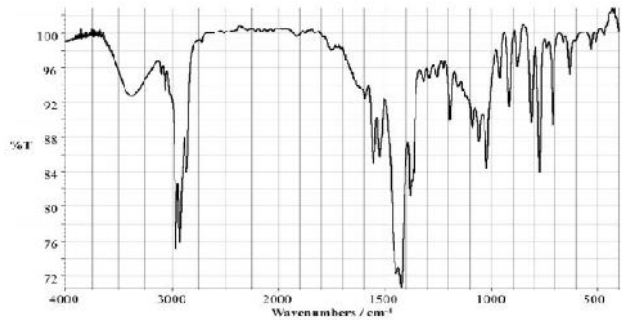
The results of the calculated and experimental harmonic frequencies of chamazulene are collected in Table 2.

Table 2. Experimental and theoretical IR spectral data (cm^{-1}) for chamazulene

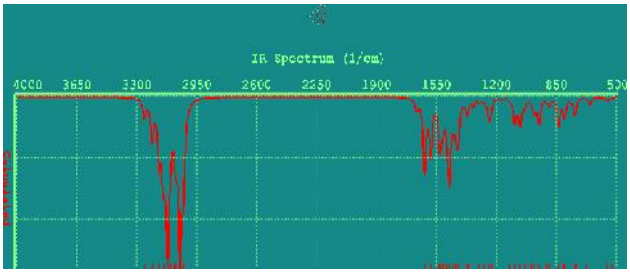
vibrations	[cm^{-1}] (Exp.)*	[cm^{-1}] (Calcd.)
sp^2 CH	3200	3170
sp^3 CH	3000	3027
C=C	1600	1620

∗ Experimental values from literature (Berger and Sicker 2009)

Theoretical IR spectra of chamazulene shows similar characteristic infrared band frequencies. The correlation factor from the linear regression was 0.9990.
Chamazulene gives the two very smallsp^2 CH valence bands at about 3200 cm^{-1} (calculated 3170 cm^{-1}) and strong sp^3 CH vibrations below 3000 cm^{-1}(calculated 3027) cm^{-1}. The C=C vibration at1600 cm^{-1} (calculated 1620 cm^{-1})is very weak displayed in Figure 4.



(a)



(b)

Figure 4: Experimental values from literature (Berger and Sicker 2009)**(a)** and theoretical **(b)** calculation of IR spectra of chamazulene

Experimental and theoretical NMR spectral data

Chemical shifts calculated using the B3LYP level with the 6-31G (d) basis sets can be utilized to eliminate the uncertainties in the fundamental assignments ofthe spectra. The ^1H and ^{13}C theoretical and experimental chemical shifts,isotropic shielding tensors and assignments of chamazulene are presented in Tables 3 and 4, respectively.

DFT calculations for structural and electronic properties

Optimized molecular structures of chamazulene of the most stable form are shown in Figure 2. Molecular orbital calculations provide a detailed description of orbitals including spatial characteristics, nodal patterns and individual atom contributions.

Table 3. The experimental and calculated ^1H-isotropic chemical shifts, ppm, with respect to $SDCl_3$ for chamazulene

	Chemical shift δ (ppm)	
Assignment	**(Exp.)***	**(Calcd.)**
H2	7.62	7.49
H3	7.22	7.04
H5	6.98	6.92
H6	7.39	7.50
H8	8.16	8.12
H9	2.66	2.51
H10	2.83	2.66
H11	2.85	2.81
H12	1.35	1.34

∗ Experimental values from literature (Berger and Sicker 2009)

The aromatic ring gave resonances in the region from 7.0–8.0 ppm in the ^1H NMR spectra of chamazulene. The edited ^{13}C NMR spectrum displays in the aromatic region five CH signals and five signals of quaternary carbons; from the carbon spectrum alone.The calculated and experimental chemical shift values showed good correspondence.The correlation factors for^1H NMR is 0.9989 and ^{13}C NMR is 0.9952.

Table 4. The experimental and calculated ^{13}C-isotropic chemical shifts, ppm, with respect to $SDCl_3$ for chamazulene

Assignment	Chemical shift δ (ppm) (Exp.)*	(Calcd.)
C1	137.4	138.08
C2	136.2	131.15
C3	112.8	105.87
C3a	136.2	129.38
C4	144.3	138.17
C5	125.0	119.01
C6	136.4	130.12
C7	135.7	129.20
C8	134.7	129.43
C8a	125.1	130.40
C9	12.9	14.39
C10	24.1	25.70
C11	33.8	37.50
C12	17.4	20.78

* Eksperimental values from literature (Berger and Sicker 2009)

The contour plots of the frontier orbitals for the ground state are shown in Figure 5, including the Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO). The both orbitals are substantially distributed over the conjugation plane. It can be seen from the Figure 5 that the HOMO orbitals are located on the substituted molecule while LUMO orbitals resemble those obtained for the unsubstituted molecule and therefore the substitution has an influence on the electron donation ability, but only a small impact on electron acceptance ability. It can be seen that the energy gaps between HOMO and LUMO of chamazulene is 3.19 Hartree. The values of the HOMO and LUMO energy gap explained the eventual charge transfer interaction taking place within the molecules. The lower the HOMO–LUMO energy gap, the lighter and less stable/more reactive the molecule (Kadhum, Mohamad, Al-Amiery, et al., 2011).

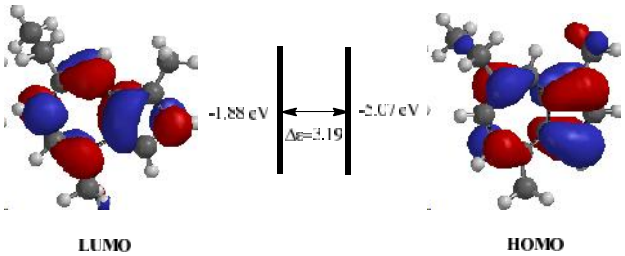


Figure 5: Frontier molecular orbitals of chamazulene

The energy gaps between HOMO and LUMO of guaiazulene is 3.20 Hartree (Safayhi, Sabieraj, Sailer, et al., 1994). Chamazulene is more reactive than guaiazulene (Špirtović-Halilović, S., Salihović, M., Osmanović, et al., 2014) because it has lower value of the HOMO and LUMO energy gap.

Atomic charges for chamazulene

Atomic charges of chamazulene showed in Table 5. These data show that the atomic charge has been affected by the presence of substituent of rings (Kadhum, Al-Amiery, Shikara, et al., 2011; Kadhum, Wasmi,Mohamad, et al., 2012)as shown in the Table 5.

The data (Table 5.) show that the high estatomic charge in molecule is at [C(3) -0.418)] and the next charge value is at [C(5) -0.249]. These data show clearly that these two atoms are the most reactive toward the substitution reactions.

Table 5. Atomic charges of chamazulene

Atom	Atom type (MM2)	Charge Huckel	Atom	Atom type (MM2)	Charge Huckel	Atom	Atom type (MM2)	Charge Huckel
C(1)	C Alkene	-0.095	C(13)	C Alkane	0.184	H(25)	H	0.047
C(2)	C Alkene	-0.0434	C(14)	C Alkane	-0.109	H(26)	H	0.248
C(3)	C Alkene	-0.418	H(15)	H	0.028	H(27)	H	0.035
C(4)	C Alkene	-0.074	H(16)	H	0.028	H(28)	H	0.036
C(5)	C Alkene	-0.249	H(17)	H	0.286	H(29)	H	0.042
C(6)	C Alkene	0.249	H(18)	H	0.029	H(30)	H	0.043
C(7)	C Alkene	0.142	H(19)	H	0.021	H(31)		
C(8)	C Alkene	0.417	H(20)	H	0.037			
C(9)	C Alkene	-0.177	H(21)	H	0.040			
C(10)	C Alkene	-0.182	H(22)	H	0.041			
C(11)	C Alkane	-0.146	H(23)	H	0.039			
C(12)	C Alkane	-0.15	H(24)	H	0.046			

CONCLUSIONS

Selected structural parameters of the optimized geometries of the chamazulene have been obtained by DFT calculations. The electronic spectra of the chamazulene fundamental modes have been precisely assigned and analyzed and the theoretical results were compared with the experimental values. Conducted research provides data about electronic spectra and structural information of chamazulene. The calculations yielded reliable results that were in good correlation with experimental data. This study is a good basis for collaboration between experimentalists and theoretical chemists.

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Summary/Sažetak

Predstavljena je kombinovana teorijska i eksperimentalna studija strukture, IR, UV-Vis, ^{1}H i ^{13}C NMR spektara hamazulena. Teorijska geometrijska optimizacija i neka molekulska svojstva hamazulena kao i njegovi IR, UV-VIS, NMR spektri su izračunati pomoću DFT B3LYP / 6-31G(d) niva. Proračuni su urađeni pomoću softvera Spartan 10. Eksperimentalni podaci pokazuju da hamazulen ima maksimum apsorpcije na 340 nm do 530 nm.Položaj λmax se ne razlikuje mnogo od teorijsko dobivenog. Maksimum apsorpcije za hamazulen je dobiven na 310 nm (izračunato 332 nm) i na 530 nm (izračunato 516 nm) što pokazuje odlično slaganje. Na IR spektru hamazulena se vide dva jako mala apsorpciona maksimuma koji potiču od vibracija istezanja sp^2 CH veze i jaki koji potiče od vibracija istezanja sp^3 CH veze.Apsorpcioni maksimum koji potiče od aromatskih vibracija teško se može otkriti, a za C=C vibracije je vrlo slab.^{1}H NMR spektroskopija, pokazala je signaleza protone prstena između 7 i 8 ppm.Signali protona metilne i metilenske grupe uočeni su na 2.7 and 2.9 ppm. Nađeno je da su rezultati pouzdani i u dobroj saglasnosti sa eksperimentalnim podacima. Ova studija je dobra osnova za saradnju između eksperimentatora i kvantnih hemičara

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Solvent-free Synthesis and Antibacterial Activity of 14-Aryl Substituted Dibenzoxanthene Derivatives

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Abstract: Xanthene derivatives are important compounds because of their proven biological activities. Seven 14-aryl-14*H*-dibenzoxanthene derivatives were synthesized by reliable solvent-free synthesis procedure using iron (III) chloride hexahydrate as a catalyst. Three synthesized derivatives possess antibacterial activities against different bacteria. Compound 14-(2',5'-dimethoxyphenyl)-14*H*-dibenzo[*a,j*]xanthene (**3**) showed best activity against *Escherichia coli* and *Staphylococcus aureus* with MIC 0.616 mg/mL. Docking study for the most potent compound was carried out by taking amino terminal domain of enzyme I as a target for antibacterial activity against *Escherichia coli* and it was shown that binding energy of **3** was similar to amikacin's (around –4.2 kcal/mol) used as a referent drug, although bound on a different sites on enzyme.

