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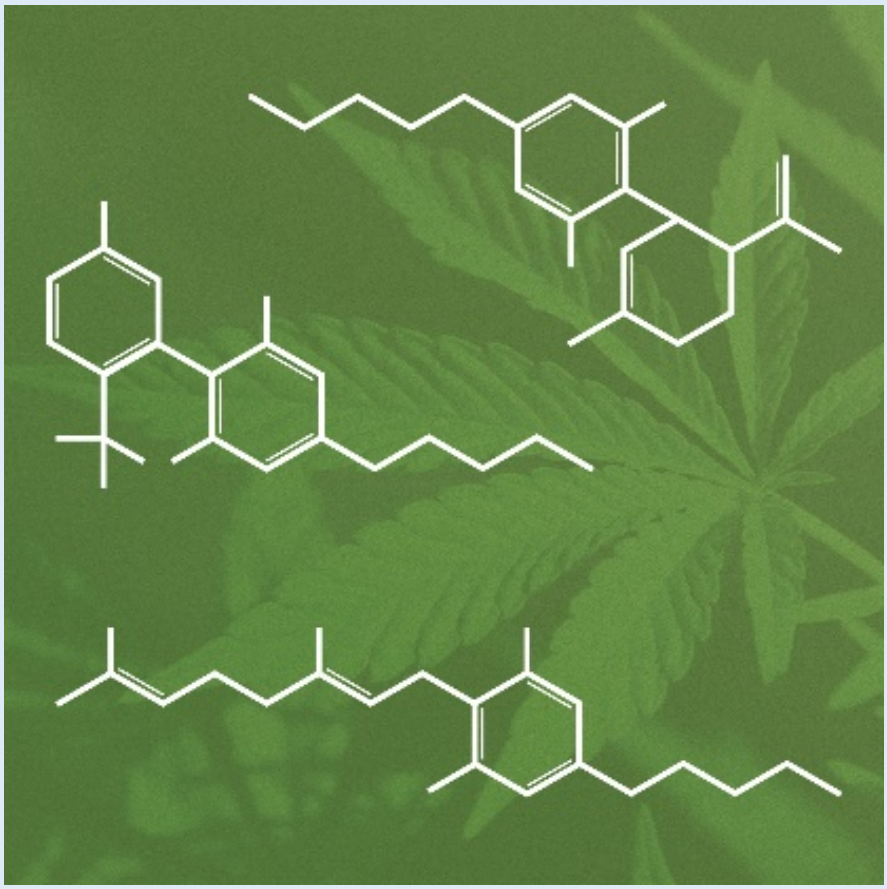

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Investigation of Inhibitory Effect of the *Rubus idealis* L. Extract on Corrosion of Copper

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Abstract: The aim of this work was to examine the impact of raspberry extract (*Rubus idaeus* L.) on copper corrosion characteristics. The raspberry leaf extract was prepared using Soxhlet extraction with ethanol as solvent. The estimation of the total polyphenol content in the obtained sample was determined by UV/Vis spectrophotometric method. The identification and quantification of phenolic acids was performed using HPLC analytical method. In addition, the aim of this work was to examine the impact of individual phenolic compounds (rutin, gallic acid, quercetin, and catechin hydrate) on the corrosion properties of copper. The corrosion rate of copper with the extract of the raspberry leaf of the Polka variety was tested. A copper corrosion test was performed in a 3% NaCl solution without and in the presence of the extract. The copper polarization resistance (Rp) values in 3% NaCl solution without and in the presence of the extract were determined by the linear polarization method. The corrosion behavior of copper in 3% NaCl solution without and in the presence of extract was determined by specific electrochemical parameters: corrosion potential (E_{corr}), corrosion current density (I_{corr}), and slopes of the anode (β_a) and cathode (β_k). The electrochemical impedance spectroscopy method was used to examine the corrosion behavior of copper in 3% NaCl solution without and in the presence of extract. The results obtained by Tafel extrapolation showed that the corrosion rate decreases in the presence of the tested extract. Studies conducted by the electrochemical impedance spectroscopy method show that the tested extract slows down the kinetics of the corrosion process, which is visible through an increase in resistance. The results confirm that the examined extract can be used for protection in an aggressive medium, such as a 3% NaCl solution.

INTRODUCTION

Copper is a metal that has extensive application due to its good properties. It is used in electrical engineering for the production of wires, sheets, pipes, and in the production of alloys. It is relatively resistant to the effects of the atmosphere and many chemicals. However, it is known to be susceptible to corrosion in an aggressive environment. The use of copper corrosion inhibitors is necessary in these cases because the formation of a passive protective layer cannot be expected (Kasapović, Korać, and Bikić, 2022). Corrosion inhibitors are divided into two groups: those that enhance the formation of a protective oxide film through an oxidizing effect, and those that inhibit corrosion by selective adsorption to the metal surface and forming a barrier that prevents corrosive agents from reaching the metal surface. Several approaches have been used in corrosion prevention science and engineering. Material selection, electrochemical techniques, coating deposition, and corrosion inhibitor application are some of the most common. Corrosion inhibitors are one of the most cost-effective and practical approaches to use. Corrosion inhibitors are compounds that, when introduced in small amounts to a corrosive medium, can slow down the rate of metal corrosion.

Inorganic compounds, for example, are known for their strong inhibitory characteristics, particularly chromates and their derivatives. Despite these environmental laws have restricted their use due to their toxicity and negative

impact on human life and ecosystems. One of the key approaches to controlling corrosion in modern society is the use of green corrosion inhibitors, which reduce corrosion rates to a suitable level with minimal environmental impact On the other hand, natural products, such as plant extracts, are readily available and inexpensive. As a result, green chemistry research and its use in corrosion inhibitors has exploded in the twenty-first century. There has been a rush of research on corrosion inhibition utilizing extracts from various plant parts. Plant extracts contain bioactive substances that have been demonstrated to be as effective as commercially available inhibitors (Radošević, 2012; Zakeri, Bahmani and Aghdam, 2022). For example, tobacco extracts are rich in chemical components such as alcohols, polyphenols, nitrogen-containing compounds, terpenes, carboxylic acids, and alkaloids, which may have electrochemical properties such as corrosion inhibition (Patni, Agarwal and Shah, 2019). Many studies have focused on natural organic compounds from plant material. Natural antioxidants are affordable, available, and renewable compounds obtained by extraction from plant material or synthesized. Studies have shown that many of these compounds can be used as effective inhibitors of copper corrosion (Grudić, Bošković, and Gezović, 2018; Al-Nami, 2019; Ahmed and Zhang , 2019; El-Tantawy, *et al.,* 2021).

According to previous reports, there are two forms of corrosion inhibition:
1. one that coats a metal surface by adsorption to it to protect the active areas from acid attack.
2. An alternative is to use an oxidation mechanism to build a protective coating on the metal surface.

The presence of phenolic components in the plant extract, as well as heteroatoms such as S, P, N, and O groups, may be responsible for both effects. Leaves of plants such as *Olea europaea* L., *Citrus aurantifolia*, and *Hibiscus sabdariffa* have been found to reduce the mild steel corrosion in an acid medium (Patni, *et al.,* 2013 Chitra, *et al.,* 2019). It is assumed that *R. idaeus* leaf extracts could have a similar effect on copper corrosion.
Effective environmental corrosion inhibitors show a high tendency for adsorption. The adsorption mechanism of organic inhibitors at the metal/solution interface can consist of one or more steps. In the first step, the adsorption of an organic inhibitor on the metal surface usually involves the replacement of one or more water molecules that were initially adsorbed on the metal surface (Bikić, 2017):

$$Inh(sol) + xH_2O(ads) \rightarrow Inh(ads) + H_2O(sol)$$

where:
Inh(sol) - inhibitor in solution,
Inh(ads) – adsorbed inhibitor,
x – number of water molecules displaced by the inhibitor.

The inhibitor combined with the metal ion M^{2+} formed on the metal surface as a result of metal oxidation or dissolution process, creates a metal-inhibitor complex (Bikić, 2017):

$$M \rightarrow M^{2+} + 2e^-$$

$$M^{2+} + Inh(ads) \rightarrow [M\text{-}Inh]_{(ads)}^{2+}$$

Depending on the relative solubility of the resulting complex, further dissolution of the metal can be inhibited or catalyzed (Bikić, 2017).
According to previous research on the dissolution of copper in chloride medium, the anodic reaction is reversible, mainly due to the strong, thermodynamically more favorable complexation of copper ions with chloride ions (Lee and Noble, 1986; Deslouis, *et al.,* 1988, Barcia, *et al.,*1993). The cathodic response is dominated by oxygen reduction, which is irreversible. Copper with chloride ions can form several complexes (Lee and Noble, 1986): CuCl, $CuCl_2^-$, $CuCl_3^{2-}$ or $CuCl_4^{3-}$. The formation of the CuCl layer takes place according to the reaction:

$$Cu + Cl^- \rightarrow CuCl$$

CuCl is poorly soluble in NaCl solution, resulting in the formation of an ion $CuCl_2^-$ complex:

$$CuCl + Cl^- \rightarrow CuCl_2^-$$

It is generally accepted that the anodic dissolution of Cu depends on the concentration of Cl^- ions and does not depend on the pH of the solution. At concentrations of Cl^- ions higher than 1 mol/dm^3, it is possible to form more complex complexes such as $CuCl_3^{2-}$ and $CuCl_4^{3-}$ (Otmačić and Stupnišek-Lisac, 2003; Kear, Barker, and Walsh, 2004; Otmačić Ćurković, Stupnišek-Lisac and Takenouti, 2010).
The cathodic reaction in neutral solutions is:

$$O_2 + H_2O + 4e^- \rightarrow 4OH^-$$

During hydrolysis, $CuCl_2^-$ ions in NaCl solution can cause precipitation of copper (l) oxide:

$$2CuCl_2^- + H_2O \rightarrow Cu_2O + 2H^+ + 4Cl^-$$

or by direct oxidation of copper (Barcia, *et al.,* 1993):

$$2Cu + H_2O \rightarrow Cu_2O + 2H^+ + 2e^-$$

When a passive film is created on a metal (e.g., Cu_2O) that does not have good protective properties, pitting corrosion will occur in the presence of aggressive ions, which is very dangerous because it quickly penetrates deep into the metal mass and can lead to cracking of the structure under stress (Winston Revie, 2000). Pitting corrosion most often occurs during the transition from an active to a passive state. The stability of Cu_2O depends on the concentration of chloride ions. The use of inhibitors and alloying reduces the possibility of pitting corrosion.
In this work, electrochemical methods were used to demonstrate the effect of the Polka raspberry leaf extract from the locality of Moševac near Maglaj. Raspberry Polka is one of the best varieties of raspberry. It is a perennial raspberry, a newer variety of raspberry originating in Poland.Raspberry leaf extract is a relatively cheap, readily available, and renewable natural product rich in various organic compounds such as polyphenolic compounds, organic acids, vitamins, etc., which makes it

a potential corrosion inhibitor (Milenković Anđelković, 2016).

EXPERIMENTAL

Materials preparation

For the application of the Electrochemical Impedance Spectroscopy method, copper samples with dimensions 13x13 mm were used. Before each measurement, the copper working surface was mechanically prepared with sandpaper of different grits, placed on the device, degreased in 97% ethanol, and washed with distilled water. Two types of materials were used to test the electrochemical characteristics of selected metal materials in 3% NaCl solution without and in the presence of the extract. Copper samples with of dimensions d = 15 mm and ranging δ from 1 to 2 mm were used for polarization measurements.

Plant extract preparation

The raspberry leaves were dried in the shade for a few days to remove all moisture content. The dried leaves were then ground in a blender to obtain a fine powder, which was later used for Soxhlet extraction with 96% ethanol as the solvent. The extraction lasted 6 hours, after which the obtained extract was dried in a rotatory evaporator. The extract was stored in a dark bottle in a refrigerator at a temperature of +4 ºC. The extracted sample was of resinous consistency and well soluble in 96% ethanol.

Total phenolic content

The total phenolic content of *R. idaeus* was analyzed spectrophotometrically by the Folin-Ciocalteu method according to Singleton and Rossi (1965) and Singleton, Orthofer, and Lamuela-Raventós (1999), using a Perkin-Elmer Lambda 650 spectrophotometer. Gallic acid was used as a reference standard. Solutions of gallic acid were prepared in a series of concentrations from 0.00125 to 0.008 mg/ml. A calibration curve was constructed based on the measured absorbance values depending on the different concentrations of gallic acid. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

Analysis of phenolic acids and flavonoids using high-performance liquid chromatography

The HPLC analysis of the raspberry extract was performed on Shimadzu Prominence (modular HPLC) with a UV/Vis detector. The separation of phenolic acid and flavonoids was done using the Nucleosil C18 column (250 mm x 4.6 mm, particle size 5 μm; Macherey-Nagel) with absorbance measurements at 280 nm (for hydroxybenzoic acid derivates, gallic acid) and 360 nm (for flavonoids, quercetin, rutin, and catechin). Standards of phenolic compounds were dissolved in 50% methanol. The mobile phases used for the analysis were Solvent A (1% formic acid) and Solvent B (acetonitrile) at a flow rate of 1 ml/min and using the following linear gradient: 0–10 minute linear rise from 10 to 25% A; 10–20 minute linear rise to 60% A; and 20–30 minute linear rise to 70% A (Vinčić, 2017). The concentrations of standards solutions of phenolic compounds for the formation of calibration curves were in the following range: 4-100 mg/l for rutin, 52.5-420 mg/l for gallic acid, 13.13-52.50 for quercetin and 5.4-540 mg/l for catechin hydrate.

Based on the obtained chromatograms and calibration curves of standard solutions of phenolic compounds, the concentrations of identified phenolic acids and flavonoids (μg/ml or mg/ml) were calculated.

Electrochemical analysis

Copper with a purity of 99.8% was used to examine the effect of raspberry (*Rubus idaeus* L.) leaf extract on the corrosion characteristics of copper. The chemical composition of copper was tested at the Kemal Kapetanović Institute in Zenica on the atomic absorption spectrometer.

The following polarization measurement methods were used in this research during the electrochemical tests of the corrosion process using DC techniques:

- linear polarization method;
- potentiodynamic polarization method.

The following method was used when testing the corrosion process by AC - techniques:

- Electrochemical Impedance Spectroscopy (EIS).

Copper polarization measurements were performed in a corrosion cell using a Potentiostat/Galvanostat device, PAR, model 263A-2, and PowerCORR® software package. An electrochemical cell contains three electrodes. A carbon electrode is used as an auxiliary electrode, and a saturated calomel electrode (SCE) with a potential of 0.2415 V is used as a reference electrode. All results listed in the paper are in relation to the SCE. The working electrode is a cylindrical body (disk) placed inside a space made of glass and metal. The dimension of the working electrode is d=15 mm and □□from 1 to 2 mm. Sample preparation and care were done according to the ASTM G5 standard (ASTM G5-94). The Electrochemical Impedance Spectroscopy method (EIS) was used to determine the kinetic parameters of the electrochemical reaction of copper corrosion in a 3% NaCl solution without and in the presence of plant extracts. Measurements were performed using the IviumSoft software package on an IVIUM® Vertex One potentiostat/galvanostat. The corrosion kinetic parameters were deduced using IviumSoft software package and PowerCORR® software package.

RESULTS AND DISCUSSION

The yield of Soxhlet extraction of raspberry leaves (12.0 g) with ethanol was 2.55 g or 21.25%.

Total phenolic content

The blue color was created by the effect of plant polyphenols on the Folin-Ciocalteu reagent components, which were detected at 725 nm (Chitra, et al., 2019).

To estimate the TPC, an increase in absorbance was measured, indicating an increase in phenol concentration, The TPC of *R. idaeus* leaf extract was 24.39±2.64 mg GA/g DW.
Many factors influence the concentration of phenolic compounds in plants, including cultivar, cultivation method, and location of occurrence, climatic conditions, and harvest time. Also, extraction and analysis procedures influence the final results (Staszowska-Karkut and Materska, 2020).

These findings are consistent with evidence from the literature. According to Pavlović, et al. (2016), the highest TPC concentration was detected in the *R. idaeus* cultivars 'Meeker' (144.20±1.58 mg GAE/g DW) and 'Willamette' (143.38 4.68 mg GAE/g DW), while the lowest TPC content was observed in the 'Tulameen' cultivar (84.64±2.05 mg GAE/g DW). Different phenolic compounds have different responses to the Folin-Ciocalteu reagent. The possible presence of interfering compounds (sugars, aromatic amines, sulfur dioxide, vitamin C, organic acids, Fe (II), and other substances that are not of polyphenolic origin), affects the unrealistic increase in results (Vinčić, 2017). According to Winston Revie, (2000), the total phenols for the domestic raspberry leaf was 811.75 mg GA / kg of a dried sample, which is significantly less than the obtained result.
According to the literature, several other plant products, such as gallic acid, quercetin, and caffeic acid from Syzygium cumini, rutin from Piper longum, myricetin and chlorogenic acid from Cyamopsis tetragonoloba, and flavonoids from Azadirachta indica (flavonoids), have excellent corrosion inhibition efficiency (Singh, Ebenso, and Quraishi , 2012). These findings initiated the search for leaf extracts of *R. idaeus* to determine its corrosion resistance against copper corrosion in 3% NaCl. These findings prompted researchers to look for *R. idaeus* leaf extracts to test their corrosion resistance against copper corrosion in 3% NaCl.

