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CONTENT


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REMOVAL OF LEAD AND ZINC IONS FROM THEIR MONOCOMPONENT AND TWO-COMPONENT AQUEOUS SOLUTIONS USING SODIUM HYDROXIDE

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: The hydroxide precipitation method, using NaOH as a precipitant agent, was conducted to treat synthetic monocomponent and two-component water solutions of Pb^{2+} and Zn^{2+} with initial concentrations of 50 and 500 mg/l of each metal. The effect of pH and initial concentration of lead and zinc ions on their removal were investigated. The precipitation experiments were carried out by batch method that involves the mixing of NaOH with solutions containing metal ions to occur nucleation, solid growth and subsequent separation of precipitates from solution by filtration. The results showed that the removal efficiency was increased by increasing of pH and initial concentration of metal ions in their water solutions. Hydroxide precipitation method using NaOH is an efficient technique for the removal of lead and zinc ions from their monocomponent and two-component water solutions of different concentrations, with maximum removal efficiency in the pH range of 10.32 to 11.39.

KEYWORDS: heavy metal, chemical precipitation, wastewater, sodium hydroxide.

INTRODUCTION

Comprising over 70% of the Earth's surface, water is undeniably the most valuable natural resource existing on our planet [1]. However clean water supplies are under threat from urbanisation, industry and agricultural development [2]. Among various types of water pollutants, heavy metals are the largest class of contaminants and also the most difficult to treat [3].

Toxic metal compounds coming to the earth's surface not only reach the earth's waters (seas, lakes, ponds and reservoirs), but can also contaminate underground water in trace amounts by leaking from the soil after rain and snow [4]. With the rapid development of industries such as metal plating facilities, mining operations, fertilizer industries, tanneries, batteries, paper industries and pesticides, etc., heavy metals wastewaters are directly or indirectly discharged into the environment increasingly, especially in developing countries [5].

Because of their high solubility in the aquatic environments, heavy metals can be absorbed by living organisms [6]. The heavy metals linked most often to human poisoning are lead, mercury, arsenic and cadmium, while other heavy metals, including copper, zinc and chromium are actually required by the body in small amounts, but can also be toxic in larger doses [7]. The toxic metals and their ions are not only potential human health hazards but also to another life forms [8]. Therefore, the application of appropriate treatment methods is necessary for their removal from contaminated water.

Water and wastewater treatment has been widely investigated with different available techniques including precipitation, sedimentation, reverse osmosis, ion-exchange, membrane process, electrochemical and adsorption [9]. Among them, precipitation process is one of the common treatment methods that used for removal of heavy metals and other pollutants [10]. Precipitation has been long used for heavy metal removal, based on the addition of chemical reagents to induce an increase of pH value, in order to manage a destabilization of the electrical charges responsible for the retention of such cations in leachates and metal containing effluents [11]. In the precipitation processes, chemicals react with heavy metal ions to form insoluble precipitates that can be separated from the water by sedimentation or filtration and the treated water is then decanted and appropriately discharged or reused [12]. The precipitation can be carried out using hydroxide, carbonate or sulphide, depending on which type of precipitant is added to the waste water. Of all the treatment techniques, heavy metal hydroxide precipitation is the most commonly employed because of its low-cost and simplicity [13]. The solubility of the precipitation products and therefore the removal degree of heavy

metals from the waste water depends on the value of the pH, on the initial concentration of metallic ions in the solution, on the nature of precipitation agent and also on the nature and concentration of other chemical species which are present in the solution [14]. The solubility of metal hydroxides, depending on the water pH, is presented in Figure 1.

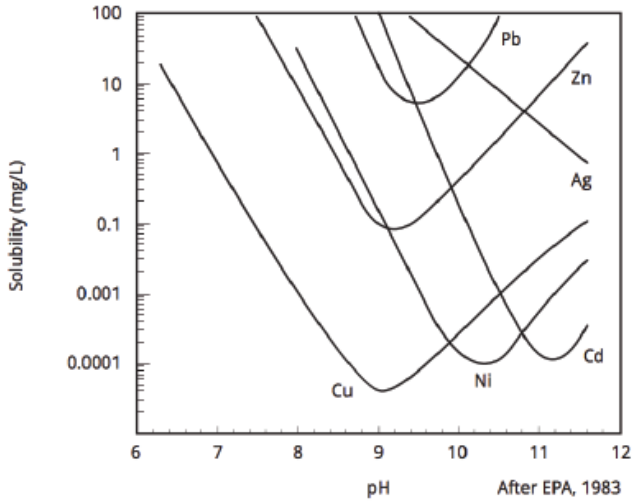

Figure 1. Solubility of metal hydroxides [15]

Given the above, the aim of this study was to determine the effects of pH and initial concentrations of Pb(II) and Zn(II) in water on the efficiency of lead and zinc removal from their monocomponent and two-component aqueous solutions using sodium hydroxide (NaOH).

MATERIAL AND METHODS

All chemicals used for the experimental part of the work were of analytical grade: Fluka sodium hydroxide, 1 mol/L, lead(II) nitrate (Alkaloid AD Skopje, Republic of Macedonia), zinc nitrate hexahydrate (Kemika, Zagreb, Croatia), lead standard solution 1,000 mg/L Pb in 0.5 M nitric acid (from $Pb(NO_3)_2$) and zinc standard solution 1,000 mg/L Zn in 0.5 M nitric acid (from $Zn(NO_3)_2$) from Merck, Nitric acid, min. 65% (Lach-Ner, Czech Republic). All glassware was first washed with detergent and rinsed with tap water, then soaked in water solution of HNO_3, and rinsed with deionized water.

Aqueous solution of sodium hydroxide with molar concentration of 0.1 mol/L was used as precipitant. The solution was prepared by dilution with deionized water. For each heavy metal, its monocomponent aqueous solutions of high and low initial concentrations (500 and 50 mg/L) were prepared. In addition, a two-component aqueous solution was prepared in which each metal had a concentration of 500 mg/L. Preparation of metal ion aqueous solutions was performed as follows. Metals, which are in the form of nitrate salts, were accurately weighed, then quantitatively transferred to volumetric flasks of 1L and diluted to the mark with deionized water. Each aqueous solution was homogenised, its initial pH value was measured and the chemical precipitation process was performed with these samples.

In order to evaluate the effect of pH on the removal efficiency, precipitation experiments were conducted with different pH conditions, by adding increasing quantities of the precipitation agent to monocomponent and two-component aqueous solutions of Pb^{2+} i Zn^{2+}. Sodium hydroxide volumes used in experiments are given in Table 1. The precipitation procedure was carried out by transferring 100 mL of metal ion solution of the appropriate concentration to a 250 mL glass. Then, a specific volume of precipitant was added to the glass and mixed with the solution by a magnetic stirrer at a rate of 300 rpm, and total mixing time was 5 min. After the required mixing time, the pH of the solution was measured and the filtration of aqueous solution of heavy metals was carried. The Whatman NO.42 filter paper was used to remove precipitates. Each filtrated sample was stored in polyethylene bottle till analysis.

Table 1. Volumes of NaOH added to monocomponent and two-component aqueous solutions of Pb^{2+} i Zn^{2+}

Initial concentrations of heavy metals in water	Volumes of 0,1 mol/L NaOH (ml) for precipitation of heavy metals					
500 mg/L Pb^{2+}	3.00	3.50	4.00	5.00	7.00	10.00
50 mg/L Pb^{2+}	0.06	0.10	0.20	0.60	3.00	6.00
500 mg/L Zn^{2+}	5.00	12.00	13.50	14.50	15.00	20.00
50 mg/L Zn^{2+}	1.00	1.30	1.50	2.00	5.00	10.00
500 mg/L Pb^{2+} 500 mg/L Zn^{2+}	1.00	5.00	10.00	20.00	50.00	100.00

Removal of lead and zinc from their aqueous solutions using sodium hydroxide was determined by analysis of initial Pb2+ and Zn2+ concentrations in samples before the treatment and their concentrations after the treatment with NaOH and filtration of samples.

Concentrations of metal ions were quantified by flame atomic absorption spectroscopy (FAAS), with air/acetylene type of flame. FAAS is of use in any analytical laboratory where elemental determinations are made [16] and generally, with air/acetylene flame lead and zinc can be determined [8]. The drawing of the calibration curve was performed using a 0.2, 1, 5, 7 and 10 mg/L standard solutions of Pb^{2+} and 0.2, 0.5, 1, 1.5 and 2 mg/L standard solutions of Zn^{2+} and measuring their apsorbance by FAAS. Obtained equations of calibration curves were

y = 0,0196x + 0,0035 for lead and

y = 0,4394x + 0,1256 for zinc.

The following equation was used for calculation of removal efficiency:

$$Er = \frac{C_0 - C_1}{C_0} \cdot 100$$

Where Er (%) is the removal efficiency, C0 (mg/L) is the initial concentration of heavy metal in untreated sample and C1 (mg/L) is the final concentration of heavy metal, after precipitation and filtration of the sample.

The effect of initial concentration on the removal efficiency was assessed by determining the removal efficiency from monocomponent aqueous solutions of 50 and 500 mg/L for each heavy metal, and from two-component aqueous solutions in which each metal had a concentration of 500 mg/L.

RESULTS AND DISCUSSION

In this study, the lead and zinc ion removal experiments from their monocomponent and two-component aqueous solutions were carried out using NaOH as a precipitant. Sodium hydroxide is commercially available low cost chemical, so it can be easily utilized as precipitating agent [17]. The efficiency results of removal of lead and zinc ions from their monocomponent aqueous solutions of high initial concentrations (500 mg/L) are presented in Fig. 2.

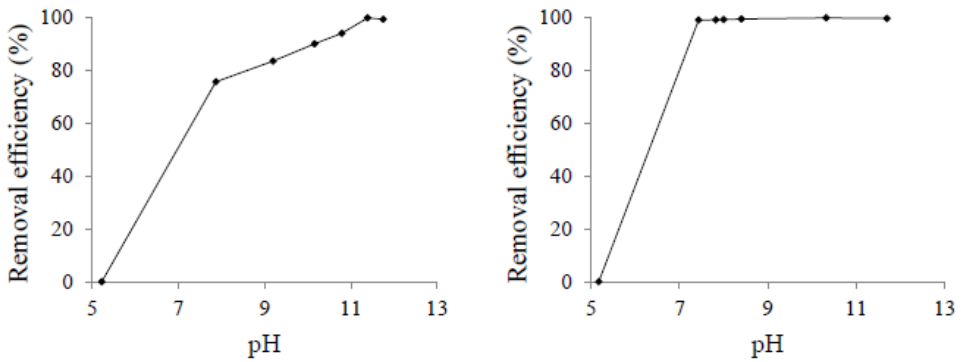

Figure 2. Effect of pH on the efficiency of removing a) Pb^{2+} and b) Zn^{2+} from their monocomponent aqueous solutions of initial concentrations 500 mg/L

Based on data from Tab. 1 and results from Fig 2. it can be seen that the increase in volume of precipitant added to samples of heavy metal aqueous solutions resulted in increased pH of the treated samples, which in turn resulted in increased efficiency of heavy metal removal. The higher removal efficiencies at high pH are related to high concentrations of OH^- ions in solution, which react with metal ions and convert them to insoluble precipitates that can be removed from the solution by filtration.

The removal efficiency for lead ions was above 90% at pH values beyond 10, while for zinc ions the efficiency was already 99.149% at pH of 7.43. This is in correlation with solubility of their hydroxides, presented at Fig.1. as zinc form insoluble precipitates at lower pH values compared to lead ions. The maximum removal efficiencies using NaOH were obtained for Pb^{2+} (99.866%) at pH of 11.39 and for Zn^{2+} (99.959%) at pH of 10,32. Chen et al. [12] conducted a hydroxide precipitation of heavy metals from their aqueous solutions of 500 mg/L concentrations. They found that the optimum pH values for chemical precipitation by sodium hydroxide were 10.5 for Pb^{2+} and 10 for Zn^{2+}. Adjustment of pH to the basic conditions (pH 9-11) is the major parameter that significantly improves heavy metal removal by chemical

precipitation [6]. In order to evaluate the effect of pH on the lead removal efficiency, Karimi [15] conducted hydroxide precipitation experiments with different pH conditions in a range from 3 to 11 and using $Ca(OH)_2$. He concluded that the optimum removal efficiency for actual and synthetic wastewater was at pH 9 to 11.

However, as the pH changed beyond the optimal value, removal efficiency for Pb^{2+} and Zn^{2+} was somewhat lower (99.397% and 07.047%). That is due to amphoteric properties of metal hydroxides, as precipitates of amphoteric metals like zinc and lead, tend to redissolve as the pH changes beyond the optimal range.

Efficiency results of removal of lead and zinc ions from their monocomponent aqueous solutions of low initial concentrations (50 mg/L) are presented in Fig. 3.

When hydroxide precipitation of heavy metals was carried out at lower initial concentrations of lead and zinc ions in their monovalent aqueous solutions (50 mg/L), maximum removal efficiencies were 98.256% for Pb^{2+} at pH of 10.63 and 99.694% for Zn^{2+} at pH of 10.83, which are lower values compared to those obtained at high initial concentrations of both metal ions (500 mg/L).

Similar observations are found in other works. Pang et al. [18] performed hydroxide precipitation on the selected heavy metal ions, using NaOH. The percent removals of Pb^{2+} were 96.9, 93.3, 69.0, and 98.3% for the initial concentrations of 14, 7, 3 and 1.5, mg/L, respectively.

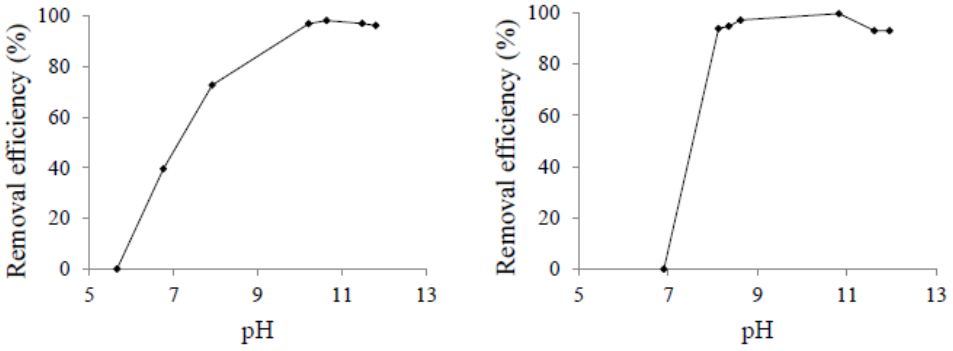

Figure 3. Effect of pH on the efficiency of removing a) Pb2+ and b) Zn2+ from their monocomponent aqueous solutions of initial concentrations 50 mg/L

Their results for percent removals of Zn^{2+} showed similar trend. Karimi [15] obtained the removal efficiencies of 88.8% and 75.5% at pH of 11, using $Ca(OH)_2$ for 600 and 300 mg/L Pb^{2+} initial concentrations. He proposed that the higher removal efficiency at higher concentrations relate to the formation of more and larger precipitates and agglomeration of these solids together.