INTRODUCTION

Xanthenes derivatives are interesting due to their wide range of biological and pharmacological properties, such as agricultural bactericide (Hideo, Teruomi, 1981), anti-inflammatory (Poupelin, Saint-Rut, Foussard-Blanpin, et al., 1978) and antiviral activities (Lambert , Martin, Merrett, et al., 1997). In addition to their biological applications they are also used in industry as dyes, in laser technology and as fluorescent materials for visualization of biomolecules (Knight and Stephens, 1989). Xanthenes are also available from natural sources, such as Santalin pigments wich have been isolated from a number of plant species (Kinjo, Uemura, Nohara; 1995). Thus, the synthesis of xanthenes is of continuing interest. Many synthetic methods exist for the synthesis of xanthenes and dibenzoxanthenes such as the cyclocondensation reaction of 2-hydroxyaromatic aldehydes and 2-tetralone (Jha and Beal, 2004), the reaction of benzaldehydes and acetophenones (Kuo and Fang, 2001), 2-naphtol with formamide (Papini and Cimmarusti, 1947), 2-hydroxynaphthyl carbinol with resorcinol (Sen and Sarkar, 1925) and from the reaction of hot alkali on 2-naphthyl oxide (Ota and Kito, 1976). Furthermore, 14-aryl-14*H*-dibenzoxanthenes are synthesized by cyclocondensation of β-naphtol with aldehydes in the presence of various catalysts such as silica sulfuric acid (Rajitha, Kumar, Reddy, *et al.*, 2005), AcOH-H_2SO_4 (Sarma and Baruah, 2005), p-TSA (Khosropour, Khodaei and Moghannian, 2005), $MeSO_3H$, sulfamic acid, cyanuric chloride, LiBr, $Yb(Otf)_3$ (Saini, Kumar and Sandhu, 2006), $TaCl_5$ and $BiCl_3$ (Soleimani, Khodaei and Koshvandi, 2011). However, these methods show varying degrees of success as well as limitations

such as prolonged reaction times, laborious work-up procedures, harsh reaction conditions, use of an excess of expensive reagents and use of toxic organic solvents (Kumar, Sunil Kumar and Narsimha Reddy, 2006). Thus, the development of an alternative milder and clean procedure is highly demanding for the synthesis of new and/or known compounds, wich surpasses those limitations. Convenient and solvent-free synthesis of 14-aryl-14*H*-dibenzoxanthenes catalyzed by iron (III) chloride hexahydrate has been described in literature (Liu, Zhou, Gao, *et al.*, 2013). Using this solvent-free method we synthesized seven 14-aryl-14*H*-dibenzoxanthene derivatives already reported but with iron (III) chloride hexahydrate as a catalyst.

In our work we conducted research of antimicrobial activity for synthesized 14-aryl-14*H*-dibenzoxanthenes. According to literature, this is the first time reporting on the antimicrobial activity of these dibenzoxanthene derivatives. In order to investigate potential mechanism of antibacterial activity for the most potent compound, docking study was performed. Molecular docking is a very useful method introduced to investigate molecular association and is particularly important in the drug discovery field to study the binding of small molecules (ligands) to macromolecules (receptor) (Barril and Morley 2005).

MATERIAL AND METHODS

General procedure for synthesis 14-aryl-14*H*-dibenzoxanthenes

A mixture of aldehyde (5 mmol), 2-naphthol (10 mmol) and $FeCl_3x6H_2O$ (1 mmol) was finely ground and heated at 90°C. The reaction was monitored by thin-layer chromatography (TLC). After completion, the system was cooled to room temperature, reaction mixture was washed with 60% aqueous EtOH and filtered to afford the crude product. Further purification was followed by crystallization from 96% ethanol (Liu, Zhou, Gao et al., 2013).

Melting points of synthesized compounds were determined by Melting Point Meter KSP1D, A.Krüss Optronic, Germany. Infrared (IR) spectra were recorded by Perkin Elmer BX FTIR using KBr pellets. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600 and 150 MHz, respectively, in deuterated dimethyl sulfoxide (DMSO-*d*6) at 25°C using NMR spectrometer Bruker AV600, with tetramethylsilane (TMS) as internal reference. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz.

Antimicrobial activity

Antibacterial activity was tested by the diffusion method against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027. The results were presented as the inhibition zones, given in millimeters (mm). Compounds that showed good antimicrobial activity by diffusion method were further tested by dilution method. Amikacin was used as a referent drug.

For determination of antimicrobial activity (diffusion method) Müller-Hinton nutritious base was used, while Sabouraud dextrose broth was used in the dilution method. When using the diffusion method, the test samples were dissolved in 99.5% dimethyl sulfoxide (DMSO) to obtain a 1 mg/100 μL stock solution. The inhibition zones were measured in millimeters at the end of an incubation period of 24 h at 37°C. In dilution method microtiter plates with 96 sites were used. In first well on 100 μL of nutrient broth was added 100 μL solution of the test compound and the content was mixed. From this well was taken 100 μL of solution, which is transferred to the next well and the contents were mixed. The process of double dilution was repeated until they fulfill all the wells in one row. The same procedure was used when microtiter-plate was filled with other compounds. DMSO was used as blank control and amikacin as a referent compound. In each of the wells was added 10 μL of the appropriate bacterial culture ($1x10^6$ cells/mL) and thermostated for 24 hours at 37°C. After thermostating, turbidimetric method was used for reading results. Minimum inhibitory concentration (MIC) corresponds to the concentration of the compound in well, where for the first time does not occur growth of bacterial culture. The concentration is expressed in mg/mL.

Docking study

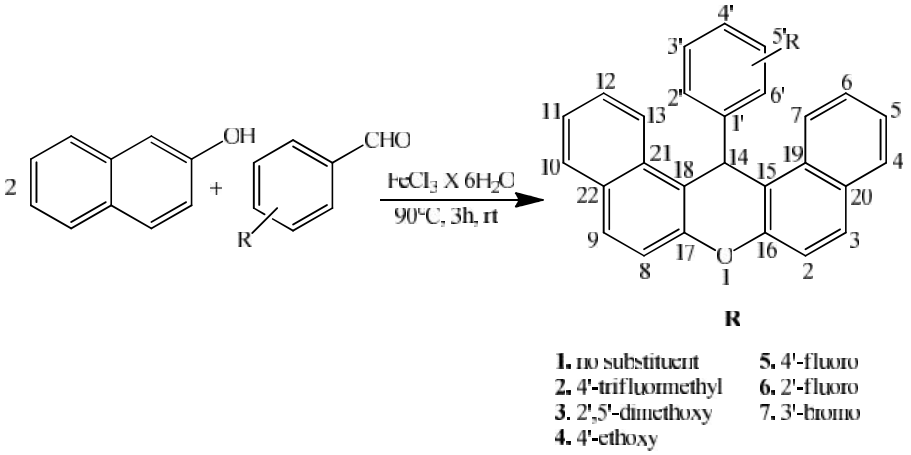
Lamarckian Genetic Algorithm of the AutoDock 4.0 program was used to perform the flexible-ligand docking studies (Morris, Huey, Lindstrom, 2009). Receptors X-ray crystal structures obtained from the Brookhaven protein data bank was applied in docking studies (http://www.pdb.org/).

Prior to actual docking run, AutoGrid 4.0 was introduced to precalculate grid maps of interaction energies of various atom types. In all dockings, a grid map with 126*126*126 points, a grid spacing of 1.000 Å. In an AutoGrid procedure, the protein is embedded in a 3D grid and a probe atom is placed at each grid point. The energy of interaction of this single atom with the protein is assigned to the grid point. Autodock 4.0 uses these interaction maps to generate ensemble of low energy conformations. It uses a scoring function based on AMBER force field, and estimates the free energy of binding of a ligand to its target. For all dockings, 10 independent runs with step sizes of 0.2 Å for translations and 5 Å for orientations and torsions, an initial population of random individuals with a population size of 150 individuals, a maximum number of 250000 energy evaluations and 27000 maximum generations.

Bindings between docked potent agents and related macromolecule were analyzed using Autodock tools program (ADT, Version 1.5.4) and PyMol-1.1 software was used for graphical visualization, analyzing interactions of ligands and receptors and producing quality of images (Lill and Danielson 2011).

RESULTS AND DISCUSSION

Seven dibenxanthene derivatives were synthesized according to procedure given in Scheme 1



Scheme 1. Solvent-free synthesis of dibenzoxanthene derivatives.

Six different benzaldehydes with electron donating and electron withdrawing substituents along with unsubstituted benzaldehyde were used in the procedure. Syntheses lasted for 3 hours and yields ranged from 82 to 90% indicating good catalytic action of iron (III) chloride hexahydrate in this solvent-free conditions.
Analytical data of synthesized compounds are given below:

14-phenyl-14*H*-dibenzo[*a,j*]xanthene (1)

Yield: 86%; mp 186°C.

IR (KBr) n 3057, 3022, 1593, 1515, 1457, 963, 829, 744, 701 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 6.46 (s, 1H, H-14), 6.97 (t, *J* 7.85 Hz, 1H, H-4'), 7.12 (t, *J* 7.85 Hz, 1H, H-3' and H-5'), 7.38 (t, *J* 7.55 Hz, 1H, H-5 and H-11), 7.46 (d, *J* 8.70 Hz, 1H, H-3 and H-9), 7.51 (d, *J* 7.85 Hz, 1H, H-2' and H-6'), 7.55 (t, *J* 7.55 Hz, 1H, H-6 and H-12), 7.76 (d, *J* 8.70 Hz, 1H, H-2 and H-8), 7.80 (d, *J* 7.55 Hz, 1H, H-4 and H-10), 8.37 (d, *J* 7.55 Hz, 1H, H-7 and H-13).

^{13}C NMR (150 MHz, DMSO-d_6) d 38.2 (C-14), 117.5 (C-15 and C-18), 118.2 (C-3 and C-9), 122.9 (C-7 and C-13), 124.4 (C-5 and C-11), 126.6 (C-4'), 127.0 (C-6 and C-12), 128.4 (C-2' and C-6'), 128.6 (C-3' and C-5'), 128.97 (C-4 and C-10), 129.03 (C-2 and C-8), 131.3 or 131.7 (C-19/21), 131.3 or 131.7 (C-20/22), 145.2 (C-1'), 148.9 (C-16 and C-17).

14-(4'-trifluoromethylphenyl)-14*H*-dibenzo[*a,j*]xanthene (2)

Yield: 88%; mp 259°C.

IR (KBr) n 1594, 1516, 1460, 1118, 1067, 744, 202 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 6.55 (s, 1H, H-14), 7.39 (d, *J* 8.10 Hz, 1H, H-3' and H-5'), 7.42 (t, *J* 7.50 Hz, 1H, H-5 and H-11), 7.58 (t, *J* 7.50 Hz, 1H, H-6 and H-12), 7.62 (d, *J* 8.10 Hz, 1H, H-2' and H-6'), 7.81 (d, *J* 8.40 Hz, 1H, H-3 and H-9), 7.84 (t, *J* 7.50 Hz, 1H, H-4 and H-10), 8.32 (d, *J* 7.50 Hz, 1H, H-7 and H-13).

^{13}C NMR (150 MHz, DMSO-d_6) d 38.0 (C-14), 116.6 (C-15 and C-18), 118.2 (C-3 and C-9), 122.5 (C-7 and C-13), 124.1 (CF_3), 124.7 (C-5 and C-11), 125.7 (C-3' and C-5'), 149.03 (C-16 and C-17), 127.2 (C-6 and C-12), 128.7 (C-2' and C-6'), 129.2 (C-4 and C-10), 129.5 (C-2 and C-8), 131.3 or 131.4 (C-19/21), 131.3 or 131.4 (C-20/22), 136.2 (C-4'), 148.97 (C-1').

14-(2',5'-dimethoxyphenyl)-14*H*-dibenzo[*a,j*]xanthene (3)

Yield: 90%; mp 170°C.

IR (KBr) n 3057, 1622, 1592, 1516, 1457, 1431, 1401, 1265, 1239, 1136, 1018, 962, 822, 751 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 4.23 (s, 3H, 6'-OCH_3), 3.46 (s, 3H, 3'-OCH_3), 6.48 (d, *J* 7.95 Hz, 1H, H-4'), 6.72 (s, 1H, H-2'), 6.78 (d, *J* 7.95 Hz, 1H, H-5'), 6.86 (s, 1H, H-14), 7.38 (t, *J* 7.50 Hz, 1H, H-5 and H-11), 7.45 (d, *J* 7.10 Hz, 1H, H-3 and H-9), 7.52 (t, *J* 7.50 Hz, 1H, H-6 and H-12), 7.75 (d, *J* 7.10 Hz, 1H, H-2 and H-8), 7.91 (d, *J* 7.50 Hz, 1H, H-4 and H-10), 8.56 (d, *J* 7.50 Hz, 1H, H-7 and H-13).