Analysis of phenolic compounds by HPLC

The corrosion inhibition properties of plant extracts are to the result of the synergistic activity of phytochemical components such as phenols, flavonoids, alkaloids, tannins, and other compounds found in them. The qualitative and quantitative analysis of *R. idaeus* leaf extract was determined using HPLC with UV/Vis detection. Figure 1 shows the HPLC chromatogram of phenolic acids and flavonoids detected in *R.idaeus* leaf extract.
Gallic acid (12.60±6.19 mg/100g DW, quercetin (33.04±0.15 mg/100g DW) and rutin (71.90±5.65 mg/100g DW) were identified in the analyzed leaf extract. The analysis did not show the presence of catechin in the analyzed sample.
According to Krauze-Baranowska, et al., 2014., among the identified polyphenols in *R. idaeus* ″Willamente″ dry shoot extract are gallic acid (722±6.5 mg/100 g DW), catechin (129.3±11.6 mg/100g DW), epicatechin (791±70.7 mg/100 g DW), isoquercetin and quercetin 3-O-glucuronide (717.57±64.1 mg/100g DW), chlorogenic acid (177.4±15.9 mg/100 g DW), etc. Li, et al. (2015) identified 16 flavonoids, including 4 quercetin derivatives, 2 luteolin derivatives, 8 kaempferol, and 2 isorhamnetin derivatives in leaf extract of *R. idaeus*. Authors Yang et al., (2020) identified 12 polyphenols in extracts of *R. idaeus*. Among them are chlorogenic acid (145.83±120.3 mg/100g DW), catechin (2.320.3±3.25 mg/100g DW), rutin (484.048±129.12 mg/100g DW), quercetin (11.190±12.59 mg/100g DW), kaempferol (0.714±0.89 mg/100g DW), etc. Staszowska-Karkut and Materska, (2020) reported 20 different phenolic compounds in the leaves of *R. idaeus*. Among them are caffeic acid (0.3-77 mg/100g DW), chlorogenic acid (2.9-23 mg/100g DW), gallic acid (2.3-31 mg/100g DM), luteolin (49 mg/100g DW), rutin (5-59 mg/100g DW, catechin (92 mg/100g DW etc. By comparing the obtained results with the literature data, we see that the content of gallic acid agrees with the data of the authors Staszowska-Karkut and Materska. All other results for the identified phenolic components in the analyzed sample were lower compared to the literature, except for rutin which was slightly higher than in the analyzed samples of Staszowska-Karkut and Materska.

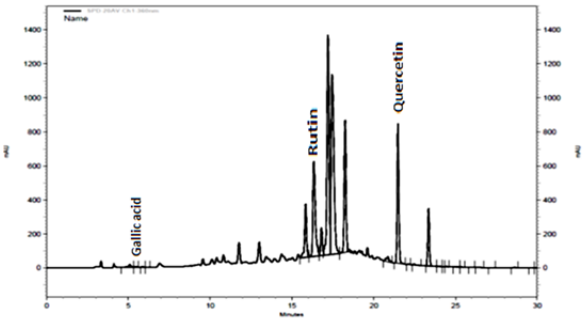

Figure 1. *HPLC chromatogram of R. idaeus leaf extract*

Effect of extract on copper corrosion characteristics using potentiodynamic polarization method

Figure 2 shows the polarization curves of copper in 3% NaCl without and with the addition of extract in different concentrations, and the obtained corrosion parameters are shown in Table 1.

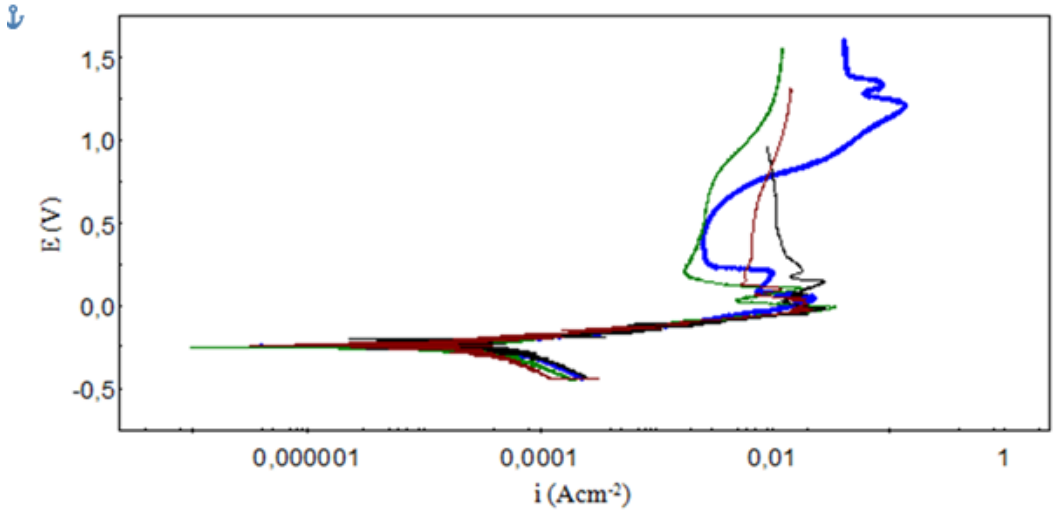

Figure 2. Anode and cathode curves of copper polarization in 3% NaCl without and with the addition of extract in different concentrations
----3% NaCl; ----3% NaCl+0,03221g/l; ---- 3% NaCl+0,04828 g/l; ----3% NaCl+0,06432 g/l

Table 1. Corrosion parameters determined by Tafel extrapolation method of copper in 3% NaCl without and with the addition of extract in different concentrations

Sample	***R.idaeus* leaf extract (g/l)**	**E_{corr} (mV)**	**i_{corr} (μAcm^{-2})**	**β_k ($mVdec^{-1}$)**	**β_A ($mVdec^{-1}$)**
Copper sample	Without extract	-226.807	9.978	309.41	67.106
	0.01612	-216.815	6.756	215.847	61.897
	0.03221	-239.573	1.078	128.844	49.793
	0.04828	-216.676	1.418	135.187	42.191
	0.06432	-223.303	1.347	120.318	46.423
	0.08033	-226.613	2.071	143.594	50.871

With increasing extract concentration, the corrosion potential shifts towards more positive values, except for the extract concentration of 0.03221 g/l. The results obtained by the Tafel extrapolation method showed that the corrosion rate decreases in the presence of almost all tested concentrations of extract. In the tested interval, the lowest corrosion rate is at the inhibitor concentration of 0.03221 g/l.

The linear polarization method shows the results obtained by the potentiostatic polarization of copper in a narrow potential range. Table 2 shows that the addition of different concentrations of the extract reduces the corrosion rate, which means that the polarization resistance increases. Polarization resistance is a measure of a material resistance, and the higher the value, the more corrosion resistant the material is. Corrosion parameters are shown in Table 1.

Polarization measurements performed in a narrow range of potentials confirmed that the extract provides the highest protection at a relatively low concentration of 0.04828 g/l in copper. Therefore, at this concentration, the corrosion rate is the lowes.

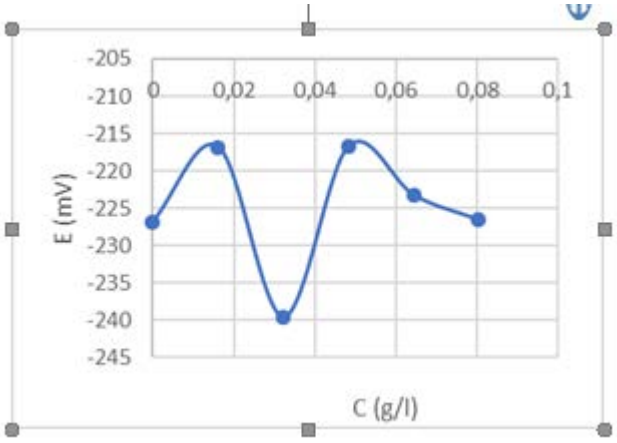

Figure 3. Dependence of the Ecor on concentration of extract

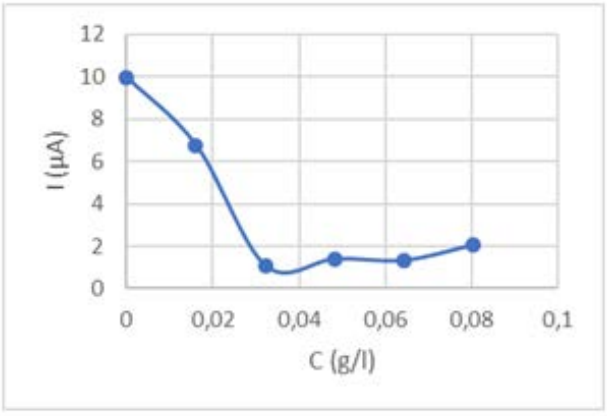

Figure 4. Dependence of the icorr on concentration of extract

Table 2. Corrosion parameters determined by Linear polarization method of copper in 3% NaCl without and with the addition of extract in different concentrations

Sample	*R.idaeus* leaf extract (g/l)	R (Ω)	Ecorr (mV)	icorr (μAcm^{-2})
	Without extract	3042.618	-205.166	7.145
	0.01612	3453.884	-202.546	6.294
	0.03221	3341.418	-211.554	6.506
	0.04828	4051.622	-208.973	5.366
	0.06432	4018.02	-219.27	5.410
Copper sample	0.08033	2804.507	-219.119	7.751

Effect of gallic acid (GA), rutin and quercetin on copper corrosion

The effect of individual components of the extract on the corrosion properties of copper was tested. The measurements were performed by adding a solution of individual components at a concentration of 0.032 g/l in 3% NaCl solution.

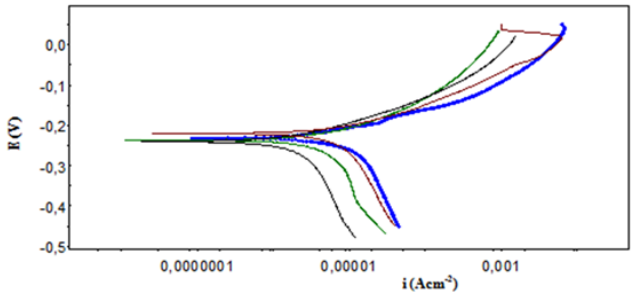

Figure 5. Anode and cathode curves of copper polarization in 3% NaCl without and with the addition of standard solution of gallic acid, rutin and quercetin ---- 3% NaCl; ---- 3% NaCl+0.03221g/l gallic acid; ---- 3% NaCl+0.03221g/l rutin; ---- 3% NaCl+0.03221g/l quercetin

Curve profiles in 3% NaCl solution and with the addition of standards they do not differ. There was a decrease in the corrosion current as well as the corrosion potential except for quercetin.

Corrosion parameters that show tendencies can be obtained from these curves.

Figure 6. Ecorr of copper in 3% NaCl solution with the addition of GA, rutin and quercetin in concentration 0.03221 g/l

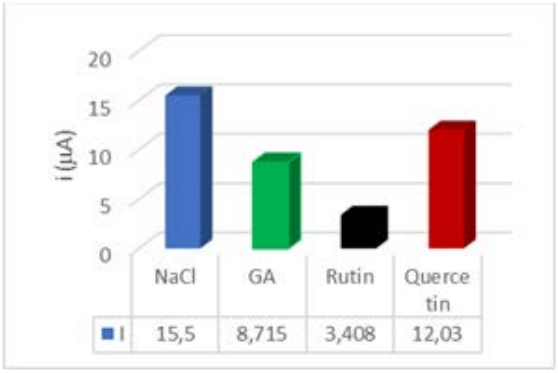

Figure 7. icorr of copper in 3% NaCl solution with the addition of GA, rutin and quercetin in concentration 0.03221 g/l

Based on the corrosion parameters obtained from the Tafel curves, it can be seen that gallic acid and rutin show inhibitory effects on the action of NaCl solution on copper. Quercetin increases the corrosion effect when NaCl acts on copper. Effect of extract on copper corrosion characteristics using electrochemical impedance spectroscopy method. The results obtained by electrochemical impedance spectroscopy can be represented by an equivalent electrical circuit, Figure 8. The results of the electrochemical impedance spectroscopy test are shown in the Nyquist diagram, Figure 9, and were analyzed using an equivalent electrical circuit, and the obtained parameters in Table 3.

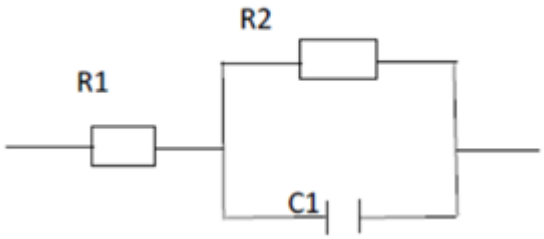

Figure 8. Scheme of the equivalent circuit of a simple electrochemical cell C1-capacitor, R1-electrolyte resistance, R2-resistance of charge transmission

From Figure 9 it can be seen that the addition of the tested concentrations of the extract increases the diameter of the impedance curves in copper compared to the one obtained

without the addition of extract. It can be concluded that the addition of the extract reduces the corrosion rate. EIS parameters for the extract as well as without it are shown in Table 3 and based on these results it is observed that the highest inhibitor resistance in copper is given by the extract concentration of 0.11227 g/l.

Table 3. Parameters obtained by electrochemical impedance spectroscopy of copper in 3% NaCl without and with the addition of extract in different concentrations

Sample	***R. idaeus* leaf extract (g/l)**	**R1 (Ω)**	**R2(Ω)**	**C(F)**
Copper sample	Without inhibitor	110.1	2776	$3.890 \cdot 10^{-4}$
	0.01612	134.7	3998	$5.061 \cdot 10^{-4}$
	0.03221	196.8	6168	$7.314 \cdot 10^{-4}$
	0.04828	219.3	6792	$7.240 \cdot 10^{-4}$
	0.06432	257.6	6930	$8.346 \cdot 10^{-4}$
	0.08033	236.0	8044	$7.889 \cdot 10^{-4}$
	0,09631	309.4	9360	$6.664 \cdot 10^{-4}$
	0,11227	742.3	10210	$5.112 \cdot 10^{-4}$

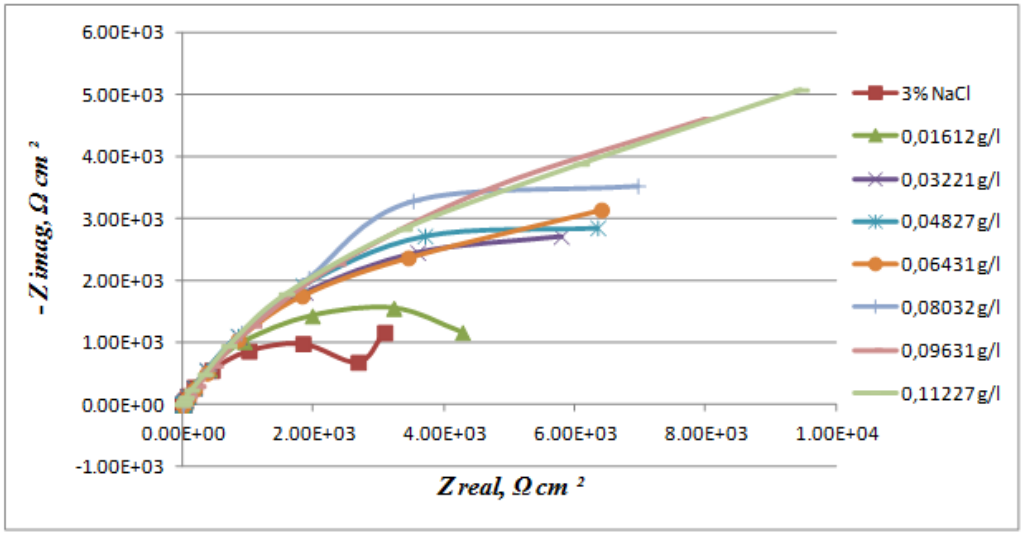

Figure 9. Nyquist copper curves in 3% NaCl without and with the addition of extract in different concentrations

CONCLUSION

The results obtained by DC - techniques (Tafel extrapolation method and linear polarization method) showed that the corrosion rate decreases in the presence of almost all tested extract concentrations Three current peaks appearon the polarization curve recorded for pure copper in 3% NaCl, , which are attributed to the formation of copper chloride and copper oxide Cu2O. In all anode branches of copper polarization curves in the presence of different concentration extracts, it was detected that the areas where copper dissolution occurs and the formation of a soluble $CuCl2^{-}$ complex and its diffusions from the metal surface into the solution and the areas of corrosion product formation occur earlier, which leads to a certain decrease in current density.

Studies conducted by the electrochemical impedance spectroscopy method show that almost all tested extract concentrations slow down the corrosion process kinetics, which is visible through an increase in resistance. Based on the results for the EIS parameters with and without the extract, it was observed that the highest inhibitor resistance in copper was given by the extract concentration of 0.112 g/l.

These results confirm that in an aggressive medium, such as a 3% NaCl solution, the test extract in a concentration of 0.032 g/l can be used for protection, as this concentration met the protection requirements for all test methods. The results of the conducted tests prove that in an aggressive medium, such as a 3% NaCl solution, the extract of raspberry Polka leaf can be used as an inhibitor of copper's corrosion at room temperature.