From the Fig. 3. it can be seen that, in the case of zinc, significant increase in the efficiency of removal was achieved in the narrow pH range. Pang et al. [18] observed that the percent removal of Zn^{2+} increased tremendously from pH 5 to 7, i.e. an average increment of 53.6% removal was observed in that pH range for 5, 8, and 10 mg/L of Zn^{2+}. In this study, due to high initial pH of metal solutions of Zn^{2+}, the removal efficiency increase of 93.836% was achieved in pH range 6.9 – 8.13 50 mg/L. For both metals, due to the amphoteric properties of their hydroxides, the increase in pH above the optimum values resulted in reduced removal efficiency.

Efficiency results of removal of lead and zinc ions from their two-component aqueous solutions with 500 mg/L initial concentrations of each metal are presented in Fig. 4.

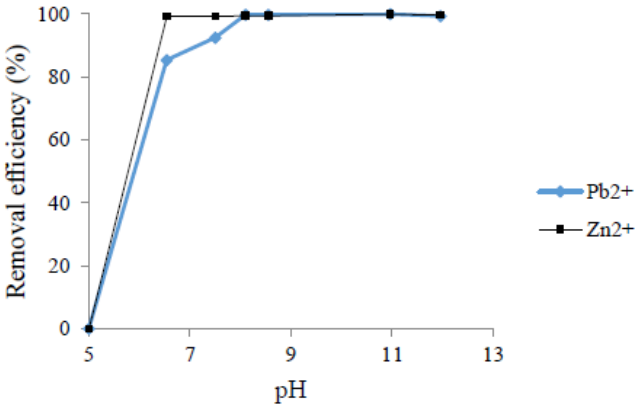

Figure 4. Effect of pH on the efficiency of removing Pb^{2+} and Zn^{2+} with initial concentrations of 500 mg/L from their two-component aqueous solutions

The results show a higher removal efficiency for Zn^{2+} at lower pH values , while with further pH increase, the percentage of Pb^{2+} removed is increased compared to the Zn^{2+}. These could be explained by amphoteric nature of lead and zinc. Mixed metals create a problem using hydroxide precipitation since the ideal pH for one metal may put another metal back into solution [19]. Maximum removal efficiencies for heavy metal ions from their two-component aqueous solutions were obtained at pH value of 10.96, and they were 99.983 % for Pb^{2+} and 99.930% for Zn^{2+}. Compared to the results obtained by precipitation of heavy metals in their low-concentration monocomponent aqueous solutions, the efficiency of removal was higher for both metals, which is consistent with the observations of higher removal efficiency for metals from their monocomponent aqueous solutions of high concentration.

CONCLUSION

Chemical precipitation of soluble metal ions as insoluble metal hydroxides is simple method for removing metal contaminants from water based on changing its pH value. Sodium hydroxide was found to be an effective and relatively low cost precipitant agent for the removal of Pb^{2+} and Zn^{2+} from their monocomponent and two-component synthetic water solutions of 50 and 500 mg/l initial concentrations of both metals. Maximum removal efficiencies for lead and zinc can be obtained in pH range of 10.32 to 11.39. Removal efficiency for both metals is higher when their initial concentrations in water are higher, and compared to lead, zinc can efficiently precipitate at lower pH values, i.e. with lower volume of added precipitant. However, amphoteric mixed metals such as lead and zinc, may each other influence on efficiency of their removal from water, since the ideal pH for precipitation of one metal, may re-dissolve another metal precipitate back into water solution.

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OPTIMIZATION OF HIGH PRESSURE HOMOGENIZATION IN THE PRODUCTION OF LIPOSOMAL DISPERSIONS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: Liposomes are spherical, biodegradable, and biocompatible vesicular systems. These vesicles are built from phospholipid double layers (membranes) surrounding the inner water phase. Liposomes are highly desirable as drug carriers because they can incorporate hydrophilic, hydrophobic and amphipathic drug substances (drugs). The physicochemical properties of liposomes such as size, charge, surface properties and encapsulation efficiency can highly influence their in vivo stability and kinetics.
The aim of our study was to prepare liposomal dispersions and to determine the influence of cycles of high pressure homogenization on some parameters, such as vesicle size and polydispersity index (PDI). Higher homogenization pressures and repeated recirculation led to further reduction in vesicle diameter and heterogeneity.
For preparing liposomal dispersions Phosal IP 40 and Phosal 75 SA were used (Lipoid, Germany). Liposomal dispersions were prepared according to the thin film hydration method. By sampling after each cycle, an estimate was made of how many cycles are needed for the dispersion to have satisfactory parameters (size and PDI).
The size and PDI analysis of the liposomes were carried out by using Zetasizer (Nano series) ZS 90, Malvern Instruments. . High pressure homogenization was carried out in 10 cycles and based on the obtained liposome size values and PDI, was determined how many cycles are needed in the process of homogenization. With each cycle, the size of the liposomes decreased and PDI value was reduced. It has been observed that after 5 cycles of homogenization there is no significant decrease in the size of the liposomes and PDI.
Therefore, in the further production of liposomes with active substances with these raw materials, is recommended to use only 5 cycles of homogenization

KEYWORDS: High pressure homogenization, phospholipids, liposomes, diameter and PDI

INTRODUCTION

The use of liposomes has been quite common lately due to their tendency to enhance the bioavailability and stability of drugs and cosmeticals. Liposomes are spherical, biodegradable, and biocompatible vesicular systems [1]. These vesicles are built from phospholipid double layers (membranes) surrounding the inner water phase. Due to the number of concentrically deposited bilayers, liposomes may be unilamellar, oligolamellar (several phospholipid bilayers) or multilamellar (many phospholipid bilayers). Their diameter ranges from 20 nm to 10 and more μm and they are forming spontaneously, by hydrating the phospholipids in an aqueous medium.

Liposomes are highly desirable as drug carriers because they can incorporate hydrophilic, hydrophobic and amphipathic drug substances (drugs), and they are also physiologically acceptable due to their similarity with biological membranes and biodegradability [2, 3]. Moreover, they are biodegradable, nontoxic, non-immunogenic and biocompatible compounds which can provide several advantages as carriers for the encapsulated molecules such as enhancing their pharmacokinetic and bio-distribution, decreasing their toxicity and providing target selectivity for them [4].

The physicochemical properties of liposomes such as size, surface properties and encapsulation efficiency can highly influence their in vivo stability and kinetics [5,6,7]. In addition, these properties can be modified simply by adding new ingredients to the lipid mixture before liposome preparation and/or by variation of preparation methods.

Various methods have been used for the preparations of liposomes which include mechanical methods like film and ultrasonic method, methods involving replacement of organic solvent, methods involving fusion of prepared vesicles or transformation of size by freeze thaw extrusion (FTE) and the dehydration-rehydration (DR) method [8].

Liposomes can therefore be manufactured with different size, ranging from several nanometers to micrometers [9]. The range of liposome preparation methods has recently been extended by a number of techniques which are based on the use of homogenizers. High pressure homogenizers are efficient multipurpose tools in pharmaceutical industry. Many stud-

ies have proven their convenience for the preparation of small and uniform vesicles. Higher homogenization pressures and repeated recirculation led to further reduction in vesicle diameter and heterogeneity.

According to Bernoulli´s law, static pressure within a fluid decreases at high velocity of flow. If the local pressure is falling below the steam pressure, bubbles filled with steam or gas arise and grow until re-elevation of the pressure causes their implosion. The surrounding water and lipid bilayers are accelerated toward the middle of the buble, followed by shock waves. This effects high local stress and the main results are reduction in the number of bilayers and decreasing diameters of the vesicles [10].

The reduction in size, broadness of the size-distribution, and lamellarity of „handshaken liposomes" during high-pressure homogenization depends on lipid composition, homogenization pressure and number of passages.

Preparation of liposomes with high-pressure homogenizers has many advantages. It avoids organic solvents or tensides, it is versatile in regard to choice of lipids and drugs, it achieves homogeneous distribution of binary lipid mixtures without prior dissolution in organic solvents, any concentration of lipids can be processed, vesicle formation is not dependent on salt concentration or pH of the aqueous solution, labile proteins and peptides can be entraped with low risk of loss of biological activity, the process may easily be scaled up, and the physicochemical stability of vesicles is not affected over at least 5 months [10].

The aim of our study was to prepare liposomal dispersions and to determine the influence of cycles of high pressure homogenization on some parameters, such as vesicle size and polydispersity index (PDI).

MATERIAL AND METHODS

MATERIALS

For preparing liposomal dispersions, phospholipids Phosal IP 40 and Phosal 75 SA were used (Lipoid, Germany).

PHOSAL IP 40	Phospholipids: Phosphatidylcholine + Lysophosphatidylcholine [%] n.l.t. 37; Non-polar lipids [%] 35-45; Ethanol [%] n.m.t. 5; Tocopherols [%] n.l.t. 0.05
PHOSAL 75 SA	Phosphatidylcholine [%] 72.0-78.0; Lysophosphatidylcholine [%] n.m.t. 6.0 Ethanol [%] 8.0-10.0

PREPARATION OF LIPOSOMAL DISPERSIONS

Formulation 1 – Phosal IP 40

Phosal IP 40	10 g
Aqua ad injectabilia	90 g

Formulation 2 – Phosal 75 SA

Phosal 75 SA	10 g
Aqua ad injectabilia	90 g

Liposomal dispersions were prepared by dissolving precise amounts of Phosal IP 40 or Phosal 75 SA in Aqua ad injectabilia. Phase mixing and homogenization was performed by Ultra Turrax Ika T25-Digital, 15 minutes at 5000 rpm.

After that, dispersions were homogenized in a high pressure homogenizer (Emulsiflex-C3, Avestin, Canada) at 500 bar in 10 cycles to optimize homogenization .

The samples were taken after each cycle . By sampling after each cycle, an estimate was made of how many cycles are needed for the dispersion to have satisfactory parameters (based on measurements of liposome size and PDI).

MICROSCOPY

The structures of liposomes were evaluated with optical microscope (BA310 Motic optical microscope, Speed Fair Co. Ltd., Hong Kong, China) at a magnification of 100 x. Microphotographs were taken with Optika Pro 3LT camera processed with Optika Vision Pro Software (Optika Microscopes, Ponteranica, Italy).

SIZE ANALYSIS AND PDI

The vesicle size and PDI analysis of the liposomes were carried out by using Zetasizer (Nano series) ZS 90, Malvern Instruments. The width of the size distribution was indicated by the polydispersity index (PDI). Samples were analyzed 24 h after preparation and diluted with Aqua purificata in a ratio of 1: 1000. All measurements were performed in triplicate.

RESULTS AND DISCUSSION

Both liposomal dispersions were milky dispersions, and the presence of liposomes was demonstrated by observation under a microscope at a magnification of 100 x (Figure 1).

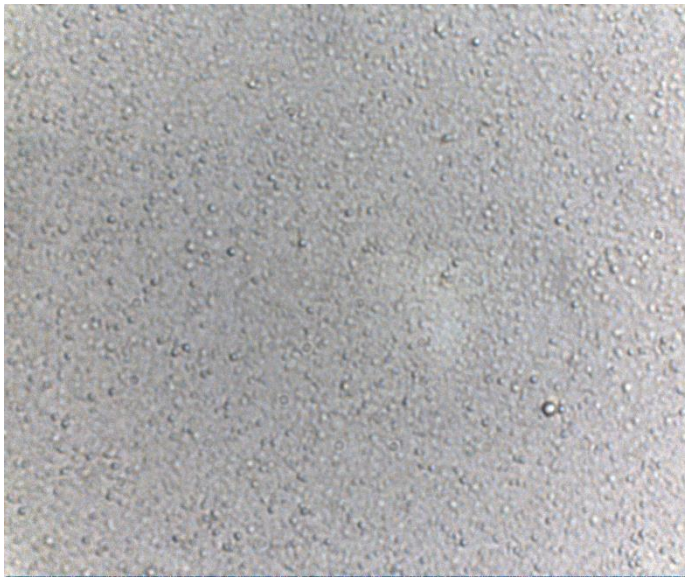

Figure 1. Liposomes under microscope at a magnification of 100 x (small round creatures are actually formed liposomes of different sizes)

Results of the characterization studies (size, polydisperzity index (PDI)) of liposomal dispersions are shown in Tables 1 and 2.

Table 1. Size analysis and PDI of liposomes with Phosal IP 40

Phosal IP40 (1:1000)	Size (nm)	PDI	PDI width
Cycle	385.4 ± 2.629	0.382 ± 0.013	238.1 ± 2.52
Cycle	340.9 ± 5.43	0.302 ± 0.033	187.2 ± 1.20
Cycle	319.1 ± 6.74	0.261 ± 0.010	162.9 ± 1.66
Cycle	306.3 ± 7.85	0.295 ± 0.028	220.6 ± 13.46
Cycle	294.9 ± 0.568	0.258 ± 0.019	149.8 ± 1.84
Cycle	288.1 ± 0.850	0.241 ± 0.006	141.5 ± 1.84
Cycle	283.5 ± 5.122	0.235 ± 0.015	137.4 ± 6.17
Cycle	279.6 ± 5.52	0.229 ± 0.012	133.6 ± 3.46
Cycle	270.9 ± 2.139	0.236 ± 0.014	131.5 ± 4.0
Cycle	257.6 ± 2.066	0.232 ± 0.021	124.0 ± 4.979

Table 2. Size analysis and PDI of liposomes with Phosal 75 SA

Phosal 75 SA (1:1000)	Size (nm)	PDI	PDI width
Cycle	269.8 ± 7.0577	0.395 ± 0.031	169.3 ± 2.25
Cycle	239.1 ± 2.074	0.293 ± 0.027	129.4 ± 7.012
Cycle	220.2 ± 1.102	0.291 ± 0.033	118.7 ± 6.514
Cycle	212.1 ± 5.749	0.283 ± 0.010	112.9 ± 1.626
Cycle	202.8 ± 5.424	0.260 ± 0.005	103.5 ± 3.58
Cycle	198.8 ± 0.723	0.263 ± 0.005	101.9 ± 0.862
Cycle	194.5 ± 1.793	0.249 ± 0.005	96.92 ± 1.269
Cycle	191.1 ± 2.136	0.243 ± 0.007	94.18 ± 1.67
Cycle	190.1 ± 2.042	0.246 ± 0.016	94.24 ± 3.99
cycle	190.7 ± 5.034	0.254 ± 0.012	96.11 ± 4.018

The aim of homogenization under high pressure is to reduce the size of the liposomes and to achieve homogeneity, as well as to increase the stability of liposomal dispersions. High pressure homogenization was carried out in 10 cycles and based on the obtained liposome size and PDI values, was determined how many cycles are needed in the process of homogenization.

The size of liposomes and PDI decreased significantly to 5 cycles, and the following values after fifth cycle were obtained. For Phosal IP 40 the size of liposomes was 294.9 ± 0.568, and PDI was 0.258 ± 0.019. For Phosal 75 SA the size of liposomes was 202.8 ± 5.424, and PDI was 0.260 ± 0.005.

The average size and size distribution of liposomes are important parameters especially when the liposomes are intended for therapeutic use by inhalation or parenteral route [11]. The liposomes with size ≤ 300 nm are able to deliver their contents to some extent into the deeper layers of the skin [12]. So for example, these liposomal dispersions could be used in the preparation of dermal preparations.

