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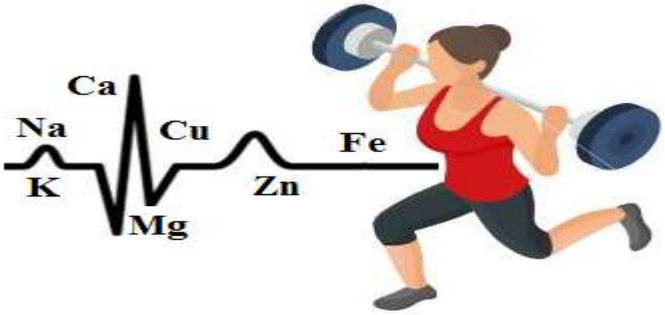

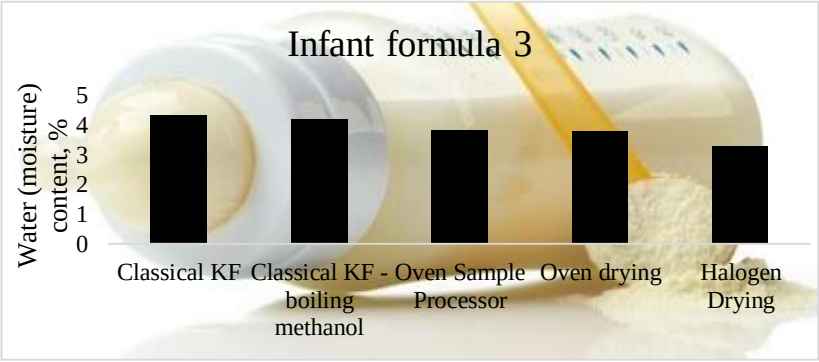

Editorial

Design of new active materials for electrochemical systems is the major contribution of chemists to the quest for new and efficient routes for energy storage and conversion. This long and unfair battle against The Second Law of Thermodynamics was finally recognized by The Nobel Comittee for Chemistry: John Goodenough, M. Stanley Whittingham and Akira Yoshino received 2019 Nobel Prize for Chemistry "for the development of lithium-ion batteries". This award is especially celebrated among electrochemists, since it is the first Nobel prize for electrochemistry-related topic after Jaroslav Heyrovský received one, 60 years ago.

Secret of lithium-ion batteries global success lies in their ability to be discharged and charged repeatedly, over several hundred cycles. They can also be partially discharged, because these batteries do not suffer from the memory effect. Their secret from scientific standpoint, however, lies in the possibility to „insert" an electron into the Li ion, without formation of lithium metallic phase during battery charge, which is achieved by the use of graphite-based materials as negative electrodes. Change from metallic lithium (used in primary lithium cells) to graphite-based materials reduced cell potential to some extent, thus reducing the amount of stored energy, but enabled multiple charging and discharging. Development of these systems also included development of different materials for positive electrodes, most famous of them being based on $LiCoO_2$ and $LiFePO_4$ structures. Although there is not much room left for the development of new materials and electrolytes for Li-ion batteries, the present work is focused on their optimization for fast charging, increased cycle life, safety, durability in wide range of temperatures and environmental friendliness.

Editors

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1-8

Protection of fuel filter with alkaline and acid zinc coatings

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Abstract: Galvanic coatings are applied so that the surface of the base material obtains appropriate properties, corrosion resistance, durability, aesthetic appearance, and long-term application in the appropriate industry. In this paper, the aim was to protect steel fuel filters with alkaline and acid zinc coatings of different thicknesses. The coating of zinc, which is applied from the alkaline electrolyte, provides good corrosion protection with excellent coating flexibility. The thickness of the coating by the X-ray fluorescence method was tested, followed by coating tests, corrosion resistance, and electrochemical tests. The results of adhesion showed a high quality coating, as no corrosion occurred during the test. The corrosion resistance tested by the salt chamber method speaks of the appearance of white and red corrosion. On alkaline electrolyte coatings, white corrosion occurred after 168 hours of exposure to the salt test, while on white zinc samples there was a white corrosion after 240 hours of exposure. Tafel polarization diagrams have been determined: corrosion potentials, current intensities, anode and cathode Tafel coefficients and calculated corrosion rates. The active and passive corrosion zone is determined by the cyclic voltammetry.

INTRODUCTION

Galvanic coatings protect the metal from the appearance of corrosion by not allowing the corrosive environment / electrolyte contact with metal to touch (Zhang, 1996). Zinc becomes anode, while the steel substrate becomes cathode and does not corrode (Vourlias et al., 2006). There are several ways to protect the sample with zinc coating, and the most common is hot galvanization, especially for large samples used in the automotive industry (Zand, Verbeken and Adriaens, 2013). Hot dipping includes dipping the steel in a bath of molten zinc (Hosking et al., 2007). In this process, aluminum has a higher affinity for iron, resulting in the formation of an intermediate Fe2Al5, and the created inhibitory layer slows down the reaction between zinc and iron (Parvini and Rafiezadeh, 2009). The samples are protected by the electrodeposition method, i.e. by depositing metal on the surface of the base material (steel) under the influence of direct current (Zand, Verbeken and Adriaens, 2012). The quality of the coating depends on a number of factors, and galvanic coating tests are mainly based on the metal bond with the base material (Pu et al., 2013). The most common tests are: the examination of the outer appearance of the coating, the methods for determining the coating thickness, the methods for testing the coating adhesion (adhesion) and methods for corrosion testing. Electrochemical methods are used to test the reactions and coating mechanisms, as well as the corrosion rate corrosion test (Xu et al., 2012; In this research, the polarization resistance of the unprotected sample as well as the protected samples by the linear polarization method was investigated, and polarization parameters were determined by the Tafel extrapolation method for the purpose of determining the corrosion parameters. The results of the cyclic voltammetry show the active and passive corrosion state on coatings of thickness from 5 to 15 microns. An unprotected sample has an accelerated corrosion time.

EXPERIMENTAL

In this work, the steel covers (Delphi) of the surface area of 2.06 dm^2 were used, as well as steel plates measuring 7 cm x 2 cm, 1 mm thick. All samples were subjected to alkaline and acidic zinc plating. The methods used to assess the quality of the coating are: coat thickness testing, coating adhesion testing (adhesion), saline test and electrochemical methods, linear polarization and cyclic voltammetry. Samples of alkaline (A) and acid (K) elecrolytes had coat thicknesses: 5, 10, 15, 20 and 25 μm and one unprotected sample (N). Table 1.

Table 1. Symbols for samples

Sample	Description	Sample	Description
A5	Alkaline electrolyte 5 μm	K5	Acid electrolyte 5 μm
A10	Alkaline electrolyte 10 μm	K10	Acid electrolyte 10 μm
A15	Alkaline electrolyte 15 μm	K15	Acid electrolyte 15 μm
A20	Alkaline electrolyte 20μm	K20	Acid electrolyte 20 μm
A25	Alkaline electrolyte 25 μm	K25	Acid electrolyte 25 μm
A51	Alkaline electrolyte 5 μm- damaged sample	K51	Acid electrolyte 5 μm- damaged sample
A52	Alkaline electrolyte 10 μm- damaged sample	K52	Acid electrolyte 10 μm- damaged sample
A53	Alkaline electrolyte 15 μm- damaged sample	K53	Acid electrolyte 15 μm- damaged sample
N	Unprotected sample		

RESULTS AND DISCUSSION

Preparation of the sample

Prior to the application of the protective coating, the preparation of the material was carried out in several steps: chemical degreasing, flow rinsing, bypassing, flushing, electrochemical degreasing, flushing, pickling, flushing. After preparation of the material, a protective coating is applied. The time of immersion and the intensity of the current depend on the surface of the material and the desired thickness of the coating. The electrolyte for the application of acid electrolyte coating contained a zinc concentration of 18-35 g/l, total chlorides 125 g/l, and boric acid 18-25 g/ l, a pH of 5, 2-5, 5. Coating electrolyte alkaline zinc contained a zinc concentration of 8-15 g/l, sodium hydroxide of 110-150 g/l sodium carbonate of less than 80 g/l. After application of the protective coating, the samples were slightly grown in nitric acid, followed by the process of blue thick-layer passivation, after which the samples were silenced. An acid electrolyte is used with pure zinc anodes (purity 99.99%), while in the alkaline electrolyte, inert steel anodes, nickel plated with 15 μm semiconductor nickel at a current density of 1 A/dm^2, are used. Protected samples are dried for 15 minutes at a temperature of 100 ° C. Figure 1-2.

Figure 1. Acid electrolyte coating (Delphi)

Figure 2. Alkaline electrolyte coatings (Delphi)

Measurement of coating thickness

Delphi fuel filter covers are composed of parts, which, obtained by deformation processing, are assembled into one unit together with the products obtained by machining by cutting. Such parts constituting the assembly (lid) go to the process of hard soldering and to the surface protection process. The thickness of the coating is the distance between the surface of the base material and the surface of the coating. Measurements were performed on the X-RAY instrument, and the principle of the operation of this instrument is based on the occurrence of a secondary radiation emission (fluorescence) as a consequence of electrons being triggered under the influence of the primary (incident) electron beam. The thickness of the coating was measured three times on each sample, in different places. Depending on the desired coating thickness, the retention time of the samples in the electrolyte is different. For samples subjected to alkaline zincing electrolytes for a period of 40 minutes, a thickness of 5 μm was obtained, and for each subsequent increase in the thickness of the coating (10, 15, 20, and 25 μm), this period increased for 20 minutes. For samples subjected to acidic zincing

electrolytes over a period of 20 minutes, a thickness of 5 μm was obtained, and for each subsequent increase in the thickness of the coating (10, 15, 20 and 25 μm), this period increased for 20 minutes. Table 2 shows the mean value of the coating thickness results for the alkaline and acidic sampling of the sample.

Table 2. Thickness of alkaline and acid electrolytes coating on the Delphi sample

Sample	Alkaline electrolytes coating μm	Acid electrolytes coating μm
1.	5, 218	5, 210
2.	9, 610	9, 951
3.	14, 98	15, 25
4.	20, 83	20, 39
5.	25, 01	25, 19

With acid electrolytes for a short time, the desired thickness of the coating can be achieved, due to the rapid separation of the zinc coating, it is uneven. When obtaining galvanic coatings it is required that they have a certain thickness, which depends on their purpose. If the coatings are exposed to aggressive medium and wear, then they must be thicker than in case of lack of corrosion and wear. A thickness of 5 μm is considered to be the smallest thickness needed, and a thickness of 45 μm thickness of the coating that can satisfy even the most difficult conditions of exploitation. The thickness of the coating is not even on a flat surface at each location is equal, the difference in the thickness of the coating on the relief surface from place to place is even greater. Thus, these methods describe the method of determining the local and medium thickness of the coating. The local thickness refers to the thickness of the coating at a particular site, and the mean thickness is the mean value of the coating thickness over the entire surface.

Coating Adhesion Testing (Adhesion)

To test the adhesion of the coating, a heating method is used, which is based on different linear coefficients of the spreading of the base material and the coating metal, resulting in the heating of the sample at the boundary surface creating stresses that tend to separate the coating from the base material. Samples were heated in a laboratory dryer for 1 hour at a temperature of 180 ° C. After heating, the samples were immersed in cold water without any change (bubbles, peeling of the coating) on the alkaline and acid electrolytes coatings, nor on the incised samples. Figure 3-6. In the unprotected sample, after submerging in cold water and drying, corrosion occurred after a few minutes. Figure 7.

Figure 3. Sample A10 i A15 before and after

Figure 4. Sample A52 i A53 before and after the adhesion test

Figure 5. Sample K10 i K15 before and after

Figure 6. Sample K52 i K53 before and after the adhesion test

Figure 7. Unprotected sample before and after the adhesion test

Adhesion methods define the method and conditions for determining the adhesion of the coating onto the base material. It is very important that in the electrolytic deposition of metal coatings firmly bind to the base material, so as not to partially or completely peel off. The poor bonding of the coating to the substrate occurs when the surface is not well prepared, the fat or oxide layer is not completely removed. If it is known that metal coatings do not have good adhesion to the substrate, they are deposited on the intermediate layer, most commonly copper, which is well bonded to all metals and for which all coatings are well grown. There is no method for determining the value of adhesion that would yield quantitative results, but by different methods it can be determined only if adhesion is satisfactory or not.

Corrosion testing

The test method in the salt chamber was used for corrosion testing. It is a standard test method used to test the metal resistance to corrosion. In the saline chamber, the samples were exposed to the fog of sodium chloride solution (3-5%) at a temperature (35 ± 2°C). Upon completion of the test, the samples are carefully removed from the exposure zone, a mild jet of running water is washed, or in a water not higher than 40°C, to remove it deposited on the examined surface. Then the samples are dried (compressed air). Products obtained by testing in the salt chamber are hydroxides and oxides of metal, which protects the base material, this change has been recorded as the appearance of white corrosion and iron (III) oxide change has been recorded as a phenomenon of red corrosion. Table 3. Under atmospheric conditions, the first product of the corrosion is the metal hydroxide, which protects the base material. For example, if the zinc surface if exposed to a humid environment (snow, rain, mist) zinc will react with water and create a corrosion product of zinc hydroxide, which is voluminous, porous and unstable. Protected material exposed to this environment, where there is the lack of oxygen and carbon dioxide, continues the reaction of the formation of zinc hydroxide and gradually spills coating. The presence of a large amount of zinc hydroxide prevents the formation of a stable zinc carbonate product, which is passive to environmental influences.

In Table 3, we can notice that samples of alkaline and acid electrolytes (A5 and K5) that were longer in the saline chamber, have almost the same thickness of the coating, which is expected because they are samples in which the coating is not cut and that it is necessary longer time to appear iron (III) oxide. The damaged samples, keept in both alkaline and acidic electrolytes, were submerged the same number of hours in the salt chamber until the appearance of white corrosion.

Table 3. Results of white and red corrosion for alkaline and acid electrolytes

Sample	Coating thickness (μm)	The appearance of white corrosion (h)	The appearance of red corrosion (h)
A5	**5,20**	**240**	**936**
A51	**5,40**	**240**	**768**
K5	**5,47**	**168**	**720**
K51	**5,30**	**168**	**648**

This is expected, since both samples were passivated in the same passivation and silenced under the same conditions. In the case of the damaged sample, the electrochemical process and the oxidation of zinc occurred, which further protects the basic construction material.

Figure 8. The results of the salted chamber of the unprotected sample

In Figure 8, we see that red corrosion has occurred on the unprotected sample, the formation of iron (III) oxide already after 24 hours.

Electrochemical testing of corrosion

The electrochemical test was carried out on the device potentiostat/galvanostat 263 A and the lock-in amplifier 5210 by Princeton Applied Research and using the software Power CV. The system consisted of three electrodes: working electrodes, reference Ag/AgCl and platinum electrodes, and all the shots were performed in 3% sodium chloride, linear polarization and cyclic voltammetry within the various limits of the potential of -1.2 to 1 V, with the previous stabilization of the system-potentials in time 2h. Figure 9 shows the linear polarization method for alkaline electrolyte of different thicknesses of the coating, as well as the galvanized sample. In Table 4 we see the values of the corrosion rate for different thicknesses. The maximum corrosion rate is at a thickness of 5 microns. Under the conditions examined, we received the lowest corrosion rate at a layer thickness of 15 microns in the alkaline electrolyte. We conclude that the reason is the mass transport that is of primary importance for the rate of corrosion in environments with limited cathode reactant. The maximum corrosion rate in such a situation is given by

the limiting current density of the cathodic reactant to the surface. When we compare the values of the corrosion current and the corrosion rate in Tables 4 and 5, we see that there are higher values for the thickness of the alkaline coating of 5 microns. We assume that the chemical composition of the electrolyte is caused the high alkaline conditions result in the formation of a rough coating. The figure10 shows the results obtained by the linear polarization method for acid electrolyte of different thicknesses of the coating as well as the frozen zinc sample. In both diagrams we see that the corrosion potential of the 5 μm coating is the highest in comparison with other coatings. Also in the alkaline coating of 10 μm thick in the anodic region of the potential, we can see the appearance of a passive, as well as the appearance of a transpasive form, with a polarization resistance value of 5737 Ω. As the thickness of the coating grows, the polarization resistance of the test sample increases.

Anode passivation and transpasition were detected on all thicknesses (5-25 μm). Also, in the case of alkaline and acidic galvanizing, the production of white corrosion that is easily deposited on the surface of the sample due to the high electrochemical reactivity of zinc, as confirmed by the work of the author An, et al., 2017.