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Summary/Sažetak

Ova studija ispituje utjecaj ekstrakta maline (*Rubus idaeus* L.) na karakteristike korozije bakra. Ekstrakt lista maline pripremljen je Soxhlet ekstrakcijom s etanolom kao otapalom. Procjena ukupnog sadržaja polifenola u dobivenom uzorku određena je UV/Vis spektrofotometrijskom metodom. HPLC analitičkom metodom izvršena je identifikacija i kvantifikacija fenolnih kiselina i flavonoida (rutin, galna kiselina, kvercetin i katehin hidrat). Nakon dobivanja i ispitivanja ekstrakta lista maline Polka, ispitana je brzina korozije bakra. Ispitivanje korozije bakra u 3% otopini NaCl bez i u prisutnosti ekstrakta.Vrijednosti otpora polarizacije bakra (Rp) u 3% otopini NaCl bez i u prisutnosti ekstrakta, određene su metodom linearne polarizacije. Korozijsko ponašanje bakra u 3% otopini NaCl bez i u prisutnosti ekstrakta i specifični elektrokemijski parametri: potencijal korozije (Ecorr), gustoća struje korozije (Icorr) i nagib anode (βa) i katode (βk). Metodom spektroskopije elektrokemijske impedancije ispitano je korozijsko ponašanje bakra u 3% otopini NaCl bez i u prisutnosti ekstrakta. Rezultati dobiveni Tafelovom metodom ekstrapolacije, pokazali su da se brzina korozije smanjuje u prisutnosti ispitivanog ekstrakta. Istraživanja provedena metodom elektrokemijske impedančne spektroskopije pokazuju da ispitivani ekstrakt usporava kinetiku procesa korozije, što je vidljivo kroz povećanje otpora. Rezultati potvrđuju da se testni ekstrakt može koristiti za zaštitu u agresivnom mediju, kao što je 3% otopina NaCl.

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11-16

Optimization and Validation of Europium-Sensitized Fluorescence Method for Determination of Tetracycline Antibiotics in Water from Fish Farms

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Abstract: Sensitized europium fluorescence was used for simple, fast and efficient determination of tetracycline residues in water from fish farms. Tetracycline antibiotics: oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) were extracted from water samples using polymeric hydrophilic–lipophilic balanced cartridges. After evaporation and pre-concentration, tetracyclines form a complex with europium and citric acid as coligand at pH 8.5. The complex formed has a wide absorption spectrum at 388 nm and a narrow emission maximum at 619 nm resulting from the $5D_0$ - $7F_2$ transition within the europium ion. The complex is stable with intensive fluorescence and linear in the concentration range of 5-2500 µg/L for tetracycline and oxytetracycline and 5-1000 µg/L for chlortetracycline. The detection limit was 0.68 µg/L for OTC, 1.29 µg/L for TC and 0.65 µg/L for CTC, respectively. The proposed method is very sensitive and particularly applicable to samples where low concentrations of tetracycline antibiotics are expected.

INTRODUCTION

Nowadays, fish farming represents a significant percentage of the population's food supply. Intensive fish farming is related to the increased usage of antimicrobial agents applied for the treatment and prevention of bacterial fish (Troell, Naylor, Metian*et al.*, 2014). Currently, due to the countries' differences in distribution and registration systems, the determination of the quantities of antimicrobials applied worldwide in aquaculture is very challenging. In each country, various legal restrictions regulate the usage of medicines in aquaculture, and compliance monitoring also varies from country to country (Miranda, Godoy, Lee, 2018; Quesada, Paschoal, Reyes, 2013; Heuer, Kruse, Grave *et al.*, 2009). In aquaculture, therapeutic doses of antibiotics are administered orally for a short time, mixed with specially formulated food or added directly into the water in the form of therapeutic baths. As fish farms are most commonly located in rivers or lakes, residues of directly added antibiotics, unconsumed feed pellets and toxic feces are distributed throughout the entire ecosystem. It is estimated that 75% of the antibiotics administered through feed are excreted into water (Kemper, 2008). The tetracycline antibiotic, oxytetracycline (OTC) is one of the most commonly used antibiotics in fish farms. Due to the low intestinal absorption of fish, which results in slow excretion of large amounts of this antibiotic, its application has to be followed by high doses of 100-150 mg per kg of fish per day for 10-15 days. (Capone, Weston, Miller, 1996). It is estimated that 70–80% of OTC in feces is intact (Miranda, Godoy, Lee, 2018). Although expected, the presence of OTC's in surface waters or sediments is not frequently confirmed. A possible explanation could be based on the fact that OTC undergoes photolysis and hydrolysis, which makes it difficult to detect in water. In addition, OTC binds easily to cations such as calcium and magnesium or binds to proteins as well. Detection of antibiotics is more challenging due to the complexes' environmental immobility and significant differences in properties (Gothwal, Shashidhar, 2014; Havelkova, Beklova, Kovacova, 2016). In order to determine the extent to which the ecosystem has been exposed to OTC, but also

to other tetracyclines, it is of crucial importance to identify and quantify them in surface and groundwater, sludge and sediments (Chen, Chen Y, Ding, *et al.,* 2015; Ahmad, Zhu, Sun, (2021). In addition to OTC, which was the first antibiotic approved by the Food and Drug Administration (FDA) for use in fish farms, tetracycline (TC) and chlortetracycline (CTC) can also be used in aquaculture (Olatoye, Basiru, 2013). To ensure the safety of food for human consumption, the Food and Agriculture Organization (FAO) has defined an acceptable daily intake of 0-30 μg/kg for these three antibiotics (FAO, 2015).

Microbiological method enzyme-linked immunosorbent ELISA (Shahbaz, Ahmadi, Karami, 2015; Aga, Goldfish, Kulshrestha, 2003), UV/VIS spectroscopy, thin layer chromatography (TLC), liquid chromatography (LC), (Bečić, Imamović, Dedić, 2014; Alanazi, Almugbel, Maher *et al.,* 2021; Pérez-Rodríguez, Pellerano, Pezza, 2018), liquid chromatography/mass spectrometry (LC/MS), liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods (Zhu, Snow, Cassada *et al.*, 2001; Cháfer-Pericás, Maquieira, Puchades, 2010; Kang, Lee, Shin *et al.*, 2018) were used for the screening and determination of tetracycline antibiotics residues. Sensitized lanthanide fluorescence is a very sensitive method based on energy transfer from a ligand to a lanthanide ion with a characteristic emission at the lanthanide emission wavelength. This phenomenon has been used to determine tetracycline antibiotics OTC, TC and CTC in various samples e.g. milk, blood plasma, serum (Hongliang, Yang, 2012; Shtykov, Smirnova, Bylinkin, *et.al*., 2005; Arnaud, Geoges, 2001) where tetracycline is used as a ligand. The present study evaluated the possible application of europium ion fluorescence as an indicator for the tetracycline antibiotics determination in the fish farms water and other surface water.

EXPERIMENTAL

ll solvents and chemicals used were p.a. and analytical reagent grade (Panreac, Italy; Merck, Germany). The ultrapure water was from a Sartorius purification system. $EuCl_3 \times 6H_2O$ (Sigma Aldrich, St. Louis, MO, USA) was used to prepare a standard solution of Eu^{3+}($1{,}6 \times 10^{-3}$ M). Tetracycline hydrochloride, Stock standard solutions of oxytetracycline hydrochloride and chlortetracycline hydrochloride (Sigma Aldrich, Germany) (500 μg/mL) were prepared by weighing accurate quantities of the standards, dissolved in 1 mL of ultra-pure water and diluted with acetonitrile. For the calibration curve, the stock solutions were diluted with acetonitrile in the concentration range 5–2500 μg/L for OTC and TC and 5-1000 μg/L for CTC.

For analysis, solutions of 1 mM citric acid, oxalic acid and tartaric acid in ultra-pure water were prepared. Tris and borate buffer solutions pH 6-9 were prepared according to Ph. Eur. Procedure (European Pharmacopoeia, 10th Edition, 2020). All prepared solutions were stored at 4°C. A Shimadzu RF-5301-PC spectrofluorometer (Kyoto, Japan) with Panorama fluorescence 1.1 software was used to measure fluorescence intensity

Sample preparation (water samples). Water samples from two different fish farms were taken in two-liter amber-colored glass bottles and used for analysis. All collected samples were filtered through Whatman filter paper to remove suspended matter and then filtered through a 0.45μm membrane filter. The samples were stored in a refrigerator at +4°C until solid phase extraction (SPE) and further analysis. Before extraction, the total concentration of calcium and magnesium was determined. An appropriate amount of EDTA was added to prevent the binding of the antibiotics to calcium and magnesium.

Blank sample matrix preparation. Water samples used as blanks were collected in amber colored glass bottles, upstream from the fish farms. These samples were extracted in the same way as spiked water samples and environmental samples to detect possible endogenous interferences and method selectivity .

Spiked water samples. The spiked water samples were prepared with an appropriate amount of tetracycline standard solution in the blank sample matrix. The concentration of tetracycline antibiotics added was 50 μg/L, 500 μg/L and 1000 μg/L.The recoveries were measured by the optimized and validated method described below.

Environmental samples. Environmental samples *w*ere collected from two fish farms, extracted and measured as the blank sample matrix and spiked water samples.

SPE extraction

Extractions of 250 mL acidified (pH 3) matrix sample, spiked water samples and environmental samples were performed on a hydrophilic-lipophilic balance (Oasis, HLB, Waters) 6 mL/500 mg cartridge at a flow rate of about 3 mL min^{-1} with Supelco vacuum manifold system connected to the vacuum pump. The cartridges were pre-conditioned with 5 mL methanol and 5 mL ultrapure water pH 3. After extraction, the cartridges were washed with 2 mL of ultrapure water to remove EDTA residues and dried by vacuum for 5 minutes to remove excess water. Tetracycline elution was performed with 3 mL of methanol. The filtrates were evaporated in a nitrogen stream after which the residues were dissolved in 1 mL of acetonitrile (Bečić, Imamović, Dedić, 2014).

Fluorescence assay

Fluorescence measurements for the calibration curves were performed as follows: one milliliter of each solution: europium ($5x10^{-5}$M), OTC or TC (5-2500 μg/L) or CTC (5-1000 μg/L) standard solutions, Tris (0.1M, pH 8.5) and citric acid (1 mM) were mixed, stirred vigorously and left for 10 minutes at room temperature. After 10 minutes, fluorescence was measured at an excitation wavelength of 388 nm and an emission wavelength of 619 nm. After SPE extraction, 1 mL of spiked water and environmental samples were used instead of standard antibiotic solutions to measure the fluorescence of these samples.

RESULTS AND DISCUSSION

Due to the high fluorescence sensitivity of the europium - tetracycline complex, it is essential to optimize the influencing parameters (Kaczmarek, 2020).

The spectroscopic properties of tetracycline are influenced by two separate chromophores in the molecule. Depending on the pH, these chromophores are protonated, which enables binding to the europium ion. Increasing the pH value increases the possibility of chromophore protonation. Therefore, it is very important to find an appropriate buffer solution and pH value. In addition, the pH value influences the fluorescence intensity of the complex. At pH values up to 5 fluorescence is very low and reaches a maximum at pH around 9 (Courrol, Samad, 2008; Shtykov, Smirnova, Yu, 2005).

In our previous studies, we have optimized the following parameters: tetracycline and europium concentration, pH and coligands (Bečić, Mušanović, Imamović, 2016).

It is known that a high concentration of Eu (III) can cause quenching of fluorescence upon collision in the singlet excited state of tetracycline (Courrol, Samad, 2008). Therefore, we optimized europium concentration by measuring the fluorescence intensity at different europium concentrations of $1.6x10^{-3}$M-$1x10^{-6}$M while tetracycline concentration was constant. Following the results obtained, europium concentration 5×10^{-5} M was selected. A linear increase in fluorescence intensity was found in the following concentrations of TC, OTC 5-2500 µg/L and CTC 5-1000 µg/L. The influence of pH on the fluorescence of Eu (III)-tetracycline complex was also optimized. The results showed that with an increase in the pH of both used buffers to 8.5, the fluorescence intensity increased. This was expected due to the deprotonation of the tetracycline molecule. (Figure 1.)

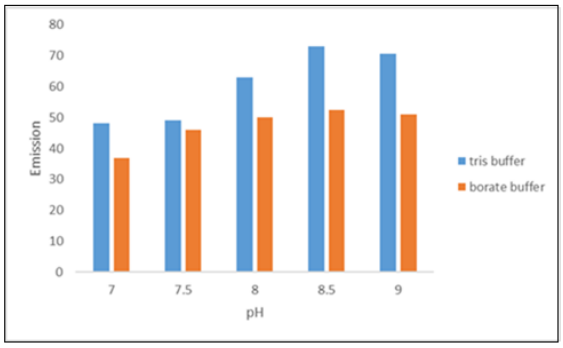

Figure 1. Fluorescence intensity with tris and borate buffer

Due to the possibility of europium hydroxide formation at pH 9, tris buffer pH 8.5 was selected for further measurements.

Citric acid was selected as a coligand, which resulted in a significant increase in fluorescence intensity. Due to the importance of the influence of pH on complex formation as well as the possibility of lanthanide precipitation, the order of addition of reactants was optimized. As seen in Figure 2, the highest fluorescence intensity was demonstrated by the complex formed by the reactants added in the following order: europium - tetracycline – tris – citric acid.

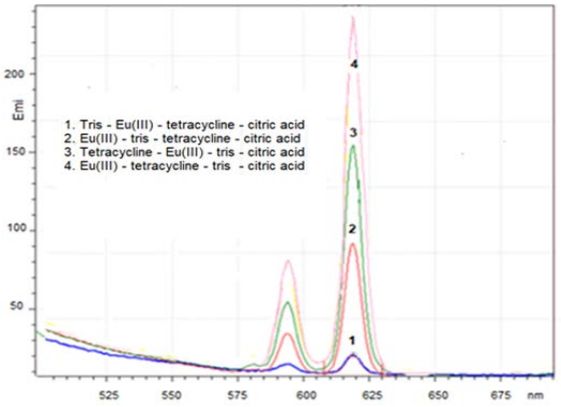

Figure 2. Influence of reactant addition order on the Eu(III) sensitized fluorescence

Method validation

To evaluate and confirm the validity of the method, analytical parameters were measured based on the ICH Guidelines (ICH, 2019).

Specificity

After SPE extracting of the blank sample matrix, specificity was determined and analyzed with endogenous interference. No interference was detected.

Linear dynamic range

The linearity of the method was determined by analyzing six solutions with antibiotic concentrations in the range of 5-2500 µg/L for TC and OTC and 5-1000 µg/L for CTC, respectively. Fluorescence measurements for each concentration were repeated six times. Calibration standard curves were constructed by plotting the mean values of fluorescence measurements against the concentrations of the standard (Figure 3.). Under optimized experimental conditions, good linearity was found between concentration and fluorescence emission with r^2=0.9987 and 0.9986for OTC and CTC and 0.9991 for TC.

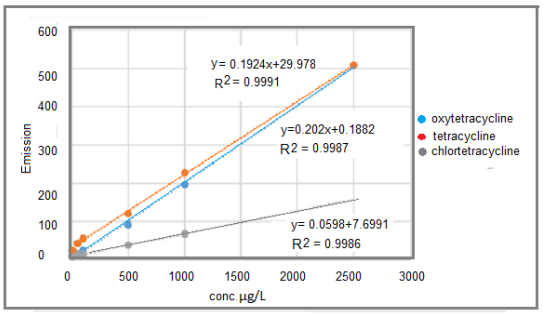

Figure 3. Calibration curve for OTC, TC and CTC

Precision

Precision (repeatability) was tested by analyzing 6 samples of the sample matrix spiked with 500 µg/L OTC, TC and CTC. The coefficient of variation was calculated by the formula:

$$CV\ (\%) = SD \times 100/mean$$

Accuracy

The accuracy of the method was expressed as the average recovery factor (%) calculated at three concentration levels using six replicates for each concentration. The

obtained results for the analysis of spiked sample matrix water containing 50, 500 and 1000 µg/L tetracycline antibiotics were higher than 90% for all compounds (Table 1).

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the blank and for OTC, TC and CTC were found to be 0.68, 1.29, 0.65 µg/L, and 1.61, 4.78 2.03, µg/L, respectively.

Table 1. shows all the parameters of method validation

Table 1. Method validation parameters

Validation parameter	Value
OTC	
Linearity	R^2= 0.9987
Precision	CV (%) = 0.92
Accuracy	Recovery (%)
Spiked sample (50 µgL)	94.46
Spiked sample (100 µg/L)	97.35
Spiked sample (1000 µg/L)	100.97
LOD	0.68 µg/L
LOQ	1.61 µg/L
TC	
Linearity	R^2= 0.9991
Precision	CV (%) = 0.97
Accuracy	Recovery (%)
Spiked sample (50 µg/L)	93.53
Spiked sample (100 µg/L)	96.35
Spiked sample (1000 µg/L)	101.65
LOD	1.29 µg/L
LOQ	4.78 µg/L
CTC	
Linearity	R^2= 0.9986
Precision	CV (%) = 0.91
Accuracy	Recovery (%)
Spiked sample (50 µg/L)	92.15
Spiked sample (100 µg/L)	96.55
Spiked sample(1000 µg/L)	97.22
LOD	0.65 µg/L
LOQ	2.03 µg/L

Analysis of environmental samples

After SPE extraction, water samples taken from the fish farms were tested by the proposed method. No residues of tetracycline antibiotics TC, OTC, CTC were found in the tested samples. A possible explanation for such results could be that the tetracycline antibiotics were photodegraded or that antibiotics were not applied during fish farming. Furthermore, the continuous flow of water can also affect the detection of antibiotic residues, if they are, due to dilution, of low concentration in the fish farm water.

