Lecture Notes in Bioengineering

Muhamed Brka · Enisa Omanović-Mikličanin · Jasmin Grahić · Samir Muhamedagić · Alen Mujčinović · Almir Toroman · Vedad Falan *Editors*

32nd Scientific-Expert Conference of Agriculture and Food Industry

Local Food Production Systems in the Era of Global Challenges

Springer

Lecture Notes in Bioengineering

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Editors
Muhamed Brka
Faculty of Agriculture and Food Sciences
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

Jasmin Grahić
Faculty of Agriculture and Food Sciences
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

Alen Mujčinović
Faculty of Agriculture and Food Sciences
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

Vedad Falan
Faculty of Agriculture and Food Sciences
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

Enisa Omanović-Mikličanin
Faculty of Agriculture and Food Sciences
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

Samir Muhamedagić
Faculty of Agriculture and Food Science
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

Almir Toroman
Faculty of Agriculture and Food Sciences
University of Sarajevo
Sarajevo, Bosnia and Herzegovina

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Preface

The collection of papers before you contains carefully selected papers presented at the 32nd International Scientific-Expert Conference of Agriculture and Food Industry "Local Food Production Systems in the Era of Global Challenges." The conference was held in Sarajevo on December 1–2, 2022, and gathered many local and international institutions and representatives who have established themselves as leaders in their respective fields. The papers presented have addressed current trending topics and challenges in animal and plant production, food technology and nutrition, agricultural economics, and innovative technologies centered around the common goal of transforming the food value chain. The double-blind review process has been conducted with a minimum of three assignees per paper, ensuring only high-quality papers are submitted and published.

Contents

Technical Correctness of Sprayer Nozzles for Pesticide Applications in Bosnia and Herzegovina

Zuhdija Omerović(✉), Nermin Rakita, Muamer Bezdrob, and Edin Omerhodžić

Faculty of Agriculture and Food Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
z.omerovic@ppf.unsa.ba

Abstract. Nozzles are supplies that are deformed during the work process and can affect the quality of pesticide application. The quality of pesticide application is reflected in coverage, flow, drift and droplet size, which mostly depends on the technical correctness of the nozzle. The goal of the paper is to determine the technical correctness of the new nozzles and deviations of the nozzle flow from the declaration values, for pesticide application in Bosnia and Herzegovina (B&H). The examination was conducted on different types and brands of nozzles that can be found in the B&H market. The examination included three different working pressures, 3, 5 and 8 bars and six different nozzle brands. Nozzle flow measurement was done with special equipment for testing devices for pesticide application, model "*Schachtner tip control B20*" and portable agricultural tractor sprayer, model "*Agromehanika 330*", applying European normative EN ISO 16122. For statistical analyses, *IBM SPSS Statistics* software, version 20, was used. Some nozzle brands had statistically significant deviations from declaration flow, while some brands satisfied ISO 16122 standards. The flow deviation of nozzle brands, marked with D, B and F didn't satisfy the mentioned standard. The largest average deviation had nozzles of brand A made of metal (69,6%) and the smallest average deviation had nozzles of brand B made of metal (2,9%). Conducted research indicates, in the B&H market are nozzles whose flow rate didn't correspond to declaration flow and there need to intensify inspection supervision in the Bosnia and Herzegovina market.

Keywords: Nozzle · Flow · Application of pesticides · Deviations

1 Introduction

Agricultural engineering is one of the main preconditions for high-quality, efficient and modern agricultural production. Machines and devices for pesticide application have a very important role in agricultural production for purpose of protecting crops from various diseases, harmful insects and weeds. Today's intensive agricultural production is unimaginable without using pesticides. Therefore, agricultural producers and consumers need to be aware of many dangers which can be caused unprofessional and irregular

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application of pesticides. For timely, precise and efficient crop protection, it is necessary to have correct and adequate devices for pesticide application. Defective and inadequate devices and their improper use leads to harmful consequences for human health and environmental pollution but also affects on increasing the total costs of farmers. The key role in pesticide application has the correctness of all components and parts of the tractor unit, not only the sprayer [1]. The failure of any part affects the pollution of the human environment and the quality of agricultural products. For these reasons, it is necessary to control and calibrate all devices for pesticide application to reduce the negative impact of pesticides on agricultural products, people and the environment. Calibration is the process of measuring and adjusting the amount of pesticides that are applied to the target area through the application device and properly performed calibration is one of the most important preconditions for effective pesticide application [2]. The calibration procedure is as follows: a) determining the amount of pesticides in the tank used to spray a certain area, b) operating the sprayer in a stationary position and measuring the amount of pesticides released for a certain time, c) collecting the pesticides from the nozzles for a certain time and determining nozzle flow [3].

For the mentioned reasons, many legal regulations have been adopted and various methods are used that will enable the production of healthy food and contribute to the reduction of environmental pollution. The majority of agricultural producers in Europe strive for agricultural production following the principles of good agricultural practice (GAP). Devices for the application of pesticides have an important place in GAP, and for their inspection, GAP used the recommendations of the first and second European symposiums on the standardization of inspection procedures for devices for the application of pesticides in Europe [4]. Since it isn't possible to check every agricultural producer, the GAP consortia have developed commercial standards that have crossed the borders of Europe and received the name Global GAP. Clause 8.4 of this standard refers to equipment and devices for the application of pesticides and defines the obligation of agricultural producers who apply Global GAP to perform a mandatory inspection of their machines and devices once a year, as well as their calibration, which will be conducted by an authorized laboratory for testing these devices. The authorized laboratory for testing devices for the application of pesticides on the territory of the Federation of Bosnia and Herzegovina is the *Laboratory for Agricultural Machinery* within the Faculty of Agriculture and Food Science, University of Sarajevo. The rulebook on devices for pesticide application started to apply on 29.10.2013, which aligned with Directive 2009/128/EC. As part of this directive, there is also a standard related to the inspection of pesticide application devices that are in use.

The new standard that has been applied since 2015 is EN ISO 16122:2015 and consists of five parts covering general provisions, field sprayers, sprinklers, fixed and semi-mobile sprayers and aerial spraying systems. This standard is exclusively related to devices of larger dimensions and working procedures, while EN ISO 19932–2, which regulates the inspection of knapsack sprayers, is also applied. According to ISO 16122, all nozzles located on the wing of the sprayer must be identical (type, size, material and manufacturer), five seconds after turning off the nozzle mustn't continue to drip, the flow of each nozzle must not deviate more than 10% of the declaration flow for used

nozzles, 5% for new nozzles. The new standard has been applied since 2015 and applies in the member states of the European Union.

Testing devices for pesticide application has already begun in many countries across the planet. Field studies of pesticide applicators on golf fields in Nebraska, USA, found more than 25% of applicators which weren't working with properly serviced and calibrated devices, whose nozzles varied in flow rate by more than 10% [5]. Also, according to other research, only one in three pesticide applicators in the USA applied pesticides within 5% of the prescribed amounts [6]. As for Europe, tests of these devices in the European Union began at the end of the seventies of the last century. Out of 70,000 tested devices in Germany, 10% of them were found to have defective nozzles [7]. Also, in Belgium, about 18,000 devices were tested, of which 86% were defective due to manometers and nozzles [8]. All these, point to a big problem in the correctness of the nozzles as the final emitters of pesticides to the treated areas, because according to earlier research, during the technical inspection of plant protection devices, the nozzles are the main factor that is tested, and must achieve the declared flow according to the ISO standard for the device to satisfy the necessary conditions at the technical inspection [9].

The research in Croatia was conducted by the Institute for Agricultural Machinery of the Faculty of Agriculture in Osijek, as a part of this project, 84 devices were tested in several cities in Croatia. The results showed that about 55% of the nozzles were in a defective condition and the devices used in these cities in Croatia don't satisfy the EN ISO 16122 standard [10]. Similar research has never been conducted in Bosnia and Herzegovina, but there have been some experiments related to nozzle flow.

Nozzles are supplies that are deformed during the working process and can significantly affect the quality of the pesticide application, which is reflected in coverage, flow, drift and the number of droplets. The basic task of nozzles on devices for the application of pesticides is to eject the spray under pressure through small openings, forming a special form of jet to break the liquid into small droplets [11]. According to the European standard ISO 16122, a new nozzle is considered good if its flow doesn't deviate by more than 5% from the manufacturer's declared value [12]. Similar problems were observed with the knapsack sprayer, where, according to the research of a group of authors, determined deviations from the declared flow from 18% to 40%, but it is a sprayer that has already been used [13]. Even brand-new nozzles can show variations in flow, where it was found that as many as 20% of new nozzles on average deviated from the allowable tolerance [14]. The nozzles that exceed the mentioned deviations shouldn't be in use. New nozzles that can be found on the B&H market are usually made of plastic or metal and they are made by different manufacturers. The research in B&H was focused on this topic, where they examined several manufacturers of new nozzles in the B&H market, i.e. their deviation from the declared flow rate. The results of that research showed the majority of tested new nozzles from different manufacturers showed significant, positive or negative deviations from the declared flows. Deviations from the declared norms ranged from -28.6% to + 64.24% [15]. In that case, slightly smaller deviations were shown by metal nozzles compared to plastic nozzles. The research concluded in the B&H market there are nozzles of suspicious quality and quality without declarations, whose flow significantly deviates from the declared one. According to the same research, the nozzle flow deviations were negative with the brands *Kosmos* (-5.54%), *AG*

(-7.95%), *Lechler* (-5.04%), and *Mlaz* (-29.08%), while the nozzles of the *Kovina* brand ejected more liquid than the declared norm (+6.75%). If it is taken into account that the allowed flow tolerance can be ± 5%, then the nozzles, from their research, *Kosmos* and *Lechler* were slightly above the upper limit of the allowed and the remaining *AG*, *Mlaz* and *Kovina* were out the allowed tolerance limits. Also, research by Tadić et al. (2012) says that they had a nozzle flow deviation, with brands *Mlaz* (+ 0.81%), *Laznik* (-1.01%), *Andrić* (- 64.44%) and *AG* (+3.70%) at the pressure of 3 bars.

For these reasons, it is necessary to examine the technical correctness of the new nozzles in the B&H market that are available to farmers. Accordingly, the goal of this paper is to examine the technical correctness of new nozzles and deviations of nozzle flow from the declared values for pesticide application in B&H. The results of the research should indicate the real quality of the new nozzles to the sellers who purchase those nozzles and sell them to farmers in B&H.

2 Material and Methods

The testing of the flow rate and the quality of protection was conducted in the authorized "*Laboratory for Agricultural Machinery*" of the Faculty of Agriculture and Food Science, University of Sarajevo. The nozzle flow test was performed using special equipment for testing sprayers, model "*Schachtner type control B20*" with the use of the agricultural tractor sprayer "*Agromehanika 330*" following the EN ISO 16122 standard. In the progress of testing, the capacity of the sprayer tank was 330 l, the spray height of 50 cm and pressures of 3, 5 and 8 bars. The test was conducted with new nozzles, of six different brands, which are the most commonly present in the B&H market. Fan-shaped nozzles were used, six brands, 30 nozzles per sample, with a spray angle of 110˚ and with different declared flow rates of 02, 03 and 04 gallons. The examined brands are marked with capital letters of the alphabet, and the material in which they are made is marked with numbers 1 and 2. Number 1 represents a nozzle made of metal (*CuZn*) and number 2 represents a nozzle made of PVC material.

By applying the EN ISO 16122 standard, the technical correctness of the sprayer and nozzle flow testing device was checked. The revolutions per minute (rpm) of the drive shaft of the sprayer were also checked, which was 540 rpm. The requirements that had to be fulfilled in advance are: the protection of the PTO shaft and the cardan shaft must be in perfect condition, the correct individual parts of the shaft, the joints and the separation securing elements mustn't show attrition and must function properly. The functionality of the pump was checked following the European standard, where for proper testing of the nozzle flow, the volume flow of the pump must be higher than the maximum flow of all nozzles, and it was determined that the pump has no visible pulsation. The specified normative also requires the correctness of the manometer, the capacity of the pump and other parts that are necessary for the functioning of the sprayer. The correctness of the tested parts of the sprayer according to EN ISO 16122 is very important for the high-quality application of pesticides on plants, therefore it is necessary to pay attention to it. Following the mentioned standard, the valves for switching on individual sections of nozzles, which must function as prescribed and mustn't show liquid leakage, were checked. Before testing the nozzles, the wings of the sprayer were secured, their stability

was determined without visible damage, and the hoses of the test equipment that were connected to the nozzles were checked. To measure the flow for each tested nozzle on the sprayer, the already mentioned equipment for testing sprayers, "*Schachtner type control B20*" was used.

To do the examination professionally and with high quality, various equipment, and tools were used during the experiment, which enable the recording and processing of data to determine possible deviations in the nozzle flow. New nozzles, which are the most represented in the B&H market, were tested for possible deviations from the declaration flow at certain pressures.

The obtained data were statistically analyzed using *IBM SPSS Statistics v20*, statistical data processing software. To examine the influence of the nozzle brands and different working pressures, as well as the influence of their interaction, on the flow deviations from the declared values, the exposed samples to the Shapiro-Wilk normality test were first analyzed to examine the suitability of the data for parametric tests and then were analyzed using two-way ANOVA parametric test. Furthermore, to compare the differences in flow deviations of different nozzle brands and different working pressures, the post hoc Tukey test was used. *Microsoft Office 2019* package was used for the graphs and tables.

3 Results and Discussion

To achieve the set goals and tasks of the paper, this chapter will present the results of the flow of six different brands of new nozzles at three different working pressures and their deviation from the declaration flow. After the presented results, adequate conclusions will be made.

3.1 Nozzles 02 Gallons at Working Pressures of 3 and 5 Bars.

In the B&H market, five different nozzles were found, which were declared for 02 gallons and were exposed to flow testing at working pressures of 3 and 5 bars. Table 1. Shows descriptive statistics of nozzle flow deviations from declared values. The declared flow rate for nozzles of 02 gallons at a working pressure of 3 bars is 800 ml/min and at a working pressure of 5 bars, it is 1030 ml/min.

Table 1 shows two nozzle brands had the measured average flow higher than declared, while for the other three brands, the flow was lower than declared. Looking at the nozzle brand, the average deviation from the declared flow was + 69.6% for brand A1, + 6.9% for brand B1, -16.2% for brand C2, -4.5% for brand D2 and -22.6% for brand E2.

At working pressure of 3 bars, the average flow rate of the tested nozzles ranged from 592.3 ml/min to 1340.3 ml/min. The lowest average flow was measured by brand E2, while the highest was measured by brand A1. The declared flow rate for the 02-gallon nozzle type at a working pressure of 3 bars is 800 ml/min, so it can be concluded that some brands had negative some positive deviations from the declared flow rate. Deviations from the declared flow ranged from -26.0% to + 67.5%. The smallest deviation from the declared flow was measured by brands B1 (+6.1%) and D2 (-4.9%), while the largest deviation was measured by brand A1 (+67.5%). When testing the same nozzles at a

Table 1. Descriptive statistics of nozzles with a declared flow of 02 gallons

Nozzle brand	Working pressure	Average flow (ml/min)	The average deviation from declaration (%)	Total average brand deviation (%)
A1	3 bars	1340,3	+ 67,5	+ 69,6
	5 bars	1769,0	+ 71,7	
B1	3 bars	849,0	+ 6,1	+ 6,9
	5 bars	1110,0	+ 7,8	
C2	3 bars	656,0	-18,0	-16,2
	5 bars	882,3	-14,3	
D2	3 bars	766,3	-4,9	-4,5
	5 bars	987,3	-4,1	
E2	3 bars	592,3	-26,0	-22,6
	5 bars	833,7	-19,1	

working pressure of 5 bars, very similar results were obtained. The measured average flow, in this case, ranged from 833.7 ml/min to 1769.7 ml/min. The lowest average flow was recorded by brand E2, while the highest was by brand A1. Considering the declared flow rate of these nozzles at a working pressure of 5 bars (1030 ml/min), it can be concluded, again, some nozzles on average throw out more liquids and some less than the declared values. Average deviations from the declared flow ranged from -19.1% to + 71.7%. Nozzle brand D2 showed the smallest average deviations from the declaration (-4.1%), while brand A1 again showed the largest deviations (+71.7%). The results indicate brands of nozzles A1, C2, and E2 declared for 02 gallons, don't satisfy the permitted deviations for new nozzles according to the EN ISO 16122 standard and as such aren't recommended for use. Brand B1 showed higher deviations than the permitted of 5%, but such a result doesn't necessarily indicate that all nozzles of this brand don't satisfy the standards because their average deviations are slightly higher than the permitted ones. The best result showed the nozzle brand D2, which was the only one with average deviations within the limits allowed according to EN ISO 16122 standards.

At both working pressures, the samples of all tested nozzle brands satisfy the conditions of normality (sig $>$ 0.05) and a parametric test, two-way ANOVA, can be performed. Based on the results of the two-way ANOVA, it can be concluded there is a statistically significant influence of the interaction of nozzle brand and working pressure on the deviation of nozzle flow from the declared value (sig $<$ 0.05). Therefore, there is a statistically significant influence of the nozzle brand and working pressure as separate factors. The result indicates different nozzle brands don't show the same deviations from the declared flow at the same working pressure, and it is necessary to compare the flow deviations of different nozzle brands and different working pressures to get clearer results. Differences in nozzle flow deviation were tested using a post hoc Tukey test and the results are shown in Fig. 1.

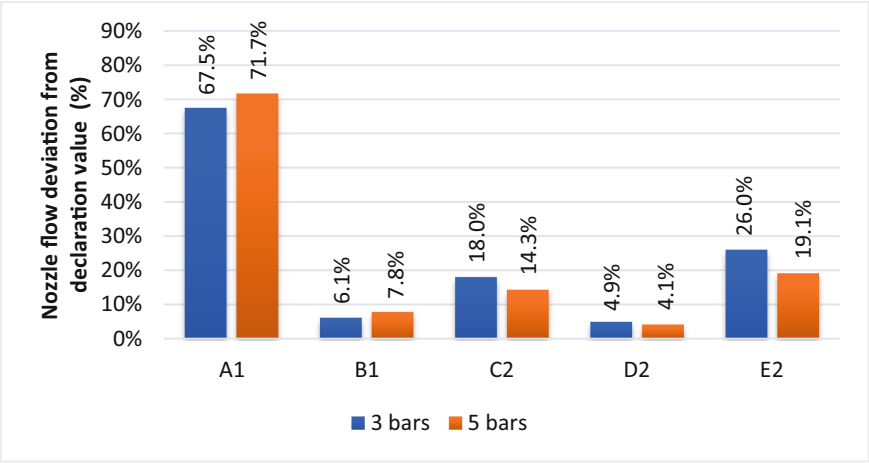

Fig. 1. Average flow deviation of different nozzles at different working pressures (02 gallons)

Looking at Fig. 1 and the results of the Tukey test, it can be concluded, all nozzle brands differ significantly in the flow deviation compared to the declared flow (sig $<$ 0.05). Also, within the brand, at different pressures, there are statistically significant differences in the flow deviation from the declaration value. Nozzle brands A1 and B1 showed higher deviations at higher working pressure compared to the lower pressure, while other brands showed higher deviations at lower working pressure compared to higher pressure. Such large deviations in flow can cause very significant damage to treated crops, contribute to higher environmental pollution, and contribute to much higher financial expenses for farmers who use these nozzles.

3.2 Nozzles 03 Gallons at Working Pressures of 3, 5 and 8 Bars

The next group of nozzles (03 gallons) are the nozzles with a slightly higher declared flow rate than the previous ones. The following Table 2. Shows the descriptive statistics of the nozzle flow deviations from the declared flow of different brands. All nozzles had a declared flow of 03 gallons, i.e. 1190 ml/min at 3 bars, 1530 ml/min at 5 bars and 1940 ml/min at 8 bars.

Table 2 shows for two nozzle brands, the measured average flow was higher than declared, while for the other two brands, the flow was lower than declared. Looking at the nozzle brand in general, the average deviation from the declared flow was + 43.8% for brand A1, + 2.9% for brand B1, -36.5% for brand C2 and -4.6% for brand D2.

Nozzles with a declared flow rate of 03 gallons are the most commonly used at three different working pressures 3, 5 and 8 bars. When testing the nozzles at an operating pressure of 3 bars, average flows ranged from 695.3 ml/min to 1712.7 ml/min. The lowest average flow was measured by nozzle brand C2, while the highest was measured by nozzle brand A1. The declared flow of these nozzles at a working pressure of 3 bars is 1190 ml/min, so it can be concluded the tested nozzles deviated in both directions (positive and negative). The average nozzle flow deviation from the declared value ranged

Table 2. Descriptive statistics of nozzles with a declared flow of 03 gallons

Nozzle brand	Working pressure	Average flow (ml/min)	The average deviation from declaration (%)	Total average brand deviation (%)
A1	3 bars	1712,7	+ 43,9	+ 43,8
	5 bars	2186,0	+ 42,9	
	8 bars	2808,0	+ 44,7	
B1	3 bars	1146,7	+ 4,3	+ 2,9
	5 bars	1560,7	+ 2,6	
	8 bars	1976,0	+ 1,9	
C2	3 bars	695,3	-41,6	-36,5
	5 bars	968,7	-36,7	
	8 bars	1332,1	-31,3	
D2	3 bars	1142,0	-6,2	-4,6
	5 bars	1471,7	-4,1	
	8 bars	1878,3	-3,4	

from -41.6% to + 43.9%. The smallest deviations from the declared flow rate were recorded by nozzle brand B1 and the largest by nozzle brand A1. When testing the same nozzles at a higher pressure of 5 bars, the results obtained were very similar. The average flow ranged from 968.7 ml/min to 2186 ml/min, while the average deviations from the declared flow ranged from -36.7% to + 42.9%. In this case, too, the smallest and the largest deviations in the flow from the declared one were shown by nozzles of the same brands as at the working pressure of 3 bars. At the highest tested pressure of 8 bars, the average flow ranged from 1322.1 ml/min to 2808 ml/min. Also, the deviations from the declared flow ranged from -31.3% to + 44.7%, where, again, the nozzles of the same brands showed the smallest and the largest deviations. Based on the presented results, it can be concluded the nozzles of brands A1 and C2 don't satisfy the criteria of the EN ISO 16122 standard, while the other two brands, B1 and D2, showed extremely small deviations that are within the allowed 5%.

In this case, too, all samples were exposed to the Shapiro-Wilk normality test and satisfied the conditions for using parametric tests.

Based on the results of two-way ANOVA, it can be concluded there is a statistically significant interaction influence on the deviation of the nozzle flow from the declared value for nozzles of 03 gallons (sig < 0.05). The result indicates that different brands of the nozzle, even for nozzles with a declared flow of 03 gallons, don't show the same deviations from the declared flow at the same working pressures, and it is necessary to compare the flow deviations of different brands of nozzles and different working pressures to get clearer results. Differences in nozzle flow deviation were tested using a post hoc Tukey test and the results are shown in Fig. 2.

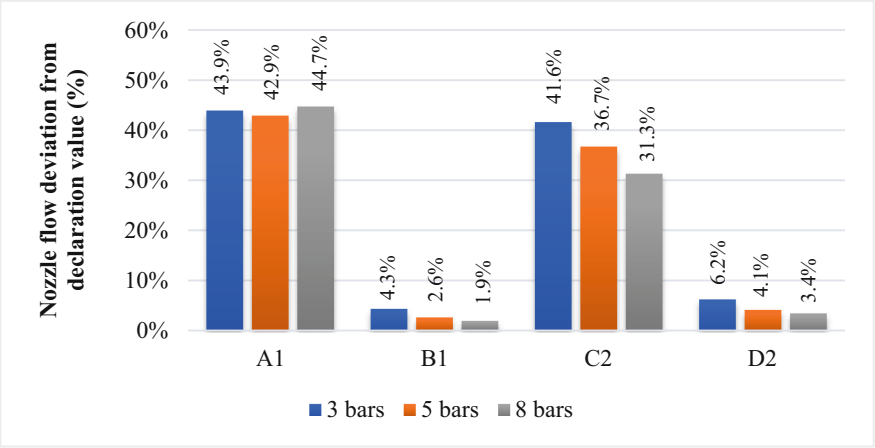

Fig. 2. Average flow deviation of different nozzles at different working pressures (03 gallons)

Looking at Fig. 2 and the results of the post hoc test, it can be concluded all nozzle brands differ significantly from each other in the flow deviation from the declared flow (sig $<$ 0.05), except for brands B1 and D2, which don't differ significantly from each other (sig $>$ 0.05), Also within the brand, at different pressures, there are statistically significant differences in flow deviation from the declared value, except between nozzle flow at 5 and 8 bars where the differences aren't statistically significant (sig $>$ 0.05). With most brands of the nozzle, the largest deviations were recorded at a working pressure of 3 bars and the smallest at a working pressure of 8 bars, except in the case of the nozzle brand A1 where the largest deviations were recorded at a working pressure of 8 bars and the smallest at 5 bars.

3.3 Nozzles 04 Gallons at Working Pressures of 3, 5 and 8 Bars

The next group is a group of nozzles with the highest declared flow of all tested nozzles of 04 gallons. Table 3 shows the descriptive statistics of the flow deviation of new nozzles from the declared flow of different brands. All nozzles had a declared flow of 04 gallons, i.e. 1580 ml/min at 3 bars, 2040 ml/min at 5 bars and 2580 ml/min at 8 bars.

Table 3 shows that for all nozzle brands, the measured average flow was lower than the declared one. Looking at the nozzle brand in general, the average deviation from the declared flow was -3.5% for the F1 brand, -6.7% for the F2 brand, -8.8% for the C2 brand and -4.1% for the D2 brand.

As in the previous case, the tested nozzle brands were exposed to testing at the three most commonly used working pressures of 3, 5 and 8 bars. The results of measuring the average flow at a working pressure of 3 bars were very similar for all tested brands. The average flow ranged from 1474.7 ml/min to 1524.3 ml/min. Therefore, the measured deviations from the declared flow were very small and ranged from -3.5% to -6.7%. The largest deviations were measured by nozzle brand C2 and the smallest by nozzle brand D2. Very similar results were obtained when testing the flow at a working pressure of 5 bars. The average flow ranged from 1860.7 ml/min to 1991 ml/min. Also, the average

Table 3. Descriptive statistics of nozzles with a declared flow of 04 gallons

Nozzle brand	Working pressure	Average flow (ml/min)	The average deviation from declaration (%)	Total average brand deviation (%)
F1	3 bars	1506,3	-4,7	-3,5
	5 bars	1991,0	-2,9	
	8 bars	2518,3	-2,9	
F2	3 bars	1490,0	-5,7	-6,7
	5 bars	1897,7	-7,0	
	8 bars	2389,3	-7,4	
C2	3 bars	1474,7	-6,7	-8,8
	5 bars	1860,7	-8,8	
	8 bars	2302,3	-10,8	
D2	3 bars	1524,3	-3,5	-4,1
	5 bars	1958,7	-4,0	
	8 bars	2464,7	-4,7	

deviations from the declared flow ranged from -2.9% to -8.8%, with the nozzles of brand F1 showing the most minor average deviations and the nozzles of brand C2 the largest. At working pressure of 8 bars, the situation is similar. The average flow of tested nozzles ranged from 2302.3 ml/min to 2518.3 ml/min. Accordingly, the average deviations from the declared flow ranged from -2.9% to -10.8%. The largest average deviations were shown by the nozzle C2 brand, while the smallest deviations were shown by the F1 nozzle brand. According to the presented results, it can be concluded the nozzles of brands F1 and D2 at all tested working pressures satisfy the criteria of the EN ISO 16122 standard and proved to be of high quality and reliable, while the nozzles of brands F2 and C2 showed deviations out of the allowed limits, but these deviations aren't significantly higher, so the results should be accepted with the questionnaire because additional tests of these nozzles are needed, which might show minor deviations within the permitted limits in other studies.

In this case, too, the samples were exposed to the same test and satisfied the conditions for using the parametric test. Based on the results of two-way ANOVA, it can be concluded there is a statistically significant interaction influence on the deviation of the nozzle flow from the declared value for nozzles of 04 gallons ($sig < 0.05$). The results, again, indicate that different nozzle brands, even for nozzles with a declared flow of 04 gallons, don't show the same deviations from the declared flow at the same working pressures, and it is necessary to compare the flow deviations of different brands of the nozzle and different working pressures. Differences in nozzle flow deviation were tested using a post hoc Tukey test and the results are shown in Fig. 3.

Looking at Fig. 3 and the results of the Tukey test, it can be concluded all nozzle brands differ significantly from each other in the flow deviation from the declared flow

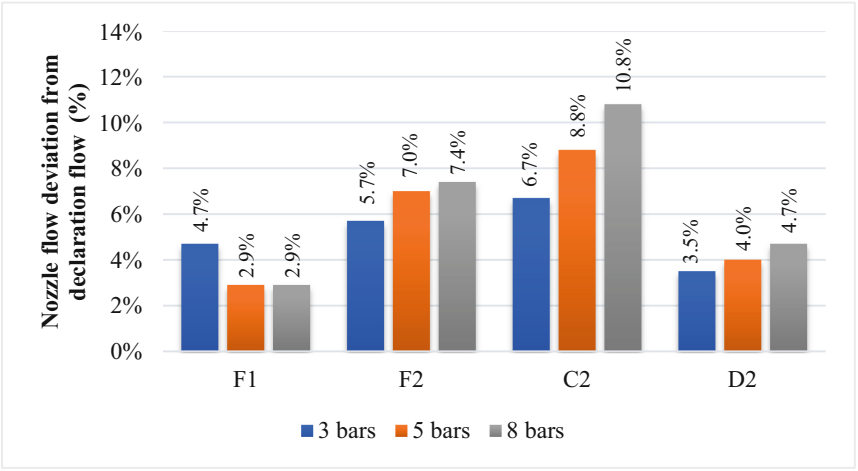

Fig. 3. Average flow deviation of different nozzles at different working pressures (04 gallons)

(sig < 0.05), except for brands F1 and D2, which don't differ significantly from each other (sig > 0,05), Also within the brand, at different pressures, there are statistically significant differences in the flow deviation from the declared value, except between the flows at 3 and 5 bars where the differences aren't statistically significant (sig > 0.05). All nozzle brands showed the largest deviations from the declared flow at the highest tested working pressure and the smallest deviations at the lowest tested working pressure. The exception is the nozzles of the F1 brand, where the largest average deviations from the declaration were recorded at a pressure of 3 bars, while the same deviations were recorded at pressures of 5 and 8 bars.

The research in B&H examined several brands of new nozzles in the B&H market, based on very similar nozzle brands [15]. The results of their research showed deviations from the declared norms ranged from -28,6% to + 64,24%. Accordingly, we can conclude the results of this study confirmed those results because the deviations in this study ranged from -36,5% to + 69,5%. According to their research, some nozzle brands deviated similarly in both studies but some deviated much more or less. Also, another group of authors tested some similar nozzle brands and got similar deviations [10].

Table 4 shows data on the percentage of nozzles that satisfy the ISO 16122 standard, ie, the 5% flow deviation tolerance threshold, by categories 02, 03 and 04 gallons.

Based on the results shown in Table 4, it can be concluded the most nozzles that don't satisfy the ISO 16122 standard were in the sample of nozzles with a declared flow of 02 gallons, about 85%, followed by nozzles with a declared flow of 03 gallons, 62.5% and at least from a nozzle sample with a declared flow rate of 04 gallons 55%. In total, of all the tested nozzles, 67.5% of nozzles didn't satisfy the permitted deviations for new nozzles of 5%, while only 32.5% of the tested nozzles satisfy the criteria of the mentioned standard. According to research [14] where it was discovered that 20% of new nozzles on average deviated from the allowed tolerance, we can conclude that this percentage is much higher in the B&H market. The reasons for this can be numerous, such as the procurement of the cheapest and low-grade nozzles in the B&H market,

Table 4. Percentual representation of nozzles that satisfy the ISO 16122 standard

Declared flow	Deviations below 5%	Deviations above 5%
02 gallons	15%	85%
03 gallons	37,5%	62,5%
04 gallons	45%	55%
TOTAL	**32,5%**	**67,5%**

which is affordable for domestic producers, but also the lack of control of standards on the goods sold in the B&H market.

4 Conclusions

Successful protection of crops from pesticides is possible only if it is done with the correct parts of the sprayer, correct adjustment and at the appropriate time. The key role in the correct application of pesticides depends on the correctness of all assemblies and parts of the tractor unit. The failure of any part or assembly affects the pollution of the human environment, and thus the quality of crops. Nozzles in the market should have the declared flow because otherwise, they cause incalculable consequences. Conducted research indicates in the B&H market, there are nozzles in the retail trade without declaration, of unknown origin and quality, and whose flow rate doesn't correspond to the declared norms. In the B&H market, several nozzle brands are available to farmers. Some of them can be found made from metal, but most of them are made of PVC plastic. Most of the examined nozzle brands had significant deviations from the declared norms and didn't satisfy the prescribed standards according to ISO 16122. Three nozzle brands showed significantly better results than the others and the average deviations of these nozzles were within or slightly over the allowed 5%. The other three tested brands showed significant deviations over the allowed 5% and didn't satisfy the ISO 16122 standard and as such shouldn't be on the market. Also, the result that 67.5% of all the tested nozzles did not satisfy the standard raises a big question mark on the quality and origin of the nozzles found on the B&H market. The obtained results indicate that it is necessary to strengthen the inspection supervision of the nozzle sales for pesticide application, to intensify the obligation of farmers to periodically calibrate devices for application following the provisions "Regulations on control of devices for the application of the pesticide" (Official Gazette of B&H No. 84/2013), and when they want to replace some nozzles they should buy more reliable brands to reduce the possibility of incorrect application of pesticides.

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Determination of Water Extractable Chloride in the Greenhouse Soil and Minimizing Interferences Caused by the Presence of the Iron Ions

Josip Jurković[1(✉)], Elmedina Bristrić[1], Sanel Haseljić[1], Sabrija Čadro[1], Fejzo Bašić[1,2], Rasim Omanović[2], and Jasmina Sulejmanović[2]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, Sarajevo, Bosnia and Herzegovina
j.jurkovic@ppf.unsa.ba

[2] Faculty of Science, University of Sarajevo, Zmaja Od Bosne 33-35, Sarajevo, Bosnia and Herzegovina

Abstract. Chloride ion (Cl^-) is one of the most abundant inorganic anions in water. In the soil, it originates from many chlorine minerals. Chlorides in soil can be released also as constituent of fertilizers. Fertilizers in greenhouses are used in high amount, where salinization of soil often happens. Increased chloride content on arable land can significantly affect the cultivation of certain agricultural crops, and the first step towards solving this problem must be to determine the chloride content as accurately as possible. The aim of this research is to determine the chloride content in soil samples from greenhouses by use of automatic titration, and to record possible disturbances caused by certain cations (Fe^{3+}), and to propose a way to minimize them. The following samples were used as material: soil from five greenhouses, soil samples outside the greenhouse - control, and irrigation water samples. In the samples following basic parameters were measured: hygroscopic water content, pH value in water and $CaCl_2$, redox potential and electrical conductivity. Chloride content was measured with automatic titrator, and iron content with atomic absorption spectrophotometry with flame atomization (Shimadzu AA 7000 instrument). Irrigation water had a neutral pH value, while the soil samples were slightly acidic (6.74) to alkaline (8.36). Irrigation water does not contribute in chloride content in soils. The highest chloride content was 226.7 mg/kg, and at this chloride value, moderately resistant plants can show negative changes. There is a certain difference in chloride content with and without EDTA addition, but amount of iron was not high enough to cause significant interference (average: 9.86 mg/kg).

Keywords: chloride · fertilizers · automatic titration · greenhouse · interferences

1 Introduction

Chloride ion (Cl^-) is one of the most abundant inorganic anions in water and wastewater. In the soil, concentration of chloride depends on many factors: depositions trough irrigation water, depositions of airborne Cl^-, input by fertilization, basin or saline springs or

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pumping of seawater. In the soil, Cl^- also originates from parent bedrock [1]. Chloride in soils bedrock is constituent of minerals such as chlor-apatite, boracite, halite, etc. The decomposition of these minerals releases chlorine and is found in the solution in the form of Cl^- anions. In this form, plants adopt it. The chloride ion is very mobile in nature, and its transfer from water to soil is easy and fast. Chlorides present in the soil are usually very soluble in water (except if they are bound in AgCl, $PbCl_2$, $CuCl(OH)_3$, etc. An increased level of soil salinity is a result of the accumulation of various water-soluble salts in the soil profile, and it can be said that those agricultural soils are saline in which the concentrations of dissolved salts are so great that they can cause limitations in the growth, development and/or productivity of cultivated agricultural crops. However, how salt stress will affect a certain agricultural crop depends on the type, variety and phase of crop development (vegetative, generative), as well as on other environmental conditions (moisture, temperature) and on ions (Na^+, Ca^{2+}, Mg^{2+}, Cl^-, $SO_4{}^{2-}$ etc.) which are found in increased concentrations in the soil [2]. According to the origin, soil salinization can be natural (primary) or anthropogenic (secondary). In arid and semi-arid climate zones and in coastal areas, high salt concentrations naturally occur in soils, while the amount of precipitation is insufficient to wash the salt into deeper soil horizons [3]. In the case of primary salinization, the salts reach the surface of the ground water by capillary rise, where they accumulate through evaporation or evapotranspiration. Excessive amounts of salt in the soil are a limiting factor in the cultivation of many agricultural crops. In conditions of increased amount of salt in the soil, there are three main factors that adversely affect plants: lack of water, ion toxicity - excessive intake of Cl^- and Na^+, and disturbance in the intake and transport of nutrients [4, 5]. Factors that affect salinity of agricultural soils are: the application of mineral fertilizers, the quality of irrigation water and the method of irrigation, climatic conditions and the type of land. Irrigation as an agrotechnical measure can affect the salinization of land in two ways: directly - when water containing more mineral substances is applied and indirectly - when larger quantities of irrigation water are applied and the level of mineralized underground water rises. The amount of chloride in the irrigation water below 70 mg/L is generally considered safe for all plants [6]. Increased amounts of chlorides (4–7 mg/g for sensitive species) are toxic to plants [7]. Increased chloride content on arable land can significantly affect the cultivation of certain agricultural crops, and the first step towards solving this problem must be to determine the chloride content as accurately as possible. One of the most accurate and precise methods for determining chloride is automatic titration with an ion-selective chloride electrode. When determining chloride by automatic titration, larger amounts of trivalent cations can interfere. Polyvalent cations (e.g., Fe^{3+} and Al^{3+}) interfere by forming complexes with chloride which are not measured by the chloride ion selective electrode [8]. Trivalent cations cause lower result than correct one. The minimization of the cation affect will be conducted with complexing agent (EDTA) which complex the cations in stable complex. Taking into account all these facts, the goal of this work was derived, which is: using automatic titration to determine the chloride content in soil samples from greenhouses, to record possible disturbances caused by certain cations (iron cations or aluminum), and propose a way to minimize them.

2 Materials and Methods

Five greenhouses were included in the research, from which three soil samples were taken (total of 15 samples). Soil samples were taken with plastic scoop, until the depth of 15 cm was reached. At a distance of 20 m from the greenhouses, samples were taken and served as a control (total of 5 samples). In addition to soil samples, irrigation water samples were also taken from each greenhouse, a total of 5 samples. All samples were collected in the area in the northeastern part of Bosnia and Herzegovina, in the city of Gradačac. In the greenhouses where the samples were taken, vegetable production is carried out, namely the production of: cucumbers, tomatoes and peppers. Sampling was done during vegetation rest.

2.1 Sample Preparation

After the soil samples were brought to the laboratory, they were air dried, homogenized and sieved trough 2 – mm sieve. Irrigation water samples in which the Fe content was measured were preserved with concentrated nitric acid.

2.2 Methods Used for Water Analysis

Irrigation water samples from the areas were taken from the city water supply and from a well. The water samples were subjected to the following analyses: pH value, electrical conductivity, redox potential, and determination of chloride and iron content. The pH value was measured using a pH meter, which was calibrated with buffers of known pH value (7.00 and 4.00) by means of Mettler Toledo, MP 230 device. Electrical conductivity was measured with a conductometer with standards of known electrical conductivity (12.88 mS/cm and 1043 μS/cm), with Mettler Toledo MC 126 instrument. The redox potential was measured using an ORP device that was calibrated with standards of known redox potential. The chloride content was measured by automatic titration with an ion-selective chloride electrode, where silver nitrate solution, concentration 0.01 mol/L, was used as the titrant. For determination of chloride content Mettler Toledo G20 used.

2.3 Methods Used for Soil Analysis

In order to determine the basic and specific quality parameters of the soil samples, several analytical methods were used:

Basic Quality Parameters

pH value

In air dried, homogenized and sieved samples, the pH value was measured in two different solutions: distilled water and a $CaCl_2$ x $2H_2O$. The pH value is measured in the ratio soil:extractant 1:5 (ISO 10390:2021 method). The measurement was performed using a pH meter (MP 230) that was previously calibrated with buffers of known pH value.

Electrical conductivity

The electrical conductivity was measured in a water suspension of the soil, in a ratio of 1:5. A conductometer (MC 126) was used for the measurement, which was calibrated with standards of known electrical conductivity.

Redox potential

The redox potential was measured with a HANNA, ORP meter (Reference electrode was Ag/AgCl). The redox potential was also measured in the water suspension (1:5 ratio) of the soil in accordance to US EPA method. The ORP device is previously calibrated with standards of known redox potential.

Specific Soil Parameters

Determination of iron content in water extract using atomic absorption spectrometry

The iron content was determined by atomic absorption spectrometry with flame atomization, using the external standard method. In order to determine the amount of iron ions that could interfere with an electrode, the measurement of the deionized water extractable iron was measured. The water extractable iron was measured in the same solution in which the chloride content was determined, just in different aliquot. The limit of detection was 1 mg/L. A Shimadzu AA-7000 device was used to measure this parameter.

Determination of chloride content

Determination of chloride was carried out in the water extract of the soil, without and with EDTA addition, as an iron complexing agent. The complexing agent was used to minimize the iron content interference on the electrode. Chloride content was measured by automatic titration with an ion-selective chloride electrode (Mettler Toledo, DM 141-SC). The titration was carried out by transferring 30 mL of the sample with the help of a burette to the container for stirring the sample, and with constant mixing, the titration was carried out with a standard solution of silver nitrate. The device was a Mettler Toledo, G20. The end point of the titration was determined potentiometrically. Analytical determinations were conducted in the laboratories of Faculty of Agriculture and Food Sciences in Sarajevo.

2.4 Statistical Evaluation

Results of this research are statistically evaluated with usage of statistical program SPSS 20.0 (SPSS Inc., Chicago, IL, USA) through next analysis: 1. Descriptive statistics (mean, maximum, minimum and median). 2. Correlation is used to see similarity between two groups of results. Description of correlation coefficients which describe strength of connection between two variables is as follows: 0.0– 0.19 very weak connection, 0.2 – 0.39 weak connection, 0.40 – 0.59 moderate connection, 0.60 – 0.79 strong connection, 0.80 – 1.0 very strong connection. Correlation can be uphill (coefficient of correlation (r) is positive), or downhill (coefficient of correlation (r) is negative).

3 Results and Discussion

3.1 Analysis of the Irrigation Water Samples

The descriptive statistics results of water samples analysis are given in the Table 1.

Table 1. Results of the water samples analysis

	pH value	ORP (mV)	EC mS/cm	Cl^- (mg/L)	Fe (mg/kg)
Minimum	6.91	294	4.16	3.65	< 1
Maximum	7.46	313	6.41	27.60	< 1
Median	7.15	294	6.05	19.96	< 1
Average	7.17	294	5.67	7.44	< 1

Analysis of the irrigation water samples showed the pH value close to neutral. The oxidation – reduction potential was in oxidative range (positive values). The electrical conductivity was quite uniform, meaning that all samples had similar number of dissolved solids. The amounts of dissolved chlorides were low enough to not contribute significantly to amounts of chlorides in soil. These amounts of chloride are considered generally safe for all plants [6, 9, 10]. Concentrations of iron was below the detection limit of the AAS instrument.

3.2 Analysis of Soil Samples

Basic Quality Parameters

The descriptive statistics of basic quality parameter of the soil samples are shown by the Table 2.

Table 2. The results of the basic quality parameters of the soil samples

	pH (Water)	pH ($CaCl_2$)	ORP (mV)	EC (mS/cm)
Minimum	6.74	6.14	176	0.64
Maximum	8.36	7.64	261	8.87
Median	7.91	7.37	228	1.70
Average	7.78	7.28	223	2.92

pH value was in some cases slightly acidic and in others moderately basic. Oxidation-reduction potential values ware positive, which is similar to irrigation water results. Electrical conductivity was in contrast to irrigation water unevenly distributed, ranging from 0.64 to 8.87 mS/cm.

Chloride Content

The chloride content of the soil samples is presented by the Fig. 1.

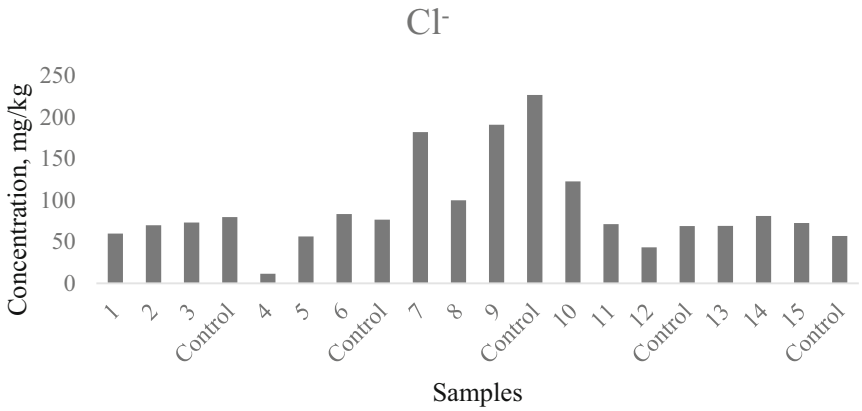

Fig. 1. Chloride content

The chloride content in soil samples was quite uneven inside each greenhouse. Amounts of chlorides in controls actually showed higher amounts of chlorides, than those found inside of greenhouses, meaning that greenhouses under investigation do not have significantly higher amounts of chlorides brought by e.g., fertilization, or the green houses are very well irrigated, so chlorides (as soluble salts) are easily.

mobilizable, and thus removed from the rhizosphere layer of greenhouse soil. The amounts of chlorides found in soil samples do not have a negative effect on the plant growth and development.

Iron Content

The iron content of the soil samples is presented by the Fig. 2.

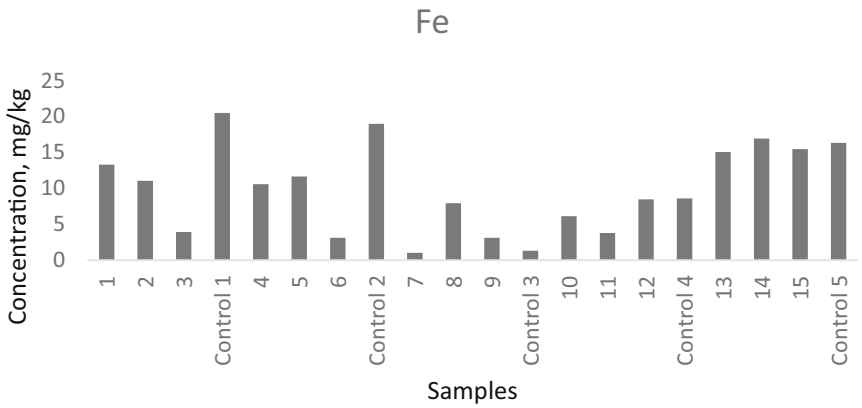

Fig. 2. Iron content

The water extractable iron content in investigated samples of was low and uneven.

In four of five cases, iron was higher in controls than in greenhouse soil samples. This means that samples inside of greenhouses is lacking in iron content. We believe that the reason is intensive agricultural production. Water extractable amounts of iron were similar to previous research [11].

Chloride Content with and Without EDTA Addition

The comparison of chloride content, with and without EDTA addition are presented by the Fig. 3**.**

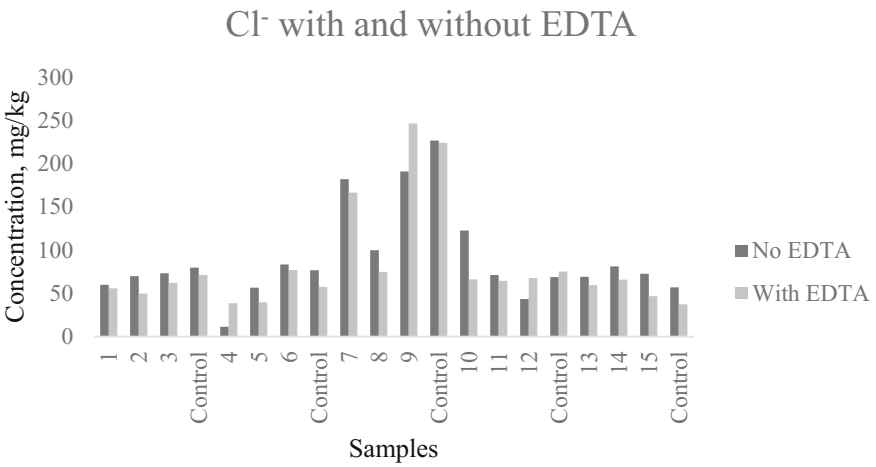

Fig. 3. Chloride content with and without EDTA addition

When the results of chloride content with and without EDTA are considered, we can conclude that in majority of samples (16 of 20), after addition of EDTA decreasing of chloride content is observed. It is expected that after the addition of EDTA, we do not have interferences anymore. So, the results should be higher. It means that, this small difference is probably derived by the heterogenicity of soil. Although certain difference is found, the amount of iron is not high enough to interfere with electrode.

Correlations

Table 3

The highest observed correlation was one found between chloride content with and without EDTA addition. This very strong connection is expected because the same parameter is measured with slightly different conditions. Iron showed a strong, and negative correlation with chloride content. Meaning that higher amount of iron corresponds to lower amount of chloride and *vice versa.* Iron compounds in soils, especially the iron oxyhydroxides are well known as a sequesters of cations from solution. The Cl^- anion shows little affinity (or specificity) in its adsorption to soil components (iron compounds are one of them) [12]. Oxidation – reduction potential shows moderate and positive correlation with chloride content. It means that higher (oxidative) ORP is accompanied with higher Cl- content. The very strong correlation is found between electrical conductivity

Table 3. Pearson's correlations among measured parameters

	Cl^-	Cl^- (EDTA)	Fe	ORP	EC	pH_{H2O}	pH_{CaCl2}
Cl^-	1						
Cl^- (EDTA)	0.920	1					
Fe	−0.562	−0.590	1				
ORP	0.605	0.622	−0.544	1			
EC	0.843	0.854	−0.741	0.549	1		
pH_{H2O}	−0.381	−0.398	0.128	−0.374	−0.275	1	
pH_{CaCl2}	−0.180	−0.195	−0.149	−0.395	0.031	0.878	1

and chloride content. Cl^- movement within the soil is largely determined by water flows [12]. Higher amount of chlorides gives a higher electrical conductivity. This fact was used to predict amounts of chlorides based on electrical conductivity [13].

4 Conclusions

The amount of chlorides found in researched samples do not threaten the wellbeing of cultivated plants inside of greenhouses.

The soil in greenhouses lacks in available iron.

Chloride is not bonded to iron particulate matter in soil, but dissolved into water fraction.

The water extractable amounts of iron are not high enough to interfere with ion-selective chloride electrode.

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Detection of Blueberry Latent Virus on Highbush Blueberries in Montenegro

Darko Jevremović[1](✉), Dejan Zejak[2], Bojana Vasilijević[1], and Aleksandar Leposavić[1]

[1] Fruit Research Institute, Kralja Petra I 9, 32102 Čačak, Serbia
darkoj@ftn.kg.ac.rs
[2] Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Zemun, Serbia

Abstract. Highbush blueberries (*Vaccinium corymbosum* L.) are a recent addition to Montenegro's fruit producing repertoire. The northern region of the country is home to new, dense plantations.

To investigate the presence of viruses in blueberry plantations in Montenegro during 2022, visual inspections of plantations were carried out. Seventeen leaf samples of five cultivars were collected and analyzed. The samples were tested by ELISA for the presence of: blueberry shock virus (BlShV), blueberry scorch virus (BlScV), blueberry shoestring virus (BSSV), blueberry leaf mottle virus (BLMoV), tomato ringspot virus (ToRSV), and tobacco ringspot virus (TRSV). Assays were conducted according to the recommended protocols. The samples were further tested for the presence of blueberry mosaic-associated virus and blueberry latent virus using RT-PCR and for the presence of the blueberry red ringspot virus using PCR. Extraction of total nucleic acids was performed with 2% CTAB buffer. Reverse transcription was done with pd$(N)_6$ random primers, and PCR was done with virus-specific primers. PCR products were analyzed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide.

The presence of blueberry latent virus was confirmed in 10 blueberry samples (59%) in cultivars ''Duke', 'Bluecrop', and 'Earlyblue'. Virus identification was confirmed by sequencing the amplified fusion protein gene fragments of all 10 isolates. No other viruses were confirmed in the tested samples.

This is the first survey for virus presence in blueberries in Montenegro and the first finding of blueberry latent virus in the country.

Keywords: *Vaccinium corymbosum* L. · Viruses · ELISA · PCR · Latent infection

1 Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is a relatively new fruit species for the Balkan countries. The intensive growing of blueberries started in Serbia in the 2000s and has reached more than 2,500 ha today (Leposavić and Jevremović, 2020). In Montenegro, growing highbush blueberries is at the very beginning, with an estimated 5 ha of orchards. Blueberry expansion may favor the emergence or spread of diseases and pests

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that could threatening production. Highbush blueberry is a host to more than 15 viruses and virus-like agents (Martin and Tzanetakis, 2018; Saad et al., 2021). Economically, the most important viruses of blueberries are: blueberry scorch virus (BlScV), blueberry shock virus (BlShV), blueberry shoestring virus (BSSV), blueberry red ringspot virus (BRRV), blueberry leaf mottle virus (BLMoV), tobacco ringspot virus (TRSV), and tomato ringspot virus (ToRSV) (Martin et al., 2012). These viruses originate in North America, but they are also present in numerous countries throughout the world. Other viruses of lesser importance include: peach rosette mosaic virus (PRMV), blueberry mosaic-associated virus (BlMaV), blueberry latent virus (BlLV), blueberry virus A (BVA), blueberry latent spherical virus (BLSV), cherry leaf roll virus (CLRV), strawberry latent ringspot virus (SLRSV), blueberry necrotic ring blotch virus (BNRBV), blueberry green mosaic-associated virus (BGMaV), and blueberry virus T (BlVT) (Saad et al., 2021). Blueberry latent virus has been reported in the USA, Japan, Serbia, and Bosnia and Herzegovina (Jevremović and Paunović, 2021, Delić et al., 2022).

In sensitive cultivars, viruses can cause significant yield losses, reduce fruit quality or destroy entire plants. Viruses can also induce latent infections in tolerant cultivars. They spread through infected plant material and vectors (nematodes and insects). Preventive virus disease control measures include the use of virus-free planting material and spatial isolation from other blueberry plantations. In practice, eradication of symptomatic or diseased plants may not give satisfactory results due to the long latency of some viruses (Saad et al., 2021).

This paper presents the results of the first survey for virus presence in highbush blueberry plantations in Montenegro.

2 Material and Methods

2.1 Plant Material

Material for this study was collected during a survey performed in June 2022 in highbush blueberry orchards in Montenegro. A total of 17 leaf samples from five blueberry cultivars (‘Aurora’, ‘Bluecrop’, ‘Brigita Blue’, ‘Duke’, and ‘Earlyblue’) were collected from commercial orchards in north Montenegro (Table 1). Each sample consisted of ten youngest but fully expanded leaves, picked from several branches of a single bush. Collected samples were stored at 4 °C (for ELISA) and at -80 °C (for PCR).

2.2 ELISA Test

All collected samples were serologically tested for the presence of six viruses: blueberry scorch virus (BlScV), blueberry shock virus (BlShV), blueberry leaf mottle virus (BlMoV), blueberry shoestring virus (BSSV), tobacco ringspot virus (TRSV), and tomato ringspot virus (ToRSV). Assays were performed by enzyme-linked immunosorbent assay (ELISA) with the reagents of AGDIA Inc. (USA) (BlScV, BlShV BlMoV and BSSV), and BIOREBA AG (Switzerland) (TRSV and ToRSV) according to the manufacturers’ recommendations. Fresh leaf samples were ground at 1:10 ratio in the general extraction buffer (GEB) (AGDIA Inc). Color development was measured at 405 nm for

Table 1. List of analyzed blueberry samples from Montenegro.

Cultivar	Number of analyzed samples	Number of BlLV positive samples	%
Aurora	1	0	/
Bluecrop	6	5	83,3
Brigita Blue	1	0	/
Duke	8	4	50
Earlyblue	1	1	100
Total	**17**	**10**	**58,8**

all viruses except BSSV (650 nm) on ELISA plate reader (MULTISKAN MCC/340, Finland) after 20 – 120 min. Samples were considered positive when recorded optical density (OD) values were at least three times higher than the OD values of the negative control.

2.3 TNA Extraction

Total nucleic acids (TNA) were extracted following a modified CTAB protocol (Li et al., 2008). A leaf tissue sample of 200 mg taken from ten leaves of a single plant was ground in extraction bags (Flexo Duga, Serbia) with 2 ml of 2% cetyltrimethylammonium-bromide (CTAB) buffer. One ml of the extract was incubated for 15 min at 65 °C and centrifuged at 10,400 rpm for 5 min. The obtained supernatant (650 µl) was transferred to a new 1.5 ml tube and vortexed with an equal volume of chloroform/isoamyl alcohol (24:1). The mixture was centrifuged for 10 min at 12,800 rpm. The supernatant (500 µl) was transferred to another tube with 350 µl ice-cold isopropanol, mixed and centrifuged at 12,800 rpm for 10 min. The aqueous phase was removed and the TNA pellet was washed with 1 ml ice-cold 70% ethanol by centrifugation for 5 min at 12,800 rpm, dried at room temperature, and dissolved in 100 µl Tris-EDTA.

2.4 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Samples were analyzed for the presence of BlLV and BlMaV by reverse transcription-polymerase chain reaction (RT-PCR) using virus-specific primers. Reverse transcription was performed with random pd(N)6 primers and Maxima Reverse Transcriptase (Thermo Scientific, USA). The obtained complementary DNA (cDNA) was then used as a template in a PCR reaction with BlLV and BLMaV specific primer sets (Martin et al., 2011; Thekke-Veetil et al., 2014). Extracted TNA was used for PCR reactions with specific primers for BRRV detection (Glasheen et al., 2002). PCR reactions were carried out in a TPersonal thermal cycler (Biometra, Germany) with Green Taq DNA Polymerase (Thermo Scientific, USA).

Horizontal electrophoresis in 1.5% agarose gel was used to analyze PCR products, which were stained with ethidium bromide and visualized with the Gel Doc EZ System (Biorad, USA) using a UV tray. The presence of a fragment of the expected size was considered a positive reaction.

2.5 Sequence Analysis

PCR products of all 10 detected BlLV isolates were sequenced. Phylogenetic relationships were reconstructed by a Maximum Likelihood (ML) tree with a Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution using MEGA11 software (Tamura et al., 2021).

3 Results

During the survey, we did not observe blueberry plants with the symptoms that could be linked to viral infections. Collected blueberry leaf samples of five cultivars were analyzed for the presence of nine viruses. None of the analyzed six viruses (BlScV, BlShV, BlMoV, BSSV, TRSV, and ToRSV) were detected in the ELISA test. PCR reaction with BRRV-specific primer pair confirmed the absence of BRRV in the analyzed samples. RT-PCR analysis was performed for BlMaV and BlLV detection. BlMaV was not detected in analyzed samples, whereas the presence of BlLV was confirmed in 58.8% samples. The expected 391 bp fragment was obtained in 10 out of 17 tested samples in cultivars 'Bluecrop' (5), 'Duke' (4), and 'Earlyblue' (1) (Table 1). BlLV was confirmed in all five surveyed localities (Fig. 1). The percentage of infected from total number of tested samples in surveyed localities varied from 50 to 75%.

Fig. 1. Sampling locations and the occurrence of BlLV in analyzed samples

To further characterize detected BlLV isolates, we partially sequenced a fragment of fusion protein (F) gene of all ten isolates. Obtained sequences were deposited in the nucleotide sequence database (https://www.ncbi.nlm.nih.gov/) and assigned the accession numbers OP561432 – OP561441. When compared, Montenegrin isolates showed 100% nucleotide (nt) sequence identity. The sequences of these isolates were compared with 31 sequences of other BlLV isolates available in the NCBI GenBank. BLAST analysis showed 99.71% nt sequence identity with isolates from South Korea (LC440652 and LC437352), Serbia (MW177582), and the USA (HM029247). Complete (100%) nt sequence similarity Montenegrin isolates share with isolates from Serbia (MW177576 – MW177581, MW177583 – MW177589), USA (HM029246, HM029248, EF442779, NC014593, and MN416031), Japan (AB608991), and Bosnia and Herzegovina (MK511321 – MK511327 and MK519231). Phylogenetic analysis of the partial nucleotide sequences of the fragment from the fusion protein gene showed that all BlLV virus isolates were grouped into a single cluster (Fig. 2).

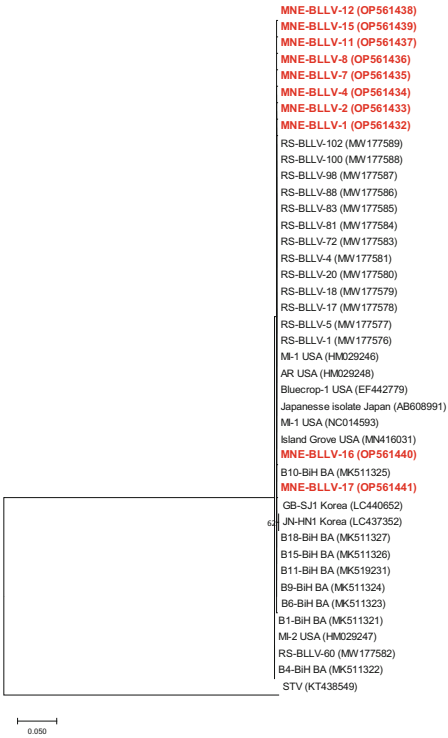

Fig. 2. Phylogenetic analysis of 41 BlLV sequences of isolates reconstructed from partial F gene sequences. The analysis was performed with MEGA11 software using ML method based on the HKY model. Phylogeny was inferred after 1000 bootstrap replications. Bootstrap support of > 60% are shown at nodes. Isolates from Montenegro are presented in red. The NCBI accession numbers are in parentheses. The tree was rooted with southern tomato virus (STV) as an outgroup.

4 Discussion

Highbush blueberry is a new species for fruit production in Montenegro, and in this study, we presented the results of the first survey for blueberry viruses in the country. Only the blueberry latent virus was detected in analyzed samples out of nine tested viruses. Blueberry latent virus is a member of the genus Amalgavirus, family Amal-gaviridae. It is a virus of minor importance that causes latent infections in blueberries, both in single or mixed infections (Martin et al., 2011).

A high percentage (58.8%) of BlLV-infected samples were confirmed during our study. Similar results were obtained in the study of Jevremović and Paunović (2021) con-ducted in Serbia, where almost 63% of the tested samples were BlLV positive. Significant BlLV incidence in analyzed samples was reported in the surveys in the USA (about 50%) and Japan (28%) (Martin et al., 2011; Isogai et al., 2011). On the other hand, the latest survey in the USA revealed a very low percentage of BlLV-infected samples (3.6%) (Martin and Tzanetakis, 2018). According to Martin et al. (2012), the prevalence of the BlLV should not be of major concern though as no symptoms have been observed on several highbush cultivars for almost 10 years. Its role in mixed infections is to be determined.

The analysis of nucleotide sequences of a partial fusion protein gene revealed a high similarity (99.71–100%) of Montenegrin with isolates from other countries. According to the studies of Martin et al. (2011), Jevremović and Paunović (2021), and Delić et al. (2022) BlLV is very homogeneous, that was also confirmed in our study.

Introducing a new plant species into the country poses the risk of introducing new diseases that can endanger production. Therefore, the most critical step in developing a control strategy is the early diagnosis of the causal agent.

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Compliance with Ethical Standards.

Conflict of Interest. The authors declare that they have no conflict of interest.

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Leaf Diseases of Wild Barley (*Hordeum spontaneum*) in Bingöl University Campus, Turkey

Işıl Saraç Sivrikaya[1], Arzu Çelik Oğuz[2], and Aziz Karakaya[2(✉)]

[1] Faculty of Agriculture, Department of Plant Protection, Bingöl University, Bingöl, Turkey
[2] Faculty of Agriculture, Department of Plant Protection, Ankara University, Dışkapı, Ankara, Turkey
karakaya@agri.ankara.edu.tr

Abstract. In May 2022, a survey was accomplished in the campus area of Bingöl University, Turkey, and diseases in wild barley (*Hordeum spontaneum*) populations were determined. A total of 15 *H. spontaneum* populations were examined. In these populations, powdery mildew incited by *Blumeria graminis* f. sp. *hordei*, scald incited by *Rhynchosporium commune*, the net form of net blotch incited by *Pyrenophora teres* f. *teres*, and spot form of net blotch incited by *Pyrenophora teres* f. *maculata* were determined by visual identification. Powdery mildew was observed in all 15 populations. Scald, the net form of net blotch, and spot form of net blotch were observed in 7, 3, and 2 populations, respectively. In these areas, the percentages of these diseases varied between 50–90% for powdery mildew, 10–30% for scald, 2–3% for the net form of net blotch, and 1–2% for the spot form of net blotch. Scale values for disease severity were higher in powdery mildew (between 5–9), 3–5 for scald, and low in both forms of net blotch (1–3, and 1–2).

Keywords: *Hordeum spontaneum* · barley leaf diseases · Bingöl University · Turkey

1 Introduction

Wild barley, *Hordeum vulgare* subsp. *spontaneum* (=*H. spontaneum*) is the ancestor of cultivated barley (*Hordeum vulgare*). *H. spontaneum* commonly grows in the Fertile Crescent region (von Bothmer et al., 2003; Karakaya et al., 2016). Chromosome numbers of both *H. spontaneum* and *H. vulgare* are the same (2n = 14). Since these two species are in the same gene pool, they can be crossed easily. Thus, the transfer of important genes from wild barley into cultured barley can be achieved (Lehmann, 1991; Fischbeck and Jahoor, 1992). Natural hybrids are possible when *H. spontaneum* and *H. vulgare* coexist in the same field (von Bothmer et al., 1995). *H. spontaneum* is common in Turkey (Karakaya et al., 2016; Saraç Sivrikaya et al., 2021). There are different biotic and abiotic disease factors affecting barley plants (Mathre, 1997). In different studies conducted in Turkey among barley and wild barley plants, several disease agents including *Drechslera teres* (teleomorph: *Pyrenophora teres*), *Rhynchosporium commune* (formerly named

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Rhynchosporium secalis (Zaffarano et al. (2011), *Drechslera graminea* (teleomorph: *Pyrenophora graminea*), *Puccinia hordei*, *Blumeria graminis* f. sp. *hordei*, and *Ustilago* spp. Were reported (Çelik and Karakaya, 2015; Karakaya et al., 2016; Özdemir et al., 2017; İlgen et al., 2017; Saraç et al., 2019; Saraç Sivrikaya et al., 2021). In this survey study, fungal leaf diseases present on wild barley plants in the campus area of Bingöl University, Turkey were determined.

2 Materials and Methods

In May 2022, a survey was accomplished at the campus area of Bingöl University, Turkey where the dense wild barley populations were present. The area consisted of 3,115,616.85 m2, and the leaf diseases observed in wild barley (*Hordeum spontaneum*) plants in this area were determined visually (Fig. 1).

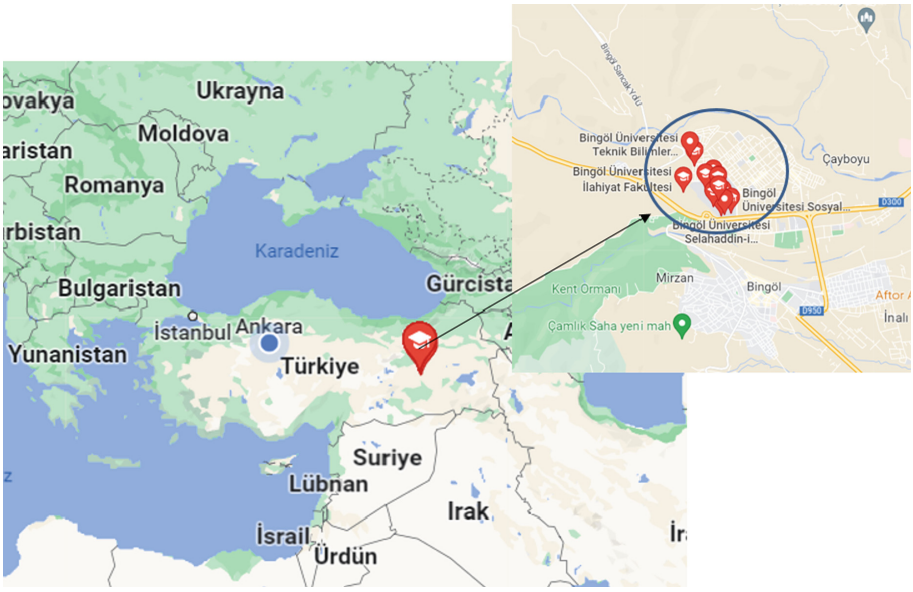

Fig. 1. The location of Bingöl University, Bingöl, Turkey (https://www.google.com/maps/search/bing%C3%B6l+%C3%BCniversitesi+kamp%C3%BCs%C3%BC/@38.8981853,40.4609192,13z)

A total of 15 locations and at least 10 plants from each location were examined (Fig. 2). For disease identification, Zillinsky (1983), Mathre (1997), and Zaffarano et al. (2011) were used. For the identification of *Rhynchosporium commune,* the nomenclature of Zaffarano et al. (2011) was followed. For determination of the severity of the diseases, a 1–9 scale was used (Saari and Prescott, 1975). In calculating the average prevalence of diseases, the populations with and without diseases were evaluated together. Bingöl province, located in eastern Turkey, is a region rich in wild barley resources because of its closeness to the Fertile Crescent region. The main reason for choosing the campus area was the widespread occurrence of *Hordeum spontaneum.*

3 Results and Discussion

In the wild barley populations at the Bingöl University campus, powdery mildew disease incited by *Blumeria graminis* f. sp. *hordei,* barley scald disease incited by *Rhynchosporium commune*, the net form of net blotch incited by *Pyrenophora teres* f. *teres* and spot form of net blotch incited by *Pyrenophora teres* f. *maculata* were determined (Table 1, Fig. 3). *B. graminis* f. sp. *hordei* was present in all 15 wild barley populations examined. *R. commune* was detected in 7 populations. *P. teres* f. *teres* was found in 3 populations and *P. teres* f. *maculata* was found in 2 populations. Percentages of diseased plants in locations where diseases were detected ranged between 50–90, 10–30, 1–3, and 1–2 for *B. graminis* f. sp. *hordei*, *R. commune*, *P. teres* f. *teres*, and *P. teres* f. *maculata*, respectively. Disease severity values ranged between 5–9 for powdery mildew, 3–5 for scald, and 1–3 for the net and spot forms of net blotch.

Table 1. Disease agents, average disease prevalences, number of infected fields, percentage range of diseased plants, and disease severity values in wild barley (*H. spontaneum*) plants observed in the survey conducted in the campus area of Bingöl University in May 2022.

Wild Barley Disease Agents	Mean Disease Prevalence	Number of Fields with Disease	Percentage Range of Diseased Plants	Disease Severity Values
Blumeria graminis f. sp. *hordei*	70	15	50–90	5–9
Rhynchosporium commune	8.67	7	10–30	3–5
Pyrenophora teres f. *teres*	0.6	3	1–3	1–3
Pyrenophora teres f. *maculate*	0.13	2	1–2	1–3

Powdery mildew disease incited by *B. graminis* f. sp. *hordei* was observed in all locations studied. The mean disease prevalence of the powdery mildew disease was 70%. Barley scald disease incited by *R. commune* was present in 7 locations with a mean prevalence of 8.67%. The net form of net blotch disease incited by *P. teres* f. *teres* was found in 3 locations with a prevalence of 0.6%. The spot form of net blotch incited by *P. teres* f. *maculata* was seen only in 2 locations with a mean prevalence of 0.13%.

In the Bingöl University campus area, powdery mildew incited by *B. graminis* f. sp. *hordei* was the most commonly encountered disease. Barley scald disease incited by *R. commune*, the net form of net blotch incited by *P. teres* f. *teres*, and the spot form of net blotch incited by *P. teres* f. *maculata* followed the powdery mildew.

These diseases detected in wild barley (*H. spontaneum*) populations in the Bingöl University campus area are the diseases that are frequently seen in barley and wild barley plants in Turkey and they are previously reported by other researchers (Akan et al., 2006;

Fig. 2. *Hordeum spontaneum* plants growing in the Bingöl University campus area, Bingöl, Turkey

Karakaya et al., 2014; Çelik and Karakaya, 2015; Karakaya et al., 2016; Özdemir et al., 2017; Karakaya et al., 2020; İlgen et al., 2017; Ertürk et al., 2018; Saraç et al., 2019; Saraç Sivrikaya et al., 2021)**.**

Karakaya et al. (2016) investigated naturally growing *Hordeum spontaneum* field populations in Mardin, Şanlıurfa, Siirt, Şırnak, Gaziantep, Diyarbakır, Hatay, and Kilis provinces of Turkey for the presence of diseases and their severities. No disease was observed in 9 *Hordeum spontaneum* populations. Powdery mildew incited by *B. graminis* f. sp. *hordei*, barley scald incited by *R. commune*, brown rust incited by *P. hordei*, both

forms of net blotch incited by *D. teres* f. *maculata* and *D. teres* f. *teres,* loose smut incited by *Ustilago nuda*, barley stripe incited by *Drechslera graminea,* and semi-loose smut incited by *Ustilago nigra* were found in these provinces. Barley scald was the most commonly observed disease. Powdery mildew and net blotch diseases followed

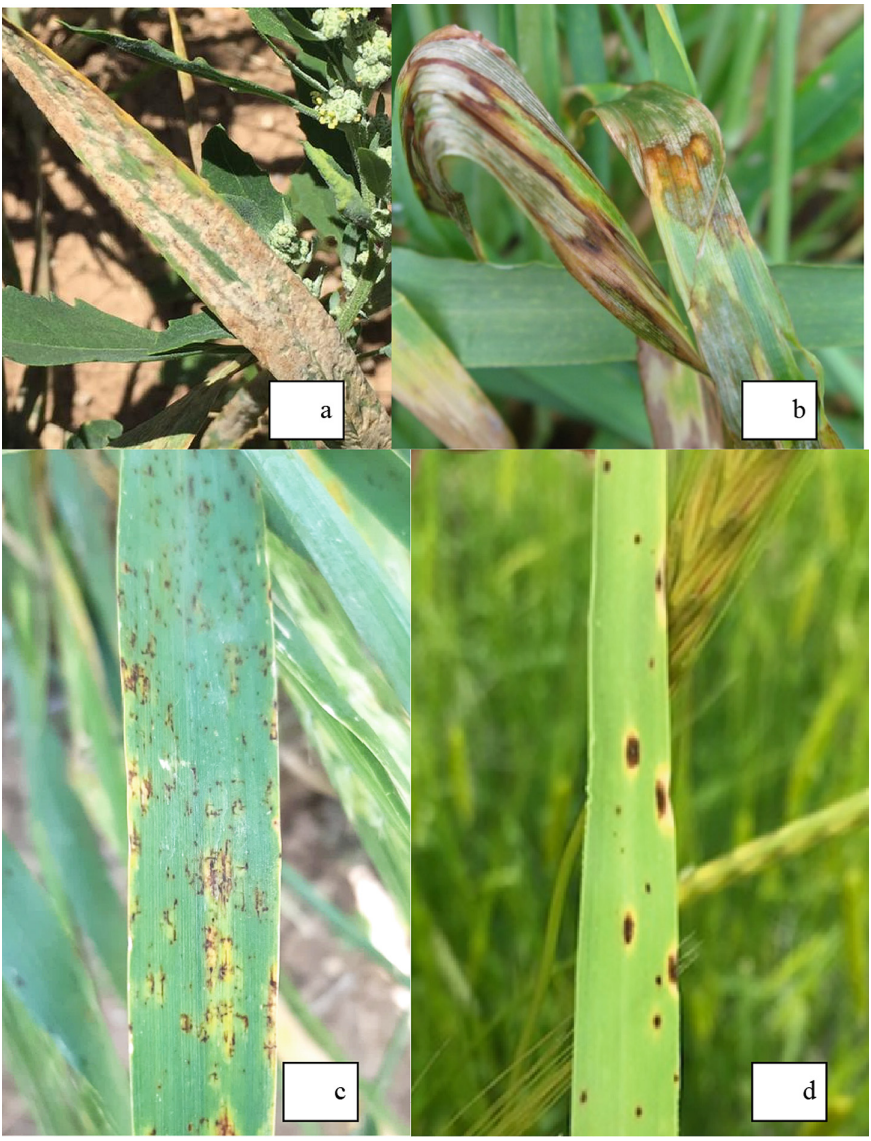

Fig. 3. A) Powdery mildew disease incited by *Blumeria graminis* f. sp. *hordei*, b) barley scald disease incited by *Rhynchosporium commune*, c) net form of barley net blotch disease incited by *Pyrenophora teres* f. *teres*, d) spot form of barley net blotch disease incited by *Pyrenophora teres* f. *maculata*

the barley scald. The severity and incidence values of diseases showed variation. In our current study, powdery mildew was the most commonly observed disease. Barley scald, the net form of net blotch, and spot form of net blotch diseases followed the powdery mildew disease.