Polydispersity index is usually considered as an indicator of particles diameter distribution in a colloidal system. The lower level of this index, the more likely the particle diameter distribution is narrower, so the diameter of particles is more uniform. The value of less than 0.1 for this index shows a homogeneous population and a value greater than 0.3 indicates a high degree of heterogeneity [13]. The values of PDI were lower than 0.3, and the liposomal dispersions can be considered homogeneous.

In our research, with each cycle the size of the liposomes decreased and PDI value was reduced. It has been observed that after 5 cycles of homogenization there is no significant decrease in the size of the liposomes and PDI. Further recirculation can cause vesicle re-growth and therefore, no further homogenization is required after 5 cycles.

The length of homogenization is also very important, and should not be carried out in too many cycles, especially for dispersions with thermolabile active substances. Therefore, we recomend only 5 cycles of high pressure homogenization in preparation of these liposomal dispersions and their use in preparation of dermal products.

CONCLUSION

Based on the results of this research, it can be concluded:

- High pressure homogenization reduced the size of the liposomes and achieved homogeneity.

- The size of the liposomes and PDI values were suitable for further use in the preparation of pharmaceutical and cosmetic products.
- For obtaining liposomal dispersions of satisfactory characteristics, it was sufficient to do 5 cycles of high pressure homogenization, and therefore, in the further production of liposomes with active substances with these raw materials, is recommended to use 5 cycles of homogenization.

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THE EFFECTIVENESS OF NOVEL CHLORINE DIOXIDE IN DRINKING WATER DISINFECTION

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: The presence of *E. coli* in drinking water is not very common, however drinking water polluted with *E. coli* can lead to infection and could cause serious illness. Water contamination can lead to adverse health effects, including gastrointestinal illness, reproductive problems, and neurological disorders. More than 200 diseases are derived from polluted water. The main objective of present research was to evaluate the effectiveness of novel chlorine dioxide for the inactivation of *E. coli* in drinking water. Chlorine dioxide is made of two compounds: liquid sodium chlorite and solid sodium-peroxodisulphate »in situ«. Chlorine dioxide composition is in accordance with water treatment regulation [1]. In this experiment, different concentrations of chlorine dioxide were added at different temperatures in order to determine the optimal conditions for *E. coli* removal from drinking water. Results showed that optimal dose is 0.2 mg/L of chlorine dioxide at room temperature, while the same dose was effective at increased temperatures at 30 °C and 40 °C. The contact time was less than 1 min.

KEYWORDS: drinking water disinfection, *E. coli*, chlorine dioxide

INTRODUCTION

Chlorine dioxide is known disinfectant agent. It is formed after the reaction between $NaClO_2$ and HCl. Beside chlorine dioxide also, NaCl is formed. The chlorine dioxide solution is not stable due to ClO_2 volatility. ClO_2 is very reactive in gaseous state therefore, it is used as aqueous solution [2]. Chlorine dioxide is more effective at lower temperature at certain contact time. Experiments showed that it is effective already after 1 min. Besides temperature, other factors such as pH, contact time and load of organic matter may influence the actual biocidal efficiency of ClO_2 [3] The same authors also found that the efficacy of ClO_2 is decreasing with increasing organic load. Factor that affect the efficiency is also pH value dependent, however in neutral range around pH = 7 the effectiveness does not change much as seen from Figure 1.

Chlorine dioxide is an effective compound capable of destroying bacteria, viruses, fungi and other cellular pathogens. It is used for drinking water disinfection as well as for disinfection of fruit in food industry. [4] Since chlorine dioxide destroys pathogens by eliminating vital proteins, the microorganisms cannot resist it by nor of their resistance mechanisms. [4]. Human and animal cells are not free from the cell-killing effect of chlorine dioxide, however, it is not harmful to the animal organism in low doses.

Table 1 presents the comparison between different disinfection products. Despite their efficacy, the main limit of the currently applied disinfectants (e.g. ozone, hydrogen peroxide, peracetic acid) is that they are toxic even at low concentrations [4]. It is seen that ClO_2 has some advantages regarding pH value and low production of disinfection byproducts (DBP). Most of DBP are formed by using chlorine, such as trihalomethanes (THM), haloacetic acid (HAAs), chloramines, etc.

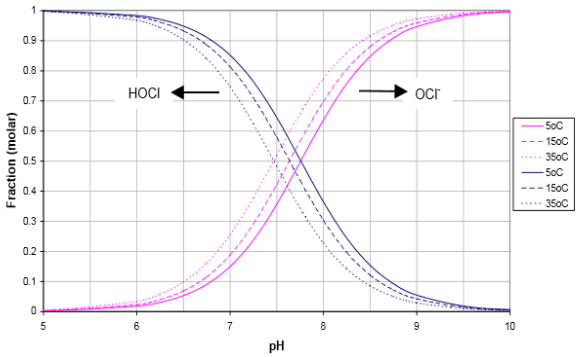

Figure 1. Effect of pH for the disinfection [5]

Table 1. Comparison of different disinfectant properties [6]

Chlorine	ClO_2	Ozone
residual	residual	-
High pH dependence	low pH dependence	middle pH dependence
DBP: THM, HAAs, etc.	chlorites	bromates
High bacteria inactivation	Very high bacteria inactivation	Very high bacteria inactivation

In drinking water, some coliform bacteria are present, among them *E. coli* in concentrations less than 50 CFU/100 mL. The amount of total organic halogen (TOCl) produced during ClO_2 oxidation was generally over ten times lower than that produced in chlorination [7] It was stated that humic acid (HA) could react with chlorine dioxide [7]. Phenolic moieties in humic acids were found to be the dominant fast-reacting precursors, responsible for the massive ClO_2^- formation in the first 5-min reaction if the concentration of humic acid was above 2 mg/L [7]. ClO_2 preferentially reacts with hydrophobic fractions of humic acids and decomposes high molecular weight fractions. Aromatic precursors (e.g., non-phenolic lignins or benzoquinones) contributed to ClO_2^- formation over longer reaction time (up to 24 h).

The aim of the work was to determine the efficiency of chlorine dioxide made in-situ on *Escherichia Coli* inhibition *(E. coli*). The bacteria waere chosen based on the fact that it is an indicator for water quality and the lifecycle of *E. coli* is longer than that of other bacteria [8]. A certain concentration of chlorine dioxide was added to constant number of *E. coli* colonies under different conditions, dependent on water temperature, chlorine dose and the concentration of organic compounds in water.

MATERIAL AND METHODS

MICROBIOLOGICAL EXPERIMENTS

The microbiological experiments were done in cooperation with National laboratory for health, environment and food (Maribor, Slovenia). Standard method ISO 9308-1 (2014) was used for determination of *E. coli*.

100 ml of water was filtered through 0.45 μm membranes. Membrane is placed on the surface of CCA medium and incubated at 36 ºC and 44 ºC for 24 h. The plates were examined for blue green colonies (indicating production of β-D-galactosidase positive and β-D-glucoronidase positive colonies have to be counted as *E. coli*. In the untreated water solution 200 colonies of *E. coli* were added. After different time units (1, 5, 10, 15 min) samples were taken and the number of colonies was measured as (CFU/100 mL).

PREPARATION OF CLO_2

The novelty of disinfection agent is that it could be prepared »in situ« very quickly and no safety precautions are necessary. Chlorine dioxide was made of two compounds: liquid sodium chlorite and solid sodium-peroxodisulphate »in situ«. Chlorine dioxide composition is in accordance with water treatment regulation [1]. In 100 mL of $NaClO_2$ 5,2 g $Na_2S_2O_8$ was dissolved. Such solution is suitable for disinfection of 1500 L of H_2O. The reaction is:

$$2\ NaClO_2 + Na_2S_2O_8 \rightarrow ClO_2 + 2\ Na_2SO_4 \qquad (1)$$

Producer Biostream (Zero, Germany) claims that the CLO_2 product does not depend of pH value.

CHEMICAL ANALYSES

Free and bound chlorine was determined with colorimetric DPD method, while the pH was measured using pH meter WTW, Germany. The absorbance (at 515 nm) using Agilent 8453 UV-Visible Spectrophotometer was measured after mixing regent with samples. Based on calibration curve the ClO_2 concentrations were determined. Excessive amount of NH_4Cl (21 mg) was added to another 10 mL of samples to stop free Cl_2 reactions before measurement of total chlorine concentrations. The total residual chlorine concentrations were also determined via absorbance at 515 nm after mixing total chlorine regent with samples. The free chlorine level was calculated by subtracting the residual ClO_2 from the total residual chlorine. The LOD of ClO_2 and free chlorine were 40 and 10 μg/ L, respectively. The analyses were performed at room temperature.

RESULTS AND DISCUSSION

Chemical parameters (total chlorine, free chlorine and ClO_2) did not change if disinfectant was added. The pH, as well as the concentrations of total and free chlorine remained the same after the disinfection. The pH value of the water was between 7.4 and 7.5. The values of total and free chlorine were below 0.1 mg/L. The value of chlorine dioxide remained the same after the disinfection: if 0.2 mg/L of chlorine dioxide was added it remained 0.2 mg/L after disinfection.

To the initial water solution was spiked with 200 colonies of *E. coli* were added. For disinfection 0.2 mg/L ClO_2 was used. The results are presented in Table 2.

From Table 2 it can be seen that already after 1 min, the disinfection was efficient and total *E. coli* colonies were inactivated. The results are consistent with another study where inactivation was reached after 1 min [3]. From Figure 2 it is seen that no *E. coli* colonies were detected.

Table 2. The results after disinfection at 0.2 mg/L ClO_2 at 20 ºC

Time (min)	*E. coli* (CFU/100 mL), 20 ºC
0	200
0.5	100
1	0
5	0
10	0
15	0

Figure 2. Plate with no detected *E. coli*.

In the following experiments, the dose was lowered at 0.1 mg/L ClO_2. The results are presented in Table 3. As seen from Table 3 the results are the same as presented in Table 2 at higher dose. *E. coli* colonies were not totally removed by lower dose. The optimum dose 0.2 mg/L ClO_2 is in accordance with previously reported values [8]

Table 3. Results after disinfection at 0.1 mg/L ClO_2 at 20 ºC

Time (min)	*E. coli* (CFU/100 mL)
0	200
1	3
5	1
10	1
15	1

Therefore, the experiments were performed at higher temperature to prove if the temperature effects the disinfection efficiency. The results with the 0.2 mg/L ClO_2 at 30 and at 40 ºC are presented in Table 4.

Table 4. Results after disinfection at 0.2 mg/L ClO_2 at 30 ºC and 40 ºC

Time (min)	*E. coli* (CFU/100 mL), 30 ºC	*E. coli* (CFU/100 mL), 40 ºC
0	200	200
1	1	1
5	0	0
10	0	0
15	0	0

As seen from Table 4 the results are similar as with higher dose and lower temperature. Since the initial value of 200 colonies is very low, it cannot be assured that the same concentration of ClO_2 would provide efficient inactivation at higher initial CFU. In the next experiment the temperature was increased to 40 ºC. The results were identical to those presented in Table 4. However, due to cost limitation we could not perform experiment with higher temperatures (above 40 ºC).

The literature claims that organic pollution could affect the disinfection efficiency [5]. The experiment was performed with water spiked with 0.1 mg/L and 0.2 mg/L of humic acid. The dose of 0.2 mg/L ClO_2 was added to each sample. The results are presented in Table 5.

Table 5. Results after disinfection at 0.2 mg/L ClO_2 and 0.2 mg/L and 0.1 mg/L humic acid

Time (min)	*E. coli* (0.1 mg HA) (CFU/100 mL)	*E. coli* (0.2 mg HA) (CFU/100 mL)
0	200	200
1	0	0
5	0	0
10	0	0
15	0	0

The results showed that low doses of humic acid do not affect the disinfection in low concentrations at 0.2 mg/L. More studies should be done in higher concentration range of humic acid since it was reported in literature that concentration above 2 mg/L of humic acid have major effect on ClO_2 efficiency [6]

CONCLUSION

In this experiment, different concentrations of chlorine dioxide were added at different temperatures in order to determine the optimal conditions for *E. coli* removal from drinking water. Results showed that optimal dose is 0.2 mg/L of chlorine dioxide at room temperature. The same dose was effective at increased temperatures at 30 °C and 40 °C. The contact time for efficient disinfection was less than 1 min. Humic acid did not affect the disinfectant efficiency in concentrations below 0.2 mg/L.

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SYNTHESIS OF GEL AIR FRESHENER AND ITS STABILITY

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT: Fragrance compounds have been used since antiquity to freshen the air or to mask the odours. Different types of air fresheners are known such as electric air fresheners with 30% market share, sprays, including aerosol air fresheners with 27%, car air fresheners with 16%, gel air fresheners with 9%, candle air fresheners and wax melts with 7 %, liquid air fresheners with 6% and others. According to research studies, in the United States, 34.7% of the population reported health problems, such as migraine headaches and respiratory problems, when exposed to fragranced products. Thus, there are numerous studies with strong evidence that fragmented products can trigger adverse health effects in the general population. Considering that air fresheners have been associated with adverse negative health effects that was the motive for proposing an alternative way of synthesis of gel air fresheners that is more green and more healthy. In this paper the gel air fresheners were synthesized by a simple and green sol-gel reaction using natural biodegradable polymer gelatin and lavender essential oil, as well as with natural banana aroma. The 3D structures of gel air fresheners of desired shapes and odours were obtained. The change in the 3D structure at room temperature was evident, probably as a result of thermal degradation and water evaporation. Anyway, the gel air freshener obtained with lavender essential oil is more acceptable to human health than commercially available ones. The results obtained in this study suggest that further improvement in stability should take place.

KEYWORDS: gel air freshener, synthesis, gelatin, lavender essential oil, natural banana aroma, health impact

INTRODUCTION

Fragrance compounds have been used since antiquity to fresh air and mask odours. For example, the ancient Egyptians were known to use musks and other natural materials to scent their tombs. Over the last 2,000 years a variety of compounds, including numerous spices and floral extracts, have been used for their ability to impart a pleasant aroma. However, the first modern air freshener was introduced in 1948. This product, using technology developed by the military to dispense insecticides, was a pressurized spray containing about 1% perfume, 24% alcohol or other solvents, and 75% chlorofluorocarbon (CFC) propellant.This was able to deliver a fine mist of fragrance that remained suspended in the air for a long period of time. This format of the product became the standard in the industry and sales grew tremendously. In the early 1950s, many companies began to add odour-counter-act ant chemicals to their formulas. These were chemicals that were intended to actually destroy or neutralize offensive odours, as opposed to simply masking them with fragrance. Perfumery houses showed these active chemicals were capable of reducing a variety of unpleasant odours, such as cigarette smoke, urine and faecal odours, cooking smells, and amine odours typically associated with fish. Compounds used for this purpose included various unsaturated esters, long-chain aldehydes and a few pre-polymers. In modern society, nearly all the people are familiar with air freshener. Air freshener is made from two major components. The first component is deodorant, which generally includes propellants, condensed gases, and substances which can have chemical reaction with sulphur compounds, ammonia, amines, formaldehyde, such as ferrous sulphate.

And the second component is freshener, which generally includes polyols, peppermint oil, essence and other substances.