CONCLUSION

The sensitized fluorescence of europium (III) ion can be used for the determination of OTC, TC or CTC residues individually or as the total amount of residues in water from the fish farm or other surface water. This method is simple, with high sensitivity, selectivity and accuracy. Due to its simplicity, it can be used as a rapid screening method to assess environmental exposure to tetracycline antibiotics residues.

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Summary/Sažetak

U radu je optimizirana i validirana metoda u kojoj se pobuđena fluorescencija europijuma koristi za jednostavno, brzo i učinkovito određivanje rezidua tetraciklinskih antibiotika u vodi iz ribogojilišta. Tetraciklinski antibiotici oksitetraciklin (OTC), tetraciklin (TC) i hlortetraciklin (CTC) ekstrahirani su iz uzoraka vode ekstrakcijom na čvrstim fazama. Nakon ekstrakcije i prekoncentracije uzorci vode su pomiješani sa europijumom i limunskom kiselinom kao koligandom pri pH 8,5. Formirani kompleksi imali su maksimum ekscitacijena 388 nm i emisije na 619 nm koji je rezultat prijelaza $5D_0$ - $7F_2$ unutar jona europijuma. Kompleks je bio stabilan s intenzivnom fluorescencijom i linearan u rasponu koncentracija od 5-2500 µg/L za tetraciklin oksitetraciklin i 5-1000 µg/L za hlortetraciklin. Metoda ima limit detekcije za OTC 0,68 µg/L, TC 1.29 µg/L i 0.65 µg/L za CTC. Predložena metoda je osjetljiva i jednostavna. Posebno je primjenjiva na uzorke gdje se očekuju niske koncentracije rezidua tetraciklinskih antibiotika.

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Correlation between antioxidant and physicochemical parameters of honey samples from Bosnia and Herzegovina and Turkey

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Abstract: The aim of this study was to determine the association value between the following parameters and the antioxidant properties of honey: colour, phenolics content (TPC), flavonoid content (TFC), proline and ascorbic acid content. The samples were collected in Bosnia and Herzegovina and Turkey and included honeydew, monofloral and polyfloral honey. The antioxidant activity of honey samples was determined using the ABTS and DPPH assays. Based on the correlation matrix, the main findings revealed a strong correlation between antioxidant activity and TPC, TFC, proline content and colour. Honey colour was in best correlation with the TFC ($r = 0.910$, $p < 0.001$) where dark coloured honeys showed a higher TFC, however antioxidant activity showed a highly significant dependence on the TPC (DPPH-TPC: $r = - 0.872$; ABTS-TPC: $r = - 0.783$, $p < 0.001$). Ascorbic acid was not established as a predictive parameter that can be used to estimate the antioxidant properties of honey and did not significantly correlate with any of the remaining variables.

INTRODUCTION

Honey is a natural dietary antioxidant that has been in use since ancient times. The biological activities of honey are derived from its chemical composition and that mainly depends on the botanical origin, climatic and environmental conditions. The honey composition is also influenced by production methods, handling, and storage, however only to a lesser extent (Kıvrak and Kıvrak, 2017; Manyi-Loh, Ndip and Clarke, 2011; Al-Mamary, Al-Meeri and Al-Habori, 2009; Beretta *et al.*, 2005). A typical honey composition includes a mixture of sugars, various phenolic compounds, minerals, proteins, enzymes, vitamins and volatile compounds (Manyi-Loh *et al.*, 2011). Special reference is given to phenolic constituents as they have been shown to possess antioxidant properties by acting through various mechanisms (Alvarez-Suarez *et al.*, 2009).

Several authors have reported the honey colour to be correlated with the polyphenol content of honey. Dark honeys, being richer in polyphenols are consequently better antioxidants (Beretta *et al.*, 2005; Ferreira *et al.*, 2009; Alvarez-Suarez *et al.*, 2010; Perna *et al.*, 2013). The antioxidant properties have been reported to be effective in reducing the risk of heart disease, cancer and immune-system decline (The National Honey Board, 2002). In addition to that, honey is known for its antimicrobial, antiviral, anti-inflammatory activities (Bogdanov *et al.*, 2008). This study evaluated the correlation between the antioxidant activity and various biochemical and physicochemical parameters in monofloral and polyfloral honey samples from two geographical regions, obtained commercially or directly from beekeepers. The selected parameters that were correlated to the results obtained by antioxidant assays included colour, the total phenolic (TPC) and total flavonoid content (TFC).

In addition, proline, which is used as a determinant of the honey quality, was reported to contribute to the reducing ability and radical scavenging potential of honey (Khalil *et al.*, 2012) and was therefore included in our study. Finally, ascorbic acid, previously used as a simple marker to discriminate the botanical origin of honeys (León-Ruiz, Vera, González-Porto, & Andrés, 2011), has been assessed as a predictive parameter useful in the evaluation of antioxidant activity.

To the best of our knowledge, although numerous studies worldwide evaluated the antioxidant properties of honey, no study reported a characterization of Bosnian honey by the selected parameters, nor their interrelation evaluated by their correlation coefficients.

EXPERIMENTAL

Chemicals

All chemicals and solvents used were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbezothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic acid, quercetin (≥95%), *o*-phenylenediamine (99.5%), 2-propanol (99.9%) and DL-proline (99%) were obtained from Sigma Aldrich, Co. (St. Louis, MO, USA). L(+)-Ascorbic acid and ninhydrin were obtained from Merck (Germany). Folin-Ciocalteau reagent, aluminium chloride hexahydrate, ammonium chloride, ammonium hydroxide, potassium persulfate, sodium carbonate anhydrous, acetone, ethanol (96%), methanol (99.5%) and formic acid were sourced from Semikem (B&H).

Honey samples

Honey samples were collected from different regions in Bosnia and Herzegovina (B&H), either as commercially available products or directly from beekeepers. Honey samples from Turkey were all commercially available products purchased in Istanbul. The honey samples were prepared as aqueous solutions in various concentrations as required by the utilised methods.

Colour

Samples were diluted in water (1:1; w/v) and the absorbance was measured at 635 nm. Results were calculated using the following formula: Pfund = -38.70 + 371.39 x ABS (Ferreira *et al.*, 2009) and expressed in millimetres on a Pfund scale (Fell, 1978).

pH

The pH was determined using the method described by Bogdanov, Martin and Lullmann (2002). Honey samples were weighed (10 g), dissolved and homogenised in 75mL of distilled water. Direct readings were taken for each honey sample using a calibrated pH meter (pH meter PH-20W).

Total phenolic content

The Folin-Ciocalteu method was used to determine the TPC as reported by Singleton and Rossi (1965), with some modifications. The volume of 200 µL of honey solution (100-300 mg/mL) was mixed with 1 mL of Folin-Ciocalteu reagent. After 5 minutes of incubation at room temperature, 800 µL of sodium carbonate solution (7.5%, w/v) was added. The mixture was incubated for 30 minutes at room temperature, and the absorbance was measured at 734 nm by using a UV-Visible spectrophotometer. Calibration curve was prepared using known concentrations of gallic acid (10-110 mg/L) and results expressed in milligrams of gallic acid equivalents (mg GAE/100 g of honey).

Total flavonoid content

The TFC was determined by using the Dowd method as adapted by Arvouet-Grand *et al.* (1994). 500 µL of honey solution (300-500 mg/mL) was mixed with 500 µL of 2% aluminium trichloride solution in methanol. The mixture was incubated for 30 minutes at room temperature, and the absorbance was measured at 415 nm by using a UV-Visible spectrophotometer. Calibration curve was prepared using known concentrations of quercetin (2.5-20 mg/L) and results expressed in milligrams of quercetin equivalents (mg QE/100 g of honey).

DPPH and ABTS

The DPPH assay was performed as described by Blois (1958). The volume of 100 µL of honey solutions at various concentrations were mixed with 1.9 mL of a DPPH solution (0.05 mmol/L in methanol) and kept in dark. After 30 min of reaction at room temperature, the absorbance of the solution was measured at 517 nm.

ABTS radical-scavenging activity of honey samples was determined according to Re *et al.* (1999). The volume of 100 µl of honey solutions at various concentrations were mixed with 900 µl of ABTS solution (7 mmol/L in ethanol) and the absorbance was recorded at 734 nm. The ABTS and DPPH scavenging ability were expressed as IC_{50} (mg/ml). The scavenging activity in both assays was calculated using the following formula:

$$\text{scavenging activity (\%)} = [(A0 - A1)/A0] \times 100$$

Where A0 is the absorbance of the control, and A1 is the absorbance of the sample/standard solution.

Ascorbic acid content

Ascorbic acid was determined using the method described by Wu *et al.* (2003), with some modifications. The volume of 500 µL of honey solution (20-30 mg/mL) was mixed with 500 µL of o-phenylenediamine solution in 0.1 mol/L hydrochloric acid (0.5%, w/v) and 750 µL of NH_3-NH_4Cl buffer solution (pH 9.4). The mixture was then diluted to 5 mL with water and thoroughly mixed by shaking. After 5 minutes, the fluorescence intensity was measured using a 1 cm quartz cell at excitation and emission wavelengths of 330 and 430 nm, respectively. Calibration curve was prepared using known concentrations of ascorbic acid (1 – 70 µg/mL) and results expressed in milligrams of ascorbic acid equivalents (mg AAE/100 g of honey).

Proline content

The proline content was determined using the method described by Bogdanov, Martin and Lullmann (2002), based on the original method of Ough (1969). The volume of 375 µL of honey solution (50-130 mg/mL) was mixed with 750 µL of formic acid and 750 µL of ninhydrin solution (3% in acetone). The tube containing the mixture was shaken vigorously for 15 minutes and placed in a boiling water bath. After 15 minutes, the tube was transferred to a 70 °C water bath for an additional 10 minutes. 3.75 mL of the 50% 2-propanol water solution was added to the mixture and left to cool. The absorbance was measured at 510 nm, 45 minutes after removal from the 70 °C water bath. Water was used as the blank. Calibration curve was prepared using the standard proline solution (200 mg/L), and the result expressed as mg of proline/kg of honey.

Statistical analysis

Except for the determination of proline content, all measurements were performed in triplicates and results are reported as mean ± SD. The Pearson's correlation coefficient (r) was calculated to find the interdependence between the variables and draw conclusions whether there is a significant association between the investigated parameters describing the colour, TPC, TFC, proline content, ascorbic acid content and antioxidant activity. Statistical significance was set at two levels: $p < 0.05$ (significant) and $p < 0.001$ (highly significant).

RESULTS AND DISCUSSION

The current study investigated 15 honey samples of different botanical and geographical origin, as presented in Table 1. The samples were classified as monofloral honey, polyfloral honey and honeydew. The pH value is a useful parameter that aids the determination of origin. According to Gomes *et al.* (2011) forest honey has higher pH values than flower honey. This study included only one sample of forest honey, which is insufficient for further interpretation. In general, depending on the botanical source, the pH of the nectar, soil and the presence of acids and minerals, the pH of honey ranges between 3.5 and 5.5 (Bogdanov, Martin and Lüllmann, 1997). The honey samples presented pH values ranging from 3.7 - 4.6. The average pH value of monofloral samples (4.15) was very similar to the pH value of polyfloral samples (4.00), however honeydew samples showed slightly lower acidity (4.43).

Botanical and geographical origin are also determinants of another parameter - colour. The colour of honey is due to pigments but also other factors such as beekeeper's interventions, aging and storage conditions may affect it (Khalil *et al.*., 2012). The international markets demand specific honey colour since American consumers prefer light honey with delicate, light taste while the population in some European areas asks for dark honey that has a stronger taste (Delmoro *et al.*, 2010). The consumer's colour preference is not necessarily a reflection of the honey quality. The colour of the honey samples in this study varied from extra white to dark amber. The majority of samples was in the range of 119.73 - 239.06 mm, which corresponds to dark amber.

Table 1. Origin, pH and colour of honey samples

Sample ID	Honey description	Country	pH	mmPfund	Colour
		Monofloral			
1 #	Sage honey	B&H	4.1 ± 0.01	164.1	dark amber
2 #	Chestnut honey	B&H	4.3 ± 0.00	119.73	dark amber
3 ##	Extracted chestnut honey	B&H	4.5 ± 0.00	54.7	light amber
4 ##	Raspberry and goji honey	B&H	3.7 ± 0.01	123.04	dark amber
		Polyfloral			
5 ##	Forest honey	B&H	4.1 ± 0.02	124.28	dark amber
6 ##	Meadow honey	B&H	3.8 ± 0.00	136.69	dark amber
7 #	Mountain honey	Turkey	3.9 ± 0.00	10.55	extra white
8 #	Mountain honey	Turkey	3.8 ± 0.01	17.86	white
9 #	Floral honey	Turkey	4.4 ± 0.00	80.04	light amber
10 #	Polyfloral honey	Turkey	3.8 ± 0.01	68.63	light amber
11 #	Foral honey	Turkey	4.0 ± 0.02	90.23	amber
12 #	Floral honey	Turkey	4.2 ± 0.02	140.72	dark amber
		Honeydew			
13 ##	Honeydew	B&H	4.6 ± 0.00	239.06	dark amber
14 #	Cedar	Turkey	4.2 ± 0.01	89.94	amber
15 #	Pine	Turkey	4.5 ± 0.01	195.03	dark amber

 # commercially obtained, ## obtained from beekeepers

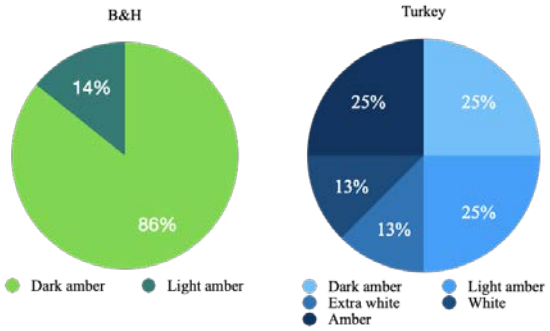

Figure 1. Colour of the investigated honey samples classified according to geographical origin.

The content of single phenolic or other compounds present in honey is too low to have a major antioxidant significance (Gheldof, Wang and Engeseth, 2002), it is rather the combination and interaction of enzymes, vitamins, amino acids, organic acids and other components that contribute to the overall antioxidant capacity (Nayik *et al.* 2016; Ferreira *et al.*, 2009). Therefore, to evaluate the antioxidant activity of the selected honey samples, a series of parameters have been determined, as shown in Table 2.

It has been demonstrated that some amino acids have antioxidant properties (Wu *et al.*, 2003; Udenigwe and Aluko, 2011). A study on Burkina Fasan honey confirmed the radical scavenging potential of proline (Meda *et al.*, 2005). Proline is the prevalent among the described free amino acids in honey (Belitz, Grosch and Schieberle, 2009) and its content is commonly used to estimate the quality and maturity of honey, as well as in detection of sugar adulteration. Genuine honey will have a minimum value of 180 mg proline per kg, however it should be kept in mind that the proline content varies in different types of honey (Bogdanov *et al.*, 1999). To illustrate that, 6719 honey samples of European origin were analysed and it was found that the proline content ranged from 222 to 956 mg/kg depending on the plant species (Oddo and Piro, 2004; Piazza and Oddo, 2004). Except for three flower honey samples from Turkey (sample 10-12), all other samples of this study had a proline content above the minimum prescribed value and can therefore be considered genuine. The raspberry honey had the highest proline content of 1305.29 mg/kg.