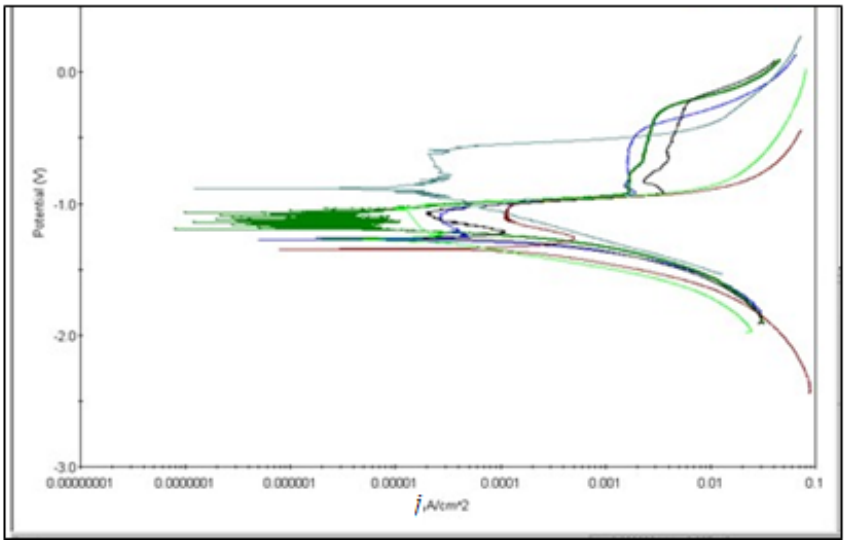

Figure 9. Polarization curve for the sample of alkaline electrolytes filters of different thicknesses: — 5; — 10; — 15; —20; — 25 μm; — damaged sample 5 μm

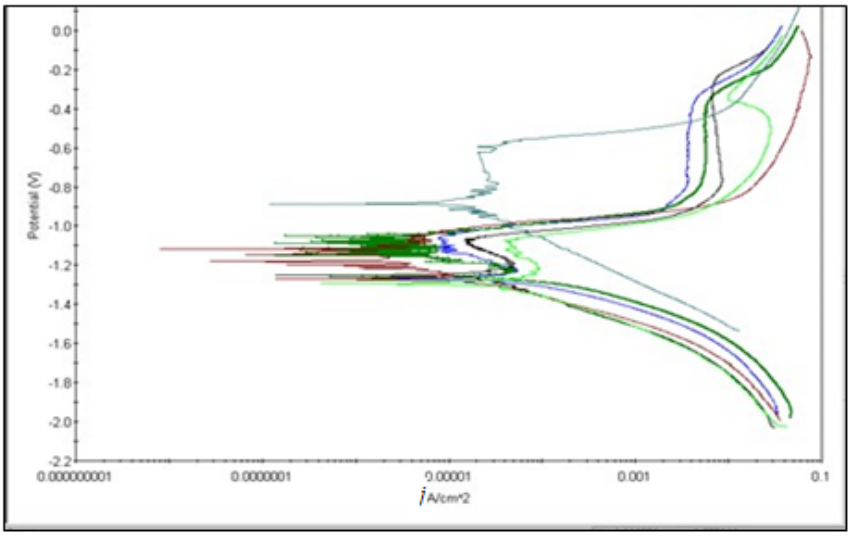

Figure 10. Polarization curve for the sample of acid electrolytes filters of different thicknesses: — 5; — 10; — 15; —20; — 25 μm; — damaged sample 5 μm

Table 4. Electrochemical data of alkaline electrolytes filters of different thicknesses from Tafel extrapolation.

Thickness (μm)	E (mV)	I_{corr} (μAcm^{-2})	Corrosion rate (mmpy)	β_A ($mVdec^{-1}$)	β_k ($mVdec^{-1}$)
5	-1027	3.56	1.5	117	64
10	-1204	1.36	0.57	99	112
15	-1262	1.01	0.43	108	49
20	-1357	1.30	0.55	86	33
25	-1279	1.51	0.64	206	99

In the acid electrolyte coating diagram 10, we see that a good sample protection is also performed, and that the same sequence of polarization resistance increases as the thickness of the coating is increased by the scanning velocity of 0.166 mV/s. Table 5. If we compare the value of corrosion rate in Table 4 and Table 5, we can conclude that it is better and more stable coating of acid electrolyte. In table 4 we see that the rate of corrosion at the same coating thickness of 10 and 20 microns. This rate of corrosion (0.11 mmpy) can be explained by simple adsorption on the electrode surface and by blocking active sites on the surface. This process takes place until the establishment of the dynamic equilibrium between the concentration of the residual solute in the solution and its concentration on the metal surface.

Table 5. Electrochemical data of acid electrolyte filters of different thicknesses from Tafel extrapolation.

Thickness (μm)	E (mV)	I_{corr} (μAcm^{-2})	Corrosion rate (mmpy)	β_A ($mVdec^{-1}$)	β_k ($mVdec^{-1}$)
5	-1271	1.47	0.62	134	59
10	-1251	2.75	0.11	98	74
15	-1252	3.94	0.16	106	129
20	-1118	2.81	0.11	66	81
25	-1292	1.76	0.74	73	87

Ponte et al., 2002 also notes the oxidation peaks of Fe^{2+} and Fe^{3+} ions in their work. In his research, the passivation potential starts at around - 950 mV, which is approximately similar in our research where we obtained the values of the potential from about - 1100 mV to about - 1300 mV and for the alkaline and acidic coating of the sample. Liu et al., 2013 examined the corrosion of pure zinc and zinc with different amounts of aluminum, and also obtained values of the potential of about -1050 mV and a small current value which proves a low sample rate in the investigated environment of sodium chloride.
The mechanism of zinc behavior in the corrosion environment is described in Hosking et al:

$Zn_{(s)} \rightarrow Zn^{2+} + 2e^-$

$O_2 + 2H_2O + 4e^- \rightarrow 4OH^-_{(aq)}$

$Zn^{2+} + 2OH^- \rightarrow Zn(OH)_{2(s)} \rightarrow ZnO_{(s)} + H_2O$

In the first step, zinc corrosion is controlled by zinc dissolution, and the cathode reaction occurs in the second step, where the hydroxide ions are responsible for the formation of zinc hydroxides (Mahdy et al., 2013). Chloride ions migrate to anodic sites in the presence of sodium chloride. This mechanism has also been proven by cyclic voltametrium where an activation and passivation zone is observed, depending on the thickness of the coating Figure 11.

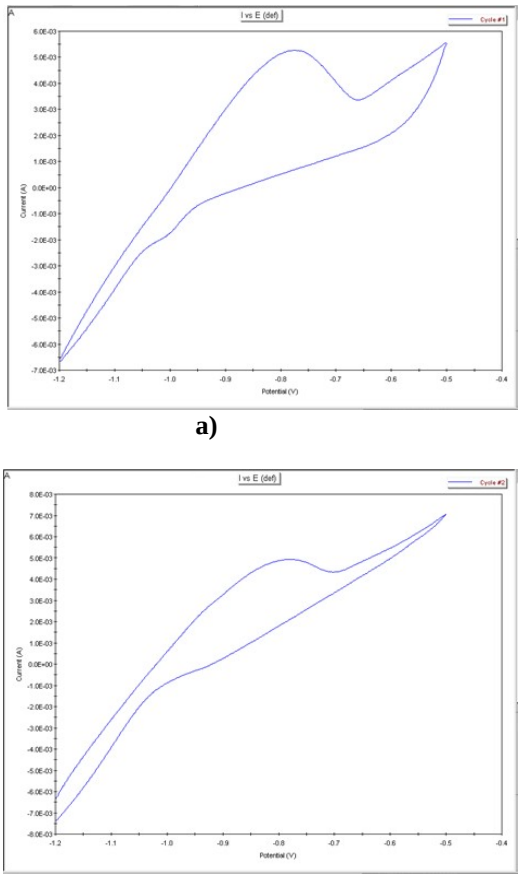

Figure 11. A cyclic voltamogram of an alkaline electrolytes of different coat thicknesses as follows: a) 5 μm and b) 15 μm

In Figure 11, we see that depending on the coating thickness, we have the formation of reduction peaks that are more pronounced with a coating thickness of 5 microns (- 0,78 V) because the diffusion of the ions and the solubility of the zinc are facilitated. As the thickness of the coating increases, the surface area between the separation peaks, and also the corrosion current, precisely because of the migration and diffusion of the ions in the chloride environment, which confirms the previously explained mechanism. *Abdel-Gawad et al.* which examined the phosphate coating of steel in sodium chloride, also received a lower current density as the thickness and coating time increased.

CONCLUSION

Based on the results of the saline chamber, we can conclude:

• The time until red corrosion occurs in the alkaline electrolytes coatings, the thickness of the 5 μm coating is 936 h, while in the frozen sample the red corrosion occurred after 768 h.
• The time before the appearance of red corrosion in an acid electrolytes sample, the thickness of the 5 μm coating is after 720 h, while in the damaged sample the red corrosion occurred after 648 h.
• By comparing the alkaline and acid electrolytes coatings with and without damage to the coating, we can conclude that better corrosion resistance provides the protection of the alkaline electrolytes coating.
• An unprotected sample was corroded already after 24 h.

Based on electrochemical measurements we can conclude:

• Alkaline electrolyte coatings with a thickness up to 15 microns thick show oxidation and reduction peaks on cycloltamograms.
• In the case of alkaline electrolytes coatings of 20 and 25 microns, the diffusion process is difficult, and therefore the corrosion process.
• Acid electrolyte coatings also show anodic passivation and transpasification on Tafel diagrams.
• Deformed samples of alkaline electrolytes, as well as acid electrolytes coatings with a thickness of 5 microns show the tendency of corrosive formation on the surface of the material.

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Summary/Sažetak

Upotreba prevlaka na bazi cinka za zaštitu od korozije čeličnih podloga je veoma široko rasprostranjena. Galvanske prevlake se nanose da bi površina osnovnog materijala dobila odgovarajuća svojstva, otpornost prema koroziji, postojanost, estetski izgled, kao i dugotrajnu primjenu u odgovarajućoj industriji. U ovom radu cilj je bio zaštititi čelične filtere goriva sa alkalnim i kiselim prevlakama cinka različitih debljina. Prevlaka cinka koja se nanosi iz alkalnog elektrolita, pruža dobru zaštitu od korozije uz odličnu savitljivost prevlake. Ispitana je debljina prevlake metodom rendgenske fluorescencije, nakon toga vršena su ispitivanja prijanjanja prevlake, te korozione postojanosti, kao i elektrohemijska ispitivanja. Rezultati adhezije su pokazali visok kvalitet prevlake, jer ni na jednom uzorku tokom ispitivanja nije došlo do pojave korozije. Koroziona postojanost ispitana metodom slane komore govori o pojavi bijele i crvene korozije. Na uzorcima kiselog cinka, došlo je do pojave bijele korozije nakon 168 h izloženosti slanoj komori, dok je na uzorcima alkalnog cinka došlo do pojave bijele korozije nakon 240 h izloženosti. Tafelovim polarizacionim dijagramima su određeni korozioni potencali i struje korozije te izračunate brzine korozije. Cikličnom voltametrijom je određeno aktivno i pasivno područje korozije (oksidacioni redukcioni pikovi) u području potencijala od -1.2 do 1 V. Najveću brzinu korozije pokazao je uzorak alkalnog cinka od 5 mikrona, jer je najmanja prevlaka. Dok kod kiselog cinak najveća brzina korozije je kod debljine prevlake od 25 mikrona.

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Calibration bath uncertainty in precision temperature measurements

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Abstract: Calibration baths are widely used in many laboratories worldwide for the calibration of thermometers by comparison. They come in different shapes and sizes and use different media (water, alcohol, silicon oil etc.), but a certain nonuniformity of the used media is always present. During precision temperature measurements, uniformity (homogeneity and temporal stability) of a calibration bath is of the utmost importance since its contribution to total measurement uncertainty is the largest. The temperature gradient can be described as a change of temperature at different positions inside the bath working volume. Temporal temperature stability depends on many factors such as: the bath temperature, control system, bath construction and the flow of the used media inside the bath, just to name a few. In this paper, we investigate uniformity of a cylindrical calibration bath. Homogeneity is determined by measurement of axial and radial temperature gradient inside the bath. Stability is examined by observation of the change in temperature reading during certain period of time. The obtained results are compared with manufacturer specification and can be used for determination of calibration bath contribution to total measurement uncertainty during calibration of thermometers.

INTRODUCTION

In the modern world, there is a growing need for precise temperature measurements in many fields of science, medicine, industry and related disciplines. The thermometers used nowadays are supposed to make the smallest possible error during measurements. However, only a limited amount of instruments is as precise as claimed in manufacturer specification, and furthermore their precision deteriorates with time. That is why calibration of thermometers is needed. Calibration of thermometers by comparison is a procedure in which readings from a thermometer with an unknown accuracy are compared to readings from a reference thermometer placed at the same temperature inside a temperature controlled calibration medium (Batagelj and Bojkovski, 2011). Because the characteristics of the instrument change over time, this procedure has to be repeated at intervals appropriate for keeping the confidence in the measurement results (Parali, Durmaz and Aydin, 2018).
The measurements for calibration of thermometers by comparison are usually performed inside a calibration bath in the range from -80 ºC to 300 ºC. For calibration by comparison method, a minimum of two thermometers is needed, i.e. a reference thermometer and a thermometer to be calibrated.
There are several uncertainty sources in the process of calibration of thermometers by comparison. Some of them can be determined from calibration certificates of the used equipment, but some have to be measured or estimated (Bojkovski, Batagelj, Drnovšek, *et al.*, 2009; Drnovšek, Puškin and Bojkovski, 1999). A major contribution to overall uncertainty during calibration of thermometers by comparison comes from a calibration bath (Bojkovski, Batagelj, Drnovšek, *et al.*, 2009; Drnovšek, Puškin and Bojkovski, 1999; Pušnik, Drnovšek and Bojkovski, 1998). To calculate uncertainty of the calibration bath it is necessary to investigate its uniformity, since calibration bath cannot be considered to be completely stable with time nor homogeneous all over its volume. In order to decrease this uncertainty contribution, equalizing blocks with

holes for positioning thermometers inside of the calibration bath can be used (Bojkovski, Batagelj, Drnovšek, *et al.*, 2009).

EXPERIMENTAL

The experiment described in this paper was conducted with the aim of investigating uniformity of a cylindrical calibration bath and its uncertainty contribution to the total calibration uncertainty. We used the Parallel Tube Liquid Bath Model 915 (Isothermal Technology) with an equalizing block inside, as presented in Figure 1.

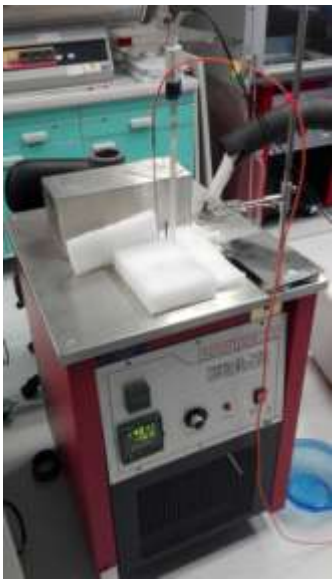

Figure 1: Parallel Tube Liquid Bath Model 915 (Isothermal Technology)

The bath working volume is 3017 cm^3, while its liquid capacity is approximately 7 l. The equalizing block contains four bores with diameter of 8 mm and depth of 120 mm, in which temperature sensors can be placed. Both, the fixed and the mobile thermometer were standard platinum resistance thermometers SPRT 159 and SPRT 154 model 670SQ respectively (Isothermal Technology).

Figure 2: A water triple point cell (WTP)

Radial gradient was measured with both thermometers fixed at the maximum depth. In order to determine axial gradient, the reference thermometer was fixed at a maximum depth while the mobile thermometer was moved axially in steps of 2 cm inside the equalizing block of the calibration bath. Thermometry bridge Fluke 1594A (Fluke Calibration) was used for simultaneous measurements on both thermometers since it provides possibility of data storage. Temporal stability was examined by monitoring the change in the reference thermometer reading during 20 minutes. A water triple point cell (WTP), presented in Figure 2, was realized with dry ice method, and it was used for checking the stability of the reference thermometer. In order to investigate homogeneity of the calibration bath, radial and axial gradient were calculated (Drnovšek, Bojkovski and Pušnik 1997; Drnovšek, Bojkovski and Pušnik 2000). We conducted 10 measurements at three different temperatures with three different media as follows: at -40 °C we used ethanol, at 50 °C we used distilled water and at 150 °C we used oil. Environmental air temperature and humidity, recorded during the measurements, were as follows: 21,3 °C and 34%, 22,4 °C and 43% and 22,4 °C and 54% respectively. The coverage factor or probability level for all the equations in this paper is k=1. Uncertainty contribution caused by the radial gradient g_r can be calculated using following equation

$$g_r = \frac{1}{\sqrt{3}} \max\left|t_{r,i} - t_{m,i}\right| = \frac{1}{\sqrt{3}} \max \Delta t;\ i = 1 \text{ to } n \quad (1)$$

where n is the number of measurements, $t_{r,i}$ and $t_{m,i}$ are temperature readings of the reference thermometer and of the mobile thermometer respectively. The gradient represents maximum temperature difference $\max \Delta t$ between the reference thermometer and the mobile thermometer when both of them are at the maximum depth. In general, the axial gradients g_a can be derived by calculating the maximum change in temperature difference registered between the reference and the mobile thermometer (maximum registered at any two positions of the mobile thermometer).
The homogeneity caused by both gradients is determined from the formula:

$$h = \sqrt{g_r^2 + g_a^2} \quad (2)$$

During stability investigation, the change in the reference thermometer reading was observed for 20 minutes. Maximum difference between the observed temperatures is used for stability determination as follows

$$s = \frac{1}{2\sqrt{3}}\left|t_{r,max} - t_{r,min}\right| \quad (3)$$

Total measurement uncertainty contribution of the calibration bath due to gradients and temporal stability is calculated from

$$u_{bath} = \sqrt{h^2 + s^2} \quad (4)$$

RESULTS AND DISCUSSION

In order to calculate the contribution of the measurement uncertainty of the bath, we determined its radial gradient, axial gradient and temporal stability. The results of the radial gradient measurements are presented in Figure 3, as differences in temperature readings between the reference and the mobile thermometer. We can notice small variations in temperature, of order 10^{-3} °C.

Axial gradient inside the calibration bath at three different temperatures is presented in Figure 4, as a difference in temperature reading of the reference thermometer and the mobile thermometer. The results also show small temperature variations, of order 10^{-3} °C.