^{13}C NMR (150 MHz, DMSO-d_6) d 30.6 (C-14), 55.4 (3'-O<u>C</u>H$_3$), 56.4 (6'-O<u>C</u>H$_3$), 111.4 (C-5'), 112.2 (C-4'), 117.1 (C-2'), 118.2 (C-3 and C-9), 118.5 (C-15 and C-18), 123.5 (C-7 and C-13), 124.4 (C-5 and C-11), 126.8 (C-6 and C-12), 128.6 (C-4 and C-10), 128.7 (C-2 and C-8), 131.0 or 132.2 (C-19 and C-21), 131.0 or 132.2 (C-20 and C-22), 135.8 (C-1'), 148.4 (C-6'), 149.0 (C-16 and C-17), 154.3 (C-3').

14-(4'-ethoxyphenyl)-14*H*-dibenzo[*a,j*]xanthene (4)

Yield: 89%; mp 174°C.

IR (KBr) n 3060, 2927, 1594, 1460, 1246, 1115, 963, 812, 745 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 8.37 (d, *J* 7.65 Hz, 1H, H-7 and H-13), 7.77 (d, *J* 9.00 Hz, 1H, H-2 and H-8), 7.81 (d, *J* 7.65 Hz, 1H, H-4 and H-10), 7.56 (t, *J* 7.65 Hz, 1H, H-6 and H-12), 7.46 (d, *J* 9.00 Hz, 1H, H-3 and H-9), 7.40 (m, 1H, H-5 and H-11), 7.40 (m, 1H, H-2' and H-6'), 6.64 (d, *J* 7.20 Hz, 1H, H-3' and H-5'), 6.37 (s, 1H, H-14), 3.82 (q, J = 7.00 Hz, 2H, OCH_2CH_3), 1.25 (t, *J* 7.00 Hz, 3H, OCH_2CH_3).

^{13}C NMR (150 MHz, DMSO-d_6) d 15.0 (OCH_2CH_3), 37.3 (C-14), 63.4 (OCH_2CH_3), 114.6 (C-3' and C-5'), 117.8 (C-15 and C-18), 118.2 (C-3 and C-9), 122.9 (C-7 and C-13), 124.4 (C-5 and C-11), 126.9 (C-6 and C-12), 128.9 (C-2 and C-8), 129.0 (C-4 and C-10), 129.3 (C-2' and C-6'), 131.3 or 131.6 (C-19/21), 131.3 or 131.6 (C-20/22), 137.4 (C-1'), 148.9 (C-16 and C-17), 157.4 (C-4').

14-(4'-fluorophenyl)-14*H*-dibenzo[*a,j*]xanthene (5)

Yield: 83%; mp 241°C.

IR (KBr) n 3071, 2361, 1624, 1594, 1503, 1460, 1433, 1401, 835, 745 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 6.47 (s, 1H, H-14), 6.81 (t, *J* 8.57 Hz, 2H, H-3' and H5'), 7.41 (t, *J* 7.49 Hz, 2H, H-5 and H-11), 7.50-7.43 (m, 4H, H-3 and H-9), 7.50-7.43 (, 4H, H-2' and H-6'), 7.57 (t, *J* 7.73 Hz, 2H, H-6 and H-12), 7.79 (d, *J* 8.88 Hz, 2H, H-2 and H-8), 7.83 (d, *J* 8.07 Hz, 2H, H-4 and H-10), 8.33 (d, *J* 8.53 Hz, 2H, H-7 and H-13).

^{13}C NMR (150 MHz, DMSO-d_6) d 37.4 (C-14), 114.8 (C-3' and C-5'), 117.3 (C-15 and C-18), 118.2 (C-3 and C-9), 122.7 (C-7 and C-13), 124.5 (C-5 and C-11), 127.1 (C-6 and C-12), 129.1 (C-4 and C-10), 129.2 (C-2 and C-8), 129.8 (C-2' and C-6'), 131.3 (C-20 and C-22), 131.3 (C-19 and C-21), 141.0 (C-1'), 148.8 (C-16 and C-17), 161.4 (C-4')

14-(2'-fluorophenyl)-14*H*-dibenzo[*a,j*]xanthene (6)

Yield: 82%; mp 233°C.

IR (KBr) n 3080, 2209, 1624, 1594, 1516, 1483, 1460, 749 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 6.80 (s, 1H, H-14), 7.03-6.91 (m, 1H, H-5'), 7.15-7.03 (m, 1H, H-4'), 7.15-7.03 (m, 1H, H-3'), 7.51-7.40 (m, 1H, H-6'), 7.51-7.40 (m, 2H, H-5 and H-11), 7.56 (d, *J* 8.56 Hz, 2H, H-3 and H-9), 7.64 (t, *J* 7.07 Hz, 2H, H-6 and H-12), 7.95 (d, *J* 7.45 Hz, 2H, H-4 and H-10), 7.95 (d, *J* 7.45 Hz, 2H, H-2 and H-8), 8.41 (d, *J* 7.98 Hz, 2H, H-7 and H-13).

^{13}C NMR (150 MHz, DMSO-d_6) d 30.6 (C-14), 115.3 (C-15 and C-18), 115.5 (C-3'), 117.6 (C-3 and C-9), 122.1 (C-7 and C-13), 124.5 (C-5 and C-11), 125.0 (C-5'), 127.2 (C-6 and C-12), 128.8 (C-4'), 129.3 (C-20 and C-22), 129.3 (C-19 and C-21), 129.3 (C-4 and C-10), 129.31 (C-8 and C-2), 130.8 (C-6'), 131.8 (C-1'), 148.1 (C-16 and C-17), 158.1 (C-2').

14-(3'-bromophenyl)-14*H*-dibenzo[*a,j*]xanthene (7)

Yield: 85%; mp 192°C.
IR (KBr) n 3066, 2923, 1624, 1591, 1457, 1430, 1399, 1243, 1065, 961, 811, 774, 690 cm^{-1}.

^{1}H NMR (600 MHz, DMSO-d_6) d 6.45 (s, 1H, H-14), 7.03 (t, *J* 7.85 Hz, 1H, H-5'), 7.15 (d, *J* 7.50 Hz, 1H, H-4'), 7.44 (t, *J* 7.50 Hz, 2H, H-5 and H-11), 7.54-7.49 (m, 1H, H-6'), 7.54-7.49 (m, 2H, H-3 and H-9), 7.62 (t, *J* 7.85 Hz, 2H, H-6 and H-12), 7.65 (s, 1H, H-2'), 7.81 (d, *J* 9.05 Hz, 2H, H-2 and H-8), 7.85 (d, *J* 7.50 Hz, 2H, H-4 and H-10), 8.33 (d, *J* 8.20 Hz, 2H, H-7 and H-13).

^{13}C NMR (150 MHz, DMSO-d_6) d 37.9 (C-14), 116.7 (C-15 and C-18), 118.2 (C-3 and C-9), 122.5 (C-7 and C-13), 122.9 (C-3'), 124.5 (C-5 and C-11), 127.0 (C-6'), 127.1 (C-6 and C-12), 129.1 (C-4 and C-10), 129.1 (C-4 and C-10), 129.3 (C-2 and C-8), 129.8 (C-4'), 130.1 (C-5'), 131.2 (C-20 and C-22), 131.2 (C-19 and C-21), 131.3 (C-2'), 147.3 (C-1').

Synthesized compounds differ in the substituents bound to the phenyl ring. Due similar dibenzoxanthene structure, all observed IR spectra contained absorption bands above 3000 cm^{-1}. On the IR spectrum of the compound **1**, which has no substituents on the phenyl ring, characteristic band was visible at 690 cm^{-1} indicating monosubstituted benzene derivative.
Derivative with two methoxy groups (**3**) on the phenyl ring showed bands at 1360-1390cm^{-1} characteristic for the O-CH_3 group.
IR spectra of compounds containing fluorine (**2**, **5** and **6**) showed absorption at 1000-1200 cm^{-1} originating from the C-F stretching. Compound with bromine substituent (**7**) showed characteristic band at 515-690 cm^{-1} from C-Br stretching.
^{1}H NMR spectra of synthesized compound revealed singlets from 6.37 to 6.86 ppm derived from aromatic protons from xanthene ring. ^{1}H NMR spectrum of **3** showed characteristic singlets at 4.23 and 3.46 ppm corresponding to the protons of the methoxy groups. For the derivative with ethoxy group, ^{1}H NMR spectrum showed characteristic shifts at 3.82 ppm and 1.25 ppm derived from the protons of the ethoxy group.
^{13}C NMR spectra of all compounds revelaed signals higher than 100 ppm for the aromatic carbons from xanthene molecule. Compound **3** on ^{13}C NMR showed signals of methoxy carbons and these signals, as expected, had small shifts in ppm. Signals for the secondary carbon atom at 63.4 ppm is visible only on ^{13}C NMR spectra of derivative with ethoxy group (**4**).

Antibacterial activity

Antibacterial activity of synthesized compounds was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* (Table 1).

Table 1. Inhibition zone (mm) of the investigated compounds.

	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus subtilis*
Compound	Inhibition zone (mm)			
1	/	/	/	/
2	18	16	18	16
3	26	10	26	10
4	12	12	12	12
5	/	/	/	/
6	/	/	/	/
7	/	/	/	/
DMSO	/	/	/	/
Amikacin	42	20	32	22

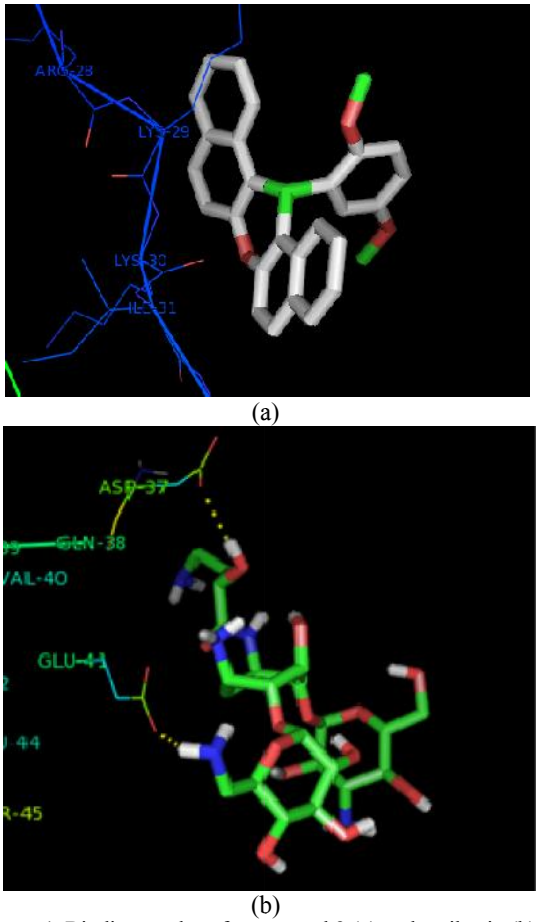
Unsubstituted compound **1** and derivatives with halogen atoms directly bound to phenyl ring (**5**, **6** and **7**) did not show any activity against tested microorganisms. The introduction of oxygen and alkyl substituents on the phenyl ring increased antimicrobial activity. 14-(4'-Trifluoromethylphenyl)-14*H*-dibenzo[*a,j*] xanthene (**2**) showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, with inhibition zone of 18 mm, while against *Pseudomonas aeruginosa* and *Bacillus subtilis* inhibition zone was somewhat smaller (16 mm). The best antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* showed 14-(2',5'-dimethoxyphenyl)-14*H*-dibenzo[*a,j*]xanthene (**3**) with inhibition zone of 26 mm. The same compound against *Pseudomonas aeruginosa* and *Bacillus subtilis* showed inhibition zone of only 10 mm. The most potent antibacterial derivative **3** assessed by diffusion method was further tested by dillution method. Results are shown in Table 2.

Table 2. Minimum inhibitory concentration (MIC, mg/mL) of the most potent compound **3**.