Saraç Sivrikaya et al. (2021) inspected the leaf diseases of naturally grown wild barley (*H. spontaneum*) populations in the Batman central district and Beşiri (Batman), Hasankeyf (Batman), Kurtalan (Siirt), and Midyat (Mardin) districts of Turkey. Barley leaf scald disease was the most common disease among the *H. spontaneum* populations. Spot form of net blotch, the net form of net blotch, powdery mildew, and brown rust diseases followed the barley leaf scald. In addition, 37 barley fields in Batman central district and Kozluk (Batman), Hasankeyf (Batman), Beşiri (Batman), Gercüş (Batman), Sason (Batman), Midyat (Mardin), Kocaköy (Diyarbakır), and Kurtalan (Siirt) districts of Turkey were investigated for the presence of leaf diseases. In barley fields, scald was the most commonly encountered disease. Spot form of net blotch, barley stripe, the net form of net blotch, spot blotch, powdery mildew, and brown rust followed the barley scald.

4 Conclusion

Powdery mildew disease incited by *B. graminis* f. sp. *hordei*, barley scald disease incited by *R. commune*, net and spot forms of barley net blotch disease incited by *P. teres* f. *teres*, and *P. teres* f. *maculata* were determined in the wild barley (*H. spontaneum*) plants grown in the campus area of Bingöl University, Turkey. The most common disease at Bingöl University campus was powdery mildew followed by barley scald, the net form of net blotch, and the spot form of net blotch.

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The Effect of Biofertilization on Potato Yield Components

Teofil Gavrić(✉), Emra Bašić, Drena Gadžo, Saud Hamidović, and Mirha Đikić

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, Sarajevo, Bosnia and Herzegovina
t.gavric@ppf.unsa.ba

Abstract. The potatoes (*Solanum tuberosum* L.) are the third most important food crop in Bosnia and Herzegovina, right after maize and wheat. It was grown on an area of 39,051 ha with an average yield of 11.3 t ha^{-1} in 2020. The potato root system is shallow, with a poor ability to extract nutrients from the soil. Therefore, potatoes should be provided with enough nutrients during cultivation to achieve high yields. This work aimed to examine the impact of biofertilizers on potato yield in the environment of central Bosnia and Herzegovina. The treatments used in this research consisted of several different fertilizers (mineral fertilizer, mineral fertilizer + biofertilizer, organic fertilizer and organic fertilizer + biofertilizer). The Carrera variety was used in the research. The research results showed that biofertilizer increased the number of tubers per plant. Biofertilizer variants had more tubers per plant (7.12) than those without it (6.93). The range of tuber mass was between 115.80 g (organic fertilizer) to 134.91 g. (mineral fertilizer). Depending on the year of research, potato yield ranged from 34.83 t ha^{-1} (2018) to 37.51 t ha^{-1} (2019). The applied fertilizers did not significantly affect the content of dry matter in the potato tuber.

Keywords: Potatoes · Fertilizer · Biofertilizer · Manure · Yield

1 Introduction

Potatoes (*Solanum tuberosum* L.) are the fourth most significant crop in the world in production, harvested areas, and consumption after wheat, maize, and rice [1]. Potatoes have been vital in preventing hunger and malnutrition worldwide [2]. Also, this crop is still an essential source of income for developing countries [3]. Furthermore, potatoes in human nutrition have gained popularity thanks to the more favourable overall nutrient-to-price ratio than many other crops [4]. In other words, potatoes are cheap and an excellent source of nutrients such as vitamin C, potassium, magnesium, and vitamin B6 [5]. Furthermore, potatoes can be used to prepare various food products using many preparation methods, including baking, boiling, and steaming. In addition to their importance in human nutrition, potatoes are also valuable in animal nutrition. Furthermore, potatoes are a useful raw material for industry, where they are used to produce products such as starch, alcohol, paper, etc. [6–8].

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In Bosnia and Herzegovina (B&H), potatoes are grown in an area of 39,051 ha with an average yield of 11.3 t ha^{-1} [9]. A lower yield than surrounding countries characterizes its production in B&H. One of the reasons for low potato yields is unfavorable environmental conditions. However, to a certain degree, growers can reduce the adverse effects of the environment on the crop by using various agronomic practices. In addition to the choice of cultivars, crop rotation, plant protection, and irrigation, crop nutrition is another important agronomic practice for potato production. This agronomic practice is even more significant because the potato root system is shallow and poorly extracts nutrients from the soil [10]. Therefore, a sufficient supply of nutrients is one of the necessary factors for achieving a high yield of potatoes [11]. Organic and mineral fertilizers are mainly used in growing potatoes. Both types of fertilizers have advantages and disadvantages. Organic fertilizers contain all macro and micronutrients and positively affect soil structure [12, 13]. However, these fertilizers' main disadvantages are their low nutrient content and slow decomposition of organic matter [14]. On the other hand, mineral fertilizers have a high content of macronutrients available to plants to achieve high yields [15, 16]. However, using these fertilizers, especially in large doses, causes some environmental issues, such as surface and underground water pollution and the reduction of soil microbial biodiversity [14]. Therefore, in addition to the mentioned fertilizers, biofertilizers have also been used in plant production. They are substances that contain various soil microbes (e.g., Bacillus, Pseudomonas, Azotobacter and Azospirillum) that colonize the rhizosphere and make nutrients easily available to plants [17]. Various benefits are associated with these fertilizers, such as the decomposition of organic matter, increasing the availability of nutrients, production of phytohormones, and contribution towards mitigation of abiotic and biotic stresses [18]. Although their use has increased significantly in the last two decades in some parts of the world [19], they are insufficiently researched in the environmental conditions of B&H [17]. Therefore, the aim of this work was to examine the influence of biofertilizers on potato yield in the environmental conditions of central Bosnia and Herzegovina.

2 Materials and Methods

2.1 Experiment Location

A field experiment was conducted at a private farm Gavrić in Kakanj (44°07′27.8″N 18°07′42.3″E) during the 2018 and 2019 growing seasons.

2.2 Weather Condition Analysis

Meteorological data from meteorological station Zenica were used in the research. The data was collected by the Federal Hydrometeorological Institute, Sarajevo, B&H [20]. The data for average monthly air temperature and amount of precipitation for 2018 and 2019 were used in the research.

2.3 Soil Analysis

Soil samples were collected at 0–20 cm depth before setting up the experiment. Each collected soil sample was a combination of four subsamples. The following soil properties were measured according to standard methods: soil pH [21], organic matter content [22], and the contents of available K and available P [23]. According to soil analysis, the experimental plots had the following chemical characteristics: pH 6.8, 5.8% organic matter, $P_2O_5 = 25.8$ mg 100 g^{-1}, $K_2O = 70.0$ mg 100 g^{-1}.

2.4 Experimental Design

A cultivar of potatoes called "Carrera" was used for the research. The treatments used in the research consisted of a combination of different fertilizers (organic and mineral) and biofertilizers (biofertilized and non-biofertilized). The experiment was set up by randomized block method in four repetitions. The size of the basic plot was 4.9 m^2.

2.5 Properties of Fertilizer

The mineral fertilizer applied in the research was produced by INA Kutina, Croatia. Mineral fertilizer was used in the form of 7:20:30 at a rate of 500 kg ha^{-1}. The second applied fertilizer was organic (manure). Manure was applied at the rate of 50 t ha^{-1}. The chemical composition of the manure was as follows: pH = 7.5; Organic Matter = 20.66%, $P_2O_5 = 0.18\%$; $K_2O = 0.6\%$. Biofertilizer called "Megaflu" was used in the research. According to the product declaration, this preparation contained the following microbes: *Bacillus megaterium, Pantoea agglomerans and Pseudomonas fluorescens.*

2.6 Crop Management

Potatoes were planted on April 16 in the first year of research (2018) and April 4 in the second year of research (2019). The distance from row to row was 70 cm and from plant to plant 33 cm. Fertilizer and biofertilizer were applied to the intended plots before planting. In addition, chemical plant protection agents were used according to the need against the Colorado potato beetle (*Leptinotarsa decemlineata*) and Potato late blight (*Phytophthora infestans*). Potatoes were harvested on September 17, 2018, and on September 14, 2019.

2.7 Data Collection

Yield components included the number of tubers per plant, the average mass of the tuber, the percent of large, medium, and small tubers, yield per hectare, and dry mass. Potato tubers from each plot were manually harvested, counted, and separated into large (>55 mm), medium (35–55 mm), and small (<35 mm) classes according to their size. Tubers of each category were weighed to calculate tuber yield. The dry matter was determined by drying the samples at 105 °C for 16 h.

2.8 Statistical Methods

All experiments were performed in quadruplicate. One-way ANOVA was used to examine the data and Tukey's multiple tests statistically evaluated the comparison of the means between treatments at a significance level of 0.05. The research findings were statistically analyzed using the SPSS 22 software.

3 Results and Discussions

3.1 Analysis of Weather Conditions

Data for the average monthly temperature and total precipitation collected from the regional meteorological stations in Zenica are presented in Fig. 1. Data analysis shows that weather conditions in 2018 and 2019 differed from the reference climate period. (1961–1990). Average monthly temperatures during both years of the research were higher than that of the reference period (1961–1990). Hot weather was registered in May 2018, when the average monthly temperature was higher by 3.4 °C compared to the reference climate period. June and August were the warmest months this year, with recorded temperatures of 4.4 °C and 3.8 °C higher than the climatic reference period. These recorded high monthly temperatures are not desirable for growing potatoes. Namely, potatoes have the highest yields when the average monthly temperature is up to 20 °C [24]. High temperatures can reduce potato yield due to physiological changes in the plant, such as respiration and photosynthesis [25]. The monthly amount of precipitation in the same period was similar to the climatic reference period (1961–1990), except for July 2018 and June 2019. In July 2018, 208 mm of precipitation was recorded, 145 mm more than the climatic reference period. In June 2019, 14.7 mm of rainfall was recorded.

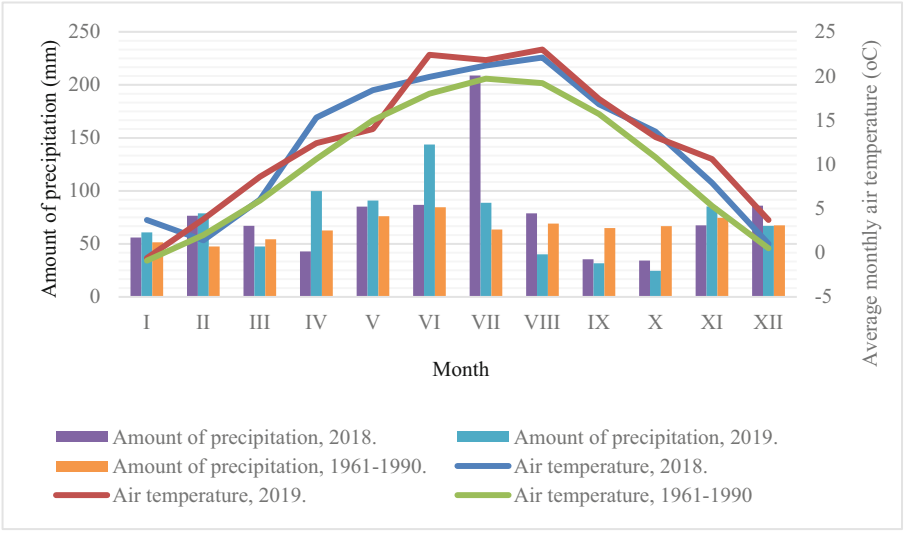

Fig. 1. Average monthly temperature and amount of rainfall

3.2 Potato Yield

The influence of biofertilizer, fertilizer type, and research year on potato yield components is presented in Tables 1, 2, and 3 and explained in detail below. The number of tubers per plant significantly depended on the application of biofertilizer, the type of fertilizer, and the year of research. The variant with biofertilizer had the most tubers per plant (7.12) compared to the variant without this preparation (6.93). (Table 1). A similar influence of biofertilizers on the number of tubers was recorded in research by many authors [26, 27]. Also, in our research, the type of fertilizer significantly impacted the number of tubers. It was recorded that the organic fertilizer treatment had a higher number of tubers per plant (7.67) compared to the mineral fertilizer treatment (6.37) (Table 2).

The year of research also significantly affected the tested traits (Table 3). Potatoes grown in 2019 had a higher number of tubers per plant (8.79) than those from 2018. According to Margus et al. [28], the number of tubers per plant mostly depends on the growing conditions at the beginning of the growing season. Based on the weather conditions observed in our research, it can concluded that more precipitation was recorded in April, May, and June 2019 than at the same time in 2018 (Fig. 1). Therefore, the 2019 research year had a more favourable beginning of the growing season, positively affecting the number of tubers per plant.

Table 1. Effect of biofertilizer application on the number of tubers per plant, the mass of the tuber, percent of large, medium, and small tubers, potato yield, and dry matter content

Biofertilizer	Number of tubers per plant	Mass of the tuber	Large tubers	Medium tubers	Small tubers	Yield	Dry matter
		–g–	–%–	–%–	–%–	–t ha^{-1}-	–%–
not applied	6.93b	128.03 ns	50.03 ns	43.66 ns	6.08 ns	35.39 ns	17.44 ns
applied	7.12a	122.68 ns	49.99 ns	43.90 ns	6.12 ns	37.03 ns	17.21 ns
Average	7.02	125.35	50.01	43.78	6.10	36.21	17.28

Different letters indicate significant differences at the 0.05 level; ns: nonsignificant differences.

The mass of the tuber was significantly dependent on the type of fertilizers and year of research, while application of biofertilizer did not significantly affect this trait. As can be observed from Table 2, the mass ranged from 115.80 g (organic fertilizer) to 134.91 g (mineral fertilizer). In contrast to organic fertilizer, mineral fertilizer treatment increased the abundance of tubers. The obtained results are, to some extent, expected due to the differences in the chemical composition of the applied fertilizers. Mineral fertilizers have a higher content of macronutrients readily available to plants than organic fertilizers [14, 16]. Also, our experiment indicated that the year of research also influenced the mass of the tuber (Table 3). A greater mass of tubers was determined in 2018 (149.63 g) compared to 2019 (101.08 g). The resulting differences are most likely the result of a different distribution of precipitation in specific years (experimental years) during the

tuber bulking stage. Thus, during June, July, and August 2018, 374.4 mm of precipitation was recorded compared to 2019, when 272.7 mm of rainfall was recorded in the same period. These observations are consistent with many authors [29–31], who found that the lack of precipitation leads to a low mass of tubers.

Table 2. Effect of type of fertilizers on number of tubers per plant, mass of the tuber, percent of large, medium and small tubers, potato yield and dry matter content

Fertilizer	Number of tubers per plant	Mass of the tuber	Large tubers	Medium tubers	Small tubers	Yield	Dry matter
		–g–	–%–	–%–	–%–	–t ha-1-	–%–
mineral	6.37b	134.91a	49.17 ns	44.97 ns	5.91 ns	34.82b	17.08 ns
organic	7.68a	115.80b	50.86 ns	42.59 ns	6.29 ns	37.44a	17.48 ns
Average	7.02	125.35	50.01	43.78	6.10	36.13	17.28

Different letters indicate significant differences at the 0.05 level; ns: nonsignificant differences.

The research results revealed that the proportion of large, medium, and small tubers significantly depends only on the year of the research. (Table 3). A significantly higher proportion of large tubers (>55 mm) was recorded in 2018 (67.25%) compared to 2019 (32.78%). On the other hand, a higher proportion of medium tubers (35–55 mm) was recorded in 2019 (57.32%) compared to 2018 (30.24%). Therefore, the proportion of medium tubers was inversely proportional to that of large tubers, which was confirmed by a negative correlation ($r = -0.987$). A tuber mass analysis partly explained the increased proportion of large tubers observed in 2018. The presumption is that the larger precipitation in 2018 is the main reason for this. Another reason is the number of tubers per plant in 2018. In particular, fewer tubers were noted in 2018 than in 2019. This means that a smaller number of tubers per plant were better filled with assimilates in 2018. This conclusion is supported by the negative correlation ($r = -0.716$) between the number of tubers per plant and large tubers (Table 4). Our results are in harmony with those obtained by many authors [32–34], who also recorded a higher proportion of large tubers in wetter soil conditions. Badr et al. [35] believe that tuber mass is a yield component more sensitive to water deficit than tuber number.

Based on the yield data, it can be concluded that the values did not change significantly due to the biofertilizer application. (Table 1). In contrast, it significantly depended on the type of fertilizer (Table 2) and the year of cultivation (Table 3). Thus, depending on the application of microbiological fertilizer, the yield ranged from 35.39 (not applied) to 37.03 t ha^{-1} (applied). There were certain differences, but they were not statistically significant. Variants treated with organic fertilizer had a significantly higher yield than those treated with mineral fertilizer (Table 2). Differences in yield were due to differences in the number of tubers per plant between treatments. Namely, the variant treated with organic fertilizer had a significantly higher number of tubers per plant. Organic fertilizers, in addition to containing micro and macronutrients, have a positive effect on improving soil structure, bulk density, aeration, porosity, and water movement [34, 36].

Table 3. Effect of years of research on the number of tubers per plant, the mass of the tuber, percent of large, medium, and small tubers, potato yield, and dry matter content

Year	Number of tubers per plant	Mass of the tuber	Large tubers	Medium tubers	Small tubers	Yield	Dry matter
		–g–	–%–	–%–	–%–	–t ha^{-1}-	–%–
2018	5.26b	149.63a	67.25a	30.24b	2.30b	34.83b	16.85 ns
2019	8.79a	101.08b	32.78b	57.32a	9.90a	37.51a	17.72 ns
Average	7.02	125.35	50.01	43.87	6.10	36.17	17.28

Different letters indicate significant differences at the 0.05 level; ns: nonsignificant differences

That is,organic fertilizer creates soil characteristics most suitable for growing potatoes [37]. Our research showed that high doses of organic fertilizers have a higher yield than plots fertilized with mineral fertilizers. This research is no exception, as earlier research on organic and mineral fertilizers indicates that the yield can be the same or higher [36, 38]. In our research, the potato yield depended on the year of study and ranged from a minimum of 34.83 t ha^{-1} (2018) to a maximum of 37.51 t ha^{-1} (2019). The recorded higher number of tubers per plant during 2019 is the reason for the higher yield in this year. This opinion is supported by the positive correlation found between these traits ($r = 0.536$) (Table 4).

Although our research has provided glimpses into the influence of biofertilizers in combination with mineral and organic fertilizers on the components of potato yield, this study has certain limitations. A limitation of this study is that it does not contain information on the nutritional value of potatoes. Thus, future research should consider the potential impact of biofertilizers on these properties. Furthermore, our research showed certain benefits that could be applied in practice. Specifically, organic fertilizers and biofertilizers positively impacted one of the main yield components (the number of tubers per plant). Therefore, the more intensive application of these fertilizers directly affects the yield components and yield. Likewise, considering that the second component of yield (tuber mass) is significantly dependent on the amount of precipitation during the tuber bulking stage, future research is needed to address water conservation and irrigation of potatoes at this stage of development.

Table 4. Coefficients of the linear correlation between the number of tubers per plant, mass of the tuber, percent of large, medium, and small tubers, potato yield, and dry matter content

	Number of tubers per plant	Mass of the tuber	Large tubers	Medium tubers	Small tubers	Yield	Dry matter
Number of tubers per plant	1						
Mass of the tuber	-0.869**	1					
Large tubers	-0.716**	0.702**	1				
Medium tubers	0.726**	-0.691**	-0.987**	1			
Small tubers	0.587**	-0.621**	-0.900**	0.823**	1		
Yield	0.536**	-0.184	-0.166	0.193	0.071	1	
Dry matter	0.457*	-0.487**	-0.349	0.348	0.276	0.069	1

*, ** Significant differences at the 0.05 and 0.01 levels, respectively

4 Conclusions

Based on the experiment results, it can be concluded that biofertilization had a positive effect on potatoes yield components in the environmental conditions of central Bosnia and Herzegovina. Biofertiliser positively affected the number of tubers per plant and directly correlated with potato yield. Therefore, applying such fertilizers can be a useful agrotechnical practice to increase potato yields.

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Effects of Mulching and Irrigation on Antioxidant Activity and Antimicrobial Properties of Basil (*Ocimum basilicum* L.)

Ajla Šakonjić, Anamarija Matijević, Saud Hamidović, Lejla Čengić, and Teofil Gavrić(✉)

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, Sarajevo, Bosnia and Herzegovina

t.gavric@ppf.unsa.ba

Abstract. Basil (*Ocimum basilicum* L.) is an annual herb from the mint family. It is used as a fresh and dry spice, and in the form of essential oil. The antioxidant activity and antimicrobial properties of basil depend on genetic properties, environmental conditions, and growing technology. Therefore, the aim of this research was to determine the effects of mulching and irrigation on the antioxidant activity and antimicrobial properties of basil. The field experiment was conducted at Butmir, Bosnia and Herzegovina. The treatments used consisted of a combination of different mulches (wheat straw mulch, black mulch film, and control) and irrigation (irrigated and non-irrigated). Total phenolics, flavonoids, antioxidant activity, and essential oil antimicrobial activity were tested. Experimental results suggested that growing technology impacts the researched traits. The high content of total phenolic (69.94 mg GAE g^{-1}), flavonoids (35.77 mg CAE g^{-1}), and antioxidant activity (45.13 μM Fe^{2+} g^{-1}) were recorded in the treatment with black mulch film. The research showed that the essential oil has a growth inhibition zone diameter of 10.88 mm for *Salmonella spp.* and 8.72 mm for *E. coli.*

Keywords: basil · mulch · essential oil · antioxidant activity · antimicrobial activity

1 Introduction

Since the beginning of time, people have been interested in medicinal and spice plants because of their beneficial effects on human health. Basil (*Ocimum basilicum* L.) is the most abundant herb in the world, particularly in Mediterranean countries. It is originally from India, but today it is cultivated worldwide [1]. Basil is used as a spice in many foods, such as soups, salads, meat specialties, and some types of cheese [2, 3]. Furthermore, basil essential oil is an additive in food products, perfume, and cosmetics [4, 5]. In addition, leaves, plant juice extracts, and seeds are used in indigenous medicine for headaches, colds, toothaches, and bronchitis [5, 6]. Recently, it was found that basil has antimicrobial properties [7, 8] and that the intensity of this effect depends on essential oil quality [9].

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The amount of phenols, flavonoids, alkaloids, tannins, and essential oils in basil is often a good indicator of basil quality [7]. Although genetic processes control the amount of these components in the plant [1, 10, 11], numerous other factors also significantly impact. For example, during cultivation, environmental factors such as temperature, soil, light, wind, humidity, weeds, animals, and microorganisms can significantly impact the synthesis of secondary metabolites [12–17]. On the other hand, different agricultural practices such as planting date, tillage, crop nutrition, irrigation, and mulching affect the change in environmental conditions, which also indirectly affects the quality and yield of basil [18–21].

Mulching is one of the agricultural practices that has been used in different crops since ancient times [22]. The main goals of using mulch are to preserve soil humidity and suppress weeds [23]. Its use leads to certain changes in environmental conditions. For example, mulch increases soil humidity and temperature and improves microbial activities [22, 24]. Furthermore, it reduces evaporation and modifies the soil's water retention capacity [25]. Therefore, mulching changes the environmental conditions that can affect the growth and development of the plant.

Considering all the above, the aim of this work was to determine the effects of mulching and irrigation on the antioxidant capacity and essential oil antimicrobial activity.

2 Materials and Methods

2.1 Field Experiment

A field experiment was conducted at Butmir, B&H. The experiment was set up during the growing season of 2022. A basil cultivar called "Genovese" was used. The treatments consisted of a combination of different mulches (wheat straw mulch, black mulch film, and control) and irrigation (irrigated and non-irrigated). Basil was sown on April 24, 2022. Seeds of sweet basil were sown in polystyrene trays (104 cells) filled with a substrate (Klasmann Potgrond H). After 30 days from sowing, seedlings were transplanted in an open field (May 24, 2022). Seedlings were planted at 70cm × 30cm plant density.

2.2 Materials for Analysis

The content of total phenols, flavonoids, and antioxidant capacity were determined in aerial parts of basil. Plant materials were collected at the flowering stage (BBCH 60–65). Collected plant samples were dried in a dark room at room temperature for 30 days. After drying, samples were milled to 1 mm and homogenized.

2.3 Extraction for Antioxidant Activity

Plant aqueous ethanolic extracts were prepared as described previously [1]. The extract was prepared as follows: approximately 1 g of dried and powdered aerial parts of basil was added to the flask. Then, 50 mL of 60% ethanol was added and mixed. After incubation at room temperature for 24 h, all extracts were filtered and stored in the fridge (4 °C) for further use.

2.4 Extraction for Antimicrobial Activity

Essential oil extraction was performed with a Clevenger type apparatus [26]. In brief, the dried plant material was weighed (50 g), then transferred to a volumetric flask with a volume of 1000 mL, and 500 mL of distilled water was added. After distillation, the isolated essential oils were dried using sodium sulfate anhydrous.

2.5 Determination of Total Phenols Content

The total phenolic content in extracts was determined by the modified Folin-Ciocalteu method [6], and the results were expressed as mg gallic acid equivalent (GAE) per g sample.

2.6 Determination of Total Flavonoid Contents

The content of flavonoids was determined by a modified spectrophotometric method based on the colored reaction of flavonoids with $AlCl_3$ [27], and the results were expressed as mg catechin acid equivalents (GAE).

2.7 Determination of Antioxidant Capacity

The antioxidant capacity of the extract was measured using the modified FRAP method [28]. The results were expressed as $\mu M\ Fe^{2+}\ g^{-1}$.

2.8 Antimicrobial Activity of Essential Oils

In this study, *Escherichia coli* and *Salmonella spp.* were used as test bacteria. The antimicrobial activity of essential oils was determined using the paper-disc diffusion method [29]. Briefly, the bacterial inoculum (1×10^8 CFU mL^{-1}) was spread on Muller-Hinton agar using a sterile swab. After that, three sterile paper discs were pre-soaked with essential oil (10 µl/disc) and placed on agar. Incubation was performed in an incubator at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition around discs and comparing it with the standard values for the inhibition zones of gentamicin *Salmonella spp.* and *Escherichia coli* [29].

2.9 Statistical Methods

The research results were statistically analyzed by analyzing variance in the SPSS 22 software program. The average values of determined data were tested using analysis of variance at $p = 0.05$.

3 Results and Discussions

3.1 Analysis of Total Phenols Content, Total Flavonoid Contents, and Antioxidant Capacity

The influence of mulch type and irrigation on the content of total phenolic, flavonoids, and antioxidant capacity is presented in Tables 1 and 2. Total phenolic contents and antioxidant capacity significantly depended on mulching. The treatments with ˝black mulch film˝ had the highest level of total phenols (69.94 mg/g GAE), followed by the treatments with ˝wheat straw mulch˝ (62.94 mg/g GAE), and the ˝control˝ had the lowest content (57.24 mg/g GAE). A similar influence of mulch, but without statistical differences, was also recorded with flavonoids. They ranged from 32.94 mg CAE g^{-1} (control) to 35.77 mg CAE g^{-1} (black mulch film). While measuring antioxidant capacity, it was found that mulch had a similar impact on antioxidant capacity.

Table 1. Effect of mulch type on total phenolic, flavonoid, and antioxidant capacity.

Mulch type	Total phenolics	Total flavonoids	Antioxidant capacity
	mg GAE g^{-1}	mg CAE g^{-1}	μM Fe^{2+} g^{-1}
Wheat straw mulch	62.81b	33.88ns	43.62a
Black mulch film	69.94a	35.77ns	45.13a
Control	57.24b	32.94ns	35.76b
Average	**63.33**	**34.19**	**41.50**

Different letters indicate significant differences at the 0.05 level; ns: nonsignificant differences. GAE – gallic acid equivalent. CAE – cathetin acid equivalent.

Phenolics and flavonoids are secondary plant metabolites that protect plants from environmental stressors [30, 31]. Therefore, their high concentrations indicate that the plant grows in stressful conditions [6]. Based on these facts and our results, it can be said that ˝black mulch film˝ changed the growing conditions, which caused the plants to react and synthesize an additional amount of secondary metabolites to protect themselves. Furthermore, this mulch type increases soil moisture [6, 8], which is most likely why basil had stressful conditions in our study. The data from table 2 support this opinion. The highest values of total phenols, flavonoids, and antioxidant capacity were recorded with irrigation (68.14 mg GAE g^{-1}, 38.81 mg CAE g^{-1}, and 48.39 μM Fe^{2+} g^{-1}, respectively) compared to non-irrigation (58.51 mg GAE g^{-1}, 29.57 mg CAE g^{-1}, and 34.61 μM Fe^{2+} g^{-1}, respectively). The largest number of researchers found that lack of water (drought) causes an increase in phenol content and antioxidant capacity [32–34]. However, some have found that soil water does not affect the content of bioactive compounds [35–38] or that drought causes their reduction [39]. Differences in that research are most likely due to different agricultural practices and environmental conditions.

The relationships between the examined parameters were determinated with the Pearson correlation coefficient. According to the results given in Table 3, there was a

Table 2. Effect of irrigation on total phenolic contents, total flavonoid contents, and antioxidant capacity.

Irrigation	Total phenolics	Total flavonoids	Antioxidant capacity
	mg GAE g^{-1}	mg CAE g^{-1}	μM Fe^{2+} g^{-1}
Irrigated	68.14a	38.81a	48.39a
Non-irrigated	58.51b	29.57b	34.61b
Average	**63.33**	**34.19**	**41.50**

Different letters indicate significant differences at the 0.05 level; ns: nonsignificant differences
GAE – gallic acid equivalent, CAE – cathetin acid equivalent

positive correlation between total phenols and antioxidant capacity (r = 0.487). Additionally, a positive correlation between flavonoids and antioxidant capacity was found. (r = 0.753). The findings of this study are consistent with the results of many other researchers [1, 6, 31], who also found a relationship between the content of total phenols and antioxidant capacity and flavonoids and antioxidant capacity. In contrast, Ghasemi et al. [40] observed no correlation between total flavonoid concentration and antioxidant capacity.

Table 3. Coefficients of the linear correlation between total phenolic contents, total flavonoids contents, and antioxidant capacity.

	Total phenolics	Total flavonoids	Antioxidant capacity
Total phenolics	1		
Total flavonoids	0.313	1	
Antioxidant capacity	0.487*	0.753**	1

*, ** Significant differences at the 0.05 and 0.01 levels, respectively.

3.2 Analysis of the Antimicrobial Activity of Essential Oils

Tables 4 and 5 show the results on the influence of the mulch and irrigation type on the essential oil's antibacterial effects. The results were interpreted based on standard values for inhibition zones for the antibiotic gentamicin (Table 6) [29]. The type of mulch did not affect essential oil antimicrobial activity (Table 4). In other words, the inhibition zone for *Escherichia coli* was between 7.83 mm (black mulch film) and 9.34 mm (wheat straw mulch), while for *Salmonella spp.*, it ranged from 9.33 mm (black mulch film) to 11.84 mm (control).

Based on the data from Table 5, it can be said that irrigation did not affect essential oil antimicrobial activity. The inhibition zone of the essential oil for *Escherichia coli* was 8.66 mm (non-irrigated) and 8.77 mm (irrigated). The essential oil's zone of inhibition for *Salmonella* spp. Ranged from 11.11 mm (non-irrigated) to 10.66 mm (irrigated).

Table 4. Effect of mulch type on antimicrobial activity of essential oils

Mulch type	*Escherichia coli*	*Salmonella spp.*
	Inhibition zone (mm)	
Wheat straw mulch	9.34 ns	11.50 ns
Black mulch film	7.83 ns	9.33 ns
Control	9.00 ns	11.84 ns
Average	8.72	10.88

ns: nonsignificant differences.

Table 5. Effect of irrigation on antimicrobial activity of essential oils

Irrigation	*Escherichia coli*	*Salmonella spp.*
	Inhibition zone (mm)	
Irrigated	8.77ns	10.66ns
Non-irrigated	8.66ns	11.11ns
Average	8.72	10.88

ns: nonsignificant differences

From the above, essential oil has antimicrobial activity. However, this effect is weaker than the antibiotic gentamicin. For example, for *Escherichia coli*, basil essential oil has an average zone of inhibition of 8.72 mm on Escherichia coli which belongs to the R category (resistant) compared to gentamicin antibiotics (Table 6). On the other hand, the essential oil had similar antimicrobial activity against on *Salmonella spp.* It has been classified in classified in the R category (resistant) compared to gentamicin antibiotics. Numerous studies on the antibacterial activity of essential oils and their constituents are available nowadays [29, 41, 42]. Researchers showed that the antimicrobial effect depends on the type of essential oil, the essential oil's chemical composition, and microorganism structure (Gram-positive and Gram-negative bacteria). In other words, it was found that some organisms are sensitive to certain essential oils but resistant to other essential oils.

Zareen et al. [7] studied the antimicrobial effect of basil essential oil, and they found that basil essential oils showed good antibacterial activity against gram-positive bacteria compared to gram negative bacteria. They consider that gram positive bacteria are sensitive to essential oil due to the absence of the lipopolysaccharide layer, a barrier to the entry of molecules from essential oil. Our study focused on gram negative bacteria with a lipopolysaccharide layer (*Salmonella spp.* and *E. coli*), which is most likely why the examined organisms were resistant to essential oils. Therefore, to obtain as much information as possible about the potential sensitivity of bacteria to basil essential oil, future research on a similar topic should also include representatives of the group of gram-positive bacteria.

Table 6. Standard values for the inhibition zones of gentamicin antibiotic for *Salmonella spp.* and *Escherichia coli* [29].

Test organism	Antibiotic	Inhibition zone (mm)		
		S	I	R
Salmonella spp.	Gentamicin	≥ 17	13–16	≤ 14
Escherchia coli	Gentamicin	≥ 15	13–14	≤ 12

S – sensitive; I – moderately sensitive; R – resistant.

4 Conclusions

The results of the experiments have demonstrated that mulching and irrigation can affect basil's total phenols, flavonoids, and antioxidant activity. Using ˝black mulch film˝ and irrigation increased antioxidant capacity of basil. Therefore, using these agricultural practices could increase basil antioxidant capacity, which is especially important if we use basil as medicinal plant. From the above, it can be concluded that essential oil has antimicrobial activity. However, the antimicrobial effect of the essential oil was weaker compared to the antibiotic gentamicin.

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Impact of Climate Change on the Soil Water Balance Components in the Area of Sanski Most (Bosnia and Herzegovina)

Ajla Smajlović, Ahmed Gudić, Ajdin Avdović, Faruk Hadžić, Sara Tatarević, Vanesa Ibraković, Zejneba Kermo, and Sabrija Čadro(✉)

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
s.cadro@ppf.unsa.ba

Abstract. Given that global climate change affects the agricultural sector, often negatively affecting yields and food quality, it is essential to understand these impacts at the local level. The area of Sanski Most belongs to the Peripannon macroregion and it is located in the northwestern part of Bosnia and Herzegovina, where agriculture represents one of the most important economic sectors. Therefore, it is essential to conduct a detailed analysis of the agroclimatic conditions and soil water balance to determine the current situation and adapt agriculture and society to the coming changes. The impact of climate change was analyzed by observing two climate periods, namely the reference climate period (1961–1990) and the current climate period (1991–2020). Comparing these two clime periods an increase in the annual amount of precipitation by 38 mm was found. Although there is a positive trend at the year's level, it is negative during the summer months (−14.73 mm per decade), when the plant needs water the most. Also, the mean air temperature shows a positive trend in all seasons, with the most significant increase in the summer months (0.49 °C per decade). In summer, the water deficit in the soil becomes more and more present; that is, the need for irrigation increases. The difference in the average amount of soil water deficit between the two climatic periods is 48 mm, with a positive trend of 14.49 mm per decade. Also, one of the more visible differences is the increase in reference evapotranspiration (ET_0), which increased by 50 mm between these two climatic periods.

Keywords: Climate change · agriculture · soil water balance · BiH

1 Introduction

Bosnia and Herzegovina (BiH) can be considered as a country rich in water, but also among the countries with a wide range of water-related problems, especially in the field of agriculture [1]. In such conditions, sustainable development cannot be achieved without the application of sustainable water management in agriculture, strengthening the capacities of all actors in the sector, implementing and improving the existing legislative framework and applying innovative solutions and tools that belong to the domain of smart

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technologies [1–6]. Regarding climate change vulnerability, most vulnerable municipalities in BiH are located across the north, with a gradual decrease in vulnerability towards the central, north, and east of the country.

The area of Sanski Most is located in the northwestern part of BiH, from a macro-regional point of view it belongs to the Peripannon macro-region, while from a meso-regional point it belongs to the Bosnian Krajina meso-region [7].

The city of Sanski Most has an area of 12.90 km^2, while the municipality size is 781.17 km^2 [8]. The relief of Sanski Most is complex, abounding in predominantly hilly areas, with a plain along the Sana River, as well as high mountains: Grmeč (highest, 1500 m.a.s.l.), Mrežnica, Čelić kosa, Mulež and Behramaginica. The altitude of the urban part of the municipality is 160 m, and the average altitude for populated places is 500 m [7, 9]. Sanski Most is located in the area of continental climate, according to the Köppen-Geiger climate classification [10] in type Cfb x"s, which represents temperate warm and humid climates [11].

The municipality has 1,238 agricultural holdings in its register, and 75 registered tradesmen are engaged in agricultural production and contribute to the development of the Sana municipality [9, 12].

This area has very favorable conditions for the development of intensive agriculture, both in the plains in the Sana river valley from 150 to 250 m.a.s.l. with the construction of hydro-melioration systems (drainage and irrigation), as well as at hills of up to 500 m, which are suitable for the development of fruit growing [13]. There is a tradition of cattle breeding in this municipality [14], and some of the largest areas of arable land in BiH are also located here [7].

Although there are great potentials, especially for the development of agricultural production, according to the methodology and criteria for determining the level of development of municipalities in Federation of BiH (FBiH), Sanski Most belongs to the underdeveloped municipalities and is in 63rd place out of 79 municipalities in the FBiH [9]. Also, vulnerability index of this municipality is high [15]. Among other problems, the sensitivity to climate change, the effects of which are becoming more pronounced in the entire area of BiH, is highlighted [16–19].

Many studies carried out for the area of BiH agree on the present changes in the climate on an annual basis [17, 18, 20–22]. In such studies, the need to apply a local approach as well as a more detailed analyses at the changes within each season is often highlighted. Therefore, the goal of this research is to determine the impact of climate changes in different time periods (1961 – 1990 and 1991 – 2020) and seasons (winter, spring, summer, autumn, vegetation and annually) on the parameters of the agro-hydrological balance of the municipality area Sanski Most, to better understand the state of water resources in this area and conclude their more rational use with particular reference to agriculture as a water user.

2 Material and Methods

The research was carried out for the area of Sanski Most, town and municipality located in the Una-Sana Canton of the Federation of Bosnia and Herzegovina, BiH. Climate of this region is represented with one weather station (WS) located at the 158 m above sea

level (44°45′N; 16°40′E). It is the only WS in this area that has long-term climate data and continuity as well as all the necessary data for the water balance calculation. Also, the station is located in an urban area, but in the immediate vicinity of agricultural areas, so the data from it can be considered representative.

Daily weather data, including mean (T_{mean}), maximum (T_{max}) and minimum (T_{min}) air temperature, sum of precipitation (P), mean relative humidity (RH_{mean}) and sunshine hours (n) for the period 1961 – 2020 were collected for this WS. Daily climate data were provided by the Federal Hydrometeorological Institute Sarajevo.

The 60-year period is divided into two climatic periods, (I) the climatic period of the reference normal: 1961–1990 and (II) the last climatological standard normal 1991–2020 [23].

Monthly water balance was calculated using the modified Thornthwaite-Mather method [24–26]. Thornthwaite-Mather method (TM) required data on monthly precipitation (P), average monthly air temperature (T), reference evapotranspiration (ET_0) and soil available water content ($SOIL_{max}$). The value $SOIL_{max} = 100$ mm was used [27] since this is the most commonly used value for the types of soil that are found in the area of Sanski Most [28, 29].

Reference evapotranspiration (ET_0) was calculated using the standard FAO Penman-Monteith 56 equation [30]. All necessary parameters required for the calculation of ET_0 were computed following the procedure developed in FAO-56 [30] via REF-ET: reference Evapotranspiration Calculator [31] model.

All parameters are divided into climate parameters including precipitation (P), temperature (T), insolation (n), relative air humidity (RH_{mean}), amount of snow and water balance components (WB) including calculated values of soil moisture deficit (SMD), Total runoff (TRO), reference evapotranspiration (ET_0) and actual evapotranspiration (AET).

All data are presented by seasons, i.e. winter (XII–II), spring (III–V), summer (VI–VIII), autumn (IX–XI), vegetation (VI–IX) and the entire year. After the calculation of annual means (μ) and the standard deviation (σ) for all analysed water balance components, a statistical measure of the dispersion of data points and the coefficient of variation (CV) were calculated. To detect the trends (b) within time series of climate parameters and water balance components parametric method of linear regression was used.

3 Results and Discussion

3.1 Climate Parameters

Before the calculations of the components of the soil water balance were carried out, an analysis of all collected climate data was carried out. (P, T, n, RH_{mean}). The wind speed parameter was not analyzed because data for a large number of years were missing.

Precipitation (P). Precipitation is a very important climate parameter characterized by high spatial and temporal variability. The following table (Table 1) shows the obtained statistical data for the sum of precipitation by seasons, in vegetation and on an annual basis, for the entire analyzed period (1961–2020), as well as especially for I (1961–1990) and II (1991–2020) climatic period. Also, differences between these two analyzed periods were determined.

Table 1. Statistical data for precipitation sums (P in mm) in the area of Sanski Most period: 1961 – 2020.

Precipitation (mm)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	$\bar{x}$	227	265	269	282	556	1042
	σ	70.3	75.2	96.3	85.5	136.4	154.9
	CV	31.0	28.4	35.8	30.4	24.5	14.9
	b	0.609	0.500	−1.473	1.076	0.031	0.712
I 1961–1990	$\bar{x}$	214	262	293	254	557	1024
	σ	75.2	68.7	101.7	65.2	122.0	125.1
	CV	35.2	26.2	34.7	25.6	21.9	12.2
	b	−1.698	1.000	−1.388	−0.616	−0.759	−2.702
II 1991–2020	$\bar{x}$	239	268	245	309	555	1061
	σ	63.7	82.3	86.6	95.2	151.5	180.2
	CV	26.6	30.7	34.9	30.8	27.3	17.0
	b	1.461	1.953	−0.789	−1.785	1.421	0.840
Difference	$\bar{x}$	26	5	−48	55	−2	38
	σ	11.5	−13.5	16.2	−30.0	−29.4	−55.1
	CV	8.5	−4.5	−0.2	−5.1	−5.4	−4.8

Note: $\bar{x}$ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

In the area of Sanski Most, the annual average amount of precipitation was determined to be 1042 mm. Based on the analysis of two climatic periods, an increase in precipitation of 38 mm was determined, with a trend of 7.12 mm per decade. Of the total precipitation, 53% falls during the vegetation period (growing season), which indicates a uniform seasonal distribution of precipitation in this area (from 227 mm in winter to 282 mm in autumn). However, there is a negative trend of precipitation during the summer period for all three analyzed time periods, i.e. the sum of summer precipitation is 48 mm less in the second period. In contrast to the summer period, during winter and autumn there is an increase in the sum of average precipitation, which amounts to 26 mm (winter) and 55 mm (autumn). It is also important to emphasize that in the second climatic period we have a greater variation in precipitation during the vegetation period and annually.

Although the annual amount of precipitation in the area of Sanski Most is increasing, which is also visible in the positive trend in the II analyzed period, where during the vegetation season and annually we have a trend of increasing (14.21 and 8.40 mm per decade), during the summer months, when water is most needed for plant production, negative trend was determined (−7.89 mm per decade).

Previously, for the territory of Bosnia and Herzegovina, it was determined that the trends of annual, seasonal and monthly precipitation are weak and do not have statistical significance. [18]. The results obtained for the area of Sanski Most agree with the results of other researchers in BiH. So, in the period 1961–2017, the annual amount of precipitation showed a tendency to increase in most areas in the north of the territory (the Peripannon region) from 5.3 mm per decade in Sanski Most to 20.5 mm per decade in Doboj (except in Banja Luka, where the annual amount of precipitation decreased by – 4.4 mm per decade) [18, 32]. Also, in the research of Popov (2020), except for the summer period, positive trends were determined throughout the territory of BiH in the autumn season – from 2.0 mm per decade in Mostar to 12.5 mm per decade in Sanski Most [32, 33].

Air Temperature (T). Air temperature is a climatic parameter that provides energy for evaporation (E) and movement of water vapor from the evaporating surface and significantly affects the value of evapotranspiration (ET). Almost all ET calculation methods base their calculations on this climate parameter, and some methods only require the air temperature for the ET calculation [34].

The following table (Table 2) shows the obtained statistical data and differences between the average air temperature in the entire analyzed period (1961–2020), as well as for I (1961–1990) and II (1991–2020) climatic period. The data are presented by season (winter, spring, summer, autumn), vegetation (April – September) and annually.

In the area of Sanski Most, the determined annual average temperature is 10.64 °C. Based on the analysis of two climatic periods, an increase in temperature by 1.0 °C was determined, with a trend of 0.34 °C per decade.

Analyzing period II (1991–2020), we can conclude that the increase in air temperature is present in all seasons with the most pronounced increase in the winter (0.71 °C per decade) and summer (0.52 °C per decade) periods. It is interesting to note that in the second period the average air temperature during vegetation period increased by as much as 1.2 °C.

These results are in agreement with earlier research related to the territory of BiH. The increase in annual air temperature of BiH ranges from 0.4 to 1.0 °C, while the increase in temperature during the growing season (April–September) even reaches 1,2 °C [22]. These changes are more pronounced in the continental part [22], where the increase in temperatures is generally greatest during the summer, while in the autumn season the temperatures in most areas increased slightly. Analyzes of trends in average, maximum and minimum air temperatures show that the warming of the climate system is present throughout the territory of BiH [18, 21, 35, 36].

Table 2. Statistical data for the average air temperature (T in °C) in the area of Sanski Most period: 1961–2020.

Temperature (°C)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	$\bar{x}$	1.14	10.78	19.75	10.91	16.82	10.64
	σ	1.66	1.08	1.15	1.09	0.89	0.80
	CV	145.62	10.05	5.84	10.04	5.32	7.56
	b	0.043	0.029	0.049	0.016	0.036	0.034
I 1961–1990	$\bar{x}$	0.64	10.33	18.91	10.61	16.23	10.12
	σ	1.72	1.04	0.62	1.12	0.60	0.52
	CV	269.47	10.08	3.26	10.56	3.70	5.15
	b	0.072	0.008	0.007	−0.032	0.003	0.014
II 1991–2020	$\bar{x}$	1.63	11.22	20.59	11.21	17.42	11.16
	σ	1.45	0.94	0.93	1.00	0.73	0.69
	CV	88.61	8.42	4.53	8.90	4.19	6.22
	b	0.071	0.044	0.052	0.038	0.047	0.051
Difference	$\bar{x}$	1.0	0.9	1.7	0.6	1.2	1.0
	σ	0.3	0.1	−0.3	0.1	−0.1	−0.2
	CV	180.9	1.7	−1.3	1.7	−0.5	−1.1

Note: $\bar{x}$ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

Insolation (n). The most important factor in the formation of the climate of an area is solar radiation. Insolation represents the amount of energy that the earth receives with the sun's rays and is expressed in units of time, that is, in hours of sunshine during one day (h/day). Like other climatic parameters, insolation changes in space and time. The following table (Table 3) shows the obtained statistical data for the entire analyzed period (1961–2020), as well as separately for the I (1961–1990) and II (1991–2020) climatic periods. Also, differences between these two analyzed periods were determined.

In the area of Sanski Most, the average annual insolation was determined to be 5.12 h per day. Analyzing the first climatic period (1961–1990), the average value of insolation is 4.82 h per day, which is below the 60-year average, while the second period has an average of 5.42 h per day, that is, between the two climatic periods there was increase in the amount of sunshine, i.e. 0.6 h or there is 11% more sunshine in the area of Sanski Most.

Table 3. Statistical data for insolation (h per day) in the area of Sanski Most period: 1961–2020.

Insolation (h/day)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	$\bar{x}$	2.37	5.65	8.31	4.13	7.21	5.12
	σ	0.67	0.76	0.91	0.56	0.69	0.53
	CV	28.27	13.37	11.00	13.57	9.62	10.31
	b	0.017	0.021	0.027	0.009	0.021	0.019
I 1961–1990	$\bar{x}$	2.09	5.29	7.84	4.05	6.86	4.82
	σ	0.65	0.65	0.67	0.50	0.49	0.38
	CV	31.25	12.35	8.50	12.39	7.14	7.89
	b	0.026	0.005	−0.005	0.004	−0.002	0.008
II 1991–2020	$\bar{x}$	2.64	6.01	8.79	4.21	7.57	5.42
	σ	0.57	0.69	0.89	0.61	0.69	0.49
	CV	21.61	11.40	10.07	14.51	9.17	8.98
	b	0.001	0.024	0.028	0.035	0.027	0.022
Difference	$\bar{x}$	0.6	0.7	1.0	0.2	0.7	0.6
	σ	0.1	0.0	−0.2	−0.1	−0.2	−0.1
	CV	9.6	0.9	−1.6	−2.1	−2.0	−1.1

Note: $\bar{x}$ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

Similar to the temperature, in all seasons there is a noticeable trend of increasing the amount of sunshine and it ranges from 0.9 h to 0.27 h per day per decade. The most pronounced changes are in the summer period, when the differences between the two climatic periods amount to as much as 1 additional hour of sunlight.

Air humidity (RH_{mean}). Air humidity is generally one of the most important parameters in agricultural production due to the relation to occurrence of diseases and their prevention, as well as defining the need for irrigation. The climate in the area of Sanski Most is such that the air circulation is significantly influenced by the inflow of maritime air from the Adriatic Sea and nearby mountain ranges [37] therefore, the area is characterized by moderate air humidity. Data on relative air humidity for different seasons are shown in the following table (Table 4).

The average air humidity for the entire analyzed period is 78.83%, where in the first climatic period we have an annual average of 79.48%, and in the second a decrease of 1.3% or the average relative humidity of 78.18%. This decrease is present in all analyzed seasons, i.e. there is a trend of decreasing air humidity in the area of Sanski Most. The most pronounced change is in autumn, when air humidity dropped from an average of 82.39% to 79.63%. The decrease in air humidity leads to a whole series of other phenomena, of which perhaps the most significant for agriculture and the environment is the increase in evapotranspiration.

Table 4. Statistical data for average air humidity (%) in the area of Sanski Most period: 1961–2020.

Air humidity (%)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	x̄	82.39	74.25	76.01	81.01	76.19	78.83
	σ	4.50	2.45	3.61	10.82	2.48	1.84
	CV	5.46	3.30	4.75	13.35	3.26	2.34
	b	−0.040	−0.033	−0.073	0.007	−0.040	−0.033
I 1961–1990	x̄	83.48	74.86	77.18	82.39	76.89	79.48
	σ	2.13	2.39	2.67	1.88	1.89	1.33
	CV	2.55	3.19	3.45	2.28	2.46	1.67
	b	−0.142	−0.051	0.013	0.053	0.013	−0.032
II 1991–2020	x̄	81.30	73.65	74.85	79.63	75.48	78.18
	σ	5.85	2.40	4.07	15.18	2.82	2.07
	CV	7.19	3.25	5.44	19.07	3.73	2.65
	b	0.260	0.025	−0.130	0.555	−0.051	0.029
Difference	x̄	−2.2	−1.2	−2.3	−2.8	−1.4	−1.3
	σ	−3.7	0.0	−1.4	−13.3	−0.9	−0.7
	CV	−4.6	−0.1	−2.0	−16.8	−1.3	−1.0

Note: x̄ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

Snow. The role of snow in hydrological processes can be of great importance, above all in the cold period of the year, in higher mountain areas and higher latitudes. Snow represents a critical storage component in the hydrologic cycle. Large waters, and the floods caused by them, occur as a consequence of rapid snow melting. In climatological and meteorological research, especially in the last few decades, snow gained a very significant place, this fact can be explained by the great interest in studying global climate change, especially the consequences of the global warming on snow-related processes.

The annual average amount of snow for the entire analyzed period was determined to be 183 mm. By far, the largest amount of snow is during the three winter months (December, January, and February), whose sum is 159 mm. It is very important to note the negative trend that snow has for all three analyzed periods, i.e. the amount of snow in winter in period II is 9 mm less, while in spring in period II it is less by 10 mm.

The trend of this reduction in the amount of snow for the entire analyzed period is 11.23 mm per decade, while this reduction is even more pronounced in period II, when it amounts to 22.67 mm per decade. A negative trend in the amount of snow was also determined for other parts of BiH, in northern BiH from 11.58 to 16.64 per decade [17], and up to 35.45 mm in the Čemerno mountain range [38] (Table 5).

Table 5. Statistical data for snow in the area of Sanski Most period: 1961–2020

Snow (mm)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	x̄	159	12	-	12	-	183
	σ	74.7	19.2	-	23.5	-	82.8
	CV	47.1	162.0	-	194.0	-	45.3
	b	−0.712	−0.339	-	−0.071	-	−1.123
I 1961–1990	x̄	163	17	-	12	-	192
	σ	79.4	23.4	-	18.2	-	84.1
	CV	48.6	139.9	-	152.7	-	43.8
	b	−3.070	−0.350	-	0.456	-	−2.965
II 1991–2020	x̄	154	7	-	12	-	173
	σ	70.7	12.2	-	28.1	-	81.8
	CV	45.9	176.4	-	228.6	-	47.2
	b	−0.762	−0.398	-	−1.107	-	−2.267
Difference	x̄	−9	−10	-	0	-	−19
	σ	8.7	11.2	-	−9.9	-	2.3
	CV	2.7	−36.6	-	−75.9	-	−3.4

Note: x̄ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

3.2 Water Balance Components

The components of the water balance are the result of climate, so their values change following the change of climate. In the following text, the four most important components of the soil water balance will be presented: Soil moisture deficit (SMD), total runoff (TRO), reference evapotranspiration (ET_0) and actual evapotranspiration (AET).

Soil Moisture Deficit (SMD). Soil moisture deficit is a very important parameter in agricultural production, because its value shows to what extent irrigation is necessary and to what extent the lack of water affects the yield of agricultural crops. The following table (Table 6) shows the obtained statistical data for the entire analyzed period (1961–2020), as well as separately for the I (1961–1990) and II (1991–2020) climate periods.

In the area of Sanski Most, the annual average amount of soil moisture deficit for the entire analyzed period was determined to be 111 mm, with a positive trend of 13.34 mm per decade. Observed by seasons, during the summer period we have an average of 86 mm, while there is a positive trend during the summer period for all three analyzed time periods, that is, the soil moisture deficit in the II period is greater by 48 mm.

The SMD obtained through the water balance procedure is the result of the determined increase in air temperature and decrease in air humidity and the amount of precipitation, that is, it can indicate the increased dryness in the area of Sanski Most. Based on the obtained statistical data, the SMD in the II period (1991–2020) is more pronounced than in the I period (1961–1990), precisely in that period when there were major droughts in BiH [39–41].

Table 6. Statistical data for soil moisture deficit (SMD in mm) in the area of Sanski Most period: 1961 – 2020.

SMD (mm)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	$\bar{x}$	2	9	86	14	105	111
	σ	2.6	11.2	69.4	19.8	76.3	77.0
	CV	103.3	125.4	80.7	145.5	72.4	69.3
	b	−0.044	0.058	1.479	−0.160	1.420	1.334
I 1961–1990	$\bar{x}$	3	8	62	15	82	88
	σ	2.9	10.3	53.0	20.5	59.6	62.3
	CV	91.2	134.0	85.4	132.7	73.1	70.4
	b	0.020	0.192	1.201	−0.297	1.306	1.116
II 1991–2020	$\bar{x}$	2	10	110	12	129	134
	σ	2.0	12.1	76.1	19.2	84.4	84.3
	CV	113.0	118.7	69.4	163.5	65.3	63.1
	b	−0.107	−0.226	1.090	−0.231	0.546	0.525
Difference	$\bar{x}$	−1	3	48	−4	48	45
	σ	0.8	−1.8	−23.1	1.3	−24.7	−22.1
	CV	−21.8	15.3	16.1	−30.8	7.7	7.3

Note: $\bar{x}$ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

Total Runoff (TRO). Excess water defined here as total runoff, is very important for the agriculture. In contrast to water deficit problems, excess water can also have some negative impacts on agricultural production, causing floods, erosion, landslides or increasing soil drainage requirements. The following table (Table 7) shows the obtained statistical data for the entire analyzed period (1961–2020), as well as for the I (1961–1990) and II (1991–2020) climate periods separately.

The annual average TRO for the entire analyzed period was determined to be 375 mm, with a positive trend of 7.19 mm per decade. Observed by season, the largest TRO is in spring when it amounts to 139 mm. In the first climatic period (1961–1990) there is a negative trend in all seasons and annually. However, in the second climatic period (1991–2020) a positive trend, the exception is autumn with a slight negative (−0.92 mm per decade), was determined. The values of these changes are much smaller in the II period. The obtained values indicate the incoherence of this data.

The period after 1991 is characterized by an increase in the amount of TRO and a higher coefficient of variation, this is precisely the period in which large floods occur in BiH, more precisely in 2004, 2006, 2009, 2010, 2014 [1, 36, 42] as well as the last one in 2021.

Table 7. Statistical data for the average value of total runoff (TRO in mm) in the area of Sanski Most period: 1961 – 2020.

TRO (mm)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	x̄	74	139	87	75	202	375
	σ	31.3	47.2	31.9	39.6	68.2	105.3
	CV	42.5	34.0	36.5	53.0	33.7	28.1
	b	0.650	0.080	−0.219	0.207	−0.150	0.719
I 1961–1990	x̄	61	136	91	69	204	357
	σ	30.5	47.9	31.2	34.3	62.3	92.3
	CV	49.9	35.4	34.2	49.8	30.6	25.9
	b	−0.113	−0.959	−0.507	−0.650	−1.187	−2.229
II 1991–2020	x̄	86	142	84	81	201	393
	σ	27.2	47.0	32.7	44.1	74.7	115.7
	CV	31.4	33.2	39.1	54.6	37.2	29.5
	b	0.244	0.341	0.241	−0.092	0.562	0.734
Difference	x̄	25	6	−7	12	−3	36
	σ	3.3	0.9	−1.6	−9.8	−12.4	−23.4
	CV	18.5	2.2	−4.9	−4.7	−6.6	−3.6

Note: x̄ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

Reference Evapotranspiration (ET_0). Reference evapotranspiration (ET_0) is a very important element of the water balance. In hydro-reclamation practice, the calculation of evapotranspiration is one of the basic steps in the design of irrigation and drainage systems. ET_0 is necessary for calculation of crop and irrigation water requirements, determination of the correct irrigation regime and sizing the irrigation system [28, 43, 44]. Reference evapotranspiration for the area of Sanski Most was calculated using FAO Penman-Monteith 56 equation [30]. The following table (Table 8) shows the obtained statistical data of reference evapotranspiration in the area of Sanski Most.

The sum of ET_0 for the analyzed period (1961–2020) is 777 mm. Comparing the I (1961–1990) and II (1991–2020) climate periods, an increase of 50 mm was found, with a positive trend of 14.49 mm per decade.

The biggest changes (8.9 mm per decade) are during the summer when the ET_0 value is also the highest (361 mm). In all seasons and all climatic periods, a certain degree of increase in ET_0 was determined, the exception being autumn, with a particularly pronounced negative trend during the II climatic period. In that period, ET_0 in the area of Sanski Most decreases slightly by 2.15 mm per decade.

For agriculture, the vegetation period is the most important, and in that period the sum of ET_0 for the entire analyzed period (1961–2020) is 607 mm, i.e. 78.1% of the total annual ET_0.

Table 8. Statistical data for reference evapotranspiration (ET_0 in mm) in the area of Sanski Most period: 1961 – 2020.

ET_0 (mm)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	x̄	52	216	361	147	607	777
	σ	7.7	18.3	28.9	11.9	41.2	50.0
	CV	14.8	8.5	8.0	8.1	6.8	6.4
	b	0.132	0.418	0.897	0.001	1.213	1.449
I 1961–1990	x̄	50	209	346	147	586	752
	σ	8.7	19.1	21.4	13.1	32.0	43.4
	CV	17.4	9.1	6.2	9.0	5.5	5.8
	b	0.489	0.268	0.496	−0.028	0.589	1.226
II 1991–2020	x̄	54	223	377	148	628	802
	σ	6.1	14.4	27.4	10.7	38.9	43.7
	CV	11.3	6.4	7.3	7.2	6.2	5.5
	b	−0.166	0.203	0.526	−0.215	0.693	0.348
Difference	x̄	4	14	31	1	42	50
	σ	2.6	4.7	−6.0	2.4	−6.9	−0.3
	CV	6.1	2.7	−1.1	1.7	−0.7	0.3

Note: x̄ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

Actual Evapotranspiration (AET). Actual evapotranspiration (AET) indicates the actual amount of water that evaporates from the surface through the processes of evaporation and transpiration, it is the main component of the hydrological cycle and one of the most important physical processes in natural ecosystems. Estimation of AET can represent an important alternative for agricultural or hydrological studies, for example when measurement techniques are not available due to their high cost, complex installation or intensive maintenance [43, 45].

The following table (Table 9) shows statistical data for AET in the area of Sanski Most observed in certain climatic periods, among which we can see differences.

In the area of Sanski Most, the sum of AET for the entire analyzed period (1961–2020) was determined to be 666 mm, with a slight increasing trend of 1.15 mm per decade. Based on the analysis of the I (1961–1990) and II (1991–2020) climate periods, the determined difference in the average annual amount of AET is only 5 mm.

In research by Čadro and Marković [17] as well as Čadro and Uzunovic [38] carried out in the area of northern BiH where Sanski Most is located, the determined value of the annual AET ranges from 578 mm (Bijeljina) to 667 mm (Banja Luka), and the values obtained for Sanski Most coincide with the upper limit of previously determined values.

Variations of this component of the water balance are not high, however, there are significant differences between the trend of changes by seasons. So, during winter, spring and autumn, we have a slightly positive trend of AET, which ranges from 0.7 to

4.7 mm per decade. On the other hand, during the summer we have a more pronounced negative trend, the value of which varies from 5.6 to 7.0 mm per decade, depending on the observed period. These results confirm the previously explained problem of more pronounced climate changes during the summer period. The differences between the two climatic periods indicate a decrease in the mean annual AET of 17.1 mm, with all that in the summer period, the AET variations are the most pronounced.

Table 9. Statistical data for Actual evapotranspiration (AET in mm) in the area of Sanski Most period: 1961 – 2020.

AET (mm)		Wi	Sp	Sum	Au	Veg	Annual
1961–2020	$\bar{x}$	50	207	275	134	502	666
	σ	8.4	15.0	45.8	16.2	48.0	50.2
	CV	16.9	7.3	16.6	12.1	9.6	7.5
	b	0.176	0.360	−0.582	0.160	−0.207	0.115
I 1961–1990	$\bar{x}$	47	201	284	131	505	663
	σ	9.3	15.6	36.1	17.9	40.6	46.6
	CV	19.7	7.8	12.7	13.7	8.0	7.0
	b	0.470	0.076	−0.705	0.269	−0.717	0.110
II 1991–2020	$\bar{x}$	52	213	267	136	499	668
	σ	6.6	11.9	53.1	14.0	55.0	54.2
	CV	12.7	5.6	19.9	10.3	11.0	8.1
	b	−0.059	0.429	−0.564	0.016	0.147	−0.178
Difference	$\bar{x}$	5	12	−17	5	−5	5
	σ	2.7	3.7	−17.1	3.9	−14.4	−7.7
	CV	7.0	2.2	−7.2	3.4	−3.0	−1.1

Note: $\bar{x}$ – Arithmetic mean; σ – Standard deviation; CV – Coefficient of Variation; b – decline of the trend curve; Wi – Winter; Sp – Spring, Su – Summer, Au – Autumn, V – Vegetation

4 Conclusion

Based on a detailed and long-term analysis of climate data and soil water balance in the Sanski Most area, we can conclude that the changes are in line with those of the national or regional level. It is especially important to point out the increase in air temperature (0.34 °C per decade) the duration of sunshine (0.19 h per day per decade), and soil moisture deficit (13.34 mm per decade) i.e. the decrease in the amount of snow (−11.23 mm per decade) and relative humidity (−0.33% per decade). Observed by seasons, the period of summer shows the most intense changes, which are hardly noticeable if only the annual level is observed.

Such data should be a kind of alarm, that is, a warning that adaptation to new challenges is necessary. The responsible institutions, that is, the authorities of this area should provide certain funds and create functional systems for the fight and adaptation to climate change. Institutions should be able to establish early warning systems and decision support systems taking into account the state of all actors in the area.

BiH, as a signatory to various initiatives and international agreements related to the climate, including the latest Green Agenda for the Western Balkans, has accepted certain concepts, among which sustainable agriculture takes a special place. Observing the upcoming trends, certain steps must already be taken.

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Effect of Pruning on Mechanical Composition of Bunch of Table Grape Varieties (*Vitis Vinifera* L.)

Mersija Delić[1(✉)], Fikreta Behmen[1], Semira Sefo[2], Pakeza Drkenda[1], Saša Matijašević[3], and Ana Mandić[4]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
m.delic@ppf.unsa.ba
[2] Agromediterian Faculty, University "Džemal Bijedić" of Mostar, Mostar, Bosnia and Herzegovina
[3] Faculty of Agriculture, University of Belgrade, Belgrade, Serbia
[4] Faculty of Agriculture and Food Technology, University of Mostar, Mostar, Bosnia and Herzegovina

Abstract. The aim of the research was to study the effect of pruning on the mechanical composition of the bunch of three introduced table grape varieties (Black Magic, Muscat Bleu and Alphonse Lavallee) in the area of Mostar. The research lasted three years (2011–2013), each variety had three variants of pruning, and each variant had four repetitions. The bud load levels studied in this research were 28 (variant I), 32 (variant II) and 40 (variant III) buds per vine. The following parameters were measured: bunch, berries and stem weight, number of berries in a bunch and single berry weight, length and width of bunch and berry. The highest values of the parameters: bunch, berries and stem weight, single berry weight and berry width were in the variety Alphonse Lavallee variant II, while the longest bunch was in the variety Black Magic variant II, and the bunch widest in variant III, as well as the berry length. The largest number of berries in a bunch was recorded in the variety Muscat Bleu variant II.

Keywords: Mechanical composition · Pruning · Black Magic · Muscat Bleu · Alphonse Lavallee

1 Introduction

Table grape varieties constitute a special group of varieties that are exclusively intended for fresh consumption. The market demands for grapes of the highest quality imposed made it necessary to design new table grape varieties. This resulted in the current abundance of new varieties whose qualitative characteristics need to be checked and compared with the conditions of the variety's origin. New varieties challenge grape producers in doubt, and therefore checking the quality of newly created varieties is a necessity, so that they can subsequently be recommended for cultivation in new agro-ecological conditions [1].

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After the introduction of the variety it is necessary to monitor its adaptation to new ecological conditions. In recent years, in the territory of Herzegovina, a large number of newly created table grape varieties, with different biological and economic-technological characteristics, have been introduced. So far these varieties have not been studied from the viewpoint of their adaptation to the ecological conditions of Herzegovina [2].

An important factor in the adaptation of the variety to new ecological conditions is the correct selection of the cultivation system. Cultivation system is of great importance in the cultivation of table grapes as attention is focused on the external appearance of the bunch and the total yield. The cultivation system consists of the planting distances, the training system, the pruning methods, the construction of trellis, and ampelo-technical measures that regulate the vegetative potential of vines [3], and the load of the buds is especially important. Understanding the correlation between the bud load and the yield is very important for grape producers in view of choosing the adequate pruning for each variety in specific ecological conditions [4].

The height of the yield affects the mechanical composition of the bunch. The bunch size depends on the environment and growing conditions. Even if these conditions are the same, there are big differences between the varieties. The size of the bunch is determined by its length and width, that is, its mass [5]. Bunch architecture is controlled by environmental and genetic factors [6]. Some of these sub-traits are under genetic control as reported for berry size and berry weight [7, 8]. Parameters, berry number in the bunch [9] and stem sub – traits [10] have the high sensitiveness to seasonal environmental changes. Accordingly, it is necessary to achieve an optimal bud load of vine for each variety in order to provide conditions for the growth and development of the vine, and thus for the production of grapes.

The aim of this work was to study in detail the influence of pruning, i.e. the bud load levels of vine on the mechanical composition of bunch of table grape varieties: Black Magic, Muscat Bleu and Alphonse Lavallee, being some of the large number of introduced table grape cultivars, in the conditions of Herzegovina.

The tested hypotheses are: (1) smaller bud loads can significantly increase the weight of the bunch; (2) higher bunch weight implies greater bunch length and width.

2 Research Methods

Material: Table grape varieties Black Magic, Muscat Bleu: Garnier 15/6 (Villard Noir × Muller Thurgau) × Seyve Villard 20–347 (Perle Noire), and Alphonse Lavallee grafted on the rootstock Paulsen 1103. The experiment lasted three years (2011–2013), and was set up at the facility "Vinogradi" doo Mostar, by the method of random selection. The vineyard is in full fruition, with intensive cultivation, drip irrigation system, and regular application of agro-technical measures. It was planted in 2008, with a planting distance of 3 m × 1.2 m, and the Moser cordon as a vine-training system. Three variants of pruning were investigated is shown in Table 1, on 72 vines in four repetitions (6 vines per repetition) for each variety. A total of 216 vines were included in the trial. Laboratory research was carried out at the Faculty of Agriculture and Food Sciences, University of Sarajevo.

Table 1. Pruning variants with the spacified number of buds

Variant	Pruning	Shoot or spur	Shoot or arc	Number of buds per vine
Variant I	Short	4 (5) + 4(2)	0	28
Variant II	Mixed	4 (2)	4 (6)	32
Variant III	Mixed	4 (2)	4 (8)	40

Mechanical composition (method by Prostoserdov): Bunches were taken at full maturity, 10 representative bunches for 4 repetitions each, within each pruning type, for all three investigated varieties and during three years (2011, 2012 and 2013). The bunch mass (g) was obtained by weighing, and the length (cm) and width (cm) by measuring on graph paper. Berries were manually removed from each bunch, counted and weighed as well as the peduncle. After that, the length (cm) and width (cm) of the berry were measured and the weight of one berry (g) was determined [11].

Analysis of the results: A two-factor analysis of variance (ANOVA) was performed for each variety and each year of the study. The obtained parameters were processed by the SPSS (IBM SPSS Statistics) computer program. The standard error was determined using the Tukey test for a significance level of 0.05. Principal component analysis (PCA) was used to identify the differentiation factor of grape variety and pruning on analyzed mechanical properties using Statgraph 3.14. The visualization of overall parameters was obtained by a heatmap function, using the ClustVis program package [12].

2.1 Ecological Conditions

Vine varieties have certain requirements in terms of the external environment conditions and that has to be taken into consideration when choosing a variety that is best suited to the given conditions. In order to make a decision on the appropriate varieties for a given region, as well as on the application of certain agro-technical and ampelotechnical measures, it is necessary to understand both the current state of temperatures and atmospheric phenomena, and their multi-year averages and extremes.

Anticipated climate change in European regions can significantly alter viticulture in the coming decades, in terms of both the spectrum and the distribution of grape varieties that are currently in use [13].

The climate of the Mostar vine-growing area is decisively influenced by the openness to the sea, along the Neretva river valley, and the separation from the northern areas by mountain massifs. The data of the Mostar meteorological station, obtained from the FBiH Hydro-meteorological Institute, were used for analyzing climatic conditions (Table 2).

Table 2. Basic hydro – meteorological factors in Mostar winegrowing region

Indicator	1961 – 1990	2011	2012	2013
Mean annual air temperature (°C)	14.1	16.2	16.1	15.9
Mean vegetative air temperature (°C)	18.83	21.76	22.1	21.16
Absolute minimum air temperature (°C)	-14.2	-1.6	-7.4	-2.4
Absolute maximum temperature (°C)	41	40.4	41.8	41.1
Precipitation during vegetation (mm)	518	396.5	744.4	931.1
Annual precipitation (mm)	1102	872.5	1394.9	2188.3
Length of the vegetation period (days)	239	235	236	237
Length of insolation (hours)	2287	2629.9	2656.9	2465.2

Mean vegetative air temperature and precipitation during vegetation.

3 Results and Discussion

The results of the analysis of the mechanical composition of the bunches of the varieties of Black Magic, Muscat Bleu and Alphonse Lavallee, depending on the bud load levels during three consecutive years, are shown in the Table 3.

The results of the study show that statistically significant differences were found in most of the characteristics of the bunches of the tested table grapes varieties. Bunch mass was statistically significantly highest in the variety Alphonse Lavallee, variant II (32 buds/vine) (458.43 g), and the lowest in the variety Muscat Bleu, variant III (40 buds/vine) (242.24 g). This was to be expected as the largest bunch mass of the studied varieties was produced by the Alphonse Lavallee variety, and the smallest by the Muscat Bleu variety [2, 14–16]. Also, with an increase in the yield, the mass of the bunch decreases, which is why it is the smallest in variant III where the highest number of buds per vine were left which is consistent with our hypothesis (1). Observed by the years of study, it was determined that there is a statistically significant difference in the same variety during the three years of study. The mass of the berries and the mass of the stem in the bunches of the examined varieties during three years of the research using three types of pruning, as parameters, followed the correlations established during the cluster mass analysis. There were statistically significant differences in the levels of these parameters between varieties, types of pruning within a given variety, and between research years.

Based on the results of the research it becomes apparent that the levels of the bunch length and width parameters of the tested varieties were statistically significant, when differences between the varieties in all three years of the research are observed. This was expected considering that the studied varieties have different bunch sizes. The largest bunch mass was in the variety Alphonse Lavallee, and the smallest in the variety Muscat Bleu. The highest values of the bunch length and width parameters were recorded in the Alphonse Lavallee variety, and the lowest in the Muscat Bleu variety, which is in accordance with our hypothesis (2). The effect of pruning on the length and width of bunches in the varieties Black Magic, Muscat Bleu and Alphonse Lavallee was not

statistically significant within the variety, therefore, we can say that in this study, the bud load in all three studied varieties did not have a statistically significant effect on the values of the bunch length and width parameters. The length and width of the berry was statistically significantly different between varieties during all three years of research. If we observe the values of these parameters within the same variety, and depending of the type of pruning, we can see that it was statistically significant in the Muscat Bleu variety for both parameters, and in the Alphonse Lavallee variety for the berry width parameter. Other values were not statistically significant.

In this study, there were statistically significant differences in the number of berries per bunch between the varieties during the years of the study. There was also a statistically significant difference in the value of this parameter and between the pruning types within the same variety, so that the pruning types with a lower bud load had a higher number of berries in the varieties Muscat Bleu and Alphonse Lavallee, while the result was the opposite in the variety Black Magic. The weight of one berry was statistically significantly different between all three tested varieties during the years of the study. Observation made by the type of pruning within one variety, gave us an unexpected result. In the case of the Black Magic variety, the highest weight of one berry was recorded in the lowest bud load. In the Muscat Bleu variety, the values of this parameter were higher with a higher bud load, while in the Alphonse Lavallee variety, the weight of one berry was not statistically significant in different types of pruning.

There was a statistically significant difference in the amount of precipitation during all three years of research. A lower amount of precipitation can negatively affect the yield, and an excessive amount of precipitation during grape ripening negatively affects its quality. The amount of precipitation during the growing season in 2011 was extremely low, and in 2013 it was too high, while the average air temperature differed by 0.6 °C.

The results of this study show that the variety Black Magic belongs to the group of varietis with medium and large bunch [14, 17]; the bunch of the Muscat Bleu variety belongs to the category of medium-sized bunches [18], and the bunch of the Alphonse Lavallee variety to the group of large bunch varieties [14, 19, 20]. Also, the size of the bunches obtained in this study is consistent with the classification of bunches of table grape varieties by size, as classified by Korać [21].

The average bunch mass in the variety Black Magic in the conditions of the Tikveš vine-growing area (Macedonia) was 369.00 g [22], in the variety Muscat Bleu in the ecological conditions of Poland it was 181.00 g [23], while in the variety Alphonse Lavallee it was 480.00 g in the conditions of northern Greece [24], and 253.9 g in the conditions of the Niš vine-growing sub-region [25].

The length of the bunch of the Black Magic variety (19.42 cm) in this study was consistent with the three-year study conducted on this variety in the conditions of the Tikveš vine-growing area (Macedonia), where the average bunch length of 20.9 cm was recorded, and the width of the bunch was smaller compared to this study [22].