In the last 25 years, aerosol air freshener formulas were modified to improve performance and reduce formula costs. The market significantly shifted away from aerosols, due to concerns about destruction of the ozone layer by chlorofluoro-carbons (CFCs).

While reformulation by the aerosol industry has kept this product form from disappearing completely, alternate air freshener delivery forms have become increasingly popular. In the 1990s, a resurgence in potpourri and candles lead to a host of new air freshening products. For example, Kalib Enterprises Ltd.'s Potpourri (Figure 1), which contains a blend of dry spices and herbs, uses a battery-operated fan to circulate fragrance throughout the room.

Figure 1. Potpourri packet [1].

Arizona Natural Resources Inc.'s Crystal Candle division has introduced candles that kill odours, as well as aromatherapy candles that have specific therapeutic uses.

One of the most innovative, and popular, new formats is Glade Plug-Ins, manufactured by S. C. Johnson of Racine, Wisconsin. By the late 1990s, sales of air fresheners in the United States had exceeded several hundred million dollars per year. One the most successful new products are Glade Plug-Ins (Figure 2), which use heat generated by electric current to vaporize air-freshening ingredients. It consists of a tiny plastic tray containing a gel-like fragrance concentrate.

Figure 2. Glade Plug-Ins fragrance. [2]

The consumer simply peels a multilayer barrier film from the top of the tray, leaving a permanent membrane layer that allows the fragrance to diffuse into the air. The tray is inserted into a warmer unit, which then is plugged into an electrical outlet. As the warmer unit heats up, fragrance permeates at a controlled rate through the film membrane, dispersing into the air [3].

The home air freshening sector is a multi-billion dollar industry, which isn't hard to believe given the staggeringly high number of air freshening devices available to consumers these days. From aerosols, sprays, and candles, to plug-ins, oils, time releasers, each with dozens of different scents to choose from, the options are overwhelming.

Getting a fresh and graceful fragrance is very important as soon as the brain receives smells there will be immediate memories and invocation create some distressing mood.

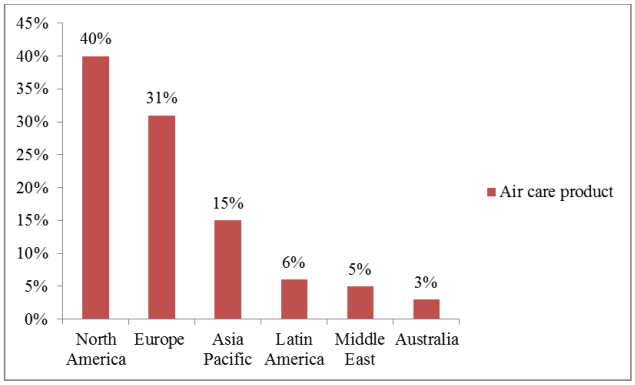

Figure 3. The worldwide representation of air care products

Air care product is a category which accounts for **9.5 billion USD globally (2016)**: North America – 40%, Europe 31%, Asia Pacific 15%, Latin America 6%, Middle East 5% and Australia 3% [4] (Figure 3). There are many different formats of air care products.

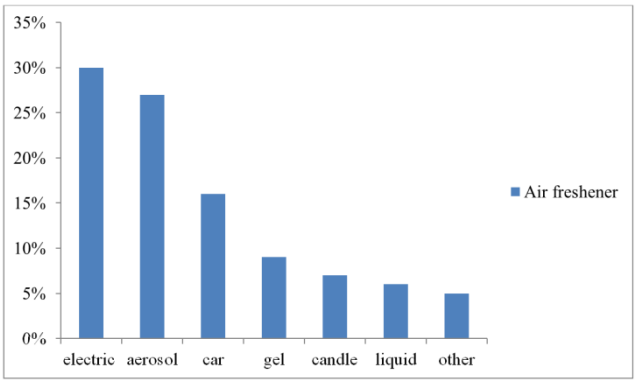

Figure 3. Different types of air fresheners

In figure 4 the percent of different types of air fresheners are shown. Electric air fresheners with 30% market share, now are fast growing because of increasing popularity of ultrasonic diffusers with some natural essential oils for this format. Sprays, including aerosol air fresheners 27% where we can observe growing importance of non-aerosol air fresheners and odour neutralizers. Car air fresheners with 16% share where especially new solid solutions

launched. Gel air fresheners – 9%, stable product with very good perspective in Asia where Indian market is the most important for category. Candle air fresheners and wax melts with 7%, where wax melts products are very popular in North America and gaining popularity in Europe and Asia Pacific. Liquid air fresheners with 6% are the category with time-honoured clients. Other category is about 5%. [4]

According to one survey, the most popular reason to use air fresheners is because users like the fragrance with response of 51% of answers. The next important reason to use air freshener is refreshing the air in room with 44%. Also, to create specific atmosphere with 28% and to connect functionality of air freshener as a decorative item in house.

Even 52% do not use air fresheners and the most common reason is preference to open the window. Second important reason not to use air fresheners is because the fragrances are too strong with 31% and conviction that these products are not environmentally friendly with 23%. For 20% air fresheners are expensive, while 18% argues that air fresheners are not good for health [4].

1.1 HEALTH IMPACTS

Frequent use of aerosols and air fresheners in the home may make babies and pregnant women ill. British researchers have found that chemicals in these products could be linked to headaches and depression in mothers, and to ear infections and diarrhoea in babies. Aerosols and air fresheners contain dozens of volatile organic compounds (VOCs) such as xylene, ketones and aldehydes, which can be toxic in high doses. VOCs are compounds with a low boiling point that form gas or vapour at room temperature. This make them excellent scent dispersal agent. Unfortunately, aerosols and air fresheners have been linked to an increased risk of asthma, particularly in children [5].

Britain is the biggest producer and user of aerosols in Europe, with the average British household buying 36 aerosol spray cans a year. In a survey of 14,000 pregnant women, epidemiologists at the University of Bristol found that those who used aerosols and air fresheners most days suffered 25 % more headaches than those who used them less than once a week. There was also a 19 % increase in postnatal depression among women who frequently used air fresheners. The study, which was presented at an international conference on indoor air pollution in Edinburgh, found that babies under six months old who were exposed on most days to air fresheners had 30 % more ear infections than those exposed less than once a week. There was also a 22 % increase in diarrhoea among babies frequently exposed to aerosols.
The biological mechanisms by which the chemicals may make people more susceptible to diseases still need to be worked out. However, experiments on mice suggest that the chemicals in air fresheners may weaken the body's defences by making the skin more permeable. A study in the US, also reported that mice exposed to VOCs from a solid air freshener experienced breathing difficulties. Rosalind Anderson from Anderson Laboratories, a private research facility in Vermont, believes that humans could be similarly affected.

According to Steinemann [6] research, who has extensively studied the health impacts of fragranced household products, one-quarter of the ingredients in air fresheners are classified as toxic or hazardous. Steinemann [6] also studied the multiple dimensions of exposures related to fragranced products and effects in the US population. 34.7 % of the population reported health problems, such as migraine headaches and respiratory difficulties, when exposed to fragranced products. Further, 15.1 % have lost workdays or a job due to fragranced product exposure in the workplace. Also, 20.2 % would enter a business but then leave as quickly as possible if they smell air fresheners or some fragranced product. Over 50% of the population would prefer that workplaces, health care facilities and professionals, hotels, and airplanes were fragrance-free. While prior research found that common fragranced products, even those called green and organic, emitted hazardous air pollutants, more than two thirds of the population were not aware of this, and over 60% would not continue to use a fragranced product if they knew it emitted such pollutants. Results from this study provide strong evidence that fragranced products can trigger adverse health effects in the general population. The study also indicates that reducing exposure to fragranced products, such as through fragrance-free policies, can provide cost-effective and relatively simple ways to reduce risks and improve air quality and health.

1.2 TOXICITY OF CHEMICAL INGREDIENTS IN AIR COMMERCIAL FRESHENERS

In 2010, the International Fragrance Association released a master list of over 3,100 chemicals that are used by most manufacturers. Chemicals on that list include carcinogens like *p*-dichlorobenzene and styrene oxide, endocrine disruptors like galaxolide and tonalide, reproductive toxicants like phthalates, problematic disinfectants like triclosan and ammonium quaternary compounds, and numerous allergens. A

fragrance can be made up of more than 100 chemicals and could include any of those harmful chemicals.

The Natural Resources Defence Council (NRDC) conducted the research and reveal that 86% of air fresheners tested contained phthalates. Phthalates are versatile chemicals, used as solvents in perfumes and fragrances, as softeners in plastics, as anti-foam agents in aerosols, and as sealants and adhesives. Given their many uses, phthalates are found in a wide array of consumer products, including cosmetics and fragrances, pesticides, pharmaceuticals, vinyl children's toys, automobiles, paints, and interior finishes. Phthalates are used in air fresheners to dissolve and carry the smell of fragrances. When people use air fresheners, the phthalates are released into the air. They may then be inhaled, or the aerosol particles may land on the skin and be absorbed. Unfortunately, the rise in popularity of air fresheners has outpaced awareness of the potential health threats from exposure to the chemicals they may contain. Most phthalates are well known to interfere with production of the male hormone, testosterone, and have been associated with reproductive abnormalities. Numerous animal studies have linked prenatal exposure to certain phthalates with decreases in testosterone, malformations of the genitalia, and reduced sperm production [7].

Even if phthalates are found not to be highly carcinogenic in humans, however, the UK's Public Health Centre for Radiation, Chemical and Environmental Hazards has revealed that air fresheners typically contain formaldehyde which is toxic compound.

The lowest concentration reported to cause sensory irritation of the eyes in humans is 0.36 mg/m^3 for four hours. Increases in eye blink frequency and conjunctival redness appear at 0.6 mg/m^3, which are considered equal to the no observed adverse effect level (NOAEL). A short-term (30-minute) guideline of 0.1 mg/m^3 is recommended as preventing sensory irritation in the general population. Evaluations of long-term effects, including cancer, based on a NOAEL and assessment factor approach, as well as estimates from the biologically motivated models, yield similar results, with values of approximately 0.2 mg/m^3 [8]. Formaldehyde is a well-known human carcinogen that has been definitively linked to cancers of the nose and throat. It is also known to cause ongoing irritation of the throat and airways, potentially leading to dangerous infections, frequent nosebleeds, asthma, and other respiratory ailments, reported the US government's National Toxicology Program. These risks are particularly elevated in the elderly, infants, and people with compromised immune systems. In fact, in 2013 has been reported in the International Journal of Public Health the study of more than 2,000 pregnant women and were found that women who used plug-in air fresheners during gestation were statistically far more likely to have babies that suffered from serious lung infections.

Wolkoff and Nielsen in 2017 [9] reviewed how the four major abundant and common airborne fragrances α-pinene (APN), limonene (LIM), linalool (LIL), and eugenol (EUG) impact the perceived indoor air quality as odour annoyance, sensory irritation and sensitization in the airways. They assessed breathing and cardiovascular effects, and work performance, and the impact in the airways of ozone-initiated gas- and particle phase reactions products.

Human exposure studies with mixtures of APN and LIM and supported by animal inhalation models do not support sensitization of the airways at indoor levels by inhalation that include other selected fragrances. Human exposure studies, in general, indicate that reported lung function effects are likely due to the perception rather than toxic effects of the fragrances. In general, effects on the breathing rate and mood by exposure to the fragrances are inconclusive. The fragrances may increase the high-frequency heart rate variability, but aerosol exposure during cleaning activities may result in a reduction. Distractive effects influencing the work performance by fragrance/odour exposure are consistently reported, but their persistence over time is unknown. Mice inhalation studies indicate that LIM or its reaction mixture may possess anti-inflammatory properties. There is insufficient information that ozone-initiated reactions with APN or LIM at typical indoor levels cause airway effects in humans. Limited experimental information is available on long-term effects of ozone-initiated reaction products of APN and LIM at typical indoor levels.

1.3 AN ALTERNATIVE WAY OF SYNTHESIS OF AIR FRESHENERS

Considering that air fresheners have been associated with adverse health effects, such as migraine headaches, asthma attacks, mucosal symptoms, infant illness, and breathing difficulties, it is challenge and need to find an alternative way of synthesis more green and more healthy.

1.4 GELATIN AS THE MAIN NATURAL POLYMER COMPOUND IN THIS SYNTHESIS

In this study, gelatin has been selected as the main ingredient in the recipe. Gelatin is a natural bi-

odegradable polymer. Gelatin, one of the most versatile, naturally occurring biopolymers, is widely used in food products and pharmaceutical dosage forms. Gelatin is composed of 50.5% carbon, 6.8% hydrogen, 17% nitrogen and 25.2% oxygen [10]. Gelatin is an animal protein prepared by the partial hydrolysis of collagen which is fibrous insoluble protein, isolated from animal skin and bones, with very dilute acid.

The rigid bar-like molecules which build collagen are arranged in fibres is interconnected by covalent bonds [11]. These molecules have three polypeptide chains arranged in a triple helix that is stabilized by hydrogen and hydrophobic bonds. The particular structure of the triple helix is due to repeating Gly-X-Y sequence (Glycine-proline-Hydroproline). Only the very short N- and C- terminal regions, called telopeptides, do not form the triple helical structure [12]. Lysine and hydroxylysine (Hyl) residues and aldehyde derivatives from them makes intra- and inter-molecular covalent crosslinks [13] which bonds hold the atoms or ions together as a compound. Gelatin is heterogeneous mixture of of α-chains (one polymer/single chain), β-chains (two α-chains covalently crosslinked) and γ-chains (three covalently crosslinked α-chains) [14] and containing between 50 – 1000 amino acids.

Typical gelatin structure is Ala-Gly-Pro-Arg-Gy-Glu-4Hyp-Gly-Pro- [15].

The triple helix of type I collagen extracted from skin and bones, as a source for gelatin, is composed of α1 (I) and one α2(I) chains, each with molecular mass ≈ 95 kD, with 1.5 nm and length ≈ 0.3μm. The triple helices are coated by a cylinder of hydration with their grooves filled with solvent molecules, and this coating maintains the collagen's conformation and mechanical properties

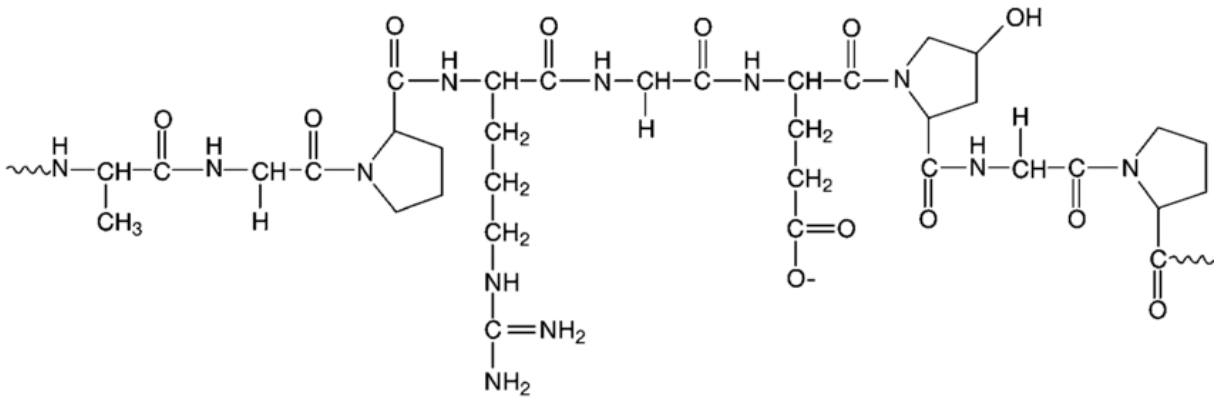

Figure 5. Chemical structure of gelatin [16]

1.5 LAVENDER ESSENTIAL OIL

Lavender (*lavandula angustifolia*) is the most versatile of all essential oils. The fragrance is calming, relaxing and balancing physically and emotionally.