	Escherichia coli	*Staphylococcus aureus*
Compound	MIC (mg/mL)	
3	0.616	0.616
Amikacin	0.005	0.011

Docking study

Possible target in microbial population include the phosphoenolpyruvate phosphotransferase system (PTS). PTS is ubiquitous in eubacteria and absent from eukaryotes. The system consists of two phosphoryl carriers, enzyme I (EI) and the histidine-containing phosphoryl carrier protein (HPr), and several PTS transporters, catalyzing the concomitant uptake and phosphorylation of several carbohydrates. Since a deficiency of EI in bacterial mutants lead to severe growth defects, EI could be a drug target to develop antimicrobial agents (Huang, Lin, Lin, *et al.*, 2013).



(a)

(b)

Figure 1. Binding modes of compound **3** (a) and amikacin (b) at the active site of amino terminal domain of enzyme I from *Escherichia coli* (PDB ID: 1ZYM) as assessed by molecular docking study.

In order to investigate a plausible mechanism of action of the most active compound (**3**) against *Escherichia coli*, docking study was performed using amino terminal domain of enzyme I as a target for antibacterial activity (pdb: 1ZYM). Binding mode of compound **3** into the active site of amino terminal domain of enzyme I from *Escherichia coli* is shown in Figure 1.

Compound **3** binds at the acitve site of enzyme with binding energy of -4.23 kcal/mol while forming no hydrogen bonds. Binding energy of amikacin on the same receptor is -4.20 kcal/mol and forms two hydrogen bonds with Glu 41 and Asp 37.

CONCLUSIONS

Using solvent-free method, catalyzed by iron (III) chloride hexahydrate, seven 14-aryl-14*H*-dibenzoxanthene derivatives were synthesized. Derivatives with CF_3, OCH_3 and OC_2H_5 groups showed good antimicrobial activity, while none of the synthesized derivatives with halogen substituents on phenyl ring showed activity against tested microorganisms. Docking study showed that the most potent compound binds at the active site of investigated enzyme with binding energy similar to amikacin's, but with no hydrogen bonds. These compounds are interesting in future investigations for antimicrobial agens.

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Summary/Sažetak

Ksantenski derivati predstavljaju značajne spojeve zbog svojih mnogostrukih bioloških djelovanja. Prema pouzdanoj proceduri suhe hemije sintetizirano je sedam 14-aril-14*H*-dibenzoksantenskih derivata uz željezo (III) hlorid heksahidrat kao katalizator. Tri sintetizirana derivata pokazala su antibakterijski učinak prema testiranim bakterijskim sojevima. Spoj 14-(2',5'-dimetoksifenil)-14*H*-dibenzo[*a,j*]ksanten (**3**) pokazao je najbolje djelovanje prema *Escherichia coli* i *Staphylococcus aureus* s minimalnom inhibitornom koncentracijom (MIC) od 0.616 mg/mL. U doking studiji za najpotentniji spoj prema *Escherichia coli* kao receptor je korišten amino terminalni dio enzima I. Rezultati doking studija pokazali su da najpotentniji spoj **3** i amikacin, korišten kao referentni antibiotik, imaju slične energije vezivanja za receptor (oko –4.2 kcal/mol), pri čemu se vežu na različitom dijelu enzima.

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Original Scientific Paper

Determination of Pesticide Residues in Honey using GC-MS Technique

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Abstract: Ten samples of honey *(Mountain Honey, Mountain flower honey, Flower honey and Chestnut honey)* were analyses for 26 organochlorine, carbamate and orgaphosphorus pesticides residues. An analytical procedure was based on QuEChERS extraction with acetonitrile followed by gas chromatography-mass spectrometry. The calibration curves constructed were linear over the range from 0.01-0.25 mlL^{-1}. The correlation coefficient were ≥ 0.995 for all pesticides standards. The mean recoveries for extractions were 70-125% for lower concentration range (0.02 mg/kg) and 62-135% for the higher concentration range (0.10 mg/kg) for pesticides analysed. Six different pesticides, propham and carbofuran (carbamate pesticides), methyl parathion, dichlorvos sulfotep and malathion (organophosphate pesticides) were detected in the analysed honeys samples.

INTRODUCTION

Pesticides are widely used in producing food. Their residues may remain in small amounts in or on fruits, vegetables, grains, and other foods (Echobichon, 2001). The way in which pesticides are used varies from country to country. There is evidence that pesticides or their residues have a negative impact on the environment and human and animal health since they are exposed to them through their diets. To ensure the safety of food, most governments regulate the maximum level of each permitted pesticide residue (MRLs). These values also vary according to the intake of different types of food in different countries. In 2008 the European Commission set new MRLs of some pesticides in honey, which are within the range of 10 and 50 ng/g (Regulation EC, 2005) Therefore, determination of these contaminants in honey is essential, since the use of pesticides has increased significantly in recent decades because of the growing demand for food production.

Besides common extraction techniques for the extraction of pesticides from different samples, other more receant approaches such as QuEChERS, solid-phase extraction (SPE), solid-phase microextraction (SPME), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE). These techniques have results in new possibilities in sample treatment and a lot of advantages suh as reduction of extraction time and volume of solvents, which is also ecologically acceptable (Lambropoulou and Albanis, 2007; Nadaf, Yadav and Kumari, 2015; Pang, Fan, Liu *et al.,* 2006)

The most widely used technique for the determinatition of pesticides is gas chromatography (GC), due to its high separation power and avilability of selective detectors such as electroncapture detector (ECD), mass spectrometry detector (MS) and nitrogen phosphorus, (NPD). Also, liquid chromatography (LC) is good alternative technique to GC because of its aplication to polar, non-volatile and thermolabile pesticides (Tette, Roche, Gloria *et al.,* 2016; Bargańska, Ślebioda, and Namieśnik,, 2014; Bargańska, Olkowska, Dymerski, *et al.,* 2014; Rial-Otero, Gaspar, Moura *et al.*, 2007).

The aim of this study was to analyse 26 pesticides residue in 10 samples of honey, mostly origin from Bosnia, using QuEChERS extraction method and GC-MS for their detection.

EXPERIMENTAL

Chemicals and Reagens: The Certified Reference Materials (CRMs) standard solutions of all pesticides (aldrine, ethyl-azinphos, methyl-bromophos, carbofuran, chlorfenvinphos, chlorpyrifos, diazinon, dichlorvos, dimethoate, α-endosulfan, ethion, fenthion, lindane, malathion, methazachlor, methoprotryne, metholachlor, methyl-parathion, pendimethalin, procymidone, propham, simazin, sulfotep, terbufos, terbutrin, triadimefon) were purchased from Dr Ehrenstorfer GmBH (Augsburg, Germany). The stock standard solutions were prepared in the concentration of 200 mg/L and were stored at −20°C. The calibration standards and working standards were prepared by dilution with acetonitrile on the day of analysis. Acetonitrile, acetone and *n*-hexane (pro analysis) were obtained from J.T.Baker (U.S.A) HPLC grade. Water was purified with a Milli-Q water system (Millipore Corporation, Billerica, MA, USA). The QuEChERS kits with salt packets containing 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 1 g trisodium citrate dihydrate, and 0.5 g, trisodium citrate hemihydrate) as well as tubes with 900 mg anhydrous magnesium sulphate and 150 mg primary-secondary amine (PSA) for dispersive solid phase extraction (dSPE) were purchased from Restek (USA).

Sample Collection. Ten honeybee samples were purchased in 2011 and 2012. Eight samples were from Bosnia, one from FYR of Macedonia, and one from Germany. All samples were stored at room temperature in the dark until analysis.
Four types of honey were analyzed. Three samples of *Mountain honey* (season 2011) from Bosnia (Olovo, Vareš and Sokolac), three samples *Mountain flower honey* (season 2012), two of them from Bosnia (Olovo, Kupres) and one obtained from FYR of Macedonia (2012). Three *Flower honey samples* (2011) from Bosnia (Gradačac), Germany, and one collected 2012 from Bosnia (Ljubuški). A *Chestnut honey* (2012) sample from Bosnia was collected from Velika Kladuša area (2012).

Sample Preparation. The honey samples (5 g) were thoroughly homogenized with 10 mL ultrapure water and approximately 5 g of the homogenate was transferred into polypropylene centrifuge tube (50 mL), ultrapure water (10 mL) was added and hand-shaken for 5 minutes. Thereafter 10 mL of acetonitrile (ACN) was added and mixed for 2 minutes, and the QuEChERS salt kit was added. The samples were immediately hand shaken and for 2 minutes and subsequently centrifuged at 3500 rpm for 5 minutes. Thereafter, 6 mL of supernatant was transfered in a 15 mL dSPE polypropilene tube. The tube was hand-shaken for 30 seconds and subsequently centrifuged at 3500 rpm or 5 minutes. Finally, 0.5 mL or 1 μL of supernatant was taken into glass auto sampler vial.

Instrumentation. GC analysis was performed on a GC-MS Agilent (GC 7890A i MS 5975) with colum backflush capability. The injector tepmerature was 280°C. The samples was injected in the splitless mode, and the splitless was opened after 2 minutes. Injection volume was 2 μL. A capillary column HP-5MS (5%-phenyl, 95%-dimethylpolysiloxane), 30 m x 0.25 mm x 0.25μm, was used. Gas career was He with constant flow of 1.90 mL/min. The oven temperature was as follows: initial temperature of 60°C, held for 2 min, increased to 25°C/min up to 150°C, at 3°C /min up to 200°C held for 1 min, and then increased to 290°C at 8°C/ and held for 43 min.
The MS ionization potential was 70 eV, and the temperatures were as follows: interface 280°C, MS source 230°C (ion source), MS Quad 150°C , electron multiplier 1200 V, in both SCAN and SIM modes 250°, transfer line 200°C, and analyzer 230°C. Analysis was performed in SIM mode monitoring specific ions of each analyte as it is shown in Table 1.

Table 1. SIM Conditions of Pesticides Detected byGC-MS

Pesticide	SIM ions (m/z)
Aldrine	262.80; 264.80; 260.80; 291.00
Ethyl Azinphos	128.60; 132.00; 159.80; 206.60
Methyl-bromophos	124.80; 328.80; 331.00; 333.00
Carbofuran	123.00; 131.00; 149.00; 164.00
Chlorfenvinphos	266.80; 268.80; 323.00; 325.00
Chlorpyrifos	196.80; 198.80; 257.80; 314.00
Diazinon	179.00; 199.00; 275.80; 304.00
Dichlorvos	109.00; 185.00; 187.00; 219.60
Dimethoate	92.80; 124.80; 142.60
α -Endosulfan	238.80; 264.80; 268.80; 276.80
Ethion	124.80; 153.00; 231.00; 384.00
Fenthion	153.00; 168.80; 124.80; 278.00
Lindane	180.80; 182.80; 216.80; 218.80
Malathion	157.80; 124.80; 126.80; 173.00
Methazachlor	132.00; 133.00; 134.00; 209.00
Methoprotryne	213.00; 226.00; 240.20; 256.00
Metholachlor	162.00; 238.00; 240.00
Methyl parathion	109.00; 125.00; 263.00
Pendimethalin	162.00; 190.80; 219.80; 252.00
Procymidone	254.80; 286.80; 283.00; 285.00
Propham	93.00; 120.00; 137.00; 179.00
Simazin	137.80; 173.00; 186.00; 201.00
Sulfotep	202.00; 245.00; 265.80; 322.00
Terbufos	153.00; 232.80; 231.00; 287.80
Terbutrin	241.20; 170.00; 185.00; 226.00
Triadimefon	128.00; 180.80; 208.00; 210.00

The most intense ion was used for quantification and the second and third ion for confirmation. Identification criteria was based on (a) the chromatographic retention data, and (b) the relative peak heights of the three characteristic masses in the sample peak that must be within ±20% of the relative intensity of these masses in the mass spectrum of the standard analyzed in the GC-MS system.

RESULTS AND DISCUSSION

Ten samples of honey were analyses for 26 organochlorine, carbamate and organochlorous pesticides residues. The calibration curves constructed were linear over the range from 0.01-0.25 mLL^{-1}. The correlation coefficient were ≥ 0.995 for all pesticides standards. The mean recoveries for extractions were 70-125% for lower concentration range (0.02 mg/kg) and 62-135% for the higher concentration range (0.10 mg/kg) for analysed pesticides.