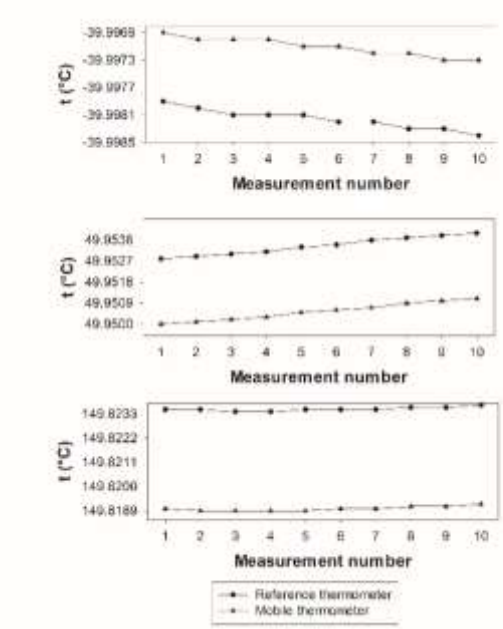

Figure 3: The radial gradient inside a calibration bath at -40 °C, 50 °C and 150 °C respectively. Lines are just guide for the eye.

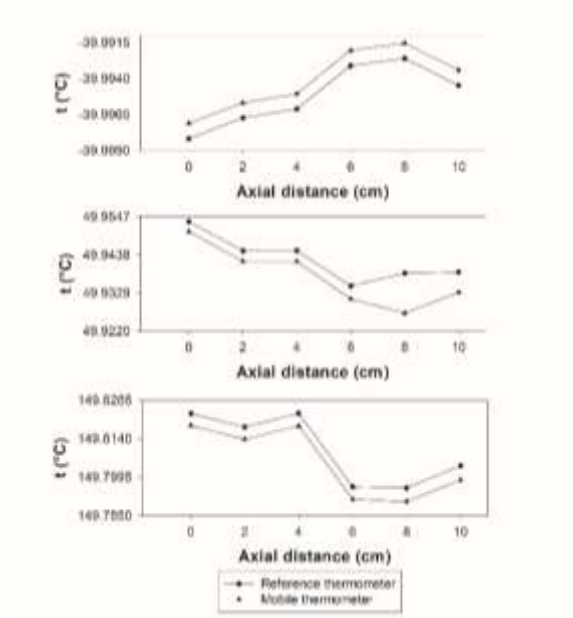

Figure 4: The axial gradient inside the calibration bath at -40 °C, 50 °C and 150 °C respectively. The lines are just a guide for the eye.

The stability of the calibration bath at three different temperatures is presented in Figure 5. As before, we can notice small temperature variations of the reference thermometer readings over time.

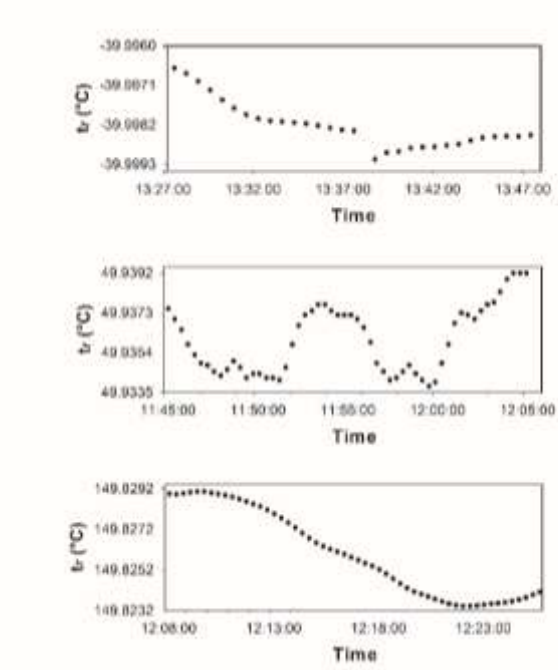

Figure 5: Temporal stability of the calibration bath at -40 °C, 50 °C and 150 °C respectively.

Homogeneity caused by axial and radial gradient, stability and uncertainty of the bath were calculated from (2), (3) and (4) respectively. The results are presented in Table 1.

Table 1: Radial g_r and axial g_a gradients, homogeneity h, stability s and total uncertainty of the bath u_{bath} at three different temperatures.

T (°C)	g_r (°C)	g_a (°C)	h (°C)	s (°C)	u_{bath} (°C)
-40	±0.0006	±0.0006	±0.0009	±0.0015	±0.0017
50	±0.0016	±0.0034	±0.0037	±0.0031	±0.0049
150	±0.0026	±0.0031	±0.0041	±0.0033	±0.0052

Our results show that uncertainty slightly increases with temperature. However, we cannot compare these values, since measurements were taken with different media. We stipulate that one of the reasons for the bath to be the most stable at -40 °C could be caused by the fact that it was left at that temperature overnight and the calibration bath probably reached better stability before the beginning of the measurements.

Manufacturer specification (IsoTech, *Parallel Tube Liquid Bath Model 915*; IsoTech, *Evaluation Report Parallel Tube Liquid Bath:Model 915*) contained relevant information of temporal stability of the calibration bath with water, oil and methanol, which enabled us to compare these data with our results as presented in Table 2. We can notice that measured stability values are smaller than those provided by the manufacturer. However, these values cannot be directly

compared since the measurements were taken at different temperatures from those in the manufacturer specification. In addition, stability at the lowest temperature depends on the type of media that was used.

Table 2: Stability of the calibration bath obtained from the measurements s_m and the stability from the manufacturer specification s_s

T (°C)	Medium	s_m (°C)	T (°C)	Medium	s_s (°C)
-40	Ethanol	± 0.001	-65	Methanol	± 0.005
50	Water	±0.002	35	Water	±0.004
150	Silicon oil	± 0.002	125	Silicon oil	± 0.007

Total uncertainty stipulated by the manufacturer was compared with measured uncertainty for two different media as presented in Table 3. For that purpose, our results were rounded to three decimal places.

Table 3: Calibration bath uncertainty obtained from the measurements u_{bathm} and the uncertainty from the manufacturer specification u_{baths}

T (°C)	Medium	u_{bathm} (°C)	T (°C)	Medium	u_{baths} (°C)
50	Water	± 0.005	50	Water	± 0.004
150	Silicon oil	± 0.005	100	Oil	± 0.007

The results obtained for water only slightly differ from the manufacturer specification data. Differences for silicon oil are a little bit bigger which can be explained by the fact that the measurements were taken at different temperature. From manufacturer specification we can also notice that uncertainty was smaller for the lower temperature, so the correlation between uncertainty and temperature ought to be further investigated.

CONCLUSIONS

The conducted investigation of homogeneity and stability of the calibration bath enables us to determine its total uncertainty contribution. This information is crucial for any laboratory dealing with calibration of thermometers by comparison method. On the basis of the calibration laboratories data, one out of five instruments either gives false reading or a possible error is not declared in the manufacturer specification (Nicholas and White 2001).

Our preliminary results indicate that it is advisable to leave the calibration bath at the temperature of measurement overnight in order to achieve better stability before the measurements are conducted. We are planning to verify this conclusion with further investigations for higher temperatures. The amount of uncertainty as determined in our experiment is not very different from the one given in the manufacturer specification, though there are some small differences. We came to a conclusion that it is justified to determine measurement uncertainty of the calibration bath regardless of manufacturer specification, especially in the laboratories dealing with precise temperature measurements.

This is particularly so since laboratories have different working conditions, use different thermometers or possibly different procedures for determination of stability and/or homogeneity of the calibration bath.

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Summary/Sažetak

Kalibraciona kupatila se koriste u mnogim laboratorijama širom svijeta za kalibraciju termometara metodom poređenja. Mogu se naći kupatila različitog oblika i veličine i koriste različite medije (voda, alcohol, silikonsko ulje itd.), ali određena neuniformnost unutar medija je uvijek prisutna. Za vrijeme preciznih mjerenja temperature, uniformnost (homogenost i vremenska stabilnost) kalibracionog kupatila je od najveće važnosti pošto je njen doprinos ukupnoj mjernoj nesigurnosti najveći. Temperaturni gradijent se može opaziti kao promjena u očitanju termometra u skladu sa promjenom njegovog položaja unutar kupatila. Vremenska stabilnost zavisi od protoka korištenog medija unutar kupatila. U ovom radu, ispitaćemo uniformnost cilindričnog kalibracionog kupatila. Homogenost se može odrediti mjerenjem aksijalnog I radijalnog temperaturnog gradijenta unutar kupatila. Stabilnost se može odrediti mjerenjem promjene očitanja termometra u određenom vremenskom intervalu. Dobijeni rezultati se mogu uporediti sa specifikacijom proizvođača i iskoristiti za određivanje doprinosa kalibracionog kupatila ukupnoj mjernoj nesigurnosti tokom kalibracije termometara.

Pigment analysis by portable XRF of the painting of artist Vojo Dimitrijevic from the collection of the History museum of B&H

Master student of interdisciplinary study conservation and restoration
(Academy of fine arts, Faculty of Architecture and Faculty of science) - Ajla Alijagić

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15-21

Phenolics content and antioxidant activity of three *Sorbus* species

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Abstract: The phenolic content and antioxidant activity of *Sorbus aucuparia* L., *Sorbus aria* (L.) Crantz and *Sorbus austriaca* (Beck) Hedlund leaves and fruit were investigated. The quantification of total phenolics, flavonoids and phenolic acids was performed using the Folin–Ciocalteu, Dowd and Arnow methods, respectively. The antioxidant activity of the extracts was evaluated using DPPH, TEAC and FRAP methods with Trolox as a standard. Leaves had a higher content of phenolic compounds and antioxidant activity than the fruits for all species. The highest content of phenolics (76.11 mg gallic acid equivalents (GAE)/g plant), flavonoids (15.86 mg rutin equivalents (RE)/g plant) and phenolic acids (44.54 mg caffeic acid equivalents (CAE)/g plant) was determined for *S. austriaca* leaves. *Sorbus austriaca* fruit had the highest content of phenolics (13.21 mg GAE/g plant), flavonoids (1.82 mg RE/g plant) and *S. aucuparia* fruit had the highest content of phenolic acids (9.05 mg CAE/g plant). The antioxidant activity was in the range: DPPH=38.42–274.52 μmol Trolox equivalents (TE)/g plant; TEAC=43.23–403.02 μmol TE/g plant; FRAP=47.13–706.96 μmol TE/g plant. The highest values of antioxidant activity were found for *S. austriaca* leaf and fruit extracts while the lowest values were determined for *S. aucuparia* leaves and *S. aria* fruit. The antioxidant activity was highly correlated with total phenolics and phenolic acids.

INTRODUCTION

Sorbus L. genus includes about 250 species of deciduous trees and shrubs mainly widespread in the northern hemisphere (Olszewska and Michel, 2009). It is estimated that about one-third of *Sorbus* diversity in Europe is located in the Balkan Peninsula, including 18 species and 12 subspecies of the genus (Hajrudinović, Siljak-Yakovlev, Brown, *et al.*, 2015b and references therein). So far, eight *Sorbus* species have been recognized in Bosnia and Herzegovina including *Sorbus aria* (L.) Crantz, *Sorbus aucuparia* L., and *Sorbus austriaca* (Beck) Hedl. (Beck-Mannagetta, 1927; Hajrudinović, *et al.*, 2015b). Fruit of *S. aucuparia, S. aria, S. domestica and S. torminalis* have been used for medicinal purposes and as food ingredients in the production of jams and juices. (Mikulić-Petkovsek, Krska, Kiprovski *et al.*, 2017 and references therein). Extracts of leaves, inflorescences, fruit and bark of various *Sorbus* species are used for their hypoglycaemic, diuretic, vasoprotective, anti-inflammatory and antidiarrhoeal properties (Raudoné, Raudonis, Gaivelyte, *et al.*, 2015 and references therein). In particular, inflorescences of *S. aucuparia* are used in traditional and European medicine as a diuretic and antiinflammatory agent. The fruit of *S. aucuparia and S. aria* is used as a vitamin and antioxidant agent and for the treatment of diarrhoea (Olszewska and Michel, 2009 and references therein). Some species such as *S. aucuparia, S. aria* and *S. domestica* have been identified as possible rich sources of phenolics (Olszsewska and Michel, 2009). The pharmacological properties of *Sorbus* species have been related to the presence of different phenolic compounds as the main antioxidants (Hukkanen, Pölönen, Kärenlampi, *et al.*, 2006). The presence of polyphenols,

flavonoids (quercetin derivatives rutin, hyperoside, isoquercitrin), phenolic acids (chlorogenic, neochlorogenic, caffeic acids), proanthocyanidins in leaves and fruit has already been mentioned by several authors (Olszewska, Nowak, Michel, *et al*., 2010; Gaivelyte, Jakstas, Razukus, *et al*., 2014; Raudoné, *et al.*, 2015). The fruit is also rich in tocopherols, ascorbic acid, carotenoids and anthocyanins (Mrkonjić, Nađpal, Beara, *et al*., 2017; Šavikin, Zdunić, Krstić-Milošević, *et al*., 2017, Mikulić-Petkovsek, *et al*., 2017 and references therein). Most research on the antioxidant activity of various plant materials has been associated with their application in the food and pharmaceutical industries as a possible source of new natural additives and antioxidants that could replace synthetic ones (Finley, Kong, Hintze, *et al*., 2011; Surwesvaran, Cai, Corke, *et al*., 2007). Therefore, the aim of this study was to estimate the phenolic content and antioxidant activity of leaves and fruits of *S. aucuparia*, *S. aria* and *S. austriaca* from Bosnia and Herzegovina. Antioxidant activity was estimated using three different methods: DPPH, TEAC and FRAP with Trolox as a standard. The determination of total phenolics, total flavonoids, and total phenolic acids was conducted using the Folin–Ciocalteu, Dowd, and Arnow methods, respectively. Relationships between phenolic compounds and antioxidant activity were investigated. To our knowledge, this is the first report on phenolic content and antioxidant activity of selected *Sorbus* species from Bosnia and Herzegovina.

EXPERIMENTAL

All chemicals were of analytical grade. Caffeic acid was purchased from Merck Chemical Suppliers (Germany), and potassium peroxysulfate from Fluka (Germany). Sodium acetate, sodium nitrite and sodium hydroxide were purchased from Kemika, (Croatia), and sodium molybdate from Acros Organics (USA). All other chemicals were obtained from Sigma-Aldrich (Germany).

Plant material

Leaf and fruit samples of *S. aucuparia*, *S. austriaca* and *S. aria* were collected in October 2016, in the area of Sarajevo, Bosnia and Herzegovina. Samples were identified by a plant taxonomist (one of the authors), and voucher specimens were stored in the Herbarium of Forest Ecology at the Faculty of Forestry. The samples were dried in a ventilated room for 15–20 days, and they were stored in paper bags in a dry and dark place until analysis.

Extraction procedure

Dried fruits and leaves were pulverised in an electric mill (Gorenje, Slovenia), and then extracted with 80% (*v/v*) aqueous methanol by ultrasound extraction (Ultrasound bath, Elmasonic, Italy). A modified extraction method previously described by Memon, Memon and Luthria (2010) and Raudonis, *et al*. (2014) was used. Briefly, plant material (0.5 g) was extracted twice with 25 mL of methanol (80%, *v/v*) for 10 minutes at 30°C. After the centrifugation (3000 rpm, 10 min), the resulting supernatants were combined, filtered (Millipore nylon filters, 0.45 μm) in a 50 mL volumetric flask and supplemented with the extraction medium to the mark. The extracts were stored at -20°C until use. Yields were determined by evaporation of extracts (5 mL) to dryness.

Determination of total phenolics

Total phenolics were determined by the Folin–Ciocalteu method as modified and described by Luthria, Mukhopadhyay and Krizek (2004). Sample solution (0.1 mL) was mixed with distilled water (7.9 mL) and Folin–Ciocalteu reagent (500 μL) was added. After 5 minutes, Na_2CO_3 (20%, 1.5 mL) was added, and the total volume was adjusted with distilled water up to 10 mL. Samples were left for 30 minutes at 40°C in a water bath (INKO 1935, Zagreb). Absorbance was read at 765 nm against a blank. Gallic acid was used to prepare the standard curve, and the results were expressed as milligrams of gallic acid equivalents per gram of dry plant (mg GAE/g plant) and per gram of extract (mg GAE/g extract). A Shimadzu UV-mini 1240 spectrophotometer was used for all spectrophotometric determinations.

Determination of total flavonoids

A modified Dowd method was used to determine total flavonoids as described by Quettier-Deleu, Gressier, Vasseur (2000). Sample solution (1 mL) and $AlCl_3$ solution (2% in absolute methanol, 1 mL) were mixed. The mixture was incubated at room temperature for 1 h, and absorbance was measured at 415 nm against a blank. The standard curve was prepared with rutin, and the results were expressed as milligrams of rutin equivalents per gram of dry plant (mg RE/g plant) and per gram of extract (mg RE/g extract).

Determination of total phenolic acids

For the determination of total phenolic acids, the modified Arnow method given by Gawlic-Dziki (2012) was used. Sample solution (1 mL) was mixed with water (5 mL), HCl (0.5 M, 1 mL), Arnow reagent (1 mL) and NaOH (1 M, 1 mL), followed by the addition of distilled water to a total volume of 10 mL. The samples were incubated for 20 min at room temperature. Absorbance was measured at 490 nm against a blank. Total phenolic acids were calculated from the standard curve, and the results expressed as milligrams of caffeic acid equivalents per gram of dry plant (mg CAE/g plant) and per gram of extract (mg CAE/g extract).

2,2-Diphenyl-1-pycrylhydrazyl (DPPH) assay

Determination of the antioxidant activity with DPPH reagent (2,2-diphenyl-1-pycrylhydrazyl radical) was carried out according to Thaipong, Boonprakob, Crosby (2006). The working solution of DPPH was prepared immediately before measurements by diluting the stock solution (3 mM) to the absorbance of A=1.1±0.02 at 515 nm. Sample solution (0.1 mL) and methanolic DPPH (1.9

mL) were mixed and left in the dark for 30 min. The decrease in absorbance was measured at 515 nm against methanol as a blank. The results were expressed as μmol of Trolox equivalents per gram of dry plant material (μmol TE/g plant) and per gram of extract (μmol TE/g extract) using the standard curve of Trolox.