Table 2. Proline, ascorbic acid, phenolic and flavonoid content in honey samples and their antioxidant activity

Sample ID	Sample description	Proline mg/kg	Ascorbic acid mg AAE/100g	TPC mg GAE/100g	TFC mg QE/100g	DPPH IC_{50} mg/mL	ABTS IC_{50} mg/mL
			Monofloral				
1B	Sage honey	768.08	22.01 ± 0.14	83.68 ± 1.69	4.65 ± 0.02	480.13 ± 11.89	51.87 ± 2.52
2B	Chestnut honey	461.98	96.46 ± 2.09	57.98 ± 0.54	4.1 ± 0.04	745.36 ± 12.14	69.70 ± 1.79
3B	Extracted chestnut honey	330.92	110.78 ± 1.88	48.8 ± 0.90	2.82 ± 0.08	1132.33 ± 7.75	93.09 ± 2.82
4B	Raspberry and goji honey	1305.29	41.81 ± 1.77	87.85 ± 1.21	4.69 ± 0.02	670.31 ± 20.49	78.99 ± 2.84
			Polyfloral				
5B	Forest honey	721.35	22.18 ± 1.28	73.17 ± 1.29	4.58 ± 0.03	464.94 ± 15.67	63.94 ± 3.37
6B	Meadow honey	984.30	38.19 ± 2.52	58.32 ± 1.81	4.69 ± 0.09	567.52 ± 30.34	84.93 ± 1.83
7T	Mountain honey	324.15	4.57 ± 0.40	12.72 ± 0.26	1.28 ± 0.01	2891.78 ± 1.76	174.60 ± 1.02
8T	Mountain honey	282.28	3.75 ± 1.01	28.06 ± 0.07	0.96 ± 0.12	2645.90 ± 28.14	169.75 ± 8.19
9T	Floral honey	377.49	12.46 ± 0.36	70.57 ± 3.07	3.34 ± 0.02	443.57 ± 10.64	49.67 ± 0.58
10T	Polyfloral honey	145.44	7.97 ± 0.44	26.11 ± 0.30	1.76 ± 0.04	**	335.06 ± 13.13
11T	Foral honey	76.88	6.06 ± 0.41	21.07 ± 0.47	1.46 ± 0.03	**	173.88 ± 8.47
12T	Floral honey	85.54	9.56 ± 0.89	29.36 ± 0.74	3.31 ± 0.05	**	232.75 ± 4.96
			Honeydew				
13B	Honeydew	677.17	30.46 ± 0.63	89.8 ± 2.35	6.87 ± 0.08	264.04 ± 5.36	37.22 ± 2.31
14T	Cedar	232.26	21.18 ± 1.36	56.06 ± 0.60	3.21 ± 0.02	564.61 ± 15.02	64.59 ± 1.83
15T	Pine	1233.58	22.18 ± 1.85	54.86 ± 0.64	4.27 ± 0.04	599.58 ± 6.40	69.73 ± 0.43

*** Honey solution in the concentration of 1 mg/mL was insufficient to obtain the IC_{50} values. Higher concentrations lead to inhomogeneous turbid solutions inappropriate for the assay.*

B – samples from B&H; T- samples from Turkey

Ascorbic acid is a naturally occurring antioxidant that has been analysed to describe both the nutritional value and antioxidant properties of honey. The number of studies is rather scarce, as ascorbic acid is unstable and prone to chemical and enzymatic oxidation (León-Ruiz *et al.*, 2013). The use of different analytical methods makes the comparison of results difficult. In the present study, the ascorbic acid content was determined spectrofluorimetrically, based on its condensation reaction with *o*-phenylenediamine. The obtained results were in the range between 22.01 - 110.78 mg AAE/100 g and 3.75 - 22.18 AAE mg/100 g in Bosnian and Turkish honey, respectively. Two studies (Kesic *et al.*, 2009; León-Ruiz *et al.*, 2013), based on a volumetric method using 2,6-dichlorophenolindophenol, reported a different concentration range in honey from B&H (37.22 - 378.3 mg/100g) and honey from Spain (0.77 – 57.15 mg/100 g). Phenolics are one of the most important classes of compounds found in honey (Khalil *et al.*, 2012) and flavonoids as a subclass represent the main functional components of scientific and therapeutic significance (Yao *et al.*, 2004; Alvarez-Suarez *et al.*., 2012). The mechanisms by which phenolic compounds exhibit their antioxidant activity include free radical-scavenging, metal ion chelation and hydrogen donation (Havsteen, 2002). The TPC values of the analysed honey samples were in the range between 12.72 - 89.8 mg GAE/100 g, with a mean value of 71.37 mg GAE/100 g for honey from B&H and 37.35 mg GAE/100 g for Turkish honey. Similar values (12.64 - 90.57 mg GAE/100 g) were obtained for Croatian honey (Piljac-Žegarac, Stipčević and Belščak, 2009) but also for African honey sorts (32.59 - 114.75 mg GAE/100 g), Brazilian honey (28.9 - 69.0 mg GAE/100 g), Greek honey (55.0 – 92.0 mg GAE/100 g) and Portuguese honey (22.62 – 72.77 mg GAE/100 g) (Meda *et al.*., 2005; Cruz *et al.*, 2014; Stagos *et al.*., 2018; Ferreira *et al.*, 2009). Honeydew followed by raspberry and goji honey had the highest TPC, while mountain honey (sample 7) had the lowest TPC. The TFC values varied between 0.96 - 6.87 mg QE/100 g, with the following mean values for Bosnian and Turkish honey respectively: 4.63 mg QE/100 g and 2.45 mg QE/100 g. Compared to other studies, the obtained values were similar to the TFC in African honey (0.17 - 8.35 mg QE/100 g) and Brazilian honey (0.90 – 4.86 mg QE/100 g) (Meda *et al.*, 2005; Pontis *et al.*, 2014).

Until today, there is no universal method that can be used to determine the i*n vitro* antioxidant activity. The two most commonly employed methods are the DPPH and ABTS assays and there is no equivocal view as for which of the two assays is more appropriate for honey samples (Lachman *et al.*, 2010; Piljac-Zegarec *et al.*, 2009; Meda *et al.*, 2005; Turkmen *et al.*, 2006). The results describing the antioxidant activity were expressed as IC_{50}, where a lower IC_{50} value implies a stronger antioxidant activity. The antioxidant activity determined by the DPPH assay was stronger in polyfloral than in monofloral honey collected in both countries, however the Bosnian honey samples appeared to have a superior radical scavenging capacity than Turkish honey. This is also true when observing the AA obtained by the ABTS assay. The IC_{50} ranged between 37.22 - 93.09 mg/mL and 49.68 - 335.06 mg/mL for Bosnian and Turkish honey, respectively. The IC_{50} values of Bosnian honey are similar to those of Greek honey reported by Stagos *et al.* (2018), however direct comparison of AA from literature data is difficult given the various reaction conditions used by different authors.
The main differences from honey samples collected in Turkey and B&H are presented in Fig. 2.
According to the obtained results, among the Bosnian honey samples, honeydew had the best antioxidant properties, whereas the same is true for the polyfloral honey (sample 9) among the Turkish honeys.
The correlation matrix (Table 3) was calculated taking into account all samples of this study, regardless of botanical and geographical origin. Clearly, there is a highly significant correlation between the TPC and TFC ($r = 0.8709$, $p < 0.001$). Findings from previous studies regarding the TPC and AA have been contradictory. Some authors found no significant correlation between these parameters (Bueno Costa *et al.*, 2016; Stagos *et al.*, 2018), while others found a significant correlation (Al *et al.*, 2009; Alvarez-Suarez *et al.*, 2010; Pontis *et al.*, 2014). The reasoning behind is that for some plants, the AA depends not only on the quantity of phenolics, but mainly on the chemical composition of phenolic compounds (Stagos *et al.*, 2012), which is then reflected onto the chemical profile of honey. In this study, the TPC and TFC showed a highly significant or significant correlation to the results obtained by both antioxidant assays.

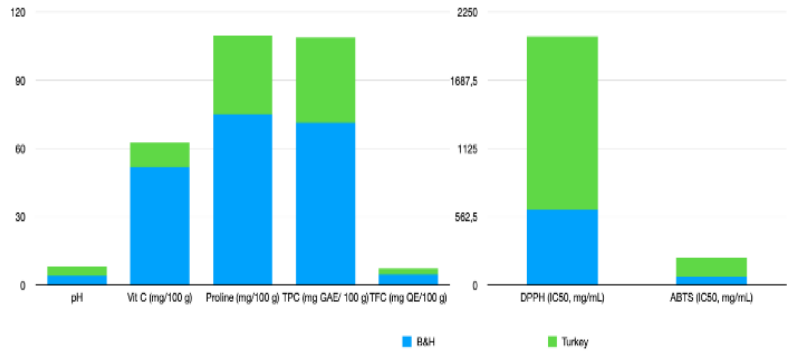

Figure 2. Comparison of physicochemical parameters in honey samples from B&H and Turkey.

Table 3. Pearson's correlation coefficients among antioxidant activity and other investigated parameters

	TPC	TFC	DPPH	ABTS	Colour	Proline	Ascorbic acid
TPC	1						
TFC	**0.879***	1					
DPPH	**-0.872***	**-0.847***	1				
ABTS	**-0.783***	-0.676**	**0.979***	1			
Colour	0.678**	**0.910***	-0.772**	-0.449	1		
Proline	0.628**	0.616**	-0.447	-0.547**	0.557**	1	
Ascorbic acid	0.297	0.295	-0.262	-0.381	0.065	0.109	1

** - highly significant ($p < 0.001$), ** - significant ($p < 0.05$)*

Judging by the correlation coefficient between the ABTS and DPPH ($r = 0.979$, $p < 0.001$), both assays are equally suitable for measurements of the radical scavenging capacity in honey. The possible relationship between phenolics, flavonoids, AA and colour has also been commonly investigated and demonstrated (Pontis *et al.*, 2014; Ferreira *et al.*, 2009; Khalil *et al.*, 2012). Numerous studies have shown that in comparison to light coloured honeys, dark honeys demonstrate a higher TPC and are therefore characterised by a stronger AA (Pontis *et al.*, 2014; Perna *et al.*, 2013; Cabrera *et al.*, 2017). At the significance level of $p < 0.05$, the TPC and the colour of the analysed samples had a correlation of $r = 0.678$, however there was a stronger association ($r = 0.910$, $p < 0.001$) between the colour and the TFC content, as shown in Fig. 3.

The colour of honeys is due to various pigments that are chemically classified as flavonoids (Havsteen, 2002), which explains the obtained results. Interestingly, the AA was significantly correlated to the honey colour only when determined by the DPPH assay. When interpreting honey samples from Turkey it is important to note that they were predominantly light-coloured honeys which, as can be seen from the correlation matrix, implies a lower phenolics content and limited antioxidant defence.

Several authors suggested the total proline content to be a critical factor responsible for the AA of honey (Khalil *et al.*, 2012; Saxena, Gautam and Sharma, 2010). Our study confirmed these findings showing lower but significant correlation between proline content and TPC, TFC, colour and the AA determined by the DPPH assay. Ascorbic acid content in Algerian honey has been found to be associated with flavonoids, colour and DPPH scavenging activity (Khalil *et al.*, 2012), however our results differ. There was no correlation between ascorbic acid and any of the variables presented in Table 3. Among the investigated samples, the best example that follows the correlation matrix is honeydew, which is dark amber in colour, and characterised by the highest TPC and TFC values and the strongest AA. Concluding from the same parameters, polyfloral honeys were of better quality than monofloral honeys.

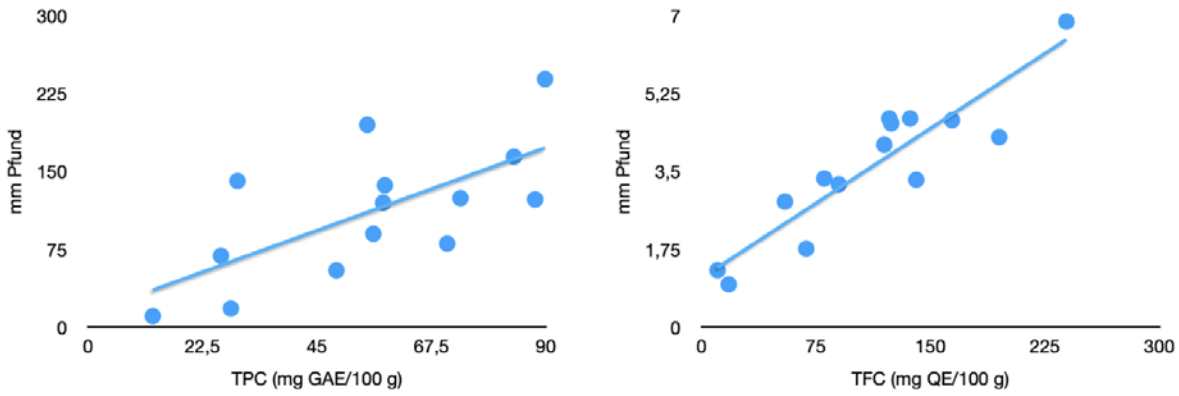

Figure 3. Correlation between colour and A) TPC and B) TFC.

CONCLUSION

Based on the results obtained from the analysis of 15 honey samples of different botanical and geographical origin it can be concluded that the following parameters are strongly associated with the antioxidant activity: colour, TPC, TFC and proline. The colour of honey is influenced by the pigments which are in chemical terms classified as flavonoids. The obtained results confirmed that dark honeys are rich in phenolics and particularly in flavonoids. There was an excellent correlation between the DPPH and ABTS antioxidant assays ($r = 0.979$, $p < 0.001$), however DPPH showed a better correlation with other parameters and can therefore be used preferentially. Our study also confirmed the total proline content is a parameter directly associated with the AA of honey, however ascorbic acid was not found to be in correlation with any of the remaining variables related to AA. The honey samples from Bosnia were richer in phenolics and of stronger AA compared to the samples from Turkey, which may be a consequence of the variety of the collected samples. Dark amber was the predominant colour of Bosnian honeys, whereas the Turkish honey had a higher share in light coloured samples. The novelty of the study is also in the description of the antioxidant properties of Bosnian honey evaluated by the selected parameters. Bosnian honey, containing significant amounts of compounds related to the AA, can consequently be recommended for use for its beneficial health effects. Polyfloral samples were found to have better antioxidant properties compared to monofloral honeys, whereas such a difference was not evident between the commercially purchased honeys and honeys obtained from beekeepers.

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Summary/Sažetak

Cilj ovog rada je bilo odrediti korelaciju između antioksidativne aktivnosti uzoraka meda i sljedećih parametara: boja, ukupan sadržaj fenola (TPC), ukupan sadržaj flavonoida (TFC), sadržaj prolina i askorbinske kiseline. Uzorci su sakupljeni u Bosni i Hercegovini i Turskoj, a obuhvatali su mednu rosu, monofloralne i polifloralne medne vrste. Antioksidativna aktivnost je određena ABTS i DPPH metodom. Na osnovu korelacionog matriksa utvrđena je dobra korelacija antioksidativne aktivnosti sa TPC, TFC, sadržajem prolina i bojom meda. Boja meda je bila u najboljoj korelaciji sa TFC ($r = 0.910$, $p < 0.001$), pri čemu su se tamniji medovi odlikovali višim TFC vrijednostima. Antioksidativna aktivnost je bila značajno ovisna o TPC (DPPH-TPC: $r = - 0.872$; ABTS-TPC: $r = - 0.783$, $p < 0.001$). Sadržaj askorbinske kiseline se nije pokazao kao prediktivan parametar koji bi se koristio za procjenu antioksidativnih svojstava meda, te nije značajno korelirao ni sa drugim varijablama.

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Bioaccumulation of metals in fish from hydro-accumulations on the Neretva River, Bosnia and Herzegovina used in different diets

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Abstract: There is a growing need to assess the level of contaminants in fish as bioindicators of the health and well-being of fish and humans as its consumers. Contamination by heavy metals (Cd, Cr, Cu, Fe, Mn, Pb, Zn) was evaluated using atomic absorption spectrometer, flame and graphite furnace technique in the water samples and fish muscle tissues of *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca* of four hydro-accumulation lakes on the Neretva River, Bosnia and Herzegovina. Samples were collected during two seasons: autumn-winter and spring-summer (2019). It has been shown that iron (Fe) was the highest accumulating metal in fish, whilst cadmium (Cd) and lead (Pb) were the lowest. Heavy metals contents were below the maximum permissible for drinking water and for fish as prescribed by national legislation. According to correlation matrix between metals content in all fish during both fishing seasons, the highest values of the Pearson coefficient were obtained in the case of essential elements (Fe, Mn, Cu and Zn) and Fe and Mn also had a statistically significant correlation with Cd and Pb. Furthermore, potential health risk assessment exposure of the adult population in B&H revealed that none of the seven heavy metals pose risk to human health, based on the estimated daily intake via consumption of these fish species as well as target hazard quotient and hazard index values less than 1.

INTRODUCTION

Fish has been used in human nutrition since ancient times, because people were able to obtain food simply and easily by fishing. With the increase of the world's population and the increase of the standard of living, especially in the developing countries, the needs for fish meat are significantly increasing (Tepe, et al. 2008). Fish catches increased 20 times from the beginning to the end of the 20th century and reached a maximum of 93 million tons per year (Baltić, Dokmanović, Bašić, et al. 2015). As fish are constantly exposed to pollutants in water, they can also be used as an excellent biological indicator of pollution in aquatic ecosystems (Benson, Essien, Akan, et al. 2007). In recent years, there has been a lot of research on the content of heavy metals in fish in different parts of the world. Most of this research has been done in the muscle tissue of fish, although, some other organs such as the liver, gills, kidneys, bones are also interesting (Karadede and Ünlü 2000, Vicente-Martorell, Galindo-Riano, Garsia-Vargas, et al. 2009, Malik, Biswas, Qureshi, et al. 2010).

Aquatic ecosystems are exposed to many substances, including heavy metals that are non-degradable and have a high capacity for bioaccumulation in water, sediment and aquatic organisms that inhabit this ecosystem. In general, the study of heavy metals can be viewed through two main aspects. The first aspect is from the point of view of health safety, ie. attention to the bioaccumulation of heavy metals that can affect human health. The second aspect is from the point of view of bioaccumulation of heavy metals in the aquatic ecosystem, which can affect the balance of the ecosystem.

Toxicity and bioaccumulation of heavy metals in aquatic ecosystems may depend on numerous factors such as: dissolved oxygen content, pH, alkalinity, and temperature (Adhikari, et al. 2006). Similarly, metals bioaccumulation in fish tissues depends on i.e., fish size, fish age, eating habits (Khalid 2004). Laboratory experiments have shown that fish that ingest heavy metals through water have a higher content of metals in their

gills, while fish that ingest higher concentrations of metals through their diet have a higher content of metals in their digestive tract and muscle tissue. Also, if the concentration of heavy metals in the water is low, the main mode of metal intake is through the food that fish ingests (Clements 1997, Khalid 2004).