In this three-year study, the variety Alphonse Lavallee had an average length of the bunch, amounting to 21.57 cm in all three years, which is consistent with the study carried out in the conditions of northern Greece on this variety, where the length of the bunch was 21.8 cm, while the average width of the bunch was by approximately 1 cm smaller compared to this study [23] (Fig. 1).

Table 3. Average values of bunch dimension at harvest of table grape varieties

Variety		Bunch weight (g)	Stem weight (g)	Berries weight (g)	Bunch length (cm)	Bunch width (cm)	Number of berries	Single berry weight (g)	Berry length (cm)	Berry width (cm)
Black Magic (BM)	Variant I	308.57 ± 90.6^{ns}	8.80 ± 0.9^{ns}	302.02 ± 92.3^{ns}	19.58 ± 0.8^{ns}	15.94 ± 2.3^{b}	64.67 ± 32.2^{b}	4.97 ± 1.4^{a}	2.69 ± 0.6^{ns}	1.93 ± 0.5^{ns}
	Variant II	330.39 ± 162.6^{ns}	9.27 ± 3.7^{ns}	321.12 ± 159^{ns}	19.66 ± 1.7^{ns}	16.86 ± 2.4^{ab}	73.10 ± 44.3^{a}	4.37 ± 0.3^{b}	2.64 ± 0.3^{ns}	1.98 ± 0.5^{ns}
	Variant III	311.59 ± 136.6^{ns}	10.10 ± 4.9^{ns}	301.49 ± 131.6^{ns}	19.02 ± 1.1^{ns}	17.10 ± 3.8^{a}	$72.10 \pm 42.7a$	4.30 ± 0.2^{b}	2.70 ± 0.4^{ns}	1.95 ± 0.4^{ns}
Muscat Bleu (MB)	Variant I	254.62 ± 91^{a}	9.49 ± 3.2^{ab}	245.13 ± 87.6^{a}	18.02 ± 0.1^{ns}	13.72 ± 1.6^{ns}	84.81 ± 17.8^{ab}	2.73 ± 0.6^{b}	1.92 ± 0.3^{ab}	1.69 ± 0.3^{b}
	Variant II	263.67 ± 95.2^{a}	9.83 ± 3.1^{a}	253.84 ± 92^{a}	18.47 ± 1.8^{ns}	13.37 ± 1.5^{ns}	90.61 ± 27^{a}	2.95 ± 0.7^{a}	1.86 ± 0.2^{b}	1.65 ± 0.2^{b}
	Variant III	242.24 ± 104.1^{b}	8.83 ± 4.0^{b}	233.41 ± 100.1^{b}	18.00 ± 1.9^{ns}	13.33 ± 2.5^{ns}	82.60 ± 24.9^{b}	3.04 ± 0.9^{a}	1.97 ± 0.1^{a}	1.75 ± 0.2^{a}
Aphonse Lavallee (AL)	Variant I	435.89 ± 221.3^{a}	11.84 ± 9.7^{ns}	424.05 ± 211.5^{a}	21.82 ± 1.0^{ns}	14.54 ± 2.8^{ns}	76.54 ± 41.5^{b}	5.86 ± 0.8^{ns}	2.36 ± 0.2^{ns}	2.05 ± 0.6^{b}
	Variant II	458.43 ± 223^{a}	13.33 ± 11.1^{ns}	445.10 ± 211.9^{a}	21.74 ± 1.2^{ns}	15.59 ± 3.0^{ns}	81.40 ± 52.5^{a}	5.98 ± 1.7^{ns}	2.39 ± 0.1^{ns}	2.11 ± 0.6^{a}
	Variant III	407.99 ± 125.9^{b}	11.64 ± 3.8^{ns}	396.35 ± 122.1^{b}	21.17 ± 2.8^{ns}	14.27 ± 0.7^{ns}	72.50 ± 26.6^{b}	3.01 ± 0.8^{ns}	2.37 ± 0.2^{ns}	2.10 ± 0.5^{a}
Year 2011	BM	247.91 ± 18.7^{b}	7.78 ± 1.0^{ns}	240.14 ± 18.2^{b}	19.20 ± 1.5^{b}	17.03 ± 0.3^{a}	50.82 ± 2^{b}	4.67 ± 0.9^{b}	2.90 ± 0.2^{a}	1.90 ± 0.1^{ab}
	MB	225.65 ± 25.5^{c}	8.42 ± 1.6^{ns}	217.20 ± 24.17^{c}	18.03 ± 1.5^{c}	14.39 ± 0.9^{b}	73.89 ± 8.21^{a}	3.13 ± 0.2^{c}	1.93 ± 0.2^{b}	1.79 ± 0.1^{b}
	AL	336.91 ± 10.9^{a}	8.80 ± 2.2^{ns}	328.11 ± 11.0^{a}	21.55 ± 0.9^{a}	13.79 ± 1.4^{b}	54.80 ± 3^{b}	6.47 ± 0.8^{a}	2.35 ± 0.1^{ab}	2.31 ± 0.1^{a}
Year 2012	BM	377.85 ± 65.3^{b}	10.82 ± 3.8^{a}	367.78 ± 60.9^{b}	19.97 ± 0.9^{b}	17.71 ± 2.6^{a}	90.61 ± 14.1^{a}	4.18 ± 0.1^{b}	2.47 ± 0.1^{a}	1.76 ± 0.1^{b}
	MB	224.28 ± 48.9^{c}	8.63 ± 3.5^{b}	215.65 ± 45.3^{c}	18.46 ± 1.1^{c}	12.52 ± 1.2^{b}	88.30 ± 20.2^{a}	2.48 ± 0.0^{c}	1.81 ± 0.1^{b}	1.54 ± 0.2^{c}
	AL	438.44 ± 44^{a}	10.99 ± 1.7^{a}	427.44 ± 42.3^{a}	20.96 ± 2.8^{a}	13.99 ± 0.7^{b}	80.60 ± 7.1^{b}	5.76 ± 0.3^{a}	2.31 ± 0.1^{a}	2.20 ± 0.1^{a}
Year 2013	BM	324.79 ± 7^{b}	9.58 ± 0.8^{c}	316.72 ± 7.1^{b}	19.09 ± 0.3^{b}	15.13 ± 1.0^{a}	68.38 ± 9.3^{b}	4.77 ± 1.2^{b}	2.65 ± 0.1^{a}	2.20 ± 0.0^{a}
	MB	310.61 ± 9.62^{b}	11.10 ± 0.3^{b}	299.51 ± 9.8^{b}	18.00 ± 2.1^{c}	13.50 ± 0.7^{b}	95.80 ± 2.4^{a}	3.10 ± 0.7^{c}	2.00 ± 0.0^{b}	1.77 ± 0.1^{b}
	AL	526.97 ± 106.6^{a}	17.02 ± 5.8^{a}	509.95 ± 100.7^{a}	22.21 ± 0.2^{a}	15.62 ± 2.0^{a}	95.0 ± 22.9^{a}	5.70 ± 1.2^{a}	2.47 ± 0.1^{a}	1.74 ± 0.1^{b}

Data represent mean values (± SD) of four repetitions. The standard error was determined using the Tukey test for a significance level of 0.05. Values (a-c, ns) are significantly different mean values of bunch dimension.

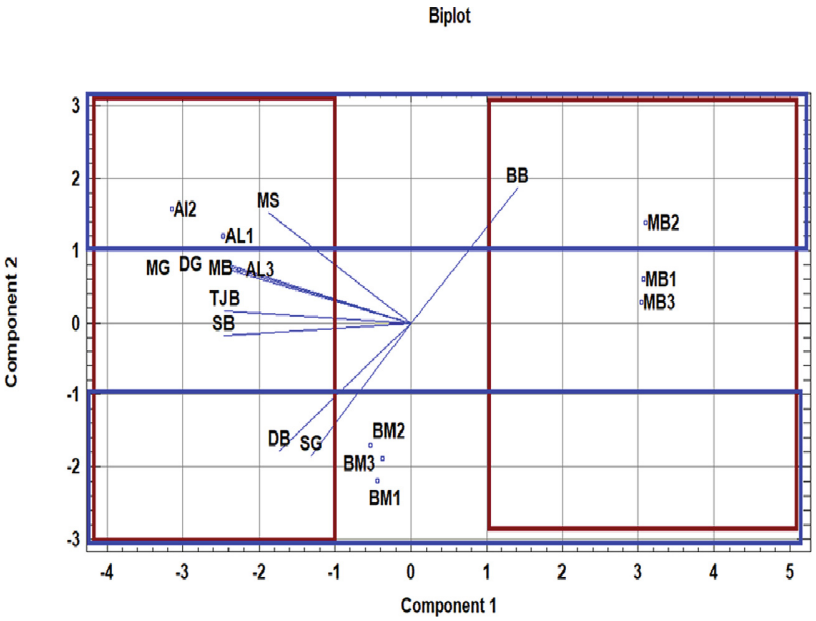

Fig. 1. Projection bunch characteristics of the table grape varieties on the PCA. MG-bunch weight, SG- bunch width, MS – stem weight, MB – berries weight, DG – bunch length, DB – berry length, SB – berry width, BB – number of berries, TJB – single berry weight, BM1– Black Magic variant 1, BM2 – Black Magic variant 2, BM2 – Black Magic variant 3, MB1 – Muscat Bleu variant 1, MB2 – Muscat Bleu variant 2, MB3 – Muscat Bleu variant 3, AL1 – Alphonse Lavallee variant 1, AL2 – Alphonse Lavallee variant 2, AL3 – Alphonse Lavallee variant 3)

The variety Muscat Blue, regardless of the pruning variant, is in the positive part of the first component and dominated by the number of berries, compared to the other two tested varieties. In the case of the Muscat Blue variety, the fruits from Variant II of the pruning stood out due to the stem weight, compared to the other two pruning models.

The Black Magic variety was distinguished by the length of the berry and the width of the bunch.

The fruits of the Alphonse Lavallee variety from the first and second pruning variants were distinguished by the stem weight and the number of berries compared to the fruits of this variety from the third pruning variant (Fig. 2).

The data of the average values of the bunch characteristics of the varieties Black Magic, Muscat Bleu and Alphonse Lavallee shown on the heatmap are divided into two main bunches. The greatest variation in the mean values of the investigated parameters was recorded in the number of berries and the mass of berries in a bunch.

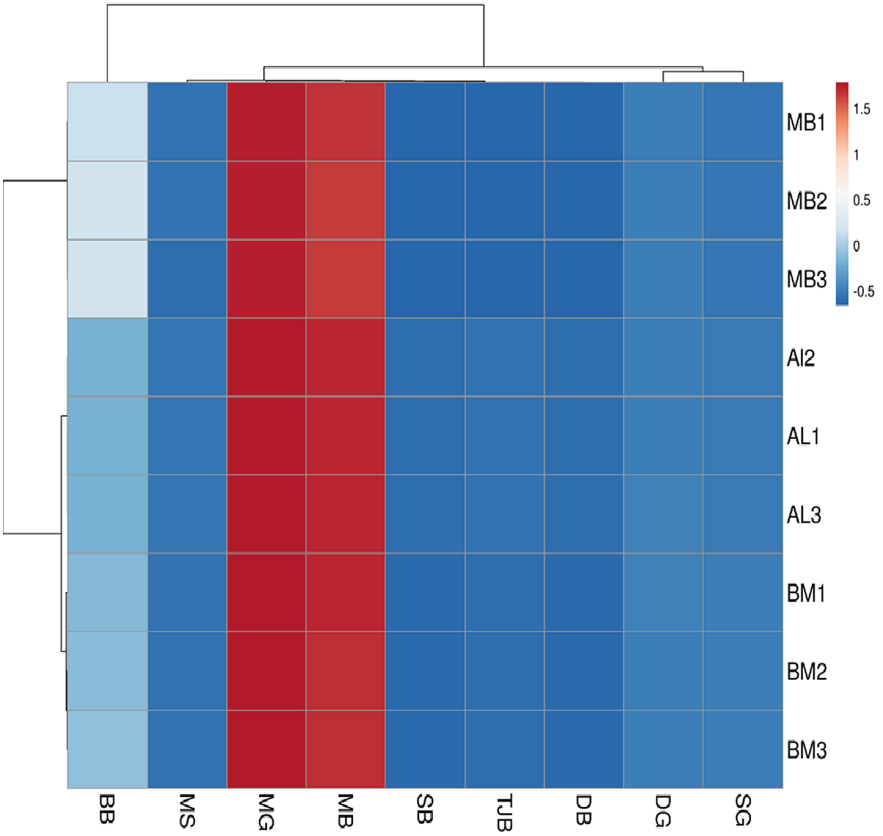

Fig. 2. Projection bunch characteristics of the table grape varieties on the Heatmap. MG-bunch weight, SG- bunch width, MS – stem weight, MB – berries weight, DG – bunch length, DB – berry length, SB – berry width, BB – number of berries, TJB – single berry weight, BM1– Black Magic variant 1, BM2 – Black Magic variant 2, BM2 – Black Magic variant 3, MB1 – Muscat Bleu variant 1, MB2 – Muscat Bleu variant 2, MB3 – Muscat Bleu variant 3, AL1 – Alphonse Lavallee variant 1, AL2 – Alphonse Lavallee variant 2, AL3 – Alphonse Lavallee variant 3)

4 Conclusion

The study of the influence of the type of pruning, i.e. the bud load on the mechanical composition of the bunch of the Black Magic, Muscat Bleu and Alphonse Lavallee varieties was carried out at the site of Vrapčići – Mostar. Based on the three-year study, it can be concluded that there was a statistically significant difference in the value of almost all examined characteristics of the bunches of the subject table grape varieties. The most favorable mechanical composition of the bunches in all three varieties, in the agro-ecological conditions of Mostar, was recorded in variant II, where pruning left 32 buds/vine, and which we recommend.

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Vascular Flora of Medieval Fortresses of Bosnia and Herzegovina

Nermina Sarajlić[1](✉), Semir Maslo[2], Biljana Lubarda[3], Nejc Jogan[4], Šemso Šarić[5], Borut Bosančić[6], and Vladimir Ranđelović[1]

[1] Department of Biology and Ecology, Faculty of Sciences and Mathematics, Niš, Serbia
nermina.sarajlic@ptice.ba
[2] Primary School, Lundåkerskolan, Gislaved, Sweden
[3] Department of Biology, Faculty of Natural Sciences and Mathematics, University of Banja Luka, Banja Luka, Bosnia and Herzegovina
[4] Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia
[5] Jelaške, Olovo, Bosnia and Herzegovina
[6] Faculty of Agriculture, University of Banja Luka, Banja Luka, Bosnia and Herzegovina

Abstract. With the objective to add to the knowledge of the vascular flora of medieval sites in Bosnia and Herzegovina, eight limestone fortresses situated in different regions and subjected to different intensity of anthropogenic impact were selected. All native and subspontaneous alien vascular plant taxa were recorded, resulting in a total of 714 taxa, ranging from 82 to 384 in individual sites. The share of most numerous families and life forms was in accordance with the situation recorded in similar habitats across Europe. Despite many common features, the flora composition of surveyed fortresses varies significantly, mainly as a result of their geographical location, local environmental factors as well as the intensity of anthropogenic pressure. The main reason for the differences in floristic composition is the fact that only ten taxa were found in all eight fortresses, and a presence of numerous "site-specific" species that were found at only one of the surveyed fortresses. Although the indicators of anthropogenic changes indicate the strong anthropogenic influence on the flora, expressed through urbanization and maintenance degree, which influenced the presence and number of endemic and alien taxa, as well as the high percentage of therophytes and widespread plants, the analysis of the overall floristic assemblages showed the major significance of biogeographical position, reflected in preferences for the environmental factors in general floral composition and supported by the distribution of floristic elements.

Keywords: Bosnia and Herzegovina · Fortress Flora · Ecological Factors · Urbanization

1 Introduction

Fortresses are generally large closed structures combining the dominant defensive function with residential and economic functions [1]. They are usually situated on rocks and, together with their impressive fortification walls, create a specific environment for plant

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life in many respects [2]. The walls are similar to natural rocks and rock crevices [3, 4], but even in cases when they are built of natural stone, the effect of mortar and other binding substances is noticeable, in a sense that different chemical composition allows the development of certain species which are not found on surrounding rocks [5, 6]. Generally, the walls are characterized by low availability of room for plant development, hardness and alkalinity of the substratum, scarcity of soil and humus, high inclination, extreme temperatures and low humidity [3, 7]. The flora of fortresses is generally composed of native species adapted to rocky habitats and species with a wide ecological amplitude, which colonize the walls due to a mass-effect from the surroundings [2, 8, 9]. There is also a significant share of alien species, most of which originate from warmer regions [9]. The study of the wall flora is also of special importance for the maintenance and preservation of archaeological monuments [2]: on one hand, roots of some plants can damage the buildings, and on the other, the walls can have a role as a habitat for some rare specialized plants not present in the vicinity of the fortress.

The flora of castle and fortress ruins – "Burgbergflora", as Sukopp [10] refers to it – and flora of city walls have been studied in Germany [11–14], Poland [15], Czech Republic [4], Bulgaria [16–18], France [3], Italy [8], Albania [19], Greece [2, 7, 20], Turkey [6], Morocco [21] and Algeria [22]. In Bosnia and Herzegovina, the flora of several individual fortresses was surveyed in recent years [23–25], but no comparison work has been done so far.

Considering the ecological differences between eight fortresses situated in different regions of Bosnia and Herzegovina and the fact that they are subjected to different intensity of anthropogenic impact, we wanted to compare their vascular flora with regard to the following questions: 1) Is there a general pattern of species composition regarding the most frequent species and species diversity? 2) Does the intensity level of human factor, expressed through the indicators of anthropogenic changes of flora, have effect on overall similarity of vascular flora of selected fortresses? 3) Can the flora composition and differences between individual fortresses be explained on the basis of local ecological factors?

2 Materials and Methods

2.1 Surveyed Area

Eight medieval limestone fortresses located in three distinct phytogeographic regions of Bosnia and Herzegovina [26] were selected: four (Blagaj, Ljubuški, Počitelj and Stolac) in Mediterranean region with predominant vegetation of Ostryo-Carpinion, two (Sarajevo, Vranduk) in Euro-Siberian region with predominant vegetation of Fagion illyricum and two (Srebrenik, Banja Luka) in the same region, but within the vegetation belt of Carpinion betuli. This delimitation is roughly mirrored in division of Bosnia and Herzegovina in three biogeographic regions [27] (Fig. 1).

Kastel Fortress (Banja Luka) is situated in the very center of the city of Banja Luka. Most part of the site is regularly maintained and used for various cultural manifestations, while some sections of walls and corners inside the fortress complex are almost completely neglected.

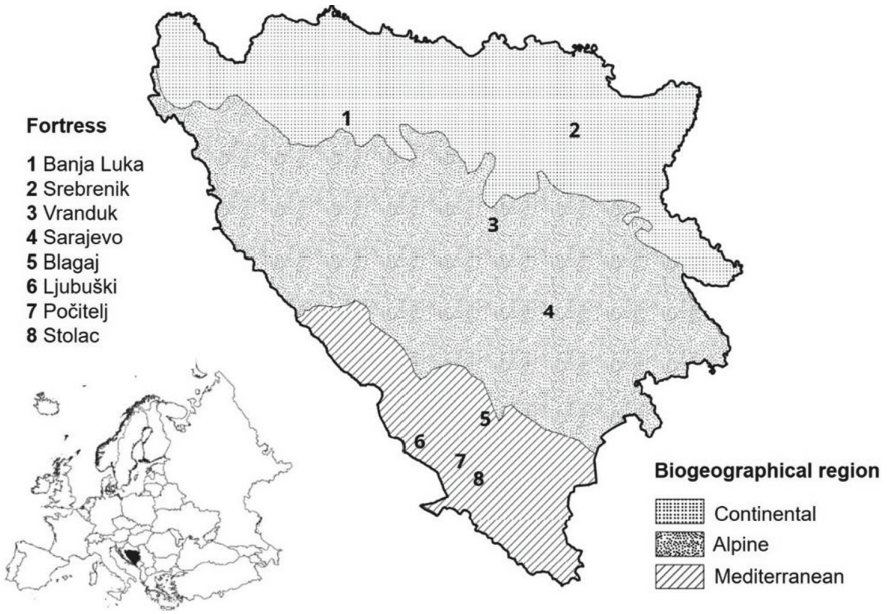

Fig. 1. Biogeographical position of surveyed sites

Gradina Fortress (Srebrenik) is located atop a steep cliff, completely surrounded by forest. Although not easily accessible, it is a popular tourist spot. A part of a fortress located near the main entrance is occasionally cleared of weeds, and other parts are derelict.

Vranduk Fortress is located on a hill about 10 km from the center of the city of Zenica. The fortress complex, which is turned into a museum and regularly maintained, is surrounded by numerous private houses and small-scale agricultural areas.

White Fortress (Sarajevo) is located on a cliff on the eastern entrance to the city of Sarajevo. Due to the lack of maintenance, the inside of the fortress is derelict. It is currently closed for public.

Old Town Blagaj – Stjepan Grad (Blagaj) is located on top of a rocky hill above the city, and is surrounded by garrigue and rocky pastures. Although it is located near the urban area, the fortress is accessible only by a narrow uphill trail, and is partially derelict.

The Fortress of Herzog Stjepan Vukčić Kosača (Ljubuški) is located on a rocky hill, surrounded by garrigue and accessible only by gravel road. The major part of the fortress is derelict.

Kula Fortress (Počitelj) is situated on a cliff in the middle of the historic village of Počitelj. It is a popular tourist spot, but no regular maintenance works are taking place.

Stolac Old Town – Vidoški (Stolac) is located on a hill above the city. The central part of the complex is frequented by tourists and used for hosting various events, and most marginal parts are derelict and damaged by illegal construction works.

2.2 Field Investigation

The vascular flora of the selected fortresses was surveyed individually, during the 2010–2021 period. All plants found on and inside the walls of the fortresses, as well in their immediate vicinity, were recorded. The taxa that are cultivated in the area were recorded only if they were found subspontaneously growing, i.e., it could positively be identified that they developed from seedlings and were not deliberately planted in the surveyed sites.

2.3 Data Analysis

The plants were identified according to the available determination keys [28–30]. The nomenclature follows the Euro + Med PlantBase [31], except for taxa marked with an asterisk (*), where the World Flora Online [32] was used.

In the list (Appendix 1), the taxa are listed in alphabetic order (by family, then by genus), followed by designations for life form, floral element, endemic status, degree of naturalization and time of introduction for alien taxa.

The data on the life forms is taken from Flora of Italy [33, 34], with categories based on the classification of Raunkiaer [35]. The taxa were classified into floral elements [36], and assigned endemic [37], and alien status [38, 39]. The alien taxa were classified as archaeophytes or neophytes according to their residence time [39, 40]. To indicate the degree of naturalization of a taxon, the terms casual, naturalized and invasive were used [41]. For the analysis of ecological preferences of the flora, the values of six ecological indices [34, 42] were considered: light (L), temperature (T), continentality (C), humidity (H), soil acidity (R) and available nitrogen quantity (N).

The overall condition of each fortress was estimated on the basis of subjective perception, and its distance from the urban area was measured by measuring the aerial distance from the fortress and the main administrative building of the nearest compact urban area (Table 1).

The scope of anthropogenic changes of the flora of each site was assessed with the use of indicators based on proportions between individual geographical-historical groups [1, 43]:

1. Indicators of anthropization.
   1.1. Indicator of total anthropization

$$\mathrm{IAnt} = \mathrm{An}/(\mathrm{Sp} + \mathrm{An}) \times 100$$

   1.2. Indicator of permanent anthropization

$$\mathrm{IAnp} = \mathrm{Mt}/(\mathrm{Sp} + \mathrm{Mt}) \times 100$$

2. Indicators of archaeophytization.
   2.1. Indicator of total archaeophytization

$$\mathrm{IArt} = \mathrm{Ar}/(\mathrm{Sp} + \mathrm{An}) \times 100$$

Table 1. General data on surveyed fortresses (geographical position, ecological characteristics, disturbance level).

Fortress	Coordinates	Phytogeographic region/vegetation alliance	Distance from the urban area (km)	Condition
Banja Luka	44.766806, 17.189648	Euro-Siberian/Carpinion betuli	<1	Regularly maintained
Srebrenik	44.703430, 18.530938	Euro-Siberian/Carpinion betuli	3.34	Occasionally maintained
Vranduk	44.291687, 17.903757	Euro-Siberian/Fagion illyricum	9.94	Regularly maintained
Sarajevo	43.859260, 18.444485	Euro-Siberian/Fagion illyricum	1.59	Partly derelict
Blagaj	43.259918, 17.902484	Mediterranean/Ostryo-Carpinion	<1	Partly derelict
Ljubuški	43.200987, 17.558122	Mediterranean/Ostryo-Carpinion	1.05	Partly derelict
Počitelj	43.135335, 17.731690	Mediterranean/Ostryo-Carpinion	3.27	Partly derelict
Stolac	43.081333, 17.954334	Mediterranean/Ostryo-Carpinion	<1	Partly derelict

2.2. Indicator of permanent archaeophytization

$$\mathrm{IArp} = \mathrm{Ar}/(\mathrm{Sp} + \mathrm{Mt}) \times 100$$

3. Indicators of kenophytization.
   3.1. Indicator of total kenophytization

$$\mathrm{IKnt} = \mathrm{Kn}/(\mathrm{Sp} + \mathrm{An}) \times 100$$

   3.2. Indicator of permanent kenophytization

$$\mathrm{IKnp} = \mathrm{Kn}/(\mathrm{Sp} + \mathrm{Mt}) \times 100$$

4. Indicator of flora modernization

$$\mathrm{IM} = (\mathrm{Kn}/\mathrm{Mt}) \times 100$$

5. Indicator of fluctuation changes

$$\mathrm{IF} = \mathrm{Df}/(\mathrm{Sp} + \mathrm{An}) \times 100$$

where An is number of alien species; Sp – number of native species; Mt – number of permanently established alien species (Ar + Kn − Df), Ar – number of archaeophytes, Kn – number of neophytes, Df – number of casual alien species.

The similarity of floral composition was calculated based on the Bray-Curtis cluster analysis using the PAST [44] and Principal Component Analysis (PCA) in SPSS Statistics 22 software.

3 Results and Discussion

A total of 714 vascular plant taxa belonging to 91 families were registered in eight surveyed fortresses. The number of species varied from 82 in Vranduk to 384 in Stolac (Appendix 1). This is similar to the number of species observed in similar habitats by other authors, which was between 59 in Elbasan fortress in Albania [19] and 418 in the ruins of Akrokorinthos Castle in Greece [2], but also to the number of species in walls – 288 in southern and western Moravia [45].

Families with highest number of species in the flora of Blagaj and Ljubuški are *Compositae*, *Poaceae* and *Brassicaceae*. In floras of Počitelj, Stolac, Sarajevo and Banja Luka, *Brassicaceae* are replaced by *Fabaceae*, and in Srebrenik by *Lamiaceae*. *Compositae* and *Poaceae* were among top four most dominant families in floras of walls and fortresses in Central Europe [1], East Bohemia [4], Bulgaria [17], Albania [16], Turkey [6, 46], Morocco [21] and Algeria [22]. However, in Vranduk, *Compositae* account only 2.44% of total flora, and the most numerous families are *Brassicaceae*, *Poaceae* and *Rosaceae* (Table 2). This can be explained by the fact that this fortress is located far from the urban area, as *Compositae* are more common in more urbanized areas, due to the fact that they are adapted to anthropogenic pressure [47].

Table 2. Most numerous families (%) in eight surveyed fortresses in Bosnia and Herzegovina.

	Blagaj	Ljubuški	Počitelj	Stolac	Sarajevo	Banja Luka	Srebrenik	Vranduk
Compositae	12.99	9.86	12.14	11.72	15.48	13.97	10.59	2.44
Poaceae	10.39	10.33	11.08	10.68	12.55	10.04	10.59	9.76
Brassicaceae	9.52	9.86	7.39	6.77	5.44	3.93	4.24	12.19
Lamiaceae	8.22	8.45	5.54	6.26	5.44	6.55	7.63	2.44
Caryophyllaceae	5.63	3.75	3.96	3.91	5.02	3.49	3.81	2.44
Fabaceae	5.63	6.57	8.18	8.33	7.53	8.73	5.93	3.66
Apiaceae	2.16	4.22	3.69	4.17	3.35	3.49	4.24	4.88
Rosaceae	1.29	2.35	2.37	2.34	5.44	4.8	5.93	9.76

Only ten taxa (*Anagallis arvensis* L., *Asplenium ceterach* L., *Asplenium ruta-muraria* L., *Capsella bursa-pastoris* (L.) Med., *Draba verna* L., *Ochlopoa annua* (L.) H. Scholz, *Veronica persica* Poir., *Senecio vulgaris* L., *Hedera helix* L. and *Petrorhagia saxifraga* (L.) Link) were found in all eight fortresses, and only one of them (*Veronica persica*) is a neophyte. Some of the typical plants of wall crevices (*Cymbalaria muralis* P. Gaertn, Mey. et Scherb and *Parietaria judaica* L.) were not found in all sites, but it is interesting to

note that *Parietaria judaica* L. was replaced by *P. officinalis* L. in those fortresses which are surrounded by forest (Banja Luka, Srebrenik and Vranduk). It has been previously observed that a number of species considered to be regular elements of wall vegetation are not as common in walls in montane regions as they are in the lowlands [3]. Also, *P. judaica*, which is a typical wall-dwelling plant, was present on only 10% of walls in the sub-urban areas in Trabzon (Turkey) [46]. The average number of "unique" taxa (those found at only one of the surveyed fortresses) is 238, and varies from 3 (Ljubuški) to 59 (Srebrenik).

Therophytes and hemicryptophytes were the dominant life forms in flora of all surveyed fortresses (Fig. 2). They were also the most numerous in walls and fortresses in across Europe [1, 2, 4, 6, 7, 13, 15, 16, 20].

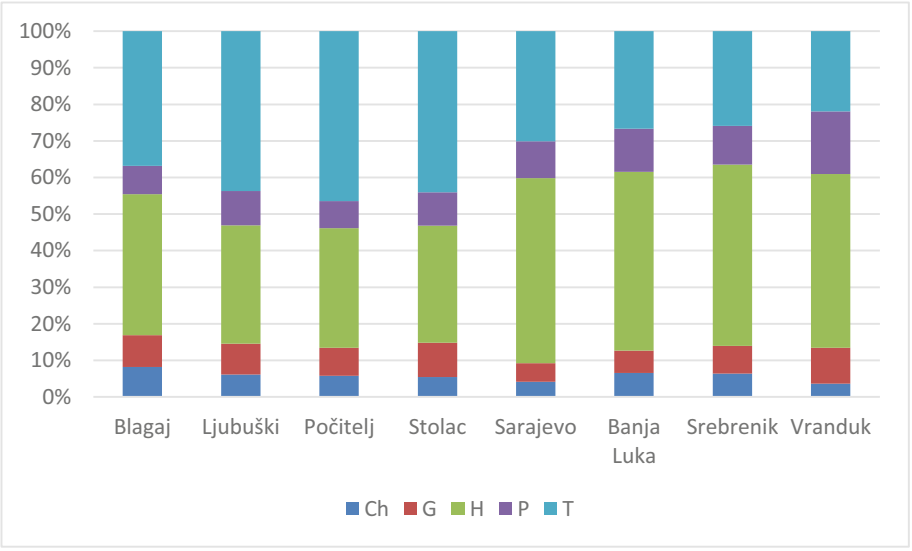

Fig. 2. Life spectrum (%) of flora of surveyed fortresses (Ch – chamaephytes, G – geophytes, H – hemicryptophytes, P – phanerophytes, T – therophytes)

Therophytes were especially numerous in sites under the influence of a slight Mediterranean climate, namely Ljubuški, Počitelj and Stolac. They were also the most numerous in floras in parts of Europe characterized by more xeric microclimatic conditions, in Greece [2, 7, 20] and Turkey [6]. Hemicryptophytes, on the other side, were the most numerous life form in central Europe: in several castle ruins across Germany [13], remains of West Slavic settlements [1], walls in East Bohemia [4] and Poland [15], but also in Bulgaria [16]. The share of phanerophytes, mostly seedlings, in the surveyed fortresses ranges from 7.39% in Počitelj to 17.07% in Vranduk. As expected, geophytes were the least dominant life form in all surveyed fortresses.

The share of endemic plant taxa in the total fortress flora is below 4%. Their number is higher in fortresses situated in the south (17 or 7.36% in Blagaj, 13 or 3.38% in Stolac and 6 or 2.82. in Ljubuški). This can be explained by the fact that the number of endemic

taxa in Bosnia and Herzegovina, as well as in the Balkan Peninsula, decreases from south towards the north, or more precisely, towards the periphery of the Balkan Peninsula and the Pannonian Plain [37, 48]. There was not a single endemic taxon at the Kastel fortress in Banja Luka, probably due to the fact that it is located in the very center of the urban area, and under strong anthropogenic pressure from visitors and regular maintenance.

Alien plants are represented by 65 taxa or about 9% of total fortress flora. The largest number of aliens were recorded it the fortress in Stolac and Počitelj (41, or slightly less than 11%), whereas the highest percentage (12.19%) was recorded in Vranduk Fortress. It can be explained by the small number of recorded taxa in the flora of this fortress, but also by the higher percentage of both invasive (6.1%) and casual (2.44%) alien species than in any other site. The smallest number of alien plants was recorded at the fortress in Ljubuški (only 4 species or less than 2%) which is explained by the fact that this fortress is situated on the hill outside the settlement and is not under strong anthropogenic influence. The percentage of alien plants in fortresses in Bosnia and Herzegovina is smaller than in fortresses and walls in central and northern Europe, where their share was more than 25% [9, 45]. The higher percentage of alien plants is also typical for the Mediterranean [8, 19]. These discrepancies can be explained by the methodological frame which is not completely clear-cut and different spectra of habitat types in fortress area are covered by certain census.

The analysis of chorological spectra (Fig. 3) shows the dominance of widespread plants and plants belonging to the Eurasian floral element in all surveyed sites. The fortresses situated in the central and northern part of the country were dominated by Eurasian (Sarajevo, Banja Luka) and European (Srebrenik) floral elements, and cultivated and adventitious plants (Vranduk). The Mediterranean floral element is was the most dominant in fortresses situated in the southern part of the country (Blagaj, Ljubuški, Počitelj and Stolac), as was the case in comparable habitat types in Greece [2, 7, 20] and Bulgaria [16].

The indicators of anthropogenic changes of flora are given in Table 3. As expected, the highest values of the indicators of anthropization (both total and permanent) were calculated for fortresses which are under the highest anthropogenic impact due to regular maintenance (Vranduk and Banja Luka), as well as in those that have the largest number of species (Počitelj and Stolac), and the lowest in Ljubuški and Blagaj. This is the result of a small share of neophytes (smaller than the share of archaeophytes) in these sites, as indicated by the indicators of kenophytization, which are very low in these sites (0.94 and 1.3, respectively). In all other sites, the indicators of archaeophytization were on average two times lower than indicators of kenophytization, indicating that their flora is more influenced by neophytes than by archaeophytes. The indicator of flora modernization (IM) was the highest in Banja Luka (88.23%), and lowest in Ljubuški (50%), which is also true for the indicator of fluctuation changes (2.62 in Banja Luka, and 0 in Ljubuški). The similar values of total and permanent values of indices indicate that alien flora is well established, with a small share of casual species. This is confirmed by the low value of the indicator of fluctuating changes. High indicator of modernization calculated for each site (from 50% in Ljubuški to 88.23% in Banja Luka) is in accordance with the high share of alien species, indicating the strong anthropogenic influence on the flora. The minimum values of the flora modernization index were calculated for the flora

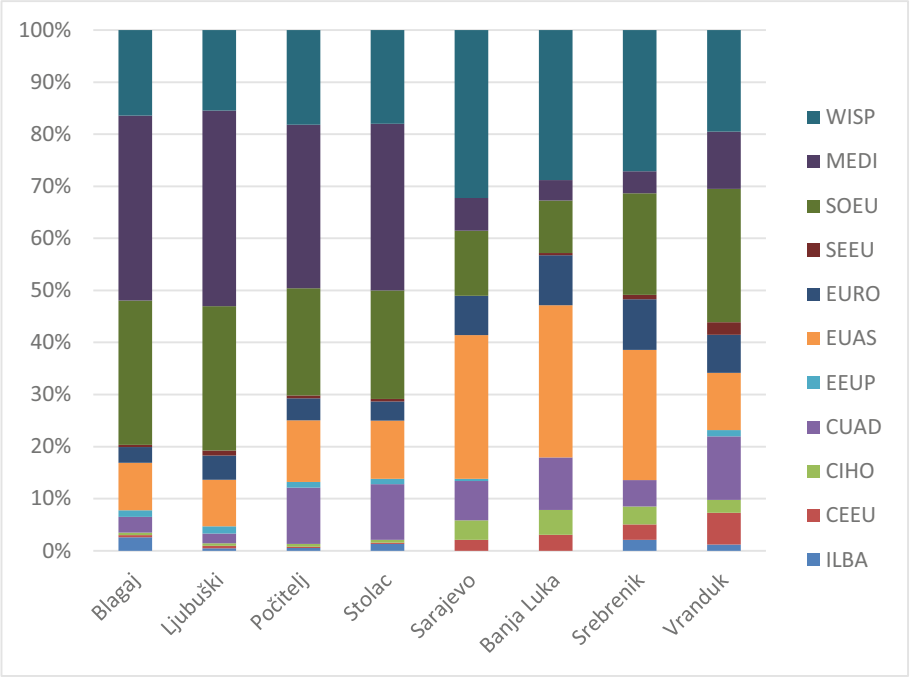

Fig. 3. Spectrum of floral elements (%) of eight surveyed fortresses (MEDI – Mediterranean floral element, ILBA – Illyrian-Balkan floral element, SOEU – South European floral element, EEUP – East European-Pontic floral element, SEEU – Southeast European floral element, CEEU – Central European floral element, EURO – European floral element, EUAS – Eurasian floral element, CIHO – Circum-Holartic floral element, WISP – widespread plants, CUAD – cultivated and adventitious plants).

of fortresses situated in areas dominated by arable land (Blagaj, Ljubuški) and forest (Srebrenik).

Table 3. Indicators of anthropogenic changes in flora of eight surveyed fortresses

	Blagaj	Ljubuški	Počitelj	Stolac	Sarajevo	Banja Luka	Srebrenik	Vranduk
IAnt	3.03	1.88	10.82	10.68	7.53	10.04	5.08	12.19
IAnp	2.18	1.88	8.89	9.02	6.35	7.62	4.27	10
IArt	1.73	0.94	3.43	3.64	2.09	3.49	2.12	4.88
IArp	1.75	0.94	3.5	3.71	2.12	3.59	2.14	5
IKnt	1.3	0.94	7.39	7.03	5.44	6.55	2.97	7.32
IKnp	1.31	0.94	7.55	7.16	5.51	6.73	2.99	7.5
IM	60	50	84.85	79.41	86.67	88.23	70	75
IF	0.86	0	2.11	1.82	1.25	2.62	0.85	2.44

The analysis of the ecological preferences of the flora [34] showed no major differences in preference for light intensity between the analyzed localities. The same applies to temperature and soil acidity. As for humidity, slightly higher values were determined for fortresses situated in Continental (Banja Luka and Srebrenik) and Alpine (Sarajevo and Vranduk) biogeographical regions than in those situated in the Mediterranean region. The biggest difference between the localities was recorded in relation to nutrients: higher values were recorded for fortresses in the northern part of the country (Banja Luka, Sarajevo, Srebrenik and Vranduk), and lower in ones situated in the south (Blagaj, Ljubuški, Počitelj and Stolac).

Analysis of the studied locations by Principal Components Analysis (PCA) explained 92.17% of variation and indicated grouping among both environmental factors and studied sites. PCA was made on the basis of environmental characteristics of individual plant species found on each studied site. Calculations were done on the basis of eigenvalues and covariance matrix, hence the analysis was directed towards correlation among locations along principal components and among studied environmental factors.

First principal component (75.35% of explained variation) is predominantly influenced in positive direction by humidity, nitrogen content and soil reaction, while in negative direction it was light and temperature. Second principal component (16.82% of variation) is predominantly defined by continentality in positive direction (Fig. 4).

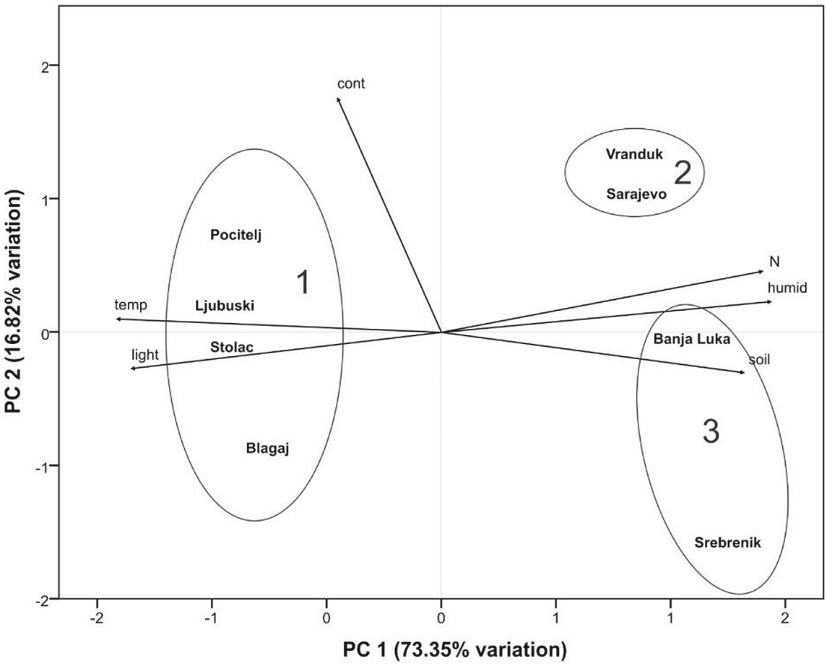

Fig. 4. Principal Components Analysis of surveyed sites with ecological preferences of the taxa towards the environmental factors

The sites are grouped based on the values of ecological indices, but the grouping also reflects their geographical distribution. Group 1 consists of Počitelj, Ljubuški, Stolac and Blagaj, which are highly marked in light and temperature indicator species, while poor in nitrogen and humidity. Group 2 is consisted of Vranduk and Sarajevo, which are less marked by light and temperature, and higher with nitrogen and humidity indicator species. Group 3 is also lower in temperature and light while higher in humidity and nitrogen, but generally lower in continentality in comparison to Group 2, which is on the same side of the figure.

The comparison of the flora composition (based on presence-absence data of complete lists of taxa), identified two broad groups of sites with different floristic assemblages (Fig. 5): one, located in the southern part of the country, in the Mediterranean biogeographical region (Blagaj, Ljubuški, Počitelj and Stolac) and another, situated in the other two regions (Sarajevo, Banja Luka and Srebrenik). Considering the fact that the cluster is made on the basis of total number of species, the dissimilarity of Vranduk in comparison to the other fortresses is expected.

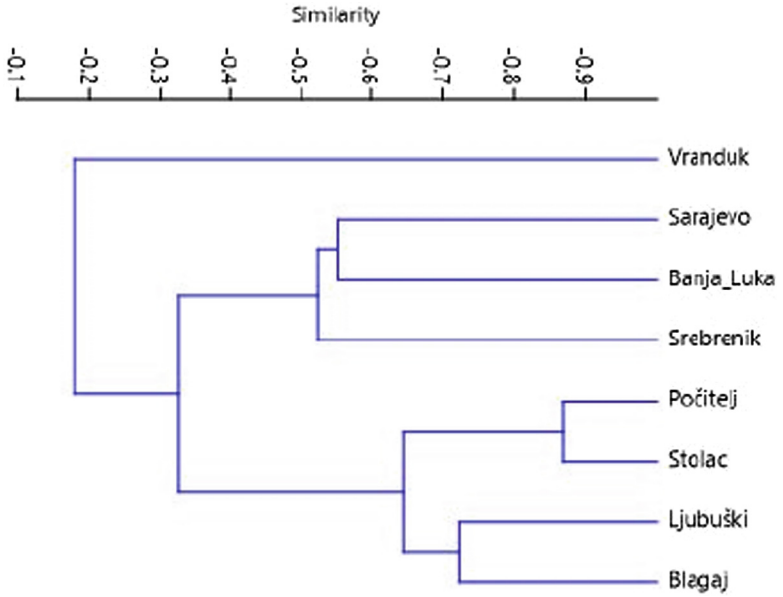

Fig. 5. Dendrogram (Bray-Curtis cluster analysis) constructed according to list of plant taxa identified at the fortresses (similarity ratio, absence-presence data).

It is evident that grouping of fortresses which was made in accordance with environmental factors (PCA) and another one by sole species abundance (Cluster Analysis) is closely matched. This clearly indicates major significance of environmental factors in general floral composition in studied fortresses.

4 Conclusions

Despite many common features, the flora composition of surveyed fortresses varies significantly, mainly as a result of their geographical location, local environmental factors as well as the intensity of anthropogenic pressure. The main reason for the differences in floristic composition is the fact that share of species common to all sites was very low, and a presence of numerous unique species. The floristic similarity among individual fortresses is a consequence of their biogeographical position. In that sense, the flora of the fortresses that belong to the Mediterranean region differs from the ones located elsewhere. The values of the indicators of anthropization and modernization, based on the share of alien species, are in line with urbanization and maintenance degree, and the anthropogenic factor also influenced the higher percentage of therophytes and widespread plants. Given the fact that the fortresses play a significant role as a habitat for native species as well as archaeophytes, the future research may assess the conservation potential of these semi-natural habitats.

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Soil Pollution by Heavy Metals Near the Lukavac Coke Factory and Models of Its Protection and Remediation

Hamdija Čivić[1](✉), Emina Sijahović[1], Senad Murtić[1], and Nermina Sarajlić[2]

[1] University of Sarajevo-Faculty of Agriculture and Food Sciences, Zmaja Od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
h.civic@ppf.unsa.ba

[2] Faculty of Natural Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia

Abstract. Soil represents a limited and invaluable natural resource that has multiple functions: production, ecological (biotope, transformation, regulatory), filtration-buffering, and provision of raw construction materials. However, industrial, technical-technological development and certain activities that humans carry out today causes numerous forms of soil degradation, including pollution and contamination of the soil with agrochemicals and heavy metals.

Bearing in mind the mentioned functions and importance of soil on the one hand and human activities that can lead to different forms of soil degradation on the other hand, the need to preserve land from degradation processes has taken on a global character.

The aim of this work was to determine the level of contamination of agricultural soils with heavy metals in the narrow zone of the coke industry in Lukavac, (44°49′23"N, 18°41′21"E). After that, in accordance with the obtained results, appropriate remedial measures are taken on the contaminated soils in order to reduce the degree of their contamination.

The results of the research showed that according to the classification of soil contamination with heavy metals, the soil in the vicinity of the Lukavac coke industry is classified as class III. It means that this soil is characterized by a high degree of contamination with heavy metals, but it can be used for growing agricultural crops, with implementation of certain protection measures.

For the purpose of chemical stabilization of heavy metals in this soil and their reduced accessibility for plants, natural aluminosilicate materials zeolite and pyrophyllite were used in this experiment. Research has shown that pyrophyllite had a significant impact on reducing the content of accessible forms of zinc (Zn), cobalt (Co) and manganese (Mn).

Keywords: soil · heavy metals · pollution · contamination · zeolite · pyrophyllite

1 Introduction

Any economic development, especially the one accompanied by the construction of industrial plants, inevitably leads to an increase in the standard of living, but at the same time it leads to an increase in pollution of the land with toxic substances. Through his

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reckless use of soil, the man has brought a large part of the land into a state of high pollution. From numerous pollutants that reach the soil due to human activity, heavy metals represent a significant problem due to their toxic effect on the environment, growth, development and quality of plants. As a result of these circumstances it is possible for humans to develop various diseases in case of exposure to harmful concentrations of heavy metals in the environment (Bates et al., 1995; Vratuša, 1999; Blake & Goulding, 2002; He Z.L. et al., 2005).

Taking into account the aforementioned facts, the preservation of fertile and remediation of agricultural land contaminated with heavy metals is simply imposed as a necessity if one wants to protect land as a primary resource for food production (Jakovljević & Antić-Mladenović, 2000; Malhotra et al., 2014). This need is particularly pronounced in the industrial areas of the Federation of Bosnia and Herzegovina (FBiH), where the possibility of soil contamination with heavy metals is extremely high (Pranjić, 2017). During 2019, the Faculty of Agriculture and Food implemented a project called "Models of protection and remediation of land contaminated with heavy metals in industrial areas of the Federation of Bosnia and Herzegovina", commissioned by the FBiH Environmental Protection Fund. This paper represents part of the results from the mentioned project which are related to the land in the vicinity of the Lukavac coke industry in the Tuzla Canton. The goal of these studies was to determine the degree of soil contamination with heavy metals in the soils in the immediate vicinity of the Lukavac coke industry. Then, on the land found to be the most polluted or contaminated with heavy metals, appropriate remedial measures were implemented with the intention of bringing the content of heavy metals to a level that does not pose a danger to the environment and human health. Through these researches, we tried to confirm the possibility and effectiveness of applying the chemical model of remediation through the use of natural aluminosilicates, zeolite and pyrophyllite. We also tested the effects of using the phytoremediation model through sowing and the cultivation of corn plants on polluted soils.

Previous research shows that the effectiveness of pyrophyllite in the immobilization of heavy metals in the soil depends on the properties of the soil, the pH reaction and the distribution coefficient of heavy metal ions in the soil. Although numerous studies confirm that pyrophyllite has a high efficiency in removing heavy metals from soil and water (Keren and Sparks, 1994; Saxena et al., 2001; Gücek et al., 2005; Kulović, 2017; Halilović, 2018; Panda et al., 2018), the binding mechanisms of heavy metals on pyrophile have not been scientifically fully explained. In the research conducted by Murtić et al. (2019) the use of pyrophyllite showed a significant effect in reducing the mobility of lead and cadmium in the soil. The aluminosilicate mineral zeolite shows similar properties and effects.

2 Material and Method

In the spring of 2019, in the vicinity of the Lukavac coke industry, average soil samples were first taken from three plots of land with an area of 1000 m^2, with the aim of determining the content of heavy metals. Only the plots that are used or can potentially be used for the purpose of agricultural production, which had no major slopes and/or depressions, were selected. On those plots the application of remedial measures is easily feasible.

Analysis of total and accessible forms of heavy metals (Cd, Cr, Pb, Zn, Cu, Mn, Fe, Ni) was performed on average soil samples in the laboratory of the Faculty of Agriculture and Food, University of Sarajevo. Also, within the framework of these studies, the type of land and the basic parameters of fertility were determined (pH, humus content, carbonate content, and the content of accessible forms of phosphorus and potassium). The plot of land, which was determined to have a higher content of certain heavy metals according to the current legislation in the Federation of Bosnia and Herzegovina, was taken into the further focus of the work. Appropriate remediation measures were carried out, such as chemical stabilization of the land using aluminosilicate minerals and phytoextraction/phytoaccumulation.

The first action during the process of chemical stabilization of contaminated soil using aluminosilicate materials (zeolite and pyrophyllite) involved the agrotechnical measure of plowing and harrowing and then setting up a trial using the method of random block arrangement with 7 variants in 3 repetitions. The variants of the experiment were as follows:

1. Variant (control; without addition of pyrophyllite and zeolite).
2. Variant (application of 200 kg/ha of 0–3 mm granulation zeolite).
3. Variant (application of 400 kg/ha of 0–3 mm granulation zeolite).
4. Variant (application of 600 kg (ha) of 0–3 mm granulation zeolite).
5. Variant (application of 200 kg/ha of pyrophyllite with a granulation of 0–3 mm).
6. Variant (application of 400 kg/ha of pyrophyllite with a granulation of 0–3 mm).
7. Variant (application of 600 kg/ha of pyrophyllite with a granulation of 0–3 mm).

The sample plots had an area of 50 m^2, with a 2 m wide distance between all plots to avoid the influence of one variant on another. Seven days after setting up the experiment, corn was sown on the tested plots, with the aim of determining whether the application of aluminosilicate materials had an effect on the reduced uptake of heavy metals by the tested plant. At the moment of technological maturity of the corn, sampling of plant material and soil was carried out on the experimental plots. The content of heavy metals was tested in the plant material (leaf), and the content of accessible forms of heavy metals in the soil.

2.1 Properties of Pyrophyllite

Pyrophyllite is a natural mineral from the group of aluminosilicates with the chemical formula $Al_2[Si_4O_{10}](OH)_2$. It got its name from the Greek words pyr - fire and philon - leaf, because it spreads in fan-shaped manner when heated. It is characterized by the following properties: hardness is between 1 and 1.5 on the Mohs scale, density between 2.7 and 2.9 g/cm^3, hydrophobic, insoluble in water and does not swell, alkaline reactions (pH between 7.5 and 7.8 (Hasanbegović et al., 2020). It has a very high effective cation exchange capacity, between 50 and 70 meq/100 g. It is soft to the touch, visually very similar to talc, and its color varies depending on the proportion of oxides and other components in the pyrophyllite ore, but as a rule it is white to gray white color.

The pyrophyllite material used in this research was created by crushing and sieving pyrophyllite ore from the Parsovići - Konjic site, Bosnia and Herzegovina. Except for silicon dioxide (SiO2) and aluminum oxide (Al2O3), which together make up 80 to 90%

of the chemical composition of pyrophyllite, it also contains various other impurities that can greatly affect its properties, especially the effective cation exchange capacity. The chemical analysis of the prifofilt material used in this research was done in the laboratory of the Faculty of Agriculture and Food Sciences, University of Sarajevo, and the obtained results are presented in Table 1.

Table 1. Chemical analysis of pyrophyllite used in this research

Tested parameter	Unit	Value
pH (H_2O)	pH	8.5
pH (KCl)	pH	8.1
SiO_2	%	67.55
Al_2O_3	%	19.10
Ca	%	3.65
K	%	0.023
Mg	%	0.135
Fe	%	0.337
Cu	mg/kg	1.40
Ni	mg/kg	2.74
Zn	mg/kg	25.68
Co	mg/kg	0.40
Mn	mg/kg	93.14
Cd	mg/kg	n.d.*
Pb	mg/kg	7.97
Cr	mg/kg	0.76

* n.d. - not detected (below the detection limit of the instrument)

The results from Table 1 show that the pyrophyllite used in this research does not deviate significantly from the basic chemical characteristics of pyrophyllite ores, except for the fact that it contains a relatively high proportion of calcium and magnesium. It can be assumed that the pyrophyllite ore from the Parsovići - Konjic site contains a certain amount of dolomite or some other natural material rich in the mentioned elements. A positive characteristic of the pyrophyllite material used in this research is the fact that it contains very low concentrations of toxic heavy metals, which makes it environmentally acceptable for use in remedial purposes.

2.2 Properties of Zeolite

Zeolite is a hydrated aluminosilicate material created by mixing volcanic lava with alkaline groundwater. Its structure is latticed, composed mostly of SiO44- and AlO45-

tetrahedra connected by common oxygen atoms. It contains both monovalent and divalent cations (Na+, K+, Ca2+) and certain amounts of water.

The zeolite used in this research was created by crushing and sieving zeolite ore from the Slanci - Veliko Selo site near Belgrade, Serbia. The basic characteristic of the zeolite used is a high cation exchange capacity (above 180 meq/100 g), which enables it to attract and bind to its structure, as well as exchange positively and negatively charged particles, i.e. cations and anions.

The complete chemical analysis of the used zeolite is shown in Table 2 (results taken from the website of the zeolite manufacturer https://zeolit.rs/).

Table 2. Chemical analysis of the zeolite used in this research

Tested parameter	Unit	Value
pH (H_2O)	pH	8.3
pH (KCl)	pH	7.4
SiO_2	%	64.55
Al2O3	%	14.49
CaO	%	4.9
K_2O	%	1.04
MgO	%	0.88
Fe_2O_3	%	2.3
Cu (bakar)	mg/kg	6.2
Ni (nikal)	mg/kg	21.0
Zn (cink)	mg/kg	42.0
Co (kobalt)	mg/kg	28.0
Cd (kadmij)	mg/kg	3.0
Pb (olovo)	mg/kg	35.0
Cr (hrom)	mg/kg	5.8

The work methods used in this research were as follows:

- a pH meter (ISO 10390, 1994) was used to determine the pH value of the soil,
- humus content in soil samples was determined by the dichromate method (ISO 14235, 1994),
- the content of easily accessible forms of potassium and phosphorus in soil samples was determined by the ammonium lactate (AL) method (Egner et al., 1960),
- the extraction of the total forms of heavy metals from the soil was done with the use of gold dust (a mixture of nitric and hydrochloric acid in a ratio of 1:3) according to the ISO 11466 (1998) method,
- the extraction of accessible forms of heavy metals from the soil was done using the so-called EDTA-solutions (a mixture of 1 M (NH4)2CO3 and 0.01 M EDTA

(ethylene-diamino-tetraacetic acid) where the pH of the solution was adjusted to 8.6 using HCl or NH4OH),
– the extraction of heavy metals from plant material was done with the use of nitric and sulfuric acid (in a ratio of 2.5:1) according to the method specified in the practicum of Lisjak et al. (2009),
– the determination of the content of heavy metals in plant material samples, and the content of total and accessible forms of heavy metals in soil samples was performed using the method of flame atomic absorption spectrophotometry on a Shimadzu 7000 AA apparatus (ISO 11047, 1998).

For the interpretation of the results related to the content of heavy metals in the soil, the Rulebook on determination of permitted amounts of harmful and dangerous substances in the soil for the FBiH (Official Gazette of the FBiH, 72/09) and the classification of soil pollution according to Bašić (1994) were used. Within the framework of the classification of land pollution according to Bašić, land is ranked in several classes, depending on the degree of land pollution with heavy metals (So).

The degree of soil contamination (So) is determined from the ratio of the determined soil contamination with a particular heavy metal to the limit value for the specified heavy metal.

So = content in soil (mg/kg) / limit value.

3 Results and Discussion

A large number of industrial facilities are located in the area of the Lukavac municipality within a distance of a few kilometers, some of which, such as coke ovens and soda factories, are major polluters of the environment. Accordingly, the development of agricultural production in the mentioned area is very questionable, especially on lands located along the Spreča river, into which waste water from various industrial plants flows, not only from the area of the Lukavac municipality, but also from the Tuzla canton as a whole.

The experimental plot was located in the immediate vicinity of the Lukavac coking plant. The type of soil on the mentioned plot is humofluvisol fluvial, meadow - brown non-carbonate clay soil.

After the analysis of the average soil sample from the said plot, the content of heavy metals was determined, which is shown in Table 3.

Table 3. Results of the analysis of the content of heavy metals in the tested soils

No			Content of heavy metals (mg/kg soil)					
	Cu	Zn	Mn	Cd	Pb	Ni	Cr	Co
1	13.3	41.93	249.29	n.d	16.50	54.31	69.91	9.03

Before carrying out the process of chemical stabilization of the tested land, the degree of its contamination with heavy metals was determined based on the ratio of the

determined contamination of the soil with an individual heavy metal to the limit value for the specified heavy metal (So), i.e. based on the classification of soil contamination (Bašić, 1994). The threshold values needed to calculate the So factor are taken from the Rulebook on determining the permitted amounts of harmful and dangerous substances in soil for FBiH. According to the obtained results, the examined land is classified in the III class of land pollution, i.e. in the land of heavy pollution with heavy metals (the land belonging to this class can be used for growing plants, but enhanced protection measures are required).

As part of determining the fertility of the tested soil, the values of the following parameters were determined: humus content, carbonate content, content of accessible forms of phosphorus and potassium, and the pH value of the tested soil. The obtained values for selected soil fertility parameters are shown in Table 4.

Table 4. Presentation of the results obtained from the chemical analysis of the tested soil

Tested parameter	Unit	Value	Soil characteristics on basis of measured values
pH (H_2O) pH (KCl)	-	4.8	acidic reaction
	-	3.9	
Humus	%	2.5	moderately humous
Phosphorus (P_2O_5)	mg $100g^{-1}$	12.6	medium
Potasium (K_2O)	mg $100g^{-1}$	14.6	medium
Carbonates	%	0	-

The results of the analysis showed that the tested soil is extremely acidic with a moderate content of organic matter. The content of accessible forms of phosphorus and potassium in the examined soil was at a medium level, and the content of carbonates was not determined.

The average contents of accessible forms of heavy metals in the examined soil (mg/kg soil), depending on the treatment with aluminosilicate materials, are shown in Table 5.

From the results shown in Table 5, it can be seen that the content of accessible forms of Cd and Pb was not determined in any of the examined plots of land. It can also be seen that the content of accessible forms of all other tested heavy metals in the soil, with the exception of Cr, was always lower in the variants where the soil was treated with aluminosilicate materials. With the aim of determining whether the mentioned influence of pyrophyllite and zeolite on reducing the accessibility of heavy metals in the investigated soil located near the coke plant in Lukavac was statistically significant, an analysis of variance (F test) was carried out, as well as testing the significance of the differences between the environments for the content of those heavy metals in which F test proved significant.

The results of the analysis of variance showed that the influence of pyrophyllite and zeolite on reducing the availability of Cu, Pb and Cr in the tested soil near the Lukavac coking plant was not statistically significant. Likewise, zeolite did not show a statistically

Table 5. Results of testing the significance of the differences of the environments for the content of accessible forms of heavy metals in the soil, near the coke oven in Lukavac.

Variant*	Content of accessible forms of heavy metals in soil (mg/kg soil)							
	Cu	Zn	Mn	Cd	Pb	Ni	Cr	Co
V_1 (control)	1.6	0.53^{a}	10.14^{a}	n.d.**	n.d	9.53	0.27	0.042^{a}
V_2 (Z200)	1.59	0.52^{ab}	9.65^{ab}	n.d	n.d	8.88	0.27	0.031^{abc}
V_3 (Z400)	1.47	0.52^{abcd}	7.13^{abc}	n.d	n.d	8.14	0.27	0.027^{bc}
V_4 (Z600)	1.48	0.52^{abc}	7.62^{abc}	n.d	n.d	8.63	0.29	0.038^{ab}
V_5 (P200)	1.46	0.42^{e}	5.60^{c}	n.d	n.d	8.1	0.28	0.000^{d}
V_6 (P400)	1.53	0.43^{e}	6.05^{bc}	n.d	n.d	8.7	0.28	0.021^{c}
V_7 (P600)	1.54	0.46^{bcde}	7.57^{abc}	n.d	n.d	8.98	0.26	0.023^{c}
$LSD_{0.05}$	-	0.069	3.61	-	-	-	-	0.014

* V1 - Control (untreated variant); V2 - Zeolite 200 kg/ha; V3 - Zeolite 400 kg/ha; V4 - Zeolite 600 kg/ha; V5 - Pyrophyllite 200 kg/ha; V6 - Pyrophyllite 400 kg/ha; V7 - Pyrophyllite 600 kg/ha
** (not detected: below device detection level)

significant effect on the reduction of Zn and Mn availability in the tested soil. The only element whose accessibility in the soil is reduced by the application of zeolite is Co, and only when zeolite was applied in a dose of 400 kg/ha.

The use of pyrophyllite, in contrast to zeolite, had a statistically significant effect on reducing the content of accessible forms of Zn and Co in the tested soil, regardless of the applied dose. Varieties of 200 kg/ha and 400 kg/ha of pyrophyllite had a positive effect on the reduction of Mn availability as well.

The average content of the tested heavy metals in the leaves of corn (mg/kg dry matter) grown on the tested soil, depending on the treatment of the soil with aluminosilicate materials, are shown in Table 6.

The content of Cd and Pb was not determined in any sample of corn leaves, regardless of the land plot from which they were collected. When it comes to other elements, it can be seen from the Table 6 that the content of Zn, Mn and Ni in corn leaves was always lower in leaves taken from plots that were previously treated with aluminosilicates, regardless of their type or application dose. This sequence of results was not fully confirmed when the subject of the test was the content of Cu and Cr in corn leaves.

With the aim of determining whether the differences in the content of heavy metals in corn leaves between the tested varieties were significant, the F test was performed, and the significance testing of the differences between the means for the content of those heavy metals in which the F test proved to be significant. The obtained results are also shown in Table 6.

The content of Cd and Pb was not determined in the leaves of corn, which was expected if we take into account the fact that in the land where corn was grown, the content of accessible forms of the mentioned elements was not determined either.

The used aluminosilicate zeolite did not show a statistically significant effect on the reduction of the content of almost any tested element in corn leaves. The only exception

Table 6. Results of testing the significance of the differences between the environments for the content of heavy metals in the leaves of corn grown on the tested soil located near the Lukavac coking plant.

Variant*	Content of heavy metals in corn leaves (mg/kg dry matter)						
	Cu	Zn	Mn	Cd	Pb	Ni	Cr
V_1 (cont.)	7.18	14.53^{a}	29.06^{a}	n.d	n.d	1.77	0.45
V_2 (Z200)	7.29	13.63^{ab}	29.01^{ab}	n.d	n.d	1.63	0.46
V_3 (Z400)	6.82	13.26^{bc}	26.31^{c}	n.d	n.d	1.56	0.47
V_4 (Z600)	6.12	12.12^{cd}	26.58^{bc}	n.d	n.d	1.47	0.36
V_5 (P200)	6.09	11.49^{de}	27.48^{abc}	n.d	n.d	1.51	0.47
V_6 (P400)	6.11	11.27^{de}	26.20^{c}	n.d	n.d	1.56	0.43
V_7 (P600)	6.17	10.21^{e}	23.20^{d}	n.d	n.d	1.62	0.43
$LSD_{0.05}$	-	1.184		-	-	-	-

* V1 - Control (untreated variant); V2 - Zeolite 200 kg/ha; V3 - Zeolite 400 kg/ha; V4 - Zeolite 600 kg/ha; V5 - Pyrophyllite 200 kg/ha; V6 - Pyrophyllite 400 kg/ha; V7 - Pyrophyllite 600 kg/ha

is the content of Zn in the leaves of corn applied at a dose of 400 kg/ha and 600 kg/ha and Mn at a dose of 400 kg/ha, compared to the control variant. Pyrophyllite, regardless of the application dose, showed a statistically significant effect on the reduced Zn content in corn leaves when compared to the control, untreated variant. A statistically significant effect of pyrophyllite in doses of 400 and 600 kg/ha on the reduced Mn content in corn leaves was also recorded.

4 Conclusion

- According to the classification of land contamination with heavy metals, the tested soil in the vicinity of the Lukavac coke plant belongs to the III class of land (So from 0.5–1), which means that agricultural production is allowed on the mentioned soil, but with enhanced protection measures.
- Soil treatment with zeolite and pyrophyllite did not have a statistically significant effect on reducing the accessibility of Cu, Ni, and Cr, regardless of the applied amount of these aluminosilicates.
- Soil treatment with zeolite only showed a statistically significant effect on reducing the content of the accessible form of Co compared to the control variant, but only at the application dose of 400 kg/ha
- Soil treatment with pyrophyllite showed a statistically significant effect on reducing the availability of Zn, Mn and Co, regardless of the applied dose.
- In the conditions of the conducted experiment, the application of pyrophyllite at a dose of 200 kg/ha reduced the accessibility of Zn by 20.7% and Mn by 44.8% in the examined soil, the application of pyrophyllite at a dose of 400 kg/ha reduced the accessibility of Zn by 18.9% and Mn by 40.3%, and the application of pyrophyllite in a dose of 600 kg/ha reduced the availability of Zn by 13.2% and Mn by 25.3%.

- The application of zeolite affected the reduction of the accumulation of Zn and Mn content in corn leaves compared to the control variant and only if it was used in a dose of 400 kg/ha and 600 kg/ha.
- The treatment of soil with pyrophyllite, regardless of the applied dose, significantly reduced the accumulation of Zn in corn leaves when compared to the control (untreated) variant. The application of pyrophyllite also had an effect on the reduction of the accumulation of Mn content in corn leaves, and only if it was used in doses of 400 kg/ha and 600 kg/ha. When it comes to the accumulation of other examined heavy metals in corn leaves, the treatment of soil with pyrophyllite did not show its justification.

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Effectiveness of Chlorantraniliprole, Methoxyfenozide and Emamectin Benzoate Insecticides in Controlling *Tuta Absoluta*

Sanel Haseljić[1](✉), Snježana Hrnčić[2], Fejzo Bašić[1], Josip Jurković[1], and Siniša Mitrić[3]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina
s.haseljic@ppf.unsa.ba

[2] Biotechnical Faculty, University of Montenegro, 81000 Pogorica, Montenegro

[3] Faculty of Agriculture, University of Banja Luka, Banja Luka, Bosnia and Herzegovina

Abstract. *Tuta absoluta* is the most abundant pest of vegetable species from the *Solanaceae* family, which has been member of Bosnia and Herzegovina (B&H) entomofauna since 2010. It is widespread in the protected production spaces of the Mediterranean and continental areas of B&H, and has become a serious problem in tomato protection. According to the many studies frequent use of older generation insecticides has led to increased resistance of *Tuta absoluta.* Therefore, the aim of this work was to investigate the effectiveness of the newer insectides generation such as: Chlorantraniliprole, Methoxyfenozide and Emamectin benzoate in suppressing the tomato leaf miner population collected from the southern area of B&H. Insecticides were applied in different concentrations to three tomato hybrids (Matias F1, Belle F1, Rally F1) under controlled conditions. Test was performed according to the modified „leaf-deep method“ (IRAC). The concentrations of applied insecticides had different effectiveness against *Tuta absoluta.* Insecticides based on active substances: Chlorantraniliprole, Methoxyfenozide and Emamectin benzoate, which were applied in two different concentrations, had no significant effect on reducing the resistance of *Tuta absoluta.*

Keywords: *Tuta absoluta* · Chemical control · Tomato · Resistance · Hybrid

1 Introduction

Tuta absoluta or the tomato leaf miner is a pest from the Lepidoptera family, which causes significant damage to tomatoes both in protected areas and in the open field. After it was first observed in Spain in 2006 [1], *Tuta absoluta* was identified in many other European countries. Thus, it was registered for the first time in Italy in 2008 [2, 3], the Netherlands [4], and France [5, 6]. It is considered that *Tuta absoluta*, as a new member of the BiH entomofauna, represents a serious problem of tomato production. Controlling *Tuta absoluta* is extremely demanding due to its development cycle and lifestyle. Large number of eggs that can be laid by one female and many generations greatly limit its

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control. Therefore, in the planning of protection against this pest, all preventive plant protection measures must be carried out, such as: crop rotation, destruction of attacked plant parts, high-quality soil cultivation and planting of healthy planting material. In order to protect tomatoes from the attack of this pest, it is necessary to establish integral principles of production based on cultural, biotechnical, biological and chemical measures, which include appropriate resistance management strategies. Chemical control as part of the strategy to control this pest was already used in the early eighties of the last century [7]. In that period, chemical agents based on organophosphates and pyrethroids were mostly used to control *Tuta absoluta.* Pyrethroids as neurotoxic insecticides act on sodium channels in the cell membranes of the nervous system, causing disruption of the flow of nerve impulses, resulting in paralysis and death of the pest, and on the function of the nervous system of *Tuta absoluta.* When it comes to chemical measures to control *Tuta absoluta*, the very nature of the pest, as well as its ability to quickly develop resistance to many insecticides, significantly limit their effectiveness. In the work [8] state that chemical insecticides are the main means of controlling *Tuta absoluta*, and that effective control of this pest can be carried out by applying different active substances in combination with biological measures. The frequent use of insecticides with different active substances has led to increased resistance *Tuta absoluta.* In the study of the effectiveness of the active substances deltamethrin, abamectin and methamidophos, the resistance larvae of *Tuta absoluta* was determined [9]. Insecticides based on indoxacarb, lufenuron, spinosad, thiacloprid, imidacloprid showed a positive effect in controlling *Tuta absoluta* in Malta [10], while in Italy products based on chlorpyrifos and pyrethrins showed good results [2]. Bearing in mind all the above, the aim of this work was to investigate the effectiveness of the newer insectides generation such as: Chlorantraniliprole, Methoxyfenozide and Emamectin benzoate in controlling *Tuta absoluta* population collected from the southern area of B&H.

2 Materials and Methods

To test the effectiveness of insecticides based on the active substances: Chlorantraniliprole, Methoxyfenozide and Emamectin benzoate, *Tuta absoluta* populations collected from tomato plantations in Višići, Gabela and Klepci in B&H. Larvae samples were collected during June in the phenophase of tomato fruiting. The following preparations were used to test the effectiveness of insecticides against larvae. Coragen 20 SC (a.s. Chlorantraniliprole- 200 g/l; SC; IRAC MoA: 28) – insecticide with contact and digestive action, which is used to control economically important pests from the order of Lepidoptera and Coleoptera. The active substance of the product is Chlorantraniliprole, which has an ovicidal effect on all stages of caterpillar development. Coragen causes activation of ryanodine receptors in insects, thereby stimulating the release of calcium from internal depots of smooth and striated muscles, causing weakening of muscle control, paralysis and ultimately, death of the insect. The preparation is characterized by ecological properties and low toxicity. Runner 240 SC (a.s. Methoxyfenozide-240 g/l; SC; IRAC MoA: 18)- belongs to the group of growth accelerators, so it is an antagonist of the shedding hormone. It has a digestive and contact effect. Affirm (a.s. Emamectin benzoate- 9.5 g/kg; SG; IRAC MoA: 6) – a contact insecticide that shows excellent

translaminar movement in leaves. When an insect comes into contact with emamectin benzoate, it becomes paralyzed and immediately stops feeding, after which it dies within two to four days.

Laboratory tests of Coragen 20 SC, Runner 240 SC and Affirm insecticides were conducted on three tomato hybrids (Matias F1, Belle F1, Rally F1), where the insecticides were applied in two concen trations (Table 1).

Table 1. Insecticides and concentrations applied in the laboratory

Insecticide	Active substance	Concentration 1(%)	Concentration 2(%)
Coragen	Chlorantraniliprole	0.016	0.02
Runner	Methoxyfenozide	0.04	0.06
Affirm	Emamectin benzoate	0.15	0.2

The insecticide efficiency test was done in two parts. The first part included the investigation of whether the previous application of insecticides to tomato leaf disks prevents subsequent applied caterpillars from burrowing into the leaf, which was determined 24 h after treatment. For this test, healthy leaves were separated from each hybrid, from which disks of equal surface were made. The discs were rinsed with distilled water and dried on a ribbed metal grid. Leaf discs prepared in this way were used to apply each insecticide according to the modified leaf-deep method [11]. After applying each of the insecticide concentrations, the leaf sets were left on a drying rack for one hour. They were then placed on filter paper in Petri dishes, after which caterpillars were applied, whereby five larvaes of 4–6 mm in size were placed on each leaf disc (Figs. 1 and 2).

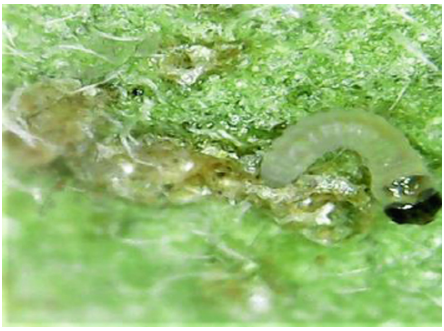

Fig. 1. Larvae on tomato leaf

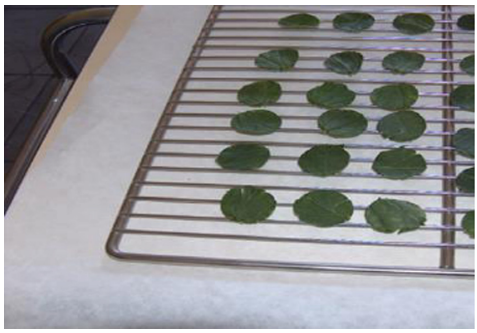

Fig. 2. Tomato leaves – discs

The second part of the experiment involved testing the effectiveness of insecticides used to control larvae applied to the leaves of three tomato hybrids. Seedlings of tomato hybrids with applied larvae were treated with insecticides in two different concentrations. Treatment was done with a hand sprayer 30 min after larvae were applied to the tomato leaves of each hybrid. The effectiveness of the insecticide was determined based on the number of live larvae registered 24 h and 48 h after treatment. Samples larvae used from tomato plant material are shown in Figs. 3 and 4.

Fig. 3. Plant material with larvae

Fig. 4. Samples larvae

Standard descriptive statistics methods were used for statistical data processing. Variable values are presented as mean ± standard deviation. The obtained data are presented in tables and graphics. All obtained results were statistically processed using the software programs SPSS (version 22) and Excel.

3 Results and discussion

3.1 Control Larvae with Affirm Insecticide

After the application of Affirm insecticide in concentrations of 0.15% and 0.2%, the average larvae number of *Tuta absoluta*, depending on the tomato hybrid and the length of the insecticide's action, is shown in Table 2. The average larvae number was derived from the total larvae number ls on the sample plants.