Trolox equivalent antioxidant capacity (TEAC) assay

Determination of the antioxidant activity with $ABTS^{\bullet+}$ reagent (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) was carried out following the method of Thaipong, *et al*. (2006). The $ABTS^{\bullet+}$ reagent was freshly prepared by mixing equal parts of the ABTS (7 mM) stock solution and $K_2S_2O_8$ (2.45 mM) solution. The mixture was left for 12 h in the dark before use. Immediately before use, the $ABTS^{\bullet+}$ solution was diluted to the absorbance of A=1.1±0.02 at 734 nm. Sample solution (0.1 mL) was mixed with the $ABTS^{\bullet+}$ radical solution (1.9 mL) and left to stand for 10 minutes (Olszewska and Michel, 2009). The decrease in absorbance was measured at 734 nm against methanol as a blank. The results were expressed as μmol of TE per gram of dry plant material (μmol TE/g plant) and per gram of extract (μmol TE/g extract) using the standard curve of Trolox.

Ferric reducing antioxidant power (FRAP) assay

A modified FRAP method was used to determine antioxidant activity as described by Thaipong, *et al*. (2006). FRAP reagent was prepared immediately before use by mixing acetate buffer (300 mM, pH=3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine) in 40 mM HCl and $FeCl_3$ (20 mM) in a volume ratio of 10:1:1. Sample solution (0.1 mL) was added to FRAP reagent (1.9 mL) in a test tube. The samples were incubated for 2 h at 37°C on a water bath (Olszewska and Michel, 2009) before measurements. Absorption of the blue-coloured complex was measured at 593 nm against a blank. The results were expressed as μmol of TE per gram of dry plant material (μmol TE/g plant) and per gram of extract (μmol TE/g extract) using the standard curve of Trolox.

Statistical analysis

All measurements were made in triplicate, and the results were expressed as the mean ± standard deviation (SD). Data were subjected to one-way analysis of variance followed by Duncan's multiple range test to separate the mean values. Statistical analysis was performed using IBM SPSS Statistics, version 20 (IBM Corp., Armonk, NY). The differences were considered statistically significant at $p<0.05$. The correlations between the contents of tested compounds and the antioxidant activity were determined by a linear regression method (Excel, Windows 10).

RESULTS AND DISCUSSION

Leaf and fruit extracts of the investigated *Sorbus* species were prepared by ultrasound extraction, and extraction yields were determined and presented in Table 1.

Table 1: Yields of extracts for *Sorbus* leaf and fruit samples

Plant species	Plant part	Mass of the sample (g)	Mass of the extract (g)	Yield (%)
S. aria	L	0.5002	0.0182	36.40
	F	0.5000	0.0352	70.40
S. austriaca	L	0.5001	0.0163	32.60
	F	0.5000	0.0242	48.40
S. aucuparia	L	0.5000	0.0176	35.20
	F	0.5002	0.0369	73.80

*L-leaves; F-fruit

The yields ranged from 48.40 to 73.80% for fruit samples, and from 32.60 to 36.40% for leaf samples. The highest yield was from *S. aucuparia* fruit, while the lowest yield was from *S. austriaca* leaves. It can be concluded that the extraction efficiency is higher for the fruit than the leaves. Similar observations were obtained in the study on the effects of extraction of *Prunus laurocerasus* leaves and fruit. Differences in extraction yields could be attributed to the presence of different compounds in each part of the plant as well as the solvent extraction activity (Karabegović, Stojičević, Veličković, *et al.*, 2014 and references therein).

The total phenolics, flavonoids and phenolic acids contents expressed as mg of standard equivalents per gram of dry plant/extract are given in Table 2. In leaf samples, the total phenolics ranged from 30.40 to 76.11 mg GAE/g plant, flavonoids from 10.94 to 15.86 mg RE/g plant, phenolic acids from 18.82 to 44.54 mg CAE/g plant. In fruit samples, determined values varied from 7.02 to 13.21 mg GAE/g plant for phenolics, from 0.87 to 1.82 mg RE/g plant for flavonoids and from 4.21 to 9.05 mg CAE/ g plant for phenolic acids.

Table 2: Total phenolics, total flavonoids and total phenolic acids in S*orbus* leaf and fruit samples.

Species	Sample	Total phenolics		Total flavonoids		Total phenolic acids	
		mg GAE/g plant	mg GAE/g extract	mg RE/g plant	mg RE/g extract	mg CAE/g plant	mg CAE/g extract
S. aria	Leaves	55.93±0.00^{e}	153.71±0.00^{d}	10.94±0.43^{c}	30.08±1.20^{c}	33.80±0.18^{d}	92.90±0.50^{e}
	Fruit	7.02±0.02^{a}	9.97±0.03^{a}	0.92±0.03^{a}	1.31±0.05^{a}	4.21±0.04a	5.98±0.06^{a}
S. austriaca	Leaves	76.11±2.70^{f}	233.54±8.30^{e}	15.86±0.32^{e}	48.66±0.97^{e}	44.54±0.65^{e}	136.70±2.01^{f}
	Fruit	13.21±0.26^{c}	27.29±0.54^{b}	1.82±0.01^{b}	3.76±0.02^{b}	8.94±0.05^{b}	18.47±0.10^{c}
S. aucuparia	Leaves	30.40±0.04^{d}	86.33±0.11^{c}	14.01±0.15^{d}	39.81±0.43^{d}	18.82±0.10^{c}	53.48±0.28^{d}
	Fruit	10.13±0.05^{b}	13.74±0.07^{a}	0.87±0.01^{a}	1.18±0.02^{a}	9.05±0.06^{b}	12.27±0.08^{b}

Values with different upper-case letters in the same column are significantly different at $p<0.05$

S*orbus austriaca* leaves had significantly higher ($p<0.05$) contents of all bioactive compounds. In general, the reported values of Olszewska and Michel (2009), Olszewska (2010, 2011) and Gaivelyte, *et al*. (2014), for total phenolics in *S. aucuparia* leaves (7.07-9.09% dry weight(DW)), are higher than in this work, and for flavonoids (0.038–1.58% DW) and phenolic acids (0.97-3.90% DW) they are comparable to the results of this study. Olszewska and Michel (2009) reported a higher content of phenols (6.06% DW) and flavonoids (1.30% DW) but lower content of phenolic acids (1.73% DW) in *S. aria* leaves than those given in this work. *Sorbus austriaca* fruit had the highest contents of phenolics and flavonoids. However, no significant difference was determined in the values of phenolic acids content between *S. austriaca* and *S. aucuparia* fruit.

In general, data for *S. austriaca* are quite limited. Raudoné, *et al*. (2015) reported similar values for total phenolic acids content between *S. aucuparia* (0.283% DW) and *S. austriaca* fruit (0.286% DW). Values for total flavonoids in *S. austriaca* fruit (0.0143% DW) and phenolic acids (0.286% DW) given by Raudonis, *et al*. (2014) were lower than the results in this study. In addition, there was no significant difference ($p<0.05$) in the values of total flavonoids content between the fruit of *S. aucuparia* and *S. aria*. According to Olszewska and Michel (2009) and Šavikin, *et al*. (2017), contents of phenolics, flavonoids and phenolic acids in *S. aucuparia* fruit yielded up to 2.68%, 0.104% and 1.52%, while for *S. aria* fruit they varied up to 2.98%, 0.093% and 0.63%, respectively. In general, the leaves have significantly higher ($p<0.05$) contents of all bioactive compounds in relation to the fruit which is in agreement with the findings of Olszewska and Michel (2009), Gaivelyte, *et al*. (2014 and references therein). Differences found between our results and previous investigations could be due to genetic factors, environmental conditions, maturity and time of harvest (Olszewska, 2011; Gaivelyte *et al*., 2014 and references therein). It is important to emphasize that the extraction method and extraction medium could influence the differences as reported by Olsezwska, Presler, Michel, *et al.* (2012), Aladedunye and Matthäus (2014). In addition, the phenolics content for *S. austriaca* and *S. aria* leaves are close to that of *Crataegus monogyna* (61.98 mg GAE/g DW), *Crataegus* x *macrocarpa* (82.44 mg GAE/g DW) (Tahirović and Bašić, 2015) and *Aloe littoralis* (62.00 mg GAE/g DW) (Surweswaran, *et al*., 2007). The values are higher than those reported for *Artemisia abrotanum* (4.9 mg GAE/g DW), *Euphorbia lathyrus* (11.50 mg GAE/g DW) and *Ocimum basilicum* (26.30 mg GAE/g DW) by Surweswaran, *et al.* (2007). On the other hand, the content of phenolics in fruit is much lower than in *Prunus spinosa* (25.14 mg GAE/g DW), *Crataegus monogyna* (28.19 mg GAE/g DW), *Rosa canina* (51.19 mg GAE/g DW) extracted with the same extraction medium (Tahirović, Bašić, Čopra-Janićijević, *et al*., 2018; Tahirović and Bašić, 2015, 2017). In general, phenolics content in leaves of S. *austriaca* and *S. aria* were higher than 5%, so we can conclude on the basis of data and references given by Olszewska and Michel (2009) that leaves of these species represent a rich source of phenolic compounds. The results expressed per gram of extracts for leaves ranged from 86.33 to 233.54 mg GAE/g extract for phenolics, from 30.08 to 48.66 mg RE/g extract for flavonoids and from 53.48 to 136.70 mg CAE/g extract for total phenolic acids. For fruit samples, obtained values were in the range of 9.97-27.29 mg GAE/g extract for phenolics, 1.18-3.76 mg RE/g extract for flavonoids and 5.98-18.47 mg CAE/g extract for phenolic acids (Table 2). *Sorbus austriaca* is the species with the highest content of investigated bioactive compounds in leaves and fruit. In addition, there are no significant differences ($p<0.05$) in the contents of total phenolics and flavonoids for *S. aria* and *S. aucuparia* fruit samples. Mrkonjić *et al*. (2017) reported for *S. aucuparia* fruit values of 0.187 mg/g extract for total flavonoids and 0.0154 mg/g extract for phenolic acids, which is lower than the data in this study. Results of total phenolics for butanol and ethyl acetate extracts of *S. aucuparia* fruit (42-103 mg/g extract) given by Aladedunye and Matthäus (2014) were generally higher. The content of phenolics in fruit was lower than values reported for cherry laurel (42.2 mgGAE/g extract; Karabegović, *et al*., 2014), rosehips (149.35 mg GAE/g extract; Barros, Carvalho, Ferreira, *et al*., 2011) and hawthorn (274.27 mg GAE/g extract; Barros, Carvalho, Ferreira, *et al.*, 2011).

Antioxidant activity

The DPPH, TEAC and FRAP assays were performed to assess the antioxidant activity of leaf and fruit extracts. The obtained results are expressed as μmol TE/g plant and μmol TE/g extract and shown in Table 3. Antioxidant activity of *Sorbus* leaf samples ranged from 121.80 to 274.52 μmol TE/g plant for DPPH, from 142.52 to 403.02 μmol TE/g plant for TEAC, and from 287.03 to 706.96 μmol TE/g plant for FRAP method.

Table 3: Antioxidant activity of *Sorbus* species leaf and fruit samples determined by DPPH, ABTS and FRAP methods

Species	Sample	DPPH		TEAC		FRAP	
		μmol(TE)/g plant	μmol(TE)/g extract	μmol(TE)/g plant	μmol(TE)/g extract	μmol(TE)/g plant	μmol(TE)/g extract
S. aria	Leaves	234.92±0.90^{e}	645.70±2.41^{e}	364.63±2.42^{e}	1002.13±6.64^{e}	551.94±0.33^{e}	1516.93±0.90^{e}
	Fruit	17.90±0.09^{a}	101.63±0.51^{a}	43.23±0.53^{a}	61.40±0.75^{a}	47.13±0.08^{a}	66.95±0.11^{a}
S. austriaca	Leaves	274.52±5.30^{f}	842.24±16.14^{f}	403.02±6.81^{f}	1236.51±20.90^{f}	706.96±29.82^{f}	2169.03±91.50^{f}
	Fruit	61.26±1.40^{c}	506.30±11.30^{d}	82.90±1.81^{c}	171.18±3.75^{c}	138.06±0.10^{c}	285.24±0.21^{c}
S. aucuparia	Leaves	121.80±0.80^{d}	346.02±2.30^{c}	142.52±2.34^{d}	404.90±6.64^{d}	287.03±0.32^{d}	815.41±0.92^{d}
	Fruit	38.42±0.72^{b}	208.32±3.90^{b}	67.64±1.70^{b}	91.70±2.24^{b}	101.10±0.70^{b}	137.04±1.30^{b}

Values with different upper-case letters in the same column are significantly different at $p<0.05$

Sorbus fruit samples gave results ranging from 17.90 to 61.26 μmol TE/g for DPPH, from 43.23 to 82.90 μmol TE/g plant for TEAC and from 47.13 to 138.06 μmol TE/g plant for FRAP. The leaves revealed significantly higher ($p<0.05$) antioxidant properties than those of fruit samples. The results agree with those observed by Cyboran, Bonarska-Kujawa, Pruchnik (2014) and Olszewska and Michel (2009). *Sorbus austriaca* leaf and fruit samples showed significantly higher ($p<0.05$) antioxidant activity in the DPPH, TEAC, and FRAP assays.

The values of antioxidant activity decreased in the order: *S. austriaca*>*S. aria*>*S. aucuparia* leaves, and in the order: *S. austriaca*>*S. aucuparia*>*S. aria* fruit. Olszewska and Michel (2009) demonstrated that leaves of *S. aucuparia* have higher antioxidant activity than *S. aria*, but fruit of *S. aria* have higher antioxidant activity than *S. aucuparia*. These results could be associated with the quantitative contents of investigated compounds in the sample. The results in this study for *S. austriaca* leaves and fruit were higher than that of Raudoné, *et al*. (2015) for *S. austrica* leaves (FRAP=78.29 μmol TE/g DW) and of Raudonis, *et al*. (2014) for *S. austrica* fruit (TEAC=9.70 μmol TE/g DW; FRAP=9.58 μmol TE/g DW). The results of antioxidant activity given by Raudoné, *et al*., (2015), Olszewska and Michel (2009), Olszewska *et al*. (2011) for S. *aucuparia* leaf (FRAP=144.31-1650 μmol TE/g DW) and fruit (ABTS=11.19-91.6; FRAP=11.83-347 μmol TE/g DW) and *S. aria* leaf (ABTS=265.5 μmol TE/g DW; FRAP=136.53–861.6 μmol TE/g DW) samples were in agreement with the results in this study. However, values of DPPH=400-628 μmol TE/g DW for *S. aucuparia* leaves, collected in summer, were also reported (Olszewska and Michel, 2009, Olszewska, 2011). In general, the antioxidant activity of *S. aria* fruit was lower than the values given by Olszewska and Michel (2009). The differences can be explained in the same way as described for the phenolic content. In addition, antioxidant activity of investigated *Sorbu*s fruit samples had lower values than blackthorn fruit (118.37-193.19 μmol TE/g DW), rosehip fruit (278.34-422.16 μmol TE/g DW), hawthorn species (0.11-0.51 mmol TE/g DW) determined by DPPH, TEAC and FRAP methods in 80% methanol extracts (Tahirović, *et al*., 2018; Tahirović and Bašić, 2015, 2017). According to Surweswaran *et al*. (2007), antioxidant activity with TEAC values between 50.10 and 1000 μg TE/g DW is considered as high. We can conclude that leaves of *Sorbus* species have high antioxidant activity, while the fruit have a medium antioxidant activity.

The antioxidant activity of *Sorbus* leaves, expressed as μmol TE/g of the extracts, ranged from 346.02 to 842.24 μmol TE/g extract for DPPH, from 404.90 to 1236.15 μmol TE/g extract for TEAC and from 815.41 to 2169.03 μmol (TE)/g extract for FRAP assay (Table 3). The results for *Sorbus* fruit samples varied from 101.63 to 506.30 μmol TE/g extract for DPPH, from 61.40 to 171.18 μmol TE/g extract for TEAC and from 66.95 to 285.24 μmol TE/g extract for FRAP assay. It should be mentioned that FRAP values of leaves are higher than those of DPPH and TEAC assay (Table 3). Vitamin C contributes significantly to the reduction properties of extracts, and its quantity in extracts of some *Sorbus* species was determined by several researchers (Egea, *et al*., 2010 and references therein). The higher reduction capability could be attributed to the higher levels of strong reductants capable of donating electrons (Sasikumar, Patharaj, Adithya, *et al*., 2012).

Correlation coefficients were investigated among the total phenolics, flavonoids, phenolic acids, and antioxidant activity with a linear regression method. The obtained results are presented in Table 4. The total phenols (r^2=0.7761-0.9981) and total phenolic acids (r^2=0.7672-0.9969) are highly correlated with DPPH, TEAC and FRAP activity. Correlations between total flavonoids (r^2=0.3984-0.7974) and antioxidant activity were lower. Higher correlation coefficients are probably associated with higher total phenolics and phenolic acids content of the samples (Table 2). Several studies suggest that phenolic compounds, including phenolic acids, can contribute to the antioxidant activity. The results obtained here were consistent with the results of other researchers confirming the correlations between different phenolic compounds and antioxidant activity (Olszewska and Michel, 2009; Raudonis *et al*., 2014; Mikulić-Petkovsek, *et al*., 2017; Šavikin *et al*., 2017).