With the increase of man's need for electricity on the one hand, and the desire to preserve a healthy environment, on the other hand, man often takes major interventions in rivers and, thus, contrary to his aspirations and essentially long-term interests, significantly disrupts normal natural life in its surroundings. The construction of dams and the formation of hydro accumulation (HA) lakes on the Neretva River, B&H marked the second half of the 20th century and clearly showed all the negative consequences of human experimentation with nature (Škrijelj 2002). With the formation of an artificial lake on river, the affected river complexes gradually pass from the stream into the standing water ecosystem. In this way, the whole complex of ecological conditions changes, which inevitably follow the changes in the composition of living communities in the aquatic ecosystem and its immediate surroundings.

Three different country-specific fish species with different eating habits: *Sander lucioperca* (carnivore), *Leuciscus svallize* (omnivore)and *Tinca tinca* (herbivore), inhabit four HAs on the Neretva River. Since there is a lack of the data for these fish species, partly due to the fast-growing aquaculture sphere in B&H, the novelty as well as the primary goal of this research is to gain valuable inputs on seven heavy metals (Cd, Cr, Cu, Fe, Mn, Pb and Zn) content. In addition, to identify possible metals sources matrix correlation analysis was applied. Although official total dietary study data is scarce in B&H, potential health risk assessment exposure of the adult population in B&H was revealed based on the estimated daily intake (EDI) via consumption of these fish species. Water samples were also taken from four HAs on the Neretva River in order to analyze heavy metals content and physico-chemical parameters (water temperature, pH and electrical conductivity).

EXPERIMENTAL

Study area

The Neretva River flows through the central, southern part of B&H. The examined fish species were collected in four artificial HAs along this river. Figure 1 shows locations of fishing sites of HA Jablaničko Lake (43°41'N, 17°44'E), HA Grabovica (43°35'N, 17°43'E), HA Salakovac (43°27'N, 17°49'E) and Mostarsko Lake (43°23'N, 17°51'E).

HA Jablaničko Lake is the largest HA on the Neretva River with seasonal water leveling and a dominant influence on the regulation of the river water regime. Due to frequent and sudden oscillations of the water level during the filling and emptying of the reservoir caused by the operation of hydropower Jablanica, the coast is destroyed, and suspended sediment is brought. The emptying of the reservoir leads to the withdrawal of sand into the depths and the formation of layers of mud and silt. The maximum length is about 30 km and the surface of the lake is 1440 ha. The maximum depth is 80 m and the water level oscillations are up to 25 m. Out of a total of 13 fish species from the ichthyofauna of the Neretva accumulation lakes, the presence of seven fish species has been found in Jablaničko Lake.

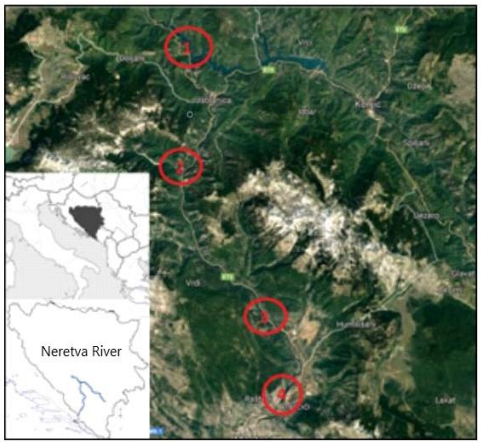

Figure 1: Sampling sites: 1 - HA Jablaničko Lake; 2 - HA Grabovica; 3 - HA Salakovac; 4 - HA Mostarsko Lake, *Google maps*).

HA Grabovica is elongated with a very small width, so it is mainly located in the canyon of the middle course of the Neretva River. The average depth of the lake is 34 m, while the water oscillations are about 4 m. During ichthyologic research of the HA Grabovica (Škrijelj 1990) the presence of six species of fish was ascertained.

HA Salakovac was formed in 1981. The maximum length of the lake is 20 km, the area is about 314 ha, and the maximum depth is about 40 m while the water oscillations are about 5 m. Studies of the ichthyic fauna of the accumulation lake Salakovac have shown that this ecosystem is inhabited by nine fish species.

HA Mostarsko Lake is the youngest HA on the Neretva River, built in 1987. The maximum length of the lake is about 10 km, and the area is about 112 ha. The greatest depth of the lake is about 20 m, while the oscillations of the water are up to 5 m. Conducted ichthyologic research of Neretva accumulation lakes included Mostarsko Lake. Research has shown that seven fish species live in this lake (Škrijelj 1990).

Sample collection and preparation

The research was conducted in two seasons: autumn - winter (September-March 2019) and spring - summer (April – August 2019), i.e. in high and low water levels of the HAs on the Neretva River. The catch was made for three fish species of different feeding habits (*Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca*) on four HAs on the Neretva River. The catch was made by local fishermen using fishing nets set up in the evening. Immediately after the fish was caught, a section of fish was performed. The fish muscle tissue was separated by section, which was then washed with Milli-Q water, weighed, packed in sterile polyethylene bags and stored at −18°C, until analysis. Table 1 shows the number of fish caught in the examined seasons. Water samples were taken in September 2019, from four HAs on the Neretva River in order to analyze heavy metals content and

physico-chemical parameters (temperature, pH and electrical conductivity). Samples of water were collected in sterile polyethylene bottles (previously washed by detergent, rinsed by Milli-Q water followed by 2 mol/L nitric acid (HNO_3), washed by Milli-Q water again and finally with sampled river water). The samples were preserved by the addition of concentrated HNO_3 (1 mL per 1 L of sample) and brought to the laboratory. Then, the samples were filtered through Whatman filter paper (No. 42) and kept in refrigerator at 4 ºC until analysis.

Table 1: Number of fish caught from four HAs on the Neretva River in the periods of spring-summer and autumn-winter.

Fish species/location	Jablaničko Lake	HA Grabovica	HA Salakovac	Mostarsko Lake
Season Spring-Summer (number of fish caught)				
Sander lucioperca	5	4	7	8
Leuciscus svallize	7	5	15	33
Tinca tinca	4	3	9	3
Season Autumn-Winter (number of fish caught)				
Sander lucioperca	4	2	6	5
Leuciscus svallize	3	5	13	28
Tinca inca	5	3	7	4

Sample preparation and heavy metal determination

Composite fish samples of the same species, the same catch locations as the same parts previously classified by the fish section were used. For microwave digestion (Milstone, Start D), 0.5 to 1.0 g of fish muscle tissue sample were weighed, then the samples were transferred to Teflon containers, and 7 mL of 65% (w/v) HNO_3 and 1 mL 30% (w/v) H_2O_2 were added. After digestion, the samples were cooled to room temperature and quantitatively transferred to 25 mL volumetric vessels and filled to the mark with 0.1 mol/L HNO_3.

The content of Cr, Cu, Fe, Mn, and Zn was determined in fish muscle samples using an flame atomic absorption spectrometer (FAAS), while Cd and Pb were quantified by graphite furnace technique (GFAAS) (AAS, model Varian AA240FS). The obtained results are expressed in units of mg/kg (wet weight of fish sample). In water samples, Zn was determined by FAAS and other metals by GFAAS. Field devices that were used to determine physico-chemical parameters of water were pH meter – Hanna Combo, HI98130 and Conductivity meter – Hanna Combo, HI8733N.

Quality Assurance

All reagents used were of analytical purity. For quality assurance, all samples were analyzed in triplicate along with blanks to minimize error. A blank was prepared in the same way as the samples and was analyzed after each batch of 15 samples. The mean result for all samples was reported, and repeatability standard deviations were calculated. Accuracy assessment was performed by spiking already analyzed fish samples with different concentrations of analyzed heavy metals, spiked at three different concentrations (low, medium, and high) covering the working range and the percent recovery was calculated. These standards were different from those used to prepare the calibration curves and were also from different stock standard solutions. The acceptable recoveries ranged between 81-107%.

The detection limit (LOD) was calculated as 3 times the standard deviation of blank (n = 10) absorbance signal. LODs for GFAAS were: 0.065 μg/L for Cd; 0.056 μg/L for Cr; 0.354 μg/L for Cu; 0.390 μg/L for Fe; 0.23 μg/L for Mn; 0.80 μg/L for Pb; 0.013 μg/L for Zn. LODs for FAAS were: 0.02 μg/mL for Cd; 0.16 μg/mL for Cr; 0.05 μg/mL for Cu; 0.98 μg/mL for Fe; 0.15 μg/mL for Mn; 1.12 μg/mL for Pb; 0.70 μg/mL for Zn.

E*xposure and potential health risk assessment evaluation*

Heavy metals exposure estimations were obtained in muscles of three fish species: *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca*. The estimated daily intake (EDI, mg kg^{-1} day^{-1}) from fish ingestion was calculated by combining the data on the consumption of fish with the determined levels (*C*, mg/kg, w.w.) of Cd, Cr, Cu, Fe, Mn, Pb and Zn for two different periods of year (spring - summer and autumn – winter). To produce exposure estimates for adult person (70 kg of body weight, BW – USEPA 2011) the fish and seafood ingestion rates (FIR) reported by Hajrić, et al. (2022) were used, since there is no available official data on dietary habits of the population in B&H. They found that the average consumption was 52.1 g day^{-1} $consumer^{-1}$ based on the conducted survey using the "Food frequency questionnaire method" (EFSA 2014) for the consumption of fish and fish products (n = 500 respondents), taking into account the proportional representation of the overall population by age, sex, education and employment status. The calculation was made according to the following expression given by Łuczyńska, et al. (2018):

$$\text{EDI (mg kg}^{-1}\text{ day}^{-1}\text{)} = \frac{C \times FIR}{BW} \quad (1)$$

The obtained EDI values of metals were compared with the oral reference dose values (Rfd) given by USEPA (2011).

A commonly used method of risk assessment estimation is the ratio between the measured concentration of metal and the oral reference dose, known as the target hazard quotient (THQ). This hazard quotient is mathematically expressed as follows (USEPA 2000; Liang et al. 2018):

$$THQ = \frac{EF \times ED \times FIR \times C}{Rfd \times BW \times TA} \times 10^{-3} \quad (2)$$

where the exposure frequency (EF, 365 days $year^{-1}$) is combined with the exposure duration (ED, 70 years), fish ingestion rate (FIR, 52.1 g day^{-1} $consumer^{-1}$), the mean concentration of Cd, Cr, Cu, Fe, Mn, Pb and Zn in fish (*C*, mg kg^{-1} w.w. given in Table 3), oral reference dose (Rfd, given by USEPA 2011), body weight of adult person (BW, 70 kg), and mean exposure time (*TA*, 365 days $year^{-1}$ x ED).

In addition, hazard index *(*HI) was calculated as sums of individual THQs obtained for each of seven heavy metals given by USEPA (2011) as:

$$HI = THQ_{Cd} + THQ_{Cr} + THQ_{Cu} + THQ_{Fe} + THQ_{Mn} + THQ_{Pb} + THQ_{Zn} \quad (3)$$

Statistical Analysis

In the statistical analysis, as basic statistical methods, descriptive statistical parameters were used. Two tests were used to test and determine statistically significant differences between the examined groups. The t-test was used to examine the significance of the differences between the mean values of the two groups examined. A group test, ANOVA, was used to examine significant differences between the three or more observed treatments. Significance of differences was determined at significance levels of 5%. Statistical analysis of the obtained results was done in the statistical package SPSS 17.

RESULTS AND DISCUSSION

Characterization of water samples

Natural and anthropogenic processes as well as the depth of the aquatic system affect the physico-chemical and chemical parameters in the water. For example, water temperature, at the time of fishing, is very important in terms of fish biological activities. The results of water physico-chemical and chemical analysis are shown in Table 2.

All observed physico-chemical parameters were slightly lower in the autumn-winter season. The lowest temperatures and pH values were recorded at HA Jablaničko Lake in both observed seasons, as well as the lowest value of conductivity recorded at the same location in the autumn-winter season. Comparing the obtained results with the national legislation, all the results were below limit value (Official Gazette of FB&H 43/07).

The concentration of Zn in the water was the highest in all four locations, while the lowest concentration was in the case of Cd at HA Mostarsko Lake and HA Jablaničko Lake, as well as Mn and Cr at HA Grabovica and Pb at HA Jablaničko Lake. Metals concentrations were slightly higher in the spring-summer season. The content of analyzed metals in water by localities followed is given in descending order: HA Mostarsko Lake: Zn>Cu>Fe>Mn>Pb>Cr>Cd, HA Salakovac: Zn>Fe>Cu>Mn>Pb>Cr>Cd, HA Grabovica: Zn>Cu>Pb>Fe>Cd>Mn>Cr and HA Jablaničko Lake: Zn>Cu>Mn>Fe>Cr>Pb>Cd. Similar results were reported by Ilie et al. (2014) for mean metal concentration in Danube River (European region), Skadar Lake, the largest lake on the Balkan Peninsula (Vemic, et al. 2014) and for Victoria Lake in East Africa (Ogoyi, et al. 2011). Comparing our results with data for, the Mn content in our study was twice lower and other metals were higher.

Heavy metals in fish

Fish muscle tissue is less active in terms of biotransformation and accumulation of heavy metals compared to other fish tissues (El Moselhy et al. 2014). The content of heavy metals (Cd, Cr, Cu, Fe, Mn, Pb, Zn) in muscle tissue of *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca* fish from HAs on the river Neretva during the spring-summer and autumn-winter seasons were determined. The results are shown in the Table 3. Concentrations of Cd and Cu were similar in the muscle tissues of all three fish species. The content of Cu in *Sander lucioperca* and *Leuciscus svallize* muscle tissue shows a statistically significant difference depending on the location of fishing ($p < 0.05$), while *Tinca tinca* caught at different locations does not show a statistically significant difference ($p > 0.05$). The highest concentrations of Fe and Mn were found in *Leuciscus svallize* and the lowest in *Sander lucioperca* muscle tissue, in both seasons. Also, the highest Pb content was determined in *Leuciscus svallize* (HA Salakovac), while *Sander lucioperca* had lower and relatively similar concentrations of this heavy metal.

Table 2: Physico-chemical and chemical parameters of water at fishing sites on the HAs of Neretva River, autumn-winter and spring-summer seasons.

Location	**HA Jablaničko Lake**		**HA Grabovica**		**HA Salakovac**		**HA Mostarsko Lake**	
Season/ parameter	**autumn/ winter**	**spring/ summer**	**autumn/ winter**	**spring/ summer**	**autumn/ winter**	**spring/ summer**	**autumn/ winter**	**spring/ summer**
Temp. (°C)	14	17	15	18	16	18	16	18
pH	6.63	6.78	6.93	6.80	6.80	6.84	6.94	6.97
Conduct. (µS/cm)	397	412	344	371	330	352	313	366
Metals (µg/L)								
Mn	3.492±0.05		9.122±0.12		<LOD*		0.690±0.02	
Cd	<LOD*		0.086±0.002		<LOD*		<LOD*	
Cr	0.360±0.14		0.821±0.10		<LOD*		0.063±0.002	
Pb	0.820±0.20		2.513±0.24		8.061±0.32		<LOD*	
Fe	9.264±0.12		45.92±2.08		1.132±0.08		0.654±0.08	
Cu	13.46±0.21		24.63±0.150		20.79±0.18		4.020±0.08	
Zn	0.075±0.005		0.067±0.002		0.060±0.002		0.069±0.004	

*LOD – limit of detection

There are a large number of fish farms on HA Salakovac, where fish food is used in large quantities. Also, the highest Pb concentration was found in water from HA Salakovac. According to the diet, *Leuciscus svallize* is an omnivore and feeds on everything it can find, from mollusks, larvae, insects, to organic waste, fruit and moss. Larger *Leuciscus svallizes* feed largely on various species of smaller fish (Škrijelj 2002). The highest concentrations of Cd, Cu, Fe and Mn were determined in *Leuciscus svallize* at the locations of HA Jablaničko Lake and HA Grabovica. The highest concentrations of these metals were also found in water samples at the HA Grabovica compared to other locations. Zrnčić, Oraić, Ćaleta, et al. (2013) point out that omnivorous fish are better biological indicators of environmental contamination, i.e., they provide a more reliable assessment of the state of the environment. *Leuciscus svallize* likes to live at the bottom of the lake, and these two sites are exposed to a number of natural and anthropogenic sources of heavy metal pollution. HA Jablaničko Lake is near the industrial town of Konjic, where a large number of metal industries that discharge their wastewater into the Neretva exist. Also, these two sites are exposed to the M-17 highway, which is used by a large number of vehicles. The order of heavy metals presence of in *Leuciscus svallize* muscle tissue was: Fe>Zn>Mn>Cu>Cr>Pb>Cd. The highest Cr content was determined in *Sander lucioperca* and the highest Zn content in *Tinca tinca* fish in both seasons both in fish from Mostarsko Lake, in both seasons. On the other hand, at the Mostarsko Lake, the lowest concentrations of all analyzed metals were found in water samples. The average Zn content in *Sander lucioperca* was statistically significantly higher in the spring-summer period ($p < 0.05$). Young *Sander lucioperca* feed on plankton and invertebrates, larvae, worms, insects. Adult *Sander lucioperca* mainly consume small fish whose size is limited by the narrowness of its esophagus. As it gets older, *Sander lucioperca* become more and more interested in dead, sick or wounded fish, mening they prefer to catch prey easily (Škrijelj 2002). Low values of all metals in *Sander lucioperca* muscle tissue can be associated with a number of factors present in the observed areas. *Sander lucioperca* feeds primarily on small, sick, and vulnerable fish, which did not have enough time for bioaccumulation of heavy metals. The order of metals presence in *Sander lucioperca* muscle tissue was as follows: Fe>Zn>Cr>Mn>Cu>Pb>Cd. *Tinca tinca* is an herbivore by diet; it feeds on different types of plants depending on the place where it lives. Plant foods contain smaller amounts of nutrients, i.e. has a lower caloric value, so *Tinca tinca* belonging to herbivores must consume larger amounts of plant food that in its composition, in addition to other ingredients, always contains a certain amount of heavy metals, or whose content varies depending on the environmental conditions in which plants grow (Škrijelj 2002). As *Tinca tinca* feeds on plants and algae, it is constantly in contact with sediment, i.e. as it likes to live in muddy parts of the lake, so is constantly exposed to heavy metals that can bioacumulate. The order of metals presence in *Tinca tinca* muscle tissue was: Zn>Fe>Mn>Cu>Cr>Pb>Cd. For Cd and Zn, in contrast to Cr, Fe, Mn and Pb, it was found that there is no statistically significant difference between fishing sites and for Cr, Fe and Pb, unlike other metals, a statistically significant difference was found depending on the fishing season ($p < 0.05$). The metal content in fish muscle tissue shows that fish caught in the autumn-winter season show a significantly smaller statistical difference compared to the spring-summer catch period ($p < 0.05$).