The limits of detection (LODs) for the pesticides detected in the investigated samples were: 0.010±0.003 (propham), 0.010±0.005 (methyl parathion), 0.010±0.005 (carbofuran), 0.010±0.008 (dichlorvos), 0.010±0.004 (sulfotep) and 0.020±0.002 mg/kg for (melathion).

Results of analyzed honey samples are shown in Table 2.

Table 2. Pesticide residues detected in honeybee samples

The variety of honey	The geographical origin and the season of collection	Detected Pesticides
Mountain honey	(Olovo, BiH 2011)	propham, methyl parathion
Mountain honey	(Vareš, BiH 2011)	nd
Mountain honey	(Sokolac, BiH 2011)	nd
Mountain flower honey	(FYR of Macedonia 2012)	propham malathion
Mountain flower honey	(Olovo, BiH 2012)	nd
Mountain flower honey	(Kupres, BiH 2012)	nd
Flower honey	(Gradačac, BiH 2011)	propham
Flower honey	(Germany 2011)	carbofuran
Flower honey	(Ljubuški, BiH 2012)	dichlorvos propham sulfotep
Chestnut honey	(Velika Kladuša, BiH 2012)	carbofuran propham

nd-not detected

The Table 2 shows that six different pesticides, propham and carbofuran (carbamate pesticides) and methyl parathion, dichlorvos, sulfotep and malathion (organophosphate pesticides) were detected.

In the three tested samples of *Mountain honey*, two pesticides, propham and parathion-methyl, were detected. Also in one of the 3 analyzed samples of *Mountain flower honey*, pesticides propham and malathion were detected. In all three samples of flower honey, pesticides were found. The sample collected from area Ljubuški, BiH, was contaminated by three different pesticides, dichlorvos, propham, sulfotep. Similary, in sample of chesnut honey, carbofuran and propham were detected. In four analysed honey samples pesticides were not found. The detected concentration of propham, methyl parathion, carbofuran, dichlorvos and sulfotep were 0.01 mg/kg, and for melathion was 0.02 mg/kg . Those values were the same as LOD values of detected pesticides.

The most common pesticide detected in even five samples of honey is *propham*. It is used as planth growth regulator in agricultural application as a selective herbicide for annual grasses and broadleaf weeds on forage crops, lettuce, spinach, sugar beets, lentils and peas. Since in this honey areas origin those vegetables are grown in large quantities, use of pesticides has increased, and is possible the reason for the contamination of honey.

Mujić, Alibabić, Jokić *et al.,* (2011) evaluated the health safety of honey (meadow, chestnut, acacia, amphorae, and honeydew) produced at 18 different locations in the region of Una-Sana Canton in the northwestern part of Bosnia and Herzegovina. They determined the pesticides, heavy metals, radioactive elements, and antibiotic residues in 46 honey samples. The content of pesticides were not found. The results of this work indicate that this area is not polluted and is suitable for the development of beekeeping. Kartalovic Jovanic, Jaksic *et al.,* (2015) from the Pannonian region in the Republic of Serbia investigated organochlorine residues in honey samples. It was found the presence of organochlorine pesticides in all samples of honey. But all of the detected concentrations of pesticides were below the maximum allowed value. Das and Kaya (2009) investigated 15 organophosphorus insecticides in 275 honey samples in 33 different cities of Turkey, using gas chromatography with electron capture detector. No insecticide residue was detected in the samples analyzed. This result is highly significant because of its impacts on public health and food safety. Bogdanov, Ryll and Roth (2003) investigated pesticide 36 organochlorine, 32 organophosphorus in 27 honey samples. The investigations discovered that detected amount of all analyzed pesticides were lower than detection limits, which varied between 0.005-0.050 mg/kg. Blasco, Fernaandez, Pena *et al.,*(2003), analysed honey from Portugal and Spain. The residues of more than one pesticide were found in honeys from both countries.

In many cases, pollution of honey is caused by pesticide application in the surrounding area or by environmental contamination. Given the use of those pesticides, it is clear why the samples of honey from rural areas consisted more types of pesticides. Fortunately, the contents of these pesticides detected in this work do not exceed the MRL values.

CONCLUSION

Based on results obtained in this work it can be concluded that the samples of honeybee analyzed are safe for human consumption, in particular mountain honeybees, with less possibility of contamination by pesticides used in farming. In general, in mountainous areas is less influence of anthropogenic factors.

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Summary/Sažetak

U deset uzorka meda (planinski, planinski cvjetni, livadski med i med od kestena) je analizirano 26 organohlornih, karbamatnih i organofosfornih ostataka pesticida. Analitička procedura je bazirana na QuEChERS ekstrakciji sa acetonitrilom, nakon čega su uzorci analizirani uz pomoć gasne hromatografije-masene spektrometrije. Linearnost za sve ispitivane pesticide je postignuta u opsegu koncentracija od 0.01-0.250 mlL^{-1}. Koeficijenti korelacije su bili ≥ 0.995 za sve ispitivane standarde pesticida. Srednje *recovery* vrijednosti ekstrakcija su iznosile 70-125% za niže koncentracijsko područje (0.02 mg/kg) i 62-135% za više koncentracijsko područje (0.10 mg/kg) za analizirane analite. Različiti pesticidi su detektovani u analiziranim uzorcima meda, i to: profam i karbofuran (karbamatni pesticidi) i metil paration, dihlorvos, malation i sulfotep (organofosfatni pesticidi).

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Original Scientific Paper

Determination of the Daily Rhythm of Cortisol in the Saliva of Women and Men

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Abstract: Saliva provides a useful and noninvasive alternative to blood for many biomedical diagnostic assays. Recently, saliva has been used as a biological sample of choice for the monitoring of hormones and other clinically important biomolecules. The assessment of cortisol in saliva has proven to be a valid and reliable reflection of the respective unbound hormone in blood and is widely accepted and a frequently employed method. Due to several advantages over blood cortisol analysis (e.g., stress-free sampling, laboratory independence, lower costs) saliva cortisol assessment can be the method of choice in basic research and clinical environments. Synthesis and secretion of cortisol has the most obvious circadian rhythm in nature. The highest concentration of cortisol in extracellular fluids is in the morning hours and the lowest one in the evening. The object of this study was to determine and compare the daily fluctuations of cortisol in saliva by measuring cortisol levels in the saliva of healthy individuals daily in certain periods. Next step was to compare the values of the concentrations of cortisol in test subjects of different sexes and determine the benefits of analysis of cortisol in saliva.

INTRODUCTION

Cortisol is the main glucocorticoid hormone which is secreted by the cortex of the adrenal gland whose release is controlled by the adrenocorticotropic hormone (ACTH), synthesized from cholesterol (Koraćević et al., 2003). ACTH is controlled by the hypothalamic peptide, corticotrophin releasing factor (CRF). High plasma concentrations of cortisol inhibit the release of CRF and ACTH through the negative feedback mechanism (Kreiger, 1975).

According to its chemical structure cortisol is a steroid hormone (Guyton and Hall, 2006). Cortisol binds to specific intracellular receptors and affects many physiological functions, including the immune system, glucose level, blood vessels and bone metabolism. Cortisol affects metabolism of carbohydrates, fats and proteins (Guyton, 1996). Cortisol is involved in the response to stress and is necessary for proper functioning of organism. Secretion of cortisol in plasma occurs periodically and starts 5-10 minutes after the secretion of ACTH. The concentration of cortisol in plasma raises gradually and reaches its highest values shortly after waking up, and then during next few hours gradually decreases and reaches the lowest value late in the afternoon and early in the evening (Gafni, et al., 2000). Synthesis and secretion of cortisol has the most obvious circadial rhythm in nature (Kirschbaum and Hellhammer, 1999). The disappearance of this circadial rhythm is present in adrenal pituitary gland, Cushing's syndrome, and Addison's disease. Neuroendocrine mechanism increases the release of cortisol up to 20 times in mental stress conditions (Ardal and Holm, 1995).

Daily rhythm, stimulation of ACTH and cortisol is affected by stress, hard injuries, burns and psychological traumas, what is trouble for diagnosis of mentioned illnesses (Kirschbaum *et al*., 1995b). Cortisol in plasma circulates in connection with proteins and individually. Most plasma cortisol is bound to corticosteroid binding

globulin (CBG, also known as transcortin) due to its high affinity. About 65% of cortisol binds for CBG, 30% for albumins and 3-5% is free (Kršljak and Gošić, 2008).
Concentration of cortisol in extracellular fluids is a reliable marker of the hypothalamic-pituitary-adrenal feedback system. Stress intensity is related to the cortisol level released in such situations because stress is a generalized body reaction aimed at protecting the organism and preventing injury (Ruzić, 2005).
Cortisol is usually determined in blood and urine, and lately, saliva has been tested for validity of cortisol assay (Nicolson *et al.,* 1997).
Saliva provides a useful and noninvasive alternative to blood for many biomedical diagnostic assays.
Salivary cortisol concentration represents 70% of unbound blood cortisol. Due to its low molecular weight and liposolubility, unbound cortisol penetrates the cellular membrane by simple diffusion which enables the level of free cortisol to be determined in all body fluids (Gafni *et al*., 2000). There is a high correlation between salivary cortisol levels and unbound free cortisol levels in plasma and serum (Hellhammer *et al*., 2009).
By determination of concentration of cortisol in serum or plasma, intensity of secretion and its effect on tissue can be defined. In this determination it is necessary to know that analysis of one sample of serum or plasma shows

EXPERIMENTAL

Materials and methods

Samples of saliva are taken from 21 healthy individuals, 11 men and 10 women, two times, 8:00-9:00 a.m. and 5:00-6:00 p.m.. Samples of saliva are centrifuged in order to remove cellular debris. Samples of saliva were keept at temperature of –24 °C.
For determination of cortisol on autoanalyzer (Vitros ECI immunodiagnostics system) it was used biochemical laboratory kit: Vitros Immunodiagnostics Products Cortisol Reagent Pack, Ortho-Clinical Diagnostics, Johnson-Johnson company.

Immunochemical method for determination of cortisol on autoanalyzer

Measuring of cortisol concentrations in serum and saliva is done on autoanalyzer, by immunochemical method, which is based on luminescent immunochemical reaction.
Determination is based on competitive binding of cortisol in sample and cortisol marked with peroxidase, on binding places with special antibodies (sheep polyclonal anti-cortisol). Antibody-antigen complexes which are formed are trapped with streptavidin at barriers of vessel. Materials that are not bound are taken out with washing. The activity of bound peroxidase is measured by luminescence reaction. Bound peroxidase catalyses oxidation of luminol forming derivatives that produce the light measured by analyser. The amount of bound peroxidase is inversely proportional to concentration of present cortisol.
Statistical analysis of the results is done using by Student's *t* test.

concentration in that particular moment. By determination of concentration of cortisol in 24 - hours urine volume, the amount of cortisol secreted during the day is defined, by which the function of the cortex of adrenal gland can be defined (Reid *et al*., 1992).
Given that, the salivary sample collection is less invasive than blood sampling, many authors agree that determination of salivary cortisol would be more appropriate than blood cortisol (Kirschbaum et al., 1995a).
The cortisol values in plasma and saliva vary during the day. The reference values for cortisol concentration are classified according to the time of sampling. These values are affected by several factors which can vary, so it is recommended that every laboratory should define its own reference values. The reference values of cortisol in saliva, determined in Laboratory of Clinical Biochemistry of Institute for Clinical Chemistry and Biochemistry of Clinical Centre at University of Sarajevo, are following: 3,5 – 49,0 nmol/L (in the morning), and 1,3 – 37,0 nmol/L (in the afternoon).
Since the daily routine (stress, physical effort, nutrition) can significantly affect cortisol concentrations, we determined cortisol concentrations in the late evening hours and compared to those determined in the morning.