Table 2. The number larvae after the application of Affirm insecticide in concentrations of 0.15 and 0.2%, depending on the tomato hybrid and the length of the insecticide's action (Tukey test)

Time after application (TAA)	Hybrid/concentration (%)									Average concentration		GA TAA
	Belle		Average Belle	Matias		Average Matias	Rally		Average Rally			
	0.15	0.2		0.15	0.2		0.15	0.2		0,15	0,2	
	Larvae number											
0 h	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}
24 h	2.27^{b}	2.27^{b}	2.27^{b}	2.80^{b}	2.80^{b}	2.27	2.40^{b}	2.20^{b}	2.20^{b}	2.49	2.24	2.37^{b}
48 h	1.60^{c}	1.40^{c}	1.50^{c}	1.13^{c}	0.93^{c}	1.03	1.07^{c}	1.00^{c}	1.03^{c}	1.27	1.11	1.11^{c}
Average	2.96^{a}	2.89^{a}		2.98^{a}	2.73^{a}		2.82^{a}	2.73^{a}		2.92^{a}	2.79^{a}	
GA hybrid	2.92^{a}			2.86^{a}			2.78^{a}					

* Time after application (TAA), * General average (GA)

After 24 h from the application of the Affirm insecticide used at a concentration of 0.15%, the average number of larvae ranged from 2.4 in the Relly hybrid to 2.8 in the Matias hybrid. Affirm insecticide applied at a concentration of 0.2% resulted in an average number larvae from 2.2 in the Rally hybrid to 2.8 in the Matias hybrid 24 h after the application. After 48 h of Affirm insecticide application at a concentration of 0.15%, the average number of larvae ranged from 1.07 in the hybrid Rally to 1.60 in the hybrid Belle. The average number of larvae 48 h after the application of Affirm insecticide at a concentration of 0.2% ranged from 0.93 in the Matias hybrid to 1.40 in the Belle hybrid.

The analysis of variance test (Table 3) showed that only the length of insecticide action had a significant impact on the average number of larvae after Affirm insecticide application. The concentration of Affirm insecticide and tomato hybrid had no significant effect on the number of larvae.

Table 3. Analysis of variance test on the influence of tomato hybrids, effect and concentration of Affirm insecticide on the number larvae of *Tuta absoluta*

Source of variability	SS	d.f	MS	F	p
Concentration (A)	1.200	1	1.200	1.680	0.196
Insecticide action (B)	685.385	2	342.69	479.770	< 0.0001
Hybrid (C)	0.941	2	0.470	0.659	0.519
A × B	0.689	2	0.344	0.482	0.618
A × C	0.422	2	0.211	0.296	0.744
B × C	4.681	4	1.170	1.639	0.165
A × B × C	0.756	4	0.189	0.264	0.901
Error	180.000	252	0.714		
Total	3070.0	270			

The results of the Tukey test (Table 2) showed that 48 h after the application of Afirma, the significantly lowest number larvae. The results of the research by Gacemi and Guenaui [12] show good efficacy of emamectin benzoate against *Tuta absoluta.*

3.2 Control Larvae with Runner Insecticide

After the application of Runner insecticide at a concentration of 0.04% and 0.06%, the average number larvae depending on the tomato hybrid and the length of the insecticide's action is presented in Table 4.

Table 4. Mortality larvae after the application of Runner insecticide concentrations of 0.04 and 0.06%, depending on the tomato hybrid and the action of the insecticide (Tukey test)

Time after application (TAA)	Hybrid/concentration (%)									Average concentration		GA TAA
	Belle		Average Belle	Matias		Average Matias	Rally		Average Rally			
	0.04	0.06		0.04	0.06		0.04	0.06		0.04	0.06	
	Larvae number											
0 h	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}
24 h	2.60^{b}	2.47^{b}	2.53^{b}	2.67^{b}	2.73^{b}	2.7^{b}	2.67^{b}	2.07^{b}	2.37^{b}	2.64^{b}	2.42^{b}	2.53^{b}
48 h	1.33^{c}	1.40^{c}	1.37^{c}	1.53^{c}	1.13^{c}	1.3^{c}	1.07^{c}	1.20^{c}	1.13^{c}	1.31^{c}	1.24^{c}	1.28^{c}
Average	2.98^{a}	2.96^{a}		4.07^{a}	2.96^{a}		2.91^{a}	2.76^{a}		2.99^{a}	2.89^{a}	
GA hybrid	2.97^{a}			3.01^{a}			2.83^{a}					

After 24 h from the application of Runner insecticide at a concentration of 0.04%, the average number of larvae ranged from 2.60 in the Belle hybrid to 2.67 in the Rally and Matias hybrids. After the application of Runner insecticide with a concentration of 0.06%, the average number of larvae 24 h after treatment ranged from 1.13 in the Matias hybrid to 1.40 in the Bella. After 48 h from the application of Runner insecticide at a concentration of 0.04%, the average number of larvae ranged from 1.07 in the Rally hybrid to 1.53 in the Matias hybrid. The average number of larvae 48 h after treatment with Runner insecticide at a concentration of 0.06% ranged from 1.13 in the Matias hybrid to 1.40 in the Belle hybrid. The results of the Tukey test (Table 4) showed that 48 h after the application of the insecticide Runner, the significantly lowest number of larvae was registered. The variance analysis test (Table 5) showed that only the length of action of the Runner insecticide had a significant effect on the number of larvae after application in each tomato hybrid.

Table 5. Analysis of variance test on the influence of tomato hybrids, length of action and concentration of insecticide Runner on the number of larvae

Source of variability	SS	d.f	MS	F	p
Concentration(A)	0.626	1	0.626	0.941	0.333
Insecticide action (B)	645.4	2	322.7	485.26	< 0.0001
Hybrid (C)	1.541	2	0.770	1.158	0.316
A × B	0.585	2	0.293	0.440	0.645
A × C	0.207	2	0.104	0.156	0.856
B × C	1.081	4	0.270	0.407	0.804
A × B × C	2.815	4	0.704	1.058	0.378
Error	167.60	252	0.665		
Total	3149.0	270			

3.3 Control Larvae with Coragen Insecticide

After the application of the Coragen insecticide at a concentration of 0.016% and 0.02%, the average number of tomato leaf miner larvae depending on the tomato hybrid and the length of the insecticide's action is presented in Table 6.

Table 6. Number larvae after the application of Coragen insecticide in a concentration of 0.016% and 0.02%, depending on the tomato hybrid and the length of the insecticide's action

Time after application (TAA)	Hybrid/concentrecation (%)									Average concentration		GA TAA
	Belle		Average Belle	Matias		Average Matias	Rally		Average Rally			
	0,016	0,02		0,016	0,02		0,016	0,02		0,016	0,02	
	Larvae number											
0 h	5.00[a]	5.00[a]	5.00[a]	5.00[a]	5.00[a]	5.0[a]	5.00[a]	5.00[a]	5.00[a]	5.00	5.00	5.00[a]
24 h	2.33[b]	1.13[b]	2.10[b]	2.80[b]	2.93[b]	2.8[b]	3.33[b]	2.93[b]	3.13[b]	2.82[b]	2.58[b]	2.7[b]
48 h	1.33[c]	1.87[c]	1.23[c]	1.40[c]	1.47[c]	1.4[c]	2.40[c]	1.13[c]	1.77[c]	1.71[c]	2.67[c]	2.19[c]
Average	2.89[a]	2.67[a]		3.07[a]	3.13[a]		3.58[a]	3.02[a]		3.41[a]	3.17[a]	
GA hybrid	2.78[a]			3.10[a]			3.30[a]					

The average number of larvae 24 h after the application of Coragen at a concentration of 0.016% on tomato hybrids ranged from 2.33 in hybrid Belle to 3.33 in Rally. The average number of larvae 24 h after the application of Coragen concentration 0.02% ranged from 1.13 in hybrid Belle to 2.93 in hybrid Matias and Rally. After 48 h from the application of Coragen concentration 0.016%, the average number of larvae ranged from 1.33 in hybrid Belle to 2.40 in hybrid Rally. The average number of larvae 48 h after the application of Coragen concentration 0.02% ranged from 1.13 in the hybrid Rally to 1.87 in the hybrid Belle.

Table 7. Analysis of variance test on the influence of tomato hybrids, length of action and concentration of Coragen insecticide on the number of larvae

Source of variability	SS	d.f	MS	F	p
Concentration(A)	3. 793	1	3. 793	5. 813	0. 017
Insecticide action (B)	575.69	2	287.85	441. 227	0. 000
Hybrid (C)	12.496	2	6.248	9. 577	0. 000
A × B	2.452	2	1.226	1. 879	0. 155
A × C	4.363	2	2.181	3. 344	0. 037
B × C	9.126	4	2.281	3. 497	0. 008
A × B × C	4.726	4	1.181	1. 811	0. 127
Error	164.4	252	0.652		
Total	3304.0	270			

The analysis of variance test (Table 7) showed that the effectiveness of Coragen in laboratory conditions was statistically significantly influenced by all experimental factors (tomato hybrid, duration of action and concentration of insecticide), as well as interactions of tomato hybrid with duration of action and concentration of the applied insecticide. From the analysis of variance test, it can be concluded that, on average, a higher concentration of Coragen resulted in a significantly lower number of larvae, that is, it was more effective. From the results presented in Table 6, it is evident that 24 h after the application of Coragen, a statistically significantly lower number of larvae was registered compared to the moment of application. Also, 48 h after the application of this agent, there was a further drop in the number of larvae, so in this term the statistically significantly lowest number of larvae was registered compared to the other two examination terms. In our research, *Tuta absoluta* showed less resistance to chlorantraniliprole, when a higher concentration was used. Also, research [13] of the *Tuta absoluta* population showed resistance to chlorantraniliprole.

4 Conclusions

From present work, it can be concluded that 48 h after application of Affirm (as. Emamectin benzoate), Runner (as. Methoxyfenozide) and Coragen (as. Chlorantraniliprole) the number of *Tuta absoluta* larvae was significantly lower. The concentration of Affirm and Runner as well as the tomato hybrid had no significant effect on the number of *Tuta absoluta* larvae. However, in the interaction of Coragen with the tomato hybrid, as well as combination the length of Coragen action and the tomato hybrid, there was a significant impact on the abundance of *Tuta absoluta* larvae. The slightly lower effectiveness of the applied insecticides in lower concentrations with different tomato hybrids is probably mainly due to the development of the larvae within the plant tissue, rapid development from egg to adult insect and a large number of *Tuta absoluta* generations.

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The Effect of Pyrophyllite on Yield Components and Accumulation of Nitrate in Lettuce (*Lactuca Sativa* L.)

Ćerima Zahirović Sinanović[1(✉)], Lutvija Karić[1], Admira Halilović[1], Dragan Žnidarčič[2], Senad Murtić[3], and Fikreta Behmen[4]

[1] Department of Vegetable Production, Faculty of Agriculture and Food Science, University of Sarajevo, Zmaja Od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina
c.zahirovic@ppf.unsa.ba
[2] Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia
[3] Department of Plant Physiology, Faculty of Agriculture and Food Science, University of Sarajevo, Zmaja Od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina
[4] Department of Fruits Production, Faculty of Agriculture and Food Science, University of Sarajevo, Zmaja Od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina

Abstract. Lettuce (*Lactuca sativa* L.) plays an important role in human nutrition. It is rich in vitamins, minerals, proteins, amino acids, phenolics, and other bioactive substances. Lettuce also contains a significant amount of natural nitrates which can cause harm to human health if they form carcinogenic nitrosamines. The nitrate concentration in lettuce is influenced by many factors such as climatic conditions, soil chemical properties, and especially fertilizer management. On the other hand, fertilizer management is crucial for lettuce growth, development, yield, and quality. Another significant aspect associated with fertilizer management is the environmental issue. Namely, more than 90% of applied fertilizers may be lost in the environment leading to water, soil, and air pollution. The main goal of this research was to determine whether the use of pyrophyllite ore as a soil conditioner can reduce the use of mineral fertilizers and thus the accumulation of nitrates in lettuce, which are potentially dangerous for human health, without reducing the growth and development of the plant. The experiment was set up in a greenhouse using the split-plot method in three repetitions with variants in which the fertilizer was replaced with pyrophyllite ore in the amount of 25%, 50%, and 75% of the recommended amount of fertilizer, and with a control variant where 100% of the recommended amount of fertilizer was used. The results of this research show that the replacement of mineral fertilizer with pyrophyllite in the amount of 25% and 50% of the recommended fertilization rate in the conditions of the experiment increases the yield of lettuce and improves the quality compared to the control variant, i.e. applying 100% of the recommended amount of mineral fertilizer.

Keywords: Lettuce · Nitrates · Pyrophyllite ore

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1 Introduction

The basis of modern vegetable production is increasing total production and improving food quality. The importance of food is increased by the fact that the natural capacity of the land for food production is limited, while the population is constantly increasing.

The most intensive form of vegetable production is in a protected area with maximum land use and irrigation system used by changing two to three vegetable gardeners during the year. This type of vegetable production, followed by the excessive use of mineral fertilizers and pesticides, gradually leads to land contamination, and consequently to the reduction of production areas and the creation of agricultural products that are questionable for the health of consumers.

The production of lettuce in different types of protected areas in Bosnia and Herzegovina is very significant. Due to the short growing season and poor use of nutrients from organic fertilizers in lettuce cultivation, mineral fertilizers are often applied. Mineral fertilizers are mostly applied in quantities greater than the needs of the cultivated plants, which equally leads to the deterioration of the physical and chemical properties of the soil, the contamination of groundwater, and the deterioration of the quality of the produced vegetables.

The use of mineral fertilizers, especially nitrogen, in the cultivation of lettuce should be strictly controlled. Lettuce is included in the group of leafy vegetables that tend to accumulate nitrates. Nitrates are compounds that often appear in nature, in living and decaying tissues of plants, animals, and humans [1, 2]. They are non-toxic by themselves, while their metabolites nitrites, nitric oxide (NO), and carcinogenic N-nitrosamines, which are formed by combining nitrites with secondary amines, have a potentially harmful effect on human health. The acceptable daily intake (ADI) of nitrate ranges from 0 to 3.7 mg/kg of body weight, and the derived ADI value for nitrites is from 0 to 0.07 mg/kg of body weight [3].

The maximum permissible values of nitrate content in lettuce are legally regulated and amount to 5000 mg NO_3/kg when growing lettuce in a protected area when harvesting from October 1 to March 31, and if the harvest was made in the period from April 1 to September 30, then the nitrate content must not exceed the limit of 4000 mg NO_3/kg lettuce [4]. The amount of nitrates in lettuce depends on a series of factors, among which nitrogen fertilization is the most important.

V. Vukadinović and V. Vukadinović [5] point out that an overdose of nitrogen fertilizers is very dangerous, regardless of whether they are of organic or mineral origin. Such activities have significantly increased the concentrations of accumulated nitrates in plants, primarily through intensive fertilization with nitrogen fertilizers, which replace the traditional use of organic fertilizers. Namely, the accumulation of the easily mobile nitrate form of nitrogen in the soil affects its increased leaching, thus contaminating groundwater and watercourses.

Lettuce is extremely sensitive to phosphorus deficiency. Charles et al., [6] state that the application of mineral fertilizer below the fertilization recommendation can lead to a significant impact on the yield, and therefore on the economic loss of the producer. On the other hand, the same authors claim in their research that lettuce producers introduce enormous amounts of phosphorus into the soil, especially if the production takes place on sandy soil (over 200 kg/ha), while the plant absorbs only 25% of the applied amounts.

This method of intensive fertilization has a negative environmental impact, and excessive exploitation of this fertilizer from ore deposits can lead to its deficit in the future. V. Vukadinović and V. Vukadinović [5] state that phosphorus fertilizers contain a certain amount of radioactive elements (radionuclides).

Considering the above, the intensive production of vegetables in a protected area imposes mandatory land conditioning, which implies maintaining optimal soil fertility in different types of protected areas. Soil with optimal fertility does not have to have a larger reserve of available nutrients, but it must have optimal physical, chemical, and biological properties that, with minimal agricultural techniques, ensure optimal dynamics of the availability of all essential nutrients and water. However, the land in the protected area generally has one or several properties that make it less fertile and require the use of soil improvers. The need to preserve and protect land has stimulated the development of many technological procedures whose goal is to prevent or at least reduce land contamination. One of the newer technological procedures involves the use of aluminosilicates such as bentonite, zeolite, and pyrophyllite. Pyrophyllite is a type of phyllosilicate mineral from the clay family, in the structure of which ions are organized into tightly bound parallel planes that build a three-layer structure. Thanks to its structure and the large contact surface, pyrophyllite has a high ion exchange capacity. It is able to retain a large number of ions needed by the plant for development, preventing their leaching from the zone of the root system, which gives it the properties of a soil improver. According to the above, the processing of pyrophyllite ore can have a positive effect on the availability of nutrients in the soil and enables the use of mineral fertilizers in smaller quantities, which is important in protecting the environment and revitalizing resources to produce health-safe food [7].

The main goal of this research was to determine whether the use of pyrophyllite ore as a soil improver can reduce the use of mineral fertilizers and thus the accumulation of nitrates in lettuce, which are potentially dangerous for human health, without reducing the growth and development of the plant. Certainly, an important goal was to examine whether pyrophyllite ore as a soil improver can increase the absorption of phosphorus and reduce the use of mineral fertilizers without having a negative effect on the productivity of lettuce.

2 Materials and Working Methods

2.1 Materials

Lettuce: (*Lactuca sativa* L.*var Sahngore*), this lettuce hybrid is widely used in production in protected areas in Bosnia and Herzegovina. It is grown from late autumn to spring in greenhouses. It is characterized by fast growth, the possibility of cultivation on different types of land, and the formation of semi-closed heads weighing 400–600 g.

Pyrophyllite: The pyrophyllite material used in this research was created by crushing and sieving pyrophyllite ore from the Parsovići – Konjic site, Bosnia and Herzegovina. Except for silicon dioxide (SiO_2) and aluminum oxide (Al_2O_3), which together make up 80 to 90% of the chemical composition of pyrophyllite, it also contains various other

substances that can greatly affect its properties, especially the effective cation exchange capacity.

The chemical analysis of the pyrophyllite ore used in this research was performed in the laboratory of the Faculty of Agriculture and Food in Sarajevo. The average total content of SiO_2, Al_2O_3, K, Ca, Mg, Cu, Ni, Zn, Co, Mn, Pb and Cr was: 67.55%, 19.10%, 0.3%, 6.65%, 0.14%, 1.40 mg kg^{-1}, 2.74 mg kg^{-1}, 25.68 mg kg^{-1}, 0.4 mg kg^{-1}, 93.14 mg kg^{-1}, 7.97 mg kg^{-1} and 0.76 mg kg^{-1}.

2.2 Study Area

The experiment was carried out in a greenhouse from November 2019 to March 2020 on a family farm in Srebrenik, Bosnia and Herzegovina (44° 41′N, 18° 26′E, and altitude 275 m). Srebrenik is located in the northeastern part of Bosnia and Herzegovina, mostly in the valley of the river Tinja, and according to geographical regionalization, it belongs to the macroregion Peripanno Bosnia. According to Keppen's climate classification, Srebrenik has a moderately warm and humid climate with hot summers (Cfb). The average annual air temperature in the area of Srebrenik is 11.1 °C, with an amount of precipitation of 856 mm, with significant precipitation also in the dry months.

The geographical structure ensures that there are no sudden temperature fluctuations during the year [8]. The land on which the experiment was conducted is classified as district cambisol (A – (B)v – C) according to the FAO land classification. In terms of mechanical composition, it is a loamy, deep soil with a favorable crumbly structure. District Cambisol is characterized by a slightly acidic pH reaction and base saturation of 50%, low to moderate water storage capacity, and medium nutrient storage capacity.

2.3 Soil Sampling and Analysis

Before setting up the experiment, an agrochemical analysis of the soil was performed at a depth of 0–30 cm, and the following parameters were determined: soil pH in water, soil pH in KCl, organic matter content, available phosphorus (P_2O_5) and potassium (K_2O). The pH value of the soil was determined by a pH meter [9], the humus content by the dichromate method [10], and the content of easily accessible potassium and phosphorus by the AL method [11]. A stainless-steel auger was used to take a soil sample.

2.4 Experimental Design and Treatments

Based on the obtained results of soil analysis and the lettuce's nutrient needs, a fertilization recommendation was made, and the same was used as the basis for conducting an experiment in which one part of the fertilizer was replaced with the appropriate amount and granulation of pyrophyllite ore.

For basic fertilization, it is recommended to apply 300 kg ha^{-1} NPK 7:20:30 (nitrogen-phosphorus-potassium). The recommended amount of fertilizer is calculated per the area of one randomized block. The experiment was set up according to a randomized block system in three repetitions. The size of the basic plot was 2 m^2. The experiment was set up according to the method of random block arrangement with four variants in

three repetitions. The area of one block was 3 m^2. The variants of the experiment were as follows:

T1 – optimal fertilization with mineral fertilizers without the addition of pyrophyllite ore, control (150 g NPK 7:20:30 for each repetition).
T2 – 75% of the recommended amount of mineral fertilizers + 25% pyrophyllite ore with 5 mm granulation (112.5 g NPK 7:20:30 + 37.5 g pyrophyllite ore).
T3 – 50% of the recommended amount of mineral fertilizers + 50% pyrophyllite ore with 5 mm granulation (75 g NPK 7:20:30 + 75 g pyrophyllite ore).
T4 – 25% of the recommended amount of mineral fertilizers + 75% pyrophyllite ore with 5 mm granulation (37.5 g NPK 7:20:30 + 112.5 g pyrophyllite ore).

Application of mineral fertilizer and pyrophyllite ore was carried out after the surface treatment of the land, a few days before planting on 20.11.2019. A "drop by drop" irrigation system is placed under the film. Also, sprinklers were installed in the greenhouse to irrigate the lettuce by raining. All other agrotechnical measures necessary for the optimal growth of lettuce (irrigation, protection against diseases, and pests) were carried out on all experimental blocks until the technological maturity of the lettuce (March 15, 2020).

2.5 Analysis of Lettuce Yield and Quality

At the technological maturity of the lettuce, samples were collected for the analytical determination of nitrate and phosphorus content. A total of 40 heads of lettuce were collected for each variant and the control (10 heads from each repetition).

Individual samples were collected following the pattern in the shape of the letter "X" through the sample square. The plants that were selected were cut at ground level. Soil and outer leaves were removed before packing the samples. The collection of samples was carried out according to the Rulebook on sampling and analysis methods for the official control of the amount of nitrates in food [4].

Nitrate content in lettuce was measured on a DR/2000 HACH device using the NitraVer 5 reagent. This method represents a modification of the cadmium reduction method using Gentisic acid instead of 1-naphthylamine [12].

Phosphorus content in lettuce was measured on a DR/2000 HACH device using the PhosVer3 phosphate reagent. The PhosVer 3 method creates a blue color with phosphate. The indicator is combined with the reducing agent ascorbic acid in a powder formulation called PhosVer 3 Phosphate Reagent [12].

2.6 Statistic Data Processing

The obtained results were processed using the statistical method of analysis of variance with the use of the SPSS software package. The significance of the differences between the average values of the tested variants and the control variant was determined using the Dunnett t-test at the significance level of $p < 0.05$.

3 Results and Discussion

The examination of basic parameters of soil fertility (Table 1) showed that the examined soil had a slight acid reaction, moderate level of organic matter (OM), and high content of the available form of phosphorous (P_2O_5) and potassium (K_2O).

Table 1. The results of soil chemical analysis

Parameter	Unit	Measured value	Recommended values for vegetable production*
pH H_2O	pH unit	5.80	5.70–7.20
pH KCl	pH unit	5.20	5.20–6.70
OM	%	3.83	3.00–5.00
P2O5	mg 100 g^{-1}	15.10	12.00–16.00
K_2O	mg 100 g^{-1}	31.30	25.00–35.00

* Values reported by [13].

Based on the obtained results we can conclude that the tested soil is suitable for vegetable production.

The results of the analysis of yield and yield contributing parameters of lettuce depending on the experimental treatment are given in Table 2.

Table 2. Yield components of lettuce

Treament[1]	The weight of the formed head (kg)	Percentage of waste leaves (%)	Market return (kg/m^2)
T1	0.43 ± 0.50	6.19 ± 0.68	6.57 ± 0.65
T2	0.60 ± 0.08*	5.74 ± 0.92	9.02 ± .75*
T3	0.57 ± 0.08*	5.36 ± 1.10	8.77 ± 0.40*
T4	0.43 ± 0.09	4.60 ± 6.16	6.50 ± 1.32

[1]Experimental treatment: T1 – recommended fertilizer rate (RFR) without pyrophyllite (PP), i.e. control treatment; T2 – 25% of RFR was replaced with PP; T3 – 50% RFR was replaced with PP; T4 – 75% of RFR was replaced with PP

* Main difference at the significance level of 0.05

Values marked with the symbol * indicate that there is a statistically significant difference compared to control variant T1

From the data shown in Table 2, the application of pyrophyllite ore in any variant did not negatively affect the weight of the formed head of cultivated lettuce in comparison to the control, moreover, the weight of the head of lettuce, and therefore the yield, were higher in the variants where pyrophyllite ore was applied as a substitute for part of the fertilizer. The highest weight of formed heads, and thus the yield, was achieved in variants

T2 where the ratio of fertilizer and pyrophyllite ore was 75%: 25%. Only the treatment in which pyrophyllite ore was applied as a substitute for 75% of the recommended amount of fertilizer did not show a positive effect on the weight of the head of lettuce.

The greater weight of formed heads of variant T2 can be explained by the specific structure of pyrophyllite, which, thanks to its three-layer structure, has a high ion exchange capacity and is able to retain a wide range of cations needed and available to the plant for development on its boundary surface, which gives this mineral the properties of a breeder. In terms of its structure, pyrophyllite is very similar to clay minerals that have the properties of colloids that are characterized by the possibility of adsorption of various substances [14–16]. We assume that the lettuce in treatments where pyrophyllite was applied as a majority replacement for fertilizer (T3 and T4) had less amount of available nutrients in the soil resulting in reduced yields.

The energy of adsorption of nutrients that are in ionic form depends on their ion hydration valence. If the valency is higher, the adsorption energy is also higher. Potassium (K), calcium (Ca), and magnesium (Mg) have lower adsorption energy compared to elements with a higher valency, i.e. heavy metals (Fe, Cu, Zn, Mn, Cd, Co, Pb, and Cr). Higher atomic mass and weaker hydration lead to higher adsorption energy [17].

Pyrophyllite ore contains a certain amount of magnesium, a high proportion of readily available phosphorus, calcium, and microelements (Mn, Zn, Fe), and thus in itself contributes to better plant nutrition, and thus plant growth and the formation of a higher yield. Accordingly, the application of NPK mineral fertilizer and pyrophyllite ore in a ratio of 75%:25% gives the plants a sufficient amount of nutrients, but with the help of pyrophyllite, the efficiency of its uptake by the plant increases at the same time.

The outer leaves of lettuce that are damaged, diseased, and overgrown are waste leaves, which must be removed after harvesting and before being offered on the market. The lowest percentage of waste leaves was in variant T4, where the ratio of fertilizer and pyrophyllite ore was 25%: 75%, while the highest percentage of waste leaves was recorded in variant T1, in which optimal fertilization with mineral fertilizers was applied without the addition of pyrophyllite ore.

However, the smaller amount of waste leaves of the variants in which pyrophyllite ore was applied was not statistically significant. Based on the obtained results, we can conclude that the variants in which pyrophyllite ore was applied in larger quantities had a positive effect on the reduction of the % of waste leaves in lettuce. This can be explained by the chemical composition of pyrophyllite ore, which contains considerable amounts of calcium (6.65%).

It should be emphasized that calcium is an essential element that participates in the construction of Ca pectinate in the cell walls and in the membrane components of the plasmalemma. Its role is very important in the structure of the cell because it affects the strength of the cell wall, and therefore the resistance of plants to mechanical damage, diseases, and pests.

Taking into consideration that the waste leaves consist of damaged and diseased leaves, we can conclude that pyrophyllite has a positive effect on the reduction of the process of leaf decay, and therefore on the reduction of the % of waste leaves in relation to the mass of the formed head.

The market yield is very important for producers, it mostly depends on the percentage of waste leaves. The highest market yield on average was achieved by the T2 variants, where 75% of the recommended amount of mineral fertilizers was applied, while 25% was replaced by pyrophyllite ore with granulation of 5 mm, and the T3 variant, where 50% of the recommended amount of mineral fertilizers was applied, and 50% was replaced by 5 mm granulation pyrophyllite. The market yield of the control variant T1 (6.57 ± 0.65) was statistically significantly different in relation to the treatments T2 (9.02 ± 0.75) and T3 (8.77 ± 0.40), while in relation to the treatment T4 (6.50 ± 1.32) differences were observed due to chance.

This result indicates that the application of pyrophyllite in greenhouses, which is characterized by a large amount of nutrients remaining in the soil, gives a positive effect even when only 25% of the recommended amount of mineral fertilizers is applied, while the rest is replaced with pyrophyllite ore. This indicates that pyrophyllite ore, thanks to its high calcium content (6 .65%) has a positive effect on the strength of the leaves, reducing their deterioration, thus increasing the market yield.

Table 3. Content of NO_3^-, P, PO_4^-, in fresh lettuce leaves

Treatment[1]	Content NO_3 (mg/kg fresh leaves)	Content P, PO_4· (mg/100 g fresh leaves)
T1	791.66 ± 7.63	29.10 ± 1.17
T2	726.66 ± 25.16	27.46 ± 0.86
T3	463.33 ± 55.07*	26.13 ± 2.28
T4	392.00 ± 10.58*	19.10 ± 1.96*

[1]Experimental treatment: T1 – recommended fertilizer rate (RFR) without pyrophyllite (PP), i.e. control treatment; T2 – 25% of RFR was replaced with PP; T3 – 50% RFR was replaced with PP; T4 – 75% of RFR was replaced with PP

* Main difference at the significance level of 0.05

Values marked with the symbol * indicate that there is a statistically significant difference compared to the control variant T1.

The highest content of nitrates was the variant T1 (control variant) with an average of 791.66 mg NO_3 in one kg of fresh leaf, and the lowest variant T4 with an average of 392 mg in one kg of fresh leaf (Table 3). The obtained results indicate that the use of pyrophyllite ore as a substitute for part of the mineral fertilizers has a positive effect on the reduction of nitrate accumulation in lettuce. Also, the presented results show that the lettuce produced in this research work had a significantly lower amount of nitrates than the upper limit allowed by the B&H rulebook [4].

Based on the presented research results (Table 3), variant T4, in which only 25% of the recommended mineral fertilizers were applied (the rest was replaced by pyrophyllite ore), adopted the smallest amount of phosphorus. This can be explained by the fact that phosphorus is a poorly mobile plant nutrient in the soil, and that the application of mineral fertilizer at only 25% of the fertilization recommendation significantly reduces the possibility of adopting this nutrient. On the other hand, if we replace 50% of the recommended dose of mineral fertilizer with pyrophyllite, we will improve the absorption of

phosphorus, reduce the excessive intake of mineral fertilizer and achieve a high yield. Mukherjee, [18] states that the increase in crop yield due to the addition of pyrophyllite to soil is due to its ability to release tightly bound phosphates already present in the soil making them available to plants.

4 Conclusions

Based on the results achieved in the conditions of the experiment, the application of pyrophyllite material from the Parsovići – Konjic site reduced the use of mineral fertilizers without negative effects on the yield of the grown lettuce.

This approach to fertilization with reduced use of mineral fertilizers makes an immeasurable contribution to environmental protection. The obtained results indicate that the application of pyrophyllite in the appropriate ratio with mineral fertilizer can increase the yield without proportionally increasing the accumulation of nitrates in lettuce leaves, which is reflected in the quality of the lettuce.

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The Use of Pyrophyllite for the Purpose of Remediation of Soil Contaminated with Heavy Metals in the Industrials Zone of Kakanj

Emina Sijahović[1(✉)], Hamdija Čivić[1], Senad Murtić[1], and Biljana Dojčinović[2]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
e.sijahovic@ppf.unsa.ba

[2] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 12, 11000 Beograd, Serbia

Abstract. From the large number of pollutants that enter the soil throughhuman activity, special attention should be paid to heavy metals due to their toxic effects on the environment. The toxic effects of heavy metals manifest themselves through the pollution of land and water systems. Through plants they enter the food chain and manifest their harmful effects on human health. The pyrophyllite as aluminosilicate material can potentially be used as a remedial measure because it reduces the presence of heavy metals in the soil, and thus its absorption into the plant. The location of the field experiment is in the immediate vicinity of the thermal power plant and the cement factory in Kakanj, which are among the biggest environmental polluters in the Zenica-Doboj Canton. Pyrophyllite particles with a size of 100 μm were added in the soil of potato field in amounts of 200, 400 and 600 kg ha^{-1}, in three repetitions. Accessible forms of heavy metals from soil samples and potato leaves were extracted with EDTA solution (0.01 mol dm^{-3} C10H16N2O8 and 1mol dm^{-3} (NH4)2CO3, adjusted to pH 8.6), and their concentrations in the obtained extract were determined by the method of atomic absorption spectrophotometry. In the conditions of the conducted experiment, the application of pyrophyllite, regardless of the applied dose, reduced the availability of Zn, Mn, Cd and Co in the tested soil.

Keywords: heavy metals · pyrophyllite · remediation

1 Introduction

With the level of human development (infrastructure, traffic, industry), the level of development of society also increases, but at the same time, the negative impact of humans on the environment also increases.

Establishing a balance between industrialization and urbanization on the one hand and environmental protection on the other is the main challenge for all modern societies. And protecting the soil quality is certainly one of the foundations on which this balance is based on [25, 26].

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Of the large number of pollutants that reach the soil due to human activity, heavy metals should be given special attention due to their toxic effects on the environment.

Because of these facts, the preservation of fertile and remediation of agricultural areas contaminated with heavy metals is simply imposed as a necessity if one wants to protect soil as a primary resource for food production [13].

All heavy metals tend to accumulate in the organism of plants and humans, which can consequently lead to toxic effects on their health.

In addition to the content and form of heavy metals in the soil, the availability of heavy metals to plants also depends on the type of soil, its chemical and physical properties, and genetic properties of the cultivated plant [20, 24, 27]. The accessibility of heavy metals to the plant, and thus its harmful effects, are most influenced by the pH value, and the capacity and state of saturation of the soil adsorption complex [5]. It is considered that the pH value of the soil is the key factor on which the uptake of heavy metals from the soil into the plant depends and that decreasing the pH value increases the accessibility of heavy metals [18, 22].

Given that conventional methods of environmental heavy metals remediation are very expensive and destructive in terms of soil composition, structure, fertility and biological diversity, and that they are limited to relatively small areas [21, 24], it is necessary to develop innovative environmental rehabilitation technologies that would be effective but also economical.

2 Material and Method

The Zenica-Doboj Canton (ZDK) occupies central and northeastern part of Bosnia and Herzegovina. Its total area is 3343.3 km^2. The largest part of ZDK stretches along the Bosna river valley, along the course of which seven cities are located within this canton: Visoko, Kakanj, Zenica, Žepče, Maglaj, Zavidovići and Doboj-jug. The relief of the ZDK in the southern hilly part stretches from Visoko to Zenica, following the course of the Bosna River. This stream is characterized by a moderately continental climate, characterized by warm summers and cold winters with moderate amounts and an even distribution of precipitation.

The special feature of ZDK, more precisely its lithosphere, is the wealth of coal, ores and minerals, which was the basis for the development of mining, metallurgy and metalworking industry in this area. In the area of ZDK, a large part of the population is engaged in agricultural production, mostly as an additional activity. This canton is also characterized by a large network of roads with a high frequency of traffic. Taking into account all the above facts; the concentrations of minerals in the lithosphere, and the strong development of the metal processing industry accompanied by the emission of fumes and dust, it can be assumed that the lands in this area, especially in the area of the municipality of Kakanj, where the industry is widespread, are more or less polluted, and thus also risky from the aspect of use in the production of healthy food.

Thermal power plant Kakanj (TEK) uses coal to generate electricity and is one of the biggest polluters of the environment in Bosnia and Herzegovina. Environmental pollution in the vicinity of TEK is primarily attributed to high concentrations of carbon dioxide (CO2), sulfur dioxide (SO2), nitrogen oxide (NO) and heavy metals resulting from coal combustion.

The release of heavy metals from TEK and their subsequent deposition in the soil is a growing problem for the social community, especially for agricultural producers whose agricultural production is prevented or at least limited due to soil pollution.

Soil treatment with aluminosilicate materials, which have been proven to have a positive effect on the immobilization of heavy metals in the soil, is certainly one of the procedures that could contribute to the achievement of this goal, which is why it was also used as part of this research.

In this research, the influence of the aluminosilicate material pyrophyllite on the content and accessibility of heavy metals in potato leaves on the plots near the thermal power plant in Kakanj is presented.

The first phase of this research was carried out in the period from 1st of March to 1st of April 2019, and its focus was the determination of the current state of heavy metals soil pollution of a plot and the land near the thermal power plant in Kakanj (Zenica-Doboj canton).

The second phase of this research was conducted in the period from 1st of April to 15th of April 2019. Its focus was the analysis of the content of total and accessible forms of heavy metals (Cd, Cr, Pb, Zn, Cu, Mn, Fe, Ni) in the collected average soil samples from the investigated locality. As part of this phase of the research, the type of land on each tested plot was determined, as well as the basic parameters of fertility (pH, humus content, carbonate content, and the content of accessible forms of phosphorus and potassium). The analyzes were performed in the laboratory of the Faculty of Agriculture and Food Sciences, University of Sarajevo.

The third phase of the research included the application of remedial measures on the selected plot of land, i.e. the application of the aluminosilicate material pyrophyllite.

A random block experiment with 4 variants in 3 repetitions was set up on the examined land. The variants of the experiment were as follows:

1. Variant (control; without addition of pyrophyllite).
2. Variant (application of 200 kg/ha of pyrophyllite with a granulation of 0–3 mm).
3. Variant (application of 400 kg/ha of pyrophyllite with a granulation of 0–3 mm).
4. Variant (application of 600 kg/ha of pyrophyllite with a granulation of 0–3 mm).

All the mentioned quantities were recalculated according to the area of the sample plots (the sample plots had an area of 20 m^2, with a 1 m wide distance between all plots to minimize the influence of one variant on another). Seven days after setting up the experiment, potatoes were sown on the tested plot, with the aim of determining whether the application of aluminosilicate materials affected the uptake of heavy metals by the tested plant. When plant reached its technological maturity, sampling of plant material and soil was carried out on the experimental plots. Heavy metals content was tested in the plant material (potato leaf), as well as the content of accessible forms of heavy metals in the soil.

The aluminosilicate material pyrophyllite was used for the chemical stabilization of soil contaminated with heavy metals. The culture on which the influence of pyrophyllite was monitored was the early variety of Agria potato.

Pyrophyllite is a natural mineral from the group of aluminosilicates with the chemical formula Al2[Si4O10](OH)2, and it is characterized by the following properties: hardness is between 1 and 1.5 on the Mohs scale, density between 2.7 and 2.9 g/cm^3,

it is hydrophobic, alkaline reactions (pH between 7.5 and 8.5). It is soft, visually very similar to talc, and its color varies depending on the proportion of oxides and other components in the pyrophyllite ore, but as a rule it is white to gray-white in color. According to available data from the literature, pure pyrophyllite consists of 60.0–67.6% SiO2 and 28.3–28.9% Al2O3 [2, 4]. The elementary cell of this mineral is electroneutral and therefore does not show any colloidal abilities [33].

The pyrophyllite material used in this research was created by crushing and sieving pyrophyllite ore from the Parsovići – Konjic site, Bosnia and Herzegovina. Except for silicon dioxide (SiO2) and aluminum oxide (Al2O3), which together make up 80 to 90% of the chemical composition of pyrophyllite, it also contains various other impurities that can greatly affect its properties, especially the effective cation exchange capacity. The chemical analysis of the pyrophyllite material used in this research was done in the laboratory of the Faculty of Agriculture and Food Sciences, University of Sarajevo, and the obtained results are presented in Table 1.

Table 1. Chemical analysis of pyrophyllite used in this research

Tested parameter	Unit	Value
pH (H2O)	pH	8.5
pH (KCl)	pH	8.1
SiO2	%	67.55
Al2O3	%	19.10
Ca	%	3.65
K	%	0.023
Mg	%	0.135
Fe	%	0.337
Cu	mg/kg	1.40
Ni	mg/kg	2.74
Zn	mg/kg	25.68
Co	mg/kg	0.40
Mn	mg/kg	93.14
Cd	mg/kg	n.d.*
Pb	mg/kg	7.97
Cr	mg/kg	0.76

* n.d. – not detected (below the detection limit of the instrument)

The results from Table 1 show that the pyrophyllite used in this research does not deviate significantly from the basic chemical characteristics of pyrophyllite ores, except for the fact that it contains a relatively high proportion of calcium and magnesium. It can be assumed that the pyrophyllite ore from the Parsovići – Konjic site contains a certain amount of dolomite or some other natural material rich in the mentioned elements. A

positive characteristic of the pyrophyllite material used in this research is the fact that it contains very low concentrations of toxic heavy metals, which makes it environmentally acceptable for use in remedial purposes.

The work methods used in this research were as follows:

- a pH meter [14] was used to determine the pH value of the soil,
- humus content in soil samples was determined by the dichromate method [15],
- the content of easily accessible forms of potassium and phosphorus in soil samples was determined by the ammonium lactate (AL) method [9].
- the extraction of the total forms of heavy metals from the soil was done with the use of gold dust (a mixture of nitric and hydrochloric acid in a ratio of 1:3) according to the ISO 11466 [17] method,
- the extraction of accessible forms of heavy metals from the soil was done using the so-called EDTA-solutions (a mixture of 1 M (NH4)2CO3 and 0.01 M EDTA (ethylene-diamino-tetraacetic acid) where the pH of the solution was adjusted to 8.6 using HCl or NH4OH),
- the extraction of heavy metals from plant material was done with the use of nitric and sulfuric acid (in a ratio of 2.5:1) according to the method specified in the practicum of Lisjak et al. [23],
- the determination of the content of heavy metals in plant material samples, and the content of total and accessible forms of heavy metals in soil samples was performed using the method of flame atomic absorption spectrophotometry on a Shimadzu 7000 AA apparatus [16].

3 Results and Discussion

The object of the work was a plot of land located in the immediate vicinity of the thermal power plant (44°5′26″N, 18°6′51″E). The type of land on the selected plot was rendzina, which was determined by visual observation of the pedological profiles made on the plots, and additionally confirmed by the review of the Pedological Map of Bosnia and Herzegovina 1:50000 authored by the Federal Institute for Agropedology in Sarajevo.

The average content of total forms of heavy metals in the tested land plots are shown in Table 2.

Table 2. Results of the analysis of the content of heavy metals in the tested soil

Content of heavy metals (mg/kg soil)								
E*	Cu	Zn	Mn	Cd	Pb	Ni	Cr	Co
V*	41.99	56.24	1091.06	0.17	64.60	127.89	16.21	13.58
LV*	65	150	-	1	80	50	80	45

E* – Elements; V* – Value; LV* – Limit value for the content of heavy metals for soils in Federation B&H above which the soil can be considered polluted [30].

Before carrying out the process of chemical stabilization of the tested land using aluminosilicate materials, the degree of its contamination with heavy metals was determined. For these purposes, the Rulebook on determination of permitted amounts of harmful and dangerous substances in soil for the Federation of Bosnia and Herzegovina, and the classification of soil pollution according to Bašić [3] were used.

According to this classification, the tested land is included in class III, i.e. heavily polluted with heavy metal contamination (land that belongs to this class can be used for growing plants, but enhanced protection measures are required).

3.1 Results of the Analysis of the Basic Fertility Parameters of the Tested land near the Thermal Power Plant in Kakanj

The results of the analysis of the basic parameters of soil fertility (pH, humus content, carbonate content, and the content of accessible forms of phosphorus and potassium) on the plot that was used to carry out the procedure of chemical stabilization of the soil are shown in Table 3.

Table 3. Presentation of the results obtained from the chemical analysis of the tested soil

Parametre	Unit	Measured value	Soil characteristic
pH (H2O) pH (KCl)	- -	7.2 6.9	nutral
Humus	%	3.63	moderately
Phosphorus(P2O5)	mg $100g^{-1}$	2.05	low
Potasium (K2O)	mg $100g^{-1}$	22.7	optimal
Carbonates	%	11.9	very high

The results of the analysis showed that the tested soil is moderately humus with a neutral reaction, which represents a favorable environment for the cultivation of most agricultural crops. The content of accessible forms of phosphorus in the tested soil was very low, while the soil was optimally provided with an accessible form of potassium. With the aim of determining whether the differences in the content of accessible forms of heavy metals between the examined variants were statistically justified, an analysis of variance was performed, and the obtained results are shown in Table 4.

Soil treatment with pyrophyllite in all used doses had a statistically significant effect on the reduction of Zn, Mn, Cd and Co availability in the soil. An interesting detail is that the doses of pyrophyllite 400 kg/ha and 600 kg/ha were not statistically significantly different from each other in terms of their influence on the accessibility of the mentioned elements.

When it comes to Pb, only the application of pyrophyllite in doses of 400 kg/ha and 600 kg/ha had a significant impact on reducing its availability in the soil. All other soil treatments with aluminosilicate materials did not show a statistically significant effect on the reduction of Pb availability in the soil.

Table 4. Results of testing the differences significance between the environments for the content of accessible forms of heavy metals in the tested soil near the thermal power plant in Kakanj

Variant*	Content of accessible forms of heavy metals in soil (mg/kg)							
	Cu	Zn	Mn	Cd	Pb	Ni	Cr	Co
V1 (control)	3.89	2.11[a]	7.56[a]	0.050[a]	2.66[a]	0.90	0.069	n.d
V2(P200)	3.62	1.61[e]	6.13[b]	0.034[d]	2.51[a]	0.86	0.070	n.d
V3 (P400)	3.48	1.33[f]	5.29[c]	0.028[f]	2.43[b]	0.85	0.071	n.d
V4 (P600)	3.49	1.32[f]	5.55[bc]	0.029[f]	2.27[b]	0.81	0.072	n.d
LSD0.05	-	0.162	0.708	0.0037	0.168	-	-	-
LV*	65	150	-	1	80	50	80	45

* V1 – Control (untreated variant); V2 – Pyrophyllite 200 kg/ha; V3 – Pyrophyllite 400 kg/ha; V4 – Pyrophyllite 600 kg/ha; n.d. – not detected; LV* – Limit value for the content of heavy metals for soils in Federation B&H above which the soil can be considered polluted[30]

3.2 Results of the Analysis of the Content of Heavy Metals in Potato Leaves Grown on Polluted Land Near the Thermal Power Plant in Kakanj

The content of Mn, Cd, Pb and Ni in potato leaves was always higher in the control (untreated) variant compared to the variants where aluminosilicate material was used, which points to the conclusion that the treatment of soil with pyrophyllite had a positive effect on reducing the accumulation of the mentioned elements in potato leaves.

Unlike the previously mentioned elements, the accumulation of Cu, Zn, Cr and Co in potato leaves was not always lower in the variants where the land treated with pyrophyllite was observed in relation to the untreated (control) variant.

To determine the statistical significance of the influence of pyrophyllite on the accumulation of heavy metals in potato leaves, the F test was performed, and the significance of the differences between the environments for the content of those heavy metals in which the F test proved significant. The obtained results are shown in Table 5.

The treatment of soil with pyrophyllite in a dose of 200 kg/ha also did not significantly affect the reduction of the content of any of the tested elements in the leaves of potatoes grown on the tested soil. Soil treatment with pyrophyllite at a dose of 400 kg/ha had a significant effect on the reduction of Cu, Zn, Mn, Ni and Cr content, while at a dose of 600 kg/ha it also significantly affected the reduction of Co in potato leaves, compared to the untreated (control) variant.

None of the soil treatments with pyrophyllite contributed to the reduction of Cd accumulation in potato leaves. This data was not expected if we consider the fact that soil treatments with aluminosilicates significantly affected the reduction of Cd availability in the soil. The assumption is that the adopted potato mostly accumulates Cd in the roots of the plant. This results in its lower transport into the leaves of the plant, and therefore there are fewer opportunities to determine the differences in the influence of the used aluminosilicates on the accumulation of Cd in the plant if the test parameter is the Cd content in leaves. Determination of Cd content in roots would be a more appropriate

Table 5. Results of testing the differences significance between environments for the content of heavy metals in the leaves of potatoes grown on the tested land near the thermal power plant in Kakanj

Variant	Heavy metals content in potato leaves (mg/kg of dry matter)							
	Cu	Zn	Mn	Cd	Pb	Ni	Cr	Co
V1(cont)	15.06[a] b	45.87[a] b	164.62[a]	0.27 9	6.39 a	12.27[a]	0.142[a] b	1.88[ab] c
V2 (P200)	15.92[a]	40.94[b] c	157.99[abc] d	0.26 8	6.12 a	12.08[ab] c	0.143[a]	1.91[ab]
V3 (P400)	12.04 d	35.86[c] d	133.62[e]	0.26 0	5.95 a	7.32[d]	0.101[d]	1.97[a]
V4 (P600)	11.85 d	28.53[e]	61.36[f]	0.22 6	6.01 a	5.29[e]	0.095[d] e	1.39[e]
LSD0.05	2.68	5.368	15.55	-		1.522	0.026	0.178

V1 – Control (untreated variant); V5 -Pyrophyllite 200 kg/ha; V6 – Pyrophyllite400 kg/ha; V7 – Pyrophyllite 600 kg/ha.

parameter for these purposes. Subhashree et al. [32] in their research came to conclusion that 75% of Cd and 98% of Pb can be removed from an acidic environment within the first 30 min of shaking wastewater samples. They stated that the adsorption capacity of Cd (II) and Pb (II) decreases with an increase in particle diameter, and because of this, smaller particle sizes have larger adsorption power. Janjićijević et al. [19] investigated the potential use of pyrophyllite for wastewater treatment and, and noted that Pb is best absorbed by pyrophyllite, and Cd and Zn were least absorbed.

Pyrophyllite has other uses in agriculture. Govedarica-Lučić et al. [12] noted the increase in yield and dry matter in cabbage grown on soil to which pyrophyllite was added, and Adamović et al. [1] concluded that the addition of pyrophyllite with a granulation of 100 μm to corn silage leads to the appearance of better organoleptic properties (color and smell), reduction of the content of milk and increasing the content of acetic acid, and decreasing the number of aerobic bacteria and yeasts.

The addition of pyrophyllite to the substrate generally leads to a decrease in the availability of heavy metals for plants [6, 7, 11, 28].

The use of clay minerals has proven to be effective for chromium immobilization [8, 10].

4 Conclusion

In the conditions of the experiment, the application of pyrophyllite in a dose of 200 kg/ha reduced the accessibility of Zn by 23.7%, Mn by 18.9%, Cd by 32.0%, and Co by 37.3% in the tested soil, in a dose of 400 kg/ha it reduced accessibility of Zn by 37.0%, Mn by 30.0%, Cd by 44.0% and Co by 66.7%, and in a dose of 600 kg/ha it reduced accessibility of Zn by 37.4%, Mn by 26.6%, Cd by 42.0% and Co by 70.5%. Application

of pyrophyllite in a dose of 400 kg/ha had a significant effect on the reduction of Cu, Zn, Mn, Ni and Cr content, while in a dose of 600 kg/ha it had asignificant effect on the reduction of Co in potato leaves.

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Spontaneous Flora of Urban Domestic Gardens of the City of Sarajevo

Nermina Sarajlić[1](✉), Nejc Jogan[2], and Vladimir Ranđelović[1]

[1] Department of Biology and Ecology, Faculty of Sciences and Mathematics, Niš, Serbia
nermina.sarajlic@ptice.ba
[2] Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Abstract. Gardens around private houses are complex urban habitats, and generally consist of a mosaic of different microhabitats including hedges, paved surfaces, lawns, flowerbeds, fruit trees, vegetable patches and areas of uncultivated land. Although their individual size is small, domestic gardens significantly contribute to the overall flora of the urban areas. The floristic composition of domestic gardens is influenced by both natural processes and by the activities of the owners, who shape them according to their own preferences, depending on culture and lifestyle. Domestic gardens have not been represented in ecological studies until recently, primarily due to lack of access and were deliberately omitted, but it has been showed that they represent the greatest source of potentially invasive alien plants. This study, conducted in the area of 32 km^2, as a part of a wider study of the urban flora of the city of Sarajevo, presents the first detailed analysis of the spontaneous flora in the domestic gardens and provides new knowledge on the flora of this, often overlooked, habitat.

Keywords: Domestic Gardens · Sarajevo · Floristic Composition · Urbanization

1 Introduction

The term domestic gardens (home gardens, backyard gardens, kitchen gardens, dooryard gardens, household gardens) refers to small patches of ground, associated with residential (owned or rented) homes, often delimited from their surroundings by hedges, walls, fences or other barriers [1]. Domestic gardens are complex habitats, and generally comprise paved paths and patios, lawns, flowerbeds, vegetable patches, various shrubs and trees, and in some cases a pond or fountain, a greenhouse, as well as uncultivated and neglected grounds, and a composting area [2, 3].

Although each garden individually occupies a small area, the total contribution of this type of habitat to the total green areas of the city can be substantial and constitute 16–36% of the total urban area [2, 4–7]. Species diversity in domestic gardens is often determined by a combination of environmental, cultural and socio-economic factors [8–10], and is directly influenced by the owners, who shape them according to their own discretion depending on culture, lifestyle and aesthetic preferences [11, 12]. Domestic gardens contribute significantly to the total number of plant taxa in urban environments,

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and are also a key site for introduction of non-native plants, including the invasive taxa [2, 13, 14].

Generally, horticultural practice promotes the use of low-maintenance plants [11], and is the main pathway for the introduction of alien taxa. Ornamental plants comprise more than 40% of widespread invasive plant taxa [15]. As domestic gardens are the places where alien taxa that escaped cultivation often occur first, the study of spontaneous domestic garden floras is interesting with respect to potential spread of alien taxa. Since most domestic gardens are located in the peripheral parts of the urban areas, close to the natural vegetation, there is a high rate of species exchange between gardens and their surroundings [16], and acquiring knowledge about the floristic composition of the domestic gardens could anticipate the spread of alien taxa and changes in biodiversity of natural plant communities. Domestic gardens are also regarded as an important habitat for managing and conserving biodiversity, including rare, endangered or endemic taxa [7, 8].

Private gardens have not been represented in ecological studies until recently [2, 17–19], primarily due to lack of access and were deliberately omitted in surveys of urban floras. The study of home gardens was initiated in the tropics of South East Asia [20], and most available data are from tropical Asia and Central America [8, 10, 21–26] and Africa [27–29], but these are mainly focused on role of domestic gardens as sustainable agroforestry systems.

To our knowledge, home-gardens in cities have not been systematically studied in Bosnia and Herzegovina, and this paper attempts to describe and analyze the flora of domestic gardens from an urban environment, with focus on alien taxa and taxa of conservational interest. We also analyzed if the floristic similarity between gardens changes according to the urbanization gradient (i.e. with the distance from the center of the urbanized area).

2 Materials and methods

2.1 Surveyed Area

The survey was performed from the summer of 2015 to the autumn of 2021, in the gardens of private, owner-occupied dwellings in the urban part of the city of Sarajevo (Fig. 1), in an area of 32 km^2 within the administrative boundary of the city, in 1 × 1 km grid cells with more than 25% coverage by residential or industrial zones [2, 3]. The plants were recorded by surveying the entire domestic garden area, where it was possible, or by observing the area from the street or nearby path, if the access to the garden was not granted by the owners. The survey was carried out using an ad-hoc method, which consists in examining the site until it is subjectively concluded that no more species can be found there [30]. Only the spontaneous flora (native taxa, and aliens that were observed growing as seedlings, in a places outside the ones where they were originally planted or sown) was recorded. Cultivars were not considered as separate taxa.

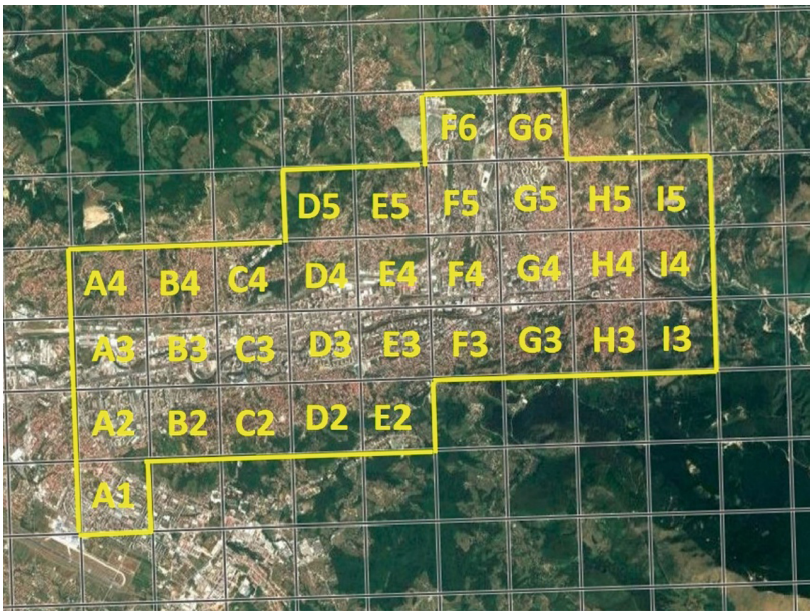

Fig. 1. Surveyed area

2.2 Data Analyss

The nomenclature follows the Euro + Med PlantBase [31]. The data on the life forms is taken from Flora of Italy [32, 33], with categories based on the classification of Raunkiaer [34].

The taxa were classified into floral elements [35], and assigned alien or native status [36, 37]. Alien taxa were further classified according to their residence time, mode of introduction and geographic origin [37, 38], as well as the degree of naturalization [38–40]. The native taxa were assigned endemic [41], and conservation status [42]. The data on the affinity towards the conditions that prevail in urban environments were taken from the BiolFlor database [43].

The scope of anthropogenic changes of the flora was assessed using the indicators based on proportions between individual geographical-historical groups[44–46]:

1. Indicators of anthropization.
   1.1. Indicator of total anthropization, IAnt = An/(Sp + An) × 100;
   1.2. Indicator of permanent anthropization, IAnp = Mt/(Sp + Mt) × 100;
2. Indicators of archaeophytization.
   2.1. Indicator of total archaeophytization, IArt = Ar/(Sp + An) × 100;
   2.2. Indicator of permanent archaeophytization, IArp = Ar/(Sp + Mt) × 100;
3. Indicators of kenophytization.
   3.1. Indicator of total kenophytization, IKnt = Kn/(Sp + An) × 100;
   3.2. Indicator of permanent kenophytization, IKnp = Kn/(Sp + Mt) × 100.
4. Indicator of flora modernization, IM = (Kn/Mt) × 100;
5. Indicator of fluctuation changes, IF = Df/(Sp + An) × 100;

where An is number of alien taxa; Sp – number of native taxa; Mt – number of permanently established alien taxa (Ar + Kn – Df), Ar – number of archaeophytes, Kn – number of neophytes, Df – number of casual alien taxa.

To examine the floristic similarity along the urbanization gradient, similarity of 1 × 1 km squares was calculated based on the Bray-Curtis cluster analysis using the PAST software [47].

3 Results and Discussion

During this research, a total of 505 species of 296 genera and 76 families were recorded. The most abundant families were *Compositae* (67 taxa, 13.27%), *Poaceae* (52 taxa, 10.3%) and *Fabaceae* (35 taxa, 6.93%). These families are the most numerous in the total vascular flora of Bosnia and Herzegovina [48]. The flora of domestic gardens in England [4] and France [49] is dominated by *Compositae* and *Rosaceae*, probably due to the fact that deliberately planted ornamentals and fruit trees and shrubs (most of which are *Rosaceae*) were included in these analyses. Out of 76 families recorded in domestic gardens in Sarajevo, 23 (30.36%) were represented by only a single species, 16 of which were native, and 7 alien. The number of taxa in domestic gardens in Sarajevo is in accordance to the data given in the available literature sources, which ranges from 208, as recorded in Marseille (France) [6], to 1166 in Sheffield (England) [4]. The large number of plant taxa in gardens is a result of the fact that in most domestic gardens, nutrients and water are constantly added to the soil, enabling the survival of plants in places where it would not otherwise be possible [18].

The analysis of life forms showed the domination of hemicryptophytes (244 taxa, 48.32%), followed by therophytes (25.54%), phanerophytes (11.68%), geophytes (8.32%), and chamaephytes (6.14%). Hydrophytes were not recorded among the spontaneous taxa. The domination of hemicryptophytes and therophytes was also noted in weed flora of gardens in settlements in around Plešivica mountain in Croatia [50].

Most of the taxa found in the domestic gardens of Sarajevo naturally occur in Bosnia and Herzegovina (86.93% of the garden flora). The share of alien taxa (13.07%) is much smaller than in this habitat type in other cities worldwide. For example, in Minneapolis–Saint Paul in Minnesota (USA), 59% of all spontaneous yard taxa were exotic [51], an average of 71% alien taxa was calculated for domestic gardens in 5 cities in Great Britain[3], and an average of 40% for 54 Central European cities [52]. In private gardens in Lauris and Marseille, there were 13% and 10% alien taxa, respectively [6]. The high ratio of alien taxa in private gardens worldwide is a result of the horticultural practices, which promote the planting of hardy and aesthetically pleasing non-native plants [1]. In Sarajevo, however, most domestic gardens are located in the peripheral part of the city, on the slopes of hills surrounding the city, where the local microclimate due to the higher altitude prevents the development of taxa originating from warmer regions. Numerous domestic gardens in Sarajevo also include a traditionally managed vegetable patches and small orchards, and parts of unmanaged or neglected ground, where the development of native vegetation is encouraged.

Out of 66 alien taxa observed during this survey, 47 (71.21%) are naturalized and 21 (31.82% of total number of alien taxa) invasive. Alien flora of domestic gardens

of Sarajevo is dominated by neophytes (38 taxa or 57.57%). The ratio of neophytes to archaeophytes increases in direct relation to the intensity of human disturbance [53], as archaeophytes are typically associated with traditional rural environments or intermediate levels of anthropogenic activities, while neophytes are more common in highly disturbed habitats, which provide distinctive environmental conditions that favor the establishment of plant taxa from warmer and drier areas [40, 52, 54, 55]. The neophyte/archaeophyte ratio in domestic garden flora of Sarajevo (1.52) indicates the high intensity of anthropogenic disturbance in this urban habitat type.

According to the mode of introduction, most alien taxa (42 or 63.64%) found during this survey were deliberately introduced in Balkan region and/or in Europe, mostly as ornamentals, or various crops, including fruit trees and vines. Some of these "planta hortifuga" [56] tend to escape the cultivation and establish their populations in fringe vegetation, as on the riparian habitats and banks, roadsides, along forest edges, on wasteyards, old walls, debris and also on arable fields.

During this survey, some of invasive neophytes introduced as ornamentals (*Reynoutria japonica* Houtt., *Acer negundo* L. *Ailanthus altissima* (Mill.) Swingle, *Helianthus tuberosus* L., *Impatiens balfourii* Hooker f., *Parthenocissus quinquefolia* (L.) Planchon, *Robinia pseudoacacia* L., *Solidago gigantea* Aiton), were observed to invade the natural or seminatural vegetation. Still, these taxa are either deliberately planted and grown in many surveyed gardens, or not removed in their seedling stages. Given the fact that most domestic gardens in Sarajevo are located in the peripheral parts of the city, it is possible that these plant taxa can become troublesome invaders particularly on dry, rocky or gravelly soils, or in riparian habitats such as are the ones found in the protected area of Bentbaša, located in the western part of the surveyed area.

The high number of seedlings of *Rhus typhina* L. was observed during this survey. This neophyte is listed as invasive in Belgium [57] and Serbia [58], and has not reached its maximum range yet, so its spread should be monitored.

Most accidentally introduced taxa are agricultural weeds (e.g. *Amaranthus retroflexus* L., *Ambrosia artemisiifolia* L., *Datura stramonium* L., *Erigeron canadensis* L., *E. annuus* (L.) Pers., *Galinsoga parviflora* Cav., *G. quadriradiata* Ruiz & Pav., *Papaver rhoeas* L., *Veronica persica* Poir.…), which can be explained by the fact that some of the surveyed domestic gardens comprise parts in which the traditional agricultural practices are still present (vegetable patches or orchards).

The analysis of floral elements of domestic garden flora of Sarajevo (Fig. 2) shows the dominance of widespread taxa (29.89%), followed by taxa of Eurasian (25.84%) and South European (13.93%) floral element. The ratio of cultivated and adventitious plants is rather low (9.89%), which is in accordance to the low percentage of alien taxa in the overall flora. Widespread and Eurasian taxa were the most numerous in garden communities in Plešivica hills in Croatia as well [50]. The domination of Eurasian taxa in both cases can be explained by the geographic location of the surveyed area, and the widespread taxa have fewer specific environmental preferences and tend to have a higher affinity to urban areas [59].

Most alien taxa (31.52%) originate from Asia, followed by taxa from other parts of Europe, mostly Mediterranean (Fig. 3). American taxa constitute 27.17% of alien flora (including 18.48 taxa from North America, 4.35 taxa from Central America and 4.35

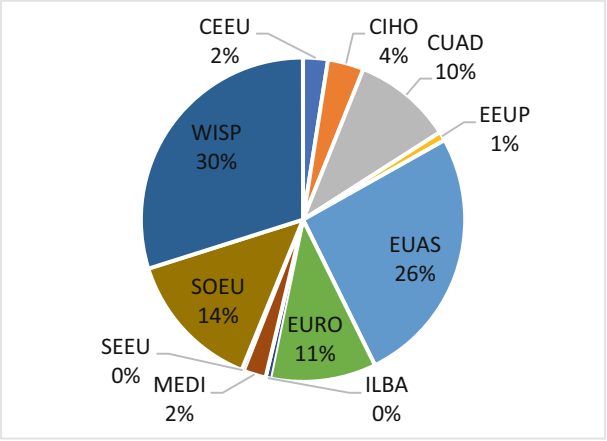

Fig. 2. Spectrum of floral elements (%) of domestic garden flora of Sarajevo (MEDI – Mediterranean floral element, ILBA – Illyrian-Balkan floral element, SOEU – South European floral element, EEUP – East European-Pontic floral element, SEEU – Southeast European floral element, CEEU – Central European floral element, EURO – European floral element, EUAS – Eura-sian floral element, CIHO – Circum-Holartic floral element, WISP – widespread plants, CUAD – cultivated and adventitious plants).

taxa from South America), and mostly include deliberately introduced ornamentals. In domestic gardens in 5 cities in the United Kingdom, the largest number of alien plant taxa was of European or Asian origin, and fewer were from North and South America, Africa and Australia [3].

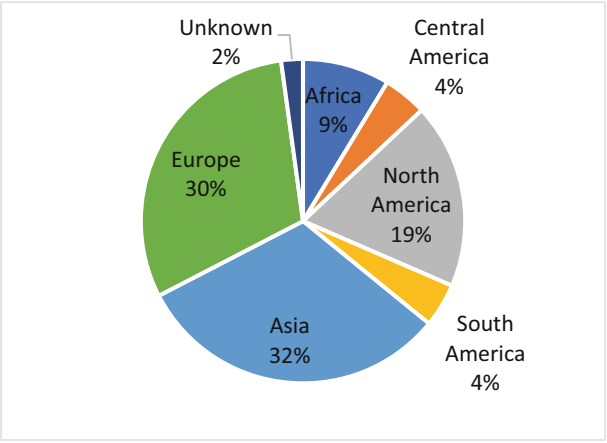

Fig. 3. Geographic origin of the alien flora observed in the domestic gardens in Sarajevo

The flora of domestic gardens is dominated by moderately urbanophobic taxa (37.04%), followed by urbanoneutral (28.27%), urbanophobic (20.99%), moderately

urbanophilic (9.85%), and only 3.85% taxa observed in this habitat type is urbanophilic (Fig. 4), which indicates the high level of naturalness in flora of domestic gardens in Sarajevo.

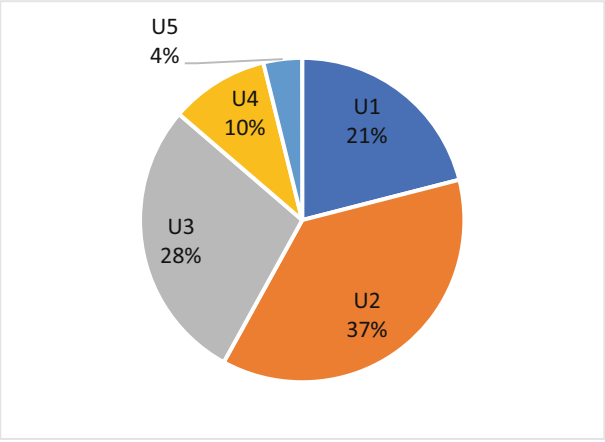

Fig. 4. The affinity of domestic garden taxa towards the conditions that prevail in urban environments (U1 – urbanophobic, U2 – moderately urbanophobic, U3 – urbanoneutral, U4 – moderately urbanophilic, U5 – urbanophilic)

The values of indicators of anthropogenic changes are presented in Table 1. The indicators of anthropophytization ($IAn_t = 13.07\%$; $IAn_p = 9.67\%$) show the considerable anthropogenic influence on the total flora of domestic gardens in Sarajevo. Higher indicator values of kenophytization ($IKn_t = 7.52\%$; $IKn_p = 7.82\%$) showed that the flora of the domestic gardens is more influenced by neophytes than by archaeophytes ($IAr_t = 4.95\%$; $IAr_p = 5.14\%$). In both archaeophytes and neophytes, the values of total and permanent indicators were similar, showing that alien flora is well established, which was confirmed by the low value of the indicator of fluctuating changes ($IF = 3.76\%$).

Endemic and endangered taxa represent only a small portion of the garden flora of Sarajevo. During this survey, one endemic taxon (*Trifolium dalmaticum* Vis.), and three taxa protected by the Red List of wild species and subspecies of plants, animals and fungi of Federation of Bosnia and Herzegovina, of which *Convallaria majalis* L. is listed as Near Threatened (NT), *Cephalanthera longifolia* (L.) Fritsch as Vulnerable (VU), and *Scabiosa cinerea* Lam. as Data Deficient (DD) species were found. The presence of these rare and endangered taxa in domestic gardens highlights the role that such land use types can fulfil in the conservation of local biodiversity. If we consider the fact that a substantial part of domestic gardens was not surveyed because of privacy issues, we can argue that this number is much higher. However small the contribution of individual domestic gardens may be towards the protection of such taxa, the collective effort of this habitat type across an entire urban ecosystem and also globally holds a large potential [2, 60].

The analysis of floristic similarity along the urbanization gradient indicates that the species richness is bigger in domestic gardens located in squares at the periphery of the

Table 1. Indicators of anthropogenic changes in the domestic garden flora of Sarajevo

Indicator	Value
IAn_t	13.07
IAn_p	9.67
IAr_t	4.95
IAr_p	5.14
IKn_t	7.52
IKn_p	7.82
IM	80.85
IF	3.76

surveyed area (Fig. 5). The exception are the squares located in the very north of the surveyed area (F6, G6), which cover mostly industrial zones, city graveyards and parks, and I4 and I5, which include substantial part of natural vegetation, and in which most houses are enclosed by tall walls, which did not allow the systematic survey of gardens. These results still demonstrate the decrease in floristic similarity along the urbanization gradient, as was found in Lauris and Marseille [6].

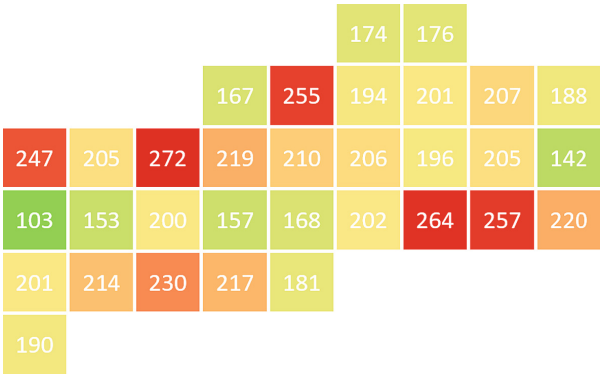

Fig. 5. Total number of taxa in gardens in 1 × 1 km squares in surveyed area

The overall floristic composition of gardens in individual squares shows that their diversity is more influenced by the geographic location within the urbanized area, as the squares which are located next to each other, and the ones that have the same distance from the urban center show higher similarity (Fig. 6).

Out of 505 taxa recorded during this survey, only 41 (8.12%) were found in all 32 1 km^2 squares. Only two of these (*Veronica persica* Poir. And *Capsella bursa-pastoris* (L.) Medik.) are alien. A total of 39 taxa (7.72%), including five aliens (*Catalpa bignonioides* Walter, *Cydonia oblonga* Mill., *Syringa vulgaris* L., *Ricinus communis* L., *Symphoricarpos albus* (L.) S.F. Blake) were observed spontaneously growing in only one

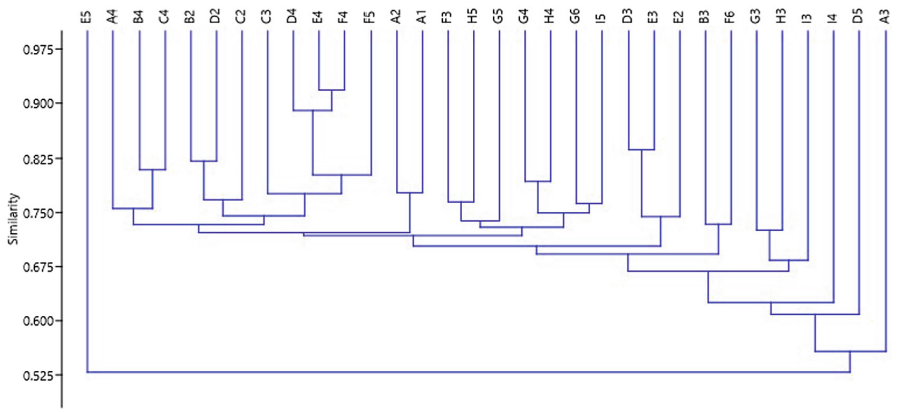

Fig. 6. Dendrogram constructed according to the list of plant taxa observed in domestic gardens in individual 1 × 1 km squares (similarity ratio, absence-presence data)

square. The fact that very small number of taxa were recorded only once indicates the low species turnover, and shows the uniformity of domestic garden flora in the surveyed area. These results differ from the ones reported for the gardens of Sheffield in England [1], which probably can be explained by the gardening practices and the fact that they included both spontaneous and cultivated taxa.

4 Conclusions

During the first systematic survey of domestic garden flora of the city of Sarajevo, a total of 505 species of 296 genera and 76 families were recorded. *Compositae*, *Poaceae* and *Fabaceae* were the most abundant families. Hemicryptophytes and therophytes are the most numerous life forms. Alien flora consists of 66 taxa, including 21 invasive, and is dominated by neophytes, which indicates high disturbance rate, and is supported by the fact that most of these taxa were deliberately introduced as ornamentals. Unlike most other cities, garden flora of Sarajevo is dominated by native taxa, and has a very low share of urbanophilic taxa, which indicates the high level of naturalness. One endemic and three protected taxa were found. However, the anthropogenic influence on the total flora of domestic gardens in Sarajevo is considerable. As some of the invasive and potentially invasive neophytes were observed to invade the natural and seminatural vegetation, but are still grown in gardens, there is the need to raise general awareness about the dangers these plant pose to the natural plant communities. More taxa were found in domestic gardens located at the edge of the urban area. Although the role of the homeowners in shaping the garden is important, the floristic diversity in Sarajevo is more influenced by the geographic location within the urbanized area. The results of this research may be used for assessing the risk of spreading cultivated plants, but also to indicate the importance of this habitat type for local biodiversity conservation.

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Analysis of the Breeding of Lipizzaner Horses at the State Stud Farm Đakovo

Barbara Perica, Maja Gregić, Tina Bobić, Pero Mijić, and Mirjana Baban(✉)

Faculty of Agrobiotechnical Sciences, University of Josip Juraj Strossmayer, Osijek, Croatia
mirjana.baban@fazos.hr

Abstract. In the Republic of Croatia, the Lipizzan horse breed is a member of the protected breeds group. The State Stud Farm Đakovo carries out its breeding and selection work according to the breeding program of the Lipizzan breed in the Republic of Croatia. It is also a Lipizzan International Federation (LIF) member, following its breeding goals and principles. The State Stud Farm Đakovo currently breeds 7 sire lines of stallions, and 11 mare families, and has 178 horses of the Lipizzan breed at two locations. Out of the total number of horses, mares are the most represented, followed by stallions, which makes up about 60% of the herd. Within the paper framework, the numerical quantity and the representation of lines and breeds of Lipizzan horses in the Đakovo State Stud Farm for the past 10 years (2012 – 2021) were analyzed. Analysis shows that the most represented sire lines are Maestoso, Siglavy and Neapolitano, followed by Conversano, Favory and Tulipan, while Pluto is the least represented sire line. By analysing the collected data on the number of mares, it is shown that the most represented mare families are Mara, Montenegro, and Mima, while the least represented is the mare family Krabbe. Observing the overall analysis of graphs and tables, according to the lines and families of stallions and mares, we can see that proper and high-quality breeding and selection planning is a very complex job, which requires a lot of effort, knowledge, and skills to preserve the representation and uniformity of lines to obtain the best possible quality offspring.