According to the Ordinance on maximum levels for certain contaminants in foodstuff (Official Gazette of B&H, No. 68/14, 79/ 16, 9/17) the maximum allowable concentrations (MAC) in B&H for certain metals in fish muscle tissue are defined as: 0.30 mg/kg for Pb, 0.050 for Cd, 30.0 for Cu, 30.0 for Fe; (values for Cu and Fe refer to whole fish). In all cases the MAC for these metals was not exceeded in all three fish species.

Regarding the metal content in the studied fish, the following descending order can be given: Fe>Zn>Cr>Mn>Cu>Pb>Cd and according to the content of analyzed metals in them, fish can be arranged in the following descending order: *Leuciscus svallize* (omnivorous) > *Tinca tinca* (herbivore) > *Sander lucioperca* (carnivore). Similar results were obtained by Abdelmoneim, et al. (1992), Chale (2002), Mendil, Uluözlü, Hasdemir, et al. (2005) and El Moselhy, et al. (2014).

Table 3: Heavy metals content (mg/kg) in muscle tissue of fish *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca* from HAs on the river Neretva during the spring-summer and autumn-winter seasons

Fish / Metals	Cd	Cr	Cu	Fe	Mn	Pb	Zn
			Spring – Summer (mean concentration / range)				
Sander lucioperca	0.0015	0.926	0.411	8.282	0.769	0.015	3.581
	0.001-0.002	0.427-1.933	0.338-0.523	4.276-11.25	0.628-0.933	0.008-0.028	2.281-4.171
Leuciscus svallize	0.002	0.283	0.549	19.60	0.983	0.033	4.242
	0.001-0.004	0.245-0.321	0.438-0.670	15.44-22.83	0.512-1.378	0.005-0.115	3.143-5.906
Tinca tinca	0.002	0.338	0.518	14.06	0.845	0.007	14.60
	0.001-0.002	0.218-0.521	0.356-0.678	13.12-15.89	0.725-0.906	0.007-0.008	5.011-25.12
			Autumn – Winter (mean concentration / range)				
Sander lucioperca	0.001	0.853	0.424	6.780	0.659	0.011	3.500
	0.001-0.001	0.215-2.314	0.347-0.491	4.325-9.604	0.412-0.897	0.005-0.022	2.344-3.904
Leuciscus svallize	0.002	0.219	0.536	13.29	0.940	0.029	4.276
	0.001-0.003	0.194-0.261	0.439-0.620	5.117-21.74	0.453-1.322	0.003-0.103	2.968-6.764
Tinca tinca	0.002	0.292	0.479	11.95	0.780	0.007	13.58
	0.001-0.003	0.222-0.444	0.342-0.622	9.435-17.05	0.708-0.828	0.005-0.011	4.634-23.81

Matrix correlation analysis

The possible metals sources can be indicated by the correlation matrix by analyzing the value which represents the linear correlation coefficient between metals. Results on all metals content in all analyzed fish at all locations during both fishing seasons were subjected to matrix correlation analysis (Pearson correlation coefficient). Correlation analysis showed that significant correlations ($p < 0.05$, $N = 72$) were obtained between the following metals: Fe-Cd (0.517), Fe-Cu (0.526), Fe-Mn (0.745), Mn-Cd (0.426), Mn-Cu (0.651), Mn-Pb (-0.509), Zn-Cu (0.558). For other metals the correlations were below 0.400 at $p < 0.05$. A graphical presentation of the most significant correlations is given in Figure 2.

According to Yılmaz, Sangün, Yağlıoğlu,et al. (2010) essential elements in fish are Fe, Zn, Cu and Mn, and non-essential Cd and Pb, later ones reflecting an exogenous influence that can be related to pollution of environment. From the correlation analysis, the highest values of the Pearson coefficient were obtained in the case of essential elements, while Fe and Mn additionally had a statistically significant correlation with Cd and Pb. Significant correlation for the metal pair Fe-Cd was also found in fish from north-west African coast, Morocco (Afandi, Talba, Benhra, et al. 2018). Similar to our results, there was a strong positive correlation between Fe and Mn as well as negative correlation between Mn and Pb for the fish samples from Nile Delta (Talab, Goher, Ghannam, et al. 2016). In the study of Bhuyan, Bakar, Islam, et al. (2016) correlation between Cd and Mn was similar to our findings but there were weak negative correlations between Cd and Fe in fish from Meghna River, Bangladesh.

Health risk assessment

EDI of Cd, Cr, Cu, Fe, Mn, Pb and Zn in adult person who consumed *Sander lucioperca*, *Leuciscus svallize* or *Tinca tinca* fish muscles from the Neretva River is shown in Table 4.

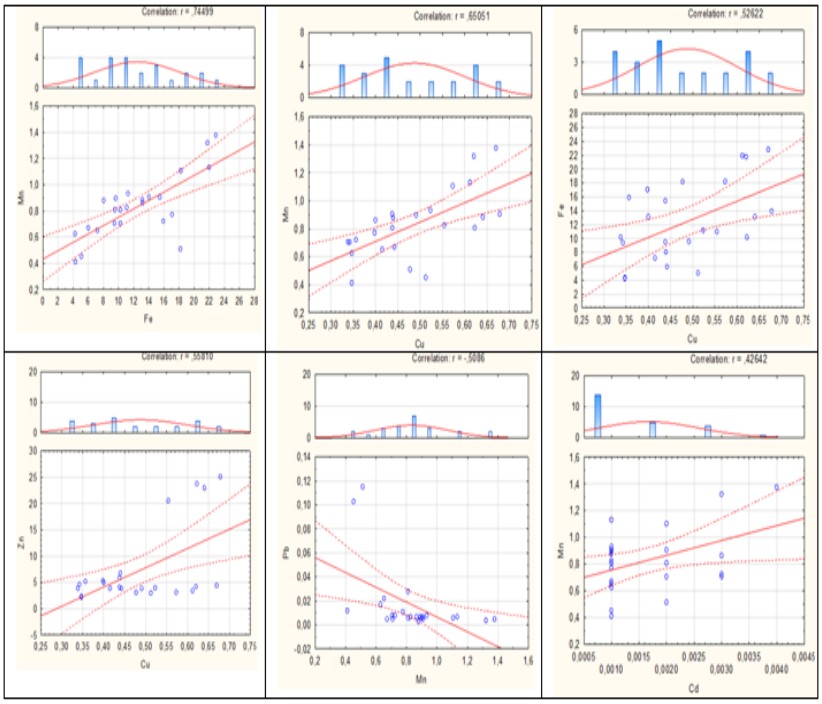

Figure 2: The most significant Pearson correlations between metals in fish from four HAs at Neretva River

Table 4: EDI (mg kg^{-1} day^{-1}) of heavy metals in *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca* fish muscles for adults.

Fish / Metals	Cd	Cr	Cu	Fe	Mn	Pb	Zn
	Spring - Summer EDI (mg kg^{-1} day^{-1})						
Sander lucioperca	1.12E-06	6.89E-04	3.06E-04	6.16E-03	5.72E-04	1.12E-05	2.67E-03
Leuciscus svallize	1.49E-06	2.11E-04	4.09E-04	1.46E-02	7.32E-04	2.46E-05	3.16E-03
Tinca tinca	1.49E-06	2.52E-04	3.86E-04	1.05E-02	6.29E-04	5.21E-06	1.09E-02
	Autumn - Winter EDI (mg kg^{-1} day^{-1})						
Sander lucioperca	7.44E-07	6.35E-04	3.16E-04	5.05E-03	4.90E-04	8.19E-06	2.61E-03
Leuciscus svallize	1.49E-06	1.63E-04	3.99E-04	9.89E-03	7.00E-04	2.16E-05	3.18E-03
Tinca tinca	1.49E-06	2.17E-04	3.57E-04	8.89E-03	5.81E-04	5.21E-06	1.01E-02
Rfd	**0.001**	**0.003**	**0.04**	**0.7**	**0.14**	**0.004**	**0.3**

For the metals examined, the calculated EDI values were in the following order: Fe≥Zn>Mn≥Cu ≥Cr>Pb>Cd for all three fish species. In general, obtained trends for EDI values for each of all of seven metals were identical in spring – summer as well as autumn – winter periods of year, indicating that season has no impact at all on final EDI value. Furthermore, none of the seven heavy metals pose risk to human health, since all obtained EDI values were significantly less than the Rfd levels (Table 4). However, heavy metal content must be monitored regularly since these pollutants can accumulate to lethal levels in fish tissue.

The THQ values for Cd, Cr, Cu, Fe, Mn, Pb and Zn as well as the hazard index per season via the consumption of the examined three fish species are shown in Table 5.

On the basis of the obtained results, daily consumption of examined three fish species will not cause any significant adverse health effects since THQ and HI levels were less than 1 (Yi, Tang, Yang, et al. 2017; Łuczyńska, et al. 2018). Therefore, all seven heavy metals examined in this study were found not to be potential health hazard for consumers.

CONCLUSION

In this research, the content of seven heavy metals (Cd, Cr, Cu, Fe, Mn, Pb, Zn) in water and three different country-specific fish species *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca* with different eating habits, during spring-summer and autumn-winter seasons, were analyzed. Heavy metals were found in the tested fish muscle tissue samples. The metal content was in all cases higher in the spring-summer season, which is probably due to the fact that the tested fish species are more active during this period of year. However, the maximum allowed concentrations prescribed by national legislation were not exceeded. From the matrix correlation analysis, the statistically significant correlations were obtained mostly in the case of essential elements (Fe, Mn, Cu and Zn). Daily consumption of examined fish species will not cause any significant adverse health effects for consumers since all calculated EDI values were lower than Rfds, as well as THQ and HI levels were less than 1.

People consume mostly large amounts of fish around the world where certain amounts of heavy metals can be detected. However, the potential benefit of consuming fish is very important due to its rich nutritional value. It is necessary that heavy metal levels do not exceed allowable amounts through fish intake.

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Table 5: Estimated target THQ and HI of seven metals in muscles for *Sander lucioperca*, *Leuciscus svallize* and *Tinca tinca* fishes for adults.

Fish / Metals	Cd	Cr	Cu	Fe	Mn	Pb	Zn	HI
	Spring - Summer THQ							**Spring - Summer**
(*Sander luciopera*)	1.12E-03	2.30E-01	7.65E-03	8.81E-03	4.09E-03	2.79E-03	8.88E-03	2.63E-01
(Leuciscus svallize)	1.49E-03	7.02E-02	1.02E-02	2.08E-02	5.23E-03	6.14E-03	1.05E-02	1.25E-01
(Tinca tinca)	1.49E-03	8.39E-02	9.64E-03	1.49E-02	4.49E-03	1.30E-03	3.62E-02	1.52E-01
	Autumn - Winter THQ							**Autumn - Winter**
(*Sander luciopera*)	7.44E-04	2.12E-01	7.89E-03	7.21E-03	3.50E-03	2.05E-03	8.68E-03	2.42E-01
(Leuciscus svallize)	1.49E-03	5.43E-02	9.97E-03	1.41E-02	5.00E-03	5.40E-03	1.06E-02	1.01E-01
(Tinca tinca)	1.49E-03	7.24E-02	8.91E-03	1.27E-02	4.15E-03	1.30E-03	3.37E-02	1.35E-01

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Summary/Sažetak

Sve je veća potreba za procjenom nivoa kontaminanata u ribi kao bioindikatora zdravlja i dobrobiti ribe kao i njenih potrošača. Kontaminacija teškim metalima (Cd, Cr, Cu, Fe, Mn, Pb, Zn) procijenjena je atomskim apsorpcionim spektrometrom, plamenom i grafitnom tehnikom u uzorcima vode i mišićnog tkiva riba *Sander lucioperca, Leuciscus svallize* i *Tinca tincana* četiri hidroakumulacijska jezera na rijeci Neretvi, Bosna i Hercegovina, sakupljena tokom dvije sezone: jesen-zima i proljeće-ljeto (2019.). Pokazalo se da je Fe najviše akumulirani metal u ribama, dok su Cd i Pb najmanje akumulirani metali u ribama. Sadržaj teških metala bio je ispod maksimalno dozvoljenog nivoa koji je propisan nacionalnim zakonodavstvom za vodu za piće i ribu. Prema korelacionoj matriks analizi sadržaja metala u svim ribama tokom obje ribolovne sezone, najveće vrijednosti Pirsonovog koeficijenta dobijene su u slučaju esencijalnih elemenata (Fe, Mn, Cu i Zn), a Fe i Mn su takođe imali statistički značajnu korelaciju sa Cd i Pb. Nadalje, procjena potencijalne izloženosti zdravstvenom riziku kod odrasle populacije u BiH pokazala je da nijedan od sedam teških metala ne predstavlja rizik za zdravlje ljudi, na osnovu procijenjenog dnevnog unosa konzumiranjem ovih vrsta riba, kao i ciljanog koeficijenta opasnosti i vrijednosti indeksa opasnosti koji su iznosili manje od 1.

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Percolation and jamming properties in limited grain growth of linear objects

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Abstract: The physical and chemical properties of the nanocrystals are highly shape dependent, and shape control has become very important. The seeded growth method enables seeds to grow in a predetermined way. We have already proposed such a model that can reproduce the granular growth on a triangular lattice and for different growth shapes. In this paper, however, we have introduced a limitation on seed growth up to a certain length. This method can be used when the growth of all seeds have to be limited to the same length, or for a mixture with the different growth limits. The main goal is to investigate how the growing limits affect the values of the percolation threshold and jamming density, and whether large objects significantly affect the percolation threshold. We used growing needle-shaped objects (k-mers) made by a self-avoiding random walk filling the nodes of the triangular lattice. Objects can grow until they reach the growth limit k' defined as the maximum number of lattice nodes belonging to one object. For $k' \geq 10$, percolation is reached for all investigated seed densities. We obtained that the values of the percolation threshold and jamming density are identical for $k' \geq 10$. Above these values, the percolation threshold and jamming remain unchanged, regardless of the growth limit. Our results also show that when significant growth is allowed, long objects are very rare and do not influence the results.

INTRODUCTION

Percolation is a second-order phase transition. The study of percolation started with the work of the chemist P. Flory in his study on gelation in polymers (Flory, 1941). The percolation theory deals with the clusters formed when each site of an infinite lattice is randomly occupied with probability p (Staufer & Aharony, 1994). A cluster (groups of neighbouring occupied sites) that connects two opposite lattice sides is called a percolation cluster, and it will appear when the probability p reaches a critical value p_c, which is called the percolation threshold (Hao, 2005). Finding the percolation threshold for a given system is one of the fundamental tasks. Percolation is used to explain, for example, the gelation of polymeric materials (Stauffer, Coniglio and Adam, 1982). the growth of rough surfaces and disordered interfaces via atomic chemisorption (Meakin, 1993), the recovery of oil from porous media (King, Buldyrev, Dokholyan, et al., 2002), ion transport in glasses and composites (Roman, Bunde, and Dieterich, 1986).

In standard percolation theory, the constituent elements of the clusters are usually randomly distributed, but correlations cannot always be neglected. Several correlated percolation models have been developed and extensively studied, such as bootstrap percolation (Adler, 1991), directed percolation (Broadbent & Hammersley, 1957), and spiral percolation (Santrs & Bose, 1992).

In the last two decades, it has become possible to synthesize many classes of nanoscale building blocks with controlled structure, size, and shape for applications in chemical engineering, medicine, electronics, etc. Seeded growth has emerged as a compelling method to create a wide variety of novel metal nanostructures (Gole & Murphy, 2004); Habas, Lee, Radmilovic, et al., 2007) and high-quality nanocrystal samples that can serve as preferential platforms for deposition of additional

material (Xia, Gilroy, Peng, et al., 2017). Dujak, Karač, Budinski-Petković, et al., (2022), proposed a model that can reproduce granular growth on a triangular lattice, from nucleation to percolation, and for different growing shapes. The object's growth was not limited. In this paper, we investigate how the growth limit of the needle-like objects (k-mers) affects the values of the percolation threshold and the jamming density.