RESULTS AND DISCUSSION

The concentration of cortisol in saliva of female subjects

The concentrations of cortisol in the saliva of women in the morning and in the afternoon are shown in Table 1, and graphical results are shown in Figure 1. The results showed that in any case, cortisol concentration was lower in the afternoon. The cortisol levels in some individuals were higher than the reference values both in the morning and in the afternoon, which can be attributed to the effect of stress. The mean concentration of cortisol in the morning was 35,8 nmol/L, and in the evening was 23,14 nmol/L.
The results showed that concentrations of cortisol in saliva of female subjects in the morning were statistically significantly higher in comparison to those in the afternoon ($p^{**}<0.01$). There are daily fluctuations in cortisol concentrations in saliva taken from female subjects.

Table 1. Concentrations of cortisol in saliva of women in the morning and in the afternoon

Number of sample	Cortisol (nmol/L)	
	In the morning	In the afternoon
1	52,1	40,8
2	37,1	26,6
3	49,1	37,4
4	67,4	57,1
5	19,1	4,9
6	24,5	11,3
7	19,5	9,2
8	32,4	12,1
9	18,9	10,1
10	31,7	21,9

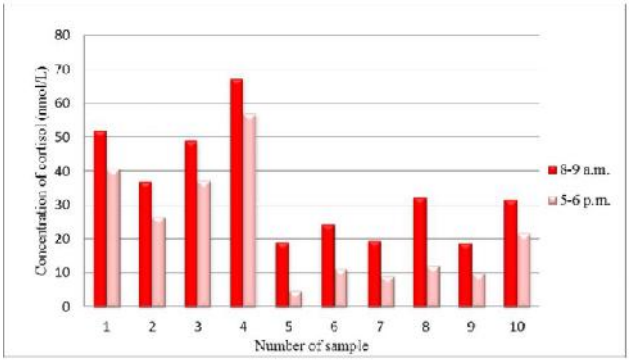

Figure 1. Concentrations of cortisol in saliva of female subjects in the morning and in the afternoon

The concentration of cortisol in saliva of male subjects

The concentrations of cortisol in the saliva of male subjects in the morning and in the afternoon are shown in Table 2, and graphical results are shown in Figure 2. The results showed that in any case, the concentration of cortisol was lower in the afternoon. Among males, there are evident individual differences in the concentration of cortisol in saliva. The mean concentration of cortisol in the morning was 32,23 nmol/L, and in the afternoon or evening 20,30 nmol/L. The results showed that concentrations of cortisol in saliva of males were statistically significantly higher in the morning in comparison to those in the afternoon/evening ($p^{**}<0.01$). Also, the results showed that there are daily fluctuations in cortisol concentrations in saliva of males.

Table 2. Concentrations of cortisol in saliva of males in the morning and in the afternoon

Number of sample	Cortisol (nmol/L)	
	In the morning	In the afternoon
1	24,7	15,9
2	65,5	40,8
3	49,9	37,4
4	67,4	47,1
5	15,5	9,6
6	41,6	17,4
7	16,9	11,6
8	13,1	5,2
9	16,5	8,5
10	21,2	9,5

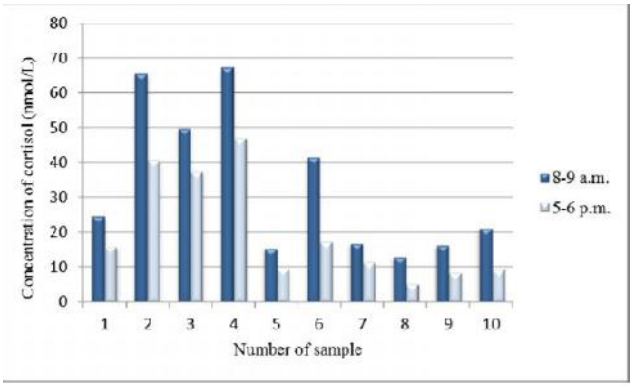

Figure 2. Concentrations of cortisol in saliva males in the morning and in the afternoon

Comparison of cortisol concentrations in saliva of female and male subjects

Results presented in Table 3., and graphically presented in Figure 3, show that there are daily fluctuations of cortisol concentrations in saliva of male as well as female subjects. The levels of cortisol in saliva in some participants were higher than the reference values which may be caused by other factors (illness, stress). The cortisol concentrations in saliva of both sexes in the morning were statistically much higher than those in the afternoon/evening (Ljubijankić *et al.,* 2008).
Regarding the female subjects, the higher mean cortisol concentrations in saliva were found in both time of sampling (in the morning and in the afternoon/evening) in comparison to males. Women seemed to have higher cortisol levels than men probably due to the fact that women are more sensitive to stress. However, these differences in cortisol concentrations between subjects of different sexes were not statistically significant.

Table 3. The middle value cortisol concentrations (and standard deviations) in saliva of female and male subjects in the morning and in the afternoon

Time of sampling	Middle value of cortisol concentration ± S.D. (nmol/L)	
	Females	Males
8-9 a. m.	35,8 ± 16,42	33,23 ± 21,30
5-6 p. m.	23,14 ± 17,13	20,30 ± 15,39

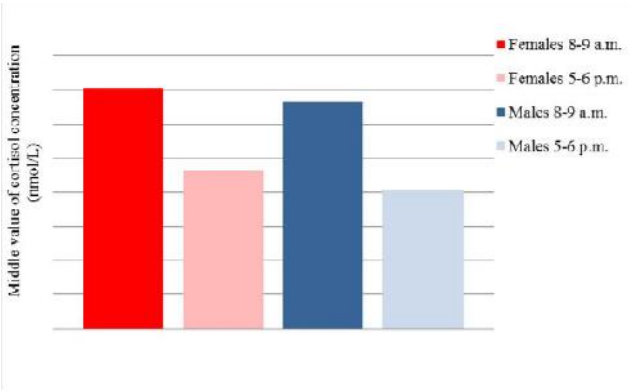

Figure 3. Comparison of cortisol concentrations in saliva of female and male subjects in the morning and in the afternoon

CONCLUSION

There are very complicated daily fluctuations of cortisol concentrations in saliva of healthy people of both sexes, because mean cortisol concentrations in the morning were statistically significantly higher in comparison to those in the afternoon/evening.
Higher cortisol concentrations in saliva of female subjects were found when compared to male subjects, but these differences were not statistically important.
Individual variability of cortisol concentration is evident during the day. The cortisol concentrations in saliva are really low, so it is recommended that in cases of metabolic disorders, sampling time of saliva should be taken in consideration.

Measurement of saliva cortisol, especially in early morning hours could be completely substitute the measurements of cortisol in serum or plasma.
The non-invasive sampling procedure allows saliva to be used for cortisol level determination in situations where blood sampling is difficult to perform.
Unlike blood, saliva contains free cortisol. Bearing in mind that the only biologically active form is free cortisol, the value obtained in saliva could be used as an objective parameter for changes in the value of cortisol in one day (morning, noon and evening).

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Summary/Sažetak

Pljuvačka predstavlja korisnu i neinvazivnu alternativu krvi za mnoge biomedicinske dijagnostičke testove. U novije vrijeme se koristi kao dijagnostička tečnost za monitoring mnogih supstanci, pa i hormona. Određivanje kortizola u pljuvački je dokazano valjan i pouzdan odraz kortizola u krvi i široko je prihvaćena i često korištena metoda. Zbog nekoliko prednosti u odnosu na analizu kortizola u krvi (uzorkovanje bez stresa, laboratorijska nezavisnost, niži troškovi) mjerenje pljuvačnog kortizola može biti metoda izbora u osnovnim kliničkim istraživanjima. Sinteza i sekrecija kortizola ima najizrazitiji cirkadijalni ritam u prirodi. Najveća koncentracija kortizola u ekstracelularnim tečnostima je u jutarnjim satima, a najniža u večernjim satima.

Cilj ovog rada je da se utvrde dnevne oscilacije kortizola u pljuvački mjerenjem nivoa kortizola u pljuvački zdravih osoba u određenim dnevnim periodima, zatim da se uporede vrijednosti koncentracije kortizola u osoba različitog spola i utvrde prednosti analize pljuvačnog kortizola.

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Rosemary as ecologically acceptable corrosion inhibitor of steel

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Abstract: In recent years, due to increasing interest and attention of the world towards environmental protection, there has been a complete reduction or use of a certain number of corrosion inhibitors. Corrosion inhibitors are substances which, added in small quantities in aggressive media, may greatly reduce the rate of corrosion of metals. In the development of corrosion inhibitors, it is necessary to pay special attention to their toxicity and impact on environmental pollution. The research of plant extracts have become a great area of interest in the study of corrosion inhibitors. Testing the ability to protect steel was performed with plant material (*Rosmarinus officinalis* L.). In order to determine the basic parameters that show the effectiveness of green inhibitors, electrochemical measurements of corrosion rate were carried out. Results obtained by DC techniques (method of Tafel extrapolation) showed that the corrosion rate decreases in the presence of the tested corrosion inhibitor. Studies have shown that, in a certain concentration, rosemary (*Rosmarinus officinalis* L.) has the effectiveness of the protection of steel in 3% NaCl, and as such, it is considered an acceptable corrosion inhibitor.

INTRODUCTION

Corrosion is a natural process by which physical-chemical interactions of metals and the environment result in the shift of metals to the thermodynamically favorable state ie. it comes to the oxidation of metals, which results in a loss of its functionality. The corrosion effect of aggressive components in the electrolyte in practice is very often reduced by using metal′s corrosion inhibitors. Within the method of protection from corrosion, the inhibitors occupy a special place, both by the specificity of protection and by the widespread application. There are several classifications of inhibitors. According to the electrochemical nature of the corrosion process, inhibitors can be classified as anodic, cathodic, or mixed.

According to the chemical nature, inhibitors are further categorized into substances of organic and inorganic origin. Finally, according to the chemical properties: oxidizing and non-oxidizing compounds and further divisions according to the pH value of the solution in which they are applied: for acidic, neutral and alkaline solutions. The main characteristics of inhibitors are: the ability to protect the metal surface, a big activity in small concentrations, low price, easy handling and storage, and low toxicity. The process of adsorption of metal surfaces is to be coated with the inhibitor, which slows down the corrosion of metals. The reduction or termination of the use of certain corrosion inhibitors has arisen due to their impact on environmental pollution. In recent years, the focus of research has shifted to the inhibitory activity of biological molecules or mixtures of natural compounds. The aim of this paper is to examine the corrosion resistance of steel in 3% NaCl solution, with the use of non-toxic "green inhibitor" (*Rosmarinus officinalis* L.). The relationship between the reaction rate, current and potential, is characteristic for the electrochemical reaction

and it is an important data in the analysis of the corrosion process by electrochemical methods on the tested system.

EXPERIMENTAL

In electrochemical corrosion, the testing process of corrosion of DC method polarization method of measurement (potentiostatic and galvanostatic) is used. The objective of measurement is to record the wrong/curved polarization of current/current polarization - voltage. For electrochemical testing of corrosion rate, the following equipment is being used - a system that consists of:

- ✓ Poteciostat/galvanostat model 263A
- ✓ PowerCorr DC Corrosion software version 2.47, PowerPulse Electroanalytical software version 1.07
- ✓ Corrosion cell K 0047, with standard, saturated calomel electrode, auxiliary electrode-graphite, and Flat Specimen Holder Kit model K0105.

Potentiostat/galvanostat is used to transfer data from a computer to a cell. The obtained data are returned from the cell to the computer. The cell is a glass container, in which auxiliary electrodes, standard calomel electrode and a working electrode with a sample are located (Figure 1). The saturated electrode is placed in Lugin capillaries, in which a saturated solution of KCl is placed.

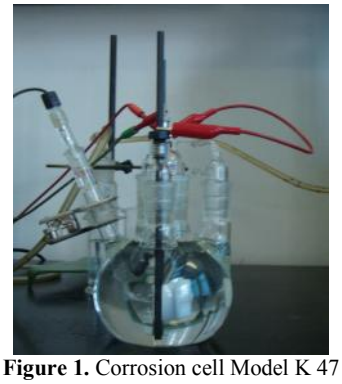

Figure 1. Corrosion cell Model K 47

In order to determine corrosion parameters, the method Tafel extrapolation, based on Butler - Volmer equation that gives the relationship between the current and overvoltage, was applied.

$$j = j_0\{\exp[(1-\alpha)zF\eta / RT] - \exp[-\alpha zF\eta / RT]\}$$

All measurements were performed in 3% NaCl solution, to which an inhibitor of plant origin has been added (*Rosmarinus officinalis* L.) at various concentrations. Work surface of tested materials (steel) is mechanically cleaned before each measurement (abrasive paper of different fineness), degreased in alcohol and washed with distilled water, chemically treated in HCl and washed with distilled water.