Keywords: Lipizzaner · Stud Farm Đakovo · numerical quantity

1 Introduction

The term “stud farm” was mentioned for the first time in 1506, which is associated with the year of the foundation of the Stud Farm in Đakovo. There are records that the stable existed even before the written records, i.e. that it was founded by the Bosnian-Srijem bishops on the properties obtained from the Croatian king Koloman, back in 1239. Horses have been mentioned for the first time on the occasion of the wedding of a Bosnian ban with a Bulgarian princess, on the occasion of which ban Tvrtko presented Bishop Peter with 10 Arabian mares and one stallion, whose breeding continued until 1806. In 1854, Bishop Josip Juraj Strossmayer founded the Lipizzaner stud farm with original breeding material from Lipica. In December of the

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same year in Stud Farm Đakovo came seven mares Sphingo (Neapolitano Waldemar-Storya), Contessa (Maestoso Erga-Kalma), Romana (Maestoso Erga-Romana), Tapia (Favory Ratisbona-Trompeta), Sorta (Neapolitano Valdemar-Stornella), Stella (Conversano Bibiana-Stornella) and Alda (Neapolitano Waldemar-Alda), and stallion Favory Casino. Further breeding resulted in the well-known Mare families: Munja (Sfinga), Karolina and Kontra (Contesa), Tera (Tapia) and Alka (Alda). Strossmayer's successor, Bishop Krapac, to fill the stud farm in 1912, brought the mares Sagan, Virtuoso, Fiorento and Are from Lipica to the stud. Today's foundation herd of Lipizzaner breeds originates from these mares (Bencevic, 1957). In 1945, the stud farm became state property, and in 1947, the Zemaljska Pastuharna was established in Đakovo. In 1957, with the abolition of the Lipik stud farm, all horses were moved to the Kutjevo military stud farm, and in 1960, after the dissolution of that stud farm, all Lipizzan mares were also moved to Đakovo. As the only breeding farm of the Lipizzaner horse breed in Croatia, it was named the Center for the Selection of Horses of the Republic of Croatia, especially influencing the improvement of the quality of breeding, spreading knowledge and awareness of their cultural value in land-based horse breeding in Slavonia. Lipizzaner stallions from the Stud farm have had a great breeding value while being correctors of terrestrial breeding. In 1997, it received the status of the State Stud.

2 Breeding of Lipizzaner Horses at the Đakovo Stud

The Lipizzan horse breed is part of the group of protected breeds in the Republic of Croatia (Ministry of Agriculture, 2021). It is spread over the entire territory of the Republic of Croatia, with the largest number in the area of Slavonia and Baranja. Đakovo Stud Farm carries out its breeding and selection work according to the Breeding Program of the Lipizzaner breed in the Republic of Croatia (stud breeding) (DEĐL, 2019). The goal of the breeding program is to breed Lipizzaner horses by the breeding principles of the Lipizzan International Federation (LIF) (LIF-establishing acts, Bruxelles 2001), respecting the traditional breeding in Croatia. The implementation of breeding is the responsibility of the State Stud Farm Đakovo and Lipik (DEĐL, 2019). The goal is to breed a horse with a lively temperament and good-natured character and a pronounced willingness to work; stallions about 155 cm to 160 cm tall, mares 153 cm to 158 cm; body mass around 550 kg; noble heads; high-set, medium-long muscular necks; well-formed withers, straight and firm back well connected by muscles; rounded and slightly lowered croup, and properly planted tail; strong and muscular legs, dry and well-formed joints, and strong hooves; extremely high elastic movements, expressive and confident in all gaits. It strives to create a horse that is suitable for all forms of recreational use, as well as achieving results in sports. The Đakovo Stud Farm breeds Lipizzaner, which is suitable for all carriage driving activities, and in recent years, Lipizzaners have proven themselves by winning high places at national competitions in dressage riding. Baban (2021) points out that the various breeding goals of individual countries and regions were changed depending on geographical-social and cultural circumstances. There was a great morphological diversity of the Lipizzaner population in appearance, type, body shape, coat colour and gait. The breeding goal at Đakovo Stud Farm was to create the largest possible frames and bones of Lipizzan breeding horses with high-quality

predispositions for driving and riding, which is why Lipizzan stallions from Hungary, Romania, Austria and Slovenia were occasionally used as fresheners of the blood (Baban et al., 2006). Today, the Lipizzaner from Đakovo belongs to the larger type of this breed. It is important to emphasize that through the breeding goal, the preservation of Croatian lines and Mare families as well as their balance in the population is carried out. The most common coat colour of an adult Lipizzaner is grey, but a very small part of the population has a darker coat colour (bay, black and very rarely chesnut). Lipizzaner foals are born with a dark coat colour, which changes during the first years of life to a lighter shade, so when reaching the age of up to ten years, the horse's coat takes on a permanent light grey shade (senile leucism). After foaling, the heads must be registered in the registry books of the Lipizzan breed in the Republic of Croatia and must be registered in the Central Register of Equids of the Republic of Croatia, which is managed by an authorized institution. The name of the male animals consists of the assigned personal number, for example: (202), the name of the stallion's line (Maestoso), the name of the dam's branch (Batosta), a Roman numeral (V) and an Arabic numeral (5) indicating the order of the registered male offspring of the dam.

Example: 202 Maestoso Batosta V-5 (Đ. 2005)

Sire: 700 Maestoso Gaetana XV-3 (D. 1991)

Mother: 765 Batosta V (D. 1993)

The naming of female animals consists of the assigned personal number (765), the name of the branch of the mother's genus (Batosta) and the Roman numeral (V) indicating the order of the registered female offspring of the mother.

Example: 765 Batosta V (Đ. 1993)

Father: 522 Tulipan Trofetta V-4 (Đ. 1986)

Mother: 557 Batosta III (D. 1987)

Newborn foals up to 6 months of age must be marked with a transporter that is implanted under the skin with a mandatory mane or tail sample taken for DNA analysis to check the origin of the head. Marking is also carried out with a hot stamp, where the letter H, which denotes the Republic of Croatia, is printed on the left saddle, and below the letter, H is the personal number, and on the left thigh the letter Đ, which denotes the State Stud Đakovo. The described form of marking is characteristic of the Đakovo Stud, while other studs and land breeding are marked in a different way (HPA, 2015).

The horse selection program at the Đakovo State Stud includes the evaluation of stallions, mares and foals. The evaluation committee consists of at least 2 members qualified for the evaluation, appointed by DEĐL for the stud breeding. To ensure breeding and selection progress, the following factors are taken into account in the evaluation:

- evaluation of origin – heads must be registered in the register of Lipizzan horses in the Republic of Croatia and must have a full pedigree that connects them to the founders of the lines and genera of the Lipizzan breed;

- evaluation of appearance – is carried out to evaluate and classify horses based on their appearance (phenotype), constitution (build), gaits, working ability, temperament and temperament following the breeding program;
- classification into class classes – defined by the overall evaluation of the appearance, the results of the working ability test and the quality of the offspring;
- working ability test (IRS) – the method of testing the horse of the Lipizzaner breed is defined according to the Ordinance of the working ability test. All horses, regardless of gender, can take the test when they are at least 4 years old, according to two protocols: IRS in riding or IRS in harness;
- evaluation of the quality of the progeny – all the results of the breeding stallion or mare that they achieved during their lifetime, as well as the results of their offspring, are entered in the registry books, and based on that they can be awarded according to the class;
- judgment of health and fertility – the breeding heads that approach the evaluation must be healthy, fertile and must not be carriers of hereditary errors.

Young stallions that are evaluated for the first time at the age of 4 and are evaluated with a minimum of 75 points or more are temporarily selected for breeding and must attend a second selection and pass the working ability test. At the age of six (if the stallion was evaluated with 75 or more points at the first and second selection and has passed the working ability test) he is permanently selected for breeding. Mares are evaluated after at least 3 years of age, while foals are evaluated in the year of birth. Before the evaluation of stallions and mares, body measurements are taken: height to the withers with a stick, height to the withers with a tape, chest girth, body length and cannon bone circumference. Characteristics that are evaluated are breed type and sexual dimorphism, head, neck, the front part of the trunk, middle part of the trunk, rear part of the trunk, front legs, hind legs, regularity of gaits, expressiveness of movements. The criteria are evaluated with marks from 1–10, the total result is obtained by adding up the average marks of all commission members for each individual factor, and the highest possible number of points is 100. Based on the results, the cattle are classified into the corresponding class (DEĐL, 2019):

- 85 and above – a typical member of the breed, desirable in breeding, top grades
- 80 to 84 – a typical member of the breed, its features are desirable in breeding
- 75 to 79 – a typical member of the breed, its characteristics can contribute to the achievement of the breeding goal
- 70 to 74 – the stallion is not recommended for breeding, and should only be used in case of preservation of genetic diversity – mares go to further breeding
- 65 to 69 – the stallion is not for breeding – the mares go to further breeding
- 64 and under declined not for breeding

When evaluating foals, the following are taken into account: breed type, head and neck, trunk, legs, regularity and generous movements. Foals with 40 or more points enter the Sect. 1, foals with 35 to 39 points belong to Sect. 2, and foals with 34 or fewer points belong to Sect. 3 (DEĐL, 2019).

3 Numerous Conditions of Lipizzaner Horses in State Stud Farm Đakovo

The numeral condition of horses is noted on the last date of the previous year (December 31, 2021), so the Đakovo State Stud currently has 178 horses of the Lipizzan breed, 56 in the pasture, and 122 Lipizzans of different ages and gender categories in Ivandvor. The Stud Farm located in the centre of Đakovo is home to stallions, who come there for training when they are 3–4 years old. In addition to them, some geldings are used for dressage and harness competitions, and participation in various events. 63 mares with offspring of different age groups are kept at Ivandvor stud farm, located outside the city.

Out of the total number, mares are the most represented, followed by stallions, which together make up about 60% of the herd (Fig. 1). Colts and fillies varying from 1 to 3 years of age make up a little more than a quarter of the herd.

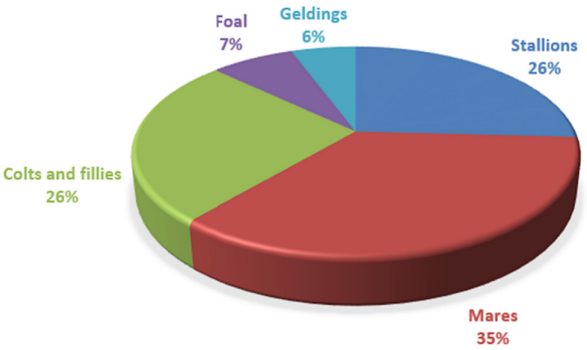

Fig. 1. Relationship of Lipizzaner heads in 2021 (Horse book of the Đakovo State Stud, 2022).

The overall numerical condition, as well as the condition of individual categories, is constantly changing due to the life cycle of the horse. The foal category includes offspring from foaling up to 6 months of age. After that, the foal moves to the weanling's category up to 1 year of age. When the youngster reaches 1 year of age, it moves to the category of yearlings from 1 to 2 years of age. After reaching the 2nd year, the youngster passes into the last category of youngsters from 2 to 3 years of age. All age groups, or categories, are classified according to gender to avoid unwanted and uncontrollable mating between youngsters. At the age of 3, fillies pass into the category of mares and then enter the mother herd as heads for further reproduction. At the age of 3–4, colts pass into the stallion category and are moved to the stud farm. In the category of geldings, we include males that do not participate in reproduction. These are horses that for certain reasons are not suitable for reproduction and have undergone the castration procedure. Stallions that are not suitable for breeding are castrated and used in equestrian sports. Their further destiny is participation in sports competitions. They are characterized by a calm and balanced character. The condition of numerous heads changes also due to the excretion of individual individuals from breeding, heads are sold taking into account the excessive condition of individuals in a line or breed, horses that were not selected

for sport or breeding due to weaker genetic value – worse breeding ratings, deaths, and unfavourable breeding seasons which results in a reduced number of future offspring. On average, about 15 foals are foaled at the Đakovo State Stud every year. The number of Lipizzaner horses at the Đakovo State Stud from 2012 to 2021 is shown in Table 1.

Table 1. Numerous conditions of Lipizzaner horses in the period from 2012 to 2021 at the Đakovo State Stud (Register of horses of the Đakovo State Stud, 2022).

Horse category	Numerous conditions of horses									
	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Lipizzan stallions	57	52	41	44	45	48	44	52	45	46
Lipizzaner mares	52	60	63	69	65	58	59	60	59	63
Lipizzaner colts from 2 to 3 years	5	4	11	12	11	2	10	3	6	7
Lipizzaner male yearling from 1 to 2 years	4	11	12	12	2	10	4	6	7	6
Lipizzaner male weanling up to 1 year	10	12	12	3	10	4	7	8	6	7
Lipizzaner male foal	10	12	12	3	10	4	7	8	6	7
Lipizzaner filly from 2 to 3 years	11	6	9	7	5	3	4	8	4	12
Lipizzaner female yearling from 1 to 2 years	6	10	7	5	3	5	8	4	12	8
Lipizzaner female weanling up to 1 year	11	7	5	5	5	8	5	14	8	6
Lipizzaner female foal	11	7	5	5	5	8	5	14	8	6
Gelding Lipizzaner	12	12	8	7	6	11	13	11	10	10
IN TOTAL	**189**	**193**	**185**	**172**	**167**	**161**	**166**	**188**	**171**	**178**

The number of Lipizzaner stallions in the observed period at the Đakovo State Stud Farm ranges from 41 in 2014 to 57 in 2012. By analyzing the number of Lipizzaner stallions over 10 years, we get an average value of 47.4 stallions. The biggest deviation from the average value was recorded in 2012. The smallest deviation from the average value was recorded in 2017 and 2021. The lowest number of Lipizzaner mares (52) was recorded in 2012, while in 2015 there were 69. The average number of Lipizzaner mares in the observed period of 10 years is 60.8 mares per year. The largest deviation was recorded in 2012 and 2015, and the smallest in 2013 and 2019. The number of Lipizzaner colts from 2 to 3 years of age ranges from 2 (in 2017) to 12 (in 2015). The average number of colts from 2 to 3 years of age is 7.1 per year. The largest deviation from the average value was recorded in 2015 and 2017, and the least in 2021. In 2021, we count 12 females aged 2 to 3 years at most, and at least 3 in 2017. If we compare the number of fillies between 2 and 3 years of age with the average annual value, which is

6.9, the largest deviation was seen in 2021, and the least in 2013 and 2015. The largest number of Lipizzaner colts from 1 to 2 years of age was recorded in 2014 and 2015, 12 of them, and the smallest number was in 2016, only 2. This is an average of 7.4 colts per year in the observed period. We recorded the largest deviation in 2016 and the smallest in 2020. Analyzing the table of the number of female yearlings from 1 to 2 years of age, it can be concluded that there were 12 of them in 2020, and only 3 in 2016. On average, that is 6.8 female yearling from 1 to 2 years of age per year. The biggest deviation from the average was in 2016, and the smallest in 2014. The number of Lipizzaner males up to 1 year of age was the highest in 2013 and 2014 (12), and the lowest in 2015 (3). In the last 10 years, the average number was 7.9 male weanling. The largest deviation from the average was recorded in 2015 when the minimum was recorded, and the least in 2019. The largest number of female weanlings up to 1 year of age was in 2019 when we counted 14, and at least 5 in 2014, 2015, 2016 and 2018. By comparison with the average value, the largest deviation is visible in 2019, when the maximum was recorded, and the minimum in 2013, 2017 and 2020. The average value is 7.4 female weanling per year. The largest number of Lipizzaner foals was recorded in 2013 and 2014 (12 foals), and the least in 2015 (3 foals). Data analysis results in an average value of 7.9 foals per year. The biggest deviation was recorded in 2015 when it was also the minimum and the least in 2019 and 2021. The number of fillies ranges from 5 (in 2014, 2015, 2016 and 2018) to 14 (in 2019). In the observed period, there were an average of 7.4 foals per year. The biggest deviation was in 2019, when the maximum was also recorded, and the least in 2013, 2017 and 2020. The number of Lipizzaner geldings ranges from 6 (in 2016) to 13 (in 2018). By comparison with the average value, the largest deviation is recorded in 2016, as well as the minimum, and the least in 2020 and 2021, when the number equaled the average value, that is, there are no deviations. In the observed period of 10 years, the average number is 10 castrates per year.

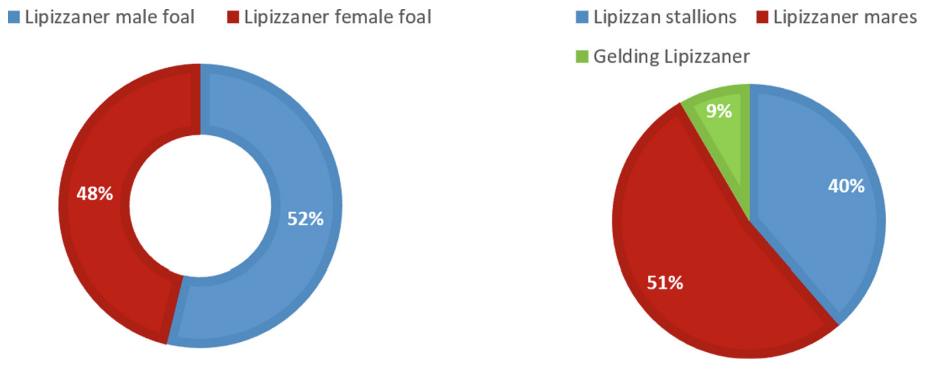

Fig. 2. The average ratio of male and female foals, and male and female foals in the period from 2012 to 2021 (Horse register of the Đakovo State Stud, 2022).

If we analyze the ratio of male and female foals in the past period, and by calculating the average value of the representation of male and female foals (Fig. 2), we can see an equal representation of both sexes, i.e., there were slightly more male foals (52%) than female foals (48%). If the above ratio is compared with the total average value of

male and female heads at the Đakovo State Stud, it can be seen that the ratios match. The average value of the total number of male heads (stallions and geldings) is 49% compared to female heads, which are represented by 51%.

4 Analysis of Male Lipizzaner Breed by Lines from 2012 to 2021

A stallion is an uncastrated sexually mature horse and, as such, is a potential future sire. Heads used for breeding must meet the evaluation of the appearance and have passed the working ability test (IRS), they must be in excellent physical and breeding condition, which is also an indicator of a favourable state of health, and they must also not be carriers of hereditary errors. In case of the appearance of morphological and physiological errors, these same heads are evaluated during the evaluation of the appearance (morphological) and veterinary examination (physiological) and are taken into account during the final evaluation. If certain errors appear in more than 10% of the offspring during breeding, the breeding stallion is excluded from breeding. Likewise, if poor fertility or complete infertility is proven, cryptorchidism (non-descended testicle in the scrotum) occurs in the offspring, which leads to the exclusion of stallions. Every year, the committee in charge of breeding and selection selects the breeding stallions that will be used in the mating season, which lasts from February 1 to June 30 of each year. The criteria according to which stallions are selected are:

- The line with the fewest individuals in the population, which is important for the preservation and representation of the lines.
- Quality and desirable morphological traits or movements with which undesirable traits in mares can be improved to obtain better quality offspring.
- Availability of individual stallions in other state studs or private breeding.

Stallions at Stud of the Đakovo (breeding male stallions that actively participate in breeding) are located in Ivandvor, where the mother herd of mares is located. When analyzing the representation of lines in the last 10 years, only male heads are taken into account, because they are representatives of lines (HPA, 2015).

Table 2. Total number of males according to stallion lines from 2012 to 2021 (Horse register of the Đakovo State Stud, 2022).

Lines of stallions	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	Σ
Pluto	11	11	8	6	5	3	3	6	6	6	**65**
Neapolitano	12	16	16	16	11	9	10	10	8	8	**116**
Favory	3	6	7	7	11	11	11	11	12	11	**90**
Conversano	11	10	12	12	9	9	8	8	7	13	**99**
Tulipan	8	5	3	4	7	8	8	10	10	8	**71**
Siglavy	20	15	14	11	11	10	15	13	10	9	**128**
Maestoso	11	16	16	15	14	14	10	11	11	11	**129**

The total number of male heads according to stallion lines at the Đakovo State Stud in the period from 2012 to 2021 is shown in Table 2. In 2012, the Siglavy line was represented the most (20 heads) and the Favory line the least (3 heads). This is why stallions of the Favory line, Neapolitano and Maestoso lines were used in the 2012 breeding season, precisely because of the increase in the number of individuals of the mentioned lines, especially the Favory line. In 2013, the highest number was recorded by the Neapolitano and Maestoso lines with 16 heads, and the least by the Tulipan line with 5 heads. Compared to the previous year, there is an increase in the number of heads in the Maestoso (5), Neapolitano (4) and Favory (3) lines, since stud stallions from those lines were used. In the breeding season of 2013, breeding stallions from the lines Conversano, Maestoso and Favory were used. In 2014, as in the previous year, the Neapolitano and Maestoso lines have the largest number of heads, and the Tulipan line has at least. In the breeding season of 2014, stallions at stud from the Favory and Tulipan lines were used, to increase the number of heads in the mentioned lines. In 2015, the most heads were recorded from the Neapolitano lines with 16 and Maestoso with 15, and the least Tulipan with 4 individuals. In the growing season of the mentioned year, nursery plants from the Favory and Tulipan lines were used, as in the previous year. In 2016, the Maestoso line has the most heads with 14, and the Pluto line has the fewest with 5 heads. Table 2 shows an increase in the number of heads of the Favory and Tulipan lines in comparison to previous years, which is extremely important for the preservation and representation of the line. Siglavy line stallions at stud were used for breeding since the number of individuals dropped by 45% from 2012 to 2016. In 2017, the largest number of heads was recorded in the Maestoso line with 14 individuals, and the least in the Pluto line with 3 individuals. In the breeding season of 2017, breeding stallions from the Siglavy line were used to continue increasing the number of individuals in the population as well as the Neapolitano line, since the number of individuals decreased by 44% in the past 3 years. In 2018, the Siglavy line records 15 head, and the Pluto line 3 (as in the previous year). The biggest increase in the number of individuals compared to the previous year was recorded by the Siglavy line, which is the result of using nurseries from the said line. For the breeding season of 2018, nursery plants from the Pluto, Favory and Tulipan lines were used. The Pluto line recorded a decrease in a number of individuals in recent years by 73%. The Siglavy line has the largest number of heads in 2019 with 13 individuals, and the Pluto line has the least with 6 individuals, although their population has increased by 100% compared to the previous year. The nurseries that were used in the 2019 breeding season are the Favory, Maestoso and Tulipan lines. In 2020, the Favory line has 12 heads, and the Pluto line has 6 (as in the previous year). That year, nursery stallions from the Conversano line were used for the mating season, since the number of stallions was in decline, as was the case from the Tulipan line. The highest number of heads in 2021 is recorded by the Conversano line with 13 individuals, and the least by the Pluto line with 6 individuals (as in the previous two years). The Conversano line recorded an increase of 46% compared to the previous year, which is the result of well-planned breeding. By analyzing Table 2 according to lines and years, it can be seen that good and high-quality planning of breeding and selection is a very complex job, which requires a lot of effort, knowledge and skills to preserve the representation and uniformity of lines with the aim of creating the best quality offspring that meet the breeding goal.

Figure 3 shows the movement of the number of individual lines in the observed period. According to what is shown, there is an unevenness in the number of individual lines in 2012 compared to 2021. In the last 10 years, this uneven representation of lines has decreased by almost 50%, which is proof of the good implementation of the given breeding plan.

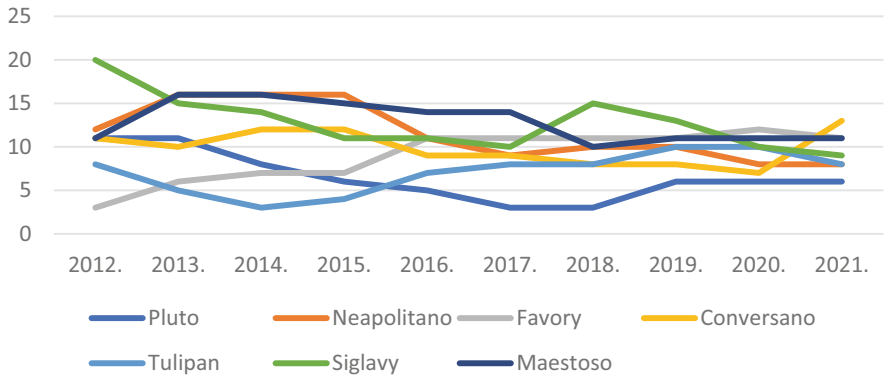

Fig. 3. The direction of movement of male necks according to stallion lines from 2012 to 2021 (Horse register of the Đakovo State Stud, 2022).

Figure 4 shows the average value of the representation of stallion lines in the last 10 years. The most represented lines are Maestoso, Siglavy and Neapolitano, then Conversano, Favory and Tulipan, while Pluto is the least represented line. Representation of

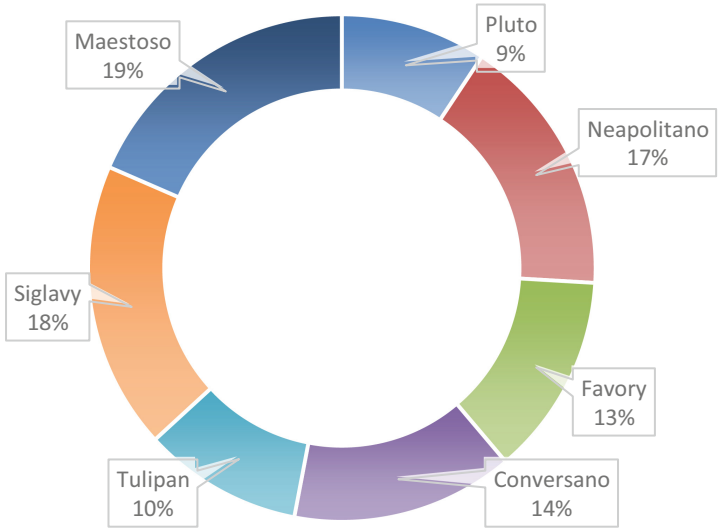

Fig. 4. Representation of stallion lines in the observed period (Stud book of horses of the Đakovo Stud Farm, 2022).

individual lines in breeding often depends on the availability of nursery stallions from other state studs, private breeding, as well as within the stud itself (blood relation with mares).

In the following text, the characteristics of each stallion at stud will be presented, which is why it was chosen for breeding.

5 Analysis of the Breeding of Female Lipizzaner Mares by Breed from 2012 to 2021

Half of the original breeds of Lipica mares belong to the old classical type whose ancestors come from Lipica and Kladrub, while the other half come from other studs, but mostly Croatian. This is confirmed by the names Zenta, Krabbe (Rendes), Trofetta (Traviata), Mara (Margit), Munja (Famosa). By lineage is meant the female offspring of one foremother, the founder of the lineage (Stipić, 1975). The total number of females by breed of mare at the Đakovo State Stud in the period from 2012 to 2021 is shown in Table 3. Appendix 1 contains tables with a detailed description of offspring for each mare from a particular breed in the observed time period. Analyzing the genus Troffeta (Table 3), it can be seen that they were the most numerous from 2012 to 2016, then the number halved by 2020, and in 2021 it increased to 4 heads. Mares 45 Trofetta XI and 137 Trofetta XIII gave birth to one female foal each. From 2012 to 2016, Rod Toplica had 3 heads, then in 2017, 2018 and 2019, it increased by one head each, and by 2021, 6 heads were recorded. Mare 222 Toplica XXI gave birth to 2 female foals, and mare 28 Toplica XXVIII gave birth to 1 female foal. The genus Allegra was equally represented during the observed period with an average of 6 heads per year. Mare 214 Allegra XXXVI gave birth to 2 female foals, and mares 776 Allegra XXXII, 215 Allegra XXXVII, 952 Allegra XXXIII and 428 Allegra XXXVIII gave birth to one female foal each. Family Mara is the most numerous family of mares in the observed period. The most numerous was in 2012 with 15 heads, then the number decreased to 12 heads by 2021. In the last 10 years, mare 117 Mara LVI gave birth to the most foals (4), followed by mare 166 Mara XIV with 3 foals, mare 221 Mara XV with 2 foals, and mares 821 Mara LII, 750 Mara LI, 352 Mara LIX and 229 Mara LVII with one female foal each. In 2012, the genus Slavonia was represented by 6 heads, then in 2016 it has one head less (5), while in the last three years there have been eight. Mares 837 Slavonia XX and 348 Slavonia XXV gave birth to 2 female foals each, and mares 248 Slavonia XXIV and 304 Slavonia XXVI gave birth to one female foal each. Family Santa had 4 heads at the beginning of the observed period, as well as in 2021, and in 2014 and 2015 it counted 5 heads. Mare 828 Santa XLVII gave birth to 3 female foals, and mare 354 Santa L one. In 2012, the Munja breed was represented by 3 heads, in 2015 it increased by 1 head, and in 2017 it increased to 6 heads, and in 2020 and 2021 there are 4 heads. Mares 96 Munja VIII and 337 Munja X gave birth to 2 female foals each. In 2012, the Gaetana breed numbered 4 heads, then in 2015 it fell by one head, in 2018 and 2019 they recorded an increase of one head each, and in 2021 they numbered 6 heads. The mare 338 Gaetana XXII gave birth to 3 female foals and contributed the most to the breeding of the mentioned breed. The Batosta breed recorded the same number of head in 2012 and 2021–6 of them, with an increase in 2015 and a decrease in 2016. The mare

19 Batosta VIII gave birth to 3 female foals in the observed period, and the mares 792 Batosta LIV and 418 Batosta LXII gave birth to one female foal each. The Montenegra genus is among the most numerous genera after the Mara genus in the last 10 years. In 2012, they numbered 9 heads, then recorded growth, and in 2021 they numbered 12 heads. Mare 114 Montenegro XLIII gave birth to 2 female offspring, while mares 129 Montenegro XLIV, 934 Montenegro XLIII, 149 Montenegro XLIII, 384 Montenegro XLVI, 343 Montenegro XLVII each had one female foal. The genus Mima is the third most represented genus in the observed period. In 2012, they numbered 10 heads, then recorded growth, in 2017 they fell to 7 heads, and in 2021 they numbered 8 heads. Mare 121 Mima XVII gave birth to 3 female offspring, mare 20 Mima XX gave birth to 2 female offspring, and mares 766 Mima XIV and 211 Mima XIX each gave birth to one female offspring. Zenta genus recorded the lowest number of head in 2012, then it increased in 2013 and 2014, in 2017 it decreased to 6 head, and in 2019 and 2020 it again recorded growth to 8 and 9 head, respectively. Mare 252 Zenta XIV gave birth to 2 female foals, and mares 951 Zenta XIII, 279 Zenta XV, 94 Zenta XV, 396 Zenta XV and 379 Zenta XIX one each. The Krabbe genus is the least represented mare genus in the observed period. From 2012 to 2019, they count 2 heads, then their number increases to 4 heads (2020 and 2021). Mares 861 Krabbe XXI and 409 Krabbe XXVI gave birth to one female foal each. Figure 5 shows the direction of movement of the number of females according to the mare's families in the last 10 years, from which the tendency to equalize the representation of all breeds of mares can be observed.

Table 3. Total number of males according to mares' families from 2012 to 2021 (Horse register of the Đakovo State Stud, 2022).

Mares' families	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	Σ
Trofetta	7	7	6	6	6	3	3	3	3	4	**48**
Toplica	3	3	3	3	3	4	5	6	6	6	**42**
Allegra	6	5	6	5	6	7	7	6	5	6	**59**
Mara	15	14	13	14	12	11	11	12	12	12	**126**
Slavonia	6	6	6	6	5	5	5	8	8	8	**63**
Santa	4	4	5	5	4	4	4	4	4	4	**42**
Munja	3	3	3	4	4	6	6	6	4	4	**43**
Gaetana	4	4	4	3	3	3	4	5	5	6	**41**
Batosta	6	6	6	7	5	5	5	5	5	6	**56**
Montenegra	9	10	10	11	11	11	11	13	12	12	**110**
Mima	10	11	11	11	10	7	7	8	7	8	**90**
Zenta	5	8	9	9	7	6	6	8	9	9	**76**
Krabbe	2	2	2	2	2	2	2	2	3	4	**23**

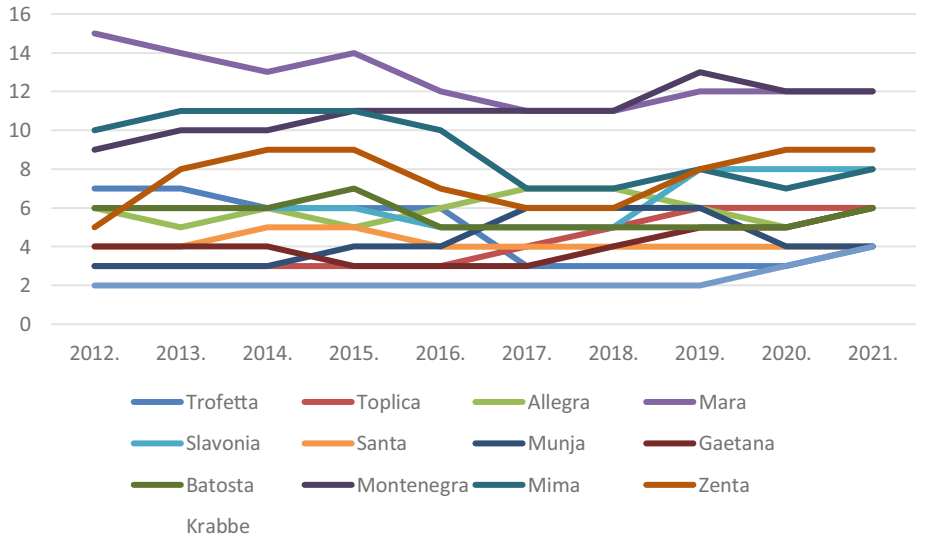

Fig. 5. Direction of movement of the number of females according to breed of mares from 2012 to 2021 (Horse register of the Đakovo State Stud, 2022).

Figure 6 Shows the average value of the representation of mares in the last 10 years. As previously stated, it can be seen that the most represented genera are Mara, Montenegro and Mima, while the least represented is the genus Krabbe. The number of

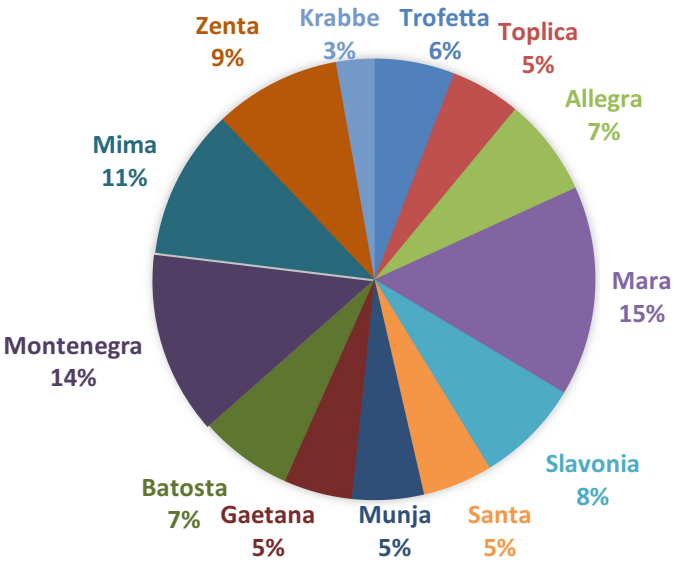

Fig. 6. Representation of breeds of mares in the observed period (Register of horses of the Đakovo State Stud, 2022).

individuals in a particular breed of mares depends on the number of female foals within the breed itself and their quality during breeding.

6 Conclusions

The State Stud Farm Đakovo has set breeding selection measures in its breeding program, based on which it carries out breeding selection work by increasing the breeding, use and economic value of the Lipizzaner population. Genetic variability is maintained by a sufficient number of selected breeding stallions and mares while maintaining an optimal proportion of lines and mare families in breeding, continuous selection, implementation of the annual allowance plan and monitoring of the level of inbreeding as prescribed by the breeding program of the Lipizzaner breed for stud breeding. Analyzing the available numerical data related to the lines of stallions and breeds of mares related to the period of the last decade, it can be concluded that the State Stud Farm in Đakovo is planning breeding and selection in a high-quality way, which is a very complex job that requires a lot of professional work, effort, knowledge and skills in order to preserve representation and uniformity of lines in order to obtain the highest quality offspring, with the tendency to increase individuals within lines and genera that are deficient. The total number of breeding Lipizzan mares and stallions, foals and foals in the Đakovo Stud was analyzed, and the data was compared with the previous ten years. The ratio of Lipizzaner foals according to stallion lines and mares is shown. With its way of working, the Đakovo Stud wants to create horses that meet the given breeding goal and needs for harness and dressage sports, as well as raising the quality of private breeding by actively participating with its own breeding stallions as carriers of quality genetic material.

Remark The paper is an excerpt from the final thesis of student Barbara Perica, titled "Analysis of the breeding of Lipizzaner horses at the Đakovo State Stud", defended on 19 September 2022 on the Faculty of Agrobiotechical Sciences Osijek.

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Influence of the Honeybee Colony Strength on Collecting Bee Venom by Electro Stimulation Method

Kosta Šumar[1], Goran Mirjanić[2], and Lejla Biber[1](✉)

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
l.biber@ppf.unsa.ba

[2] Faculty of Agriculture, University of Banja Luka, Banja Luka, Bosnia and Herzegovina

Abstract. Honey production has become lately more and more risky because of the frequent extreme climate changes. For this reason, it is necessary to develop production and other bee products. One of the bee products that deserves attention is certainly bee venom. Therefore, it is necessary to complete the knowledge on the possibilities of producing bee venom by a modern method of electro stimulation.

The study was conducted in Pale near Sarajevo in the village Podmjedenik. The experiment was carried out on a total of 6 honeybee colonies divided into three groups of two colonies. Collection of bee venom from worker bees was carried out by two electro stimulation devices. The study showed statistically significant differences in bee venom weight between strong (0.3 mg), medium strong (0.2 mg) and between medium strong and weak (0.11 mg) honeybee colonies. A statistically significant difference was also found in the amount of collected bee venom in the morning (0.3 mg) compared to the amount collected in the afternoon (0.1 mg), as well as the amount in the early evening (0.2 mg) and afternoon hours. There were no statistically significant differences between the morning and the early evening hours in the amount of collected bee venom.

Keywords: beekeeping · bee products · honeybee colony · bee venom · electro stimulation method

1 Introduction

Many products obtained from the bee colony are used nowadays, namely honey, which is the best known and most used bee product, followed by propolis, bee bread, royal jelly, beeswax and bee venom. All bee products are highly valued on the market for their medicinal properties. Beekeepers have been producing more and more bee venom because many studies have concluded that bee venom has a great impact on human health and pain relief in some diseases, so it is used in the pharmaceutical industry, but it is also used in the cosmetic industry for the production of various creams. At the age of 12–14 days, the maximum amount of venom is collected in the reservoir of the gland, after which the secretory cells degenerate. Worker bees, the most numerous members

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of the bee colony, are sexually immature females. The production of bee venom is a demanding process, and it is developing more and more over the years. Bee venom has been used in folk medicine to treat various diseases. Additionally, for example, over 400 human diseases have been treated in China and India.

Bee venom is a product of the secretion of venom glands in bees. The venom is a thick, colorless liquid with a strong odor and a very bitter taste. In its free state, bee venom (separated by bees and located in hygienic vials) is a yellowish liquid with an acidic reaction, a pleasant aroma and a bitter and spicy taste. It is easily soluble in water, aqueous solution of glycerine, vegetable oils, poorly soluble in ethyl alcohol of various concentrations and organic acids. It is heavier than water (relative density of 1.0851.131). Furthermore, it contains 30–48% solids. It dries in the air, but the dried residue immediately absorbs moisture.

Bee venom has a complex chemical composition. It contains proteins (including enzymes), peptides, amino acids (histamine, dopamine, norepinephrine), quaternary derivatives, ammonium bases - acetylcholine, lipids (fats and sterols), ash elements (mineral thiol), sugar (glucose and fructose), nucleic and ascorbic acids, hydrochloric acid and other substances. The main part of the dry matter consists of proteins and peptides (80%). Protein melatin (50% of dry matter) is the main component of the venom (Hegić, 2019).

It dries quickly in the air and loses two thirds of its weight. It is slightly acidic, resistant to temperature and freezing does not harm it. If it is not protected, the bee venom will change its color from white to brown-yellow due to oxidation, and such changes caused by oxidation reduce the quality of the bee venom.

Bee venom can be found as:

- Pure and dried bee venom - it is white, when used as a solution it is colorless.
- Dried bee venom - it can be contaminated with pollen, nectar and dust, and its color varies from yellow to brown-yellow.
- Freeze-dried venom - it is purified venom and is stored in hermetically sealed boxes (Tucak et al., 2004).

2 Material and Method

The research was conducted at the family apiary in 2022. It is located 35 km from Sarajevo at the foot of Romanija mountain at 1000m above sea level. There are 80 colonies in Dadant-Blat's beehives at the apiary. Research was done on 6 bee colonies, in the period of two months (June and July). An experiment was conducted and the aim was to see the amount of collected venom using standard electro stimulation. The venom was collected from productive communities, but of different strengths.

2.1 Collection of Bee Venom

There are a few different designed electrostimulators. All of them are essentially simple, meet the basic conditions of commercial production of bee venom and enable the availability of simultaneous collection of venom from a large number of bees, with the bees remaining alive. The standard method of venom collection was performed with an electric stimulator and an applicator (see Fig. 1).

Fig. 1. Electrical stimulator and applicator (Šumar, 2022)

Bee venom was collected simultaneously from two colonies of equal strength. Additionally, the venom was collected at different times for strong societies as well as for medium and weak ones, with an interval of four days and a duration of half an hour. An apparatus was used on which other parameters such as: frequency, pause, interval length (signal) and voltage can be adjusted, which was different at the given moment. The threshold of sensitivity depends on the duration of the stimulation and the step of their amplification. The weaker the increase in the strength of electrical impulses, the higher the stimulation threshold (accommodation effect). On the other hand, a growing impulse causes a rapid reaction in the tissue at lower strengths.

2.2 Extracting Venom from the Applicator

The venom is delivered with the applicators to a previously ventilated room where there is no air flow. The collection is done immediately or after prior drying in an air flow that does not exceed 40 °C. Drying is mostly done due to high humidity in the hive. This type of applicator is placed in boxes, the flow of hot air from the hair dryer is adjusted. To prevent the removal of venom from the glass, it is covered with a multi-layer gauze (2–3 layers). The criterion based on which it is known that the venom has dried is its brittleness during collection. Also, the venom can be dried naturally in the sun, which is soon after the end of the experiment. The venom does not lose its properties, with two thirds of the venom evaporating and one third remaining on the glass. The applicator with the dried venom is removed from the cassette and inspected. After that, we put the glass with the venom on the table in the laboratory over black paper. The venom is

removed with a razor blade at an angle of 45º, and vibrations remove the venom from the edges of the glass onto shiny white paper. The rate of removal of the venom from the glass must be such as to prevent excessive dispersal of the venom particles formed by scraping the venom from the glass. The scraped venom is concentrated on the ends of the glass applicator and on the paper. The venom is then transferred using a makeshift spatula made of tracing paper into a vial with black glass. Removing the venom from the glass of the applicator leads to a large dispersion of the powdery parts of the obtained product, which leads to their entry into the mucous membranes of the respiratory organs.

2.3 Recommendations for Bee Venom Collecting

The collection of venom in apiaries should be based on the beekeeping schedule. The time frame of the beginning, duration and intensity of the main pastures is different for different locations. The beekeeper, depending on the pasture and the location, should make a schedule and plan the time of venom collection in accordance with that schedule.

In the period of honey collection, when there is an intensive development of the bee community and the collection of honey and pollen, the collection of venom is not recommended. Only after the stabilization of the bee community and the decline in collection one can start with the collection of bee venom.

The optimal collection interval from the same bee colony is the period considered from the point of view of the development of venomous glands and the longevity of the bees. In this way, we can theoretically justify the optimal time of 20 days. Mostly empirically, it was found to be 15–20 days. Only with such a method of collection is it possible to maintain the main indicator of beekeeping - honey productivity at a normal level, and not reduce productivity. It has been established that the collection of venom carried out in accordance with the technological elements mentioned above increases the vitality of bees. First, it increases the flying activity of the bees, thereby increasing the intake of nectar. However, honey productivity does not increase due to increased honey consumption by the bee colony. In general, the effect of electrical stimulation of bees in the process of collecting venom is reflected in the activation of the physiological state of the bees and the metabolic intensification of the organism. The amount of fat in the body decreases. As a proof of this statement, there are some data of research conducted by the department (Khomutov et al., 1994), and other research (Giniytalin, 1990, Toyganbaev, 1994). The collection of venom in regimes of 5, 10 and 15 days was carried out by the authors and it was determined that electrical stimulation for 5 days reduces the intake of honey by 200-800g, pollen by 7.5%, reduces bee brood by 4–17%. In contrast, electrostimulation for 10–15 days results in an increase in the daily intake of nectar by 100-600g, pollen by 4–7% and brood by 8–42%. All this leads to an increase in the strength of the colony. It was also determined that the strength of the colonies after coming out of winter in the colonies where the venom was collected for 5 days was 1314% less than the control, while the colonies where the venom was collected for 10–15 days came out of hibernation stronger by 5–20% of control.

In the collection season, which is in the summer months, a bee colony gives an average of 0.2 to 0.3 mg of venom per day. The best results were obtained when bee venom collection was carried out every 14 days. Venom is actively extracted from one community by electrostimulation for up to 30 min a day. With the help of one electrostimulator,

venom can be extracted from three to four bee colonies in one day. Taking venom has a positive effect on bees, according to some researches, because it extends their life span and because during the extraction of venom by electrostimulation, the queen becomes stronger and egg laying increases, thereby the bee community increases.

On the treated societies, those from which the venom was extracted, a smaller occurrence of varroa (*Varroa jacobsoni*) was observed (see Fig. 2). Also, compared to other untreated societies, the amount of extracted honey is no less, which leads to the conclusion that the extraction of venom does not harm the queen and the bees (Katalinić, 1982).

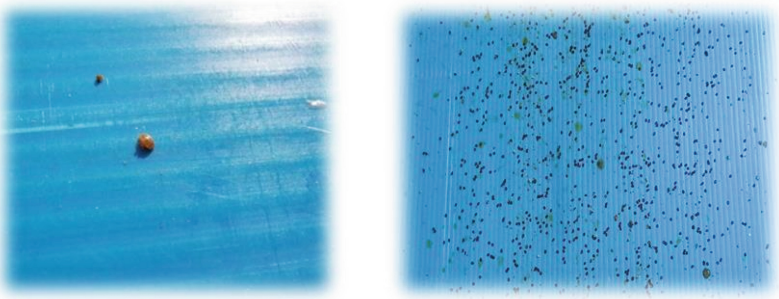

Fig. 2. Varroa (a - The hive where the venom was extracted; b - The hive where the venom was not extracted) (Šumar, 2022)

Special interest was shown in the indirect effect of electrostimulation on the bee colony. Various studies have shown that there is an increase in the strength of the society as a result of the increased production of eggs in the uterus. This increase may be a consequence of the process of collecting venom by stimulating the bees, since increasing their motor activity leads to an increase in the temperature in the hive and hence the improvement of the process in the uterus. In addition, during this period, an increased separation of lactating milk bees was noticed.

The described technology makes it possible to obtain 350 mg of bee venom in one session by using a set with two applicators in a medium-strong colony (3 kg of bees). In this way, for the entire spring-summer-autumn season (5–8 sessions), it is theoretically possible to obtain 2–4 g of raw venom without intensifying the process.

2.4 Venom Collection Problems

The biggest problem is getting the bees to sting the glass (see Fig. 3) and thus leave the venom on it. Many people think that current impulses will make them do it, but this is wrong. What makes the bees sting is their defense and the smell of the venom that has already been released. That smell signals danger and the bees become aggressive and angry. The signal irritates the bees to release the venom at that moment, and the pause allows them to change. Another big problem is the appearance of predation on the apiary, which will not happen if it is done carefully. The colonies in the apiary must be approximately equal in strength.

Fig. 3. Venom collection problems (Šumar, 2022)

3 Results and Discussion

During the research, 24 venom samples were collected using the classical method of electrostimulation, the results were subjected to statistical analysis. The obtained results were statistically processed using the analysis of variance without transformations, and the significance of the difference was assessed using the Duncan test. The calculation was performed with a significance level of $p = 0.05$.

The results of the masses of venom collected from bee colonies using electronic stimulation, depending on the strength of the colony, are shown in Fig. 4.

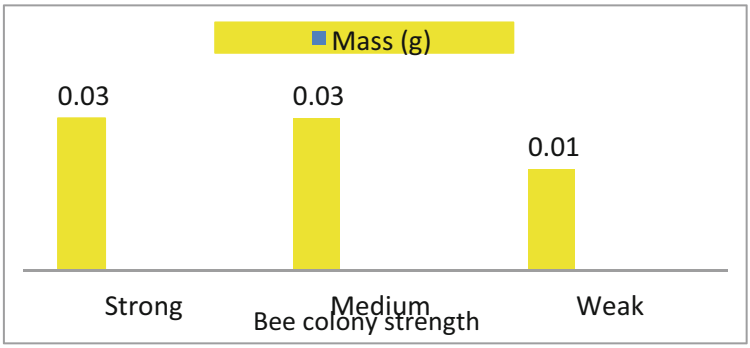

Fig. 4. Venom mass depending on the strength of the bee colony

The results obtained by the experiment show that strong communities produce a significantly higher amount of venom compared to others. Expressed as a percentage, the mass of venom collected from strong communities was 200% higher than the mass of venom collected from weak communities. There are not many literature sources on methods of collecting venom. Hence, for example Fakhimzadeh (1998) reports the effectiveness of 26 mg of dry bee venom collected from one bee colony for one hour. The device described by Nobre (1998) collects 60 mg of venom under the same conditions.

A statistically significant difference was found in the strong (0.03 g) and medium strong (0.02 g) group compared to the weak group (0.01 g).

The mass of bee venom varies depending on the time, as well as the day on which the treatment is performed. Thus, the mass of venom increased according to the days of collection, which explains that the intensity of grazing plays a big role as well as the number of bees in the hive. Therefore, the largest amount of venom was collected in the morning and evening hours, while in the afternoon it was the lowest, as shown in Fig. 5.

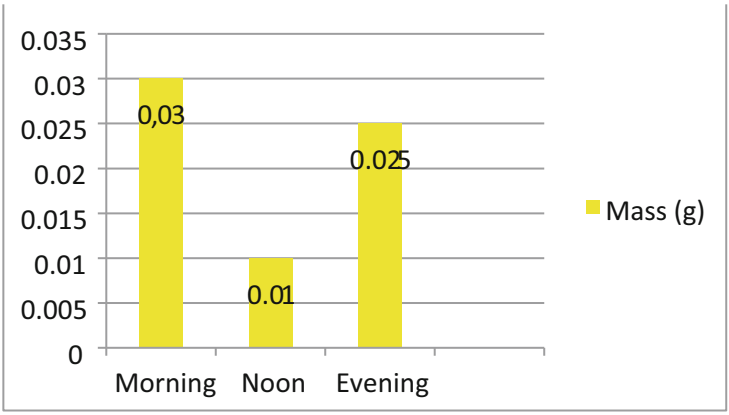

Fig. 5. The mass of the venom depending on the period of the day in which the venom was extracted

In the research by Omar (2017), the highest average mass of bee venom was collected in August, when 0.44 mg of bee venom per bee colony was determined, then in July.

0.42 mg, and the lowest average mass of 0.30 mg was found during February. Also, Hegazi et al. (2017) found that although there was no statistically significant difference in the mass of bee venom per bee colony collected during June in two consecutive years (2014 and 2015), the highest mass of bee venom was determined in the third week in both years. The mass of bee venom ranged from 48.9 ± 0.03 mg to 72.4 mg. For the purposes of the research, they used a device for collecting bee venom that works on the principle of intermittent pulses of electric current under a voltage of 12V and was placed on the top of the hive. They performed the experiment for 10 days every month for three hours a day. The bee colonies they used in their research were fed sugar syrup (1:1) and bee bread.

4 Conclusion

Bee venom is a highly valued product obtained from bees by various methods, of which the most effective and painless method is electrostimulation of bees, after which the bees remain alive and bee venom is obtained with the least impurities and in the largest quantities. Electrostimulators can be placed at the entrance of the hive and in the hive itself. A purer venom with fewer impurities is obtained when the apparatus is placed on the top bar which was impossible to test due to lack of equipment. Considering the strength of the bee colony, it was determined that strong bee colonies (0.03 g) give more venom compared to medium (0.02 g) and weak (0.01 g).

Based on the previous research, it was also determined that the colonies where the venom was extracted were healthier, due to the appearance of significantly smaller amounts of varroa. The societies in the apiary must be of approximately the same strength to avoid predation and the suffering of the entire apiary. And it is best to treat all the colonies in the apiary.

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An Overview of Using Algae Meal in Feeding Freshwater Fish Species

Senada Čengić-Džomba, Emir Džomba(✉), and Dženan Hadžić

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
s.cengic-dzomba@ppf.unsa.ba

Abstract. World aquaculture production of farmed aquatic animals grew on average at 5.3 percent per year over the period 2001–2018 (FAO, 2020). This trend imposes an increased demand for fishmeal and fish oil as the two major dietary ingredients used in compound aquafeeds. Global awareness of the need to manage aquatic ecosystems in a sustainable manner requires the use of alternative protein sources in fish nutrition as a replacement for the mentioned marine ingredients in aquafeeds. Due to the high content of proteins, whose amino acid composition is similar to the amino acid composition of fishmeal proteins, as well as the content of vitamins, natural carotenoids, polyunsaturated fatty acids and other bioactive substances, the use of algae as feed is one of the options for the complete or partial replacement of fishmeal in fish nutrition. High yields of algal biomass per unit area, their ability to grow in non-potable water, low production cost and cultivation in an environment-friendly way are crucial for development and sustainability of aquaculture. This overview highlights the impact of replacing fishmeal with blue-green algae Spirulina platensis in compound aquafeeds on the production performances of different freshwater fish species.

Keywords: algae meal · fishmeal · freshwater fish · spirulina

1 Introduction

Global production of aquatic animals was estimated at 178 million tons in 2020 (FAO, 2022), out of which 63% (112 million tons) came from oceans (70% capture fisheries and 30% from aquaculture), and 37% (66 million tons) from inland waters (83% from aquaculture and 17% from capture fisheries). An important share (around 20 million tons or 11% of overall production) is used for production of fishmeal and fish oil intended as feed for aquatic and terrestrial animals. In the last 10 years, average annual production of fishmeal and fish oil amounted to 5 million tons, while production of fish oil amounted between 0.8 and 1.3 million tons a year (EUMOFA, 2021).

Seventy percent of the produced fishmeal is used as feed for aquaculture, while a smaller part is used for pigs and chickens feeding in the early stage of their life. In aquaculture, about 30% of fishmeal is used as feed for shrimps, followed by feed for salmonids, with the largest share in the diet of young fish. 73% of fish oil is used

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in aquaculture feed industry, mostly for salmonid fish (Mallison, 2017). The trend of increased demand for aquatic organisms in the world, as well as the concerns regarding sustainability of aquaculture production, impose the need to replace the fishmeal with alternative sources of protein. Vegetable protein and proteins of animal origin from rendering and slaughtering have been used for many years as a substitute for fishmeal and have certain disadvantages. The main deficiency of animal protein is significant variation in quality, lower digestibility of protein, and in some cases, higher ash content. Although they do contain a high percentage of protein, the blood meal and the feather meal cannot satisfy the fish requirements for amino acids. In comparison with fishmeal, vegetable sources of protein have worse amino acid profile, meaning lower content of methionine, lysine and arginine. These deficiencies become even more apparent if they make a greater share in the diet of carnivorous fish, particularly younger groups that require a higher protein feed. In addition, presence of fiber and phytates in this feed negatively affects digestion. All of these are reasons that had prompted the research in seeking new sources of protein that could partially or completely replace fishmeal in the fish feed (Hardy et al., 2006).

A number of studies (Alagawany et al., 2021; Mahata et al., 2022; Nagappan et al., 2021) conducted over the past decades suggest that several kinds of microalgae: *Spirulina platensis*, Chlorella sp., *Spirulina maxima*, Dunaliella sp. And Ulva sp., could be a good source of protein, but also of other nutrients and bioactive substances in fish diet. Among the mentioned microorganisms, special attention has been devoted to blue-green algae Spirulina *(Spirulina platensis* or *Arthrospira platensis*), primarily due to high protein content which can be as much as 70% (Ramírez-Rodrigues et al., 2021), and beneficial amino acid composition (Masten Rutar et al., 2022). *Spirulina platensis*, compared to other algae, is the richest source of γ –linolenic acid (GLA) polyunsaturated fatty acid (PUFA) that has proven beneficial effect on reduction of low-density lipoprotein, treatment of eczema and mitigation of symptoms of pre-menstrual syndrome (Tanticharoen et al., 1994).

Besides protein, Spirulina also contains significant amounts of vitamins (B-vitamins) and minerals, and free-radical scavenging phycobiliproteins. Numerous scientific studies (Aber et al., 2015; Faheem et al., 2022. Zhang et al., 2020) have showed its anti-oxidant, anti-viral, anti-cancer and immune stimulating effect of Spirulina on fish health.

2 Comparative Overview of Nutritional Composition of Spirulina and Fishmeal

2.1 Basic Chemical Composition

Apart from having high content of protein (50%–70%), essential amino acids and essential fatty acids, Spirulina is also a rich source of vitamins and minerals. Thus, fish diets can include various shares of Spirulina. Comparative overview of main nutritional composition of Spirulina and fishmeal is shown in Table 1.

It is evident, from the results of the aforementioned research, that the protein content in Spirulina is quite high but also variable - the determined values ranged from 55.82% to 71.90%.

Table 1. Chemical composition of *Spirulina platensis* and fishmeal

Feed	References	Moisture, %	Crude protein, %	Gross energy, kcal/100 g	Ether extract, %	Crude fiber, %	Minerals, %	Calcium, %	Total phosphorus, %	Starch, %
Spirulina platensis	Alvarenga et al. (2011)[1]	11.92	58.20	428.60	2.60	0.78	8.44	0.48	1.06	dnr
	Saharan and Jood (2017)[2]	5.27	71.90	353.55	1.27	9.70	3.50	0.62	0.79	dnr
	Sharoba (2014)[2]	4.64	62.84	dnr	6.93	8.12	7.47	0.922	2.191	3.56
	Seghiri et al. (2019)[2]	12.66	76.65	436.18	2.45	4.07	1.56	6.00	10.088	dnr
	Raczyk et al. (2022)[2]	7.09	71.34	333.40	0.36	8.45	5.93	dnr	dnr	dnr
	Alagawany et al. (2021)[2]	6.98	56.79	dnr	8.33	4.25	10.05	0.363	0.123	dnr
	Ngakou et al. (2012)[2]	8.51	55.82	dnr	5.95	7.63	7.89	dnr	0.243	dnr
Fish meal herring*	NRC (1993))	8.00	72.00	dnr	8.40	0.60	10.40	2.20	1.67	dnr
Fish meal menhaden*	NRC (1993)	8.00	64.50	dnr	9.60	0.70	19.00	5.19	2.88	dnr
Fish meal white*	NRC (1993)	9.00	62.30	dnr	5.00	0.50	21.30	7.31	3.81	dnr
Fish meal	INRA (2004)	7.70	62.60	dnr	9.50	0.00	17.80	4.48	2.76	dnr

* mechanically extracted; [1]expressed on natural matter basis, [2]expressed on dry matter basis; dnr = did not report

Protein content may be affected by the algae growing conditions (medium and temperature). According to Yilmaz (2012), increasing temperature from 30 °C to 40 °C may, in addition to leading to growth variations, reduce the protein content in Spirulina from 64% to 59%. Matos et al. (2016) suggest that fibers in microalgae are present in the amount of 5%-18%. The crude fiber content in Spirulina, according to the authors mentioned in Table 1, range between 3.50% and 14.60%, while according to Nagapan et al. (2021), average fiber content in Spirulina is 8.5%. The characteristic of microalgae fiber is low content of hemicellulose and complete absence of lignin and cellulose in the cell walls, what significantly improves its digestibility. Predominant compounds that build the cell walls of *Spirulina platensis* include mucopeptides, acidic polysaccharides and lipopolysaccharides, and this makes Spirulina more digestible for fish compared to some other algae (Nagapan et al., 2021). Phytates and oxalates are not present in Spirulina (Masten Rutar et al., 2022).

2.2 Amino Acid Composition

Quality of protein depends on amino acid content. By comparing the amino acid profile of the Spirulina protein with amino acid profile of soybean meal, Alvarenga et al. (2011) found greater content of methionine, leucine, isoleucine and valine; approximately same content of arginine; and lower content of lysine in the Spirulina protein (Table 2). Yücetepe and Özçelik (2016) and Nagappan et al. (2021) reported similar results. The only difference found by Neumann et al. (2018), Bashira et al. (2016) and Montoya-Martínez et al. (2016) compared to the abovementioned authors is a greater content of lysine in the Spirulina protein.

By comparing the amino acid profile of Spirulina with amino acid profile of fismeal, Nagappan et al. (2021) found a greater content of aforementioned amino acids in the fishmeal. This difference is particularly significant in arginine – there is as much as 50% more of arginine in fishmeal than in Spirulina. Similar results were also found by Bashira et al. (2016), while Neumann et al. (2018) documents somewhat different results, showing greater content of valine, isoleucine, and leucine in Spirulina, and approximately the same content of lysine compared to the content of the same amino acids in fishmeal. A similar content of methionine and lysine in these feeds are also found by Montoya-Martinez et al. (2016). NRC (1993) states only the higher content of methionine in various kinds of fishmeal compared to the content of this amino acid in Spirulina, while the other amino acids were found in lower or similar amounts. In spite of the mentioned differences, it can be concluded that the amino acid profile of Spirulina is well balanced, and that Spirulina makes a much better choice than soybean meal to replace fishmeal in fish feed. Leucine and valine are major essential amino acids in Spirulina.

The research presented in Table 1 shows that the content of lipids (ether extract) in Spirulina dry matter ranges from 0.36% to 8.33%. According to Ambrozova et al. (2014), the lipid content in Spirulina ranges from 6.40% to 14.3% in dry matter. It seems that growing conditions, seasonal characteristics and technological drying processes can cause different deposition of lipids as well as differences in the fatty acids profile in Spirulina.

The profile of fatty acids in *Spirulina platensis*, as shown in Table 3, suggests that the dominant fatty acids are palmitic, linolenic and γ-linoleic (GLA) acids. Content of palmitic acid (C16:0) ranges from 33.30% to 51.54% (percentage of total fatty acid content), content of linoleic (C18:2n-6) ranges from 15.00% to 22.00%, and γ-linolenic (C18:3n-6) acid 12.90% to 27.60%. Stearic acid is also present in the amount of 1.06%-5.77%.

The total content of monounsaturated fatty acid (MUFA: C16:1 and C18:1) in *Spirulina platensis* amounted 9.45% (Widianingsih et al., 2013).

Mühling et al. (2005) finds that the Spirulina growth rate at various temperatures influences the content of three predominant fatty acids. Raising temperature from 200C to 300C increased the content of palmitic and linoleic acids (from 40.2% to 46.1% and 24.30% to 34.50%, respectively), while the content of the γ-linoleic acid decreased from 28.00% to 12.90%. Bhakar et al. (2014) examined the influence of different concentrations of $NaNO_3$ (1.0, 1.5 and 2.5 g/L) and K_2HPO_4 (0.2, 0.3 and 0.5 g/L) on the lipid

Table 2. Amino acids profile of the *Spirulina platensis*, soybean meal and fishmeals commonly used in fish feed

	Spirulina Raczyk et al. (2022)[1]	Spirulina Nagappan et al. (2021)[2]	Spirulina Alvarenga et al. (2011)[2]	Spirulina Neumann et al. (2018)[2]	Spirulina Bashira et al. (2016)[2]	Soybean meal Alvarenga et al. (2011)[2]	Fish meal Nagappan et al. (2021)[2]	Fishmeal herring[3]*	Fishmeal menhaden[3]*	Fishmeal tuna[3]*	Fishmeal white[3]*
Aspartic acid + asparagine	30.69	5.1	5.34	dnr	6.31	5.29	dnr	dnr	dnr	dnr	dnr
Glutamic acid + glutamine	49.63	7.8	8.15	dnr	8.47	8.65	dnr	dnr	dnr	dnr	dnr
Serine	20.35	2.6	2.92	dnr	dnr	2.42	dnr	dnr	dnr	dnr	dnr
Glycine	21.26	2.6	3.00	dnr	3.43	2.01	1.7	dnr	dnr	dnr	dnr
Histidine	27.39	0.8	1.00	1.28	1.13	1.38	dnr	1.05	1.45	1.75	1.34
Arginine	26.13	3.4	3.96	6.79	4.47	3.55	6.8	4.54	3.82	3.43	4.21
Threonine	39.61	2.7	2.84	4.39	3.31	1.85	3.5	2.90	2.50	2.31	2.57
Alanine	12.10	3.9	4.54	dnr	5.02	2.02	dnr	dnr	dnr	dnr	dnr
Proline	22.92	1.7	2.15	dnr	2.53	2.36	dnr	dnr	dnr	dnr	dnr
Tyrosine	20.34	2.1	2.58	dnr	3.07	1.74	dnr	2.20	1.94	1.09	1.94
Valine	27.89	3.0	3.34	5.87	4.21	2.03	4.0	4.20	3.22	2.77	3.02
Methionine	8.07	1.2	1.98	1.81	1.71	0.79	2.5	2.08	1.75	1.47	1.68
Cysteine	4.60	dnr	0.72	0.77	0.64	0.59	0.4	0.74	0.56	0.47	0.75
Isoleucine	25.34	2.9	3.06	5.07	3.64	2.04	3.7	3.13	2.66	2.45	2.67
Leucine	39.69	4.8	4.84	8.04	6.17	3.40	6.2	5.19	4.48	3.79	4.52
Phenylalanine	19.02	2.5	2.50	dnr	3.33	2.29	3.3	2.71	2.41	2.15	2.34
Lysine	22.62	2.5	2.72	3.91	3.40	2.80	3.7	5.57	4.72	4.06	4.53
Tryptophan	dnr	dnr	dnr	dnr	0.85	dnr	dnr	0.77	0.65	0.57	0.60

[1]expressed as mg AA/g dry matter, [2]expressed as g AA/100 g protein; [3]expressed as % on feed basis * NRC 1993; dnr = did not report

content and fatty acid profile of different Spirulina strains. Concerning *Spirulina platensis*, the results of this research showed that at lower initial concentrations of nitrogen and phosphorus, the amount of lipids was the highest and that $NaNO_3$ was more effective in increasing the total lipid content. The highest amount of GLA accumulated at a concentration of 1.0 g/L $NaNO_3$, while K_2HPO_4 had a greater impact on the profile of other fatty acids.

According to Turan et al. (2007) the total content of SFA in anchovy meal was 33.44%, with dominant palmitic acid in the amount of 20.31%. Average content of MUFA was 20.92% with predominant share of oleic acid (12.86%). Of the PUFA, the highest concentration of 18.3% was found for docosahexaenoic acid-DHA (C22:6 n-3), and 7.93% of eicosapentaenoic acid-EPA (C20:5 n-3). PUFA n-3 content (27.49%) was significantly higher than n-6 PUFA (4.54%) (all values are expressed as percentage of total fatty acid content).

Table 3. Fatty acids composition of *Spirulina platensis* and fishmeal

Fatty acid	Spirulina powder					Fishmeal protein, 65%[3]
	Raczyk et al. (2022)[1]	da Silva et al. (2019)[2]	Masten Rutar et al. (2022)[2]	Yordanova et al. (2015)[2]	Mühling et al. (2005)[2]	
C12:0		1.76				0.10
C14:0		1.00	0.35	0.45		6.00
C16:0	51.54	33.30	41.80	49.45	44.1–46.1	17.80
C16:1	2.88	3.24	6.93	3.20	2.9–3.9	7.20
C17:0	4.04	1.07	0.33			
C18:0	1.06	5.77	3.90	4.66	1.3–2.1	3.60
C18:1n-9	2.69	3.30	3.78	6.94	3.5–7.6	12.30
C18:2n-6	17.21	17.90	22.00	17.25	15.0–31.5	
C18:3 n-3		0.36		0.82		1.90
C18:3 n-6	19.30	27.60	19.40	17.23	12.9–28.5	
C18:4						1.50
C20:0		0.26				0.30
C20:1		0.11				6.60
C20:2		0.11				
C20:3 n-0		0.84				
C20:4 n-6		0.48				2.40
C20:5						9.00
C22:0		0.93				0.30
C22:1						7.70
C22:5						2.60
C22:6 n-3		0.55				6.60
C24:0		0.09				
∑ SFA	56.63	45.60				
∑ MUFA	5.57	6.60				
∑ PUFA	37.80	47.80				

[1]% of the spirulina powder; [2]Fatty acids are presented as percentages of the total amount of the methyl esters identified [3]INRAE-CIRAD-AFZ; SFA-saturated fatty acids; MUFA-monounsaturated fatty acids; PUFA-polyunsaturated fatty acids

3 Possibility to Replace Fishmeal with Spirulina in Feed for Freshwater Fish

Nandeesha et al. (1998) investigated the effect of replacing fishmeal protein with Spirulina (*Spirulina platensis*) protein on growth, carcass composition, activity of digestive enzymes and diet digestibility during 120 days in common carp (*Cyprinus carpio L.*) feeding. In four experimental diets fishmeal protein was replaced by Spirulina protein at amount of 25%, 50%, 75% and 100%. Control diet contained no Spirulina. In order to exam effect of Spirulina as solely source of protein they used one more (6th) experimental diet. Protein content in fishmeal and in Spirulina amounted 67% and 54.5% respectively, and fat content was 2% and 1%. All diets had approximately the same protein content (nearly isonitrogenous levels), and nearly equal energy value (14.59–14.79 kJ/g) except for the sixth diet, at which the energy value was 17.01 kJ/g. The results of this experiment showed no significant difference in body weight and length of the fish. Spirulina did not have negative effect on specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER). Net protein retention (NPR) was the best in fish fed with the diet where Spirulina was the only protein source. Negative influence of Spirulina was not found in organoleptic assessment of quality of raw and thermally processed fish. Digestibility of protein was even better than in case of fish fed with control diet. Results of this experiment show that Spirulina can be used as the sole source of protein in carp feeding.

Ramakrishnan et al. (2008) in 45 day feeding trial investigated effect of incorporation *Lactobacillus acidophilus*, *Saccharomyces cerevisiae* and *Spirulina maximus* into diets at concentrations of 1%, 2%, or 3% on common carp grow parameters. Control diet contained no supplement. Protein content in the control diet was 36.2%, while the diets enriched with Spirulina contained 40.6%, 41.4% and 43.2% protein. According to authors the best survival, growth, SGR, PER and FCR were achieved with diet containing 3% of Spirulina. There were no significant differences of carcass quality among the fish fed with different diets, although the greatest protein content in the carcass was obtained in the groups fed the diets containing Spirulina.

Faheem et al. (2022) investigated effect of supplementing diets with 0, 1, 5 or 10% of Spirulina on growth parameters, digestive enzymes, hepatic antioxidants, and immunity biomarkers of juvenile grass carp (*Ctenopharyngodon idella*). Fingerlings of average body weight 4.81 g were fed over 90 days with diets where the wheat flour, soybean meal, cottonseed meal and mustard cake were partly replaced with Spirulina. Results of the experiment showed the greatest growth, final body weight and SGR in fish fed a diet containing 5% of Spirulina. Mucus protease, lysozyme and peroxidase activity significantly increased in fish fed with 5% of Spirulina. Spirulina has significantly increased the enzyme activity. Activity of amylase was significantly higher in all experimental groups compared to the control group. Higher intestinal protease activity recorded in fish fed with 5 and 10% Spirulina and activity of lipase in fish fed diets containing 1% of Spirulina. Hepatic lipid peroxidation was significantly reduced in fish fed 1% and 5% of Spirulina, while a significant increase in hepatic superoxide dismutase was found in fish fed with 10% of Spirulina. Increase in mucosal lysozyme was also found in fish fed with 5% of Spirulina.

Replacing 15–20% of fishmeal with *cyanobacteria* decrease the growth rate of rainbow trout (Nagappan et al., 2021). Precise level of replacing the fishmeal or other feeds with microalgae depends on the fish species and pelleting conditions.

Rosenau et al. (2022) studied the possibility of total replacement of fishmeal with Spirulina in the feed of three salmonid species: rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta fario*). Initial average body weight of the fish was for rainbow trout 100.8 g, brook trout 111.8 g and brown trout 98.25 g. Brown trout was excluded from the experiment because of non-acceptance of the diet containing Spirulina, which was manifested in the loss of body weight after two weeks of experiment. The experiment results showed a significant difference in body weight among species (body weight differences among species were also found at the beginning of the experiment). In both species, body weight was lower in fish fed Spirulina containing diets, FCR was low in all groups, but lower in groups fed the fishmeal. In case of brook trout, high variation of body weight of fish fed with Spirulina was detected, suggesting that individual fish convert more efficiently the Spirulina protein or consume greater quantity of feed, which can be caused by genetic factors. Color of the filets (yellow and red) of both species was intensified as a result of adding Spirulina (the coloring was greater in brook trout). Concerning fatty acids, Spirulina containing diets reduced the content of PUFA including n3 fatty acids, increased the SFA content, and showed no significant influence on MUFA content in the muscles. The results showed significant differences in the content of different fatty acids in fish fed different diets, and negligible difference among different species. The EPA was reduced in both species, while DHA was reduced only in brook trout. Although both EPA and DHA were low in fish fed Spirulina containing diets, this difference was not detected in muscles, and this can be explained by the fact that rainbow trout can synthesize PUFA up to a certain amount.

In feeding trial with commercial (control, crude protein 39.67%) and experimental diet (crude protein 41.44%), where 10% of commercial diet was replaced with Spirulina Sirakov et al. (2012) found better weight gain, length, SGR, average daily growth and FCR in rainbow trout fed the diet containing Spirulina.

Results of another study (Hernández Flores et al., 2012) suggest that total replacement of fishmeal with 25% soybean meal and 75% of Spirulina in feed for juvenile rainbow trout improves the serum lysozyme activity and reduces the amount of phosphorus in faeces. Although there were no statistical differences among groups fed different diets (75% Spirulina and 25% soybean meal, 50% Spirulina and 50% soybean meal, and 25% Spirulina and 75% soybean meal) regarding to growth indicators, fish fed with fishmeal had strongest growth. Concerning replacement diets the best results were achieved with a mixture of 75% Spirulina and 25% soybean meal.

El-Sheekh et al. (2014) in 65 day feeding trial investigated the effect of replacing 0%, 50%, 75% and 100% of fishmeal with spirulina (*A. platensis*) in hybrid red tilapia fries (*Oreochromis niloticus* x *Oreochromis mossambicus*) diets. The control diet did not contain Spirulina. Experimental diets were iso-energetic (473 kcal/100 g) and iso-nitrogenous (36% CP).

The best growth performances (FW, WG, ADG and SGR) were obtained by replacing 75% of the fish meal with Spirulina. Slightly less favourable results were obtained by

substituting 50% of fishmeal, which were still better compared to diets without Spirulina. Lower mortality was recorded in fish fed with diets in which the replacement of fishmeal was 75% and 100%. The highest feed intake, FCR and PER value, as well as the highest red and white blood cells count were recorded in fish fed with 75% Spirulina.

Mahmoud et al. (2018) found that addition of 1% Spirulina more effectively increases bactericidal, phagocytic and lysozyme activities conferring protection against infection and significantly improves immunity of Nile tilapia against *Pseudomonas fluorescence* comparing to addition of 2% Spirulina in the diets.

Rosenau et al. (2021) explored the possibility of total replacement of fishmeal (20% in the feed) with Spirulina in the diet of African catfish (*Clarias gariepinus*) during a 10-week experimental period. The results of the experiment showed significantly lower final body weight and significantly higher values for the yellow skin and fillets color of fish fed with Spirulina. Higher values for C20:5n3 and C24:0 in fish fillet were found in the group fed with fishmeal, while C16:0 was more prevalent in fish fed with Spirulina. No significant differences were found in the content of C18:1n9 and C18:2n6 between the groups. The slight increase in n6/n3 ratio in the fillets observed in spirulina fed fish reflects the fatty acid composition of the diets.

4 Conclusion

In spite of being mildly inferior compared to standard protein (with 80% limiting amino acids), the Spirulina protein is significantly better than almost any other plant protein. One important advantage of Spirulina is that there are no limiting factors for utilization of amino acids. Full replacement of fishmeal in feed for some salmonid species results with poorer FCR (compared to the fishmeal fed fish), what can be explained by less efficient decomposition of cell walls of Spirulina in gastro-intestinal system of fish.

Significant variations in the final body weight in these species indicate a more efficient conversion of spirulina protein into body protein or a higher intake of spirulina by some individuals, which implies a possible influence of genetic factors. Therefore, breeding programs might increase the efficiency of using Spirulina in salmonid nutrition.