Table 4: Correlation coefficients between phenolics, flavonoids, phenolic acids and antioxidant activity

	Correlations for data expressed in (mg/g plant)		
Bioactive compounds	DPPH	TEAC	FRAP
Phenolics	0.985	0.9678	0.9965
Flavonoids	0.7529	0.6470	0.7488
Phenolic acids	0.9856	0.9703	0.9969
	Correlations for data expressed in (mg/g extract)		
	DPPH	TEAC	FRAP
Phenolics	0.7761	0.9753	0.9981
Flavonoids	0.3984	0.716	0.7974
Phenolic acids	0.7672	0.9775	0.9990

CONCLUSIONS

The most abundant phenolic compounds in the investigated *Sorbus* species are total phenolics and phenolic acids, while the content of total flavonoids was lower. *Sorbus austriaca* leaves and fruit had a significantly higher ($p<0.05$) content of active compounds than other species, except the total phenolic acids in fruit. However, no significant difference ($p<0.05$) was found in the phenolic acids content between *S. austriaca* and *S. aucuparia* fruit. In addition, *S. austriaca* leaf and fruit extracts had significantly higher antioxidant activity than extracts of other species. In general, the leaf samples have a significantly higher content of bioactive compounds and antioxidant activity than the fruit samples. Based on the results obtained in this study, we can conclude that the investigated *Sorbus* species, in particular, *S. austriaca*, represent a valuable source of natural antioxidants with the possibility of their application in medicine, pharmacy and the food industry. In this regard, more detailed investigations on the quantitative composition of individual phenolic compounds and their antioxidant properties are necessary.

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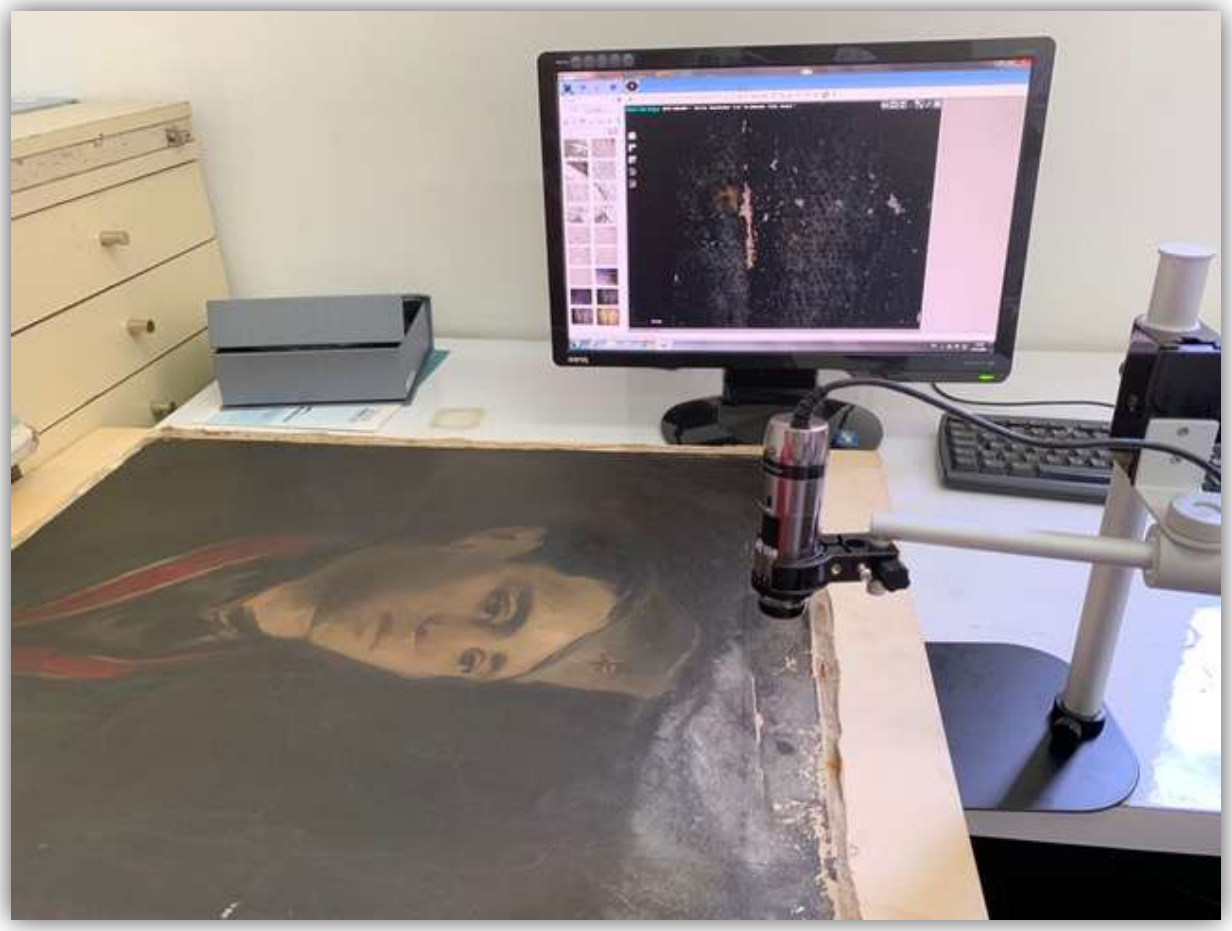
Summary/Sažetak

Ispitivan je sadržaj fenola i antioksidacijska aktivnost lišća i plodova vrsta *Sorbus aucuparia* L., *Sorbus aria* (L.) Crantz i *Sorbus austriaca* (Beck) Hedlund. Kvantifikacija ukupnih fenola, flavonoida i fenolnih kiselina provedena je Folin-Ciocalteu, Dowd-om i Arnow-om metodom. Antioksidacijska aktivnost ekstrakata je procijenjena korištenjem DPPH, ABTS i FRAP metode s Troloxom kao standardom. Lišće je imalo veći sadržaj fenolnih spojeva i veću antioksidacijsku aktivnost od plodova za sve vrste. Najveći sadržaj fenola (76,11 mg ekvivalenata galne kiseline (GAE)/g biljke), flavonoida (15,86 mg ekvivalenata rutina (RE)/g biljke) i fenolnih kiselina (44,54 mg ekvivalenata kafene kiseline (CAE)/g biljke) utvrđen je za listove *S. austriaca*. Plodovi *S. austriaca* imali su najveći sadržaj fenola (13,21 mg GAE/g biljke) i flavonoida (1,82 mg RE/g biljke), a plodovi *S. aucuparia* imali su najveći sadržaj fenolnih kiselina (9,05 mg CAE /g biljke). Antioksidacijska aktivnost kretala se u području: DPPH=38,42-274,52 μmol TE/g biljke; ABTS=43,23-403,02 μmol TE/g biljke; FRAP=47,13-706,96 μmol TE/g biljke. Najveće vrijednosti antioksidacijske aktivnosti utvrđene su za ekstrakte lista i ploda *S. austriaca*, dok su najniže vrijednosti utvrđene za listove *S. aucuparia* i plodove *S. aria*. Antioksidacijska aktivnost je bila u visokoj korelaciji sa ukupnim fenolima i fenolnim kiselinama.



Analysis of the surface morphology using a Dino-Lite digital microscope of the painting of artist Ljubo Lah from the collection of the History museum of B&H

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Toxic compounds in homemade spirits in Bosnia and Herzegovina: A pilot study

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Abstract: The main aim of this preliminary study was to investigate the presence of potentially toxic compounds (methanol, hydrocyanic acid and urea) in homemade fruit spirits produced in different parts of Bosnia and Herzegovina. A total of 15 samples of 8 different fruit spirits were analyzed (apple, apricot, cherry, grape, pear, plum, quince and juniper). Content of hydrocyanic acid was higher than maximum permissible level in 5 out of 15 samples. In general, the average content of methanol was higher in samples from Bosnia comparing to the samples from Herzegovina (874.62 vs. 563.99 g/hL of pure alcohol), but still was lower than maximum concentration proposed by national regulation (1200 g/hL of pure alcohol for fruit spirits and 1000 g/hL of pure alcohol for grape spirit) except for one grape spirit sample (1162.2 g/hL of pure alcohol). Urea was detected in all analyzed samples (5.819 to 77.82 mg/L) with the average concentration of 37.95 mg/L. Since this is, to our knowledge, the first study that included the chemical analysis of homemade spirits in BiH, these results are of great importance for the further research.

INTRODUCTION

Consumption of alcoholic beverages is a common habit of celebrations, with meals and even as a remedy in Bosnia and Herzegovina. For this reason, not only drinking but also homemade production of different kind of alcoholic beverages (wines and spirits) has a long tradition in all parts of Bosnia and Herzegovina. The most common kinds of spirits are those made of plums and grapes, but other different types of alcoholic beverages are also made (mostly of stone fruit). According to World Health Organization (WHO) homemade alcoholic beverages are considered as unregistered, which means that these products are not taxed as alcoholic beverages and also are not registered under a state provision (WHO, 2018). Globally, the most common category of unrecorded alcohol is homemade alcohol and regarding the type of alcohol, the biggest portion of unrecorded consumption can be attributed to spirits (Rehm, Larsen, Lewis-Laietmark, *et al*., 2016). In Bosnia and Herzegovina, general production, common analytical composition limits and parameters for the geographical denominations for strong distilled alcoholic beverages are defined by State Rulebook (2012). According to the results of a systematic analysis for the Global Burden of Disease for the period of 1990-2013 alcohol use is one of the ten leading risks in terms of attributable DALYs (disability-adjusted life-years) not only globally, but also in Bosnia and Herzegovina (Forouzanfar, *et al*., 2015). Based on results of a systematic analysis for the Global Burden of Disease Study 2016 there is a growing trend of alcoholic beverages consumption in B&H over the last decades, especially among men (5 L of alcohol per capita in 1990 vs. 6,5 L per capita in 2016) and it is estimated that 44 % of total alcohol stock were not recorded (Griswold *et al.*, 2018). The projection of the trend of alcohol consumption up to 2020 and 2025 in

B&H shows a strong increase (up to 1.2 L) compared to consumption in 2016 (WHO, 2018). Based on the WHO report on alcohol consumption in B&H, spirits constitute 12 % of all alcoholic beverages consumed in B&H, and 28 % of total consumed spirits have not been recorded. Data on proportion of unrecorded consumption in B&H vary depending on the sources used, but this can be explained by the fact that unrecorded consumption is derived mainly from the surveys by local experts based on fragmented data (WHO, 2018). A certain amount of compounds that are considered as contaminants, can be formed during production and present in alcoholic beverages, especially if distillation is conducted in uncontrolled home production. In addition to its potential health risk, unrecorded consumption can contribute to heavy drinking due to its low cost and availability (WHO, 2018). Most of the experts perceived unrecorded consumption as a financial, public health and social problem (Rehm, *et al*., 2016).

The main aim of this study was to investigate the presence of potentially toxic compounds (methanol, hydrocyanic acid, and urea) in homemade spirits produced in different parts of Bosnia and Herzegovina.

EXPERIMENTAL

A total of 15 samples of various homemade spirits from the different parts of Bosnia and Herzegovina were collected (Table 1.).

Table 1: Samples analyzed in this study

Sample	Type of spirit	Content of ethanol (%)	Location of production
1.	Apple spirit	40	Mostar (Herzegovina)
2.	Apricot spirit	43	Sarajevo (Bosnia)
3.	Apricot spirit	40	Mostar (Herzegovina)
4.	Cherry spirit	40	Mostar (Herzegovina)
5.	Grape spirit	40	Čitluk (Herzegovina)
6.	Grape spirit	40	Čitluk (Herzegovina)
7.	Juniper spirit	40	Čitluk (Herzegovina)
8.	Pear spirit	40	Sarajevo (Bosnia)
9.	Pear spirit	38	Čapljina (Herzegovina)
10.	Pear spirit	38	Mostar (Herzegovina)
11.	Plum spirit	40	Tuzla (Bosnia)
12.	Plum spirit	40	Tuzla (Bosnia)
13.	Plum spirit	47	Sarajevo (Bosnia)
14.	Plum spirit	46	Čapljina (Herzegovina)
15.	Quince spirit	45	Tuzla (Bosnia)

These spirits are usually made by distillation of the fermented fruit mash (apple, apricot, cherry, grape, pear, plum and quince). The juniper spirit is made by soaking the juniper berries in the plum spirit. The ethanol content of the samples analyzed is determined by measuring their density using an alcohol meter (Iberian Coppers S.A., Portugal).

All the reagents used in experimental work were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Distilled water was used for dilution and cleaning in all analytical procedures.

Determination of methanol

Methanol was determined using the spectrophotometric method after previous distillation (under atmospheric pressure). 1 mL of distillate was diluted in a volumetric flask (25 mL) with water. For the further analysis 1 mL of this solution was used.

Spectrophotometric method is based on oxidation of methanol to formaldehyde with potassium permanganate and further reaction with chromotropic acid. 2 mL of 3 % potassium permanganate was added to 1 mL of previously diluted distillate and left for 30 minutes at room temperature, with occasional shaking.

After 30 minutes, approximate 0.05 g of sodium bisulphite (p.a.) was added to reduce the excess of permanganate and also 1 mL of 5 % aqueous chromotropic acid solution. After mixing, 15 mL of concentrated sulfuric acid was added and solution was heated in boiling water bath for 20 minutes.

Upon cooling to the room temperature, absorbance was measured at 575 nm against the reagent blank, and used for calculation of methanol content from the calibration curve previously prepared.

Determination of cyanides

Hydrocyanic acid in samples was determined using a silver nitrate titration with *p*-dimethylaminobenzylidene rhodanine indicator (EPA, 1996; APHA, 2005). This is the official method used in laboratory for food analysis in Federal Institute for Public Health in B&H.

Determination of urea

Urea in samples was determined using spectrophotometric method previously published and originally proposed for the determination of urea in dermatological formulations and cosmetics (Bojic, Radovanovic, Dmitrijevic, 2008). In summary, 1 mL of native sample was transferred to volumetric flask and 1 mL of 2.33 M HCl and 1 mL of methyl orange solution (0.6 mM) was added. The solution was diluted with approximately 2 mL of water and after addition of 1 mL of potassium bromate solution (1 mM), diluted with water to volume (10 mL). Absorbance was measured at 505 nm after 20 minutes, and content of urea was calculated from the previously prepared calibration curve.

RESULTS AND DISCUSSION

Calibration curves were linear in range 100-1000 mg/L for methanol (R^2=0.999) and in range 10-100 mg/L for urea (R^2=0.994).

The content of hydrocyanic acid and methanol in samples was calculated and expressed in g/hL of pure alcohol (g/hL pa). The content of urea was calculated and expressed in mg/L of native sample (Table 2.).

The content of methanol ranged from 67.482 to 1162.2 g/hL pa, with the average of 688.24 g/hL pa.

The levels of hydrocyanic acid in analyzed samples ranged from ND to 17.1 g/hL pa, with the average content of 8.96 g/hL pa. In 3 out of 15 samples (20 %) HCN was not detected.

Urea was detected in all analyzed samples (5.819 to 77.82 mg/L) with average concentration of 37.95 mg/L.

Table 2: Summary of analytical results

Sample	HCN (g/hL pa)	Methanol (g/hL pa)	Urea (mg/L)
1.	16.2	228.73	24.16
2.	5.01	955.51	63.62
3.	16.2	471.65	66.50
4.	ND	228.73	77.82
5.	10.8	67.482	5.819
6.	5.40	1162.2	7.340
7.	ND	561.82	75.20
8.	5.40	848.38	48.88
9.	17.1	545.84	32.57
10.	5.68	1099.9	60.33
11.	10.8	1024.3	19.21
12.	5.40	806.87	18.12
13.	ND	1098.7	32.48
14.	4.69	709.57	24.12
15.	4.80	513.97	13.04
mean	**8.96**	**688.24**	**37.95**
STD	**5.03**	**349.32**	**25.14**

Legend: 1- apple; 2,3-apricot; 4-cherry; 5,6-grape;7-juniper; 8-10 pear; 11-14 plum; 15-quince; ND-not detected

When grouping the samples based on the region of their origin and production, no significant difference was observed among mean values for the different toxic components (Table 3.).

Table 3: Levels of investigated toxic components in analyzed samples grouped based on the region of production

Region		Bosnia	Herzegovina
Number of samples		6	9
HCN (g/hL pa)	range	ND-10.8	ND-17.1
	mean±STD	5.24±3.43	8.45±6.84
Methanol (g/hL pa)	range	514.97-1098.7	67.482-1162.2
	mean±STD	874.62±207.13	563.99±378.55
Urea (mg/L)	range	13.04-63.62	5.819-77.82
	mean±STD	32.56±19.99	41.54±28.63

In general, the average content of methanol was higher in samples from Bosnia comparing to the samples from Herzegovina (874.62 g/hL pa vs. 563.99 g/hL pa), but still was lower than maximum concentration proposed by national Rulebook on strong alcoholic beverages (2012) (1200 g/hL pa for fruit spirits and 1000 g/hL pa for grape spirit) except for one grape spirit sample (1162.2 g/hL pa).

Other reports on average content of methanol in samples of apple, cherry, plum and pear spirits were similar to those found in our study (Bauer-Christoph, Watchter, Christoph, *et al*., 1997; Winterová, Mikulíková, Mazáč, *et al*., 2008). Content of methanol recorded in two samples of grape spirit was 67.482 and 1162.2 g/hL pa. This variation in results is probably due to different grape processing and the distillation techniques, as well as to the different grape variety used for production of spirits analyzed. These findings are similar to those previously published (Geroyiannaki, Komaitis, Stavrakas, *et al*., 2007). Average content of methanol in samples from Bosnia (874.62 g/hL pa) was higher than methanol content previously reported for spirits from Hungary (20-616 g/hL pa), Lithuania (ND-29 g/hL pa) (Lachenmeier, Sarsh, Rehm, 2009a), Ukraine (ND-262 g/hL pa) (Lachenmeier, Samokhvalov, Leitz, *et al*., 2010) and Poland (ND-757 g/hL pa) (Lachenmeier, Ganss, Rychlak, *et al*., 2009b). The highest content of methanol was found in plum spirits (mean value 909.86 g/hL pa), which is in line with previously published results for the methanol content in plum spirits originated from this region (Filajdic and Djukovic, 1973).The average content of HCN (8.45 g/hL pa) in spirits from Herzegovina was higher than maximum permissible level (MPL) proposed by aforementioned regulation (7 g/hL pa) and in 4 out of 9 samples (apple, apricot, grape and pear) content was almost two times higher than MPL. Only one sample from Bosnia (plum spirit) had content of HCN higher than MPL (10.8 g/hL pa). The average content of HCN in plum spirits was 5.22 g/hL pa, which is bit higher than previously published data for plum brandies from Poland and Slovakia (0.1 and 0.6 g/hL pa, respectively). According to Satora and Tuszynski (2008) these differences can be due to the different technologies of production.