Figure 6. Lavender oil. [17]

MATERIAL AND METHODS

Two samples of gel air fresheners with difference in odour and colour were prepared.

2.1 PROCESS OF SOL-GEL SYNTHESIS

In 50 ml of distilled water at room temperature was dissolved 15 g of gelatine. 150 ml of distilled water was boiled and removed from the heat. The gelatine solution was added to the boiling water and mixed until yellow homogeneous solution was obtained. In the first sample, 15 drops of natural lavender essential oil were added, without any colour added. In the first sample 1% of the salt was also added. In the second sample 15 drops of natural banana aroma were added and yellow food colour (on the top of the teaspoon). After well stirring the homogeneous mixture was pouring into a mould with different shapes and then left in the freezer for 2 hours to cool.

After cooling in the freezer the samples were taken out at the room temperature. The 3D structures of gel air fresheners of desired shapes and odours were obtained.

RESULTS AND DISCUSSION

Prepared gel air fresheners are shown in figure 7. It is possible to note the transition from sol to gel state. The formation of gel state is observed after 2 hours of freezing. Considering that gelatin is a polyampholyte it gels below 35-40 °C. The heterogeneous nature of the molecular weight profile of this biopolymer is affected by pH and temperature, which affects the noncovalent interactions and phase behaviour of gelatin solution [18]. Marty et al. 1978 [19] first observed that the complex phase behaviour of gelatin was affected by temperature and other experimental conditions. In line with this finding, Figure 7 and 9 confirms the influence of temperature on gel structure. According to literature data, the molecular weight profile of gelatin remained unchanged at pH 5.0 to 7.0. However, at pH < 6.0, the gelatin particles lacked any net charge, resulting in aggregation at pH > 7.5; the increased charge on the particles contributed to increased resistance to dehydration.

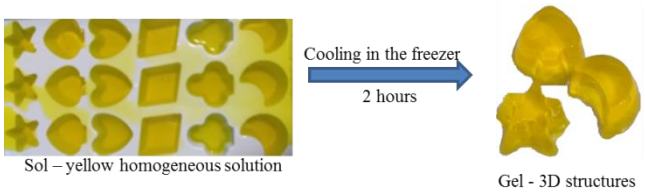

Figure 7. Sol-Gel transition and 3D structure formation.

3.1 THE TEMPERATURE INFLUENCE

At room temperature long strings of amino acids stick together in a formation called a triple matrix. This allows each chain to bond to several others, and form a complex 3D matrix. When gelatin is heated, bonds between the chains are loosen, turning the solid structure into a liquid. These gelatin chains have hydrogen atoms attached to their sides. These hydrogen branches can weakly bond with water. The oxygen atom is weakly bonded to the hydrogen atoms on the sticky-out branches of the chain. When the water receives a bit more energy by increasing the temperature, hydrogen bond will break, and the water molecule will drift away. As the water cools, it slows down until this weak bond can be re-established, linking the water to the gelatin chain again.

The stability of synthetized gel air fresheners at room temperature (around 28 °C) was studied few days. The results of the stability are shown in figure 8.

It can be observed that the 3D structure is distorted as time moves away, probably due to the loss of water by evaporation. After 84 hours at room temperature the mold starts to grow probably because of the presence of water. Knowing that tiny mold spores are all around us in the air, which is not harmful to our health in moderation. Once a spore lands on a surface, it searches for water and nutrients to feed off. Considering that mold needs water, food, suitable air quality and temperature in order to grow, this was excellent indicator that this system is not adequate and stable for the application at high temperatures. Consequently, the stability of the system synthetized has to be further improved.

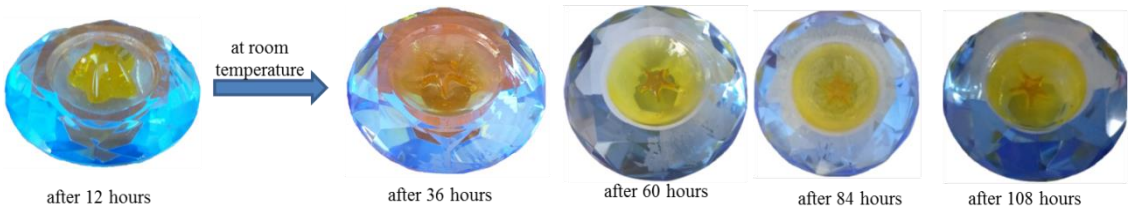

Figure 8. System stability over time.

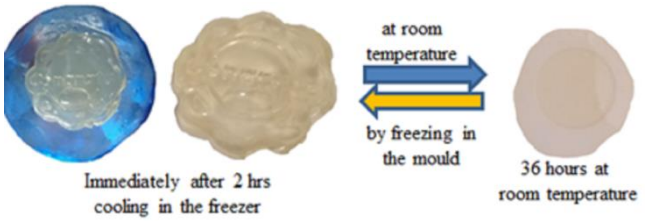

Figure 9. A reversible reaction.

It seems that the sample with lavender oil is less stable then the sample with natural banana aroma. In the figure 9 is shown that this reaction is reversible by changing the temperature.

CONCLUSION

The problem with commercially available fresheners is that they can potentially have harmful ingredients and it can be especially irritating for people

with asthma and allergies. With this in mind, the challenge for this type of research was the development of new natural air fresheners with essential oils such as lavender. In this paper the gel air fresheners were synthetized by a simple and green sol-gel reaction using natural biodegradable polymer gelatin and lavender as essential oil and natural banana aroma. The 3 D structures of gel air fresheners of desired shapes and odours were obtained. The change in the 3D structure at room temperature was evident, probably as a result of thermal degradation and water evaporation. Anyway, the gel air freshener obtained in this research is more acceptable to human health than commercially available ones. The synthetized gel air fresheners in this way should be naturally anti-fungal and anti-bacterial, very effective and easy to prepare. The results obtained in this study suggest that further improvement in stability should take place.

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FLUORIMETRIC PROFILES, FLAVONOID AND POLYPHENOLS CONTENT OF ACACIA, MEADOW AND HONEYDEW HONEY SAMPLES AND THEIR CORRELATION WITH COLOUR INTENSITY OF HONEY

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:
Introduction: Previous studies have showed that fluorimetric analysis may be used as a simple, rapid, low cost and reliable method for authentication botanical origin of honey. Primary aims of this study were to record fluorescence spactra of acacia, meadow and honeydew honey samples and to determine content of flavonoid and polyphenoles in relation to colour intensity of honey.
Material and methods: Fluorescence spectra of honey samples were recorded. Spectrophotometric analysis was used to determine flavonoid and polyphenols content. The honey color scoring was developed by the authors as the arbitrary system.
Results: Acacia honey showed high fluorescence emission intensity after an excitation at 340 nm, 390 nm and 440 nm. Meadow honey showed fluorescence after excitation at 390 nm and 440 nm, while fluorescence, caused by excitation at 340 nm, was absent. Honeydew honey showed low intensity of fluorescence at 440 nm excitation while fluorescence was absent at 340 nm and 390 nm excitation, respectively. Statistically significant difference was found for flavonoids and polyphenols levels, between honeydew and acacia honey. Statistically significant difference in polyphenols levels between meadow and acacia honey was found. There was no statistically significant difference of flavonoids and polyphenols between samples of meadow and honeydew honey.
Conclusion: Fluorimetric profiles, flavonoid and polyphenols content, together with colour intensity of honey may be useful in authenitication of botanical origin of honey.

KEYWORDS: florescence spectra, honey, botanical origin, flavonoids, polyphenols

INTRODUCTION

Honey is a natural product derived from honeybees from the nectar of flowers with a wide range of minor constituents with antioxidant properties such as flavonoids, certain enzymes (glucose oxidase, catalase), amino acids and proteins. Generally, antioxidants play an important role in food preservation and human health by combating damage caused by oxidizing agents. Over the past decades numerous methods and analyses were developed for determining botanical origin of honey and its correlation with phenolic and flavonoid contents as antioxidants of honey [1]-[5].

Numerous different methods which are considered to be the traditional methods for authenticating the botanical origin of honey require considerable sample preparation, highly skilled personnel, and are also time-consuming and costly [6]. Previous studies have showed that fluorescence spectroscopy in honey analyses has many advantages compared to other methods such as better sensitivity, little sample preparation and rapid, simple and low-cost usage [7], [8]. Primary aim of this study was to record fluorescence spectra which were, according to our previous study, critical for estimation of botanical origin analyzed acacia, honeydew and meadow honey and also to determine content of flavonoids and phenolic compounds, strong fluorophores and well established markers of botanical origin of honey [6], in relation to colour intensity of honey.

MATERIAL AND METHODS

The honey samples which fluorimetric profiles coincided with the manufacturer's statements on the botanical origin were included in the study: 73 different honey samples (11 acacia, 25 meadow, 9 honey-

dew, 4 lime, 2 heather, 1 sunflower and 21 mixed honeys). The number of 63 honey samples was collected from individual producers from Bosnia and Herzegovina and 10 samples were commercially available.

FLUORIMETRIC ANALYSIS

Fluorescence spectra of honey samples were recorded at spectrofluorimeter RF-5301 PC (Shimadzu, Japan). Fluorescence emission spectra from 290 nm to 650 nm were recorded after excitation of the honey samples at different wavelengths: 220 nm, 270 nm, 310 nm, 340 nm, 350 nm, 390 nm, 440 nm, 450 nm and 460 nm. The entrance and exit slits for the excitation light-beam were both 1.5 nm. Honey samples, used for recording fluorescence spectra, were prepared in the following manner: 20 g of honey were incubated 8 h in a water bath at 40°C, cooled at room temperature and then used for recording fluorescence spectra in 1 cm quartz cell, without dilution and filtering.

ESTIMATION OF TOTAL PHENOLIC COMPOUNDS

For the determination of total phenolic compounds, a modified method of Singleton and Rossi [9] was used. The amount of 5.0 g of honey was treated with 50 mL of distilled water, mixed and filtered through a qualitative filter. 500 μL of this solution was mixed with 2.5 mL Folin – Ciocalteau reagent (0.2N) for 5 min. and then 2 mL of Na_2CO_3 solution (75 g/L) was added. The samples were incubated at room temperature in the dark for 2 hrs and the absorbance of the samples was measured at 760 nm. For the blank solution, methanol was used in place of honey; a stock solution of gallic acid (1 mg/mL) was prepared for further dilution. The linearity obtained was R^2 = 0.9987 (Y= 6.9156x). Results were expressed as mg gallic acid equivalent (GAE) per 100 g of sample.

DETERMINATION OF FLAVONOIDS

For flavonoids determination, 1mL of honey solution (1 mg/mL) was mixed with 0.3 mL of 5% $NaNO_2$ and after 5 minutes 0.3 mL of 10% $AlCl_3$ was added. The honey samples were mixed, incubated for 6 minutes and neutralized with 2.0 mL of 1M NaOH solution. The absorbance was read at 510 nm. Quercetin was used to calculate the standard curve. A linearity of 0.998 (R^2) was obtained (Y= 0.378x – 0.002), and result expressed as mg quercetin equivalent (QE) per 100 g of sample [8].

COLOR SCORING

The honey color scoring was developed by the authors as the arbitrary system. The color intensity was estimated by four independent observers. All honey samples were classified into four groups: very bright, bright, medium dark and dark.

STATISTICAL ANALYSIS

For statistical analysis of the results SPSS software, release 20.0. (SPSS Inc., Chicago, IL, USA) was used. Mann-Whitney U test was used to compare differences in flavonoids and polyphenols levels between honey samples of different botanical origin. Correlations between flavonoids, polyphenols and color intensity were defined by Spearman rank correlation analysis. In all tests, two-sided *P* below 0.05 was considered significant.

RESULTS AND DISCUSSION

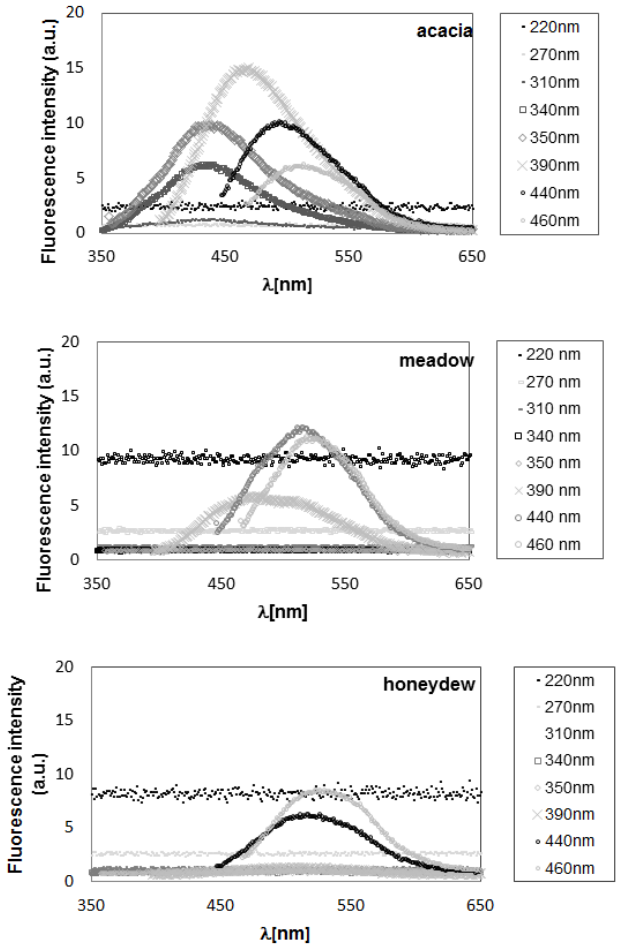

Figure 1. Fluorescence emission spectra of natural acacia, meadow and honeydew honey from Bosnia and Herzegovina recorded from 290 nm to 650 nm, at different excitation wavelengths: 220 nm, 270 nm, 310 nm, 340 nm, 350 nm, 390 nm, 440 nm, 450 nm and 460 nm.

According to our previous research, honey samples of same botanical origin had very similar set of fluorimetric emission spectra [7], [8]. In most of the tested samples, the obtained fluorimetric profiles coincided with the manufacturer's statements on the botanical origin of honey. Also, by determining the concentration of proline in honey samples, those samples in which the proline was not detected were excluded from the study as they were genuine [10]. Therefore, a further investigation included 9 samples of acacia honey, 12 meadow samples and 11 samples of honeydew honey.