Change of the filet colour to yellow, as a result of carotenoid content in Spirulina, might cause rejection of the product because in European countries the desired colour of trout filet is white or red/pink. Complete replacement of fishmeal with Spirulina also results with reduced n3-PUFA in muscles. Partial replacement of fishmeal with Spirulina, or combination of Spirulina with soybean meal achieves satisfactory performance in these species of fish. In some herbivorous fish species, the complete replacement of fishmeal with Spirulina gives satisfactory results. By partial replacement it is possible to achieve even better growth performances comparing to the control. In general, partial replacement of fishmeal with Spirulina improves growth parameters, immune status and antioxidant response in fish. Precise ratio of replacement of fishmeal or other feeds with Spirulina, varies depending on the fish species and pellet manufacturing conditions. The price of Spirulina on the market is still quite high and is one of the main obstacles to the greater use of this algae in fish nutrition.

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Agro-Ecological Zoning as the Basis for Planning Agricultural Production in the Area of the City of Tuzla

Adela Mujić(✉) and Melisa Ljuša

Faculty of Agriculture and Food Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
adela.mujic@untz.ba

Abstract. In addition to economic and social circumstances, climate conditions, soil properties and their production potential are the main initiators of agricultural production and its economic success. In the planning of agricultural production in a certain area, it should be considered that certain agricultural crops on different soil types thrive differently in the given agro-ecological conditions. The main goal of this research is to show the natural potential of certain soil types and subtypes in the City of Tuzla, to group them into agroecological zones, and to perform spatial distribution and optimization of production based on the characteristics of individual soil types and requirements of individual crops. In this research, the FAO Agro-Ecological Zoning (AEZ) methodology was applied, with a focus on soil and terrain parameters. Considering the soil parameters (pH, texture, humus) and the terrain slope, specific limitations for the cultivation of agricultural crops were identified. The average soil pH value in the Tuzla area ranges from 5.3 to 8.4, the soil is quite clayey while the average content of humus in the area of the city of Tuzla ranges from 0.93 to 9.48%. Following the requirements of the crops and combining the abovementioned soil parameters and the slope, suitability for the cultivation of chokeberry and blueberry was determined and presented on the maps. The results of this study on chokeberry indicate wide range of suitability levels from the very suitable to unsuitable. When it comes to blueberries production, suitability ranges from moderately suitable to unsuitable. The application of the AEC-GIS methodology resulted in a map of maps showing the general advantage for agricultural production from the housing content of humus, pH and texture, and slope gradients. The second set of cards, obtained by the combination of humus, pH and texture, and according to crop requirements, shows the benefits of growing aroma and blueberry. These maps can serve as a very important axis-new for planning the development of agriculture, as they are based on land resources estimates.

Keywords: agro-ecological zoning · agricultural production · planning · sustainability · soil parameters

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1 Introduction

As stated in the Draft Rural Development Strategic Plan for Bosnia and Herzegovina (2018–2021), the agriculture sector is an important link in the overall economic structure of Bosnia and Herzegovina (BiH). The share of agriculture in GDP is about 8% and is ranked as the fourth branch of the economy (BHAS, 2019). The percentage of agriculture in total employment is relatively high and amounts to about 19% of the working-age population (BHAS, 2019). In addition, a large number of the population is engaged in agricultural production as an additional occupation for the production and provision of food for their own consumption. There are three basic resource factors for agricultural production: climate, soil, and water (Čustović and Ljuša, 2017). Physical, chemical and biological properties of the land, including topography and climate, together affect soil potential, agritechniques and knowledge of people on productivity, i.e. food production capacity (Vukadinović and Vukadinović, 2018). According to the Spatial Plan of the Tuzla Canton for period 2005–2025 agriculture in the area of Tuzla is very important to the overall economic development and food supply of the population. In crop production in the area of the City of Tuzla, the leading products are cereals (corn), potatoes, vegetables (tomatoes, cucumbers, peppers), fruits (apples and stone fruits), and berries. Agricultural producers in the Tuzla area show a great interest in growing chokerberries and blueberries. Because of its healing properties, it has been increasingly sought after, so that even more farmers are responsible for its cultivation. The fruits of the aromatic are rarely consumed fresh, but they are an excellent raw material for blended juices, and as such they currently have a significant economic value. Due to the great interest in chokeberry, the city administration launched several projects through which planting material was donated. On the other hand, the demand for blueberry is quite high both on the host and on a foreign market. Due to the low-level and high ransoming prices, farmers are increasingly reluctant to grow blueberries. According to Kurtovic and César (2016), three commercially important blueberries are present: Northern high-level blueberries (V. Corymbosum L.), rbitacea - rabbit eye - blueberry (V. Ashei Reade), blueberry (V) Angustifolium Ait., V. Myrtilloides Michx.). Data on vegetables under the chokberries and blueberries in the Tuzla area do not exist although the average yield of chokeberry is 0,70 to 0,80 kg per plant in the first two years of production (Kurtović et al., 2017). When it is about blueberries, the average yeald per plant is 3.5 to 5 kg, which certainly depends on the age of the seedlings (Kurtović et al., 2016). In the Spatial Plan of the Tuzla Canton for period 2005–2025, possible strategic orientations in agriculture by the municipality are presented. Organization of agricultural production based on healthy food, encouragement of rural entrepreneurship, promotion of intensive fruit growing, livestock, vegetable growing, beekeeping, etc. This orientation for Tuzla is certainly acceptable, given that the potential of agricultural land is not the same throughout the territory and it is not intended for every type of agricultural production. The geographic information system (GIS) has proved to be an excellent tool in Agroecological zoning that enabled connecting different parameters, modelling by-Datak and the preparation of thematic maps (Ljuša and Čustović 2010). In the AEZ approach, fruit crops play a key role with their specific requirements in relation to the values of climate, soil and terrain parameters (Kurtović et al., 2013). The main goal of this research is to show the natural potential of individual types and subtypes of soil in the area of the City of Tuzla, to group

them into agro-ecological zones, and to present spatial distribution and optimization of production by combining the characteristics of individual soil types with the requirements of chokeberry and blueberry for its growth. Furthermore, potential planning is possible from the point of view of socio-economic and social aspects and the needs of the population of the City of Tuzla.

2 Material and Methods

The research was carried out based on the existing spatial data. Soil map and data in scale of 1:25000, Land use value map in scale of 1:10000, and Soil bonity map in scale of 1:10000 were used as the main sources of data. These maps are an integral part of the GIS database prepared within the project "Multipurpose evaluation of land use and protection and creation of a land use value map for the area of the City of Tuzla" implemented by the Faculty of Agriculture and Food Science, University of Sarajevo (Čustović and Ljuša, 2016). The analysis of climate parameters (temperature, rainfall, length of vegetal range hood) is based on Tuzla's meteorology station. The data were obtained from the Federal hydro-meteorological office of Sarajevo for the period 1961–2015. The FAO AEZ (Biancalani et al., 2004) methodology applied in this research is focusing on soil parameters (pH, texture, humus), and slope. The system of Agroecological zoning (AEZ) is used to determine specific limitations for the cultivation of crops in certain conditions of climate, soil, and terrain, but also in certain (assumed) conditions of investment and management (FAO, 1996). Selected soil parameters were analyzed and a set of thematic digital maps were prepared. Limitations for growth of chokeberry and blueberry were determined based on their requirement for growth (Tables 1 and 2). These parameters were compared with the requirements of the crops, which resulted in maps of the suitability for the production of selected crops. The levels of suitability for crops are as follows: S1-very suitable (no significant limitations to sustained application of a given use, or only minor limitations that will not significantly reduce productivity), S2-moderately suitable (the limitations will reduce productivity or benefits and increase required inputs), S3-marginally suitable (land having limitations which in aggregate are severe for sustained application of a given use), and N-unsuitable.

Table 1. Chokeberry and requirements according to parameters

Parameters	S1	S2	S3	N
pH	5,0–8,0		4,5–5,0	< 4,5; > 8,5
Texture	Loam, sandy loam, silt loam		Sandy clay loam, silty clay loam, sandy clay	clay
Humus	> 3	1–2	< 1	
Slope	0–30		> 30	

Table 2. Blueberry and parameter requirements

Parameters	S1	S2	S3	N
pH	3,8–4,5		4,5–5,5	< 3,8; > 5,5
Texture	loam, sandy loam, silt loam		sandy clay loam, silty clay loam, sandy clay	clay
Humus	> 3	1–2	< 1	
Slope	0–30		> 30	

3 Results

In this chapter, the results of the research are presented: soil characteristics, requirements for the cultivation of agricultural crops, the slope of the terrain, and planning agricultural production based on the results obtained.

3.1 Pedological Characteristics of the City of Tuzla

In the soil map of the City of Tuzla M 1:25000, which is shown in Fig. 1, 21 (Automorphic and Hydromorphic) soil types are represented (Čustović and Ljuša, 2016). In the territory of the city of Tuzla, the following soils can be distinguished from the group of Automorphic soils: non-carbonate and carbonate Smonica, District brown leached soil on clays, Loams and Sandstones, District brown soil on clays and clays, Distric brown soil on sands and sandstones, Eutric brown soil on Marls and Limestones, Eutric brown carbonate soil on conglomerates, Clays, Sandstones and Marls, Eutric brown soil on clays and clays, Eutric brown soil on Serpentines, Eutric brown soil on slates, Eelosols leached, Eelosols typical, Pelosol typical, Pelosol leached, Leached typical soil on clays, Clays and clayey sandstones, Primary pseudogley, Secondary pseudogley on the slope, Secondary pseudogley on the plain, Deposol, Cinerosol. When we talk about the group of hydromorphic soil types, the following are represented on the territory of the city: Alluvial carbonate sandy soil, Humofluvisol, non-carbonate, Humofluvisol carbonate deeply weathered, Eugleous-hypogleic, non-carbonate, which were described in detail by Resulović et al., 2008.

3.2 Agro-Ecological Conditions for Agriculture Production

When planning agricultural production, agroecological conditions are the most important factors. None of the factors work independently, but in interaction with others, so it is necessary to pay attention to each individually so that the agricultural production plan could work. Restrictions in the soil can be multiple, and refer to the pH reaction, texture, and humus content. Some of these restrictions can be removed or fixed.

3.3 Soil Reaction – pH Value in H_2O

For most of the crops that are grown in our country, the optimal pH value of the soil in H_2O is approx. 6.5. However, the acidic reaction of the soil, pH < 5.0 and alkaline >

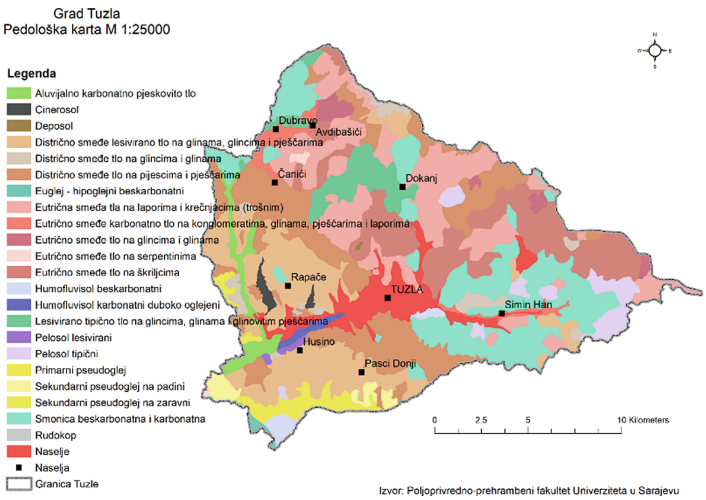

Fig. 1. Soil map of the City of Tuzla

7.5 can have a significant impact on the yields of some of the cultivated crops because in these environments specific problems of nutrition and the dynamics of macro and micronutrients occur. This is why pH plays an important role in defining the suitability of the soil for the cultivation of an agricultural crop. Table 3 shows the levels of soil suitability based on pH in H_2O, and for cultures that are sensitive to a high pH reaction.

Table 3. Soil suitability levels based on pH in H_2O

pH value	Suitability levels
6,5 – 7,0	S1 – very suitable
7 – 7,5; 5,5 – 6,5	S2 – suitable
7,5 – 8; 5 – 5,5	S3 – moderately suitable
>8; <5	N – marginally suitable

Soils that have a pH reaction in the water of 6.5 - 7.0 have almost no restrictions in terms of pH reaction (S1). Soils with a pH reaction of 7.0–7.5 and 5.5–6.5 belong to the S2 class, 7.5–8 and 5–5.5 to the S3, and $>$ 8 and $<$ 5 to the N class. Soils with a pH greater than 8.2 have serious limitations for certain crops sensitive to chlorosis or nutrition, primarily phosphorus, and some microelements. The average soil pH value in the Tuzla area ranges from 5.3 to 8.4, that is, from moderately acidic to very alkaline soils (Fig. 2). With calcification measures, acidic soils can very quickly get a neutral reaction and thus increase their fertility.

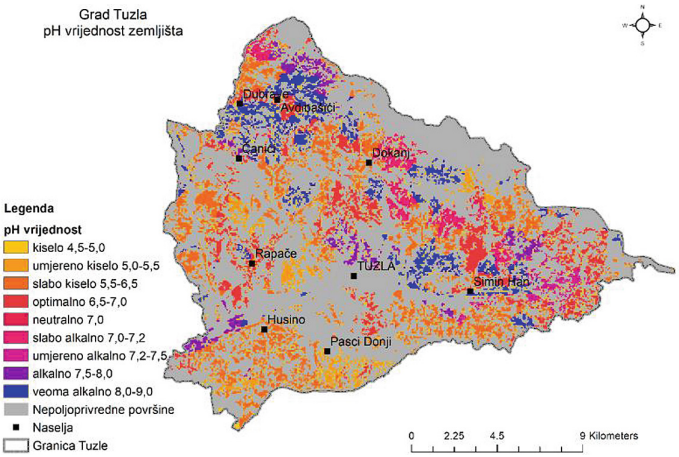

Fig. 2. pH values of soil in the City area

3.4 Soil Texture

Soil texture is defined by the percentage ratio of sand, powder and clay fractions (sand 2.0 - 0.06 mm, powder 0.06 - 0.002 mm, clay < 0.002 mm). The S1 convenience class includes lands that are loam, sandy loam, silty clay loam, and sandy clay loam. In the S2 class of convenience, silt, silty clay, silt loam, sandy clay, and clay loam are included. The S3 class includes clay and loamy sand, and the N class includes sands or surface rocky shallow and skeletal soils with $> 50\%$ skeleton in the profile. In the area of Tuzla, the soil is quite clayey, which is a limiting factor for plant production (Fig. 3).

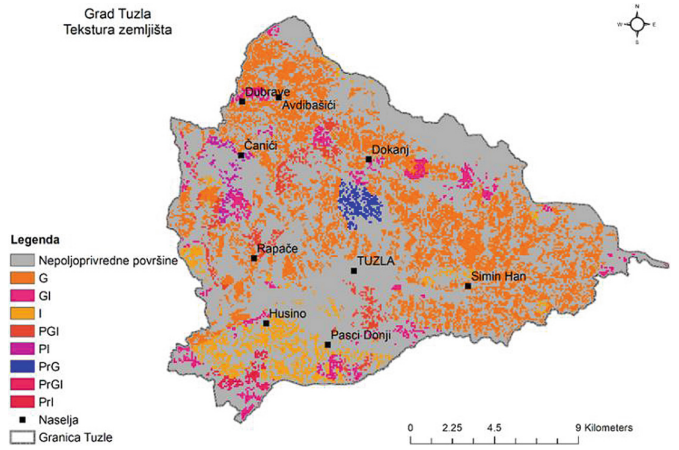

Fig. 3. Map showing the texture of the land in the area of the City of Tuzla

3.5 Humus Content

The role of humus in the soil is widely known, not only from the point of view of plant nutrition but above all the establishment of a favorable structure and biological activity of the soil. Based on the percentage of humus content of land in the City area, surfaces with the following humus content can be classified into the classes of benefits shown in table 4.

Table 4. Levels of soil suitability shown according to the humus content

Humus content	Levels of soil suitability
> 3%	S1
2–3%	S2
< 2%	S3

Humus content could not be a factor that would classify soil as unsuitable, except in the case of sand and completely skeletal soils. Organic matter can be introduced into the soil, and thus compensated. The average content of humus in the investigated soils in the area of the city of Tuzla ranges from 0.93 to 9.48% (Fig. 4). It is possible to increase the low content of humus using humidification measures, by sowing siderates or by adding organic matter to the soil, and in this way influence the increase in soil fertility.

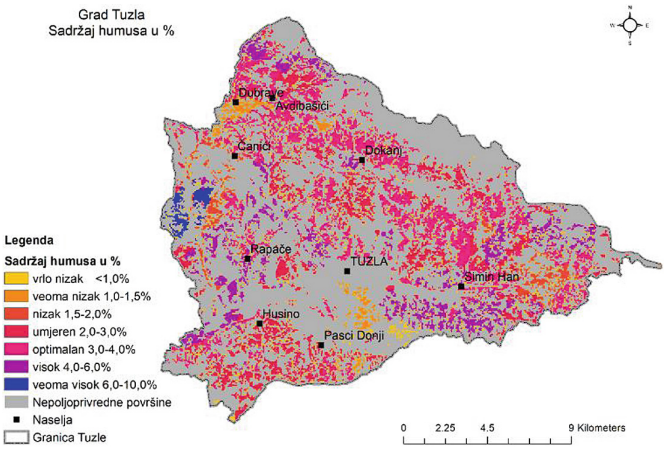

Fig. 4. Map of humus content in % in the area of the City of Tuzla

3.6 Slope of the Terrain

The slope of the terrain has a very significant role in the assessment of agroecological conditions for agricultural production and is directly related to the plant cultivation

system. The suitability of the land for the cultivation of field crops, vegetables, and fruit crops is directly related to the slope, that is, the greater the slope, the smaller the choice of cultivated crops. The mechanized system of crop cultivation rationalizes, increases the volume, and makes agricultural production cheaper, which makes it far more competitive on the market. 0–5% - flat and slightly undulating lands in terms of limitations for the cultivation of all crops, flat lands have no limitations and are ideal for all forms and systems of cultivation, i.e. LUTs. 5–10% - slightly sloping and undulating lands - have very few limitations, that is, almost no limitations for the crops that are grown in our areas. 10–15% - slightly sloping land - land with certain limitations, so that for certain crops appropriate leveling measures and leveling of the terrain are required. They have some influence on LUTs, but not enough to exclude some of the possible breeding systems. 15–30% - moderately sloping - For most cultures, these lands have limitations for certain forms of cultivation and require appropriate technical measures of land management such as terracing. 30–60% - sloping lands - lands have an eliminative character for most cultivated crops and require more extensive and complex land management measures. 60–80% - very sloping land - This slope is limiting for the mechanized cultivation system of almost all agricultural crops and has a direct impact on LUTs. > 80% - extremely sloping land - This is the slope of the terrain on which the only possible extensive use of the land as pasture is where the traditional grazing system with goats and sheep can only be applied (Biancalani et al., 2004).

In the area of the city of Tuzla, the slope of the terrain is represented in four categories: < 5%, 5–12%, 12–15%, and > 15% (Fig. 5). According to the slope of the terrain, we can say that lands suitable for cultivation (<5% and 5–12%) are represented in the area of the settlements of Rapace, Sicki Brod, Plane, Mihatovici, Brgule, Pasci Gornji, Ljubace, and some land near the settlements of Simin Han and Dokanj. Lands with higher slopes (12–15% and > 15%) have limitations for the cultivation of agricultural crops, as well as the use of mechanization.

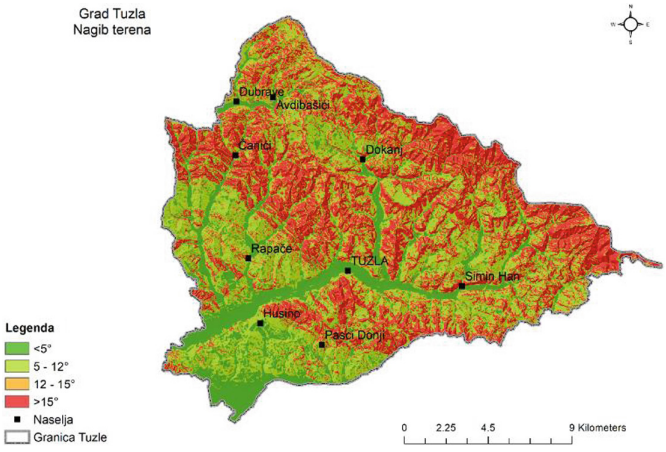

Fig. 5. Map of the slope of the terrain of the City of Tuzla

3.7 Climate Characteristics

The Tuzla area has a moderate-continental climate with certain specificities caused by the local relief and position in relation to the dominant regions in the surrounding area. The mean annual temperature for the last 40 years is 10oC. The average annual rainfall in Tuzla is 894 l / m2. The most precipitation occurs in spring and summer, so June is the month with the highest average amount of precipitation, 111 l/m2. For sum T > 50C, the length of the vegetation period lasts 253 days. The sum of active temperatures for the mentioned vegetation period is 3,494.2 °C. For sum T > 100C, the length of the vegetation period lasts 190 days. The sum of active temperatures for the mentioned vegetation period is 3,015.2 °C. As for the temperature conditions and average precipitation for the cultivation of chokeberry and blueberry, in the wider area of Tuzla they are favorable, and for that reason they are not elaborated in detail in the paper.

3.8 Suitability for Growing Agricultural Crops: Chokeberry and Blueberry

Chokeberry is an autochthonous plant of the North American ecosystem, more precisely, it originates from the area of Canada and Florida, where it grows as a wild species, on wet and acidic soils, with an annual rainfall of 1000 to 1200 mm. It includes the following species: black chokeberry (*A. Melanocarpa*) and red chokeberry (*A. Arbutifolia*), and a species created by crossing the previous two – chokeberry with purple-colored fruits (*Aronia x prunifolia*) (Kurtović et al., 2017). Black chokeberry plants can also be grown in mountainous areas since they are enviably resistant to low winter temperatures, and due to late flowering in spring, there is no danger of their generative organs freezing due to late spring frosts (Strik et al., 2013). The optimal soil pH value for the successful cultivation of chokeberry is 6, and given that it has a weakly vigorous root that penetrates the soil shallowly, it can also be grown on surfaces with a higher level of groundwater (0.6–0.7 m below the surface of the soil) which most other fruit trees cannot tolerate due to the asphyxiation of the underground system. It is a heliophyte culture, which requires a lot of light and should be planted in sunny positions (Gluhić, 2018). On the map of the benefits of chokeberry cultivation in the area of the City of Tuzla (Fig. 6). The levels of convenience that are prepared according to the parameters required by this culture are presented. Very suitable land (S1) is located in the local communities of Bukinje, Husino, Pasci, and very little in the local community of Dobrnja. The following soil texture dominates the mentioned area: loam, sandy loam, and silt loam. The pH reaction of the soil ranges from 5.0 to 8.0, the humus content is greater than 3, and the slope is from 0–30. These areas of the City of Tuzla are distinguished by the best soil for growing chokeberry, and the soil type is District brown leached soil on clay, loam, and sandstone.

Moderately suitable land (S2) and marginally suitable land (S3) for growing chokeberry is located in the local communities of Sicki Brod, Mramor, Caklovici (Donji and Gornji), Grabovica, Gornja Tuzla, Dokanj and Solina. Soils (level S2) consisting of silt, silty clay, silt loam, sandy clay and clay loam containing 1–2% humus are represented. This land and the area of the city of Tuzla is suitable for growing chokeberry, and the result of production will be a high-quality yield. Marginally suitable soils - S3 for growing chokeberry in the city area are sandy clay loam, silty clay loam and sandy clay. The

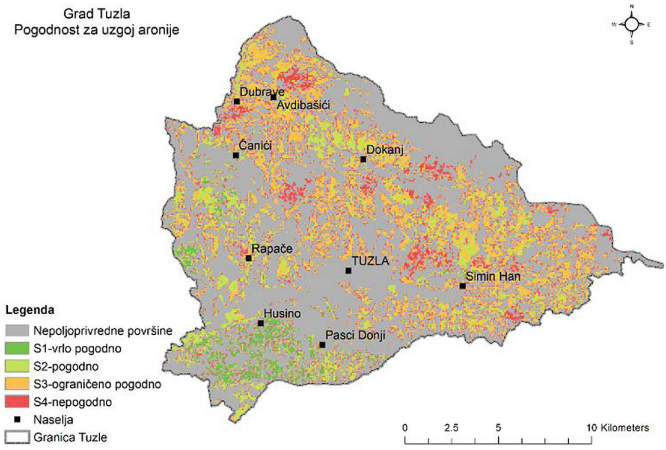

Fig. 6. Map of the advantages of chokeberry cultivation in the area of the City of Tuzla

pH value ranges from 4.5 to 5.0, the humus content is less than 1%, and the slope is greater than 30. Chokeberry can be grown on such lands with adequate agrotechnical land improvements. Unsuitable land (N) for growing chokeberry in the area of the City of Tuzla is represented from the central to the northern part of the city, namely the settlements: Avdibasici, Dubrave, Lipnica, Dokanj, Solina and the area around Simin Han. These soils are characterized by a soil pH value of less than 4.5 and greater than 8.5, and the texture of the soil is clay. Cultivation of chokeberry is not recommended on such lands, but the biggest limitation is the pronounced slope of the terrain.

In the territory of BiH, wild blueberry is the most common, which grows in the higher areas of almost all our mountains. For agricultural production, tame varieties, selected blueberries, with larger fruit are the most important. Kurtović et al., 2016 state that these genotypes are much more fertile, but their fruits have colorless juice and little or no aroma, which are not characteristics of the local forest blueberry. Regarding the cultivation of highbush blueberry on different types of land, it is important to point out that a good medium for blueberry growth is positively correlated with the amount of sand and negatively correlated with the amount of silt and clay. The optimal pH value of the land for blueberry cultivation varies in interval from 4.5 to 4.8. Relief depressions, in which cold air is trapped, should be avoided. Shallow, poorly drained land, as well as land prone to flooding, are not suitable for raising blueberry plantations (Kurtović et al., 2016). Blueberries grown in BiH have a good price and are sustainable because there are no fresh blueberries from other countries on the market during their ripening period, which certainly gives them an advantage. When we talk specifically about the area of the City of Tuzla, there are no statistical data about the areas planted with blueberries. The reason for this is that the land has very little land that is suitable or moderately suitable for the cultivation of this crop, and the repair of the land, for the producers, would require a lot of material and financial investments. Interpreting the suitability map for blueberry growing in the area of the City of Tuzla (Fig. 7), we notice that no land belongs to the S1 level of convenience, that is, there is no very suitable land for blueberry cultivation.

This tells us that there is no land with a pH reaction of 3.8–4.5, which is the ideal acidity for growing blueberries. Moderately suitable lands (S2) are mostly represented in the area of settlements of Husino, Kiseljak, and Pasci, while smaller areas of these lands are also represented in the settlements of Brgule and Avdibasici. The pH reaction of the soil in this area is 4.8, and the humus content is 1–2%. These lands in the area of the City of Tuzla represent the most favorable lands for growing blueberries. With proper cultivation technology, blueberry plants can achieve yields of 3.5 to 5 kg/plant, which certainly depends on the age of the seedlings. Marginally suitable land (S3) for growing blueberries is mostly spread over the entire surface of the City of Tuzla. The pH value of these soils ranges from 5 to 5.5 (Fig. 3). Kurtović et al. (2016) in their book "Strawberry" state that soils with higher pH values can be improved by adding acidic components. The humus content on these lands is less than 1, while the slope is greater than 30, which is a distinct limitation for intensive production. Land unsuitable for blueberry cultivation (N) in the area of the City of Tuzla is located in the area of the settlements: Caklovici Gornji, Gornja Tuzla, Solina, Lipnica Gornja, Dobrnja. These soils have a large slope (>30), soil pH is < 3.8 and > 5.5, and the texture is clay. When the soil has a pH value lower than 3.8, it can cause burns on the edge of blueberry leaves, while soils with a pH > 5.5 exhibits a strong buffering capacity, which is why, despite the addition of acidic components, the pH value of the soil cannot be permanently changed. For this reason, it is better to avoid such lands for raising blueberry plantations (Kurtović et al., 2016).

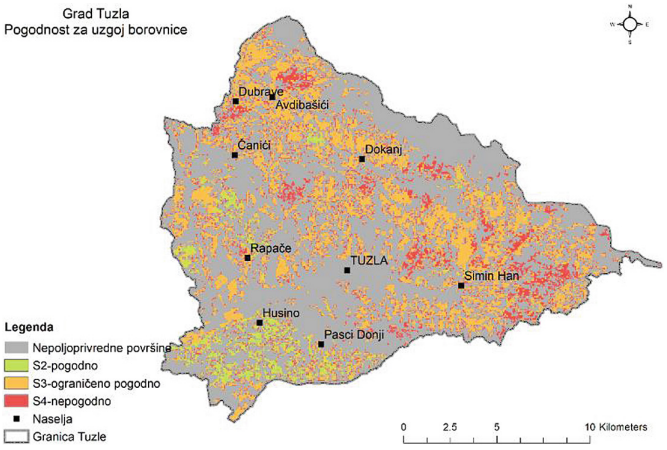

Fig. 7. Map of benefits for growing blueberries in the area of the City of Tuzla

4 Conclusion

Agriculture in the area of Tuzla is very important to the overall economic development and food supply of the population.

In addition to economic and social circumstances, climate, soil properties and their production potential are the main drivers of agricultural production and its economic

success. In the process of planning agricultural production in a certain area, it should be kept in mind that certain agricultural crops on different soil types succeed differently in given agroecological conditions. For this reason, agroecological zoning of agricultural production is one of the key factors and the basis of the success of this economic branch. When assessing the level of land suitability for the area of the City of Tuzla, special attention was paid on climate factors (temperature, precipitation, length of vegetation period), and to the following soil parameters: pH value, texture, humus content, and slope as terrain factors. To determine the limits for the cultivation of agricultural crops, the system of agroecological zoning (AEZ) is used, with the use of GIS. From the point of view of chokeberry and blueberry production, the climate is favorable. Analysis of soil parameters (ph, texture and humus, and terrain slope) determined very suitable and moderately suitable growing conditions required by chokeberry for successful production are located in the areas bordering the city of Zivinice (Local communities Husino and Pasci), the municipality of Lukavac (Local communities Sicki Brod and Bukinje), and at the foot of the Majevica mountain (Local communities Dokanj, Simin Han, Caklovici). Although chokeberry could great economic importance, which is reflected in the quick return of invested funds, but also in highly sought-after fruits on the market, that the production of chokeberry is not particularly prevalent in the area of the City of Tuzla. The results of this study on blueberries indicate that there are limitations to the cultivation of this crop. Suitable land for blueberry cultivation is in the area of Local communities Husino and Gornja Lipnica (a small area). The rest of the city's surface mostly has an alkaline pH reaction, which does not suit blueberries. Although this crop is in high demand on the market and has a good price, its cultivation is quite limited in the area of the City and there are no statistical data on the area under blueberry in this area. The application of the AEZ model in GIS resulted in a set of maps that show suitability for growing chokeberry and blueberry. The obtained research results can serve as a good basis for planning agricultural production. The application of AEZ results in the planning of agricultural development in the area of Tuzla can contribute to investment security and the long-term perspective of agricultural production in this area, which is certainly the most important motive for successful planning.

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Screening Based Approach to Rational Utilization of GMO Testing Resources: Case of DP305423 Soybean

Merima Miralem, Anesa Ahatović Hajro, Mujo Hasanović(✉), and Adaleta Durmić-Pašić

Institute for Genetic Engineering and Biotechnology, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
mujo.hasanovic@ingeb.unsa.ba

Abstract. The provisions of the Law on GMO in Bosnia and Herzegovina (B&H) are event based. The official testing laboratories should be able to detect GMO presence, to identify and quantify authorized, and to detect and identify unauthorized GMO events. The laboratories are also required to perform under ISO17025, and to introduce accredited methods according to new authorizations in the EU. They are not able to maintain financial sustainability within a limited B & H market, so they focus on the detection methods and identification of the events authorized for use in feed. Such setting justifies concerns that certain GM events evade detection and reach the market. In order to assess the risk thereof, we screened food and feed for the presence of soybean DP305423. This GM event is modified for increased production of oleic acid and is grown in a closed loop system. DP305423 is not detectable with the standard set of detection markers (p-35S, p-FMV, t-NOS, pat/bar), so the EU testing laboratories maintain the event specific method for the purposes of detection, identification and quantification. The samples of food and feed, previously screened for GMO in our laboratory, were tested for DP305423 using EURL-GMFF validated RealTime PCR based identification method (QT-EVE-GM-008). We detected no DP305423 among 95 samples of diverse food and feed products. We conclude that DP305423 is a low-risk GM event, so the introduction of event-specific method should not be a priority. Instead, we propose market screening strategy for prioritizing the future introduction of new event-specific methods.

Keywords: DP305423 soybean · GMO · RealTime PCR · event-specific method

1 Introduction

Soybean (*Glycine max* L.) is an annual plant from *Fabaceae* family and it has high nutritional value. In addition to being an important source of proteins for diabetics and vegetarians, it is an essential component of feed. Although the cultivation of soybean began in China, between the 17th and 11th centuries BC [1], it is now cultivated worldwide. FAOSTAT (Food and Agriculture Organization Corporate Statistical Database) states

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the soybean world production in 2020 at > 353 Mt [2]. According to the ISAAA (International Service for the Acquisition of Agri-biotech Applications) report from 2019, soybean is also the leading transgenic crop that occupies 48% (91.9 million hectares) of the global biotech crop area. Different types of transgenic soybean are cultivated in 8 countries: USA, Brazil, Argentina, Canada, Paraguay, Uruguay, Bolivia and South Africa [3].

The European Union (EU) has developed a comprehensive legal framework for genetically modified organisms (GMO) in order to ensure safe and responsible use of GMO food and feed. The authorizations and labeling requirements are event-based which means that the implementation of the legal provisions depends on the ability of testing laboratories to detect, identify and quantify particular event in food and feed. To that end, the EU has established EURL-GMFF (European Union Reference Laboratory for GM Food and Feed) within the Joint Research Center (JRC), with the primary task to "perform scientific assessment and validation of detection methods for GM food and feed as part of the EU authorization procedure" [4]. It also assists national reference laboratories for GMO control in the EU member states. EURL-GMFF extends its role beyond the EU by supporting implementation of harmonized analytical procedures worldwide.

In Bosnia and Herzegovina (B&H), the Law on GMO was adopted in 2009. It is harmonized with the EU legal framework on GMO [5]. The first authorizations for use of GM soybean in feed were issued in 2015 for MON-04032-6. Since then, additional six GM events of soybean were authorized for use in feed [6]. The official testing laboratories should perform at the level necessary to support the enforcement of compliance with the authorization and labeling requirements. They should be able to detect GMO presence, to identify and quantify authorized GMO events, and to detect and identify unauthorized GMO events. The laboratories are required to perform under ISO17025 and to introduce accredited methods according to the latest authorizations in the EU. With traceability provisions in place, the laboratories are not able to maintain financial sustainability within limited B & H market. Therefore, they focus on maintaining the accredited DNA based detection methods (t-NOS, p-35S CaMV and p-34S FMV) and one laboratory recently accredited identification and quantification method for one GM event (MON-04032-6). Therefore, there is concern that unauthorized GM events, which do not contain the common regulatory elements, evade the detection and reach the market. Also, due to the lack of accredited identification and quantification methods for other six GM events authorized for use in feed, the ability of the authorities to monitor the compliance with the labeling requirements remains questionable.

In order to assess the risk thereof, we screened food and feed samples for the presence of one soybean event that cannot be detected using standard DNA based detection methods available in the country. Soybean DP305423 is modified for increased oleic acid and reduced linoleic acid, and the tolerance to acetolactate synthase-inhibiting herbicides. It is approved for cultivation in Canada, Japan and the United States [7]. Since 2015, DP305423 is authorized for food and feed in the EU. In 2017, the EU authorized stacked event DP-305423-1 x MON-04032-6. EURL-GMFF has validated the event-based method QT-EVE-GM-008 [8] as a part of the authorization procedure. DP305423 is not authorized for use in B&H. Therefore, this GM event should be controlled in all

imported food and feed. In addition to the standard screening procedure, the EU based laboratories test for the presence of this event in food and feed samples.

The primary goal of this study was to determine whether transgenic soybean DP305423 was present in the samples of food and feed collected in B&H, which were previously analyzed using standard detection methods. The secondary objective was to determine the frequency of this GM event in certain types of food and to estimate the actual need for the introduction and accreditation of the event specific method for the detection/identification of DP305423 by the authorized laboratories.

2 Material and Methods

Genomic DNA extracts from 95 samples of soybean containing food and feed were retrieved from -20°C storage. The samples were previously screened for GMO using validated methods based on RealTime PCR with TaqMan probes [9]. The structure of the sample is given in Table 1. Soybean presence was previously established by analyzing lectin, a taxon specific marker (QT-TAX-GM-002) [10] .

Table 1. The structure of sample analyzed for DP305423

	Food	Feed
GMO detected	12	47
GMO not detected	23	13

RealTime PCR reactions were performed in a total volume of 25 μl with 100 ng template DNA, TaqMan Universal MasterMix x 1, 0.8 μM forward and 0.5 μM reverse primers and 0.22 μM TaqMan probe (Applied Biosystems, Foster City, CA). Primers and probe sequences, and amplification conditions were according to the validated method QT-EVE-GM-008 [8]. The method amplifies the boundary area between the host genome and the 3' end of the transgenic cassette of DP305423. The certified reference material (DP305423—10%; ERM-BF426d, JRC-IRMM) was used as a positive control, and sterile deionized water as a negative control (reagent blank). RealTime PCR reactions were run on the ABI 7300 RealTime PCR instrument (Applied Biosystems). The analyses were performed in duplicate.

3 Results and Discussion

Following the analyses of RealTime PCR runs, we concluded that none of the samples contained DP305423 soybean. Positive control was amplified as expected with an average Ct value of 25.15 (24.34–26.03). That is concurrent with the values obtained during the verification procedure. Considering the diversity of processed samples of food and feed containing soybean, we can conclude that the presence of DP305423 on the B&H market is highly improbable.

According to Mall et al. [11], DP305423 is grown in "closed loop cultivation" system which includes steps for the avoidance of commingling with export-bound crops. The crops are used to extract oil while the remaining solids are recycled back into the soil. Oil produced from DP305423 is commercially known as Plenish^{TM} and is authorized on the EU market, provided compliance with the labeling requirements. It is extremely difficult to obtain a sufficient amount of DNA from refined oil. Some successful protocols were published [12, 13], however, the DNA was suitable for detection only. Thus, the labeling relies primarily on the report by the operator.

Considering the exceedingly complex GMO universe, various research teams around the globe have devoted considerable efforts to developing more sustainable methods of GMO identification. There have been attempts to validate procedures for simultaneous identification of multiple events based on multiplex end-point PCR reaction with gel or capillary electrophoresis detection [14–17], RealTime PCR based pre-spotted plates [18] and, most recently, microarray [19] and dPCR [20, 21]. However, validated RealTime PCR based methods are still the foundation of the quantification process.

Despite the undeniable progress, most of the analytical techniques are technologically demanding and expensive, and therefore remain beyond the reach of most testing laboratories. Therefore, it is our belief that GMO testing system, in small market economies, would benefit from an integral approach that is based on the assessment of risk that a particular GMO event would reach the market. Development of such an approach is within the mandate of the GMO council of B&H. In addition to its regular task of monitoring the state and development of genetic technology and the use of GMO, the Council would require access to information on the origin of the imported high risk crops such as soybean and maize. The access can be provided by the Ministry of trade and international relations of B&H. Based on the data, the GMO Council should alert the food safety authority of potential risks, and precipitate preemptive market screening. Thus, the laboratories would accredit the method only when the risk truly exists. Prioritizing the future introductions of new event-specific methods would make the entire system more sustainable, the entire biosafety system more reliable and the testing system cost effective.

4 Conclusions

Based on the collected data, we can conclude that DP305423 represents a low-risk GM event, and it is not necessary for each authorized laboratory to maintain an accredited method for its detection/identification. Considering the costs of verification and accreditation of RealTime PCR based methods, and limited resources of the testing laboratories, a risk-based approach to prioritizing the introduction of methods should be developed. Studies of this type can contribute to more rational allocation of funds for the authorized laboratories, as well as lead to a significant decrease in the cost of analyses.

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Antioxidant Activity and Total Phenolic Content Determining in Samples of Celandine (*Chelidonium Majus* L.) by Applying Different Solvents

Zilha Ašimović(✉), Semira Hasanović, Ševal Muminović, Nermina Spaho, Selma Čorbo, and Lejla Čengić

Faculty of Agriculture and Food Sciences, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
z.asimovic@ppf.unsa.ba

Abstract. Celandine (*Chelidonium majus*) is herbaceous, medicinal herb used in traditional medicine for internal use as tea, syrup, as well for external use. Teas from celandine contain phenolic compounds with high antioxidant activity which creates positive effects on human health.

The aim of this study was to determine the content of phenolic compounds and antioxidant activity in fresh and dry parts of plants (leaf and stem) with using water, diluted methanol, diluted ethanol (as solvents), for 15 min at 70 °C. The samples of plant Celandine (*Chelidonium majus*) originates from Canton Sarajevo, Bosnia and Herzegovina. Total phenols compounds were determined by *Folin-Ciocalteu* method (600 nm), antioxidant activity was determined by using *PFRAP* (700 nm). Gallic acid was used as the standard, and the results are expressed in mg GAE/100 g. The results showed that fresh leaves have a higher content compared to samples of dried leaf, particularly when diluted ethanol was used as a solvent. Based on the obtained results it can be concluded that the samples of dried leaf have higher antioxidant activity in comparison to fresh leaf, in case when diluted methanol was used as a solvent. Also, fresh and dry Celandine leaf have higher phenolic content and antioxidant activity compared to fresh and dry stem. During the extraction it was proved that both fresh and dry parts (leaves and stem) showed the lowest phenolic compounds content and antioxidant activity when water was used as a solvent.

Keywords: Celandine · Antioxidants · Polyphenols · Leaf · Stem

1 Introduction

Chelidonium majus L. (family Papaveraceae), or greater celandine, is an important plant in western phytotherapy and in traditional Chinese medicine [1].

Celandine is a perennial herbaceous plant, with a branched stem, 30 to 80 cm high. The leaves are large, oddly pinnately divided, gray and hairy on the reverse side [2].

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The lower leaves are petiolate, and the upper ones are sessile. The flowers are yellow, gathered at the tops of the tree in rare shield inflorescences [2, 4].

The yellow juice contains a large number of different alkaloids, some of which are related to poppy opium. Due to the presence of a large number of alkaloids, many physiotherapists consider this plant poisonous and suitable only for external use. Besides alkaloids, it also contains saponins, organic acids (chelidonic, citric, malic), flavonoids, proteolytic enzymes and others. The plant has a sedative effect on the central nervous system. Some studies indicate the effect of these alkaloids on the bacteria *Staphylococcus aureus* [2]. Celandine is a reliable medicinal agent in the treatment of serious liver diseases if it is used homeopathically. As an agent that cleans the blood and liver, it has the best effect on metabolism. Celandine is almost exclusively used as an ingredient in herbal preparations against spasms of the digestive organs, biliary and urinary tracts, vascular and bronchial spasms, and externally in cases of chronic eczema, because it has antimicrobial and anti-ringing effects. The juice of the fresh plant is also used to remove some types of warts. The plant is poisonous and is used in limited quantities. It should not be taken for internal use during pregnancy and breastfeeding. For external use, it is used for malignant skin diseases, corns, warts and incurable ringworm. Toxic effects have rarely been recorded in humans, thanks to the traditional use of celandine in the form of refuse (tea). The first toxic effects were described with the use of chemical and more concentrated extracts. Celandine in a larger dose can cause liver damage, along with nausea and weakness [3].

Crude extracts of *C. majus L.,* as well as, purified compounds derived from it exhibit a broad spectrum of biological activity (anti-inflammatory, antimicrobial, antitumoral, analgesic, hepatoprotective) that support some of the traditional uses of it. On the other hand, celandine also has scientifically proven effects, e.g., ant-osteoporotic activity and radioprotection, which are not mentioned in traditional sources. Moreover, recent controversy about hepatoprotective versus hepatotoxic effects of *Chelidonium majus L.,* has renewed the interest of medical community in this plant [1].

The protective role of phenolic compounds in biological systems is attributed to their ability to pair (“capture”) free radical electrons, chelate transition metal ions, activate antioxidant enzymes and inhibit oxidases [5]. Thus, phenolic compounds, due to their pronounced antioxidant activity, reduce the risk of the appearance of tumors, chronic and vascular diseases [6–8]. The antioxidant activity of phenolic compounds is mainly due to their redox properties such as adsorbing and neutralizing free radicals quenching singlet and triplet oxygen, or decomposing peroxides. In general, flavonoids have higher antioxidative activities against peroxyl radicals than do phenolic acids due multiple hydroxyl groups [9].

The aim of this work is to determine the content of total phenolic compounds and antioxidant activity in fresh and dry parts (leaf and stem) of celandine and to determine the existing differences in their content in different solvents (water, ethanol and methanol) during extraction for 15 min at 70 °C.

2 Material and Method

2.1 Samples

The research was started by collecting samples of the plant *Chelidonium majus* L (Celandine) at different locations of Sarajevo Canton, during April, 2016. The plants were collected in the period when they began to bloom, considering that at that stage the celandine has the most active substances. Before analysis, the samples were properly stored at room temperature and in a dry place protected from light. Plants were air-dried without direct contact with sunlight. Analysis of fresh leaves and stems were conducted immediately after harvest. Analyzed parts of the plant were leaf (L) and the stem (S) (Fig. 1).

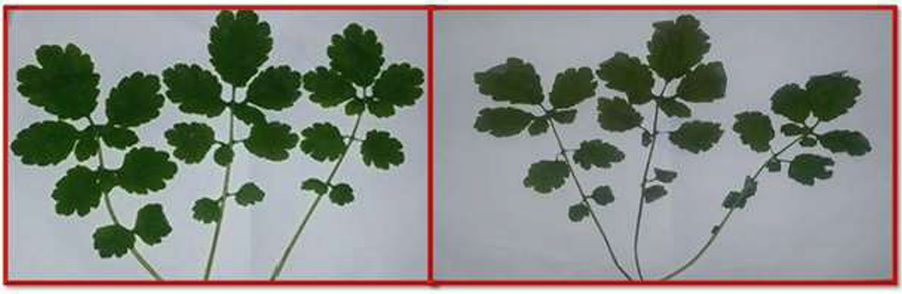

Fig. 1. Fresh and dry parts of *Chelidonium majus* L., for analysis

2.2 Extraction of Samples

For the extraction 1–2 g of samples of celandine, three different solvents: diluted ethanol (70:30 v/v); diluted methanol (70:30 v/v) and distilled water, were used. Extraction of samples with 40 mL of solvent, was carried out at 70 °C for 15 min, with reflux condenser. After cooling, extracts were filtrated through Whatman no.40 filter paper (Whatman International Ltd, Kent, UK)., and filtrates were adjusted to 50 mL with diluted ethanol, diluted methanol or distilled water. The same extract was used for the determination of total phenolic compounds and antioxidant activity.

2.3 Determination of Total Phenolic Content (TPC)

Determination of the total phenolic content in the extracts of leaves and stem is carried out spectrophotometrically using the Folin-Ciocalteau assay according to Ough and Amerine (1988); Singleton et al. (1999) with modifications [10–12]. 1 mL of the obtained sample extract, 1.5 mL of Folin-Ciocalteau reagent (diluted 2:1) to the test tube, wait for 5 min, then 7.5 mL of saturated (Na_2CO_3), was added. After that, everything is mixed well in a test tube and incubated in a water bath for 20 min at 50 °C, protected from the light. After cooling the samples, a reading of absorbance on the spectrophotometer (Cary IE

UV-VIS spectrophotometer, Agilent Technologies, USA) at a wavelength of 600 nm. Quantification of total phenols content was made by using of calibration curve of gallic acid (15 to 90 mg/L). Results were expressed mean ± standard deviation (SD), as mg GAE/100g (Gallic Acid Equivalents).

2.4 Determination of Antioxidant Activity

Antioxidant activity of celandine (leaf and stem) was determined using PFRAP (*potassium ferricyanide reducing power*) method, [13, 14] with modifications. 1mL of extracts was transferred to test tube and 1 mL of 0.1% $K_3[Fe(CN)_6]$ was added. After 5 min 1 mL of $FeCl_3$ and 7 mL distilled water was added and blue complex between phenolics and $K_3[Fe(CN)_6]$ was measured at 700 nm. Quantification of antioxidant activity was made against gallic acid calibration curve.

2.5 Statistical Analysis

Statistical processing of the data was performed in Microsoft Excel 2007. And included two-factorial ANOVA with repetition, in order to show whether or not there are statistically significant differences in the obtained results between the tested samples. Each analysis in triplicate and expressed as (mean ± SD). In order to determine a statistically significant difference between the tested samples, a non-parametric LSD test was used.

3 Results and Discussion

The results of phenolic compounds and antioxidant activity are presented from Tables 1 2, 3 and 4. Total phenolic content and antioxidant activity were calculated on the basis of gallic acid and expressed as mg GAE/100 g fresh weight (FW) and for dry samples as mg GAE/100 g DW.

From the presented data, it can be seen that the fresh leaf samples that were extracted in diluted ethanol had the statistically significantly highest average content of total phenols (159.36 ± 0.96 mg GAE/100 g FW), while the samples that were extracted with water had the statistically lowest average content of total phenols (55.22 ± 0.78 mg GAE/100 g FW).

Highly statistically significant differences were found in the average content of total phenols in fresh celandine leaf samples between content in the water (55.22 ± 0.78 mg GAE/100 g FW) and diluted methanol (150.34 ± 0.77 mg GAE/100 g FW), and water (55.22 ± 0.78 mg GAE/100 g FW) and diluted ethanol (159.36 ± 0.96 mg GAE/100 g FW).

The dry leaf samples that were extracted in diluted ethanol had the statistically significantly highest average content of total phenols (57.42 ± 0.79 mg GAE/100 g DW), while the samples extracted with water had statistically lowest average content of total phenols (13.54 ± 0.64 mg GAE/100 g DW).

A statistically significant difference in the average content of total phenols in the samples of dry leaves of celandine were between the solvents water (13.54 ± 0.64 mg

GAE/100 g DW) and diluted methanol (48.97 ± 0.40 mg GAE/100 g DW), and water and diluted ethanol (57.42 ± 0.79 mg GAE/100 g DW).

The extraction procedure of the plant material, as well as the solvent used, play a key role in the extraction of phenolic compounds. A statistically significant effect of solvent was found on the average content of total phenols in celandine leaf ($F = 4590.054 > Fcrit = 3.402826$); $LSD_{0.05} = 1.225$.

In Wojdylo et al. (2007) content of phenolic acids in pre-dried celandine plant: caffeic acid 186 ± 0.02 mg/100 g DW, neochlorogenic acid 167 ± 0.06 mg/100 gdw, p-coumaric acid 71.7 ± 0.11 mg/100 g DW [15]. According to group of researchers, the content of total phenols in the celandine (water extracts) during the first phenological stages, ranged from 16.45 ± 1.11, and for second, had a value of 12.72 ± 1.17 mg GAE/g extract. The content of total phenols in the ethyl acetate extract had a value of 28.84 ± 0.93 mg GAE/g extract, while from the methanol extract has a value of 38.89 ± 1.23 mg GAE/g extract.[16]. Nile et al. (2021) found that in the methanol extract, the pod contained polyphenol content of 23.67 mg/g, whereas the stem had lowest content of 7.67 mg/g. All the other part parts of *C. majus* L., such as the leaf, flower and root showed concentrations of 13.9 mg/g, 12.11 mg/g and 10.35 mg/g, respectively. Gallic acid was used as a standard [17].

Table 1. Total phenolic content (TPC) in celandine in the fresh samples, as mg GAE/100g FW (mean ± SD)

Extraction solvent	Leaf (L) N = 5	Stem (S) N = 5
Water	55.22 ± 0.78	30.30 ± 0.70
Methanol (70:30, v/v)	150.34 ± 0.77	67.65 ± 0.58
Ethanol (70:30, v/v)	159.36 ± 0.96	76.84 ± 0.74

The highest content of total phenolic compounds in the celandine stem was in diluted ethanol, while the lowest was in water.

The content of total phenolic compounds in the samples of fresh stem of celandine ranged from 30.30 ± 0.70 to.76.84 ± 0.73 mg GAE/100 g FW, while in dry stem they ranged from 10.00 ± 0.30 to 34.72 ± 0.59 mg GAE/100 g DW. Based on the obtained results, it can be concluded that the samples of fresh stem have a higher content of total phenolic compounds compared to the samples of dry stem.

A statistically significant influence of the solvent was found on the content of total phenolic compounds in the stem of celandine ($F = 917.3674 > Fcrit = 3.402826$); $LSD_{0.05} = 1.220$.

A statistically significant influence of the solvent was found on the antioxidative activity in the leaf of celandine ($F. = 193.7015 > Fcrit = 3.402826$); $LSD_{0.05} = 1.1464$.

It can be seen that the highest average antioxidant activity in the stem of celandine was in diluted methanol, while the lowest was in water. The content of antioxidant activity in the samples of fresh stem of celandine ranged from 2.97 ± 0.06 to 20.55 ±

Table 2. Total phenolic content (TPC) in celandine in the dry samples, as mg GAE/100g DW (mean ± SD)

Extraction solvent	Leaf (L) N = 5	Stem (S) N = 5
Water	13.54 ± 0.64	10.00 ± 0.80
Methanol (70:30, v/v)	48.97 ± 0.40	25.17 ± 0.65
Ethanol (70:30, v/v)	57.42 ± 0.79	34.72 ± 0.59

Table 3. Antioxidative activity in celandine in the fresh samples, as mg GAE/100g FW (mean ± SD)

Extraction solvent	Leaf (L) N = 5	Stem (S) N = 5
Water	17.14 ± 0.71	2.97 ± 0.04
Methanol (70:30, v/v)	38.37 ± 0.89	20.55 ± 0.94
Ethanol (70:30, v/v)	35.69 ± 0.68	14.89 ± 0.69

0.94 mg GAE/100 g FW, while in dry stem they ranged from 11.01 ± 0.62 to 34.88 ± 0.68 mg GAE/100 g DW.

A statistically significant influence of the solvent was found on the antioxidative activity in the stem of celandine (F = 54,63527> Fcrit = 3.402826); $LSD_{0.05} = 1.274$.

The samples of fresh stem that were extracted in diluted methanol had the statistically significantly highest average antioxidant activity (20.55 ± 0.94 mg GAE/100 g FW), while the samples in the water had the statistically lowest average antioxidant activity (2.97 ± 0.06 mg GAE/100 g FW). Also, highly statistically significant differences were found in the average of antioxidant activity in samples of fresh stem of celandine between the solvents: water (2.97 ± 0.06) and diluted methanol (20.55 ± 0.94 mg GAE/100 g FW), as well as, between water (2.97 ± 0.06) and diluted ethanol (14.89 ± 0.69 mg GAE/100 g FW).

A statistically significant difference in the average antioxidant activity in the samples of dry stem of celandine is between the water (11.01 ± 0.62) and diluted methanol (34.88 ± 0.68 mg GAE/100 g), and water (11.01 ± 0.62) and diluted ethanol (24.74 ± 0.48 mg GAE/100 g DW).

According to Kazazic et al. (2016) the antioxidant activity in pre-dried celandine plant was 47.94 ± 2.06 mM Fe2+/100 g DW. Antioxidant activity was determined using the FRAP method. According to the DPPH•· Method, the antioxidant activity was 9.17 ± 0.30 mg/100 g DW, and according to ABTS•+ it was 3.13 ± 0.23 mM TE/100 g DW [18].

Antioxidant activity in previously dried celandine plant was 62.2 ± 4.33 μM Trolox/100 g DW. Antioxidant activity was determined using the FRAP method. According to the DPPH• method, the antioxidant activity was 300 ± 3.34 μM Trolox/100g DW, and according to ABTS •+ it was 9.56 ± 1.05 μM Trolox/100 g DW [15].

The antioxidant activity in dried celandine in methanol had value of 50.72 ± 1.13 μg/mL, in ethyl acetate 448.64 ± 2.04 μg/mL, and in water (428.87 ± 1.77 μg/mL). For determination antioxidant activity DPPH reagent was used [16]. According to Then et al. (2003) the content of antioxidant activity during the vegetation period in fresh greater celandine (IV. Month) was 289.5 ± 18.5 μmol/L. Antioxidant activity was determined using the FRAP method [19].

Table 4. Antioxidative activity in celandine in the dry samples, as mg GAE/100g DW (mean ± SD)

Extraction solvent	Leaf (L) N = 5	Stem (S) N = 5
Water	30.38 ± 0.80	11.01 ± 0.62
Methanol (70:30, v/v)	65.56 ± 0.48	34.88 ± 0.68
Ethanol (70:30, v/v)	56.94 ± 0.79	24.74 ± 0.48

4 Conclusion

Experimental data showed that different solvents used (water, diluted methanol, diluted ethanol) significantly affected on extraction of phenol from the leaves and stems of *Chelidonium majus* L. Fresh leaf samples had higher TPC compared to dry leaf, as well as fresh stem samples compared to dry stem. Samples of dry leaves of celandine showed higher antioxidant activity compared to fresh leaves, as well as samples of dry stem compared to fresh ones. The lowest TPC and antioxidant activity were in water, because of different polarity of extraction solvent, implicated that binary system of extraction (ethanol:water) or (methanol:water), showed better efficiency in extraction polyphenolic compounds, compared to water.

It was seen that the average TPC in fresh parts of celandine (leaf and stem) is higher compared to dry parts of celandine (leaf and stem), and that the highest content was, when diluted ethanol as solvent, was used. The antioxidant activity is higher in the dry parts of the celandine (leaf and stem) compared to the fresh parts of the celandine (leaf and stem), and the highest antioxidant activity was in diluted methanol.

In generally: the variation in the TPC in celandine samples as well as the antioxidant activity is a consequence of the action of numerous factors, such as the habitat, the physiological stage in which the plant was harvested, climatic conditions, the method of picking, the method of drying, and the method of storage and transportation.

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Determination of Elements Composition in Vranec Wines Produced with Different Maceration Time

Violeta Ivanova-Petropulos[1(✉)], Elena Bogeva[1], Katerina Bačeva Andonovska[2], Dragan Nikolić[3], and Trajče Stafilov[4]

[1] Faculty of Agriculture, Goce Delcev University, Stip, Krste Misirkov, 10A, 2000 Stip, Republic of North Macedonia
violeta.ivanova@ugd.edu.mk

[2] Research Center for Environment and Materials, Macedonian Academy of Sciences and Arts, Bul. Krste Misirkov 2, 1000 Skopje, Republic of North Macedonia

[3] Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia

[4] Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Arhimedova 5, 1000 Skopje, Republic of North Macedonia

Abstract. Vranec (*Vitis Vinifera* L.) is the most widespread red grape variety in Republic of N. Macedonia, which is grown in all vineyards, but mostly it is located in the main Tikveš wine region. In the present study, Vranec wines have been produced applying 4, 7, 14 and 30 days for maceration, in order to determine the influence of maceration time on the elements profile of wines. Inductively coupled plasma–atomic emission spectrometry (ICP-AES) technique was used to perform the analyses of 18 elements, including Al, Ba, Bi, Ca, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, S, Sr, V and Zn. The results demonstrated that K, P, Mg and Ca were the predominant elements in wines, regardless the maceration time applied for vinification. Concentration of the most of the elements, such as Al, Ba, Bi, Mg, Mn, Na, Ni, P, Sr, P and Zn, increased during the maceration. Copper was determined in a highest concentration in the wine macerated for 4 days (average value: 0.026 mg/L), followed by decreasing of the content during maceration, as it was expected. In general, the content of toxic elements Cu and Fe was lower than the maximal allowed concentrations, concluding that studied Vranec wines were safe for consumption and possessing a nutritional value as a result of the high level of K, P, Mg and Ca.

Keywords: Maceration · Elements · Vranec wine · ICP-AES

1 Introduction

Wine contains various organic and inorganic substances, such as carbohydrates, organic acids, proteins, polyphenols, volatile compounds, as well as elements (metals and non-metals), which influence the quality [1]. Elements are important constituents of wine, which has already been proven that possess nutritional value (e.a. Ca, Cr, Co, K, Mg, Mn,

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Na, Se, and Zn). Some of them have potential toxic effects on the human body, such as heavy metals As, Cd, and Pb. According to the regulation published at the International Code of Oenological Practices, established by OIV (International organization of vine and wine), maximum acceptable limits for some metals have already been set as following: 0.2 mg/L for As, 0.01 mg/L for Cd, 1 mg/L for Cu, 0.15 mg/L for Pb, 80 mg/L for Na, and 5 mg/L for Zn. Therefore, their content have to be controlled during vinification [2].

Many factors influence the concentration of elements in wines, starting from the vineyards conditions, such as vine variety, soil content, fertilization practices used, climate changes, etc., continuing to the end of fermentation and vinification (addition of selected yeasts, maceration time, content of proteins and tartarates, addition of fining agents (i.e. bentonite, especially for white wines) [2–7]. Maceration is a technological phase important for production of high quality red wines. Longer maceration leads to increased color stability and complexity, improves the taste and flavor as well as overall wine quality. During maceration, the metals concentration increases, followed by decreasing throughout the fermentation process, as a result of precipitation [3, 8]. In addition, the prolonged maceration time can increase concentrations of some elements, such as Cd, Cr, Cu, Fe, Pb and Zn, since some of them can be released from cellar equipment produced from brass and stainless steel materials.

Elements can directly or indirectly affect the clarity and sensory characteristics of wines, due to the formation of precipitates during various phases of vinification (fermentation, maturation and storage). Moreover, Ca, K, Mg, and Na are considered as important elements for effective and successful alcoholic fermentation, while Cu, Fe, Mn, Zn are important elements for yeasts activity [8].

Various analytical techniques, such as atomic absorption spectroscopy (AAS), inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) have been used for determination of metals and nonmetals content in wines from various countries in the world [3, 8–17]. Actually, inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) have been introduced for the first time in the year 2011, in the booklet of the International Organization of Vine and Wine (OIV) as recommended methods for wine elemental analysis and control [18, 19].

Several studies on the elemental composition of Macedonian wines have been performed and published applying ETAAS, ICP-OES, ICP-AES and ICP-MS techniques [3, 10, 20]. Thus, systematic study on the elemental's concentration and composition of Macedonian wines has been performed in distinguishing red and white wines as well as in determination of their geographical origin. Analyses were carried out with inductively coupled plasma–mass spectrometry (ICP-MS) and inductively coupled plasma–optical emission spectrometry (ICP-OES) for wines classification [10]. In addition, the contents of Pb and Cd have been determined in *Vitis Vinifera* L. white wines (Smederevka, Chardonnay and Riesling, vintage 2011) grown at two different territories in the Tikveš region: Disan and Negotino, using electrothermal atomic absorption spectrometry (ETAAS) [3]. Moreover, multi-element composition of red Vranec wines fermented with autochthonous and commercial yeast strains, have been quantified, applying microwave

digestion method for wine sample preparation, followed by ICP-MS analyses of the elements [20].

However, to the best of our knowledge, there has been no report on the concentration of elements in red Vranec wines produced with different maceration time, applying inductively coupled plasma-atomic emission spectroscopy (ICP-AES) for analysis. Considering this, the aims of the present work were twofold: (1) to report and use a simple and fast ICP-AES method for determination of elements, after dilution of wines (1:1 with 1M HNO_3) and (2) to study the influence of maceration time on the elements composition of red wines from Vranec grape variety, the most important variety in Republic of N. Macedonia and Balkan countries.

2 Materials and Methods

2.1 Chemicals and Reagents

All reagents and standard solutions were p.a. grade. Bi-distilled water was used to prepare all solutions. Basic standard solution with a concentration of 1,000 mg/L for 23 elements in diluted nitric acid (ICP multi-element standard solution IV was purchased from Merck. Basic standard solutions with a concentration of 1,000 mg/L for Mo, P, S and V were purchased from Solution Plus.

2.2 Grape and Wine Samples

Grapes from Vranec variety, *Vitis vinifera* L. cultivated at Disan location in the Tikveš wine region, R. N. Macedonia, were harvested at optimal maturity (23–24° Brix, vintage 2010). Grapes were collected early in the morning from nine years old vineyards (area of 6 ha, at 560–580 m altitude) and were transported to the wine cellar "Elenov", located in Demir Kapija. The distance between the rows was 2.4 m and the distance between the vines was 0.9 m.

Harvested grapes were processed with electrical inox crusher/destemmer, followed by addition of SO_2 in a form of 5% sulphurous acid (ca. 50 mg/L total concentration of SO_2), addition of commercial yeast Lalvin ICV-D254, *S. cerevisiae* (25 g/hL, supplied from Lallemand, Montreal, Canada) after two hours in order to start the fermentation and addition of nutrients (25 g/hL, Go-Ferm, obtained from Lallemand, Canada). Then, the grape mash was divided into 4 sets, applying maceration of 4, 7, 14 and 30 days (abbreviations: V-4d, V-7d, V-14d and V-30d, respectively). During the maceration and alcoholic fermentation at 25 °C, "pumping over" was applied, three times a day. When fermentation finished, wines were separated from the pomace, stabilized at temperature of 15 °C for a period of three days, bottled and stored in a wine cellar at 6–8 °C. Elements profile was determined after three years wine aging.

Wine samples were diluted in ration 1:1 with 1M HNO_3 before the ICP-AES analyses.

2.3 ICP-AES Conditions

All analyzed elements (Al, As, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sr, Tl, V, Zn) were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Varian, 715-ES). In this spectroscopic method, inductively coupled plasma was used as the source for excitation of atoms. The test solution was fed by a peristaltic pump through an atomizer into the quartz burner (torch), which consists of three concentric tubes with separate inlets for the solution and the working gas argon. The torch was located in an induction coil connected to a high-frequency generator to produce a strong electromagnetic field that allows the generation of a plasma in which excitation occurs. At a temperature of about 7000 K, excitation of all elements present in the test solution was possible.

The following emission lines were used in ICP-AES: Al: 396.152 nm, As: 188.980 nm, Ba: 493.408 nm, Bi: 223.061 nm, Ca: 370.602 nm, Cd: 214.439 nm, Co: 238.892 nm, Cr: 267.716 nm, Cu: 327.395 nm, Fe: 238.204 nm, K: 769.897 nm, Li: 670.783 nm, Mg: 883.829 nm, Mn: 257.610 nm, Mo: 202.032 nm, Na: 589.592 nm, Ni: 231.604 nm, P: 213.618 nm, Pb: 220.353, S: 181.972 nm, Sr: 407.771 nm, Tl: 351.923 nm, V: 292.401 nm, Zn: 213.857 nm. The data on the operating conditions are given in Table 1.

Table 1. Instrumental parameters for ICP-AES analyses of elements.

RF generator			
Operating frequency: 40.68 MHz free-running, air-cooled RF generator			
Power output of RF generator: 700–1700 W in 50 W increments			
Introduction area			
Sample nebulizer: V-groove			
Spray chamber: Double-pass cyclone			
Peristaltic pump: 0–50 rpm			
Spectrometer			
Optical arrangement: Echelle optical design			
Polychromator: 400 mm fo cal length			
Echelle grating: 94.74 lines/mm			
Polychromator purge: 0.5 l min^{-1}			
Megapixel CCD detector: 1.12 million pixels			
Wavelength coverage: 177 nm to 785 nm			
Conditions for the program			
RFG power	1.0 kW	Pump speed	25 rpm
Plasma Ar flow rate	15 l min^{-1}	Stabilization time	30 s
Auxiliary Ar flow rate	1.5 l min^{-1}	Rinse time	30 s
Nebulizer Ar flow rate	0.75 l min^{-1}	Sample delay	30 s

Calibration solutions were used for construction of calibration curves ($y = ax + b$) for each element. The linearity of the calibration curves was tested and correlation factor $R^2 > 0.99$ was considered as acceptable.

The lowest concentration that can be quantitatively determined with an acceptable level of accuracy is defined as limit of detection (LOD). LODs were determined for each elements: 0.25 μg/L for Al, 10 μg/L for As, 0.5 μg/L for Ba, 1 μg/L for Bi, 0.5 μg/L for Ca, 0.1 μg/L for Cd, 1 μg/L for Co, 1 μg/L for Cr, 0.25 μg/L for Cu, 0.12 μg/L for Fe, 100 μg/L for K, 1 μg/L for Li, 0.5 μg/L for Mg, 0.03 μg/L for Mn, 4 μg/L for Mo, 40 μg/L for Na, 5 μg/L for Ni, 10 μg/L for P, 10 μg/L for Pb, 50 μg/L for S, 0.5 μg/L for Sr, 10 μg/L for Tl, 1 μg/L for V and 0.06 μg/L for Zn.

2.4 Statistical Analyses

Each wine was analyzed in three replicates. Statistical analysis was performed, including calculation of means, standard deviation and relative standard deviation, using the XLSTAT software, version 7.5.2, Addinsoft (Paris, France). In addition, the ANOVA test of Student–Newman–Keuls of multiple comparisons of mean values was applied to the results for the concentration of each element in order to reveal differences or similarities between the studied wines. Significant difference was considered at level of $p < 0.05$.

3 Results and Discussion

The results related to the determination of 18 elements (Al, Ba, Bi, Ca, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, S, Sr, V, Zn) in Macedonian red Vranec wines produced with different maceration time of 4, 7, 14 and 30 days are presented in Table 2. The elements As, Cd, Co, Mo, Pb and Tl, which are heavy and harmful, were detected at concentrations lower than the determined LOD, and therefore, they are not presented in the table. Elemental analysis demonstrated that K, P, Mg, S and Ca were the dominant elements in the wines, regardless the maceration time, followed by Na, Fe, Mn, Sr and Al.

Potassium was the major element in all analyzed Vranec wines regardless the maceration time, ranging from 298 to 332 mg/L. This element is considered as a natural component present in the grapes that passes into the wine during fermentation. The content of potassium in wine is an indication for its concentration in the grape berries at the final stages of ripening [10]. High potassium contents lead to precipitation of tartaric acid and then, increasing of the pH value, causing wine instability. In our study, the content of potassium was not very high, which confirms the tartarate stability of the wines, regardless the duration of maceration time applied for winemaking. Phosphorous, which is naturally present macroelement and essential for life, was ranging between 71.3 and 105 mg/L in the wines. High content of this element in the wines confirms their nutritional value [20]. Sulfur was detected in the wines in the range of 47.3 to 61.5 mg/L. In fact, S is expected to be present since SO_2 is used in winemaking to prevent oxidation and browning of wine (enzymatic and nonenzymatic oxidation of phenolic compounds, especially phenolic acids, carbohydrates and amino acids). The content of S in the analyzed wines confirms that wines were sufficiently protected from oxidation and ensures their stability.

Table 2. Concentration (mg/L) of elements in Vranec wines produced with maceration time of 4, 7, 14 and 30 days and determined by ICP-AES.

Elements (mg/L)/Wine samples	V-4d	V-7d	V-14d	V-30d	Min	Max	Average
Al	0.33 ± 0.006	0.51a ± 0.007	0.51a ± 0.007	0.54a ± 0.008	0.33	0.54	0.474
Ba	0.097 ± 0.006	0.125a ± 0.0005	0.123a ± 0.002	0.134a ± 0.003	0.097	0.134	0.120
Bi	0.042 ± 0.001	0.056 ± 0.0028	0.081 ± 0.0021	0.112 ± 0.007	0.042	0.112	0.073
Ca	31.3a ± 0.342	33.5a ± 0.424	32.4a ± 0.326	31.9a ± 0.145	31.3	33.5	32.3
Cr	0.002a ± 0.001	0.004 ± 0.0002	0.001a ± 0.0007	0.010 ± 0.003	0.001	0.010	0.004
Cu	0.026 ± 0.003	0.009 ± 0.003	0.004 ± 0.0007	0.000 ± 0	0.000	0.026	0.010
Fe	0.87a ± 0.034	0.91a ± 0.008	0.57 ± 0.0404	0.84a ± 0.034	0.57	0.91	0.797
K	317 ± 0.598	332 ± 1.29	298 ± 1.677	305 ± 1.813	298	332	313
Li	0.010 ± 0.0002	0.004 ± 0.0002	0.000 ± 0	0.002 ± 0.0002	0.000	0.010	0.004
Mg	51.9 ± 0.143	55.1 ± 0.234	54.9 ± 0.183	61.9 ± 0.218	51.9	61.9	55.9
Mn	0.42a ± 0.003	0.45a ± 0.002	0.44a ± 0.004	0.49a ± 0.030	0.42	0.49	0.45
Na	1.93a ± 0.036	2.08b ± 0.044	1.91a ± 0.048	2.15b ± 0.022	1.91	2.15	2.02
Ni	0.000 ± 0	0.016 ± 0.0004	0.000 ± 0	0.022 ± 0.0002	0.000	0.022	0.010
P	71.3 ± 0.132	88.4 ± 0.031	91.8 ± 0.445	105 ± 0.353	71.3	105	89.2
S	61.5 ± 0.212	53.5a ± 0.084	47.3 ± 0.438	54.8a ± 0.362	47.3	61.5	54.3
Sr	0.39 ± 0.0212	0.48a ± 0.0136	0.48a ± 0.008	0.52 ± 0.043	0.39	0.52	0.467
V	0.056 ± 0.001	0.016 ± 0.002	0.000 ± 0	0.000 ± 0	0.000	0.056	0.018
Zn	0.037 ± 0.0001	0.029 ± 0.0009	0.085 ± 0.001	0.180 ± 0.002	0.029	0.180	0.083
Σ	**537**	**568**	**529**	**564**	**529**	**568**	**550**

Results represent an average value of three repeated measurements ± SD (standard deviation). Values with indicated letter within a row have been analyzed by the Student–Newman–Keul's test (ANOVA) and are considered as not significantly different at $p > 0.05$.

In general, high concentrations of macroelements calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) contribute a salty taste in wines. The concentration of Ca (average value: 32.3 mg/L), Mg (average value: 55.9 mg/L) and Na (average value: 2.02 mg/L) were very low compared to other wines from the world [21] which means that the salty character was not present in Vranec wines, as it was expected. Similar contents between elements were noticed for manganese (on average: 0.45 mg/L), strontium (on average: 0.467 mg/L) and aluminum (on average: 0.474 mg/L), regardless the maceration time.

The presence of Al, Cu, Fe, Pb, Zn is important for conducting and completion of an effective alcoholic fermentation and therefore, their contents in wine must be controlled. Moreover, excess content of dissolved iron and copper can change the flavor and appearance of wine since both elements act in oxidation and reduction reactions, changing the flavor and color of wine, detrimenting the overall quality. The iron and cupper contents in Vranec wines ranged from 0.567 to 0.912 mg/L (on average: 0.797 mg/L) and from 0.00 to 0.026 mg/L (on average: 0.01 mg/L), respectively, which were very lower compared to maximum allowed concentrations for Fe (5 mg/L) and Cu (1 mg/L) in wine [2].

Heavy metals, such as Cd and Pb, have toxic effects and they deactivate the enzymes. Therefore, their contents in wine must be monitored. In this study, both metals (Cd and Pb) have been not detected in the analysed Vranec wines. In addition, Ni, Bi and Zn were found in very low concentrations (on average: 0.01 mg/L for Ni, 0.073 mg/L for Bi and 0.083 mg/L for Zn) compared to the maximum acceptable levels (for Ni: 0.1 mg/L and for Zn: 5 mg/L). In fact, the potential source of Ni in wine is the presence of nickel alloys in stainless steel equipment.

Considering the influence of maceration time, the content of trace elements is expected to increase during fermentation, and afterwards to decrease, since they are absorbed onto the yeast cell and precipitate or coprecipitate with polyphenols and tannins and then removed. Thus, copper was present in highest concentration in the wine macerated for 4 days (0.026 mg/L), followed by decreasing of the content during maceration, as it was expected. Sr and Fe presented relatively similar concentration regardless the maceration time. In this study, for most of the elements, such as Al, Ba, Bi, Mg, Mn, Na, P, Sr and Zn, a slight increasing of their content was noticed during maceration, observing slightly higher content in wines macerated for 30 days.

In general, analyzed Vranec wines produced with different maceration time contained similar or lower levels of determined elements compared to the literature data [10–16, 21], which confirms their safety for consumption. Moreover, high level of macroelements, such as K, P, Mg and Ca, confirmed the nutritional value of Vranec wines.

4 Conclusion

In this study, fast and simple method based on ICP-AES technique was used to determine 18 elements, including Al, Ba, Bi, Ca, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, S, Sr, V and Zn, in Vranec wines produced with maceration time of 4, 7, 14 and 30 days, after simple sample preparation (dilution of wines in ratio 1:1 with 1M HNO_3). K, P, Mg, S and Ca were the dominant elements in all wines regardless the maceration duration, followed by elements Na, Fe, Mn, Sr and Al. Concentration of Cu decreased during maceration, while slight increase of Al, Ba, Bi, Mg, Mn, Na, P, Sr and Zn contents were noticed in the wines. All wines presented a good dietary source of macro and micro-nutrient elements.