RESULTS AND DISCUSSION

Corrosion behavior of steel S235JR and X5CrNi18-10 has been presented experimentally by the obtained Tafel diagrams (Figure 2). Based on the curves obtained by Tafel extrapolation the corrosion parameters have been determined (Table 1).

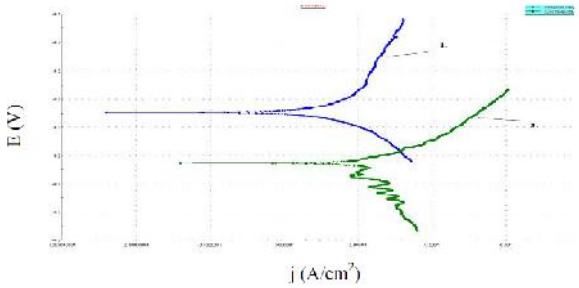

Figure 2. Tafel diagrams in 3% NaCl solution: X5CrNi18-10 (1) and S235JR (2)

Table 1. Data obtained by Tafel extrapolation

Type of steel	Electrolyte 3% NaCl		
	Corrosion potential (mV)	Corrosion current (μA)	Corrosion rate (mm/year)
X5CrNi18-10	-447	15,32	1,832
S235JR	-626	3,48	0,4166

For tested steel S235JR were used three different concentrations of rosemary extracts as a corrosion inhibitor. The results are shown in Figure 3, and the corrosion parameters have been presented in Table 2.

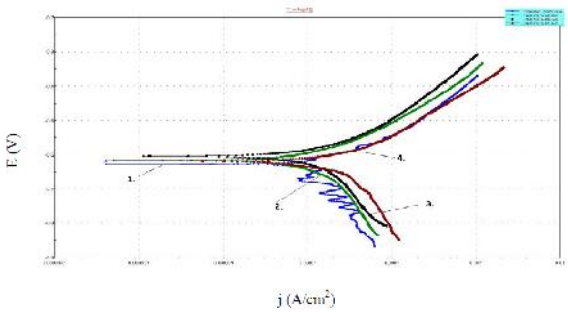

Figure 3. Tafel diagrams for steel S235JR in 3% NaCl solution with different concentrations: (1) steel without inhibitor; (2) 0.005 mg / ml;(3) of 0.01 mg/ml (4) of 0.1 mg/ml inhibitor

Table 2. Corrosion indicators for the plant extract of rosemary as an ecologically acceptable corrosion inhibitors for steel S235JR in 3% NaCl solution.

c_{inh} (mg/ml)	j_{corr} (μA/cm²)	B_k (mm/year)	E_{corr}	Z_{inh} (%)
Without inhibitor	3,48	0,4166	-626	-
0,005	2,27	0,2712	-616	34,90
0,01	3,20	0,3831	-604	8,04
0,1	8,03	0,9608	-616	-

As it can be seen from the diagram (Figure 3), polarization curve is shifted towards the left (curve 2),

which means that the presence of the inhibitor at a concentration of 0.005 mg/ml reduces the corrosion rate. On the basis of certain corrosion parameters by Tafel extrapolation method (Table 2), it can be seen that in the investigated concentration range the lowest rate of corrosion is at the concentration of inhibitors of 0.005 mg/ml and it amounts to 0.2712 mm/year and its effectiveness amounts to 34.90%. By further increasing of the concentration to 0.01 mg/ml inhibitory effect continues, because the corrosion rate (0.3831 mm/year) is still lower than the rate of corrosion of the examined steel without the presence of the inhibitor (0.4166 mm/ year). At a concentration of 0.1 mg/ml inhibitor functions as an activator because the corrosion rate is higher than the rate of corrosion without inhibitor.

Figure 4. shows the anodic and cathodic polarization curves X5CrNi18-10 in the electrolyte with inhibitor addition (rosemary) in different concentrations (Table 3). The polarization curve shifts to the left (curve 2, 3, 4, 5) ie. the presence of inhibitor at the concentration of 0,01 mg/ml; 0,1 mg/ml; 0,3 mg/ml and 0,4 mg/ml reduces the corrosion rate. On the basis of certain corrosion parameters by Tafel extrapolation method in Table 3 we can see that in the investigated concentration range the lowest rate of corrosion is at the concentration of inhibitors of 0.3 mg/ml and it amounts to 0.03642 mm/-year, and its efficiency amounts to 98.01%.

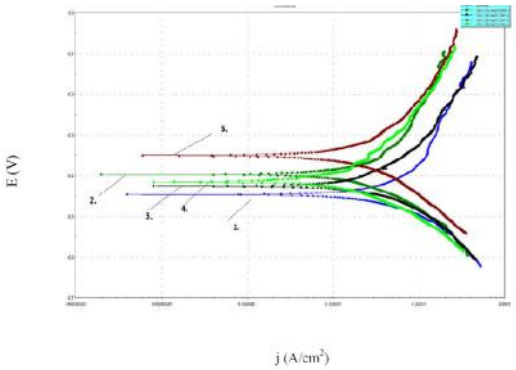

Figure 4. Tafel diagrams for steel X5CrNi18-10 in 3% NaCl solution (1) steel without inhibitor (2) 0.01 mg / ml; (3) 0.1 mg / ml; (4) 0.3 mg / ml; (5) 0.4 mg / ml;

Table 3. Corrosion indicators for plant extract of rosemary as an ecologically acceptable corrosion inhibitors for steel X5CrNi18-10 in 3% NaCl solution.

c_{inh} (mg/ml)	j_{corr} (μA/cm^2)	B_k (mm/year)	E_{corr}	Z_{inh} (%)
Without inhibitor	15,32	1,832	-447	-
0,01	6,35	0,07597	-398	95,85
0,1	5,82	0,06965	-425	96,20
0,3	3,04	0,03642	-419	98,01
0,4	5,0	0,05983	-347	96,73

CONCLUSION

As the obtained results have shown, rosemary (*Rosmarinus officinalis* L), as the "green inhibitor", can be applied in order to reduce the corrosion rate. For steel S235JR, examined by Tefal extrapolation in 3% NaCl solution with the addition of rosemary, the best results were obtained with the inhibitor concentration of 0.005 mg/ml.

It has been proven that the applied "green inhibitor" has better properties with regard to decreasing of corrosion rate for steel X5CrNi18-10. Based on the corrosion parameters obtained by Tafel extrapolation method in the investigated concentration range of rosemary as a corrosion inhibitor, it has been shown that the lowest rate of corrosion is at a concentration of 0.3 mg/ml.

Test results have shown that rosemary has a high efficiency for the protection of steel X5CrNi18-10 in 3% NaCl solution (98.01%) at a concentration of 0.3 mg/ ml.

Results obtained by DC techniques (by Tafel extrapolation method) have shown that the investigated inhibitor of certain concentration can be used as a "green inhibitor" of corrosion for both steels tested in this paper.

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Summary/Sažetak

Posljednjih godina, zbog većeg interesa i pozornosti svijeta prema zaštiti okoliša dolazi do potpunog smanjenja odnosno upotrebe određenog broja korozionih inhibitora. Inhibitori korozije su tvari koje dodane u maloj količini u agresivni medij mogu u velikoj mjeri smanjiti brzinu korozije metala. Pri razvoju korozonih inhibitora potrebno je posebnu pažnju obratiti na njihovu toksičnost te uticaj na onečišćenje okoliša. Istraživanje ekstrakata biljaka u zadnje vrijeme je područije visokog interesa kada je riječ o inhibitorima korozije. Ispitivanje mogućnost zaštite čelika je vršeno sa biljnim materijalom (*Rosmarinus officinalis* L.*)*. U cilju određivnja osnovnih parametara koji pokazuju efikasnost zelenih inhibitora izvršena su elektrohemijska ispitivanja brzine korozije. Rezultati dobiveni DC- tehnikama (metodom Tafelove ekstrapolacije) pokazali su da se brzina korozije smanjuje u prisustvu ispitivanih inhibitora korozije. Istraživanja su pokazala da ružmarin (*Rosmarinus officinalis* L.*)* ima pri određenoj koncentraciji djelotvornost zaštite čelika u 3% NaCl-u, te kao takvom se smatra prihvatljivim inhibitorom korozije.

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The effect of chlorogenic acid on the Briggs-Rauscher oscillating reaction

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Abstract: The Briggs-Rauscher oscillating reaction can be used as test for antioxidant activity of pure compounds or food extracts. Antioxidants are substances that have the ability to neutralize free radicals, which are harmful to human health. Adding the substances with antioxidant ability to the reaction mixture, oscillations temporarily stops, and after a certain time the oscillating reaction starts again. The period without oscillations is known as inhibition time, and it's proportional to the quantity of antioxidant species in reactive mixture. In this study the Briggs-Rauscher reaction was used to measure the antioxidant activity of chlorogenic acid. Inhibition time, duration of reaction and the number of oscillations was determined varying the concentration of chlorogenic acid and solvent (water, ethanol, dimethyl sulfoxide). Flow of oscillations in the Briggs-Rauscher reaction mixture was monitored as a change in potential between the platinum and silver-silver chloride electrodes at room temperature. With increasing concentrations of chlorogenic acid in all three solvents the inhibition time of oscillations is increased.

INTRODUCTION

Oxidative stress is the result of excessive production of free radicals, due to disturbances in the balance of oxidation-reduction processes in biological systems. Exposure to free radicals may be inhibited by the compounds that have antioxidant properties. Antioxidants have the ability to stabilize or deactivate free radicals before they can damage cells in a way to donate an electron or a hydrogen atom to free radicals (Brewer, 2011).

Various phenolic compounds have antioxidant properties and important role in the prevention and/or the development of various diseases caused by the action of free radicals. From the chemical structure of phenolic compounds depends on their antioxidant activity (Shalaby and Shanab, 2013).

Antioxidant activity of phenolic acids depends on the number and position of hydroxyl groups to the carboxyl functional group. Monohydroxy benzoic acids with a hydroxyl group in ortho- or para- position relative to the carboxyl group does not show antioxidant activity, while the meta-hydroxybenzoic acid show. Antioxidant activity of phenolic acids increases with the degree of hydroxylation. Trihydroxy gallic acid shows a high antioxidant activity (Rice-Evans *et al.*, 1996).

The antioxidant activity of food of plant origin stems from the cumulative and synergistic effects of a large number of antioxidants such as vitamins C and E, antioxidants, mainly phenolic acids and flavonoids, terpenoids, carotenoids and trace minerals (Rice-Evans *et al.*, 1996; Robards *et al.*, 1997).

Chlorogenic acid, ester of caffeic and quinic acid, a major phenolic compound in coffee is known as a powerful natural antioxidant. As coffee (with or without the presence of caffeine), chlorogenic acid acts as an antioxidant in neurons against hydrogen peroxide-induced stress. In one liter of coffee, chlorogenic acid is found in quantities of 500 to 800 mg. Significant amounts of chlorogenic acid is found in fruits and vegetables such as apples, pears, strawberries, eggplants, tomatoes and potatoes. Daily intake of coffee, can provide sufficient

intake of chlorogenic acid, however, the same input by using dietary supplements may be better (Beregi *et al*., 2003; Kim *et al.*, 1997; Kono *et al.*, 1997; Sondheimer, 1964).