Fluorimetric profiles of acacia, meadow and honeydew honey are presented in Figure 1. Acacia honey showed high fluorescence emission intensity after an excitation at 340 nm, 390 nm and 440 nm. Meadow honey showed fluorescence at excitation at 390 nm and 440 nm, while fluorescence emission, caused by excitation at 340 nm, was absent. Honeydew honey showed low intensity of fluorescence at excitation at 440 nm while fluorescence intensity was absent at excitation at 340 nm and 390 nm. These excitation wavelengths and resulting fluorimetric profiles represent a possible algorithm for fast characterization of botanical origin of honey samples. In further experiment, fluorescence emission profiles were used to differentiate honey samples into three groups: acacia, meadow and honeydew. Samples which fluorimetric profiles were of mixed character were not included in the further analysis.

Intrinsic fluorophores and their specific microenvironments in honey produce a complex excitation-emission pattern which varies among samples. Dramićanin and colleges have found five spectral regions of high emission intensities for natural honeys [11]. Our results of fluorescence emission spectra after excitations at 310 nm, 340 nm and 350 nm, for acacia honey were in accordance with the fourth spectral region, obtained after the excitation from 310 nm to 360 nm [11]. However, according to our results, fluorescence spectra of honeydew and meadow samples showed very low fluorescence emission when excited in this region. Moreover, the fluorescence emission after an excitation at 310 nm, 340 nm and 350 nm, together with fluorescence emission at 420 nm after the excitation between 220 nm and 400 nm [7], represents one of the key results for identification of acacia honey.

In Table 1 the descriptive statistics for proline, flavonoid and polyphenol values in three analyzed honey groups are presented.

Table 1. Descriptive statistics for proline, flavonoids and polyphenols in different types of honey

Honey		Sample No	Median ± SD	Range
Acacia	Proline (mg/kg)	9	452.60 ± 214.10	190.00 – 778.80
	Flavonoids (mg QE/100g)	7	15.49 ± 0.39	8.45 – 19.89
	Polyphenols (mg GAE/100g)	9	222.00 ± 72.92	98.00 – 298.00
Meadow	Proline (mg/kg)	12	694.60 ± 340.30	279.60 – 1229.20
	Flavonoids (mg QE/100g)	12	19.45 ± 16.23	5.81 – 67.42
	Polyphenols (mg GAE/100g)	12	443.00 ± 168.52	164.00 – 654.00
Honeydew	Proline (mg/kg)	11	665.60 ± 386.14	331.60 – 1441.80
	Flavonoids (mg QE/100g)	9	22.53 ± 9.81	11.97 – 44.54
	Polyphenols (mg GAE/100g)	11	388.00 ± 147.10	240.00 – 642.00

Acacia had the lowest levels and the honeydew honey had the highest levels of flavonoids and polyphenols (Table 1). Obtained levels of polyphenols in honeydew honey are consistent with the previously published data [12] which showed higher levels of phenolic compounds in honeydew honey in relation to other honey samples. When honeydew and acacia honey were compared, highly statistically significant difference was detected for flavonoids and polyphenols. We found statistically significant difference in polyphenol levels between meadow and acacia honey samples which is also in accordance with results ob-

tained by other authors who found that meadow honeys had significantly higher total phenolic content than acacia honey [13]. In this research we have not found statistically significant difference in the flavonoids content or in the content of polyphenols between samples of meadow and honeydew honey (Table 2).

Table 2. Comparison of levels of flavonoids and polyphenols measured in three different types of honey

	Flavonoids		Polyphenols	
Honey	Man Whitney U	P value	Man Whitney U	P value
Acacia *vs* honeydew	8.5	0.012*	6.5	0.001**
Acacia *vs* meadow	28.5	0.261	23.5	0.028*
Meadow *vs* honeydew	42	0.422	60	0.74
(*) statistically significant difference (**) highly statistically significant difference				

Table 3. Correlations of flavonoids, polyphenols and color intensity in different honey types

Sample		Spearman correlation coefficient	P value
Flavonoids *vs* polyphenols	Acacia	0.342	0.45
	Meadow	0.441	0.151
	Honeydew	0.874	0.002*
All honey samples	Color intensity *vs* flavonoids	0.608	0.001**
	Color intensity *vs* polyphenols	0.546	0.001**
(*) statistically significant difference (**) highly statistically significant difference			

The results of correlations between flavonoids, polyphenols and color intensity are presented in Table 3. Correlation between flavonoids and polyphenols was significant in the honeydew samples. On the other hand, some authors [14] found low correlation (R=-0.38) between total phenolic and total flavonoid content in the honeydew samples analyzed.

Based on the color intensity, all honey samples were classified into four groups: very bright (N=5), bright (N=8), medium dark (N=8) and dark (N=11). In all analyzed samples, statistically significant correlation was found between color intensity and the flavonoid levels, and between color intensity and polyphenols, respectively (Table 3). Our results are consistent with the results of Pontis et al. [15] who also found significant correlation between color and flavonoid and phenolic content of the honey samples which they investigated.

CONCLUSION

Obtained results suggest that fluorimetric profile, flavonoid and polyphenols content, together with colour intensity of honey may be useful in authentication of botanical origin of honey. Further investigation is necessary for developing unique algorithm for fast characterization of botanical origin of honey.

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PHYSICAL-CHEMICAL ANALYZES OF STANDARD PEAR CULTIVARS GROWN IN TURKEY WITH TURKISH VARIETIES THAT WERE DOMESTICATED IN BOSNIA AND HERZEGOVINA

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

The assortment of pear in BiH is very diverse. A significant contribution to the cultivation of a large number of sorts and varieties of pears was contributed by the constant migration of people who carried and planted fruit trees with them. The largest number of fruit varieties and pears in BiH came from two directions. From the east with the Ottoman Empire and from the west with the arrival of the Austro-Hungarian Empire. The pear, as a fruit species, takes a significant place in fruit production, considering that it can be grown in a wide range of climatic conditions, different ripen times, from early summer to late autumn, and multi-purpose use.The fact is that many varieties of pears arrived from Turkey centuries ago and that they were preserved and retained in BiH. This fact is corroborated by the names of variety / Karamut, Jeribasma, Black Izmir and others

The paper analyzes the following parameters: pH, total acidity in mmol / 100 g, pectic substance% Ca-pectinate, raw fiber (%), vitamin C in mg / 100 gr, natural invert (%), total invert (%), total phenols in mg / 100 g of fruit, soluble dry matter (° Brix).The aim of this paper is to determine the usable value and, therefore, the possibility of introducing standard cultivars grown in Turkey to our area through physical and chemical analysis of fruits of indigenous and standard pear trees.

The juice of the tested variety has a lower pH value than the reference values. Low supply of Ca pectate. Vitamin C content is above average. The tested varieties in terms of soluble dry matter content meet the applicable legal framework.

KEYWORDS: domesticated varieties, usable fruit value, migration, introduction

INTRODUCTION

In Bosnia and Herzegovina, the pear assortment is colorful. In addition to standard varieties, William, Bella di giugno, Santa Marie, Butire, Konferans, etc., varieties that have the status of autochthonous are represented in pear cultivation, although it is not yet precisely determined whether they are domesticated or indigenou. The varieties names suggest that they were introduced a long time ago and spontaneously with the migration of the population, which is characteristic of the Balkans. Thus we have Jeribasma, Karamut, Stambolka, varieties of pears whose names indicate that they are originally from Turkey.So the dilemma remains what is the autocthonous and what is the domesticated variety. Thus, some researchers have given their definitions of the term indigenous variety.Indigenous or autocthonous varieties are considered to be all those that originate in our country or have been grown in our country for a long time and are of unknown origin, but are of great economic importance and represent a general national significance. They usually have more important economic-biological and pomological properties, which make them suitable as starting material for selection[1].

Traditional apple cultivars in Bosnia and Herzegovina are a valuable source of desirable genetic characteristics including important pomological, nutritional and technological characteristics of the fruit[2].

Often the same variety is called with many different names, in different local conditions.The cultivation of domestic and domestic varieties is reduced to cultivation as solitary, single, plants[3]. Very rarely they are planted in plantations. In the last ten years there has been interest in their plantation cultivation, but this is still negligible. It should be emphasized that regardless of the cultivation system, these varieties represent significant potential from the point of view of the diversity of genetic material and are the starting point for breeding and creating new varieties. These varieties have been used for a long time and have become resistant to certain diseases

and pests, and are of interest in the production of varieties resistant or tolerant to diseases and pests.The diversity of cultivated plant species serves as a basis for overall biodiversity in agriculture[4]. It is very important to understand that by caring for biodiversity, we actually care about millions of lives, about nutrition and medicine, traditional and modern pharmacology.

MATERIAL AND METHODS

The research included the following pear varieties: Ankara, Black Izmir, Deveci, Jeribasma, Karamut, Malatya, Margarita, Takisha.Fruit analysis was done at the Food technology laboratory of the Faculty of Technology in Tuzla.As the time of ripening of the fruits is different, so the fruits of the pears were harvested and delivered at intervals in accordance with the ripening of the individual varieties, and the samples for analysis were prepared in three repetitions. Samples for analysis represented the average composition of the fruit, and the result was expressed as the mean of the three samples. Preparation of the sample for physicochemical analyzes involved the subtraction of the fruits into a homogeneous slurry from which the given parameters were determined. All analyzes were performed in accordance with the Rulebook on methods for sampling and performing chemical and physical analysis to control the quality of fruit and vegetable products.

The soluble dry matter (° Brix) and the refractive index were directly read on the Abbe refractometer scale.The method for determining the pH value is based on measuring the potential difference between two electrodes immersed in the test liquid, by immersing the electrode in a homogenized sample on the instrument directly reading the value with an accuracy of 0.03. In this paper, the pH meter Mettler-Toledo was used to determine the pH value.The determination of vitamin C in the sample was performed by dissolving the homogenized sample in metaphosphoric-acetic acid, and the volume titrated with 2.6 dichlorophenolindophenol until pink colour is appear.

The results are expressed as mg / 100 g of fresh sample.An indicator color change method was used to determine the total acidity, which is based on titration with sodium hydroxide solution in the presence of phenolphthalein indicator.Total acidity is expressed in millimoles of monobasic acid per 100 ml of product.The determination of the direct reducing and total sugars was made using a Luff solution according to the instructions explained in the Rulebook on methods of sampling and chemical and physical analysis for the control of the quality of fruit and vegetable products. The method is based on the principle that, under certain conditions, reducing sugars (natural invert) convert cupric sulfate ($CuSO_4$) from Luff's solution into copper oxide (Cu2O). An unspent amount of cupri-ion is retitrated with a thiosulphate solution. The difference between the blank and rehearsal expenditure shows the amount of sugar in the table. Non-reducing disaccharide (sucrose) must first be inverted, that is, hydrolyzed to reducing monosaccharides by acid, and then determined by Luff's solution. In this way, the total amount of sugar in the test sample (total invert) is obtained. The difference between the total invert obtained and the natural invert yields the amount of reducing sugars produced by sucrose inversion.

Determination of pectin in the form of Ca-pectate is a method by which pectin is saponified to Na-pectate and then precipitated with calcium, dried and measured by the amount of precipitate.

The proportion is a certain amount of ground and homogenized sample (depending on the amount of pectate present in the raw material) and heated with distilled water in a boiling water bath. After cooling the contents, distilled water was added and filtered. The resulting filtrate was mixed with NaOH, covered with a watch glass and allowed to stand at room temperature for 24 hours. The next day, acetic acid was added to the test solution and calcium chloride was added after 5 minutes, resulting in the formation of calcium pectate. After the addition of calcium chloride, the sample stood for 1 hour and then heated to boiling for 3 minutes. The precipitate was separated from the hot solution by filtration through pre-dried and measured filter paper. Filtration should be carried out quickly, as the cooled precipitate is draining slowly. By washing with warm water, the precipitate liberates all foreign constituents until complete removal of the chloride ions (control with AgNO3 solution). The precipitate filter paper was dried in an oven at 105 ° C to constant weight, cooled, measured, and then the Ca pectate was recalculated.

The determination of the crude fiber was done by mixing the sample with a mixture of acetic acid and nitric acid and boiling it for half an hour under reflux, then immediately while the fibers were still filtered through dried and weighed filter paper, which was precipitously dried in an oven at 105 ° C. to constant mass, cooled, measured, and then the amount of crude fiber was recalculated.

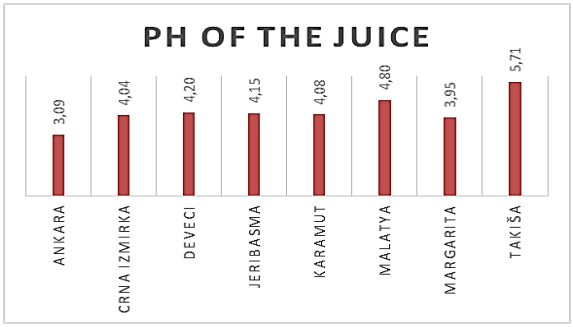
Total phenols were determined spectrophoto-metrically in an ethanol extract of the sample by

measuring the resulting color intensity at a wavelength of 765 nm.

The method is based on the color reaction of phenol with Folin-Ciocalteu reagent expressed in mg of gallic acid per 100 g of fresh sample.

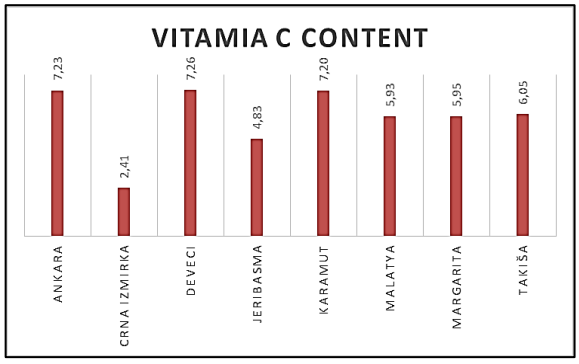
RESULTS AND DISCUSSION

From the standpoint of the pH value of the juice, Graph 1, of the tested pear varieties, it can be stated that the loweer pH value has the Ankara variety (3.09), while Takisha has the highest pH value (5.71). Generally, it can be stated that the juice of the tested varieties has a lower pH value, except for the Takisha variety, and it is not necessary to add citric acid in order to obtain the acidity of the juice. The Takisha variety is traditionally grown and used for the purpose of drying and making jam.



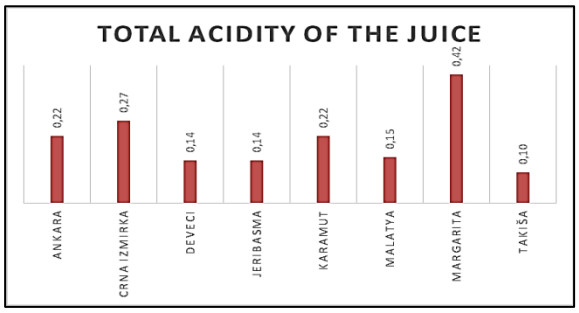
Graph 1: pH of the juice

Vitamin C content in juice, expressed as mg / 100 g of fresh sample, Graph 2, ranged from 2.41 for Black Izmir to 7.23 for Ankara.According to literature sources, pear fruit averages 4 mg / 100 g. Based on the average content of vitamin C in the fruit of the pear, it can be concluded that the tested varieties, except Ankara, have an above average content of vitamin C. This information is important because of the importance of vitamin C in human nutrition.