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The Effect of Essential Oils on the Quality and Oxidative Stability of Linseed Oil

Belmina Halilović(✉), Selma Čorbo, Teofil Gavrić, and Munevera Begić

Faculty of Agriculture and Food Science, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
belmina.halilovic@ppf.unsa.ba

Abstract. The aim of this work was examination of quality and oxidative stability of linseed oil, with and without the addition of essential oils. The content of carotenoids, chlorophylls and total phenols, the content of moisture, insoluble impurities, the composition of saturated and unsaturated fatty acids, the content of free fatty acids and the peroxide value were examined in linseed oil (initial analysis without the addition of essential oils). In the basic sample of linseed oil, garlic, thyme and oregano essential oil were added in different concentrations (0.1% and 0.5%). The samples were stored for 30 days at a temperature of 18 to 20°C in different conditions, in daylight and in the dark. With the addition of different essential oils, the content of moisture and insoluble impurities increased in proportion to the addition of a higher concentration of essential oil. PUFA are more abundant than MUFA. Linolenic fatty acid was dominant in all samples, with the content higher than 50% especially in samples with 0.1% oregano essential oil adition. The content of free fatty acids decreased and the peroxide value increased in all samples after 15 and 30 days of storage in the light and in the dark. The sample with the addition of garlic essential oil in a concentration of 0.1% had the lowest value of the peroxide value, i.e. the best oxidative stability. On the other hand sample with the addition of thyme essential oil in a concentration of 0.5% had the highest peroxide value and the lowest oxidative stability.

Keywords: linseed oil · essential oils · antioxidants · fatty acids · oxidative stability

1 Introduction

Vegetable oils and medicinal plants have a long tradition of use in Bosnia and Herzegovina. Small production plants were established in the last decade in Bosnia and Herzegovina, called "mini-oileries", that produce cold-pressed linseed oil. Linseed (*Linum usitatissimum* L.) is a plant that has various uses and is the oldest textile plant. Linseeds are used as raw materials for oil production. Linseeds are rich in oil and protein. They contain significant amounts of vitamins A, B6, D, and E and unsaturated fatty acids. Considerable amounts of mucilage, lecithin, minerals (including zinc), and free amino acids can also be found [1]. Cold-pressed vegetable oils are produced by a mechanical

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pressing process, without heating, to maintain the oil's quality and nutritional value. The process of cold pressing of linseeds produces an edible oil of appropriate quality, characterized by high nutritional value and oxidation stability. The quality of the raw material is very important for the production of oil. Before pressing, the raw material is grounded, conditioned and then pressed to obtain raw linseed oil. Oils and fats have a limited shelf life, after which undesirable changes may occur, both in their organoleptic properties and in their nutritional value. Due to the spoilage of oils and fats, biologically active substances such as vitamins, provitamins and essential fatty acids are partially lost. Furthermore, various decomposition products are created that give oils and fats unpleasant smells and tastes. The three basic types of oil and fat spoilage are enzymatic, microbiological and oxidative spoilage, i.e. autoxidation of oil and fat [2]. The high content of unsaturated fatty acids in cold-pressed oil makes it susceptible to oxidative changes, especially linseed oil, which contains linolenic acid [3]. Because linseed oil lacks a refining process, there may be components that reduce its sustainability (degradation products of oxidation, metals, etc.), and on the other hand, a higher content of natural ingredients with antioxidant properties may contribute to better sustainability of the oil (tocopherols, carotenoids, phenolic compounds, etc.) [4]. The addition of essential oils, as natural antioxidants, aims to increase the oxidative stability of vegetable oils. Ethereal or essential oils (lat. *Aetheroleum*) are produced as secondary metabolites of aromatic plants. Essential oils are liquid, mostly colorless, soluble in organic solvents, and less dense than water. Due to their volatility, they are usually isolated from plants through the distillation process. Based on the wide spectrum of pharmacological activities of essential oils, they are used in the food, pharmaceutical, cosmetic and agricultural industries [5].

This study aimed to examine the quality and oxidative stability of cold-pressed linseed oil, treated with the addition of diverse essential oils in various concentrations and stored in different conditions and lengths of storage.

2 Materials and Methods

2.1 Materials

Cold-pressed linseed oil (2L) from an individual producer (GA-ME-HA d.o.o.), were taken for analyses. The essential oils, garlic oil (Producer: Pranarom International-Belgium), thyme and oregano oil (Producer: Halilović d.o.o., BiH), were collected from the Sarajevo Canton market.

2.2 Methods

Initial analyses were made on basic cold-pressed linseed oil (without the addition of essential oils). The following quality determination methods were used for the analysis: the composition of saturated and unsaturated fatty acids on a gas chromatograph GC/MS using a Hewlett Packard 6890 II instrument, with a selective mass detector (MSD) 6890 II [6], the content of insoluble impurities using the standard ISO 663:1992 method [7], moisture content using the ISO 662:1992 method [8], free fat content acid using the

standard titration method [9], peroxide value using the Wheeler method [10], carotenoid content using the BSM method [11], chlorophyll content [12], and the content of a total of phenol [13].

After the initial analyses, the samples were prepared in such a way that it was 2 litters of linseed oil separated into pre-sterilized 500 ml dark-coloured glass bottles, to which the essential oils of garlic, thyme and oregano were individually added in the amount of 0.1% and 0.5%. The obtained mixtures of linseed and essential oils were subjected to the same quality testing methods as on the base sample (composition of saturated and unsaturated fatty acids, insoluble impurities, moisture content, free fatty acid content and peroxide value). After 15 and 30 days of storing samples mixed with different essential oils (garlic, thyme and oregano) in different concentrations of 0.1% and 0.5%, and a base sample of base oil, stored at a temperature of 18 to 20°C in daylight and in the dark, viability was determined based on peroxide value and free fatty acid content.

2.3 Statistical Analysis

The Past 3.15 program [14] was used for statistical data processing. To determine a statistically significant difference in the values of peroxide value, moisture content, insoluble impurities, composition of fatty acids and free fatty acids, under the influence of the type and concentration of the added essential oil, a two-factor analysis of variance was applied, and in the case of statistically significant differences, Tukey's post-hoc test was used. In addition to the above, a two-factor analysis of variance was used to examine the influence of time and method of storage on the values of the examined parameters. The aim was to determine whether the type and concentration of essential oil affect the oxidative stability of cold-pressed linseed oil samples. For the correlation and presentation of the results multivariate data analysis was used - analysis of the basic components or PCA analysis.

3 Results and Discussion

3.1 Determination of Oil Quality (Initial Analysis)

Table 1, shows the content of carotenoids, chlorophyll and total phenols in the base (base) sample of linseed oil. Results represent the mean value of two determinations ± standard deviation (SD).

Table 1. The average content of carotenoids, chlorophyll and total phenols in cold-pressed linseed oil

Parameter	Linseed oil
Carotenoids (β-carotenoid mg/kg)	13.50 ± 0.03
Chlorophyll (pheophytin α mg/kg)	1.85 ± 0.02
Total phenols (mg/kg GAE)	381.51 ± 1.34

The base sample of linseed oil had a content of carotenoids expressed as β-carotenoid in the value of 13.50 mg/kg. The content of carotenoids in linseed oils varies from 2 to 11.5 mg/kg of oil [15], and according to Tuberoso et al. [16] 0.7 mg/kg.

Relative to our results the base sample of linseed oil had a chlorophyll content of 1.85 mg/kg, which is in accordance with the data in the literature of 3.4 mg/kg oil reported by Tuberoso et al. [16]. The same author states that in many oilseeds, the amount of chlorophyll ranges from 1.5 mg/kg in peanut oil to 33.9 mg/kg in olive oil. Similar results to those in Table 1 are reported by Suri et al. [17], the chlorophyll content in linseed oil is 1.45 mg/kg. Obranović et al. [18], found that the mean value of chlorophyll in linseed oil expressed as pheophytin is 0.44 mg/kg of oil. According to their results, the difference is in the content of chlorophyll (from 0.23 to 0.86 mg/kg) and carotenoids (from 1.02 to 2.78 mg/kg) in the oil obtained from four varieties of linseed. Similar results (0.59 and 0.39 mg/kg) of oil were reported by Zhang et al. [19] for the oil obtained from two different varieties of linseed. Deme et al. [20] state the different content of chlorophyll in the oil, where the content primarily depends on the variety of linseed oil and was from 1.64 to 2.31 mg/l. In their research, Choo et al. [21] stated that the content of chlorophyll in cold-pressed linseed oil ranged from 0.80 to 5.76 mg/kg of oil, and the content of total phenols from 76.8 to 307.3 mg/kg. 100 g. The content of total phenols in the base sample of cold-pressed linseed oil was 381.51 mg/kg GAE. Kiralan et al. [22] report a slightly higher content of total phenols in linseed oil (554 mg/kg GAE).

In the research by Odeh et al. [23] the content of total phenols in linseed oil during storage of 180 days was examined, where the content at the beginning of the study was 5.44 mg/kg, after 60 days 5.62 mg/kg, and after 180 days 10.58 mg/kg. The content of total phenols is determined by various factors. Variety, growing conditions (soil and temperature), the interaction between genotype and growing conditions, postharvest treatments including mechanical extraction, and storage conditions can significantly alter the chemical composition of plant materials [24]. The foregoing may explain why the results of this research differ from those of other authors mentioned in the paper. The obtained values for the moisture content and the content of insoluble impurities in the tested samples are shown in Table 2.

Table 2. Content of moisture and insoluble impurities in cold-pressed linseed oil with the addition of different concentrations of essential oils

Parameter	Control	0.1%BL	0.5%BL	0.1%MD	0.5%MD	0.1%O	0.5%O	F 1*	F 2*	Interaction
Moisture (%)	0.10	0.12	0.15	0.15	0.18	0.20	0.25	**	ns	ns
Insoluble impurities (%)	0.35	0.38	0.40	0.45	0.60	0.50	0.55	**	ns	ns

** Factor 1– type of essential oil; Factor 2 – concetracion; p – significance level: * p<0.05; ** p<0.01; *** p<0.001; ns – no statistical significance*

Analysis of variance found that there is a statistically significant influence of the experimental factor (type of essential oil) on the content of moisture and insoluble impurities ($p < 0.01$), while the influence of the essential oil concentration factor and the interaction of factors on the two parameters was not statistically significant. From Table 2. it can be seen that the moisture content in all tested samples is in accordance with the Regulation on Vegetable Oils, Edible Vegetable Fats and Mayonnaise [25] and is less than 0.4%, while the content of insoluble impurities is higher than the maximum allowed values, i.e. greater than 0.05%. In the base sample of linseed oil, the moisture content is 0.10%, and the content of insoluble impurities is 0.35%. By adding essential oil of garlic, thyme, and oregano to linseed oil in different concentrations (0.1% and 0.5%), the moisture content and insoluble impurities increase. The values for both parameters are higher with higher concentrations of added essential oil. It was found that all three added essential oils affect the increase of moisture and insoluble impurities in the oil. Oregano essential oil the amount of 0.5% had the greatest influence on the increase in the values of the mentioned parameters, while garlic essential oil in the amount of 0.1% had the least influence on the change in moisture content (0.12%) and insoluble impurities (0.38%). On the other hand, thyme added in the amount of 0.1% had the same effect on the moisture content, the value of which was 0.15%, as did the addition of garlic in an added concentration of 0.5% (0.15%).

In the research of Biondić [26], the lower moisture content in linseed oil was recorded compared to the results determined in this research (0.068%), while the values of the content of insoluble impurities are in accordance with the results of this work (0.38%) and are higher than permitted according to the current Regulation. Similar results were recorded by Radanović [27] in her research, the results of which are within the allowed values. Moisture content in cold-pressed linseed oil was 0.067%, and insoluble impurities 0.16%. Accordingly, in order to reduce the content of impurities in the oil, it is good to carry out sedimentation, filtration, or centrifugation of the oil. Table 3 shows the composition of fatty acids in the examined samples of cold-pressed linseed oil with the addition of different essential oils in concentrations of 0.1% and 0.5%.

Analysis of variance revealed that there is a statistically very high significant influence of the experimental factor (type of essential oil) on almost all tested fatty acids of linseed oil ($p < 0.001$), with the content of C10:0, C16:0, C18:0, C20:0 very significant influence of the mentioned factor ($p < 0.01$), with C22:1 content and MUFA/SFA ratio statistically significant influence of the mentioned factor, while the differences determined between the values of the n-6/n-3 ratio were not statistically significant. The essential oil concentration factor showed a very high statistically significant effect on the content of C18:4 and C20:4 ($p < 0.001$), a very statistically significant effect on the content of C12:0 ($p < 0.01$), a statistically significant effect on the content of C16: 1, while the determined differences in the content of all other fatty acids were not statistically significant. The interaction of the factors had a statistically very high influence on the content of C18:4 and C20:4 ($p < 0.001$), a highly statistically significant influence on the content of C18:2 and SFA ($p < 0.01$), a statistically significant influence on the content of C12:0, C18:3, n-6 and n-3 fatty acids, while the determined differences in the content of all other fatty acids were not statistically significant. The total content of SFA

Table 3. Composition of fatty acids in cold-pressed linseed oil with the addition of different essential oils and their concentrations

Fatty acid	Control	0.1% BL	0.5% BL	0.1% MD	0.5% MD	0.1% O	0.5% O	F 1*	F 2*	Interaction
C8:0	0.50	0.53	0.52	0.10	0.12	0.30	0.35	***	ns	ns
C10:0	0.20	0.25	0.20	0.25	0.26	0.15	0.15	**	ns	ns
C12:0	0.10	0.15	0.15	0.10	0.15	0.15	0.20	***	**	*
C14:0	0.05	0.05	0.04	0.15	0.17	0.10	0.13	***	ns	ns
C15:0	0.05	0.05	0.05	0.08	0.05	0.15	0.18	***	ns	ns
C16:0	5.00	5.50	5.30	3.50	3.80	4.50	4.70	**	ns	ns
C17:0	0.05	0.05	0.05	0.05	0.10	0.50	0.55	***	ns	ns
C18:0	3.00	3.90	3.50	3.10	3.00	4.10	4.20	**	ns	ns
C20:0	0.30	0.50	0.52	0.35	0.40	0.50	0.55	**	ns	ns
C14:1	0.05	0.15	0.15	0.05	0.05	0.10	0.15	***	ns	ns
C16:1	1.05	2.15	2.50	1.00	1.20	1.85	2.00	***	*	ns
C17:1	0.30	0.35	0.38	0.35	0.20	0.50	0.50	***	ns	ns
C18:1	15.10	18.00	19.20	11.00	10.10	12.00	11.80	***	ns	ns
C22:1	0.20	0.25	0.30	0.20	0.25	0.30	0.35	*	ns	ns
C18:2	19.20	18.00	18.50	23.10	20.20	17.60	18.70	***	ns	**
C18:3	45.50	49.10	47.00	48.00	49.50	50.00	48.50	***	ns	*
C18:4	0.10	0.10	0.15	0.20	0.25	0.15	0.50	***	***	***
C20:4	0.30	0.35	0.30	0.35	0.80	0.35	0.50	***	***	***
SFA	9.25	10.98	10.33	7.68	8.05	10.45	11.01	***	ns	**
MUFA	16.70	20.90	22.53	12.6	11.80	14.75	14.80	***	ns	ns
PUFA	65.10	67.55	65.95	71.65	70.75	68.10	68.20	***	ns	ns
n-6	19.50	18.35	18.0	23.45	21.00	17.95	19.20	***	ns	*
n-3	45.60	49.20	47.15	48.20	49.75	50.15	49.00	***	ns	*
PUFA/SFA	7.04	6.15	6.38	9.33	8.79	6.52	6.19	***	ns	ns
MUFA/SFA	1.80	1.90	2.18	1.64	1.47	1.41	1.34	*	ns	ns
n-6/n-3	0.43	0.37	0.4	0.49	0.42	0.36	0.40	ns	ns	ns

** Factor 1– type of essential oil; Factor 2 – concetracion; p – significance level: * p<0.05; ** p<0.01; *** p<0.001; ns – no statistical significance; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; n–6 omega 6 fatty acids, n–3 omega 3 fatty acids*

in the tested samples ranged from 7.68 to 11.01%, while the base sample had an approximate mean value of 9.25%. With the addition of essential oil of garlic and oregano in the amount of 0.1%, the SFA content was 10.98% and 10.45%, and in the sample with

the addition of essential oil of garlic in the amount of 0.5%, it was 10.33%. Samples with the addition of thyme essential oil added in both concentrations showed slightly lower SFA values compared to the other samples. Of the saturated acids, in all samples with the addition of 0.1% thyme and garlic, the most abundant was palmitic acid, whose content ranged from 3.5% to 5.5%. According to Tuberoso et al. [16], the content of palmitic acid in linseed oil was 4.9% and 3.50% according to Kostadinovic-Velickovska and Mitrev [28]. Lewinska et al. [29] state that the content of palmitic acid in linseed oil ranged from 5.1 to 5.3%. Of the unsaturated fatty acids, PUFA acids were dominant, the content of which in all samples ranged from 65.10 to 71.65%. These acids were significantly more abundant than MUFA acids (11.80–22.53%). The most abundant acid in linseed oil was linolenic acid (18:3). The highest content of linolenic acid was recorded in the sample of oil with the addition of oregano in the amount of 0.1% (50.00%), while the lowest value was recorded in the sample with the addition of essential oil of garlic in the amount of 0.5% (47.00%). (Table 3.). Compared to the base sample, an increase in linolenic acid was observed in all samples with the addition of different essential oils and their concentrations. The highest content of linoleic acid (18:2) was found in samples with the addition of thyme essential oil in a concentration of 0.1% and 0.5% (23.10% and 20.20%). Other samples had slightly lower values compared to the base sample (19.20%). In all tested oil samples, with the addition of different essential oils and their concentrations, the content of oleic acid was lower compared to linoleic acid. The exception is the sample with added garlic essential oil in the amount of 0.5% (19.20%), while the value of linoleic acid in the same sample was lower (18.50%). The content of oleic acid is in accordance with the results reported by Lewinska et al. [29] for two linseed oils, namely 15.8% and 20%. These three acids had the highest proportion in all tested samples. According to the recommendations of the UK Department of Health [30], Wood et al. [31] and Wood et al. [32] the values of the PUFA/SFA ratio should be greater than 0.4 in order for the food to be declared as a product of high nutritional value. Based on the above, and according to the results from Table 3, it can be concluded that the examined sample of cold-pressed linseed oil, with and without the addition of essential oils in different concentrations, can be declared as such, i.e. a product of high nutritional value. Acids from group n-3 were significantly more dominant than n-6. The values ranged from 45.60% to 50.15% for group n-3, and for group n-6 from 17.95% to 23.45%. The highest content of n-3 was recorded in the sample with the addition of essential oil of oregano in the amount of 0.1%, and n-6 in the sample with the addition of essential oil of thyme, also in the amount of 0.1%. The health- recommended ratio of n-6/n-3 fatty acid groups according to Simopoulos [33] is 1:1

– 4:1, according to Simopoulos [34] around 4, and according to Wood et al. [31] and Wood et al. [32] below 4. High content of linolenic acid resulted in a low n-6/n-3 ratio ranging from 0.36 to 0.49, depending on the sample. A similar composition of fatty acids in linseed oil is reported by Savva and Kafatos [35], according to which linseed oil contains 9.0 g SFA/100 g, 18.4 g MUFA/100 g and 67.8 g PUFA/100 g, of which 53,4 g ALA. The authors state that this unusually high ALA content makes the mentioned oil oxidize quickly, which is why it is not supplied as a pure oil but supplied with suitable antioxidant preparation. Also, Grajzer et al. [36] published results stating that the content of SFA in linseed oil is about 10.7%, MUFA 20.5%, PUFA 68.8%, of which linolenic

acid is dominant (53.1%), and the n-6/n-3 ratio is 0.3%. Zhang et al. [19] investigated the characteristics of linseed oil from two different linseed plants. They found that the SFA content was 15.55% and 8.52%, and the total SFA content was 83.53% and 90.70%. They concluded that a higher content of SFA in oil did not affect its stability compared to oil with a lower content of unsaturated fatty acids. These results largely agree with the results described in the work of Herchi et al. [37] where it was reported that linseed oil contained from 59% to 69% of PUFA acids, mainly linolenic acid (38–54%).

3.2 Determination of the Oxidative Stability of the Oil at the Beginning of the Treatment (Initial Analysis), After 15 and 30 days of Storage in Different Storage Conditions (Light, Dark)

Table 4., shows the values for the content of free fatty acids for each tested sample of cold-pressed linseed oil with the addition of different essential oils (garlic, thyme and oregano) in different concentrations (0.1% and 0.5%) of essential oils, after 15 and 30 days of storage in different conditions (light and darkness).

Table 4. Average values of the content of free fatty acids ± SD in cold-pressed linseed oil with the addition of different essential oils and in various concentrations (light and dark)

Free fatty acids (%)					
Sample	Control	15 days (light)	15 days (darkness)	30 days (light)	30 days (darkness)
Control	$0.73^{x} \pm 0.00$	$0.68^{yX} \pm 0.00$	$0.56^{yY} \pm 0.00$	$0.79^{zX} \pm 0.00$	$0.73^{zY} \pm 0.00$
0.1% BL	$0.90^{aAx} \pm 0.01$	$0.68^{aAyX} \pm 0.00$	$0.73^{aAyY} \pm 0.00$	$0.85^{aAzX} \pm 0.00$	$0.90^{aAzY} \pm 0.00$
0.5% BL	$0.90^{aAx} \pm 0.00$	$0.69^{aAyX} \pm 0.02$	$0.67^{bAyY} \pm 0.00$	$0.79^{bAzX} \pm 0.00$	$0.84^{bAzY} \pm 0.00$
0.1% MD	$0.84^{aBx} \pm 0.00$	$0.67^{aAyX} \pm 0.00$	$0.67^{aByY} \pm 0.00$	$0.84^{aAzX} \pm 0.00$	$0.90^{aBzY} \pm 0.00$
0.5% MD	$0.85^{aBx} \pm 0.00$	$0.62^{aAyX} \pm 0.00$	$0.63^{bByX} \pm 0.02$	$0.96^{bBzX} \pm 0.00$	$0.85^{bBzY} \pm 0.00$
0.1% O	$0.90^{aAx} \pm 0.00$	$0.64^{aAyX} \pm 0.04$	$0.67^{aByX} \pm 0.00$	$0.84^{aAzX} \pm 0.00$	$0.79^{aCzY} \pm 0.00$
0.5% O	$0.95^{bCx} \pm 0.00$	$0.74^{bCyX} \pm 0.02$	$0.73^{bCyX} \pm 0.00$	$0.90^{bCzX} \pm 0.00$	$0.84^{bCzY} \pm 0.00$

a - b Different lowercase letters in rows indicate statistically significant differences between linseed oil samples, regarding the concentration of added essential oil; A - C Different capital letters in the rows indicate statistically significant differences between the linseed oil samples, regarding the type of added essential oil; x – z Different lowercase letters in the columns show statistically significant differences between the linseed oil samples, regarding the storage time; X – Y Different capital letters in columns show statistically significant differences between linseed oil samples, regarding storage conditions (light or dark).

From Table 4, it can be seen that the content of free fatty acids in all tested samples was within the limits allowed by the Regulation on Vegetable Oils, Edible Vegetable Fats and Mayonnaise [25]. The content of free fatty acids in the base sample in the initial analysis was 0.73% and increased with the addition of all essential oils in both concentrations. From the table, it can be seen that the content of fatty acids in all samples after the 15th day of storage both in the light and in the dark decreased compared to the initial values, with the smallest change recorded in the base sample (0.68% and 0.56%). After the 30th

day of storage, both in the light and in the dark, the content of free fatty acids increased in all samples, but mostly remained within the range of values that the samples had during the initial analyses, that is, there was no statistically significant difference compared to the initial results, which means that the essential oils used had a positive effect on the oxidative stability of the oil. Slightly higher values were recorded for samples with the addition of thyme essential oil in the amount of 0.1%. After 30 days of storing the samples in the dark, the value of free fatty acids was 0.90%, and in the light the value was almost identical to the initial analysis (0.84%). In the sample with the addition of garlic in the amount of 0.1%, higher values were recorded in the samples stored in the dark that were identified in the initial analyses (0.90%), compared to the samples stored in the light. With the addition of oregano essential oil in both concentrations, the FFA content after 30 days of storage in the light was lower (0.84% and 0.90%) compared to the value of the initial analyses (0.90% and 0.95%). Samples stored in the dark for the same period of time (30 days) had significantly lower values of FFA content (0.79% and 0.84%). Accordingly, it can be concluded that the addition of essential oils with adequate storage conditions in the dark and at the appropriate temperature has a positive effect on the oxidative stability of the oil. According to Choo et al. [21] variations in the content of free fatty acids in linseed oil range from 0.25% to 0.98%, and according to Abd-El-hady and Elsorady [15], expressed over oleic acid, they range from 0.36 to 1.16% similar to the results of Biondić [26] (0.38%). Obtained values of the peroxide value for each tested sample of cold-pressed linseed oil with the addition of essential oils (garlic, thyme, oregano) in different concentrations (0.1% and 0.5%), after 15 and 30 days of storage in different conditions (light and dark), are shown in Table 5.

Table 5. Average values of the peroxide value ± SD in cold-pressed linseed oil with the addition of different essential oils and different concentrations (light to dark)

Peroxide value (mmol O2/kg)					
Sample	Control	15 days (light)	15 days (darkness)	30 days (light)	30 days (darkness)
Control	$0.49^{x} \pm 0.00$	$2.96^{yX} \pm 0.02$	$1.49^{yY} \pm 0.01$	$4.95^{zX} \pm 0.00$	$1.97^{zY} \pm 0.01$
0.1% BL	$0.50^{aAx} \pm 0.00$	$14.95^{aAyX} \pm 0.35$	$1.97^{aAyY} \pm 0.04$	$20.45^{aAzX} \pm 0.49$	$2.71^{aAyY} \pm 0.37$
0.5%BL	$0.50^{aAx} \pm 0.00$	$16.67^{bAyX} \pm 0.23$	$3.00^{bAyY} \pm 0.00$	$19.46^{aAzX} \pm 0.21$	$3.16^{aAyY} \pm 0.34$
0.1% MD	$0.49^{aBx} \pm 0.00$	$15.39^{aAyX} \pm 0.21$	$5.26^{aByY} \pm 0.04$	$21.78^{aAzX} \pm 0.00$	$6.80^{aBzX} \pm 0.09$
0.5% MD	$1.97^{bBx} \pm 0.01$	$15.86^{aByX} \pm 0.20$	$7.88^{bByY} \pm 0.05$	$21.75^{aBzX} \pm 0.35$	$9.00^{bBzY} \pm 0.35$
0.1% O	$0.49^{aCx} \pm 0.01$	$16.42^{abcYx} \pm 0.11$	$3.56^{aCyY} \pm 0.36$	$21.75^{aAzX} \pm 1.06$	$4.70^{aCyY} \pm 0.35$
0.5% O	$1.22^{bCx} \pm 0.34$	$13.17^{bCyX} \pm 0.09$	$4.21^{aCyY} \pm 0.41$	$20.50^{aAzX} \pm 0.00$	$5.17^{aCyY} \pm 0.38$

a – b Different lowercase letters in rows indicate statistically significant differences between linseed oil samples, regarding the concentration of added essential oil; A – C Different capital letters in the rows indicate statistically significant differences between the linseed oil samples, regarding the type of added essential oil; x – z Different lowercase letters in the columns show statistically significant differences between the linseed oil samples, regarding the storage time; X – Y Different capital letters in columns show statistically significant differences between linseed oil samples, regarding storage conditions (light or dark)

According to the results shown in the previous table, it can be stated that the peroxide values on the base samples (initial analysis) are in accordance with the Regulation on Vegetable Oils, Edible Vegetable Fats and Mayonnaise [25], where the highest value compared to the base sample (0.49 mmol O2/kg) was the sample with the addition of thyme essential oil in the amount of 0.5% (1.97 mmol O2/kg). The best protection against oxidative deterioration was shown by the samples with the addition of garlic essential oil in both concentrations (0.1% and 0.5%), with the 0.5% concentration slightly better for the oil stored in the light for 30 days (19.46 mmol O2/kg), and 0.1% for oil stored in the dark (2.71 mmol O2/kg). All samples stored in the light, except the base sample, after 15 days of storage, had significantly higher values than the initial ones, which means that the essential oils used did not show antioxidant properties. However, all antioxidant-supplemented samples stored in the dark showed some protection against oxidative deterioration even after 30 days, although the values were slightly higher than those of the base samples (initial analysis). In addition, the sample with the addition of thyme essential oil in the amount of 0.5% (9.00 mmol O2/kg) had the highest peroxide value, while the sample with the addition of thyme essential oil at a concentration of 0.1% had slightly lower value (6.80 mmol O_2/kg) and better antioxidant properties.

In the case of samples stored in the dark with the addition of the essential oil of oregano, the sample with the added concentration of 0.1% (4.70 mmol O2/kg) had better protection against oxidative deterioration, compared to the concentration of 0.5% (5.17 mmol O2/kg). However, the mentioned differences are not statistically significant. Odeh et al. [23] report in their research the peroxide values for linseed oil with the addition of herbs before and during storage. Linseed oil with the addition of essential oil of oregano shows values for the initial analysis of 2.46 mmol O2/kg, and after 60 days of storage, the peroxide value was less than 1.5 mmol O2/kg. This means that oregano has strong antioxidant properties and good stability, as well as a long oil storage time in suitable storage conditions. Also, Odeh et al. [23] state that oxidative spoilage occurs due to inadequate oil storage conditions (high temperature, daylight, ionizing radiation and the presence of heavy metals Cu and Fe) which could be supported by the results of our own research, shown in Table 5. Stanković [38] determined in her research that using essential oil (winter savoury, oregano, thyme and basil), with the addition of 0.05%, there is a decrease in the peroxide value in apricot kernel oil after 25 min of microwave heating compared to the base sample. This means that the examined essential oils possess antioxidant properties and show protection against oxidative deterioration. In her work, Stokić [39] obtained similar initial peroxide values in oil without the addition of antioxidants (0.41 mmol O2/kg), similar to the one in this research. The peroxide value of the same sample as well as samples with added antioxidants (BHA, BHT, PG and α-tocopherol) increased during the 96 h of the test, which proves that not all antioxidants necessarily improve the oxidative stability of the oil [38].

3.3 Principal Component Analysis (PCA) of Fatty Acid Composition and Chemical Parameters

The analysis of the main components was carried out on the basis of a correlation matrix in which 28 parameters were included for four groups of linseed oil samples (base, with the addition of essential oils of oregano, thyme and oregano). For the analysis of the main

components, the contents and ratios of the following fatty acids were used as variables C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C14:1, C16:1, C17:1, C18:1, C22:1, C18:2, C18:3, C18:4, C20:4, SFA,

MUFA, PUFA, PUFA/SFA, MUFA/SFA, n-6/n-3, moisture content, insoluble impurities content, free fatty acids content and peroxide value. The first two components, which are the result of testing the composition of fatty acids and chemical parameters of linseed oil without and with the addition of essential oils contained 79.28% of the total variance, namely the first 43.96% and the second 35.32%, which indicates that the examined parameters were well chosen. The cumulative variance for the four principal components was 94.30%. It can be seen from the graph that octane fatty acid (C8:0) was highly positively correlated with C16:0, C18:1, MUFA and MUFA/SFA. A significant positive correlation was found between the peroxide value, the content of insoluble impurities, C14:0, C20:4 and PUFA. Also, there was a very high positive correlation of fatty acids C12:0, C15:0, C17:0, C18:4, C18:3, C22:1, C18:0, C17:1, C20:0 with moisture content and of free fatty acids. A high correlation was found between C14:1, C16:1 and SFA content, and all mentioned fatty acids were significantly negatively correlated with C18:2 content, PUFA/SFA and n-6/n-3 ratios. From the graph it can be seen that the samples of linseed oil with the addition of garlic oil were positioned in relation to the content of C8:0, C16:0, C18:1, MUFA and the MUFA/SFA ratios that were characteristic of them (blue color of the ellipsoid). The base sample of linseed oil without additives separated into a separate quadrant (purple color of the ellipsoid). The content of moisture, free fatty acids, C12:0, C17:0, C18:0 and C17:1 were characteristic for samples of linseed oil with the addition of essential oil of oregano (red color of the ellipsoids). The samples of linseed oil with the addition of essential oil of thyme were positioned in a

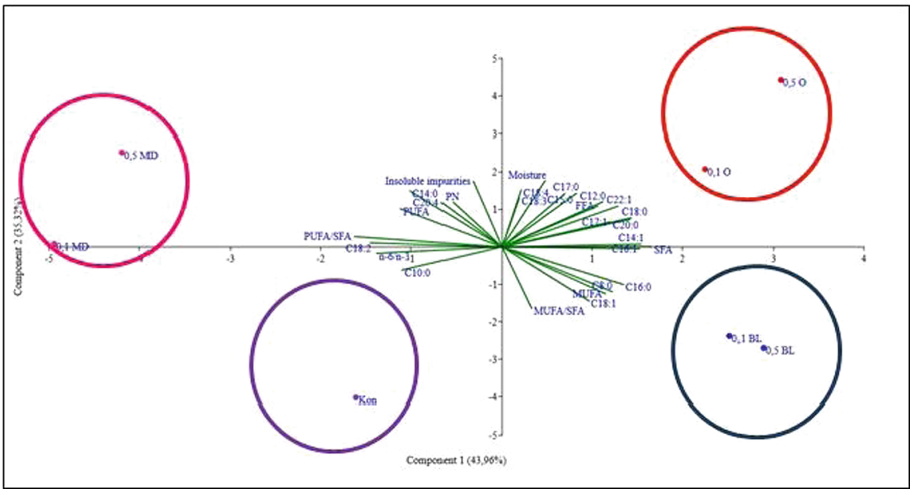

Fig. 1. The plot of principal component analysis of the composition of fatty acids and chemical parameters of linseed oil without and with the addition of essential oils (*Kon – Control; BL – a sample with the addition of garlic; MD - a sample with the addition of thyme; O - a sample with the addition of oregano; PN - peroxide value; FFA- free fatty acids*)

separate quadrant (pink color of the ellipsoid), where the content of insoluble impurities, C14:0, C20:4 and peroxide values were characteristic for the sample of linseed oil with the addition of 0.5% of the mentioned essential oil. On the other hand, the characteristic content of C18:2, C10:0 and values of the ratio between PUFA/SFA and n-6/n-3 acids had a sample of linseed oil with the addition of thyme essential oil in the amount of 0.1% (Fig. 1).

4 Conclusion

The chlorophyll content determined in the base sample of linseed oil (1.85 mg/kg) corresponds to literature data, but compared to the results of other authors, the carotenoid content (13.50 mg/kg) is slightly higher, and the phenol content (381.51mg/ kg GAE) differs both in this and in other studies. Variations in the content of total phenols in linseed oil can be explained by the fact that the chemical composition of plant materials is influenced by a number of factors that can change them. With the addition of essential oils and in higher concentrations, the value of the content of moisture and insoluble impurities increases. In all tested samples, the average content of saturated fatty acids had significantly lower values (9.68%), compared to the average content of unsaturated fatty acids (84.5%), of which polyunsaturated fatty acids were dominant (68.2%). The content of linolenic acid increased with the addition of different essential oils, and the sample with the addition of oregano in the amount of 0.1% (50.00%) had the highest value. The high content of linolenic acid resulted in a low n-6/n-3 ratio, which averaged 0.4%. The content of free fatty acids in all tested samples was within the allowed values. After the 15th day of storage, it was lower both in the light and in the dark, and after the 30th day of storage, it also increased in all samples. There was no significant difference compared to the initial results. In the initial analyses, the linseed oil samples had appropriate peroxide values (0.49–1.97 mmol O2/kg). All samples kept in the light, except for the base one, after the 15th day had peroxide values that were significantly higher compared to the initial ones. All oil samples, stored in the dark with the addition of different essential oils and concentrations, had certain antioxidant properties and better stability even after 30 days of storage. Based on the obtained results, we can state that the applied essential oils have an antioxidant effect and a beneficial effect in terms of improving the stability of edible vegetable oils. Analyses performed only on one sample of vegetable oil with the addition of different essential oils and in different concentrations, showed that it is possible to make a new and/or similar product (without the application of new technologies), which would satisfy all parameters not only of the quality of the oil but also of its organoleptic properties. We have these or similar products on the market today, but it is important to apply their different concentrations that will positively or negatively indicate how and to what extent they affect the nutritional properties on the one hand and the health aspect on the other. Regarding the health aspect, research should be continued and focused on a special phase of testing, in order to establish the concentration of the very important toxic substance pulegone, which is present in almost all essential oils and causes the appearance of allergies and a negative effect on the liver and nervous system.

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Identification of Anthocyanins and Anthocyanin-Derivatives in Vranec Wines During Aging

Violeta Ivanova-Petropulos[1(✉)], Dragan Nikolić[2], Borimir Vojnoski[3], and Isidro Hermosín-Gutiérrez[4]

[1] Faculty of Agriculture, Goce Delcev University, Stip, Krste Misirkov, 10A, 2000 Stip, Republic of North Macedonia
violeta.ivanova@ugd.edu.mk
[2] Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia
[3] Department of Enology, Institute of Agriculture, University "Ss. Cyril and Methodius", Arhimedova 5, 1000 Skopje, Republic of North Macedonia
[4] Instituto Regional de Investigación Científica Aplicada (IRICA), Escuela de Ingenieros Agrónomos, Universidad de Castilla-La Mancha, Ronda de Calatrava 7, 13071 Ciudad Real, Spain

Abstract. This study aimed to characterization of anthocyanins (3-monoglucosides, 3-acetylglucosides, and 3-coumaroylglucosides), pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins in Vranec wines during aging of three years. The HPLC-DAD–ESI-MS^n technique was applied for identification of anthocyanins and derived stable pigments pyranoanthocyanins. All anthocyanins presented mass spectra characterized with two signals, molecular ion M^+ and fragment ions $[M\text{-}162]^+$, $[M\text{-}204]^+$ and $[M\text{-}308]^+$ resulting from elimination of glucose, acetylglucose and *p*-coumaroylglucose moieties, respectively. From the group of pyranoanthocyanins, A-type and B-type vitisins, as well as hydroxyphenyl-pyranoanthocyanins have been determined according to their molecular ions (M^+) and characteristic fragments.

Keywords: anthocyanins · pyranoanthocyanins · hydroxyphenyl-pyranoanthocyanins · Vranec wine · HPLC-DAD-ESI-MS^n

1 Introduction

Colour of wine is one of the most important sensorial attribute, firstly perceived by the consumers. Anthocyanins are the responsible colored compounds, synthesized in the skins of red grape berries and transformed to the wine. The main anthocyanins in *Vitis vinifera* L. grape varieties are delphinidin, cyanidin, petunidin, peonidin and malvidin in a form of 3-*O*-glucosides, 3-*O*-acetylglucosides, 3-*O*-*p*-coumroylglucosides [1, 2]. Among all them, derivatives of malvidin are the most abundant colored compounds in red grapes and wines.

I. Hermosín-Gutiérrez—In memoriam.

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When maceration will start, anthocyanins are the first compounds which are extracted from red grape skins, reaching maximum levels after few days of maceration (usually 3 to 4 days). Afterwards, their content is followed with decreasing during the end of fermentation, stabilization and storage of wine. It has been determined that there are many reasons for decrease of anthocyanin's content, including their adsorption on yeast cell walls, co-precipitation with proteins and tartarates, participation in various chemical reactions in which new and stable compounds are formed. Moreover, their content decreases during filtration and finning process [3, 4]. In particular, anthocyanins participate in formation of anthocyanin-derived pigments during wine storage and ageing, and thus, contribute to a progressive change of the red–purple colour towards a more red–orange colour, especially evident during aging. In fact, anthocyanins participate in cycloaddition reaction at the O-5 and C-4 positions with fermentation metabolites and other grape and wine phenolic compounds [5–7], forming new stable anthocyanin-derived pigments, and named pyranoanthocyanins. This group includes vitisins, hydroxyphenyl-pyranoanthocyanins and flavanyl-pyranoanthocyanins, playing an important role in colour stabilization and sensory properties [7–10].

In the past decades, different techniques have been used for analysis of pigments and studying their structure, such as high-performance liquid chromatography (HPLC) and ultrahigh-performance liquid chromatography (UPLC) coupled to diode array detection (DAD) and/or mass spectrometry (MS) [1–8, 11], matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) [12, 13], nuclear magnetic resonance (NMR). Recently, high performance liquid chromatography combined with low-field drift tube ion mobility time-of-flight mass spectrometry (HPLCxIMS-TOFMS) was used for characterization and fingerprinting of Macedonian red wines [14].

In this study, the focus of the work was set on the identification and quantification of individual anthocyanins and pyranoanthocyanins, the most important compounds which determine the wine colour and stability. For that purpose, wines produced from Vranec variety, the most widespread and important grape variety for Macedonia and for the Balkans, characterized with deep red colour, have been analyzed with HPLC coupled to DAD and ESI-MS (Ion Trap) in order to identify and investigate the changes of pigments during wine aging.

2 Materials and Methods

2.1 Chemicals and Reagents

Commercial standard of malvidin 3-glucoside was purchased from Phytolab (Vestenbergsgreuth, Germany). Standards of pyranoathocyanins, including vitisin A or 10-carboxy-pyranomalvidin-3-glucoside, pinotin A or 10-(4‴-monohydroxyphenyl)-pyanomalvidin-3-glucoside and 10-(3‴, 4‴-dihydroxyphenyl)-pyranomalvidin-3-glucoside, have been isolated in laboratory conditions [6]. All other solvents used were of HPLC quality and purity (> 99%) and all chemicals of analytical grade (p.a). Water used for analyses was of Milli-Q quality.

2.2 Wine Samples

Red wine samples from Vranec *V. vinifera* L. variety (vintages: 2006, 2007 and 2008) were kindly provided by Tikveš Winery, Kavadarci. Wines from the three vintages have been produced by same winemaking protocol in triplicates. Thus, harvested grapes (maturity of 22 to 24 °Brix) were processed with electrical inox crusher/destemmer, then supplemented with SO_2 (ca. 60 mg/L total concentration), and after few hours, *Saccharomyces cerievisiae* yeast was inoculated to start the alcoholic fermentation. Maceration time of 8–10 days at 23 ± 2 C was applied, with pumping over and delastage once to two times per day. Wines produced with the same technological treatment, from three tanks were mixed in order to obtain representative samples for analysis.

2.3 HPLC-DAD-ESI-MS^n Analysis

Analyses of pigments have been performed with instrumentation supplied by Agilent: An Agilent 1100 Series system (Agilent, Germany) coupled to DAD (G1315B) and a LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI-MS^n) system. An Agilent ChemStation (version B.01.03) software was used for data processing and Agilent LC/MS Trap software (version 5.3) was used for mass spectra processing. Before analyses, wines were diluted with 0.1 M HCl solution (1:4, *V/V*), filtrated (0.20 μm, polyester membrane, Chromafil PET 20/25, Macherey-Nägel, Düren, Germany) and then were injected into the HPLC system. Separation of the analytes was performed on a Zorbax Eclipse XDB-C18 column (250 × 4.6 mm; 5 μm particle size; Agilent, Germany) at 40 °C. The mobile phase consisted of solvent A: water/acetonitrile/formic acid (87:3:10, *V/V/V*, solvent A) and solvent B: water/acetonitrile/formic acid (40:50:10, *V/V/V*), at flow rate of 0.63 mL/min. Proportions of solvent B were as follows: 0 min, 6%; 15 min, 30%; 30 min, 50%; 35 min, 60%; 38 min, 60%; 46 min, 6% [3].

Identification of pigments was performed in a positive ionization mode. Nitrogen was the drying gas (flow rate of 11 L/min), the drying temperature was set at 350 °C, the pressure of the nebulizer was 65 psi, the capillary at 2500 V, capillary exit offset at 70 V, skimmer 1 at 20 V; skimmer 2 of 6 V and the compound stability at 100%. The mass spectra were recorded in *m/z* range of 50–1200. DAD chromatograms were recorded at 520 nm [3].

2.4 Statistical Analyses

XLSTAT software, version 7.5.2, Addinsoft (Paris, France) was used for calculation of means, standard deviation and relative standard deviation. Each wine was analyzed in three replicates.

3 Results and Discussion

3.1 Identification of Anthocyanins and Anthocyanin-Derivatives

Identification of individual anthocyanins and anthocyanin-derivatives in Vranec wines (vintage 2006, 2007 and 2008) was performed with HPLC–DAD–ESI-MS^n technique. In total, 25 pigments were determined, including 14 anthocyanins, 5 pyranoanthocyanins and 6 hydroxyphenyl-pyranoanthocyanins. Identification of analyte peaks was performed comparing the UV/Vis spectra and the retention times of compounds for which standards were available. Moreover, obtained ESI-MS and MS/MS data were compared with those found in the relevant literature [5–10]. Table 1 contains the data for the molecular and fragment ions of the identified compounds.

Table 1. MS identification of colored compounds in Vranec wines.

Pigments	MS (m/z)	MS/MS (m/z)
Anthocyanins	M^+	Fragment ion
3-O-glucosides		
Delphinidin-3-glucoside	465	303
Cyanidin-3- glucoside	449	287
Petunidin-3- glucoside	479	317
Peonidin-3- glucoside	463	301
Malvidin-3- glucoside	493	331
3-O-Acetylglucosides		
Delphinidin -3-acetylglucoside	507	303
Petunidin -3- acetylglucoside	521	317
Peonidin -3- acetylglucoside	505	301
Malvidin -3- acetylglucoside	535	331
3-O-p-Coumaroylglucosides		
Delphinidin -3-*p*- coumaroylglucoside	611	303
Cyanidin -3- *p*-coumaroylglucoside	595	287
Petunidin -3- *p*-coumaroylglucoside	625	317
Peonidin -3- *p*-coumaroylglucoside	609	301
Malvidin - *p*-coumaroylglucoside	639	331
Pyranoanthocyanins		
Vitisins		
Vitisin A	561	399
Acetyl-vitisin A	603	399
p-Coumaroyl-vitisin A	707	399

(*continued*)

Table 1. (*continued*)

Pigments	MS (m/z)	MS/MS (m/z)
Vitisin-B	517	355
Acetyl-vitisin-B	559	355
Hydroxyphenyl-pyranoanthocyanins		
10-DHP- pyranomalvidin -3- glucoside (pinotin A)	625	463
10-DHP- pyranomalvidin -3- acetylglucoside	667	463
10-DHP- pyranomalvidin -3- *p*-coumaroylglucoside	771	463
10-MHP- pyranomalvidin -3- glucoside	609	447
10-MHP- pyranomalvidin -3- acetylglucoside	651	447
10-MHP- pyranomalvidin -3- *p*-coumaroylglucoside	755	447

Abbreviations: 10-DHP: 10-(3′′′, 4′′′-dihydroxyphenyl); 10-MHP: 10-(4′′′-monohydroxyphenyl)

Anthocyanins. In total, 14 anthocyanins were identified in analyzed Vranec wines, present in a form of 3-*O*-glucosides, 3-*O*-acetylglucosides and 3-*O*-*p*-coumaroylglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Identification was based on two characteristic signals, molecular ion M^+ and aglycone fragments $[M–162]^+$, $[M–204]^+$ and $[M–308]^+$ as a result of elimination of a glucose moiety, acetylglucoside group and *p*-coumaroylglucoside group, respectively [1–3].

Pyranoanthocyanins. Pyranoanthocyanins are formed in a reaction of cycloaddition between anthocyanins and pyruvic acid, caffeic acid, and *p*-coumaric acid. These compounds are called 10-carboxy-pyrano-anthocyanins or A-type vitisins [10]. In this study, three A-type vitisins have been identified in wines, as follows: vitisn A (10-carboxy-pyranomalvidin-3-glucoside), acetyl-vitisin A (10-carboxy-pyranomalvidin-3-acetylglucoside) and *p*-coumaroyl-vitisin A (10-carboxy-pyranomalvidin-3-*p*-coumaroylglucoside). These three compounds show same characteristic fragment ($[M + H]^+ = m/z$ 399) which corresponds to 10-carboxy-pyranomalvidin aglycone [3]. Thus, the mass spectrum of vitisin A contains the molecular ion M^+ at *m/z* 561 and fragment ion at *m/z* 399, resulting from the loss of glucose. The mass spectra of acetylvitisin A and coumaroylvitisin A show M^+ at *m/z* 603 and 707, respectively, and fragment ions at *m/z* 399, as a result of the elimination of acetyl- and *p*-coumaroyl groups, respectively.

B-type vitisins are formed in a cycloaddition reaction between anthocyanins and acetaldehyde. In this study, following B-type vitisins were identified: vitisin B (pyranomalvidin-3-glucoside) with molecular signal M^+ at *m/z* 517 and fragment ion at *m/z* 355 by loss of glucoside (162 Da) and acetyl-vitisin B (pyranomalvidin-3-acetylglucoside) with molecular signal M^+ at *m/z* 559 and fragment ion at *m/z* 355 by loss of acetylgucoside (204 Da) [3, 15]. Fragmentation of vitisin A and vitisin B is presented in Fig. 1.

Hydroxyphenyl-Pyranoanthocyanins. Hydroxyphenyl-pyranoanthocyanins are compounds form in a reaction of caffeic acid and anthocyanins. In this study,

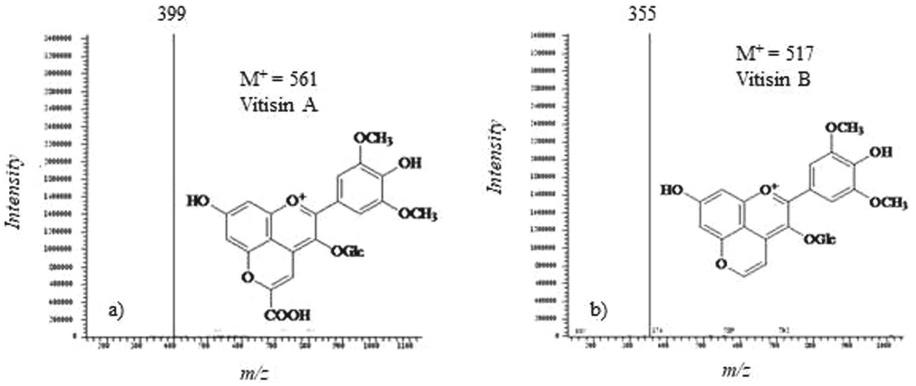

Fig. 1. MS/MS fragmentation of vitisin A (a) and vitisin B (b).

three hydroxyphenyl-pyranoanthocyanins have been determined, observing molecular ions at *m/z* 625, 667 and 771 and identified as 10-(3‴, 4‴-dihydroxyphenyl)-pyranomalvidin-3-glucoside (known as pinotin A) [6], 10-(3‴, 4‴-dihydroxyphenyl)-pyranomalvidin-3-acetylglucoside and 10-(3‴, 4‴-dihydroxyphenyl)-pyranomalvidin-3-*p*-coumaroylglucoside, respectively. MS/MS fragmentation of molecular ions gave fragment ion at *m/z* 463 corresponding on elimination of glucose, acetylglucoside and *p*-coumaroylglucoside groups, respectively [15].

Moreover, compounds originated in a reaction of *p*-coumaric acid and anthocyanins are called hydroxyphenyl-pyranoanthocyanins. MS/MS analysis of Vranec wines showed presence of molecular ions at *m/z* 609, 651 and 755, which were identified as 10-(4‴-monohydroxyphenyl) derivatives of pyranomalvidin-3-glucoside, pyranomalvidin-3-acetylglucoside and pyranomalvidin-3-*p*-coumaroylglucoside, respectively, all producing fragment ion at *m/z* 447 [15].

3.2 Influence of Aging on Pigments Content

Table 2 summarize the data for the individual anthocyanins, pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins quantified in Vranec wine. Quantitative analysis of pigments was performed on a basis of peak area calculations in the HPLC-DAD chromatograms which were recorded at 520 nm. UV/Vis chromatogram of one Vranec wine is presented in Fig. 2.

Malvidin derivatives were present in highest content in wines, as it was expected. Thus, malvidin-3-glucoside was the main anthocyanin in wines from all three years of production (47.6 to 50.2%, on a molar basis), regardless the year of production, as it is already known for most of the *V. vinifera* cultivars, followed by petunidin-3-glucoside (10.6 to 14%, on a molar basis), whereas the cyanidin-3-glucoside (1.03 to 2.34%, on a molar basis) was present in lowest content in all wines (Table 2). Obtained results were in accordance to previous work focused on phenolic analysis of Macedonian red wines [4].

With regards to pyranoanthocyanins, vitisin A was the dominant compound in all wines, present in a relatively high amount (39.5 to 69%), followed by acetyl-vitisin A

Table 2. Content of **a**nthocyanins, pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins in Vranec wines from three vintages (2006, 2007 and 2008) (molar % of each compound in its group and total content of each group of compounds in mg/L).

Compounds/Vintage	Vranec wines		
	2006	2007	2008
Anthocyanins			
Delphinidin-3-glucoside	10.9 ± 0.08	7.38 ± 0.04	6.69 ± 0.04
Cyanidin-3- glucoside	2.34 ± 0.02	2.09 ± 0.02	1.03 ± 0.01
Petunidin-3- glucoside	14.0 ± 0.11	10.6 ± 0.10	10.9 ± 0.09
Peonidin-3- glucoside	10.1 ± 0.09	9.34 ± 0.08	8.10 ± 0.08
Malvidin-3- glucoside	47.6 ± 0.39	50.2 ± 0.42	48.7 ± 0.45
Delphinidin -3-acetylglucoside	2.51 ± 0.02	1.74 ± 0.02	1.12 ± 0.01
Petunidin -3- acetylglucoside	0.47 ± 0.01	0.93 ± 0.01	1.54 ± 0.01
Peonidin -3- acetylglucoside	0.71 ± 0.01	1.14 ± 0.01	1.47 ± 0.01
Malvidin -3- acetylglucoside	3.13 ± 0.02	5.54 ± 0.04	7.24 ± 0.06
Delphinidin -3-*p*-coumaroylglucoside	0.65 ± 0.01	0.66 ± 0.01	0.92 ± 0.01
Cyanidin -3-*p*-coumaroylglucoside	0.65 ± 0.01	0.73 ± 0.01	0.78 ± 0.01
Petunidin -3-*p*-coumaroylglucoside	0.82 ± 0.01	0.75 ± 0.01	1.41 ± 0.01
Peonidin -3-*p*-coumaroylglucoside	1.62 ± 0.01	2.20 ± 0.02	2.40 ± 0.02
Malvidin -*p*-coumaroylglucoside	4.57 ± 0.03	6.80 ± 0.05	7.64 ± 0.06
Total anthocyanins*	**16.1 ± 0.2**	**53.6 ± 0.6**	**508 ± 6.2**
Vitisins			
Vitisin A	69.0 ± 0.58	59.7 ± 0.52	39.5 ± 0.36
Acetyl-vitisin A	12.6 ± 0.11	16.4 ± 0.14	19.7 ± 0.17
p-Coumaroyl-vitisin A	11.5 ± 0.12	14.9 ± 0.16	12.6 ± 0.14
Vitisin B	6.67 ± 0.05	8.82 ± 0.07	28.4 ± 0.24
Acetyl-vitisin-B	< LOD	< LOD	< LOD
Total vitisins**	**6.94 ± 0.06**	**15.5 ± 0.13**	**53.1 ± 0.57**
Hydroxyphenyl-pyranoanthocyanins			
10-DHP- pyranomalvidin -3- glucoside (pinotin A)	45.5 ± 0.45	38.1 ± 0.30	25.4 ± 0.26
10-DHP- pyranomalvidin -3- acetylglucoside	< LOD	10.8 ± 0.18	< LOD
10-DHP- pyranomalvidin -3-*p*-coumaroylglucoside	< LOD	< LOD	< LOD
10-MHP- pyranomalvidin -3- glucoside	55.1 ± 0.52	38.2 ± 0.39	49.8 ± 0.46
10-MHP- pyranomalvidin -3- acetylglucoside	< LOD	4.26 ± 0.05	11.5 ± 0.15
10-MHP- pyranomalvidin -3-*p*-coumaroylglucoside	< LOD	9.07 ± 0.09	13.6 ± 0.14

(*continued*)

Table 2. (*continued*)

Compounds/Vintage	Vranec wines		
	2006	2007	2008
Total HP-pyranoanthocyanins* **	**1.08 ± 0.03**	**3.59 ± 0.04**	**7.37 ± 0.07**

Abbreviations: 10-DHP: 10-(3′′′, 4′′′-dihydroxyphenyl); 10-MHP: 10-(4′′′-monohydroxyphenyl).
*mg/L, as malvidin 3-glucoside; **mg/L, as vitisin-A; ***mg/L, pinotin A; Limit of detection (LOD): Mv-glc 0.640 mg/L.
All results are average values of three replicates ± SD (standard deviation).

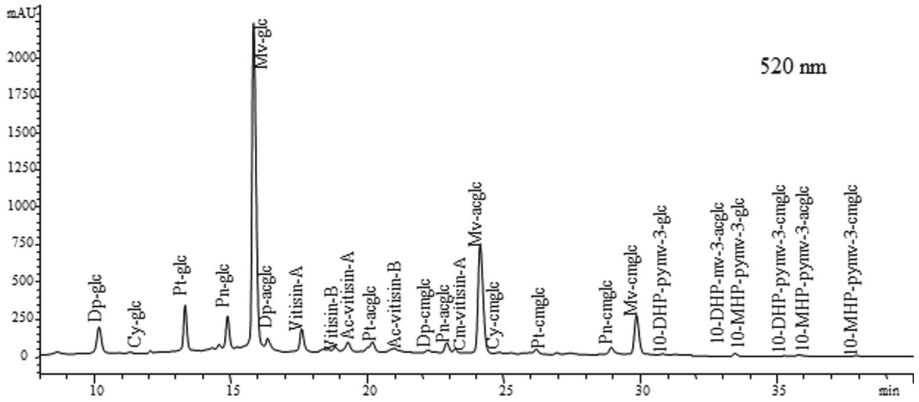

Fig. 2. UV-Vis chromatogram of Vranec wine sample (vintage 2007) recorded at 520 nm. **Abbreviations**: Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pn: peonidin; Mv: malvidin; glc: 3-glucoside; acglc: 3-(6′′-acetyl)-glucoside; cmglc: 3-(6′′-coumaroyl)-glucoside; 10-DHP: 10-(3′′′, 4′′′-dihydroxyphenyl); 10-MHP: 10-(4′′′-monohydroxyphenyl); pymv: pyranomalvidin; vitisin A: 10-carboxy-pyrmv-3-glc; vitisin B: 10-H-pymv-3-glc; A-type vitisin: 10-carboxy-pyranoanthocyanins.

(12.6 to 19.7%) and *p*-coumaroyl-vitisin A (6.67 to 28.4%). Vitisin B ranged from 6.67 to 28.4%, on a molar basis, while acetyl-vitisn B was not quantified since it was detected below the limit of detection. Concerning the group of hydroxyphenyl-pyranoanthocyanins, 10-DHP-pyranomalvidin-3-glucoside (pinotin A) and 10-MHP-pyranomalvidin-3-glucoside ranged from 25.2 to 45.3% and 38 to 54.7%, respectively (Table 2), while 10-DHP-pyranomalvidin-3-*p*-coumaroylglucoside was present in a very low amount (lower than the determined limit of detection).

Regarding the influence of aging, it is well known that anthocyanin content in red wines declines constantly, as a results of various mechanisms, such as adsorption on yeast cell, oxidation, degradation, adsorption and precipitation with tartarates, proteins, polysaccharides or condensed tannins, as well as their participation in stable and complex anthocyanin derived pigments. In this view, it was observed that total anthocyanins were highest in wine produced in 2008 (508 mg/L), followed by intense reduction in wines from 2007 (53.6 mg/L) and 2006 (16.1 mg/L). Similar trend was observed for vitisins and

hydroxyphenyl-pyranoanthocyanins content. Highest amount was noticed in the wine sample produced in 2008 (53.1 mg/L total vitisins and 7.37 mg/L total hydroxyphenyl-pyranoanthocyanins), followed by reduction of their content in wines from 2007 and 2006 (Table 2). Generally, pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins are considered as important pigments in wine, which also decrease during aging, similarly as anthocyanins.

4 Conclusion

In this study, 25 pigments (anthocyanins and anthocyanin derivatives) have been identified and quantified in Vranec wines applying HPLC-DAD-ESI-MS^n technique. Malvidin-3-glucoside and its derivatives (3-acetylglucoside and 3-*p*-coumaroylglucoside) were the major compounds in all analyzed wines. Vitisin A and 10-MHP-pyranomalvidin-3-glucoside were the dominant compounds in the group of derived pigments. Regarding the year of production, wine from vintage 2008 presented highest levels of all pigments analyzed. In general, anthocyanins dominated in all wines, regardless the year of production, followed by pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins.

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The Effect of Antioxidants on the Quality and Stability of Olive Oil

Sara Uglješa, Selma Čorbo, and Munevera Begić(✉)

Faculty of Agriculture and Food Science, University of Sarajevo, Zmaja od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina
m.begic@ppf.unsa.ba

Abstract. Lipid oxidation is significant problem during the production, processing and using of edible oils, which causing an important changes in the chemical, sensory and nutritional properties. Antioxidants are used for preventing oxidation of the edible oils (natural and synthetic). Synthetic antioxidants are cheaper than natural ones, while natural antioxidants are safer than synthetic ones. In this work, the quality and oxidation stability of olive oil with and without the addition of natural and synthetic antioxidants, were investigated. The natural antioxidants that were used were immortelle, milk thistle and smoketree extracts, while the synthetic antioxidants were: propyl gallate (PG), butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT). Four samples of olive oil were used for analysis. Different types of natural antioxidants were added with a concentration of 0.2%, while the concentration of synthetic antioxidants was 0.01%. The aim of this investigation was to monitor the influence of eleveted temperature on oil quality parameters, first of all peroxide value and acid value using the Schaal-Oven test in different time intervals for maximum 96 h. Analyses have shown that eleveted temperature has a significant influence on the change in oil quality, especially when it comes to the total acidity of the oil and the peroxide value, because there was an increase in this parameters. After completing the analysis of all tested samples, by monitoring the value of the peroxide value, the conclusion was that the antioxidants that had the most influence on reducing spoilage and improving the quality of the oil were smoketree extract from natural antioxidants and propyl gallate from synthetic antioxidants. In terms of acid value, immortelle and smoketree extracts have been shown to be good natural antioxidants, while butylhydroxytoluene has been shown to be a good synthetic antioxidant.

Keywords: olive oil · antioxidants · oil stability · peroxide value · acid value

1 Introduction

Since ancient times, olive oil has been used in the Mediterranean area, primarily for human consumption. Olive oil is rich in antioxidants, vitamins, and various nutrients, which is why many medicinal properties are attributed to it [1]. Olive oil is a functional food, whose nutritional properties have a positive effect on human health [2]. In addition to the high content of monounsaturated fatty acids, it contains biologically valuable

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components, such as α-tocopherol, phenols, phytosterols, chlorophylls and carotenoids [3]. The method of processing, olive variety, the area of cultivation, the time of harvesting olives and the method of processing, have a great influence on the mentioned components. Therefore, the process of oxidation of vegetable oils is inevitable, and the duration of the process depends on the composition of the oil itself, and on the presence of factors that accelerate or slow down oxidation [1]. It can be slowed down and even prevented by the addition of antioxidants that can be found naturally in the oil, and the best-known natural antioxidants are tocopherols, polyphenols and phytosterols. Apart from them, carotenoids and chlorophylls, natural pigments found in oils, also play a role in preventing oxidation. The addition of natural antioxidants in the form of plant extracts, aims to increase the oxidative stability of vegetable oils. The antioxidant properties of natural antioxidants in the form of plant extracts, originate from the ability of components, especially phenols, to stop or delay the aerobic oxidation of organic matter. Olive oil is an important source of natural phenolic antioxidants. Phenols in olive oil are present more compared to other vegetable oils. Their presence in the oil, even in small quantities, significantly affects the oxidation stability of the oil. According to some authors, phenols are one of the most important factors that influence the oxidative stability of olive oil and are also the most responsible for the great resistance of olive oil to oxidation. They are represented in olive oil from 50 to 500 mg/kg [3]. Vegetable oils, especially olive oil, are a rich source of phytosterols, nutritionally valuable ingredients. Dietary intake of plant sterols reduces cholesterol absorption and lowers total and LDL plasma cholesterol levels [4, 5]. The color of extra virgin olive oil is yellow to green, which comes from the presence of chlorophylls and carotenoids [2]. The decomposition of chlorophyll produces pheophytin, which is the dominant chlorophyll in the oil. The most abundant carotenoids are β-carotene and lutein. The influence of external factors, especially daylight, significantly affects the reduction of colored pigments in oil [6–8]. Factors that influence the content of chlorophylls and carotenoids in olive oil are the variety, degree of maturity, cultivation conditions, method of extraction and oil storage conditions [9]. The production of quality olive oil includes a whole series of steps, from the application of appropriate agrotechnical measures in the olive grove (fertilization, pruning, irrigation, protection against diseases and pests), through the proper harvesting of the fruit and its handling to the moment of processing in the oil mill and finally to proper storage, which are the factors that affects the content of phenols, chlorophylls and carotenoids [2]. Improper preservation and storage of olive oil results in an improper loss of product quality. That is why it is extremely important to properly store the produced oil and store it in appropriate containers in order to avoid quality deterioration [10]. The aim of the work was to determine the influence of natural and synthetic antioxidants on the quality and stability of extra virgin olive oils and the content of chlorophyll, phenol and carotenoides in oils and what their influence was.

2 Materials and Methods

2.1 Materials

Four samples of extra virgin olive oil of different origins in dark glass bottles were used for the testing. All four samples are in their original packaging (produced in 2022). The first three samples of olive oils come from the market of Bosnia and Herzegovina, while the fourth sample is from the market of the Republic of Slovenia. Olive oils were used as the control oil, subsequently treated with the addition of various types and concentrations of plant extracts, namely: smoketree, milk thistle and immortelle, with concentrations of 0.2%. The preparation of plant extracts is carried out by weighing 5 g of each plant species into a measuring flask and adding 100 ml of water. The prepared flasks with plant materials were transferred to a water bath heated to 60 °C and heating was carried out for 2 h. After that, the content was filtered. Various synthetic antioxidants were added, namely butylhydroxytoluene (BHT), butylhydroxyanisole (BHA) and propyl gallate (PG), in a concentration of 0.01%. The mentioned plant extracts are produced from the following plants: milk thistle (*Sylibum marianum*), smoketree (*Cotinus coggygria*) and immortelle (*Helicchrysum italikum*), without preservatives and artificial substances (Producer: Mobis Pharm, B&H). Plant extracts are natural substances that can be used to improve the sustainability of oils since they have significant antimicrobial, antioxidant, and other biological activities [11]. The experiment was performed in three replicates.

2.2 Methods

2.2.1 Schaal-Oven Test

To test the oxidative stability and quality of the oil, the Schaal-Oven test was applied in a drying oven at a temperature of 63 °C. This is a dynamic test, in which oxidative changes occur in the oil due to the action of heat without the influence of light. The content of free fatty acids was determined using the standard titration method BAS EN ISO 6858, 2003 [12], and the peroxide value using the Wheeler method BAS EN ISO 3960, 2001 [13]. These parameters were tested in all oil samples, before treatment and after treatment at a temperature of 63 °C. The mentioned parameters were monitored after 24, 48, 72, and 96 h of temperature treatment. The composition in total phenols, chlorophyll and carotenoid content was also monitored during the exposure of the oil to the test conditions.

2.2.2 Determination of Total Phenolic Content

The presence of total phenol was determined on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer. The method is based on the reaction of phenol with the Folin-Ciocalteau reagent. The sample for analysis is prepared in the following way: 1 ml of the sample is mixed with 15 ml of distilled water, 5 ml of Folin-Ciocalteau reagent and with 15 ml of 20% Na_2CO_3 solution. After 2 h of incubation, the absorbance of all samples is measured at 765 nm using a spectrophotometer (Perkin Elmer Lambda 25 UV/VIS, 190–1100 nm). The total amount of phenol was measured using a calibration curve, and the results were expressed as gallic acid (mg/ml). It was done according to the method [14].

2.2.3 Determination of Chlorophyll Content

Chlorophylls content was determined by measuring absorbance at 670 nm in an undiluted oil sample. The measurement results are expressed as pheophytin α. The procedure involves measuring the absorbance in an undiluted oil sample at wavelengths of 670, 630 and 710 nm with a spectrophotometer (Perkin Elmer Lambda 25 UV/VIS, 190–1100 nm) in appropriate 10 mm wide cuvettes without a blank test. The results are expressed according to the method [15].

2.2.4 Determination of Carotenoid Content

The presence of carotenoids was determined using a spectrophotometer (Perkin Elmer Lambda 25 UV/VIS, 190–1100 nm). Carotenoid content was determined by measuring absorption at 445 nm in a 10% diluted oil sample. The measurement results were expressed as β-carotenoid content [16].

2.2.5 Statistical Analysis

All determinations were carried out in triplicate, and data were reported as mean ± standard deviation. The Past 3.15 program [17] was used for statistical data processing. To determine a statistically significant difference in the peroxide values, phenolic, chlorophyll, carotenoid, and free fatty acids content in base oils a one-factor analysis of variance was applied. Also, to determine a statistically significant difference in the peroxide values and free fatty acids content, under the influence of the type of added synthetic and natural antioxidants and sampling time, a two-factor analysis of variance was applied. In case of statistically significant differences, Tukey's post-hoc test was used. Multivariate data analysis - principal component analysis or PCA analysis - was used for correlation and display of results.

3 Results and Discussion

3.1 Results of Determination of Peroxide Value and Content of Free Fatty Acids in the Control Samples

Oxidative decomposition processes can depend on several factors: the raw material from which the oil or fat is produced, the composition of the oil, storage conditions, and ingredients that can speed up or slow down the reactions [18, 19]. One-factor analysis of variance revealed a statistically significant influence of the sample factor (manufacturer) on the values of the mentioned parameters ($p < 0.05$), except for the peroxide value ($p > 0.05$). The addition of the mentioned plant extracts in the stated concentrations provides thermal stability and high resistance to oxidative degradation of the product. By examining the initial values of peroxide content of extra virgin olive oil (without the addition of plant extracts), which was used as a control, it was found that the peroxide values varied within a range 2.75–3.75 mmol O_2/kg, which correspond to the provisions of the Regulation on Vegetable Oils, Edible Vegetable Fats and Mayonnaise [20], because the values were less than 10 mmol O_2/kg. From Table 1, it can be seen that the content of free fatty acids in all tested samples were in accordance with the Regulation on

Vegetable Oils, Edible Vegetable Fats and Mayonnaise [20], because the values were less than 3% (as % oleic acid). Virgin olive oils were characterized by high antioxidant stability. High stability can be correlated with antioxidant molecules and their activity (phenolic compounds, carotenoids, pigments) and the high content of monounsaturated fatty acids in triacylglycerol molecules (about 70% oleic acid) [21]. According to Čorbo et al. [2], the peroxide values for cold-pressed olive oil are from 1.00 to 3.00 mmol O_2/kg, according to Rabrenović and Dimić [5] from 1.53 to 3.84 mmol O_2/kg, and according to Mulagić et al. [22] was 2.55 mmol O_2/kg. The stated values are in accordance with the obtained results in our research. Also, according to Stokić [23] the values of the free fatty acids for cold-pressed olive oil are from 0.59 to 0.62%, therefore, values are in accordance with the obtained results in our research.

3.2 Results of Determination of Carotenoid Content

The color of the oil is determined based on the content of chlorophyll and carotenoids. Sample M3 had the highest content of carotenoids (13.37 mg/kg), and sample M4 had the lowest content (7.32 mg/kg). According to Luaces et al. [24] the carotenoids values in extra virgin olive oils after storage (1 month) and under temperature from 60 °C to 68 °C range from 1.4 to 17.5 mg/kg, therefore, the stated values are in accordance with the results obtained in our research.

3.3 Results of Determination of Chlorophyll Content

The analysis of chlorophylls was performed on four samples of extra virgin olive oils, these were control samples, without additional antioxidants. The chlorophyll content of the tested extra virgin oils varied within a range 9.07–12.47 mg/kg. The highest content was determined in sample M3 and the lowest in sample M4. According to Anniva et al. [25] the chlorophyll values in extra virgin olive oils after storage in light and under temperature ranged from 8.6 to 68.3 mg/kg, also according to Psomiadou and Tsimidou [26] who studied Greek olive oils under the influence of light and temperature, the research showed that chlorophyll values ranged from 2.6 to 64.1 mg/kg, also according to Gómez-Alonso et al. [27], the values of the chlorophyll after 21 months at room temperature and in the dark are from 2.71 to 15.3 mg/kg. The stated values are in accordance with the obtained results in our research.

3.4 Results of Determination of Phenolic Content

Phenols are responsible for the only health claim of virgin olive oil recognized by the European Commission EU 432/2012 and the European Food Safety Authority [28]. In research Castillo-Luna et al. [29], they studied the decrease in the phenolic content of 160 extra virgin olive oil after 12 months storage in darkness at 20 °C. Phenolic concentration was decreased 42.0 ± 24.3% after this period and this reduction strongly depended on the initial phenolic profile. In our research, the content of total phenols was the highest in sample M2 (328.45 mg/kg GAE) and the lowest in sample M1 (195.86 mg/kg GAE). According to Čorbo and Đorđević [3] the values of the total phenol for olive oil are from 50 to 500 mg/kg. Therefore, the results from our research are in the values of other authors.

Table 1. Results on base oils, for the parameters listed below

Parameter	M1	M2	M3	M4
Peroxide value (mmol O_2/kg)	$3.75^{a} \pm 0.35$	$2.75^{a} \pm 0.35$	$3.25^{a} \pm 0.35$	$3.75^{a} \pm 0.35$
Free fatty acids (%)	$0.17^{a} \pm 0.00$	$0.31^{c} \pm 0.04$	$0.22^{ab} \pm 0.00$	$0.31^{bc} \pm 0.04$
Carotenoids (β-carotenoid mg/kg)	$8.51^{b} \pm 0.12$	$9.52^{c} \pm 0.11$	$13.37^{d} \pm 0.07$	$7.32^{a} \pm 0.21$
Chlorophyll (pheophytin α mg/kg)	$12.03^{b} \pm 0.04$	$11.58^{b} \pm 0.04$	$12.47^{b} \pm 0.06$	$9.07^{a} \pm 0.09$
Total phenols (mg/kg GAE)	$195.86^{a} \pm 2.62$	$328.45^{d} \pm 0.93$	$212.58^{b} \pm 5.25$	$286.20^{c} \pm 2.64$

a-b - different lowercase letters in the rows indicate statistically significant differences in the values of the examined parameters.
M1, M2, M3 and M4 - samples of extra virgin olive oil.

3.5 Results of Determining the Peroxide Value Using the Schaal-Oven Test

The Schaal-Oven test was used to test the activity of natural and synthetic antioxidants of different concentrations in examined samples of extra virgin olive oils, and the peroxide values were determined. This was observed with the addition of all antioxidants, in all concentrations. A two-factor analysis of variance revealed that there is a statistically significant influence of the sampling time on the peroxide values in all samples of olive oil with the addition of different antioxidants ($p < 0.05$). The type of antioxidants showed a statistically significant influence on the peroxide values in all samples of olive oil with the addition of different antioxidants ($p < 0.05$), except for the third tested sample ($p > 0.05$). The interaction of the factors had a statistically significant effect on the peroxide value of the M1, M3 and M4 samples of olive oil with the addition of different antioxidants ($p < 0.05$), while the determined differences in the peroxide values of the second examined sample were not statistically significant ($p > 0.05$). In the case of sample M1, it was observed that after 24 h the sample with added immortelle extract had the highest peroxide value of 5.25 ± 0.35 mmol O_2/kg. In comparison, after 48 h and 72 h, the sample with added synthetic antioxidant BHT had the highest value, $8.75 \pm 1{,}06$ mmol O_2/kg and 9.75 ± 0.35 mmol O_2/kg, respectively. Also, at the end after 96 h of exposure to temperature of 63 °C, the highest peroxide value was observed in the sample with immortelle extract, $11.00 \pm 0{,}00$ mmol O_2/kg. The sample with PG had the lowest value starting from 24 h to 96 h, so at 96 h, the value was 9.00 ± 0.00 mmol O_2/kg. In the case of sample M2, after 24 h, the causes with immortelle extract and BHA have the highest value, 4.75 ± 0.35 mmol O_2/kg, then after 72 h and 96 h, the sample with PG had the highest peroxide value, 9.50 ± 0.70 mmol O_2/kg. The lowest peroxide value was found in the sample with smoketree extract 7.75 ± 0.35 mmol O_2/kg. Sample M3, has the highest value with added milk thistle extract and at the end of 72 h and 96 h, 11.00 ± 0.00 mmol O_2/kg, 14.50 ± 0.70 mmol O_2/kg. After 72 h and 96 h, the samples with smoketree and immortelle extracts, and BHA were significantly accelerated. The lowest value was after 48 h and 96 h, in the sample with PG, so at 96 h, the value was $10.50 \pm$

1.41 mmol O_2/kg. Sample M4, had the lowest value with added PG, and at the end of 72 h and 96 h, 6.75 ± 0.35 mmol O_2/kg, 8.50 ± 0.70 mmol O_2/kg. The highest value after 24 h and 48 h was the sample with milk thistle extract 5.75 ± 0.35 mmol O_2/kg, 6.50 ± 0.70 mmol O_2/kg and after 96 h the sample with BHT 13.25 ± 1.76 mmol O_2/kg. An accelerated process was observed in the sample with PG after 24 h and 48 h, 5.25 ± 0.35 mmol O_2/kg, 6.25 ± 0.35 mmol O_2/kg. According to Čorbo and Đorđević [30] the peroxide values of olive oil are from 0.5 to 15.5 mmol O_2/kg, according to Mulagić et al. [22], the peroxide values of olive oil are from 2.18 to 13.80 mmol O_2/kg, therefore, values are in accordance with the obtained results in our research. Also, according to Gómez-Alonso et al. [27] the peroxide values ranged from 2.67 to 6.52 mmol O_2/kg for virgin olive oil after three months of storage (at room temperature), where we can conclude that high temperature (in our case 63 °C) has a bad effect on the stability and quality of extra virgin oils, because the results of our research are higher. Obtained peroxide values for each tested sample of extra virgin olive oil with the addition of different antioxidants, natural (extracts of immortelle, milk thistle and smoketree) and synthetic (PG, BHA, BHT), of different concentrations (natural antioxidants 0.2% and synthetic 0.01%), and treated in an oven at 63 °C with the Schaal-Oven test in different time intervals (24, 48, 72 and 96 h) are shown in Table 2.