The average content of urea in all analyzed samples was 37.95 mg/L, with highest content in cherry spirit (77.82 mg/L) and juniper spirit (75.20 mg/L). The lowest content was recorded for two grape spirits from Herzegovina (5.819 and 7.340 mg/L). These findings are similar to those reported by Polastro, Boso and Andrade-Sobrinho (2001) for distilled beverages from different parts of Brazil. Labanca and Gloria (2008) investigated 68 samples of sugar cane spirits from Brazil and in only 4% of the samples urea content was higher than 3 mg/L.

Urea is considered as one of the precursors to the formation of ethyl carbamate in fermented and distilled beverages. Ethyl carbamate can be also formed from the reaction of other precursors such as cyanide and ethanol (Jiao, Dong, Chen, 2014; Gowda, Sua, Karlovsky, *et a*l., 2018). According to Ayloit, Cochrane and Leonard (1990) monitoring of initial concentrations of ethyl carbamate precursors in freshly distilled spirits can be useful guide for predicting final ethyl carbamate concentration in mature spirit. The CONTAM Panel for a scientific opinion on the risks to human health related to the presence of ethyl carbamate and hydrocyanic acid in food and alcoholic beverages concluded that ethyl carbamate in alcoholic beverages indicates a health concern, particularly with respect to stone fruit brandies (EFSA, 2007). According to EFSA (2007), mitigation measures should be taken to reduce the levels of ethyl carbamate in certain alcoholic beverages such as fruit brandies, and such measures should focus on hydrocyanic acid and other precursors of ethyl carbamate to prevent the formation of ethyl carbamate

during shelf-life of these products. Despite the small number of samples, these are the first data on the toxic components of locally produced spirits. Considering that the consumption of these spirits is quite common in B&H, our findings are of great interest to the local community.

CONCLUSIONS

The toxic components that we have analyzed in our fruit spirit samples can pose a health hazard to consumers, especially if they are present in high concentrations. In one third of our samples, content of hydrocyanic acid was higher than MPL. The urea content of all the samples was relatively high, which can be of importance bearing in mind that urea is one of the most important precursors of ethyl carbamate. Since this is, to our knowledge, the first study that included a chemical analysis of homemade spirits in B&H, these results are of great importance for the further research that clearly needs to be undertaken.

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Summary/Sažetak

Glavni cilj ovog preliminarnog istraživanja bio je da se ispita prisustvo potencijalno toksičnih sastojaka (metanol, cijanidna kiselina i urea) u domaćim rakijama proizvedenim u različitim dijelovima Bosne i Hercegovine. Ukupno je analizirano 15 uzoraka voćnih rakija napravljenih od 8 vrsta voća (jabuka, kajsija, višnja, grožđe, šljiva, kruška, dunja i kleka). U pet od 15 ispitivanih uzoraka sadržaj cijanidne kiseline bio je iznad maksimalno dozvoljene koncentracije. Uopšteno, prosječni sadržaj metanola bio je viši u uzorcima sa lokaliteta u Bosni u odnosu na uzorke proizvedene u Hercegovini (874.62 prema 563.99 g/hl čistog alkohola), ali i dalje je bio ispod maksimalno dozvoljene koncentracije predložene državnom regulativom (1200 g/hl čistog alkohola za voćne rakije i 1000 g/hl čistog alkohola za lozovaču) osim za jedan uzorak lozovače (1162.2 g/hl čistog alkohola). Urea je detektovana u svim ispitivanim uzorcima (5.819 do 77.82 mg/l) sa prosječnim sadržajem od 37.95 mg/l. S obzirom da su ovo, po našem saznanju, prvi podaci o hemijskoj analizi domaćih rakija u BiH, od velikog su značaja za dalja obimnija istraživanja, koja je svakako potrebno provesti.

X-ray analysis of the paintning of artist Milenko Atanacković from the collection of the History museum of B&H

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Changes in mineral content in trainees' blood and urine due to high-intensity training

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Abstract: High-intensity training is becoming more popular nowadays when people have less time to engage in prolonged physical activity. Expertly led high intensity training is a safe way to achieve desired fitness goals. The aim of the study was to check if there were significant changes in the concentrations of sodium, potassium, calcium, magnesium, zinc, iron and copper in the blood and urine of twelve trainees after a short but intense training. Blood and urine sampling was performed before and after high intensity training where bodyweight exercises and exercises with external load were used. Statistical analysis was performed using paired t-test (2-tailed) with α=0.05 as statistical significance.

The results obtained showed that the measured mineral concentrations varied as a result of intense physical activity, but these variations were small and did not have a general trend of increase or decrease of analyzed mineral content. Based on these results, it can be concluded that, from the standpoint of the mineral concentrations loss, short high-intensity training is safe for the trainee's health.

INTRODUCTION

Inorganic compounds that are essential to the human body in terms of the metabolic support and function of various physiological processes required for life, growth and/or cellular functions are classified as minerals (Frausto da Silva and Williams, 2001; Lukaski, 2004). The insight into the relationship between health and mineral content, as a result of different types of training, has been intensively studied within the field of mineralomics (Yasuda, Yonashiro, Yoshida, et al., 2006; Uzun, 2013). Understanding of the mentioned interaction can be helpful in diagnosis and treatment of several illnesses caused by elevated or lowered mineral contents, by combining the mineralomic studies with traditional metabolomic studies (Coffey, Durkie, Hague, et al., 2013; Mainous, Wright, Hulihan, et al., 2014). The body's mineral demand is increased as a result of heavy exercise, regarding the minerals loss by kidneys or urine excretion (Keen, Gershwin, Lowney, et al., 1987; Chinevere, Kenefick, Cheuvront *et al*., 2008). A significant reduction in tissue's fat from the abdomen area, as well as a significant increase in the aerobic capacity of obese young men, is gained by high intensive interval training (HIT), 3 times a week, during 20 minutes within 12 weeks, showing the effectiveness of short, intense training (Heydari, Freund and Boutcher, 2012). Additionally, in comparison to the control group of women who performed long-term training of constant moderate intensity, a significant reduction in subcutaneous fat tissue and insulin resistance of women who performed HIT, 3 times a week within 15 weeks (both tested groups), was obtained (Trapp, Chisholm, Freund, et al., 2008). This implied that a higher intensity of short-term aerobic training was a more effective alternative to the moderate-intensity training used so far to regulate pressure. Therefore, recently HIT has gained popularity as an effective method of improving anaerobic as well as aerobic fitness (Burgomaster, Hughes, Heigenhauser, *et al*., 2005; Burgomaster,

Heigenhauser and Gibala, 2006) in only a few sessions, being widely used not only by healthy trained individuals, but also populations of patients with different metabolic disorders (Gibala, Little, Macdonald, et al., 2012). A combination of high anaerobic demand, mainly in the first bouts, and an increasingly high aerobic contribution, as the high intensity bouts are repeated, resulted in the effectiveness of this type of training stems (Bogdanis, Nevill, Boobis, et al., 1996; Parolin, Chesley, Matsos, et al., 1999). HIT is becoming popular for athletes and other populations with only a few conflicting facts about oxidative stress after an acute session (Bloomer, Falvo, Fry, et al., 2006; Deminice, Trindade, Degiovanni, et al., 2010; Farney, McCarthy, Canale, et al., 2012) or short-term training (Hellsten, Apple and Sjödin, 1996; Fisher, Schwartz, Quindry, et al., 2011). In general, the implementation of high intensity short term interval training improves the antioxidant status of healthy individuals, which supports positive effects not only on physical conditioning, but on overall health (Bogdanis, Stavrinou, Fatouros, et al., 2013). It has been proven that, unlike long running, most respondents perceive moderate intensity interval running as a training that gives greater comfort (Bartlett, Close, MacLaren, et al., 2011). The assumption is that high-intensity training is a safe way to achieve fitness goals in the case of advanced trainees. Cardio training involves exercises that are performed by repeated repetition over a long time (10-40 minutes) involving large muscle groups that require oxygen supply. The aims of this study were to get to know the extent to which changes in mineral content in the blood and urine occur after a short, but intense physical activity and to investigate the above assumption that professionally managed high-intensity training is a safe way of achieving fitness goals. The chosen minerals as the focus of this study were sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu) and zinc (Zn), while inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used for their quantification. Concentrations of seven minerals in urine and blood of twelve trainees were measured before and after high-intensity training. Statistical analysis was performed using paired t-test (2-tailed, paired).

EXPERIMENTAL

Instrumentation

Quantification of minerals in blood and urine was performed in the Laboratory of Quality Control of Rijeka Refinery, INA Oil and Gas Company by Teledyne Leeman Labs Prodigy ICP-AES instrument (Plasma source: RF generator with >78% power consumption; Generator: 27.12 MHz, 1.4 kW; Sampler: glass concentric nebulizer and glass spray chamber; Rinsing time: 20 s; Plasma gas: Argon (20 L/min); Detection system: Charge injection device (CID) cooled to -41°C; Softver: Salsa). Wavelenghts (nm) of tested minerals were: 589.592 (Na); 766.491 (K); 422.673 (Ca); 279.078 (Mg); 213.856 (Zn); 259.940 (Fe) and 324.754 (Cu). Radial plasma observing mode was applied for Na and K analysis. Digestion was performed by microwave oven (High performance Microwave digestion unit, mls 1200 mega, Gmbh). Additionally, analytical balance (Sartorius, d=0.001 g), Vacuette 2 mL (HKO medical systems) and Vacuette Quickshield Complete Plus (HKO medical systems) were used for experimental work.

Chemicals

High purity standard solutions (1000 μg/mL in 4% HNO_3) of Na, K, Ca, Mg, Fe, Cu and Zn were supplied by SCP Science, EU. Nitric acid (65%) was purchased from Zorka Pharma-Kemija, Serbia, while hydrogen peroxide (30%) was purchased from Kemika, Croatia.

Participants

Trainees who had participated in this study were healthy and fit individuals (nine women and three men) performing HIT 45 minutes once a week for last few years. Two trainees were over 48 years old and others were between 25 and 35 years old.
After a thorough explanation of the testing, training protocol, possible risks and the right to terminate participation at will; written informed consent was obtained from each participant for the use of their blood and urine samples.

Testing and training

The entire duration of the HIT had lasted 45 minutes (10 min for warming up + 30 min for main part and 5 min of recovery). A total of three training sessions were conducted with a one-week break. The high-intensity training consisted of changing the intervals of high loads and intervals of rest.
The intensity in the training process involved a load that the trainee was assigned in the form of a force to overcome or in the form of the speed at which the movement is performed. High load intervals consisted of applying great force in the case of an exercise with an external load (weights and kettle bells) or in the pursuit of achieving the highest speed in the case of bodyweight exercises (various jumps, running and as many repetitions of each exercise). Circuit type of training was performed. Work time was 30 seconds and rest time was 1 minute, regardless whether the exercises were used with external load or bodyweight. Breaks included an active rest such as light running and performing less demanding exercises. Prior to the experimental part, the optimal load (weight) which will be used for performing a number of repetitions of complex exercises was defined for each of the trainees. In the case of bodyweight exercises, trainees maintained a high level of perceived effort during the duration of the work interval with the use of heart rate monitoring, because heart rate and intensity of work are closely related.
At high intensity intervals, the heart rate was up to 80% of the maximum heart rate ($HR_{80\%}$). The maximum heart rate (HR_{max}) is the number of heart beats per minute

equal to the number obtained when the age is taken from the number 220, according to the following equation:
$HR_{80\ \%}=HR_{max}$ x 0.8=(220-age) x 0.8

Blood and urine sampling

Sampling of blood and urine was performed before and after HIT to find out whether acute electrolyte losses are caused by physical effort. The sampling procedure followed the principles of the Helsinki Declaration. Volume of single blood sample was 15 to 20 cm^3. Blood samples (n=24) and urine samples (n=24) of twelve fitness-active trainees were analyzed within three days and total of 72 blood samples and 72 urine specimens were included in the study.

Microwave digestion of blood samples

Blood samples (mass between 2 and 3 g with ± 1 mg of precision) were weighted into a Teflon vessel and 4 mL of HNO_3 (65%) and 0.25 mL H_2O_2 (30%) were added to each vessel. The containers were placed in a carousel and firmly sealed in a microwave oven at the digestion program: 5 minutes at 300 W and 2 minutes at 600 W.
Blood samples of twelve trainees were numbered from 1 to 12, while A and B marks a blood sample before training (BT) or after training (AT).

Urine samples treatment

A 5 mL volume of each urine sample was taken into a 10 mL bottle supplemented with 5 mL of HNO_3 (10%).

RESULTS AND DISCUSSION

Results of mineral concentrations in the urine of trainees before and after HIT

The concentration of analyzed minerals in the urine of individual trainees before and after HIT significantly varied, which is most likely affected by the degree of hydration, fluid consumption during and after training, the amount of excreted sweat (which depends on numerous factors with large individual differences due to physiology, diet, etc.) and similar. The obtained mineral concentrations in the tested urine samples before and after HIT in this study are shown in Table 1 and ranged as follows: sodium from 350 ppm to 2692 ppm; potassium from 157 ppm to 2785 ppm; calcium from 46 ppm to 292 ppm; magnesium from 12 ppm to 71 ppm; while the concentrations of zinc, iron and copper for all trainees were below 1 ppm. Table 1 shows the increase of sodium concentration for trainees who had less than 1000 ppm of Na before the exercises (No. 1, 2, 7, 8 and 12 trainees), except for trainee No. 9, where an insignificant decrease of sodium concentration has been obtained. For other trainees (with Na_{BT} concentration>1000 ppm), a significant decrease was obtained in the urine.
The potassium concentration was ≤1000 ppm for nine of twelve trainees before training (Table 1). The lowest concentration (<200 ppm) was recorded for trainee No. 1. In the case of trainee No. 4, K_{BT} was significantly higher than in other trainees (above 2000 ppm). In general, after training, an increase of potassium concentration in the urine of all trainees has been obtained. Decrease of potassium concentration was observed only for trainees Nos. 5 and 6, while for No. 9 it remained at the same level.
The calcium concentration in all urine samples ranged from 50 to 300 ppm. Concentration of Ca_{AT} increased for five trainees, while for seven it decreased. From the above, in general it can be concluded that HIT does not affect calcium concentrations in the urine.
Magnesium and zinc concentrations increased in the case of eleven of a total of twelve trainees, as a result of HIT. The opposite change only happened with trainee No. 10, for both minerals, which cannot be explained by any significant reason.

Table 1.Concentration (ppm) of seven minerals in 12 urine samples of trainees before (BT) and after training (AT).

Mineral	**Na** (ppm)		**K** (ppm)		**Ca** (ppm)		**Mg** (ppm)		**Zn** (ppm)		**Fe** (ppm)		**Cu** (ppm)	
	BT	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**
1	491	579	157	263	55	67	12	24	0.21	0.25	0.09	0.15	0.07	0.08
2	550	974	1222	1800	81	90	15	25	0.20	0.29	0.09	0.11	0.02	0.03
3	1997	1694	683	884	125	113	25	35	0.16	0.24	0.07	0.10	0.02	0.04
4	2054	1699	2162	2785	292	273	58	61	0.48	0.63	0.06	0.12	0.03	0.06
5	2692	2158	1557	1455	232	191	52	55	0.24	0.30	0.06	0.15	0.06	0.04
6	1124	840	633	437	82	71	14	15	0.12	0.14	0.13	0.09	0.02	0.07
7	350	615	700	1500	49	53	13	23	0.15	0.53	0.10	0.13	0.01	0.03
8	621	743	673	1001	46	64	10	30	0.11	0.15	0.07	0.07	0.02	0.02
9	741	715	673	667	175	170	62	71	0.15	0.26	0.09	0.28	0.05	0.02
10	2296	1317	1000	1398	240	140	40	36	0.24	0.13	0.09	0.08	0.02	0.02
11	1555	1282	636	660	132	104	23	23	0.34	0.36	0.08	0.08	0.01	0.04
12	915	1439	1015	1800	104	130	35	50	0.16	0.35	0.07	0.17	0.01	0.02
$\bar{x}$	1282	1171	925	1220	134	122	29	37	0.21	0.30	0.08	0.13	0.03	0.04

Calcium concentrations decreased for seven trainees, while magnesium and zinc concentrations increased in the case of eleven trainees. The difference is especially evident for trainee No. 10, so it can be noted that HIT had a much more significant effect on trainee No. 10, compared to other trainees.

The concentrations of iron in urine were less than 0.15 ppm and very uniform for all trainees, which was not the case for other analyzed minerals. After HIT, there was an increase in for seven trainees. However, for two trainees the iron concentration remained the same, while it decreased for two trainees. Characteristically, after training, a decrease of Fe, Zn, Mg, Ca and Na concentrations in the urine sample of No. 10 trainee, was obtained. It is noticeable that Cu concentrations were expectedly low and had not reached 0.1 ppm. Only trainees Nos. 5 and 9 had a decrease, while others had an increase or the copper concentration remained the same as before training. In general, the highest measured concentrations in the urine were for sodium and potassium, with almost negligible concentrations of zinc, iron and copper. Therefore, it can be concluded that after training an increase of mineral concentrations in the urine was more often observed. Increased copper concentrations were observed for ten trainees. Furthermore, nine of twelve trainees had increased potassium and iron concentrations after training, while higher concentrations of sodium and calcium were observed for five trainees.