For measurements of the antioxidant activity of pure compounds and food extracts, several different methods are used (Shalaby and Shanab, 2013). Cervellati *et al.* (2001) have developed a method for measurements of antioxidant activity based on the inhibitory effect by antioxidants on the oscillations of the Briggs-Rauscher reaction. The Briggs-Rauscher oscillating system consists of the iodination and oxidation of an organic substrate by acidic iodate it the presence of hydrogen peroxide with Mn(II) ion as catalyst (Gurel and Gurel, 1983; (Gajdoš-Kljusurić *et al.*, 2005). Oscillations occur between the periodic variation of the concentrations of intermediates and catalyst. By adding antioxidants in the Briggs-Rauscher reaction mixture the oscillations are temporarily interrupted. Inhibition time is in proportion to the amount and properties of added antioxidants. pH value of the Briggs-Rauscher reaction mixture is about 2, which is similar to that of the fluids in the human stomach. Therefore, useful information on the *in vitro* antioxidant activity under acidic conditions can be obtained using the Briggs-Rauscher reaction which is a great advantage compared to other methods for measurements of antioxidant activity (Cervellati *et al*., 2002; Hönor and Cervellati, 2002; Hönor *et al*., 2002).

An important application of the Briggs-Rauscher reaction is found in many studies concerning human health (Pejić *et al.*, 2012). Electromagnetic waves that travel through the muscle tissue similar oscillations Briggs-Rauscher reaction system, which is why these waves can help to understand of complex phenomena such as the heart beats and nerve tissue.

In this work, the antioxidant activities of different concentrations of chlorogenic acid in different solvents (water, ethanol and dimethyl sulfoxide) were analysed by inhibition of the Briggs-Rauscher reaction.

EXPERIMENTAL

Reagents

All used chemicals and reagents were of analytical grade: potassium iodate, Semikem; sulphuric acid, 96%, Semikem; hydrogen peroxide, 30%, Semikem; malonic acid, Merck; manganese(II) sulphate, Merck, starch, Merck; ethanol, 95%, Semikem; dimethyl sulfoxide, Merck and chlorogenic acid, Acros Organics.

Preparation of the solutions for the Briggs-Rauscher reaction

Three solutions (A, B and C) were prepared.

Solution A: Solution of potassium iodate (0.2 mol dm^{-3}) in sulfuric acid (0.43%).

Solution B: Solution of hydrogen peroxide (15%).

Solution C: Solution of malonic acid (0.15 mol dm^{-3}), manganese(II) sulphate (0.02 mol dm^{-3}) and starch (0.03%).

Mixture of equal volumes of the solutions A, B and C represents the Briggs-Rauscher reaction mixture, which is used for measurements of antioxidative activity (Marković and Talić, 2013).

Preparation of the solutions of chlorogenic acid

Exactly 10 mg of chlorogenic acid was dissolved in 100 cm^3 of solvent, distilled water, ethanol and dimethyl sulfoxide. From stock solution of chlorogenic acid (100 mg dm^{-3}) solutions of different concentrations: 75; 50; 25 and 10 mg dm^{-3} were prepared.

Evaluation of antioxidant activity using the Briggs-Rauscher reaction

Oscillations of the Briggs-Rauscher reaction mixture were followed potentiometrically by recording the potential of a platinum electrode and $Ag/AgCl/KCl_{(sat)}$ reference electrode (+197 mV vs. SHE). The electrode was connected to a pH multimeter (Phywe, Model 13702.93). The accurance of the multimeter was ±1 mV. All measurements were conducted at room temperature, 20±0,5°C. The mixture was stirred by a magnetic stirrer (600 r.p.m.). The Briggs-Rauscher reaction mixture were prepared by mixing the appropriate amounts of stock solutions (A, B and C) in beaker to a total volume of 30 cm^3. The order of addition was: solution A, solution C solution and B. Oscillations begin after the addition of solutuon B. The pH value of the Briggs-Rauscher reaction mixture is 1.56. Solutions of chlorogenic acid (1 cm^3) was added to 30 cm^3 active the Briggs-Rauscher reaction mixture after the third oscillation.

RESULTS AND DISCUSSION

A non-inhibited Briggs-Rauscher reaction had about 20 oscillations that could be monitored and visually based on changes in the color of the reaction mixture from colorless through yellow to dark blue and again the same changes. The color change is explained by the oscillation of the concentration of I_2 and I^- as in reaction (Gajdoš-Kljusurić *et al.*, 2005):

$$IO_3^- + 2H_2O_2 + CH_2(COOH)_2 + H^+ + ICH(COOH)_2 + 2O_2 + 3H_2O \quad (1)$$

Reaction (1) is derived from the following two reactions:

$$IO_3^- + 2H_2O_2 + H^+ \rightarrow HOI + 2O_2 + 2H_2O \quad (2)$$

$$HOI + CH_2(COOH)_2 \rightarrow ICH(COOH)_2 + H_2O \quad (3)$$

The reaction (2) occurs through two different processes, radical and non-radical.

During these process, changes in concentration of iodide ion in solution and the color change can be observed because the reaction (3) takes place in two steps:

$$I^- + HOI + H^+ \rightarrow I_2 + H_2O \quad (4)$$

$$I_2 + CH_2(COOH)_2 \rightarrow ICH(COOH)_2 + H^+ + I^- \quad (5)$$

Yellow occurs due to the formation of I_2. The emergence of I_2 is caused by the rapid production of HOI during radical process. Over time, it creates more HOI than can be utilized at any given moment. Certain amount of HOI is reduced with hydrogen peroxide to I^-. The concentration of I^- is growing, and the resulting yellow

color of the Briggs-Rauscher reaction mixture is changed to dark blue when I^- dominates more than HOI. Then I^- is combined with I_2 to form a complex with starch. When the concentration of I^- is high, reaction (2) is switched to slow non-radical process, and the color begins to fade, and the cycle is repeated. Sample of recording of the potential for non-inhibited oscillating Briggs-Rauscher mixture is shown in Figure 1.

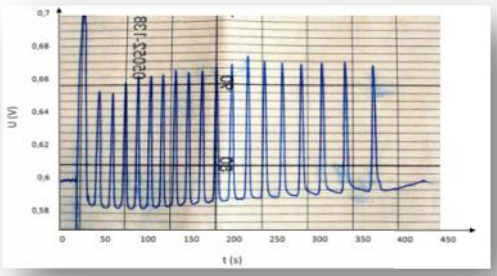

Figure 1. Oscillations for a non-inhibited Briggs-Rauscher reaction mixture

Addition of the solution of chlorogenic acid in theactive Briggs-Rauscher reaction mixture causes an immediate effect of quenching of oscillations. The oscillations stop and start again after a period because the reaction produces hydroperoxyl radicals that are quenched by antioxidants. The quenching of oscillations is measured as an inhibition time which is correlated with contents of the added antioxidant. Sample of recording of the potential when 1 cm^3 of aqueous solution of chlorogenic acid (50 mg dm^{-3}) was added to 30 cm^3 of an oscillating Briggs-Rauscher mixture after third oscillation is shown in Figure 2.

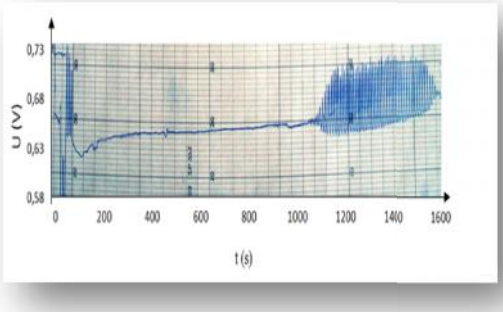

Figure 2. Oscillations for inhibited Briggs-Rauscher reaction mixture

Estimated inhibition time for measurements of the antioxidant potential is performed by subtracting the time appearance regenerated oscillations of the third oscillation. In this way, the determined value of inhibition time for all the tested solutions of chlorogenic acid (Table 1).

Table 1. Inhibition time of the Briggs-Rauscher reaction caused by different concentration of chlorogenic acid

Concentration of chlorogenic acid (mg dm^{-3})	Inhibition time (s)		
	Water	Ethanol	Dimethyl sulfoxide
10	35	25	20
25	110	135	70
50	1030	1210	855
75	1935	1635	1515
100	2395	2400	2080

An inhibition time of the Briggs-Rauscher reaction increases with increasing concentrations of chlorogenic acid in the case of all three solvents. The aqueous solutions of chlorogenic acid showed the best antioxidant activity, followed by ethanolic, and dimethyl sulfoxide solutions. Inhibition time depends linearly on the concentration of chlorogenic acid between 10 and 100 mg dm^{-3}. The correlation coefficient between the inhibition time and concentration of chlorogenic acid in aqueous and ethanolic solutions was 0.977 and 0.983 in dimethyl sulfoxide solutions.

With increasing concentrations of chlorogenic acid increases the total time of oscillations of the Briggs-Rauscher reaction, as expected (Table 2).

Table 2. Time of oscillations of the Briggs-Rauscher reaction caused by different concentrations of chlorogenic acid

Concentration of chlorogenic acid (mg dm^{-3})	Time of oscillations (s)		
	Water	Ethanol	Dimethyl sulfoxide
0	355	250	280
10	520	435	420
25	850	640	375
50	1655	2005	1125
75	2545	2275	2035
100	2800	2990	3300

Addition of 1 cm^3 of pure solvent (water, ethanol and dimethyl sulfoxide) in the Briggs-Rauscher reaction mixture had no significant effect on the number of oscillations in the system, as in the case of adding a solution of chlorogenic acid (Table 3).

Table 3. Number of oscillations of the Briggs-Rauscher reaction caused by different concentrations of chlorogenic acid

Concentration of chlorogenic acid (mg dm^{-3})	Number of oscillations		
	Water	Ethanol	Dimethyl sulfoxide
0	22	18	20
10	31	28	28
25	53	30	30
50	61	66	66
75	51	53	53
100	51	56	56

Number of oscillations of the Briggs-Rauscher reaction is increased by the addition of chlorogenic acid in higher concentrations of all three cases to a point, after which the number is reduced. It has been noted that the chlorogenic acid concentration of 50 mg dm^{-3} increased the number of oscillations, after which further increase the concentration reduces the number of oscillations.

CONCLUSIONS

The Briggs-Rauscher oscillating reaction is suitable as an analytical method for measuring the relative *in vitro* antioxidant activity of pure compounds and extracts of food at a low pH, like the pH in the human stomach. In this study a linear relationship is confirmed between the inhibition time of oscillation in the Briggs-Rauscher reaction mixture and the concentration of pure chlorogenic acid in three solvents (water, ethanol and dimethyl sulfoxide), ranging from 10 to 100 mg dm^{-3}. Application of the Briggs-Rauscher reaction has many advantages over other methods for measurements of antioxidant activity. Analysis is cheap, quick and necessary reagents and devices are commonly used in all chemical laboratories.

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Summary/Sažetak

Briggs-Rauscher oscilirajuća reakcija se primjenjuje kao nova metoda za testiranje antioksidacijske aktivnosti čistih spojeva i ekstrakata hrane. Antioksidansi su spojevi koje imaju sposobnost neutralizirati slobodne radikale, koji su štetni za ljudsko zdravlje. Dodatkom spoja koji ima antioksidacijska svojstva u Briggs-Rauscher reakcijsku smjesu, oscilacije se privremeno prekidaju, da bi se nakon određenog vremena ponovo nastavile. Vrijeme prekida oscilacija naziva se vrijeme inhibicije i proporcionalno je količini dodanog antioksidansa. U ovom radu Briggs-Rauscher reakcija je primijenjena za dokazivanje antioksidacijske aktivnosti hlorogenske kiseline. Praćeno je vrijeme inhibicije, vrijeme trajanja i broj oscilacija u zavisnosti od koncentracije hlorogenske kiseline i rastvarača (voda, etanol, dimetilsulfoksid). Tok oscilacija u Briggs-Rauscher reakcijskoj smjesi praćen je kao promjena potencijala između platinske i srebro-srebrohloridne elektrode na sobnoj temperaturi. Sa porastom koncentracije hlorogenske kiseline u sva tri rastvarača produžavalo se vrijeme inhibicije oscilacija Briggs-Rauscher reakcijske smjese.

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24. maj 2016.g
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