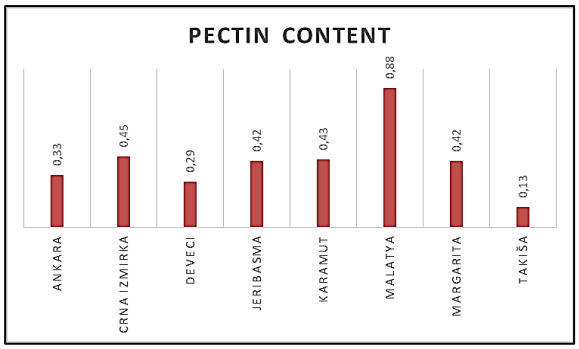
Graph 2: Vitamia C content

The total acidity of the juice, Graph 3, expressed in mmol / 100 g, recorded the lowest value in the Takisha variety (1.40 mmol / 100 g), while the highest value was recorded in the Malatya variety (4.80 mmol / 100 g)



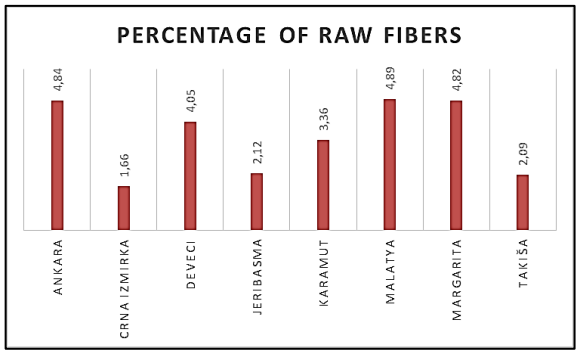
Graph 3: Total acidity of the Juice

The pectin content, graph 4, expressed through% Ca-pectate was highest in the Malatya variety (0.88%) and lowest in the Takisha variety (0.13%).The pectin content of the raw materials was investigated [5,6] and it was concluded that the pear contained 0.3 –3.8 Ca-pectate. The content of pectin, Ca-pectate ranged from 0.13 to 0.88% and it can be concluded that the tested pear varieties have low supply of Ca-pectate



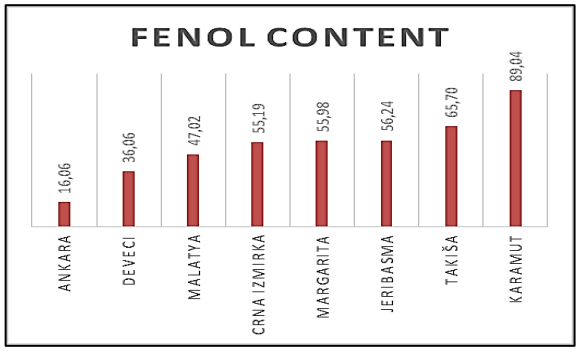
Graph 4: Pectin content

Pear is the best source of fiber. The recommended daily amount of fiber is 20 - 35 g / day.Dietary fiber is made up of edible plant cells, polysaccharides, lignin and the like that are not hydrolyzed or digestible in the human digestive tract. The components covered by this definition are cellulose, hemicellulose, lignin, inulin, gums, modified cellulose, mucus, oligosaccharides, pectins, waxes, quinine and suberin[7].The lowest percentage of crude fiber was recorded in the Black Izmirka variety (1.66%), while the Malatya variety contained the highest percentage of crude fiber (4.89%).



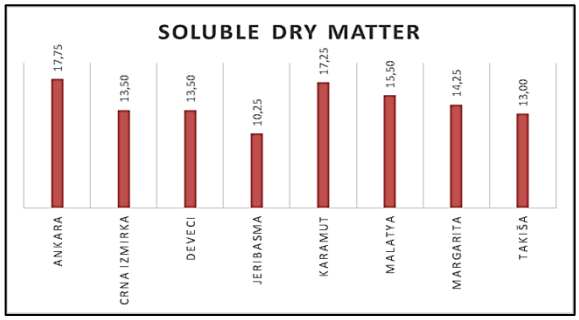
Graph 5: Percentage of raw fibers

The highest phenol content, Graph 6, was expressed in Karamut (89.04 mg / 100 g), while in Ankara (16.06 mg / 100 g), the phenol content was lowest.Due to the positive effect of phenol on the human body, great attention is paid to the assortment of fruits, and the preservation of phenols in fruits during processing and storage.



Graph 6: Fenol content

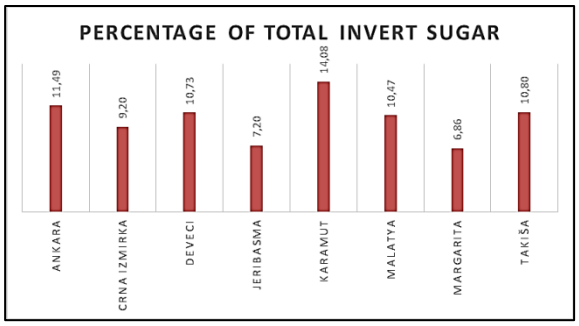
The highest soluble dry matter content, Graph 7, was determined in Ankara (17.75 ° Brix) and the lowest in the Takisha variety (13.00 ° Brix).The obtained values of soluble dry matter for all tested varieties of pears had a value greater than 10.0˚ Brix, and according to this parameter, are, according to the current legislation, suitable for marketing and processing.



Graph 7: Soluble dry matter

The total invert sugar, Graph 8, expressed in % was the highest in the Karamut (14.08 %) and the lowest percentage of total invert sugar in the Margarita variety(6.86 %).

According to research[7], the content of invert sugar in the pear was 8.1%. Based on this we can say that most of the varieties examined contain a higher percentage of invert sugar (Karamut 14,08 %, Ankara 11,49 %, Takiša 10,80 %, Deveci 10,73 %, Malatya 10,47 %, Crna Izmirka 9,20 %).



Graph 8: Percentage of total ivert sugar

CONCLUSION

1. It can generally be stated that the juice of the tested varieties has a lower pH value than the reference values.
2. Based on the average content of vitamin C in the fruit of the pear, it can be concluded that the examined varieties, except Ankara, have an above average content of vitamin C in comparison with referens values.
3. The content of Ca-pectate ranged from 0.13 to 0.88% and it can be concluded that the tested pear varieties have a low supply of Ca-pectate.
4. According to the content of soluble dry matter, all varieties had values above 10,0˚ Brix and, according to this parameter, are suitable for placing on the market and processing in accordance with the applicable legislation.
5. Of the varieties tested, 75% have above-average total invert sugars.

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DETERMINATION OF α-SOLANINE CONTENT IN TWO VARIETIES OF POTATOES BY THE DENSITOMETRIC METHOD

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:

Solanine is a glycoalkaloid found in the *Solanaceae* family, such as the potato. It is very poisonous even in small quantities because it has pesticide and fungicide effects and represents a natural plant defense mechanism. Its concentration increases when the plant is exposed to the agents that can cause plant stress (fertilization, insecticide use, etc.). This paper aims to examine the influence of three cultivation systems (conventionally, organically and naturally) on the biosynthesis of α-solanine (αS) through his quantification in young potatoes using densitometry. Two varieties of potatoes were analyzed: *Aladdin* (*Ala*) and *Mona Lisa* (*MoL*). For statistical analysis, the Student's t-test was used.

The results showed that the use of artificial insecticides caused a very intense biosynthesis of αS in the conventionally grown *Ala* variety (1.19 mg/100 g of fresh tubers (f.t.)) in comparison to the average α-solanine content (AαSC) by the organically grown *Ala* (0.62 mg/100 $g_{f.t.}$) (it is close to the statistical significance, (p=0.08)). It is difficult to explain the very high AαSC of natural *Ala* cultivation (1.62 mg/100 $g_{f.t.}$).

Analysis of potatoes of the *MoL* variety showed that the AαSC of conventionally grown potatoes (1.35 mg/100 $g_{f.t.}$) was statistically higher than the AαSC of naturally grown potatoes (0.59 mg/100 g of f.t.) ($p^*<0.05$). Also, AαSC of the organically grown *MoL* (1.40 mg/100 $g_{f.t.}$) was higher than the AαSC of naturally grown *MoL*, but without statistical significance (p>0.05).

Concentrations of αS founded in the case of conventionally, organically and naturally grown potatoes are considered safe and such potatoes are suitable for consumption. However, because of a slight reduction in toxic αS, it is recommended to consume organically grown potatoes (*Ala* variety), and naturally grown potatoes (*MoL* variety).

KEYWORDS: glycoalkaloids; α-solanine; potatoes; densitometry.

INTRODUCTION

Most plants of the *Solanaceae* family are very significant to humans. These include tobacco (*Nicotiana spp*.), sweet pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), tomatoes (*Lycopersicon esculentum*) and potatoes (*Solanum tuberosum*). Of all these, the most important are potatoes.Although potatoes are perceived as a source of carbohydrates, they are equally good sources of high-quality proteins [1], [2]. Fresh potatoes contain 2% of protein, while dried potatoes have up to 10% protein. Markakis (1975) calculated, based on amino acid composition, that the proportion of high-quality proteins in potatoes was 70%, compared to proteins contained in whole eggs. It should be emphasized that the proteins in potatoes constitute an excellent source of lysine, but not of cysteine and methionine, which limited their nutritional value [3].

Historically, solanine is the first isolated alkaloid [4] that has been recognized as a glycoalkaloid [5]. It was not until 1955 that Kuhn and Low proved that solanine was a mixture of two components, α-solanine, and α-chaconine [6]. From 300 representatives of the genus Solanum, 90 structurally distinct steroidal alkaloids were isolated and characterized [7], [8]. In conventionally grown potato varieties the primary glycoalkaloids present are α-solanine and α-chaconine. These two components show similar toxic effects on humans [9], [10]. When crossing varieties, care should be taken that the glycoalkaloid content does not reach the limit at which it would not be safe

to use potatoes in the diet. However, many factors can affect the level of glycoalkaloids in potatoes. Climate change in the cultivation area can cause variations in glycoalkaloid concentrations among representatives of the same variety [11], [12]. The tuber does not cease to produce glycoalkaloids after harvesting, and improper storage can result in a dramatic increase in these components [13]. Sinden (1972) [14] indicated a greater increase in glycoalkaloids due to mechanical damage to the tuber, and Petersen (1993) emphasized that the damaged tuber should be discarded, as should green potatoes [15].

The glycoalkaloids are not evenly distributed within the tuber. The largest content is just below the crust. Potatoes naturally produce solanine and the related glycoalkaloid chaconine, which serve as a mechanism of defense against insects, diseases, and pests. Potato leaves, stems and shoots are particularly rich in glycoalkaloids. When the potato tuber is exposed to sunlight, it becomes green and the biosynthesis of glycoalkaloids is increased. This process reveals a tuber that "protects" from being eaten. The green color is derived from chlorophyll and as such is harmless. However, it is an important indicator that solanine and chaconine are present in elevated concentrations in the tuber.

Certain diseases such as potato decay can dramatically increase the concentration of glycoalkaloids. It is believed to be a natural reaction of the plant, response to infection or mechanical damage. If green color is observed beneath the bark, this strongly suggests that solanine is rewarded, although the two processes can occur independently of one another. Second and more reliable indicator of potato toxicity is its bitter taste. Because of this appearance and the bitter taste of potatoes, solanine poisoning is rare if storage is taken into account. The most common symptoms are vomiting and diarrhea, so poisoning can be misdiagnosed as gastroenteritis. Most people who are poisoned by solanine completely recover. However, fatal cases were reported due to poor and inadequate treatment. The deaths were also due to the consumption of other plants of the willow family, such as *Solanum dulcamara* (woody willow) [10], [16], [17]. Recently, the relevance of α-solanine has increased because of its possible role as an inhibitor of breast, pancreatic, and esophageal cancers and melanoma [18].

This study aimed to examine the influence of three cultivation systems (conventionally, organically and naturally) on the biosynthesis of α-solanine (αS) through his quantification in young potatoes using densitometry.

EXPERIMENTAL

Thin-layer chromatography with densitometry detection (HPTLC) (Desaga, CD60 TLC scanner with appropriate software, and Desaga AS 30 TLC applicator, Heidelberg, Germany) was used to determine α-solanine in potatoes of the Mona Lisa and Aladdin variety.

The extraction and precipitation were done according to the protocol based on a quantitative method for solanidine glycoalkaloids [19]-[21]. The washed, unpeeled potato tubers are cut into small pieces or rendered. 30 g of ground potatoes was weighted into a 300 mL flask and 200 mL of 96% ethanol was added. The flask with the alcoholic potato pulp was placed in a water bath at 90 °C and incubated for 10 minutes. The resulting extract was filtered through Whatman filter paper no. 1 via the Bühner funnel. The filtered extract was evaporated to a volume of 20 mL on a rotavapor, at 60 °C.After evaporation, 50 mL of 10% acetic acid was added to the resulting concentrate, mixed and centrifuged at 8000 rpm for 30 minutes at 10 °C. After centrifugation, the solution is poured into an Erlenmeyer flask with a glass stopper and brought to pH 10 with ammonia. The flask is immediately sealed (to prevent evaporation of the ammonia) and heated in a water bath at 70 °C for 20 min. After heating, the solution was poured into 15 mL centrifugal tubes and cooled at 4 ºC for at least 3 h. The solution was centrifuged at 8000 rpm for 30 minutes at 10 °C.

After centrifugation, the liquid portion is carefully decanted and the glycoalkaloid precipitate remains at the bottom of the cuvette. The precipitate was dissolved in 200 μl of the mobile phase.

CHROMATOGRAPHIC PLATE PREPARATION AND CHROMATOGRAPHY

The glass is well cleaned with methanol before developing the chromatographic plates. Silica gel was applied to the plate and allowed to dry overnight. Before use, the TLC plate was activated at 110 °C. Twenty-one samples of 5 μl volume were applied at 8 mm intervals to the chromatographic plate. This sample plate was heated to 90 °C for 30 minutes and then placed in a mobile phase bath for 1 hour.

MOBILE PHASE SELECTION

Acid and neutral mobile phases did not show the good separation of α-solanine and α-chaconine. Best distributed was achieved in the alkaline mobile phase containing methanol, chloroform and ammonium hydroxide. The selected mobile phase was the di-

chloromethane-methanol-water-ammonium hydroxide system (70 + 30 + 4 + 0.4 v / v), in which the stain is well separated and the R_f values are in the range between 0.2 and 0.98.

SELECTION OF DETECTION REAGENTS

The Carr-Price reagent was chosen because of its high sensitivity. Antimony(III) builds a double bond with the steroid component, forming a red colour. The reagent must be anhydrous as water interferes with the reaction of antimony chloride with glycoalkaloids. Therefore, it is very important to heat the plates to 90 °C (between the analysis steps) and preferably keep them in a desiccator. The Carr-Price reagent was modified because the addition of acetic acid increased the sensitivity and colour remained stable for a longer period.