There was also an increase of all tested mineral concentrations in the urine of trainees Nos. 1, 2, 7 and 12, after training. For the trainee No. 8, the concentration of five minerals has also increased and the two other minerals remained at the same concentration level. It is interesting that all of these were females.

The training had a different effect on the change in mineral concentrations for trainee No. 10 compared to the others. There was a decrease in the concentration of up to five minerals after training, the concentration of copper remained at same level and the only obtained increase was for potassium concentration. This trainee was much older than others, except trainee No. 2.

Analyzing the urine sample results after training (Table 1), it can be concluded that there was a change in mineral concentrations, but without any established trend (increase or decrease in general); which indicates that training had no decisive influence to the excretion of minerals.

Results of mineral concentrations in the blood of trainees before and after HIT

Analyzing the results in Table 2, it is clear that training did not significantly affect the change of sodium concentration in the blood of the trainees. For nine of twelve trainees there was an increase of sodium concentration in blood, while for other three trainees a decrease was obtained. Therefore, it cannot be said that HIT in general decreased or increased the sodium concentration in the blood. The effect of HIT on the trend of a change in potassium concentration in the blood of the trainees was even less relevant because six trainees had an increase and other six had a decrease of potassium concentration (Table 2).

For eight trainees, there was an increase in calcium levels in the blood, while the remaining four trainees had Ca_{AT} lower than Ca_{BT}. Concentration decreases were insignificant (only a few ppm) while increases for trainees Nos. 6 and 10 were significant (55 and 75 ppm, respectively).

Unlike the increase of sodium, potassium and calcium concentrations, the concentration of magnesium, zinc and iron decreased for most of the trainees. Magnesium concentration decreased in case of eight of twelve trainees, while nine of twelve trainees had zinc and iron decreased.

Table 2. Concentration (ppm) of seven minerals in 12 blood samples of trainees before (BT) and after training (AT).

Mineral	**Na** (ppm)		**K** (ppm)		**Ca** (ppm)		**Mg** (ppm)		**Zn** (ppm)		**Fe** (ppm)		**Cu** (ppm)	
	BT	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**	**BT**	**AT**
1	1379	1451	1264	1444	249	231	10.50	10.60	2.04	2.33	275	336	0.82	0.80
2	1463	1467	1409	1414	248	245	10.55	9.42	2.56	2.48	297	302	0.76	0.81
3	1350	1435	1362	1403	207	231	10.45	9.67	2.56	2.14	313	261	0.78	0.79
4	1412	1286	1355	1285	234	235	10.85	13.20	2.53	2.80	281	335	0.79	0.83
5	1342	1292	1368	1309	206	213	11.30	10.70	2.76	2.68	353	339	0.71	0.78
6	1294	1419	1348	1361	212	265	11.75	8.94	2.20	1.64	378	320	0.89	0.97
7	1332	1373	1160	1314	248	269	8.85	8.96	1.58	1.55	184	176	0.95	0.99
8	1415	1296	1387	1155	252	244	9.60	9.21	1.84	1.79	232	202	0.88	0.92
9	1332	1399	1282	1269	235	255	9.35	9.59	1.82	2.02	204	200	0.87	0.95
10	1299	1393	1275	1208	215	289	10.15	7.99	1.90	1.52	292	240	0.61	0.63
11	1166	1258	1231	1129	235	228	8.00	7.01	1.88	1.66	186	183	0.60	0.62
12	1205	1248	1108	1121	264	289	5.84	6.11	0.86	0.84	84	53	0.84	0.85
$\bar{x}$	1332	1359	1295	1284	233	249	9.77	9.28	2.04	1.95	256	245	0.79	0.83

Analyzing the data in Table 2, unlike for the previous six minerals, there was a major increase of Cu_{AT} concentration in the blood (in the case of eleven of twelve trainees).

Observing the individual HIT influence, some trends may be noticed for specific trainees. Therefore when certain mineral concentration AT decreased or increased in the case of trainee No. 7, the same happened with trainee No. 12. The same relations were observed in the case of eleven of twelve tested minerals for trainees Nos. 2 and 11, then for trainees Nos. 5 and 10, trainees Nos. 6 and 7 as well as for the trainees Nos. 6 and 12.

Another characteristic was noticed for trainee No. 8. After training, all mineral concentrations (except Cu) in the blood of this trainee have decreased. The highest measured concentrations in the blood were for sodium and potassium, while the copper concentration was the lowest one. Analyzing the results (Table 2), a similar conclusion as for the urine samples was obtained, meaning that there is no general trend of concentrations increasing or decreasing, which indicates that HIT had no key influence on contents of minerals in blood.

Statistic results for minerals in urine and blood

The influence of HIT on a significant change of the mineral concentration in urine and blood was estimated based on the paired t-test (2-tailed). The results of the t-test for urine and blood are given in Table 3.

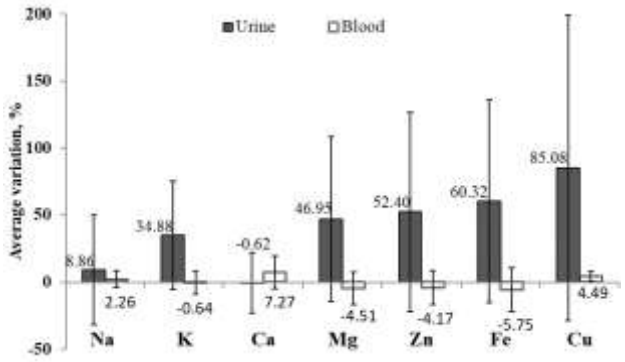

Figure 1. Average of variation percent in urine and blood.

As significance level, value α=0.05 was chosen. Therefore, only values with $p<0.05$ were considered statistically significant. Statistical data analysis obtained by determining contents of sodium, potassium, calcium, magnesium, zinc, iron and copper in the urine and blood of twelve trainees using the ICP-AES method showed that the HIT had a statistically significant effect on the change of potassium, magnesium, zinc and iron concentration in urine, as well as on the change of copper in the blood. However, due to the extremely low concentrations (<1 ppm) of zinc, iron and copper in urine, as well as for copper in blood, the statistical test cannot be considered relevant for changes in the concentration of these minerals in the given samples.

Figure 1 shows the average of variation percent of seven analyzed minerals in urine and blood after intensive training with a standard deviation.

CONCLUSIONS

The statistical data analysis obtained by determining the concentration of sodium, potassium, calcium, magnesium, zinc, iron and copper in the urine and blood of twelve trainees using the ICP-AES method showed that HIT had a statistically significant effect on the change of potassium, magnesium, zinc and iron concentration in urine, as well as on the change of copper in the blood. However, due to the extremely low concentrations (<1 ppm) of zinc, iron and copper in urine, as well as for copper in the blood, the statistical test cannot be considered relevant for changes in the concentration of these minerals in the named samples.

After the intense training, there was a change in the concentration of minerals in the urine and the blood, but the change did not have a general trend of increase or decrease of the analyzed contents of minerals. Therefore, it can be concluded that HIT does not have a key impact on the tested parameters.

In general, expertly led high-intensity training is a safe way to achieve fitness goals in the case of advanced athletes.

Table 3. Statistical values of analyzed minerals in urine and blood samples.

Mineral	Na	K	Ca	Mg	Zn	Fe	Cu
t test (urine)	0.389	0.013	0.237	0.003	0.024	0.031	0.115
t test (blood)	0.283	0.727	0.068	0.227	0.282	0.337	0.002

* the statistical analysis was performed using paired t-test (2-tailed) with α=0.05 as statistical significance

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Summary/Sažetak

Visoko intenzivni trening postaje sve popularniji u današnje vrijeme kada ljudi imaju sve manje vremena za bavljenje dugotrajnim fizičkim aktivnostima. Stručno vođen trening visokog intenziteta može biti siguran način postizanja fitnes ciljeva. Cilj istraživanja bio je da se provjeri dešavaju li se značajne promjene u koncentracijama natrija, kalija, kalcija, magnezija, cinka, željeza i bakra u krvi i urinu 12 vježbača poslije kratke, ali intenzivne fizičke aktivnosti. Uzorkovanja krvi i urina provođena su prije i poslije treninga visokog intenziteta u kojem su se koristile vježbe s vanjskim opterećenjem i vlastitom težinom tijela. Statistička analiza rezultata je napravljena korištenjem parnog t-testa (2-tailed) te je kao nivo značajnosti uzeta vrijednost α=0.05. Rezultati su pokazali da se koncentracija mjerenih minerala mijenja usljed naporne fizičke aktivnosti, ali promjene su male i nemaju generalni trend povećanja ili smanjenja koncentracije analiziranih minerala. Na osnovu ovih rezultata može se zaključiti da je, sa stanovišta gubitka minerala, kratak trening visokog intenziteta siguran za zdravlje vježbača.

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37-42

Determination of water content in infant formula

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Abstract: Water is one of the most important constituents of food, very important to be accurately quantified. Furthermore, water content affects the stability and shelf life of food. The evaluation of most chemical parameters is based on dry mass and many methods use heating which result in losing all volatile compounds, including water. Also, it is much harder to extract all of water if we have a complex matrix.

Regarding this, the aim of this study was to determine water content in different infant formula by various methods. For examination of water content in three different types of infant formula three different techniques were used (oven sample processor, drying oven and halogen drying) and compared to classical Karl Fischer titration with two different solvents. Each sample was measured in ten probes, and classical Karl Fischer titration was used as a reference. The results showed that the reference method was the best regarding speed of measurement, amount of sample needed and obtained water contents (3.01-4.35%), followed by Karl Fischer in boiling methanol (2.80-4.30), oven sample processor (2.96-4.23%), halogen drying (2.74-4.03%) and drying oven (2.38-3.52). Methods using heating could not remove all water from the sample within a reasonable time.

INTRODUCTION

The amount of water in food samples is one of the most commonly measured parameters, since it is a criterion of nutritive value, taste, shelf time, etc. (Isengard, 2001). Water is present in several different forms in almost all food. For instance, in dried products a small amount of water is present, while in beverages a very high amount of water is contained (Jurković, 2018). Determination the water content in food is not an easy task, especially if the sample has a complex matrix like infant formula. The results obtained should represent only the content of water without the content of volatile compounds, which is often not the case. On the other hand, it is difficult to evaporate all the water in complex samples. Thus, water quantification is a challenge for practical reasons. Furthermore, because of so many different methods for water content determination, it is a question which one gives the correct value (Isengard, 2001).

There are few main problems when we want to determine water content in food: loss of volatile compounds – by heating the sample (mass loss techniques); sample contamination; Maillard reaction, etc. (Jurković, 2018).

Additionally, since water could remain trapped in a complex sample, sometimes it is hard to evaporate all of the water in a reasonable period of time. Therefore, extraction water with a suitable solvent is a better way than heating it.

One of the most complex samples for determination water content is infant formula, which is a synthetic version of mother`s milk, known as a dietary substitute. The infant formula contains all compounds that are important for baby`s growth and development: including blending fats, proteins, minerals and carbohydrates (Kotb, Farahat, El-Daree, 2016). It is commonly produced on an industrial scale from cow milk (formulation of cow milk proteins).

On the other hand, the powder form is obtained by a spray drying process, which can ensure a pretty low moisture content. Depending on the children age, the formulas are divided into two basic types: a) products for newborns, b) products for children older than 4 or 6

months (Molska, Gutowska, Baranowska-Bosiacka *et al*., 2014).

According to Codex Alimentarius, infant formula is defined as a breast-milk substitute specially manufactured to satisfy the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding. An infant is a person not older than 12 months of age. Industrial product - infant formula has to meet some requirements, even it is processed by physical means it has to be adequate packaged to prevent spoilage and contamination under normal conditions of handling, storage and distribution in the country where the product is sold (Codex Alimentarius Commission, 2015). Furthermore, according to Codex Alimentarius, the moisture content of infant food has to be governed by good manufacturing practice for the individual product categories and should be at a level that provides minimum loss of nutritive value and within a level that microorganisms cannot reproduce (Codex Alimentarius Commission, 2017). For powdered milk products, moisture below 5% is recommended. Many non-European countries have standards for maximum moisture level in powdered infant food.

For instance, the allowed maximum level of moisture in India is ≤4.5% (FSSAI 2017), in China ≤5% (GB 10765 2011) and in east African countries ≤3% (EAS 78, 2006). Several studies have reported low moisture content in different infant food: 1.96% (Gasmalla Khadir, Musa et al. 2013), 0.42-2.55 (Kotb, Farahat, El-Daree *et al*. 2016) and 1.97-2.02% (Tham, Wang, Yeoh *et al.* 2016) and less than 2.5% (Semeniuc, Muste, Rotar *et al.* 2012). On the other hand, there is no literature data related to accurate water content analysis in infant food, which is the aim of this study.

EXPERIMENTAL

Material and methods

In this study we used different kinds of milk based infant formula, for different stages of baby`s development:

1) Infant formula 1 - for period after birth until six months of age
2) Infant formula 2 – for period between six and 12 months of age
3) Infant formula 3 – for babies older than twelve months

All tested kinds of infant formula had different amounts of proteins, sugars, fats and other constituents.

Oven drying; halogen drying, combined Karl Fischer titration with heating oven (oven sample processor) and Karl Fischer titration with boiling water were used for determination of water content and compared to classical Karl Fischer titration, which was used as a reference.

Karl Fischer titration – reference

This is a direct method based on a chemical reaction that is quantitative and selective for water determination (Isengard, King, Reh, 2006; Isengard, Präger, 2003). Measurements of water content were carried out on Titrando 890 Metrohm (Metrohm, Herisau, Schwitzerland) equipped with a volumetric Karl Fischer titration cell and thermostat, using Solution 1- which is a mixture of hydranal (Riedel de Haën) and formamide (Riedel de Haën), as a solvent – reference.

The second solvent used was boiling methanol (Riedel de Haën) – not a reference.

Application and optimisation of applied methods

Karl Fischer titration

The extraction of water from the sample requires the selection of the right solvent (Isengard, 2008). After several solvents used for testing (Schöffski, 2001), a mixture of hydranal and formamide was selected for the first titration and only boiling methanol for the second. Boiling methanol vapour can absorb water from all parts of the titration cell back to titration. In addition, the extraction time should be enough to ensure the quantitative extraction of water from the sample. Prior to the Karl Fischer titration, instrument calibration was performed using a standard with a known amount of water (10.00%). Liquid standards were introduced into the sample cell with a 10 cm syringe needle, while the sample (0.02 to 0.03 g) was introduced by spoon specialized for Karl Fischer titrators. The syringe mass was measured before and after the addition of the sample to the titration cell, followed by 20 s of mixing. The additions of titrant were larger at the beginning, while close to the endpoint (potentiometrically determined) the additions were smaller (0.001 mL). For infant formula samples titrations, the duration was around 450 s (7.5 min), until all water was extracted. Each sample was measured in ten probes.

Oven drying

Mass loss measurements (subtraction of sample mass measured before and after heating) were performed at 105°C using a laboratory oven, Binder FDL 115 (Binder, Mount Holly, USA). The main problems during heating could occur because of volatilization of compounds which gain higher results or the insufficient evaporation of water.

Classical Oven is an old and very well-defined method, easy to use for determination of mass loss, but time consuming. For better distribution of sample, pre-dried silicate sand was used. Lactose standard (with 5.05% of water, Fluka) was used for determination of water content.

The analyzed samples (2.000-4.000g) weighted into glass weighting bottles were mixed with pre-dried sand and dried at 105°C, until a constant mass was obtained.

Halogen drying

In order to rapidly determine the moisture content, a thermo gravimetrically halogen drying method could be used (Morales, Van Boekel, 1998). These measurements were made using a Sartorius MA 40 (Sartorius, Göttingen, Germany). The drying process is highly dependent on the radiation temperature and the distribution of the sample in the sample holder. The distribution of the sample on the sample plate should be even in all parts of the plate. In the case that samples would not be distributed in the layers of similar thickness, it is hard to evaporate the water from the thicker parts.

Therefore, halogen drying was carried out at 100°C within 60-70 min and sample was inserted in the sample holder with a plastic spoon (1.000-2.000g).

Combined Karl Fischer titration

Vaporization of water from a sample and Karl Fischer titration are combined within this direct method (Felgner, Schlink, Kirschenbühler *et al.*, 2008; Kestens, Connely, Bernreuther, 2008). A sample is heated in the oven (774 Oven Sample Processor, Metrohm, Herisau Schwitzerland) and the formed water vapor is introduced into the coulometric Karl Fischer Cell by dried air as a gas carrier. Dry air is achieved by passing of air trough molecular sieves. Water that comes out of the sample is due to heating. However, some water can remain. Inside of this cell titrant is formed by electricity.

The main parameter which should be optimized is a temperature for water vaporisation. The temperature of measurement depends on the composition of the sample. Thus, the adequate temperature (120°C) was chosen by "temperature ramping" (1°C/min) from 20 to 250°C. Additionally, it was necessary to find the best flow rate of a gas carrier. Prior to sample analysis, water content was measured in blanks and standards. Lactose with known amount of water (5.05%) was used as standard, while blank was only vial with air. Other experimental conditions were previously reported in detail by Jurković (2018): measurement duration (65-100 min); stop criteria (absolute drift of 20 μg/min); sample mass (0.1500-0.2500 g) was introduced into the vial with a plastic spoon and the vial was closed. Since this method measures water in the air formed by heating the sample, content of water was measured in the closed vial.

Five blank probes were first measured, followed by ten probes of sample measurements. Results of the blank probe were subtracted from the result of sample measurement.