EXPERIMENTAL

Definition of the model and the simulation method

The Monte Carlo simulations are performed on a two-dimensional triangular lattice of different sizes L. Periodic boundary conditions are used. Coverage of the lattice $\theta(t)$ is the fraction of the covered lattice sites by the growing objects at time t. At large times the coverage $\theta(t)$ approaches the jammed-state value called jamming coverage θ_J. In that state, none of the objects can grow to unocupied spaces. The percolation threshold θ_p^* is the coverage of the lattice when a percolating cluster appears. The lattice is filled with objects using random sequential adsorption model (RSA) (Evans, 1993), (Privman, 2000) (Cadilhe, Araújo, and Privman, 2007). The growing objects on the lattice are modeled by self-awoiding walks (Budinski-Petković, Lončarević, Dujak, et al., 2017)

Definition of the model

The point-like seeds are deposited on the sites of the planar triangular lattice at a given density ρ. Density of seeds is calculated as a fraction of sites of the lattice that are occupied by seeds. Each seed can grow only in one direction, creating a linear object called k-mer. K-mers are line-segments of lenght $l = k - 1$ where k denotes the number of the lattice sites that belongs to that particular k-mer. The formation of k-mers with corresponding percolation thresholds and jamming coverages is shown in Table I.

Table I: Formation of k-mers of different lengths $l = k - 1$ (up to $l = 3$) with corresponding percolation thresholds θ_p^* for infinitely large lattice and jamming coverages θ_J. The numbers in parentheses are the numerical values of the standard uncertainty of θ_p^* and θ_J referred to the last digits of the quoted value.

k-mer	k	l	θ_p^*	θ_J
●	1	0	0.5000(1)	1
●—●	2	1	0.4867(1)	0.9141(3)
●—●—●	3	2	0.4628(3)	0.8362(4)
●—●—●—●	4	3	0.4432(2)	0.7891(6)

As the k-mers grow, they come in contact (i.e. there is a lattice site between them) and they are merged into a single cluster. There are numerous clusters that grow simultaneously. If two clusters come into contact (i.e. occupied perimeter sites are separated by a single lattice spacing), they are amalgamated into a single cluster. Percolation is reached when a cluster connects opposite edges of the lattice and then the percolation threshold θ_p^* is reached. To determine the percolation threshold θ_p^*, the tree-based union/find algorithm is used (Newman & Zi, 2001). The jamming coverage θ_J is reached when no more growing objects can grow in growing direction on the lattice (Lončarević, Budinski-Petković and Vrhovac, 2007; Budinski-Petković, Vrhovac, and Lončarević, 2008).

Simulation method

The RSA model of seeds in two dimensions is used to prepare the initial state of the system. Monomers (k-mers with $k = 1$) that represent the point-like seeds are deposited onto lattice using the Monte Carlo procedure, up to the chosen density ρ. Then deposition is switched off and a random growing process is initiated.

At each Monte Carlo step, a lattice site occupied by seed is selected at random. An adjacent site that is not occupied by another seed or k-mer is selected randomly and the seed grows into dimer (k-mer with $k = 2$). A double occupation at any site is not allowed. Only a single step k-mer growth is allowed and only the last point of the corresponding k-mer is active for further growth. The k-mers can grow only in direction of the first step. If the corresponding adjacent site is not empty, the k-mer elongation attempt is not possible and the object remains unchanged. The growth of the k-mers is limited up to k' i.e. they can grow until they reach the lenght $l = k' - 1$ defined at the beginig of the simulation.

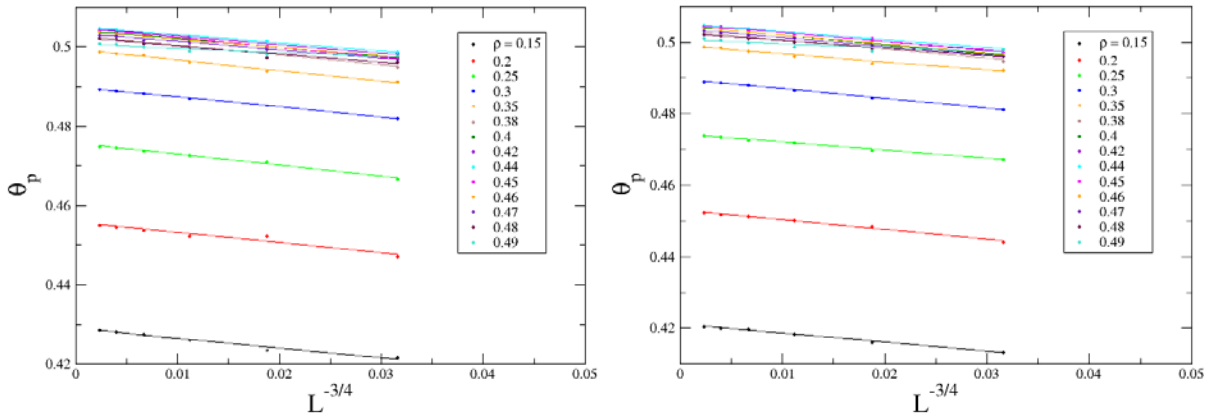
RESULTS AND DISCUSSION

The percolation threshold and jamming coverage were investigated for various seed densities $0.15 \leq \rho \leq 0.49$ on the lattice size ranging from $L = 100$ to $L = 3200$, and for various growth limits $2 \leq k' \leq 160$. The data are averaged over 500 independent runs for each lattice size.

The finite-size scaling theory of the percolation behavior on two-dimensional lattices (Staufer & Aharony, 1994) is used to obtain the percolation threshold for un infinitely large lattice θ_p^*. According to this theory, the efective percolation threshold θ_p (the mean value of threshold measured for the finite lattice) approaches the asymptotic value $\theta_p \to \theta_p^*$ for $L \to \infty$ via the power law:

$$\theta_p - \theta_p^* \propto L^{-1/\nu} \qquad (1)$$

where the constant $\nu = 4/3$ is the critical exponent (Staufer & Aharony, 1994). Equation (1) allows extrapolation of the threshold for an infinite lattice. Finite-size scaling of the lattice threshold θ_p against $L^{-3/4}$ is shown in Figure 1.a and Figure 1.b for various initial seed densities and for $k' = 4$ and $k' = 160$ as representatives.



(a) (b)

Figure 1: Finite-size scaling of the effective percolation threshold θ_p against $L^{-1/\nu}$ with $\nu = 4/3$ for growing k-mers up to a) $k' = 4$, b) $k' = 160$

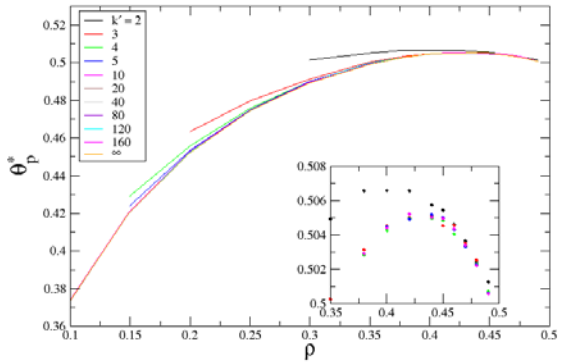


Figure 2: Dependence of the percolation threshold θ_p^* on the initial seed density ρ. The inset shows an enlarged part of this graph that displays a non-monotonic behaviour for $k' = 2, 3, 4, 5, 10$

The dependence of the percolation threshold θ_p^* on the initial seed density ρ is shown in Figure 2. It can be seen that the percolation is not reached for all seed densities depending on the growth limits k'. If $k' = 2$, the percolation is reached for $\rho \geq 0.3$, if $k' = 3$ the percolation is reached for $\rho \geq 0.2$, and for $k' = 4$ and 5 the percolation appears at $\rho \geq 0.15$. For $k' \geq 10$ the percolation was achieved for all investigated densities. However, for all k' the percolation threshold increases monotonically for low values of seed density, reaches a maximum for seed densities in the interval $0.4 < \rho < 0.45$, and then for higher values of seed density, θ_p^* decreases for all k' towards the same value $\theta_p^* = 0.5$. The results for $k' \leq 5$ differ slightly from each other showing a little bit higher values of θ_p^* for the same seed densities. For $k' \geq 10$ the results overlap.

At low values of initial seed densities, k-mers have enough space to grow, but if their growth is limited to small lengths, the surface remains very porous, and a percolation cluster can not be formed. On the other hand, if a percolating cluster is reached, the percolation threshold θ_p^* will have higher values for lower values of the growth limits k'.

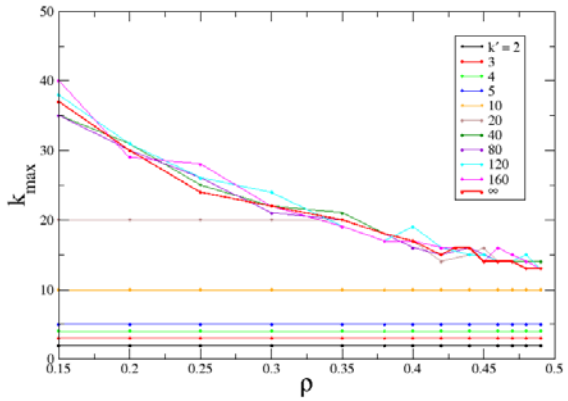


Figure 3: Largest growing objects in the jamming coverages vs. the initial seed density ρ on the lattice sizes $L = 3200$.

The maximum reached lenght limits depending on the seed density, for different growth limits is shown in Figure 3. For $k' < 20$ the set growth limit is reached for all seed densities. For $k' \geq 20$ there are critical maximum lengths of k-mer growth regardless of growth limit k', depending only on the initial density of seeds. When significant growth is allowed, long objects are very rare and do not influence results.

Unlike the results shown in Figure 3, where the mean value of the maximum length reached at least once in all 500 independent simulations is shown, Figure 4 shows the mean value of the maximum length $\bar{l}_{max}$ of the k-mers in all 500 independent simulations. It is obvious that in the cases where the k-mers have not reached the growth limit, the mean value of the maximum length is identical for all growth limits.

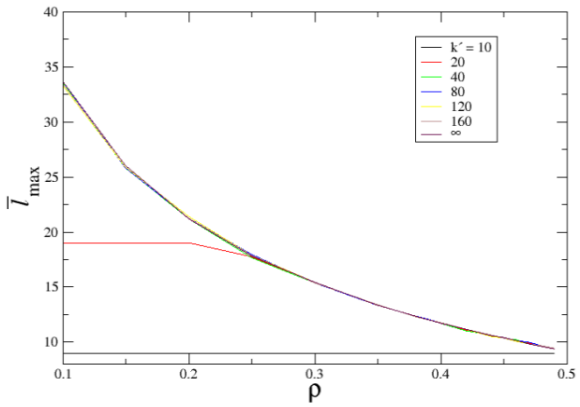


Figure 4: The mean value of the maximum length $\bar{l}_{max}$ for different seed densities ρ and growth limits k'. For $k' < 10$, $\bar{l}_{max}$ is always equal to $k' - 1$. Size of the lattice is $L = 3200$.

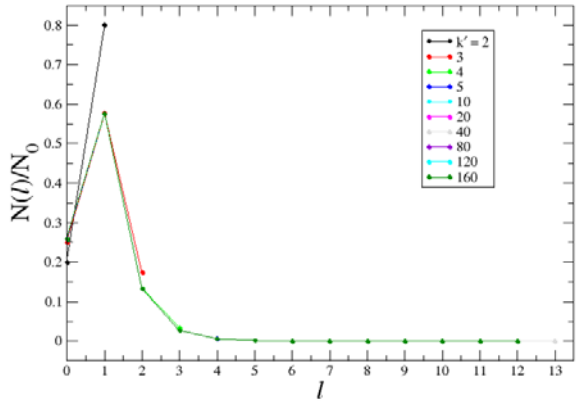


Figure 5: Dependence of the normalized number of deposited k-mers $N(l)/N_0$ on the k-mers length l, for the system in the jamming state. The results are given for seed density $\rho = 0.49$ on the lattice sizes $L = 3200$, for different k' indicated in the legend. Here, N_0 is an initial number of seeds at a given density ρ.

Figure 5 shows the dependence of the normalized number of deposited k-mers $N(l)/N_0$ on their length l. It can be seen that the results for the growth limit $k' \geq 10$ are almost identical (the results are slightly different for $k' \geq 5$ which is not noticeable in this graph).

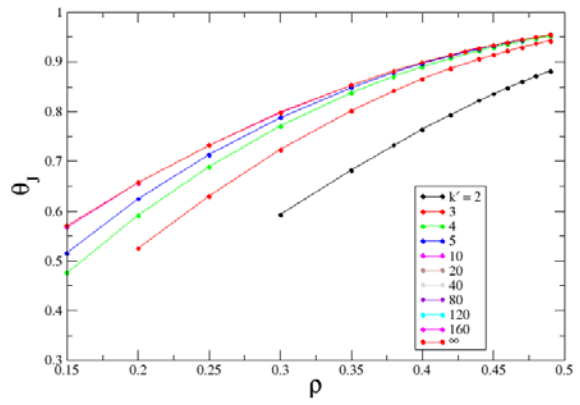


Figure 6: Dependence of the jamming coverage θ_J on the initial seed density ρ for growing k-mers.

They differ only in the maximum length reached at $k' = 40$. For larger k-mers, ratio $N(l)/N_0 \to 0$, this means that long k-mers are very rare. In all cases dimers ($k = 2$) are the most numerous k-mers.

The jamming coverage θ_J (for the cases where percolation is reached) for different growth limits and seed densities is shown in Figure 6. For lower values of the growth limit, θ_J has lower values. As the growth limit increases, the values of the jamming coverage for a particular seed density increase and become identical for $k' \geq 10$.

CONCLUSION

Dependence of the percolation threshold and the jamming coverage on the limit of k-mers growth using numerical simulations was investigated. Simulations were performd for initial states with various initial seed densities and for different growth limits.

Depending on the growth limit percolation was not reached for all seed densities. For the lowest value of the growth limit $k' = 2$, the percolation is reached for $\rho \geq 0.3$. When the growth limit increases, the seed density for which percolation appears decreases. For $k' \geq 10$ the percolation was achieved for all investigated densities. For the same seed densities, the values of the percolation threshold for $k' \leq 5$ have a slightly higher values than for $k' \geq 10$. For all growth limits k', the percolation threshold θ_p^* increases with seed density ρ, reaches a broad maximum, and then decreases. The results become identical for $k' \geq 10$.

The jamming coverage θ_J also increases with ρ for all the growth limits, and the values of θ_J become identical also for $k' \geq 10$. For lower values of the growth limit, θ_J has lower values.

The k-mers can reach a given lenght in cases where growth limit is less than 20. For $k' \geq 20$ there are critical maximum lengths of k-mer growth regardless of the growth limit k', depending only on the initial density of seeds but the mean value of the maximum length is identical.

These results suggest that there is a critical growth limit for k-mer growth, above which the percolation threshold and jamming coverage remain unchanged for all seed densities. There are also critical maximal lengths of k-mer growth regardless of the growth limit k', depending only on the initial density of seeds.

Although present, long k-mers, when significant growth is allowed, are very rare and do not influence the results. On the other hand, small k-mers have a significant role that can be further investigated by making a mixture of seeds with two or more different growth limits. It is also interesting to follow the changes in the percolation threshold if some point-like impurities are initially added to the lattice.

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Summary/Sažetak

Fizička i hemijska svojstva nanokristala u velikoj mjeri zavise od njihovog oblika, tako da je kontrola oblika postala veoma važna. Metoda rasta sjemena omogućava sjemenu da raste na unaprijed određen način. Ranije smo predložili model koji može da reprodukuje rast sjemena na triangularnoj rešetki tako da se formiraju različiti oblici. U ovom radu smo, međutim, uveli ograničenje na rast sjemena do određene dužine. Ovaj metod se može koristiti kada je rast svih sjemena ograničen na istu dužinu, ili za smjese sjemena s različitim konačnim dužinama. Glavni cilj je ispitati kako ograničenje rasta utječe na perkolacioni prag i gustinu zagušenja, te ispitati da li dugački objekti značajno utječu na perkolacioni prag. Koristili smo narastajuće objekte u obliku igle ili k-mere koji su formirani samoizbjegavajućim slučajnim šetnjama koje popunjavaju čvorove triangularne rešetke. Objekti mogu da rastu dok ne dostignu granicu rasta k' koja je definisana kao maksimalni broj čvorova rešetke koji pripadaju jednom objektu. Za $k' \geq 10$ perkolacija je postignuta za sve ispitivane početne gustine sjemena. Dobili smo da za granice rasta $k' \geq 10$ vrijednosti perkolacionog praga i gustine zagušenja se preklapaju za sve vrijednosti gustine sjemena. Iznad ovih vrijednosti perkolacioni prag i gustina zagušenja ostaju uvijek isti bez obzira na granicu rasta. Rezultati pokazuju da kada je dozvoljen znatan rast, dugački objekti su veoma rijetki i ne utječu na rezultate.

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