DETECTION AND READING

After developing in the mobile phase, the plates are air-dried for 15 minutes and then placed in the oven for 60 minutes at a temperature of 90 °C. Direct visualization is achieved when the plate is sprayed with a modified Carr-Price reagent and then dried for 5 minutes at 105 °C. Glycoalkaloids will appear as red spots. Densitometry reading is performed by reflection scanning at 507 nm. The determination is best done 20 minutes after heating the plates, as the red chromatographic zones of the glycoalkaloids turn purple over time. For all these analyses, standards of α-solanine were prepared in ethanol, with the following concentration: 0.91 μg/ml, 9.01 μg/ml, 90.1 μg/ml, and 1000 μg/ml.

RESULTS AND DISCUSSION

Three samples of conventional, organic and natural cultivation were used to determine α-solanine in the potato of Aladdin and Mona Lisa varieties. The selected samples (tubers) were of approximate size so that the samples had a close-ratio of peel to pulp as possible. The amounts of α-solanine found in potato samples of the Aladdin variety of conventional cultivation ranged from 0.47 to 1.73 mg per 100 g of the fresh tuber (f.t.), with a mean of 1.19±0.64 mg/100 $g_{f.t.}$.

Organic-grown potatoes of Aladdin cultivar had an α-solanine content of 0.07 to 1.72 mg/100 g of the fresh tuber, with a mean of 0.62±0.95 mg/100 $g_{f.t.}$. According to these results, the amount of α-solanine found in organically grown samples is almost two times smaller than the amount of α-solanine determined from samples of conventionally grown potatoes (Table 1).

Table 1. Amount of α–solanine in analyzed potato samples Aladdin and Mona Lisa varieties

Variety of potato	System of cultivation	α-solanine content (mg in 100 g of fresh tuber)	The average α-solanine±S.D. (mg/100 $g_{f.t.}$)
Aladdin	Conventional	1.71	1.19±0.64
		0.47	
		1.38	
	Organic	0.08	0.62±0.95*
		1.72	
		0.07	
	Natural	1.76	1.62±0.33**
		1.25	
		1.86	
Mona Lisa	Conventional	1.45	1.35±0.19***
		1.12	
		1.45	
	Organic	2.96	1.40±1.35
		0.57	
		0.68	
	Natural	0.98	0.59±0.56
		0.20	
		0	

* the average α-solanine content (AαSC) of Aladdin variety was statistically smaller than the AαSC of Mona Lisa variety in organic cultivation ($p^*<0.05$);
** the AαSC of Aladdin variety was statistically higher than the AαSC of Mona Lisa variety in natural cultivation ($p^*<0.05$);
*** the AαSC was statistically higher than the AαSC of natural cultivation ($p^*<0.05$).

The content of α-solanine in samples of naturally grown potatoes ranged from 1.25 to 1.86 mg/100 $g_{f.t.}$. The mean of the measured concentrations of 1.62±0.33 mg/100 g $_{f.t.}$ is 1.36 times higher than that found in conventionally grown potatoes of the same variety.

Analysis of potato samples of the conventionally grown Mona Lisa variety found α-solanine content from 1.12 to 1.45 mg/100 $g_{f.t.}$ with an average content of 1.35±0.19 mg/100 $g_{f.t.}$ (Table 1). If this content is compared to an average amount of up to 1.40±1.35 mg/100 g of fresh organic-grown tubers, it may be observed that they do not differ significantly, while the average amount of α-solanine in naturally grown potatoes 0.59±0.56 mg/100 $g_{f.t.}$ was 2.3 times smaller.

The results obtained for α-solanine in samples of different varieties but with the same cultivation system were compared. The amount of α-solanine in Aladdin and Mona Lisa varieties of conventional cultivation does not differ significantly, while in organic farming the amount of α-solanine found in Aladdin variety is 2.3 times less than that found in the sam-

ples of Mona Lisa variety. Regarding natural cultivation, the amount of α-solanine in Aladdin samples was 2.7 times higher than that of Mona Lisa potatoes.

The results showed that the use of artificial insecticides caused a very intense biosynthesis of αS in the *Ala* variety by the conventionally cultivation system [AαSC 1.19 mg/100 $g_{f.t.}$] in comparison to the AαSC by the organically grown *Ala* (0.62 mg/100 $g_{f.t.}$) [it is close to the statistical significance, (p=0.08)]. It is difficult to explain the very high AαSC of naturally grown *Ala* (1.62 mg/100 $g_{f.t.}$).

Analysis of potatoes of the *MoL* variety showed that the AαSC of conventionally cultivation system (1.35 mg/100 $g_{f.t.}$) was statistically higher than the AαSC of naturally cultivation system (0.59 mg/100 $g_{f.t.}$) ($p^{*}<0.05$). Also, the AαSC of organically grown *MoL* (1.40 mg/100 $g_{f.t.}$) was higher than the AαSC of naturally grown *MoL*, but without statistical significance (p>0.05).

CONCLUSION

From the results obtained in this study, it could be observed that the use of artificial insecticides caused a rather intense biosynthesis of α-solanine in the conventionally grown Aladdin variety, compared to those organically grown. The average content of α-solanine was two times higher in conventionally vs organically grown young samples of potatoes of Aladdin variety.

The analysis of the potatoes of Mona Lisa variety showed that the average content of α-solanine in conventionally and organically grown potatoes did not differ. However, the average content of α-solanine in naturally grown potatoes is 2.3 times lower than that of conventionally grown potatoes.

Comparing the results obtained for α-solanine in samples of different varieties, but in the same cultivation system, we can see that the average content of α-solanine in both conventional varieties does not differ significantly. In a sample of organically grown potatoes, the average content of α-solanine found in the Aladdin variety is 2.3 times less than that found in the samples of the Mona Lisa variety. For naturally grown potatoes, the average content of α-solanine in Aladdin samples was 2.7 times higher than that of Mona Lisa potatoes.

The concentrations of α-solanine that were found in the case of conventional, organic and natural cultivation of potatoes are considered safe and such potatoes are suitable for consumption. However, because of a slight reduction in toxic α-solanine, it is recommended to consume organically grown potatoes (*Aladdin* variety) and naturally grown potatoes (*Mona Lisa* variety).

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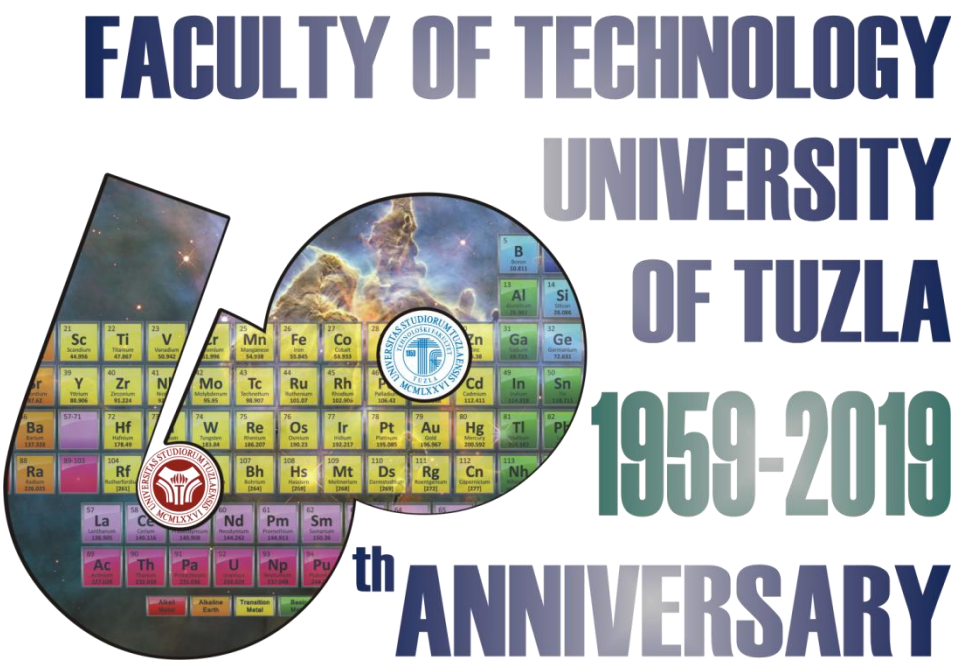

The Ceremony Academy, which was held on November 14th, 2019 in the Faculty amphitheater, marked the jubilee of 60 years of existence and work of the Faculty of Technology, University of Tuzla

In one of the lectures, the first generation of students enrolled in 1959/1960 academic year

The Faculty of Technology in Tuzla is the oldest institution of higher education in Tuzla and one of the oldest in Bosnia and Herzegovina. It was founded in 1959 and was the backbone of the founding of the University of Tuzla. The memory of that year and the review of the initial development path is an obligation imposed on the current generation.

From the first days of its existence, the Faculty has been focused on its positioning as an autonomous, modern, comparatively competitive institution that ensures the flexibility of study according to international standards that follow European trends and initiatives through teaching programs, research and professional activities. The enthusiasm and introduction of innovated curricula and technologies by a large number of young teachers, with extensive experience of senior colleagues, are valuable for the future development of the Faculty, for modern education in accordance with the Bologna process and scientific research compatible with relevant research institutions in Europe and the world.

The strength of the Faculty is based, above all, on highly motivated teachers and associates who are the carriers of all key Faculty activities. The Faculty has a total area of 7,878 m^2, which refers to the offices for teachers, and premises for teaching and research work, all of which are equipped with modern equipment, which all together creates the conditions for a high level of quality of the teaching process and scientific research work.

The Faculty of Technology in Tuzla has played a significant role in the education of scientists and experts in this part of Bosnia and Herzegovina and beyond, and our students are our greatest success. The importance of the Faculty in the region of northeastern Bosnia and in Bosnia and Herzegovina can be shown by numerous indicators, ranging from changing population structure, expanding and

building industrial capacities, increased employment of workers, etc., but it is especially important to emphasize the importance of the Faculty in establishing other higher education and scientific institutions in Tuzli, from which the University of Tuzla grew, in 1976.

For many years, through its vision and mission Faculty of Technology demonstrates its commitment to the promotion and achievement of results in its scientific research and teaching activities, as an important precondition for achieving the progress and competitiveness of the society in which it exists and operates. The rich tradition and existing intellectual potential of the Faculty can be also in the future a strong support for the development of the economy of the state of BiH.

The creation, preservation and transfer of scientific knowledge, the transformation of fundamental knowledge into applied innovations, the introduction of innovative curricula and technologies in the educational activity are also the goals of the Faculty in the future. The Faculty's strategy is the determination and implementation of the optimal ratio of European values and domestic tradition in the education of students.

May this valuable jubilee be an appreciation for the work of all former and current Faculty staff and all our students for the past 60 years.

Staff with freshmen in 2019/2020 academic year

We are particularly proud that on 14th and 15th of November, 2019 at the Faculty of Technology, University of Tuzla, and as part of the anniversary commemoration of the Faculty, was held the VI International Symposium on "Environmental Potentials, Sustainable Development and Food Production - OPORPH 2019".

The symposium gathered scientists and experts in the fields of chemistry, chemical i biochemical engineering, chemical and environmentally sustainable technologies, food technology and biotechnology, environmental potentials and waste management, and agronomy. Representatives of higher education and research institutions from Bosnia and Herzegovina and abroad were present at this gathering, as well as representatives of of business entities with whom the Faculty of Technology has many years of cooperation.

The Symposium program, in addition to preliminary lectures by invited eminent professors from Europe and Asia, included seventy-five oral and poster presentations by the registered participants. Student section, this year organized for the first time within the Symposium, attracted great interest of students who, during the presentation of the results of their researchs, showed exceptional eloquence and a high level of knowledge in the fields of their work.

Today, the Faculty of Technology is recognized and distinguished for the scientific and research achievements of its teachers, associates and students in the local and wider environment. In the process of education we develop and encourage quality research work by both professors and students, and especially application-oriented research.

Research in the fields of environmental protection, chemical engineering and technology, food technology, quality control and food safety, agricultural production and applied / engineering chemistry are fundamental development frameworks that we will continue to encourage.

Sead Ćatić, Dean of Faculty of Technology

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The manuscript must contain the title of the manuscript, full name(s) of the author(s) without abbreviation, abstract, the list of key words, the main text with all the tables and figures embedded and list of references. Can contain also the "Acknowledgement" section.

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EXAMPLES OF CITATIONS FOR DIFFERENT MATERIALS:

Material Type	Works Cited
Book in print	[1] B. Klaus and P. Horn, *Robot Vision*. Cambridge, MA: MIT Press, 1986.
Chapter in book	[2] L. Stein, "Random patterns," in *Computers and You*, J. S. Brake, Ed. New York: Wiley, 1994, pp. 55-70.
eBook	[3] L. Bass, P. Clements, and R. Kazman, *Software Architecture in Practice*, 2nd ed. Reading, MA: Addison Wesley, 2003. [E-book] Available: Safari e-book.
Journal article	[4] J. U. Duncombe, "Infrared navigation - Part I: An assessment of feasability," *IEEE Trans. Electron. Devices*, vol. ED-11, pp. 34-39, Jan. 1959.
eJournal (from database)	[5] H. K. Edwards and V. Sridhar, "Analysis of software requirements engineering exercises in a global virtual team setup," *Journal of Global Information Management*, vol. 13, no. 2, p. 21+, April-June 2005. [Online]. Available: Academic OneFile, http://find.galegroup.com. [Accessed May 31, 2005].
eJournal (from internet)	[6] A. Altun, "Understanding hypertext in the context of reading on the web: Language learners' experience," *Current Issues in Education*, vol. 6, no. 12, July 2003. [Online]. Available: http://cie.ed.asu.edu/volume6/number12/. [Accessed Dec. 2, 2004].
Conference paper	[7] L. Liu and H. Miao, "A specification based approach to testing polymorphic attributes," in *Formal Methods and Software Engineering: Proceedings of the 6th International Conference on Formal Engineering Methods, ICFEM 2004, Seattle, WA, USA, November 8-12, 2004*, J. Davies, W. Schulte, M. Barnett, Eds. Berlin: Springer, 2004. pp. 306-19.
Conference proceedings	[8] T. J. van Weert and R. K. Munro, Eds., *Informatics and the Digital Society: Social, ethical and cognitive issues*: IFIP TC3/WG3.1&3.2 Open Conference on Social, Ethical and Cognitive Issues of Informatics and ICT, July 22-26, 2002, Dortmund, Germany. Boston: Kluwer Academic, 2003.
Newspaper article (from database)	[9] J. Riley, "Call for new look at skilled migrants," *The Australian*, p. 35, May 31, 2005. [Online]. Available: Factiva, http://global.factiva.com. [Accessed May 31, 2005].
Technical report	[10] J. H. Davis and J. R. Cogdell, "Calibration program for the 16-foot antenna," Elect. Eng. Res. Lab., Univ. Texas, Austin, Tech. Memo. NGL-006-69-3, Nov. 15, 1987.
Patent	[11] J. P. Wilkinson, "Nonlinear resonant circuit devices," U.S. Patent 3 624 125, July 16, 1990.
Standard	[12] *IEEE Criteria for Class IE Electric Systems*, IEEE Standard 308, 1969.
Thesis/Dissertation	[1] J. O. Williams, "Narrow-band analyzer," Ph.D. dissertation, Dept. Elect. Eng., Harvard Univ., Cambridge, MA, 1993.

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