RESULTS AND DISCUSSION

Several different techniques were used to determine the moisture (water) content in infant formula samples, i.e. classic Karl Fischer titration with different solvents, combined Karl Fischer titration, oven drying and halogen drying. The results are shown in Figures 1, 2 and 3. All of these methods are used for moisture content, except Karl Fischer titration which represents the water content determination in food samples. The results obtained showed, as expected, that for all infant formulas analysed, the highest results were obtained when water content was determined or when Karl Fischer titrations were applied. However, analysing the results in more detail, it could be concluded that the moisture content determined by halogen drying is the closest to the Karl Fisher titration results, but is highly dependent on the radiation temperature as well as the expertise of analysts. Furthermore, analysing the results obtained by conventional oven drying, it could be concluded that the results were lower and that the 0.83% of difference in results represents only 81% of the result obtained by the reference method (classical Karl Fischer), which is a significant difference. Even more, other differences between methods were also shown in Table 1, and are related to time of measurement, mass of the sample and standard deviation. In that meaning, the best method recommended for water determination is Karl Fischer titration (classical and in boiling methanol), while the classical oven drying method is more suitable for labeling only the moisture content in different food samples.

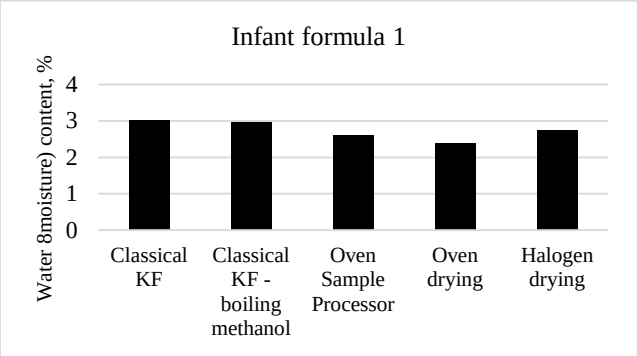

Figure 1. Water content in Infant formula 1.

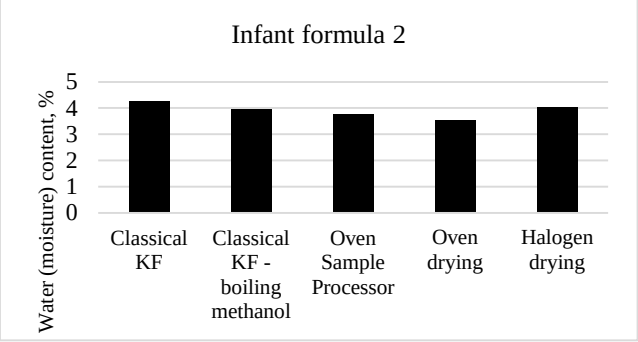

Figure 2. Water content in Infant formula 2.

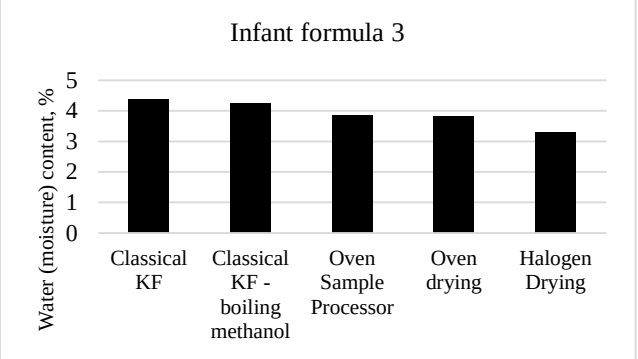

Figure 3. Water content in Infant formula 3.

The results of the water content for each sample together with main parameters of the analytical methods and the main statistical data are shown in Table 1. The results obtained by all the methods used were approved by Codex Alimentarius Commission (2015), which states that the water content of the infant formula should be below 5% and compared to the national standard in India and China where the water content should be ≤4.5 and ≤5% (FSSAI 2017; GB 10765 2011), respectively. Results of water content ranged between 2.38 and 4.35% (Table 1) and depended on the sample type and the method of water determination applied.

Table 1: Comparison of results of water content for each sample with different analytical methods

Infant formula 1					
	Classical KF	KF - boiling methanol	OSP	Oven drying	Halogen drying
Time	400 s	700 s	65-100 min	6 h	60-70 min
Mass	0.02-0.03g	0.02-0.03g	0.01-0.02 g	2-3 g	1-2 g
Maximum	3.10	3.16	2.63	2.48	2.87
Minimum	2.95	2.80	2.56	2.28	2.66
Median	3.01	2.97	2.59	2.35	2.74
Average	3.00	2.96	2.59	2.38	2.74
STDEV	0.04	0.12	0.02	0.09	0.08
Infant formula 2					
	Classical KF	KF in boiling methanol	OSP	Oven drying	Halogen drying
Time	450 s	650 s	65-100 min	6 h	60-70 min
Mass	0.02-0.03g	0.02-0.03g	0.01-0.02 g	2-3 g	1-2 g
Maximum	4.29	4.07	3.81	3.59	4.16
Minimum	4.18	3.81	3.74	3.38	3.95
Median	4.23	3.92	3.78	3.57	4.01
Average	4.24	3.93	3.78	3.52	4.03
STDEV	0.08	0.07	0.03	0.09	0.08
Infant formula 3					
	Classical KF	KF in boiling methanol	OSP	Oven drying	Halogen drying
Time	450 s	700 s	65-100 min	6 h	60-70 min
Mass	0.02-0.03g	0.02-0.03g	0.01-0.02 g	2-3 g	1-2 g
Maximum	4.45	4.30	3.87	3.34	3.93
Minimum	4.26	4.13	3.78	3.23	3.62
Median	4.36	4.23	3.83	3.27	3.86
Average	4.35	4.23	3.83	3.28	3.79
STDEV	0.05	0.06	0.03	0.05	0.13

The results obtained were much higher in comparison to the results of the water content reported in the literature, i.e., for powdered infant formulas reported by other authors, the water content varied between 0.42 and 2.55% (Kotb, Farahat, El-Daree, 2016; Tham, Wang, Yeoh *et al*. 2016; Gassmalla, Khadir, Musa *et al*. 2013; Semeniuc, Muste, Rotar et al. 2012), depending on country of origin, product type (cereal or milk based) and producer. As mentioned above, certain differences in the methods used are mainly related to the amount of water (moisture), speed of measurement and precision. Regarding the time, the fastest method was Karl Fischer titration method (measurements were up to 8 minutes), while the longest method was the Oven drying (6-8 hours). Additionally, the Classical Karl Fischer method could be approved and be even shorter, with applying higher temperature of analysis, since the extraction of water from the samples depends on temperature.

Furthermore, Karl Fischer in boiling methanol showed lower results for water content comparing to the reference method (classical Karl Fisher method) which implies that methanol is not a better solvent than Hydranal (used in classical Karl Fischer titration) for extraction of water from infant formula samples. Additionally, for the combined Karl Fisher titration method and the heating method (Oven sample processor) the main disadvantage is the time of measurement and other parameters that should be controlled what makes the method complicated. Also, it is not possible to have absolutely dried air as a carrier. Although the determination of water seems to be one of the simplest parameters that can be determined, the difficulties mentioned above by applying different methods as well as the analysis of a complex matrix, i.e. infant formula, requires more attention and expertise from analysts to distinguish which kind of method is approriate for water and moisture determination.

Precision of methods

Under the described conditions, ten portions of each sample (Infant formula 1, 2 and 3) were analysed for the determination of water content by various methods. The lowest STDEV of all analysed samples was obtained from the results of the method of Oven sample processor (0.02), followed by the Classical Karl Fischer titration method (0.04). The standard deviations obtained indicate that both methods have very good repeatability and precision.

Furthermore, a comparison of obtained results of four tested methods (KF with boiling methanol, Oven sample processor, Oven Drying and Halogen Drying) with the Classical Karl Fischer titration method for water determination was performed by t-test analysis at 95% confidence level. These values revealed that there was no good agreement for the water determination between the four methods and the Classical Karl Fischer method as the reference method. Additionally, there was a significant difference between the results by performing t-test at 95% confidence level. Unfortunately, regarding obtained results, it can be concluded that with these four tested methods, all the water from samples could not be removed.

CONCLUSION

Lower results of water content are achieved after application of methods based on sample heating, because it is hard to evaporate all the water in a reasonable period of time. Also, the formation of crust on the surface od the sample blocks water from evaporating. Due to the complex matrix of infant formula, the reference method (KF) showed the best results.

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Summary/Sažetak

Voda je jedan od najvažnijih konstituenata hrane, stoga je vrlo bitno njeno precizno kvantificiranje. Nadalje, sadržaj vode utiče i na stabilnost i rok trajanja hrane. Kako se određivanje većine hemijskih parametara temelji na promjeni suhe mase, mnoge metode upravo koriste zagrijavanje koje rezultira gubitkom svih hlapivih spojeva, uključujući i vodu. Također, mnogo je teže izdvojiti svu vodu ako je uzorak složenog matriksa. S tim u vezi, cilj ovog rada bio je utvrditi sadržaj vode u različitim formulama za dojenčad različitim metodama. Za određivanje sadržaja vode u tri različite vrste formule za dojenčad, tri različite tehnike su korištene tehnike (kombinirana Karl-Fischer-ova titracija nakon sušenja uzorka u peći, klasično sušenje uzorka u peći i sušenje halogenom) te uspoređene sa klasičnom Karl Fischer-ovom titracijom sa dva različita rastvarača. Svaki uzorak je mjeren u deset paralelki, a klasična Karl Fischer-ova titracija je korištena kao referentna metoda. Rezultati su pokazali da je klasična Karl Fischerova titracija (referentna) najbolja metoda u pogledu brzine mjerenja, količine potrebnog uzorka i dobivenog sadržaja vode (3.01-4.35%), nakon čega slijedi Karl Fischer-ova metoda u ključalom metanolu (2.80-4.30), kombinirana Karl Fischer-ova metoda nakon sušenja uzorka u sušnici (2.96-4.23%), metoda sušenja halogenom (2.74-4.03%), te metoda klasičnog sušenja u sušnici (2.38-3.52). Dobiveni rezultati potvrđuju da metode koje koriste samo sušenje ne mogu ukloniti svu vodu iz uzorka u razumnom vremenskom periodu.

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Authors are fully encouraged to use ***Cover Letter Template*.**

Manuscript preparation

The submitted articles must be prepared with Word for Windows. Manuscripts should be typed in English (either standard British or American English, but consistent throughout) with 1.5 spacing (12 points Times New Roman; Greek letters in the character font Symbol) in A4 format leaving 2.5 cm for margins. Authors are fully encouraged to use ***Manuscript Template***.

All contributions should be written in a style that addresses a wider audience than papers in more specialized journals. Manuscripts with grammar or vocabulary deficiencies are disadvantaged during the scientific review process and, even if accepted, may be returned to the author to be rewritten in idiomatic English. The authors are requested to seek the assistance of competent English language expert, if necessary, to ensure their English is of a reasonable standard. The journal maintains its policy and takes the liberty of correcting the English of manuscripts scientifically accepted for publication.

Tables and figures and/or schemes should not be embedded in the manuscript but their position in the text indicated. In electronic version (Word.doc document) tables and figures and/or schemes should follow the text, each on a separate page. Please number all pages of the manuscript including separate lists of references, tables and figures with their captions.

IUPAC and International Union of Biochemistry and Molecular Biology recommendations for the naming of compounds should be followed.

SI units, or other permissible units, should be employed. The designation of physical quantities should be in Times New Roman font. In text, graphs, and tables, brackets should be used to separate the designation of a physical quantity from the unit. Please do not use the axes of graphs for additional explanations; these should be mentioned in the figure captions and/or the manuscript (example: "pressure at the inlet of the system, kPa" should be avoided).

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Latin words, as well as the names of species, should be in *italic*, as for example: *i.e., e.g., in vivo, ibid, Artemisia annua* L., *etc*. The branching of organic compound should also be indicated in *italic*, for example, *n*-butanol, *tert*-butanol, *etc*.

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The manuscript must contain, each on a separate page, the title page, abstract in English, (abstract in Bosnian/Croatian/Serbian), graphical abstract (optional), main text,

list of references, tables (each table separately), illustrations (each separately), and legends to illustrations (all on the same page).

1. ***Title page*** must contain: the title of the paper (bold letters), full name(s) of the author(s), full mailing addresses of all authors (italic), keywords (up to 6), the phone and fax numbers and the e-mail address of the corresponding author.
2. A one-paragraph ***abstract*** written of 150–200 words in an impersonal form indicating the aims of the work, the main results and conclusions should be given and clearly set off from the text. Domestic authors should also submit, on a separate page, a Summary/Sažetak. For authors outside Bosnia and Herzegovina, the Editorial Board will provide a Bosnian/Croatian/Serbian translation of their English abstract.
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   - ***Results and Discussion***should include concisely presented results and their significance discussed and compared to relevant literature data. The results and discussion may be combined or kept separate.
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Examples of ***reference style***:

a) Reference to a journal publication:
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d) Reference to a proceeding:
Seliskar, C. J., Heineman, W.R., Shi, Y., Slaterbeck, A.F., Aryal, S., Ridgway, T.H., Nevin, J.H. (1997). *New spectroelectrochemical sensor*, in Proceedings of 37th Conference of Analytical Chemistry in Energy and Technology, Gatlinburg, Tenesee, USA, p.p. 8-11.

e) Patents:
Healey, P.J., Wright, S.M., Viltro, L.J., (2004).*Method and apparatus for the selection of oral care chemistry*, The Procter & Gamble Company Intellectual Property Division, (No.US 2004/0018475 A1).

f) Chemical Abstracts:
Habeger, C. F., Linhart, R. V., Adair, J. H. (1995). Adhesion to model surfaces in a flow through system. *Chemical Abstracts,* CA 124:25135.

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

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

- ***Tables*** are part of the text but must be given on separate pages, together with their captions. The tables should be numbered consequently in Latin numbers. Quantities should be separated from units by brackets. Footnotes to tables, in size 10 font, are to be indicated consequently (line-by-line) in superscript letters. Tables should be prepared with the aid of the Word table function, without vertical lines. Table columns must not be formatted using multiple spaces. Table rows must not be formatted using Carriage returns (enter key; ↵ key). Tables should not be incorporated as graphical objects.
- ***Figures and/or Schemes*** (in high resolution) should follow the captions, each on a separate page of the manuscript. High resolution illustrations in TIF or EPS format (JPG format is acceptable for colour and greyscale photos, only) must be uploaded as a separate archived (.zip or .rar) file.

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Journal accepts only TIF or EPS formats, as well as JPEG format (only for colour and greyscale photographs) for electronic artwork and graphic files. MS files (Word, PowerPoint, Excel, Visio) are NOT acceptable. Generally, scanned instrument data sheets should be avoided. Authors are responsible for the quality of their submitted artwork.

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The facility exist for color reproduction, however the inclusion of color photographs in a paper must be agreed with Editor in advance.

Reporting analytical and spectral data

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

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

3. ***NMR Spectroscopy***:
   ^{1}H NMR (500 MHz, DMSO-d_6) d 0.85 (s, 3H, CH_3), 1.28–1.65 (m, 8H, 4´CH_2), 4.36–4.55 (m, 2H, H-1 and H-2), 7.41 (d, *J* 8.2 Hz, 1H, ArH), 7.76 (dd, *J* 6.0, 8.2 Hz, 1H, H-1'), 8.09 (br s, 1H, NH).
   ^{13}C NMR (125 MHz, $CDCl_3$) d 12.0, 14.4, 23.7, 26.0, 30.2, 32.5, 40.6 (C-3), 47.4 (C-2'), 79.9, 82.1, 120.0 (C-7), 123.7 (C-5), 126.2 (C-4).
   Abbreviations: d, chemical shift in parts per million (ppm) downfield from the standard; *J*, coupling constant in hertz; multiplicities s, singlet; d, doublet; t, triplet; q, quartet; and br, broadened. Detailed peak assignments should not be made unless these are supported by definitive experiments such as isotopic labelling, DEPT, or two-dimensional NMR experiments.
4. ***IR Spectroscopy***:
   IR (KBr) n 3236, 2957, 2924, 1666, 1528, 1348, 1097, 743 cm^{-1}.
   Abbreviation: n, wavenumber of maximum absorption peaks in reciprocal centimetres.
5. ***Mass Spectrometry***:
   MS *m/z* (relative intensity): 305 (M^+H, 100), 128 (25).
   HRMS–FAB (*m/z*): $[M+H]^+$calcd for $C_{21}H_{38}N_4O_6$, 442.2791; found, 442.2782.
   Abbreviations: *m/z*, mass-to-charge ratio; M, molecular weight of the molecule itself; M^+, molecular ion; HRMS, high-resolution mass spectrometry; FAB, fast atom bombardment.
6. ***UV–Visible Spectroscopy***:
   UV (CH_3OH) l_{max} (log e) 220 (3.10), 425 nm (3.26).
   Abbreviations: l_{max}, wavelength of maximum absorption in nanometres; e, extinction coefficient.
7. ***Quantitative analysis***:
   Anal.calcd for $C_{17}H_{24}N_2O_3$: C 67.08, H 7.95, N 9.20. Found: C 66.82, H 7.83, N 9.16.All values are given in percentages.
8. ***Enzymes and catalytic proteins relevant data:***
   Papers reporting enzymes and catalytic proteins relevant data should include the identity of the enzymes/proteins, preparation and criteria of purity, assay conditions, methodology, activity, and any other information relevant to judging the reproducibility of the results[1]. For more details check Beilstein Institut/STRENDA (standards for reporting enzymology data) commission Web site (http://www.strenda.org/documents.html).

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

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The following list will be useful during the final checking of an article prior to sending it to the journal
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- Full postal address,
- Telephone and fax numbers,
- All figure captions,
- All tables (including title, description, footnotes),
- Manuscript has been "spellchecked" and "grammar-checked",
- References are in the correct format for the journal,
- All references mentioned in the Reference list are cited in the text, and *vice versa*.

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Submissions should be directed to the Editor by e-mail: ***glasnik@pmf.unsa.ba***, or ***glasnikhtbh@gmail.com***. All manuscripts will be acknowledged on receipt (by e-mail) and given a reference number, which should be quoted in all subsequent correspondence.

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