Table 2. Average values of peroxide value ± SD in different time intervals and with the addition of natural and synthetic antioxidants

M1						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$3.75^{Aa} \pm 0.35$	$5.00^{Aa} \pm 0.00$	$6.75^{Aab} \pm 0.35$	$9.00^{Ac} \pm 0.70$	$10.75^{Ad} \pm 1.06$
Milk thistle	0.2	$3.75^{Aa} \pm 0.35$	$4.75^{Aa} \pm 0.35$	$6.75^{Aab} \pm 1.06$	$7.75^{Ac} \pm 1.06$	$9.75^{Ad} \pm 0.35$
Smoketree	0.2	$3.75^{Aa} \pm 0.35$	$4.50^{Aa} \pm 0.00$	$7.00^{Aab} \pm 0.00$	$8.25^{Ac} \pm 1.76$	$9.00^{Ac} \pm 0.70$
Immortelle	0.2	$3.75^{Aa} \pm 0.35$	$5.25^{Aa} \pm 0.35$	$5.75^{Aa} \pm 0.35$	$8.25^{Ab} \pm 1.06$	$11.00^{Ac} \pm 0.00$
PG	0.01	$3.75^{Aa} \pm 0.35$	$4.00^{Aa} \pm 1.76$	$5.50^{Aa} \pm 0.70$	$6.25^{Ba} \pm 0.35$	$9.00^{Ab} \pm 0.00$
BHA	0.01	$3.75^{Aa} \pm 0.35$	$4.75^{Aa} \pm 0.35$	$8.75^{Bb} \pm 1.06$	$9.75^{Ac} \pm 0.35$	$10.75^{Ad} \pm 1.06$
BHT	0.01	$3.75^{Aa} \pm 0.35$	$4.25^{Aa} \pm 0.35$	$6.00^{Aa} \pm 0.00$	$8.00^{Ab} \pm 0.70$	$9.50^{Ac} \pm 0.70$
M2						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$2.75^{Aa} \pm 0.35$	$4.25^{Aa} \pm 0.35$	$7.00^{Ab} \pm 0.00$	$7.75^{Ab} \pm 0.35$	$9.50^{Ac} \pm 2.12$
Milk thistle	0.2	$2.75^{Aa} \pm 0.35$	$3.50^{Aa} \pm 0.00$	$6.75^{Ab} \pm 1.06$	$7.25^{Ab} \pm 1.06$	$8.25^{Ab} \pm 0.35$
Smoketree	0.2	$2.75^{Aa} \pm 0.35$	$3.25^{Aa} \pm 0.35$	$5.00^{Aab} \pm 0.70$	$6.50^{Ab} \pm 0.70$	$7.75^{Ab} \pm 0.35$
Immortelle	0.2	$2.75^{Aa} \pm 0.35$	$4.75^{Aa} \pm 0.35$	$6.25^{Aab} \pm 0.35$	$7.25^{Ab} \pm 1.06$	$8.25^{Ab} \pm 0.35$
PG	0.01	$2.75^{Aa} \pm 0.35$	$3.25^{Aa} \pm 0.35$	$6.00^{Ab} \pm 0.70$	$6.50^{Ab} \pm 0.70$	$9.50^{Ac} \pm 0.70$
BHA	0.01	$2.75^{Aa} \pm 0.35$	$4.75^{Aa} \pm 0.35$	$5.75^{Aab} \pm 0.35$	$7.50^{Abc} \pm 0.00$	$9.25^{Ac} \pm 0.35$
BHT	0.01	$2.75^{Aa} \pm 0.35$	$3.00^{Aa} \pm 0.00$	$6.50^{Ab} \pm 0.00$	$7.75^{Ab} \pm 0.35$	$9.00^{Ac} \pm 0.00$

(*continued*)

Table 2. (*continued*)

M3						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$3.25^{Aa} \pm 0.35$	$5.50^{Aa} \pm 0.35$	$8.00^{Ab} \pm 0.70$	$9.25^{Ab} \pm 1.06$	$11.00^{Ac} \pm 1.41$
Milk thistle	0.2	$3.25^{Aa} \pm 0.35$	$4.75^{Aa} \pm 0.35$	$7.00^{Ab} \pm 0.00$	$11.00^{Ac} \pm 0.00$	$14.50^{Bd} \pm 0.70$
Smoketree	0.2	$3.25^{Aa} \pm 0.35$	$4.00^{Aa} \pm 0.00$	$7.25^{Ab} \pm 0.35$	$9.75^{Ac} \pm 0.35$	$12.00^{Ad} \pm 0.70$
Immortelle	0.2	$3.25^{Aa} \pm 0.35$	$5.25^{Aa} \pm 0.35$	$6.25^{Aab} \pm 0.00$	$9.50^{Ac} \pm 0.70$	$12.25^{Ad} \pm 1.06$
PG	0.01	$3.25^{Aa} \pm 0.35$	$5.00^{Aa} \pm 0.00$	$5.00^{Ba} \pm 0.00$	$8.75^{Bb} \pm 0.35$	$10.50^{Ac} \pm 1.41$
BHA	0.01	$3.25^{Aa} \pm 0.35$	$5.50^{Ab} \pm 0.70$	$6.75^{Ab} \pm 0.35$	$9.75^{Ac} \pm 0.35$	$11.75^{Ad} \pm 0.35$
BHT	0.01	$3.25^{Aa} \pm 0.35$	$5.25^{Aa} \pm 0.35$	$5.75^{Bab} \pm 0.35$	$7.25^{Bab} \pm 0.35$	$11.25^{Ac} \pm 0.35$
M4						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$3.75^{Aa} \pm 0.35$	$5.00^{Aa} \pm 0.70$	$5.75^{Aa} \pm 1.06$	$8.25^{Aab} \pm 1.06$	$10.75^{Ac} \pm 1.76$
Milk thistle	0.2	$3.75^{Aa} \pm 0.35$	$5.75^{Aa} \pm 0.35$	$6.50^{Aa} \pm 0.70$	$8.00^{Aab} \pm 1.41$	$9.25^{Aab} \pm 0.35$
Smoketree	0.2	$3.75^{Aa} \pm 0.35$	$5.75^{Aa} \pm 0.70$	$6.00^{Aa} \pm 0.00$	$8.75^{Aab} \pm 1.06$	$10.75^{Ac} \pm 0.35$
Immortelle	0.2	$3.75^{Aa} \pm 0.35$	$5.75^{Aa} \pm 0.35$	$5.75^{Aa} \pm 0.35$	$8.50^{Aab} \pm 1.41$	$9.25^{Ab} \pm 0.35$
PG	0.01	$3.75^{Aa} \pm 0.35$	$5.25^{Aa} \pm 0.35$	$6.25^{Aab} \pm 0.35$	$6.75^{Aab} \pm 0.35$	$8.50^{Aac} \pm 0.70$
BHA	0.01	$3.75^{Aa} \pm 0.35$	$4.25^{Aa} \pm 0.35$	$6.00^{Aa} \pm 0.70$	$7.00^{Aab} \pm 0.70$	$9.50^{Ac} \pm 0.00$
BHT	0.01	$3.75^{Aa} \pm 0.35$	$5.25^{Aa} \pm 1.06$	$5.75^{Aa} \pm 1.76$	$7.00^{Aab} \pm 1.41$	$13.25^{Bc} \pm 1.76$

A-B - different capital letters in the columns indicate statistically significant differences between olive oil samples without and with the addition of different antioxidants.
a-b - different lowercase letters in rows show statistically significant differences between olive oil samples without and with the addition of different antioxidants sampled at different time intervals.
M1, M2, M3 and M4 - samples of extra virgin olive oil.
0, 24, 48, 72 and 96 h - sampling time (0 h - before starting the temperature treatment; sampling after 24, 48, 72 and 96 h of temperature treatment).

It can be concluded that the synthetic antioxidant propyl gallate had the best effect, but it is very interesting that the natural antioxidants showed themselves well, so smoketree extract is in step with propyl gallate. That is, in all four samples, the best where propyl gallate and smoketree extract, but on the fourth day, all but sample M2 had a peroxide value above 10 mmol O_2/kg, when we talk about the control samples, and when we talk about the causes with added antioxidants, all except sample M3, had a lower peroxide value of 10 mmol O_2/kg. Observing all the given results, it can be concluded that sample M2 is a very high-quality olive oil, while the same cannot be said for sample M3.

3.6 Results of Determining the Free Fatty Acids Content After Schaal-Oven Test

After applying the Schaal-Oven test on extra virgin olive oil samples with addition of different natural and synthetic antioxidants in different concentrations, the changes in free fatty acids contents were observed. A two-factor analysis of variance revealed that there is a statistically significant influence of the sampling time on the content of free

fatty acids in all samples of olive oil with the addition of different antioxidants ($p < 0.05$), except for the M3 sample. Also, the type of antioxidants showed a statistically significant influence on the content of free fatty acids in all samples of olive oil with the addition of different antioxidants ($p < 0.05$), except for the M3 tested sample ($p > 0.05$). The interaction of the factors had a statistically significant effect on the content of free fatty acids in the M1 and M4 samples of olive oil with the addition of different antioxidants ($p < 0.05$), while the determined differences in the content of free fatty acids in the M2 and M3 examined samples were not statistically significant ($p > 0.05$). This was confirmed with the addition of all antioxidants, in all concentrations. Analyzing the sample M1, it was noticed that during the period from 24 h to 96 h, the sample with the addition of immortelle extract had the lowest value of free fatty acids, after 24 h, the value was 0.25%, and after 96 h, 0.29%, while the sample with the addition of the synthetic antioxidant, BHA, had the highest value, after 24 h, the value was 0.62%, and after 96 h, 0.87%. Comparing sample with immortelle extract addition and the sample with BHA, during the period of 24 h to 96 h, it was noticed that the sample with BHA had almost 60% higher content of free fatty acids. Sample M2 had the highest value of free fatty acids in samples with synthetic antioxidants, BHA and BHT, throughout the entire analysis period, from 24 h to 96 h. The sample with BHA, after 24 h, had a value of 0.42%, and after 96 h, 0.54%, while with BHT, after 24 h, it had a value 0.44%, then after 96 h, 0.53%. Analyzing samples M1 and M2, it was seen that in both olive oils, BHA has the highest values of free fatty acids, but in sample M1 the values are higher by 30%. The lowest value throughout the entire analysis period was for the sample with immortelle extract, after 24 h, the amount was 0.25%, and after 96 h, 0.29%. In sample M3, samples with smoketree extract and BHT had the lowest values, where during the period from 24 h to 72 h, they had identical values, 0.28%, 0.32%, 0.34% but at 96 h the sample with BHT had a lower value (the same value as at the end of 72 h), in the amount of 0.34%. The sample with the addition of PG had the highest value of free fatty acids, throughout the entire analysis period, after 24 h, the amount was 0.50%, while at the end, 96 h, the value was 0.59%.

Table 3. Average values of free fatty acids content ± SD in different time intervals and with the addition of natural and synthetic antioxidants

M1						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$0.17^{Aa} \pm 0.00$	$0.36^{Ab} \pm 0.04$	$0.39^{Ab} \pm 0.04$	$0.40^{Ab} \pm 0.04$	$0.45^{Ab} \pm 0.04$
Milk thistle	0.2	$0.17^{Aa} \pm 0.00$	$0.31^{Aa} \pm 0.08$	$0.33^{Aa} \pm 0.04$	$0.35^{Aa} \pm 0.00$	$0.78^{Bb} \pm 0.04$
Smoketree	0.2	$0.17^{Aa} \pm 0.00$	$0.25^{Aa} \pm 0.04$	$0.25^{Aa} \pm 0.04$	$0.33^{Aa} \pm 0.04$	$0.34^{Aa} \pm 0.04$
Immortelle	0.2	$0.17^{Aa} \pm 0.00$	$0.25^{Aa} \pm 0.04$	$0.25^{Aa} \pm 0.04$	$0.28^{Aa} \pm 0.00$	$0.29^{Aa} \pm 0.04$
PG	0.01	$0.17^{Aa} \pm 0.00$	$0.39^{Ab} \pm 0.80$	$0.39^{Ab} \pm 0.04$	$0.40^{Ab} \pm 0.00$	$0.42^{Ab} \pm 0.04$
BHA	0.01	$0.17^{Aa} \pm 0.00$	$0.62^{Bb} \pm 0.40$	$0.70^{Bb} \pm 0.04$	$0.80^{Bb} \pm 0.04$	$0.87^{Bbc} \pm 0.04$
BHT	0.01	$0.17^{Aa} \pm 0.00$	$0.30^{Aa} \pm 0.12$	$0.31^{Aa} \pm 0.04$	$0.33^{Aa} \pm 0.12$	$0.34^{Aa} \pm 0.04$

(*continued*)

Table 3. (*continued*)

M2						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$0.31^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.04$	$0.39^{Aa} \pm 0.04$	$0.40^{Aa} \pm 0.08$	$0.41^{Aa} \pm 0.04$
Milk thistle	0.2	$0.31^{Aa} \pm 0.04$	$0.28^{ABa} \pm 0.08$	$0.31^{Aa} \pm 0.04$	$0.33^{Aa} \pm 0.04$	$0.42^{ABa} \pm 0.04$
Smoketree	0.2	$0.31^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.04$	$0.39^{Aa} \pm 0.04$	$0.45^{Aa} \pm 0.04$
Immortelle	0.2	$0.31^{Aa} \pm 0.04$	$0.35^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.08$	$0.38^{Aa} \pm 0.04$
PG	0.01	$0.31^{Aa} \pm 0.04$	$0.30^{Aa} \pm 0.08$	$0.36^{Aa} \pm 0.04$	$0.37^{Aa} \pm 0.00$	$0.42^{Aa} \pm 0.04$
BHA	0.01	$0.31^{Aa} \pm 0.04$	$0.42^{Aa} \pm 0.04$	$0.48^{Bb} \pm 0.04$	$0.50^{Bb} \pm 0.00$	$0.54^{Ab} \pm 0.04$
BHT	0.01	$0.31^{Aa} \pm 0.04$	$0.44^{Ba} \pm 0.12$	$0.49^{Bb} \pm 0.04$	$0.51^{Bb} \pm 0.04$	$0.53^{Bb} \pm 0.04$
M3						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$0.22^{Aa} \pm 0.00$	$0.33^{Aa} \pm 0.08$	$0.35^{Aa} \pm 0.04$	$0.35^{Aa} \pm 0.04$	$0.38^{Aa} \pm 0.04$
Milk thistle	0.2	$0.22^{Aa} \pm 0.00$	$0.28^{Aa} \pm 0.00$	$0.42^{Aa} \pm 0.04$	$0.43^{Aa} \pm 0.04$	$0.45^{Aa} \pm 0.00$
Smoketree	0.2	$0.22^{Aa} \pm 0.00$	$0.28^{Aa} \pm 0.00$	$0.32^{Aa} \pm 0.00$	$0.34^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.04$
Immortelle	0.2	$0.22^{Aa} \pm 0.00$	$0.36^{Aa} \pm 0.04$	$0.38^{Aa} \pm 0.00$	$0.39^{Aa} \pm 0.04$	$0.57^{Aa} \pm 0.04$
PG	0.01	$0.22^{Aa} \pm 0.00$	$0.50^{Aa} \pm 0.04$	$0.53^{Aa} \pm 0.04$	$0.57^{Aa} \pm 0.12$	$0.59^{Aa} \pm 0.00$
BHA	0.01	$0.22^{Aa} \pm 0.00$	$0.39^{Aa} \pm 0.08$	$0.41^{Aa} \pm 0.04$	$0.45^{Aa} \pm 0.12$	$0.47^{Aa} \pm 0.04$
BHT	0.01	$0.22^{Aa} \pm 0.00$	$0.28^{Aa} \pm 0.08$	$0.32^{Aa} \pm 0.04$	$0.34^{Aa} \pm 0.08$	$0.34^{Aa} \pm 0.08$
M4						
Antioxidants	Concentration (%)	0 h	24 h	48 h	72 h	96 h
Control	0	$0.31^{Aa} \pm 0.04$	$0.31^{Aa} \pm 0.08$	$0.39^{Aa} \pm 0.15$	$0.39^{Aa} \pm 0.04$	$0.39^{Aa} \pm 0.15$
Milk thistle	0.2	$0.31^{Aa} \pm 0.04$	$0.31^{Aa} \pm 0.12$	$0.38^{Aa} \pm 0.00$	$0.51^{Bb} \pm 0.04$	$0.67^{Bb} \pm 0.00$
Smoketree	0.2	$0.31^{Aa} \pm 0.04$	$0.31^{Aa} \pm 0.04$	$0.39^{Aa} \pm 0.00$	$0.50^{Bb} \pm 0.15$	$0.53^{Ab} \pm 0.04$
Immortelle	0.2	$0.31^{Aa} \pm 0.04$	$0.25^{Aa} \pm 0.04$	$0.29^{Aa} \pm 0.04$	$0.32^{Aa} \pm 0.12$	$0.36^{Aa} \pm 0.08$
PG	0.01	$0.31^{Aa} \pm 0.04$	$0.33^{Aa} \pm 0.04$	$0.35^{Aa} \pm 0.04$	$0.36^{Aa} \pm 0.80$	$0.38^{Aa} \pm 0.00$
BHA	0.01	$0.31^{Aa} \pm 0.04$	$0.31^{Aa} \pm 0.04$	$0.33^{Aa} \pm 0.00$	$0.45^{Aa} \pm 0.00$	$0.48^{Aa} \pm 0.00$
BHT	0.01	$0.31^{Aa} \pm 0.04$	$0.31^{Aa} \pm 0.04$	$0.31^{Aa} \pm 0.00$	$0.34^{Aa} \pm 0.00$	$0.64^{Bb} \pm 0.04$

A-B - different capital letters in the columns indicate statistically significant differences between olive oil samples without and with the addition of different antioxidants.
a-b - different lowercase letters in rows show statistically significant differences between olive oil samples without and with the addition of different antioxidants sampled at different time intervals.
M1, M2, M3 and M4 - samples of extra virgin olive oil.
0, 24, 48, 72 and 96 h - sampling time (0 h - before starting the temperature treatment; sampling after 24, 48, 72 and 96 h of temperature treatment).

After analyzing the results, it was noticed that the samples with immortelle extract, BHA and milk thistle extract accelerate reactions throughout the entire period that can affect the stability of olive oil. Analyzing the sample M4, the highest value of free fatty acid content was in the sample with added milk thistle extract, and at the end of 96 h, the value was 0.67%, and the lowest value during the entire analysis period, the sample with the addition of immortelle extract, where the amount after 24 h was 0.25%, and

after 96 h, 0.36%. According to Čorbo and Đorđević [30], the values of the free fatty acids of olive oil are from 0.148 to 1.772%, according to Bošković [31], the values of the free fatty acids of extra virgin olive oil are from 0.180 to 0.306%, therefore, values are in accordance with the obtained results in our research. Also, according to Gómez-Alonso et al. [27] values for the free fatty acids ranged from 0.29% to 0.42% for virgin olive oil after three months of storage (at room temperature), where we can conclude that high temperature (in our case 63 °C) has a bad effect on the stability and quality of extra virgin oils, because the results of our research are somewhat higher. Observing the results, it was concluded that all samples, did not have higher values than allowed by the Regulation on Vegetable Oils, Edible Vegetable Fats and Mayonnaise [20] - 3% free fatty acids expressed as oleic. All causes, along with all antioxidants, passed the Schaal-Oven test well, with the emphasis that all three natural antioxidants for all four samples did not change the stability and quality. The content of free fatty acids is an important parameter that indicates the degree of hydrolytic degradation of the oil. The obtained values for the free fatty acid content of each tested sample are presented in table 3. And represent the mean value of two determinations ± standard deviation.

3.7 Principal Component Analysis (PCA) of Sustainability Parameters

The analysis of the main components was carried out on the basis of a correlation matrix in which 10 parameters were included, that is, two parameters during sampling in five time intervals for four groups of olive oil samples (control, with the addition of natural and synthetic antioxidants). For the analysis of the main components, the values of the peroxide value and the content of free fatty acids were used as variables, when sampling five times every 24 h. The first two components that are the result of testing the sustainability parameters of olive oil without and with the addition of antioxidants contained 66.24% of the total variance, namely the first 37.08% and the second 25.16%. The cumulative variance for the four main components was 83.52%. It can be seen from the graph that the peroxide values at each sampling were in a very high positive correlation, and that all together were in a high negative correlation with the content of free fatty acids determined before the experiment was set up (FFA0). The contents of free fatty acids determined after 24 h, 48 h, 72 h and 96 h achieved a significant positive correlation. From the graph it can be seen that the samples of the second tested sample of olive oil (M2-yellow color) were separated from the other samples and positioned in the lower two quadrants in relation to the content of free fatty acids determined before setting up the experiment (FFA0), which was characteristic of them. Samples M1-BHA and M3-PG were positioned in relation to the content of free fatty acids determined after 24 h, 48 h, 72 h and 96 h, which were characteristic for them. The sample M3-MK was positioned in relation to the peroxide values determined after 72 h and 96 h that were characteristic of it, and compared to all tested samples with and without the addition of antioxidants, it had the highest peroxide values after 72 h and 96 h (Fig. 1).

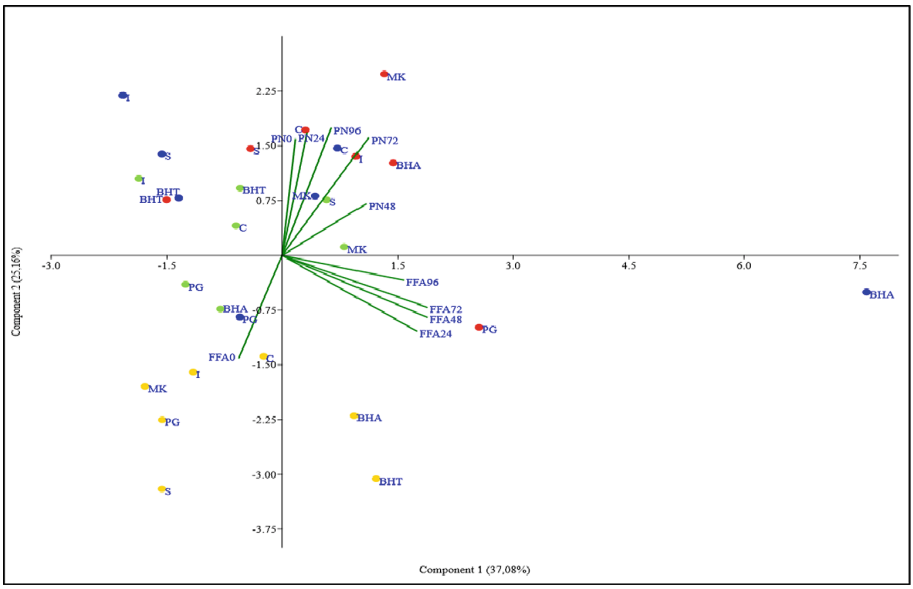

Fig. 1. The plot of principal component analysis of the sustainability parameters (M1-blue, M2-yellow, M3-red, M4-green; M1, M2, M3 and M4 - samples of extra virgin olive oil)

4 Conclusions

The tested parameters (peroxide value and free fatty acids content) reflect and indicate the quality of the oil, and the obtained initial values for determining these two parameters are within the limits within the limits prescribed by Rulebook for this type of oil. The results of the base oils have values that corrisponds to the given limits, and the amounts for the peroxide value in cold pressed olive oil are 2.75–3.75 mmol O_2/kg, and for free fatty acids content 0.17–0.31%. The addition of smoketree, milk thistle and immortelle extracts, was aimed to improve the stability of the oil, acting as antioxidants, just like synthetic antioxidants (BHA, BHT, PG). Statistical analysis of the data showed the influence of the sampling time and type of added natural and synthetic antioxidants on the peroxide values and the content of free fatty acids. It was analytically determined that all tested extra virgin olive oils are of good quality after treatment with antioxidants. Basic quality parameters (peroxide value and free fatty acids content) are in accordance with the current Rulebook. With the addition of natural and synthetic antioxidants, increased the stability, i.e. oil sustainability towards oxidative deterioration. Smoketree extract showed the best activity as a natural antioxidant, while propyl gallate (PG) was the best of the synthetic ones. Also, with the addition of immortelle extract in three oil samples, accelerated spoilage occurred, while in one sample the accelerated spoilage was caused by the addition of butylhydroxytoluene. Content of free of fatty acids was also in the values according to the valid Rulebook. Natural antioxidants showed antioxidant activity in the following order: immortelle, milk thistle and smoketree extract. Among the synthetic antioxidants, butylhydroxytoluene showed the best activity. The presence of chlorophyll in all olive oil samples was within the permissible values for fresh oil.

The presence of chlorophyll is very important because its decomposition also means a shorter shelf life of the oil. The content of carotenoids in base samples of extra virgin olive oil were on a par with other results of other studies. The carotenoid content is important because its reduction can also reduce the stability of the oil. The values of the content of total phenols were characteristic for extra virgin olive oils, and there were no deviations. If the phenol content is lower, the sustainability of the oil may be reduced. The addition of plant extracts rised the oxidative stability of examined extra virgin olive oil samples as well as acceptability from the aspect of its sensory properties.

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Plant-Based Diet as a Sustainable Diet or a New Diet Trend

Lejla Mujezin[1(✉)], Adna Salkić[2], and Irzada Taljić[3]

[1] PZU Apoteka Avicena, Branilaca 41, 71300 Visoko, Bosnia and Herzegovina
l.mujezin@avicena.ba
[2] JU Apoteke Sarajevo, Maršala Tita 1, 71000 Sarajevo, Bosnia and Herzegovina
[3] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina

Abstract. Nutrition that is based on mostly consuming plant origin food, also known as plant-based diet, generates multiple forms of dietary patterns, which in common have lower consummation of animal-based food. Plant-based diet must not be mistaken for a vegetarian diet, or subcategories of the one, even though they all have in common lower intake of animal-based food. Plant-based diet is promoted because of its' multiple health benefits.

The aim of the research is to evaluate plant-based diet as a sustainable diet or as a new diet trend. Results of this research demonstrate: positive effect on a cardiovascular health (positive effect on the decrease of lipid blood concentration, the decrease of blood pressure, the decrease of body weight, the improvement of blood sugar control, the improvement of the overall lifestyle), enablement of required protein intake among the athletes, enablement of the intake of dietary fibers, plant oils, nuts, seeds and nutritive rich food. On the contrary plant-based diet is not represented as a safe diet for children and pregnant women.

Plant-based diet satisfies all aspects of sustainability, fulfilling the regulations of environment protection, preserving humans' health, enabling easy meal preparation and respecting peoples' cultural habits. It has been shown that is not safe for pregnant women and children. Popularization of the plant-based diet would ensure industry change, improvement of peoples' overall health and satisfaction of social and ecological aspects of worlds' population.

The used study method for this research is the analysis of literatures' sources on the topic of plant-based diet.

Keywords: plant-based diet · sustainable diet · healthy diet

1 Introduction

Plant-based diet is a healthy diet which includes mostly the consummation of nutritive rich plant-based foods, and decreased consummation of refined foods, oil, and animal-based foods (including eggs and dairy), as it is mentioned in Tuso (2013). Plant-based diet refers to increased consummation of larger amounts of fruit, vegetables, legumes (beans and soya beans), seeds and nuts in smaller amounts [1].

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Sustainabilty in the area of diet and nutrition was first mentioned in 1980, at the Institute of nutrition, at the University od Giessen. Sustainable diet is the one that incorporates intake of plant-based food, such as: fruit, vegetables, whole grains, potatoes, legumes and dairy products, and decreased intake of processed foods. Sustainable diet also refers to increased intake of plant-based oils, nuts and seeds, and decreased intake of fish, eggs and meat [2].

Increased intake of plant-based foods, such as: whole grains, vegetables and fruit; correlates to decreased risk of cardiovascular diseases, where increased intake of healthy plant-based foods such as potatoes and certain sugars is related to increased risk of metabolic diseases, as it is mentioned in Satija et al. (2016) [3].

There are three plant-based indicators established, as it is mentioned in Martínez-González (2014):

- PDI (an overall plant-based diet index)
- hPDI (a healthful PDI) and
- uPDI (an unhealthful PDI),

and they can evaluate the quality of a plant-based diet [4].

The goal of this research paper is to distinguish a plant-based diet as a sustainable diet or a new diet trend.

From the research problem we have excluded the following questions, such as:

– Is a plant-based diet safe for the environment?
– Is plant-based food nutrient rich?
– Is a plant-based diet healthy and safe for the healthy and unhealthy consumers?

2 Research Methodology

Analysis of literatures' sources on the topic of plant-based diet, is the used study method.

In terms to achieve the noted goal, literature data from the following sources were used:

- Available scientific and professional literature;
- Original scientific articles from biomedicine magazines;
- Medical data bases (Medline, Embase, Science Direct), public and digital archive PubMed;
- Electronic magazines and books;
- Internet, general and specialized research programs.

Research type:

- Retrospective and
- Descritpive.

3 Results and Discussion

3.1 Results of the Studies About Effects of Plant-Based Diet on the Enviroment

Agriculture and meat consummation are responsible for 25% production of all greenhouse gases. Livestock breeding results with a 250 times greater Greenhouse gas emissions (GHGE) on a gram of produced protein in a comparison to legumes production.

Eggs, dairy, nontransportable seafood production have smaller GHGE in a comparison to the meat production [5].

Germany notes that 72% of emitted gases come from animal-based foods production, where 28% of emitted gases come from plant-based foods' production [6].

Animal-based food production engages larger financial resources, amid greater costs. In Germany, animal-based foods industry results in emitting 85% of total gas emission, which corresponds to the 44% of gas emission only from animal-based foods production [7].

Surface of used land for animal-based food production has increased during the last 20 years, on every continent, excluding Africa. Because of the lack of land for livestock breeding, many countries prefer import of animal-based foods. European Union imported 362 000 tons of beef (176 000 tons from Brasil and 106 000 tons from Argentina) during the time period from 2008. Until 2010 [8].

Water consuption is way lower for production of plant-based foods in comparison to the animal-based food production. As a confirmation of the noted, consuption of water to the kg of produced foods' products is listed: 15 415 L water for 1 kg of produced beef, 5 988 L for 1 kg of produced pork, 5 060 L water for 1 kg of produced cheese, 3 265 L water for 1 kg of produced eggs, 1 827 L water for 1 kg of produced wheat, 822 L for 1 kg of produced apples, 287 L water for 1 kg of produced potatoes, 214 L for 1 kg of produced tomatoes [9].

Waste that comes from animal-based foods processing, contains large amount of nitrogen, phosphorus and pottasium compounds, traces of metals and antibiotics. Also it is a source of more than 199 zoonotic pathogens, which can pollute certain foods' sources and water. It is estimated that 76 milion Americans yearly, are inffected by causitive agents that come from food. Worldwide 20 milion people die from diseases which are caused by the agents that come from food [10].

Production of 1 kcal of plant-based protein requires 2,2 kcal of fossil fuel. Production of 1 kcal of animal-based protein requires 25 kcal of fossil fuel, which is 11 times more in comparison to the production of plant-based protein. If livestock would only be fed by quality food, this fossil fuel use would be twice as lower [11].

Goal of the study by Friel et al. (2009), was to find the strategy for gass emission reduction for 50% until 2030. Considering the results, it is found that 30% reduction in livestock breeding could achieve the noted goal. It is also found that the livestock breeding reduction can also lower the incidence of ischemic hearth disease for 15% [12].

Results of the study by Monsivais et al. (2015), have shown that greater compliance to the Dietary Approaches to Stop Hypertension (DASH) diet, caused lower GHGE and increased financial expense. Individuals that had a better compliance, showed 16% lower GHGE a day, but 18% higher financial expense a day. Consummation of larger amounts of fruit, whole grains, and smaller amounts of salt, red and processed meat caused lower GHGE, while the consummation of vegetables, low fat dairy and highly sugared products, increased the GHGE. Data also shows that the DASH diet had 18% bigger financial cost for an individual, no matter the persons' adherence to the diet, in comparison to the unhealthy diets [13].

Table 1. Nutrients and HSR/100g for plant-based hamburgers, sausages, minced meat and animal-based hamburgers, sausages, minced meat [14].

Nutrients	*Plant-based hamburgers*	*Animal based hamburgers*	*P value*	*Plant-based sausages*	*Animal-based sausages*	*P value*	*Plant-based minced meat*	*Animal based minced meat*	*P value*
Energy (kJ)	736 ± 194	760 ± 257	0,737	735 ± 155	1157 ± 287	<0,001	574 ± 238	774 ± 162	0,035
Proteins (g)	9,7 ± 2,6	15,4 ± 2,6	<0,001	13,4 ± 6,0	16,0 ± 3,1	<0,081	13,7 ± 5,6	25,1 ± 4,0	<0,001
Fats (g)	7,2 ± 4,8	13,7 ± 7,8	0,001	7,9 ± 3,8	22,1 ± 8,4	<0,001	5,4 ± 5,2	9,4 ± 3,6	0,053
Saturated fats (g)	1,5 ± 1,6	6,2 ± 4,1	0,005	2,4 ± 2,1	8,5 ± 1,6	<0,001	2,1 ± 3,1	3,9 ± 1,7	0,108
Carbohydrates (g)	16,7 ± 7,2	5,2 ± 1,9	<0,001	11,4 ± 6,2	3,7 ± 1,5	<0,001	7,9 ± 7,3	0	
Sugars (g)	3,4 ± 3,2	1,3 ± 0,9	0,046	2,2 ± 1,9	0		1,9 ± 1,5	0	
Dietary fibers (g)	5,3 ± 2,3	NA		4,2 ± 1,8	0,6 ± 0,4	<0,001	5,9 ± 3,4	0	
Sodium (g)	372 ± 1173	463 ± 119	0,119	497 ± 136	826 ± 142	<0,001	401 ± 310	64 ± 12	0,007
Iron (g)	3,6 ± 0,8	Not registered		3,4 ± 0,4	3,6 ± 1,0	0,529	2,8 ± 1,0	2,1 ± 1,1	0,2
HSR	3,9 ± 0,4	2,9 ± 0,9	0,005	3,8 ± 0,6	1,4 ± 0,2	<0,001	4 ± 1,2	4,2 ± 0,3	0,623

Study conducted by Curtain and Grafenauer (2019), which analyzed 137 products which represent plant-based replacement for meat, shows that the number of plant-based meat products has grown 5 times in a comparison to 2015., as it is shown in Table 1. Plant-based chicken was ranked first for the protein content, while the plant-based sausages had the biggest amount of saturated fat, which content varried around 1,4–2,4 g/100. Health Star Rating (HSR) value was 46% for all products. Plant-based products had higher content of carbonhydrates and dietary fiber, and lower energy value in comparison with the animal based meat. Plant-based minced meat had 6 times higher content of sodium than animal-based minced meat, while animal-based sausages had 66% more sodium than the plant-based sausages. Difference in iron content in minced meat and sausages was not noticed, while the category of animal-based hamburgers did not show quantitative content of iron [14].

Results of the study conducted by Baden et al. (2020), have shown that during the time period of 4 years, Physical component scores (PCS) have lowered, while the Mental component scores (MCS) have increased among the participants. Results point on the correlation of growth of PDI and hPDI for more than 10 points and positive changes to PCS and MCS. It was shown that PCS increase of 10 points effected the hPDI increase in NHS group of participants, while the 10 point MCS growth resulted in the increase of hPDI in NHS II group of participants. Results of the study show the positive correlation between hPDI and Health related quality of life (HRQoL), and negative correlation between uPDI and HRQoL. Increased compliance to the plant-based diet results in the improvement of physical and mental HRQoL [15].

3.2 Results of the Studies About the Effect of Plant-Based Diet on Cardiovascular Health

A study conducted by John et al. (2002), has analyzed the effect of improved fruit and vegetable consummation on antioxidant plasma concentration and blood pressure during the time period of 6 months. Results have shown that intake of 1,4 portions of fruit and

vegetables lower the systolic blood pressure for 4 mmHg, and diastolic pressure for 1,5 mmHg [16].

Meta analysis conducted by Wang et al. (2014), had shown that the intake of 1 portion of unprocessed red meat increases the death rate from cardiovascular diseases, no matter the duration of red meat consummation [17].

Randomized clinical study conducted by Gardner et al. (2005), shows that the LDL and trygliceride reduction was more significant in "low-fat plus" group. Participants practiced the diet which was along the American Heart Association (AHA) recommendations. Control group practiced "low-fat" diet, while the study group practiced "low-fat plus diet". Among the "low-fat" group, tryglcerides levels were decreased by 0,24 mmol/L and LDL levels for 0,18 mmol/L. Tryglicerides level among "low-fat plus" group were lowered for 0,18 mmol/L, while the LDL level were decreased by 0,36 mmol/L. Tryglicerides in the "Low-fat plus" group were decreased by 8% and LDL by 9%, in comparison to the start values [18].

Ketih et al. (2015) have conducted the study, where they studied the effect of modified portofolio diet on HDL and LDL levels. Practice of the noted diet resulted in 19% LDL decrease and significant homocistein decrease. Modified portofolio diet didn't have an impact on HDL levels [19].

Results of the study conducted by Song et al. (2016), show that the replacement of animal-based proteins by 3% with plant-based proteins, causes a decrease of risk from cardiovascular disease caused deaths by 10% and the risk of cardiovascular diseases by 12% [20].

3.3 Results of the Studies About the Effect of Plant-Based Diet on Diabetes

Results of the study that was conducted by Chen et al. (2018), show that the increased intake of plant-based foods is connected to lower Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), even among the participants with increased body mass index. Results show that the increased plant-based index is connected to the lower incidence of prediabetes, as well as diabetes type 2, no matter the body mass, age, and gender [21].

Meta analysis of 13 randomized controlled studies conducted by Viguiliouk et al. (2015), studied the effect of animal-based protein replacement with plant-based protein, and its' effect on blood sugar levels. Animal protein intake in an amount of 35% of total protein intake, has resulted in hemoglobin A1c, glucose and fasting insulin decrease [22].

Results of 12 conducted cohort studies by Anue et al. (2009), show that the risk of diabetes was 17% greater for the participants who consumed greater amount of red meat, in comparison to the participants who consumed smaller amounts less often. For every 120 g of consumed red meat, risk of diabetes was greater by 20% [23].

Study that analyzed the effect of plant-based diet on blood glucose levels and body mass was conducted by Wolfram and Ismail-Beigi (2011). It showed the decrease in blood glucose level for 28% and body weight reduction for about 7,2 kg, among the participants who practiced plant-based diet, in a comparison with the control group. Control group practiced the diet for diabetics according to the American guidelines and had the same calorie intake as the group of the participants [24].

3.4 Results of the Studies About the Effect of Plant-Based Diet on Obesity

A study conductes by Shintani et al. (1991), on obese female and male participants, who consumed traditional Hawaian diet, which is a plant-based diet. This diet consisted from low intake of fish and chicken, and high intake of fruits and vegetables. Participants lowered their calorie intake from 2594 kcal to 1569 kcal a day, and increased their carbonhydrate intake from 51% to 78% of total daily energy intake. Participants reduced their body mass for an average 15 lb [25].

Results of the study conducted by Najjar et al. (2019), had shown that the intake of 11.8 portions of fruit and 16 protions of vegetable daily, had reduced energy intake by 700 kcal and noted weight loss by 14,75 lb [26].

A study conducted by Wright et al. (2017), had reviewed an impact of plant-based diet with decreased fat intake, which was about 7–15% of the total energy intake; on cardiovascular health and weight loss. Results have shown a decrease in body mass index for 4,4 kg m^{-2} after 6 months, and after 12 months 4,2 kg m^{-2}, among fat and obese participants. After three months, level of cholesterol had lowered and measured average 0,95 mmol/l, after 6 months 0,71 mmol/l, and after 12 months into the study, it measured average 0,55 mmol/l [27].

In the cohort study, conducted by Gomez-Donoso et al. (2019), effect of healthy and unhealthy plant-based diet on body weight was compared. It was found that healthy plant-based diet decreases the risk of overweight body mass for 18% during the 10 years time period, while unhealthy plant-based diet decreased the risk for only 7% [28].

3.5 Results of the Studies About the Effects of the Plant-Based Diet on Athlete Performances

Athletes often consume protein suplements, which are produced from soya, rice, peas and hemp. It is recommneded to consume protein rich foods, such as: tofu, nuts, seeds, hemp seeds, which can also be combined into smoothies and shakes. Isolated proteins in the form of powder are micronutrient poor, when compared to the wholesome foods. Because of the lack of conducted studies, there are no recommendation for increased protein intake for athletes [29].

A study shown by Frank et al. (2009), points that there is no documented advantage by consuming more than 2 g/kg body weight a day, because excessive protein intake can also result negativly on calcium depos, kidney function, bone health and cardiovascular health [30].

3.6 Results of the Studies About the Effect of Plant-Based Diet on Cognitive Health

In western countries consummation of red meat is connected to the negative impact on cognitive health, while in Asian countries this kind of effect is not reported. The cause of the mentioned statement, is that Asians consume smaller amounts of red meat, about 35 g a day, while western populations consume 128 g a day [31].

Consummation of tuna and dark meat fish has a positive effect on cognitive heath, and this effect is conected to the diets with decreased intake of animal-based foods, especially

among older females, as it is noted in the study conducted by Kim et al. (2013). Female participants who consumed 1,1–2,0 portions of tuna and dark meat fish a week, have showed better results in cognitive tests, when compared to female participants who consumed less than one portion of fish a week [32].

The study review (Kaartinen et al., 2000; Yadav et al., 2016; Rauma et al., 1993; Elkan et al., 2008) pointed out a mild positive effect of plant-based diet on the relief of migraines, multiple sclerosis, fibromialgia and rheumatoide arthritis symptoms [33].

Longitude study, which was conducted in England, by Boehm et al. (2018), shows a positive corelation of fruit and vegetables consummation on overall well-being. Also the results of the study which is conducted in New Zealand, show a positive correalation of psychological health and plant-based diet. Interventional study which was conducted on 500 participants, who were suffering from depression and anxiety, noticed the relief of symptoms after the participants consumed plant-based diet and modified their lifestyle, in a duration of 12 weeks [34].

3.7 Results of the Studies About the Effect of Plant-Based Diet on Pregnant Women and Children

Academy of Nutrition and Dietetics (ACND) had confirmed that a plant-based diet is safe during the pregnancy, and that insures the health of mother and child [35].

Plant-based diet and a diet with no consummation of animal-based foods, when correctly practiced, are safe for health of pregnant women and children. It is important to note, that correct practice of the two noted diets is possible in well developed countries, in which people are well educated about the diets and where the needed foods are easily approachable and available [36].

Gibsson et al. (2008), have conducted a crossection study on 99 pregnant women. Diet of the praticipants included the intake of corn and fermented Ethiopian bananas, while the intake of animal-based foods was less than 1% of total energy intake. Results show that 23% of pregnant women had low levels of vitamin B12, which were lower than 150 pmol/L, while 62% of them had increased level of methylmalonic acid, which was higher than 271 nmol/L. Even though they were diagnosed with vitain B12 anemia, they were not showing the symptoms of macrotic anemia [37].

Results of the study which was conducted by Haidar et al. (1999), in which the participants were the children from Ethiopia who practiced the diet with lower intake of animal-based foods; show morbidity about 22,9%, caused mostly from high fever and cough. According to the anthropometrical calculations 29,6% of children were malnourished, and 6,8% of children had lower body wight when compared to their height. 13.6% of children had lower hemoglobin level, while only two of them had the level of hemoglobine lower than 70 g/l. Iron anemia was found in 18,6% of children, who mostly consumed plant-based foods [38].

4 Conclusion

Plant-based diet has a positive effect on cardiovascular health, which is a result of lower lipid blood levels, lowered blood pressure, weight loss, improved control over blood glucose levels and improved lifestyle. Plant-based diet considers higher intake of

dietary fibers, plant oils, nuts and seeds, and nutritive rich foods. Plant-based diet is able to satisfy athletes' performances and their needs for protein intake.

We have to note the importance of plant-based indexes, as objective indicators, which can be used for evaluation of the plant-based diet.

Plant-based diet satisfies all of the norms of sustainable diet, satisfying the norms of environment preservation, peoples' health preservation, and also respecting the cultural aspects and meal preparation processes. According to the previously mentioned, we can say that plant-based diet is a sustainable diet, and as such is recommended for everyone except children and pregnant women.

Its is important to point out the relatively small "plant-based" foods' market, which is in a constant growth. In future, people should aspire to the improvement and change of agriculture and nutrition politics, as well as promotion of plant-based diet on global level. This should result in improved proccesses of plant-based foods production and distribution, which would improve the health of human population, as well as social and ecological aspects of world population.

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Characteristics of Steviol Glycosides and Their Function as Sucrose Substitutes: A Review

Adna Salkić[1(✉)], Lejla Mujezin[2], and Sanja Oručević-Žuljević[3]

[1] Public Institution Pharmacy Sarajevo, Sarajevo, Bosnia and Herzegovina
adnasalkic21@gmail.com
[2] PZU Apoteka Avicena, Visoko, Bosnia and Herzegovina
[3] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, Sarajevo, Bosnia and Herzegovina

Abstract. *Stevia rebaudiana* Bertoni or stevia represents a branched small perennial shrub from the Asteraceae family. Stevia derivates are available on the market in the form of tablets, powder and liquid. It is used in herbal medicines (diabetic tonics), food industry (confectionery, desserts, ice cream, sauces, ketchup, drinks, soft drinks, formulas for athletes) as well as cosmetics (toothpastes and mouthwashes). The feeling of sweetness in stevia is caused by steviol glycosides. The four primary steviol glycosides: stevioside, rebaudioside A, rebaudioside C, dulcoside A. Stevia is a natural non-nutritive intensive sweetener that has a significant advantage over other sweeteners (sucrose and artificial sweeteners) as a food industry ingredient. It can be used as a sucrose replacement in beverages and food products. Stevia enhances the taste of food, helps digestion, weight loss, has antioxidant and antimicrobial action, prevents tooth decay among diabetics and healthy people who care about their health. In the future, stevia is probably going to play a significant role in the supply of high strength sweeteners for the expanding natural food market.

Keywords: Steviol glycoside · stevia · sucrose substitute in food and beverages

1 Introduction

Today, there is a great interest in healthy eating throughout the world as a result of the increased incidence of obesity, which initially occurred in developed countries, and is now becoming represented in all parts of the world. Since 1975, the prevalence of obesity has increased threefold. Several other medical disorders, including steatosis, diabetes, cardiovascular illnesses, and malignant diseases, are associated with obesity as a condition. Also, the prevalence of diabetes has dramatically increased over the past few decades, which is why it is critical to act quickly to lower the disease's risk among the general population. Measures include limiting the intake of added sugars or substitution sugar with caloric or non caloric sweeteners, the application of which has the potential to reduce daily caloric intake, for the prevention and control of diabetes. The American Food and Drug Administration (FDA-Food and Drug Association) has

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approved six artificial sweeteners and two natural sweeteners, produced by plants stevia and monk fruit, which represent in addition to natural and non-nutritive sweeteners. Consumption of foods and beverages sweetened with artificial sweeteners has increased in recent years.

It is assumed that substitution nutritional sweeteners with non-nutritive sweeteners in food and drink can help control body weight. Bearing in mind the mentioned health benefits, an increasing number of companies want to follow this trend, with the intention of creating healthier products, they replace their current products with new formulations. This implies the use of non-nutritive sweeteners as the main ingredients of new formulations, which ensure sufficient sweetness of the product without increasing its energy value. As a non-nutritive sweetener, steviol glycosides (SGs) is increasingly present in food products, in numerous studies as the main substitute for sucrose as a commonly present sweeteners.

2 Steviol Glycosides in Stevia

Stevia rebaudiana Bertoni or stevia is a small branched perennial shrub from the *Asteraceae* family, native to the Amambay region in northern Paraguay. Stevia was given the names Moises Santiago Bertoni, an Italian botanist, and the Paraguayan chemist Ovid Rebaudi. It grows as an herbaceous shrub, usually 65–80 cm high. Stevia leaves are up to 5 cm long and up to 2cm wide, but the length and height vary depending on the variety of the plant itself. The plant can be cultivated and the leaves are used to sweeten food and drinks. Sweeteners made of stevia are available in liquid, powder and granulated form. From ancient times, stevia leaves have been used as medicine, to cure wounds, diabetes, and high blood pressure, as well as to sweeten a variety of beverages [1–3]. There are 154 identified species of stevia, and *Stevia Rabeudiana* Bertoni is one of them, and is particularly popular for its sweet taste. It is characterized by elongated, lanceolate or shovel-shaped leaves with jagged edges from the middle of the leaf to the tip [4]. Diterpenes, triterpenes, tannins, stigmasterol, ascorbic acid, alkaloids, steroids, saponins, flavonoids, carotene, chromium, cobalt, magnesium, iron, potassium, phosphorus, riboflavin, thiamin, zinc, apigenin, luteolin, and quercetin are just a few of the numerous substances found in stevia leaves [5]. The leaves also contain a variety of natural glycoside chemicals, such as SGs (Fig. 1), including steviolbioside, stevioside, dulcoside A, and rebaudiosides A, C. These compounds are what give the leaves their sweet flavor [4].

Steviol Glycosides

So date, more than 40 SGs have been discovered [6]. Stevioside (from 5–15% of dry matter), rebaudioside A (Reb A) F (2–6% of dry matter), rebaudioside C (Reb C) (1–2% of dry matter), and dulcoside A (0.4–0.7% dry matter) are the most prevalent SGs in stevia leaves [7]. The only difference between SGs and other diterpenes, such as steviol, is the quantity and kind of sugars connected to the R1 (OH) and R2 (H) locations of aglycone steviol. Aglycon steviol can be modified using the sugars glucose, fructose, rhamnose, xylose, and deoxyglucose. The two most significant SGs are stevioside and Reb A, which are 200–300 times sweeter than sucrose and have no calories. Stevioside

and Reb A both have two glucose molecules connected to steviol, with the difference being that Reb A has three glucose molecules attached [1, 8].

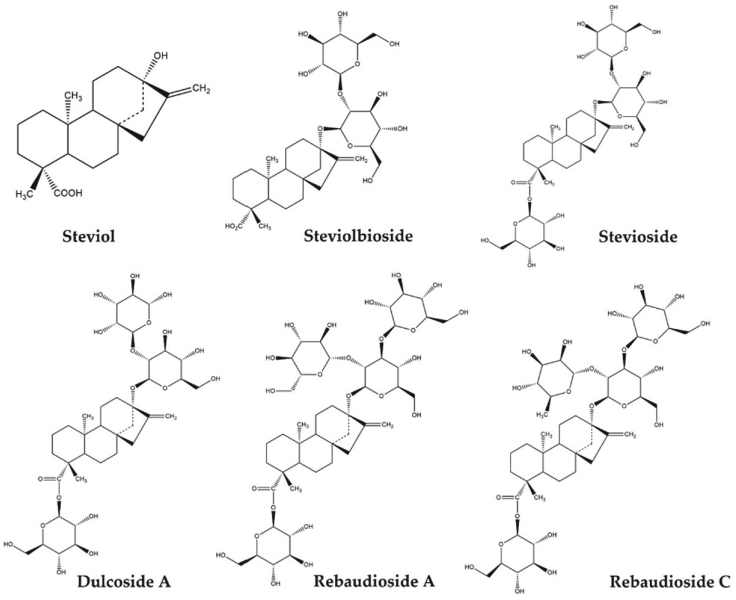

Fig.1. Chemical structure of the SGs [6].

In addition to sweetness, SGs have additional therapeutic effects including antihyperglycemic, antihypertensive, anti-inflammatory, antibacterial, antiviral, antifungal, anticancer, antidiarrheal, diuretic and immunomodulatory effects, as well as in the prevention of caries and obesity. It helps treat type 2 diabetes by promoting the release of insulin because of its effects on lowering hyperlipidemia and insulin sensitivity [2, 9].

The aforementioned characteristics of SGs allow for their widespread use in herbal medicines (tonics for diabetics), the food industry (candy, ice cream, sauces, ketchups, barbecue sauces, formulas for athletes), and cosmetics (mouthwashes and toothpastes, creams) [7].

Earlier studies have shown that the smallest polar glycoside (steviolbioside) is relatively weak in sweetness, and the two more polar glycosides (Reb A and Reb D) are among the strongest in sweetness. Table 1. Shows the relative sweetness of SGs compared to sucrose. Many studies demonstrate that Reb A's overall taste quality is superior to stevioside's due to Reb A's lower bitter taste component and lower amount of cooling in its flavor [10].

Table 1. Comparing the relative sweetness of SGs and sucrose [10].

Compound	*Relative sweetness compared to sucrose*
Stevioside	300
Rebaudioside A	250–450
Rebaudioside B	300–350
Rebaudioside C	50–120
Rebaudioside D	250–450
Rebaudioside E	150–300
Dulcoside A	50–120
Steviobioside	100–125

According to Lindley DuBois et al. examined the sweetness and bitterness of SGs depending on other commercial sweeteners. The findings revealed that Reb A has a substantially lower amount of bitterness than stevioside in addition to having a greater flavor (Table 2) [10].

Table 2. The strength and taste of stevioside and Reb A expressed as a percentage of the over all taste impression [10].

Compound	% of total taste		
	Sweet	Bitter	Other flavors
Stevioside	62	30	8
Rebaudioside A	85	12	3

Table 2. Shows the strength of sweetness and the percentage of flavors expressed: sweet and bitter, where it can be seen that Reb A is stronger than stevioside in percentage of sweetness, 85 compared to 62, and bitter in percentage of the total taste prevails with stevioside 30, and for Reb A it is 12. From this data it can be seen that other tastes (tastes that are not really desirable and can cause an aftertaste in the mouth) A Reb are more favorable, there are almost no other taste sensations - 3, compared to stevioside which is this value 8 (2.5 times higher).

Physico and Chemical Properties of SGs

SGs can withstand heat. High temperatures have no effect on the SGs' structure, preventing them from losing their sweetness. They can be used in food processing, such as pasteurization, sterilization, and frying, due to their stability at higher temperatures.

SGs Steviol glycosides do not ferment or caramelize. They are well soluble in water and water alcohol solutions. They are resistant to acids and bases [11]. Steviol glycosides are stable across a broad pH range from 3 to 9, even at 100 °C, which makes them acceptable as food additives. Food rarely displays pH values higher than 9, and

sweetness rapidly decreases above pH 9 despite this. When refrigerated at 37 °C, citric and phosphoric acid containing carbonated beverages lose sweetness by 36% and 17%, respectively. When exposed to UV light, Reb A degrades, while stevioside is stable under the same conditions [10].

Metabolism of SGs

The bacteria in the colon breaks down SGs by cleaving the glycosidic bonds, eliminating the sugar components and leaving behind the aglycone backbone, or steviol. SGs are not digested in the upper section of the gastrointestinal tract. The glycosidic linkages in stevioside cannot be broken down by human saliva, salivary alpha amylase, pepsin, pancreatin, or pancreatic alpha amylase, however human intestinal microbiota can convert stevioside to steviol. With their glucosidase activity, bacteria from the Bacteroides group cause the hydrolysis of the SGs in the intestines. The released parts of the sugar are not absorbed and the intestinal microflora uses them as a source of energy, which makes it a calorie free sweetener. Steviol is not metabolized in the intestinal microbiota, it is quickly absorbed systemically, transported to the liver where, with the help of glucuronic acid, it is conjugated into steviol glucuronide, which is excreted in urine and feces [1, 10, 12].

Regardless of the kind or quantity of sugar groups, SGs are hydrolyzed to steviol at generally similar rates. Like other SGs, stevioside and Reb A go through comparable metabolic and elimination processes [1, 13] (Fig. 2).

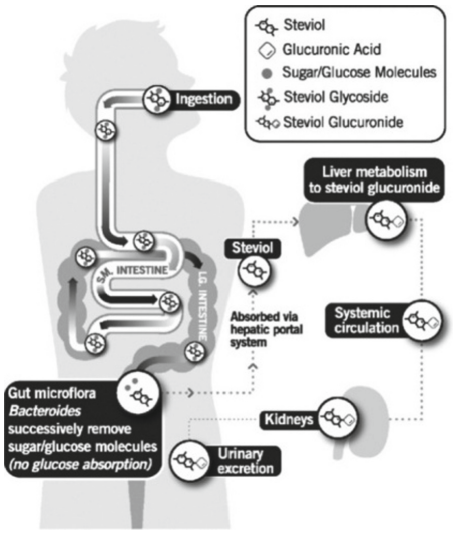

Fig.2. Metabolism of the SGs [1].

3 Substitution Sucrose with SGs

SGs are promising new raw material for the food market, with great potential for development [14]. Stevia and stevia based sweeteners are good substitutes for sucrose for several reasons. They are added in a relatively small amount and have no energy value. They have a variety of advantages over other high intensity sweeteners including acesulfame, alitame, aspartame, saccharin, and sucralose, therefore it is reasonable to anticipate that SGs will eventually replace them as the primary source of high-intensity sweeteners for the expanding natural food market [4].

Using SGs in the treatment of several chronic and non-chronic diseases, including diabetes, cardiovascular disease, cancer, kidney disease, obesity, inflammatory bowel disease, and dental caries, has a number of advantages. Regular usage of SGs has been shown to reduce blood sugar and cholesterol levels, enhance blood coagulation and cell regeneration, inhibit the formation of cancerous cells, and strengthen blood vessels [15].

Health Benefits of SGs on Glucose Level, Energy Intake, Appetite and Feeling of Satiety
Food has a significant impact on how blood glucose is regulated. SGs increases pancreatic performance and acts as a glucose stabilizer. Increased insulin sensitivity from stevioside lowers blood sugar levels. Moreover, because it is not absorbed by the body, SGs is suggested as a dietary supplement for diabetics with type 2 diabetes. It can improve glucose tolerance to consumed carbs, regulate glucagon secretion and blood sugar levels, and boost the impact of insulin on the cell membrane. It can also lower blood sugar levels. To support glucoregulation, sugars in diet can be replaced with steviol glycoside or stevioside from stevia leaves [1].

Sucrose intake has been proven to lead to a postprandial increase in glucose and insulin. Great interest has been shown in researching the influence of SGs on the postprandial level of glucose and insulin in the blood.

The effects of stevia, aspartame, and sugar on meal intake, feelings of satiety, and postprandial glucose and insulin levels were studied by Anton et al. in 2010. According to the findings, eating food sweetened with SGs significantly reduced postprandial blood glucose levels compared to eating food sweetened with sucrose and postprandial insulin levels compared to eating food sweetened with sucrose and aspartame. Also, they discovered that eating food sweetened with aspartame and SGs results in a reduced energy intake than eating food sweetened with sucrose. The Stevia group consumed significantly less food overall (including preloads) than the sucrose group did during the entire day. When the preload calories were excluded from the studies, there was, however, no observable difference in the amount of food consumed. The subjects did not overeat at lunch or supper, as the researchers discovered no differences in satiety levels between the various locations at any time [16].

Consuming SGs before a meal decreases hunger and does not result in an additional rise in postprandial glucose concentration, according to research from Farhat et al. (2019). This finding may be important for the prevention and treatment of obesity and diabetes. In comparison to sugar and water, they looked at whether stevia raised postprandial glucose levels after a meal and whether it boosted appetite and food consumption. The results showed that, when compared to water and stevia, there was no discernible difference between the preload and enhanced caloric sugar preload in terms

of energy intake throughout meals or throughout the day. The outcomes were in line with the findings of a study by Anton et al. (2010), which discovered that stevia did not, in comparison to sugar, result in a short term food replacement during meals or throughout the day. Tey et al. reported similar findings in 2016. Nonetheless, additional research must be conducted [17].

The findings of the study conducted by Tey et al. (2017) show that the caloric intake of subjects who consumed food sweetened with SGs and aspartame was lower, compared to the energy intake of subjects who consumed food sweetened with sucrose. Subjects who consumed SGs sweetened food also had a lower level of postprandial glucose in the blood, compared to subjects from the other two groups. On the contrary, the study did not show that the same effect would be achieved with the use of SGs in soft drinks. Consumption of drinks sweetened with sucrose resulted in a sharp increase in glucose and insulin concentrations in the blood one hour after consumption. The consumption of other drinks resulted in a sudden increase in the level of glucose and insulin in the period after lunch, the concentrations of which were twice as high, compared to the concentrations of glucose and insulin in subjects who consumed drinks sweetened with sucrose [18].

Tey et al. (2017) looked at the effects of stevia versus sugar on daily caloric intake from beverages in another randomized investigation. The findings revealed that compared to beverages sweetened with sucrose, aspartame, SGs, and monk fruit have no discernible impact on daily caloric intake, postprandial glucose levels, and insulin levels [19].

Ritu et al. (2016) investigated the impact of SGs (1g/day) of stevia leaf powder on type 2 diabetics' fasting blood glucose levels. The study was unique from previous research in that stevia leaf powder was employed in place of SGs, which were previously used in research. The results demonstrated that 60 days after the initiation of stevia leaf administration, SGs from stevia leaves lower fasting blood glucose levels in persons with diabetes. The levels of serum triglycerides, cholesterol, and VLDL all decreased, as did the levels of fasting and postprandial blood glucose. Results should be taken cautiously because the stevia leaves' raw powder contains a variety of other ingredients [20].

However, an earlier study conducted by Barriocanal et al. (2008) showed that the use of stevioside in diabetics did not affect the blood glucose level, as well as HbA1c in type 2 diabetes. The study showed that the use of stevioside did not cause changes in the glucose concentration in fasting blood, while in placebo subjects there was a significant increase in HbA1c and blood glucose concentration. The results indicate that SGs have the potential to maintain an unchanged diabetic state when used above the ADI value [21].

The findings of the study that was done by Stamataki et al. (2020) showed that the use of SGs didn't have a significant effect on glucose, insulin and weight change, but an increase in body weight was recorded in people who consumed glucose in contrast to people who consumed SGs. People who consumed SGs maintained their weight. Daily energy intake was lower in people who consumed SGs [22].

According to Ahmad et al. (2018), cookies sweetened with SGs cause consumers to feel less hungry after eating them than cookies sweetened with sucrose. The authors postulate that Reb A, which is present in stevia, causes the release of satiety hormones such peptide YY (PYY), cholecystokinin (CKK), and glucagon like peptide to increase (GLP-1) [23].

In contrast to yogurt sweetened with sucrose, yogurt sweetened with xylitol, SGs, or monk fruit lessens the feeling of hunger, according to a study by Chadha et al. (2022) [24].

Effect on Blood Pressure

Systolic and diastolic blood pressure have been shown to decrease using stevia leaf extract. Research have demonstrated that stevia extracts and its separated glycosides have hypotensive and diuretic effects. Similar to a class of medications known as calcium channel blockers, which are used to treat hypertension, SGs acts at the level of the cell membrane. Because SGs relaxes the arteries, it lowers blood pressure [1]. In two investigations, persons with hypertension who had not stopped taking their blood pressure medication were examined for the impact of stevioside. According to the findings of one study, stevioside (1500mg/day) usage for two years decreased both systolic and diastolic blood pressure. It's interesting to note that blood pressure began to decrease seven days after stevioside medication began. Chan et al. carried out a similar trial in which they evaluated the efficacy of giving hypertensive patients stevioside (750 mg/day). After three months, stevioside began to have a hypotensive impact [25, 26].

Another study found that SGs dramatically lowered diastolic blood pressure, however, results for systolic blood pressure were less significant. It is believed to be a result of the research' data being inconsistent and incomparable. Further clinical research and regulatory assessments are required [27].

Anticancer Activity

Studies have shown that SGs has an anticarcinogenic effect directly, but also through its antioxidant effect and its effect on lipid regulation [6]. Stevia leaf extract contains a substance called labdane sclareol that has cytotoxic and anticancer effects [1]. A study by Paul et al. (2012) demonstrated the polyphenolic components of stevia leaf extracts' inhibitory effects on tumor growth. The results proved that stevioside is a powerful apoptosis inducer and that it triggers apoptosis by creating reactive oxygen species inside of cells. This alters the transmembrane potential of the mitochondria and activates the route that leads to apoptosis through the mitochondria [28].

The findings of the research by Chen et al. (2018) show the anti-proliferative effects of steviol on six different gastrointestinal cancer cell lines, including gastric and colorectal cells. In all cell lines, steviol reduces cell viability. The study revealed the intriguing finding that the cytostatic effects of 5-fluorouracil (100–200 g/mL) and steviol (100–200 g/mL) on cell growth (60–90% inhibition) are comparable. According to the obtained results steviol may be used as a chemotherapeutic drug to treat cancer due to its metabolic properties and cytotoxicity toward human cancer cells. Human gastric cancer cells, colorectal cancer cells, and colon adenocarcinoma cells experienced a G1 phase arrest from steviol, while human poorly differentiated gastric cancer cells and ileocecal adenocarcinoma epithelial cells experienced a G2 phase arrest [29].

Gupta et al. (2017)'s study demonstrates that steviol has a potent cytotoxic effect on breast cancer cells. The purpose of the study was to examine the cytotoxicity, induction of apoptosis, and mechanism of action of steviol on human breast cancer cells. Steviol was used at its highest concentration for 48 h, and a dose dependent reduction in cell viability resulted in a 40% decrease in cell population. Reactive oxygen species (ROS) mediated arrest at the G2/M phase transition is how steviol triggered apoptosis [30].

Steviol has been shown in a study by Chen et al. (2018) to be cytotoxic in an osteosarcoma cell line. The findings demonstrated that steviol inhibited cell proliferation in a dose and time dependent manner, which reduced the cells' capacity to form colonies [31].

Stevia derivatives show cytotoxic and anti-proliferative effects on cancer cells, which is the basis for new research.

Substitution Sucrose with SGs in Flour-Based Confectionery Products

The primary sugar used to make flour based treats is sucrose, which makes up between 30 and 40 percent of the entire recipe. It is crucial to the creation and caliber of the finished product. A high dose of sucrose is bad for health reasons, hence substituting sucrose is a significant issue nowadays. In order to investigate the status of postprandial glycemia, Gao et al. (2016) conducted a study in which sucrose was replaced in the manufacturing of muffins with varying concentrations of stevia and inulin. The study's findings demonstrated that muffins completely devoid of sugar had better firmness than the test samples. Muffins whose composition included inulin as the only sweetener showed the highest firmness compared to muffins with stevianna in comparison to the test. Half substitution of sucrose, stevianna and inulin resulted in muffins of similar firmness. The results of the study indicate that muffins containing 50% inulin and muffins containing 50% stevianna had no change in softness, while muffins in which all the amount of sucrose was substituted by inulin had less softness compared to other samples. Surprising results showed that muffins with 100% substitution of sucrose with SGs, which had a higher softness than muffins with only sucrose [32].

The use of intense sweeteners, as well as stevia-based sweeteners in flour confectionery products can affect the structure, taste and especially the color of the product. Intensive sweeteners can contribute to a sweet taste; however, their use or omission of sucrose inevitably leads to changes in the texture of the product [33].

The results of one analysis noted that biscuits sweetened only with a preparation based on SGs (Reb A) could not achieve the same sensory characteristics as biscuits sweetened with natural sugars such as sucrose and fructose. Biscuits without natural sugars were lighter in color due to the lack of caramelization. In addition, the other sensory characteristics of biscuits with Reb A were inferior to samples containing sucrose or fructose. Biscuits with SGs have a lower caloric value than sucrose or fructose biscuits. According to nutrition and health claims placed on foods, these goods cannot be categorized as "reduced energy" products because the calorie reduction did not reach 30% [34]. Many studies have been conducted on the impact of stevia and SGs on dough rheology, physical and chemical quality, and flour confectionery items. In order to simulate the properties of sugar and guarantee enough moisture and texture, research frequently points in the direction of mixing stevia with some polyols or bulking agents [33].

The production of functional oat flour biscuits could potentially use stevia aqueous extract as a bioactive ingredient [35] (Table 3).

Table 3. Effects of adding stevia to flour-based confectionery products

Stevia derivates	*Products*	*Combination*	*Results*	*Ref.*
Aqueous extract of Stevia	Oatmeal cookies	Replacement 25, 50, 75 and 100% sucrose	- Due to their high fiber content, angiotensin-converting enzyme (ACE) inhibitor activity, amylase inhibitory activity, and antioxidant effect, the functional characteristics of cookie samples were suggested by partially substituting sucrose with an aqueous extract of S. rebaudiana - Samples containing 25 and 50 percent of the aqueous extract of S. rebaudiana) received the highest sensory approval	[35]
Stevia leaf powder	Biscuits based on mocaf and pedada flour	Fructose syrup	- There was no discernible substantial affect on the yield, moisture, ash, protein, fat, or carbohydrates of the samples; - The best results were obtained by substituting 10.0% fructose syrup and 1.5% stevia leaf powder	[36]
Stevioside	Biscuit	Replacing levels 50 and 100%	- Samples, particularly those containing stevioside 50%, not have negative impacts on the general quality - Significant improvement in color, chewiness, flavor, and overall acceptability	[37]
Stevioside maltodextrin mixture	Iranian traditional cookie	Replacing levels: 25%, 50%, 75%, and100%	- 25% was the acceptable maximum level of saccharose replacement - Higher levels revealed a deleterious effect of hardness and a decline in overall quality	[38]
Stevioside	Cookie	Fructose and stevioside in diferent ratio	- Reduction of glycemic index and glycemic load	[39]

(*continued*)

Table 3. (*continued*)

Stevia derivates	*Products*	*Combination*	*Results*	*Ref.*
Stevioside and Reb A	Cookie	Substitution levels: 50, 75 and 100%	- Decreasing caloric value, total sugars, fracturing power, diameter and spread ratio; - Raising the moisture, ash and thickness	[40]
Stevia	Muffins	Inulin	Inulin as the only sweetener showed the highest firmness -50% inulin and muffins containing 50% stevia had no change in softness -muffins in which all the amount of sucrose was substituted by inulin had less softness compared to other samples	[40]
SGs	Wheat dough	Erythritol	Reduces dough consistency and water absorption	[37, 38]
SGs	Cookie		Cookies sweetened with SGs reduce the feeling of hunger after consumption	[23]

The positive properties were reported by the authors Salaras et al. (2018) using stevioside in oat cakes, as well as Jariyah et al. (2021) who used a combination of SG with fructose syrup in biscuits. Vatankhah et al. (2014) completely replaced sucrose in the biscuit composition, reducing calories by up to 15%, and significant improvements in color, chewiness, taste and general acceptability were noted. According to Vazdi et al. (2016), increased SG levels had a negative impact on hardness and reduced the overall quality of the product. Popova et al. (2021) recorded a decrease in glycemic index and glycemic load in combinations of cookies with SG, while Hassan et al. (2016) recorded a decrease in caloric value, total sugars, breaking strength, diameter and spread ratio in cookies to which stevioside and Reb A were added in different proportions [35–40].

The aqueous extract of stevia is thought to have the capacity to control the glycemic response of muffins by adding either 50% stevia or 50% inulin without changing the texture of the finished product [41]. Depending on their concentration and type, erythritol and SGs as sugar substitutes have varying effects on the coagulation qualities of wheat dough. Erythritol and SGs decrease dough consistency and water absorption [42, 43].

Substitution Sucrose with SGs in Chocolate and Related Products

In addition to sweetness, sucrose in chocolate provides a number of functional properties such as mouthfeel, flavor enhancement, and preservation. According to Minali et al. (2020), the use of SGs in the production of chocolate offers a number of health advantages that may enhance the nutritional and health status of the general public [44]. Research

frequently point to the use of stevioside and a few polyols as bulking agents to replicate the properties of sugar and offer the right amount of moisture and texture [45].

The successful production of sugar-free chocolate products with the desired appearance, texture, taste, and aroma has a great deal of potential when sucrose is replaced with high intensity sweeteners like stevioside in combination with sugar alcohols and other bulking agents like polydextrose, maltodextrin, and inulin. These goods share a lot in common with sucrose-containing goods in terms of quality [46].

The impact of bulking agents on the physicochemical characteristics of chocolate products sweetened with stevia was examined by Nur et al. in 2021. The findings shown that adding inulin and sorbitol to dark chocolate that has been sweetened with stevia enhances the protein and fat content while lowering the moisture content. In comparison to reference chocolate, milk chocolate that has been bulked up with inulin and erythritol has a larger amount of protein and fat and a lower proportion of carbohydrates. The percentage of carbohydrates in stevia sweetened dark and milk chocolate rises with a larger inulin concentration. The fat content of dark chocolate that has been sweetened with stevia is decreased by a higher sorbitol concentration. An increase in erythritol concentration brightens the color and lowers dampness. Inulin concentrations increase the reduction of fat in stevia sweetened, sucrose free milk chocolate [47].

In their study, Shah et al. (2010) used polydextrose as a bulking agent in place of sucrose in a chocolate recipe that was sweetened with stevia and inulin with varied degrees of polymerization. The findings demonstrated that long chain inulin, in conjunction with stevia and polydextrose, is the most ideal ingredient for the creation of chocolate devoid of sucrose. The research also revealed that substituting stevia for sucrose as a sweetener and inulin and polydextrose for bulking agents had little to no impact on the final product's hardness [41, 48].

The ability to manufacture sugar free chocolate using bulking agents and SGs as a non caloric sweetener was demonstrated by Aguilar et al. in 2020. Maltitol, SGs, polydextrose, and inulin were the ingredients in one sample formulation while SGs and maltitol were the ingredients in the other. When compared to milk chocolate with sucrose, the second formulation had the best score and mouthfeel. Samples without sugar performed higher in sensory tests relating to overall likeability, melting speed, and flavor of chocolate and milk [49].

Homayouni et al. (2012) conducted a study to evaluate the physical characteristics of dietary chocolate milk with the addition of SGs and inulin as a substitute for sugar. The findings demonstrated that replacing sugar with SGs boosted precipitation and greatly reduced viscosity, but using inulin significantly decreased precipitation and increased viscosity. To improve the physical properties of the product in chocolate milk sweetened with SGs, a thickening agent, such as inulin, should be used [50].

Rodriguez et al. (2016) conducted research aimed at developing white chocolate formulations for diabetics where sucrose was substituted with sucralose and SGs. The results showed that combinations of SGs with sucrose have a synergistic effect and that the sensory properties of chocolate are acceptable if SGs is combined with sucrose and sucralose [51].

It has been proven that there is a greater intensity of taste when, instead of sucrose, the chocolate was sweetened with potassium acesulfame, SGs and monk fruit sweetener.

Tan et al. (2020) investigation revealed that the use of SGs, as a substitute sweetener in tea, results in the appearance of a metallic and pungent taste. In addition to the above, it is known that the sweetener from monk fruit and SGs lead to increased acidity and a "chemical" taste. Also, sucralose and SGs lead to an increased taste of acidity when they are used as sweeteners in chocolates [14].

According to Chranioti et al. (2015), to effectively reduce or mask the bitter taste of products containing steviol glycoside, the spray drying microencapsulation method can be used with encapsulating agents such as maltodextrin and inulin [52].

Azevedo et al. (2016) investigated the effects of Reb A, which is thought superiority in sweetness and taste, at various concentrations in bitter chocolate with additional stevia and insulin. The study's findings demonstrated that there were no appreciable variations between the tested concentrations and the matching Reb A values [53].

In order to solve the problem of stevia's bitter taste, new research was recently conducted that focused on sweetening ice cream with three SGs (Reb A, Reb D and Reb M) due to their different sensory characteristics. Muenprasitvej et al. (2022) observed that ice creams sweetened with Reb D and Reb M have a better taste and provide a better perception than Reb A, which is the most commonly utilized glycoside in the food sector. Ice cream sweetened with Reb M had the same intensity of sweetness as ice cream sweetened with glucose. Ice cream that has been sweetened with Reb D and Reb M has a sweet, pleasant, creamy, and milky flavor, while ice cream that has been sweetened with Reb A has an artificial, chemical flavor [54].

In their 2017 study, Torri et al. looked into the viability of making premium chocolate that has been sweetened with both raw and commercial stevioside. The findings demonstrated that raw stevia extract can be used to produce high quality chocolate with reduced sugar content that will be acceptable to customers and have much higher antioxidant activity [55].

In comparison to conventional chocolate, a bar of dark chocolate without sugar sweetened with SGs, erythritol, and inulin lowers postprandial blood glucose levels in diabetics, according to research by Oliveira et al. (2022) [56].

Considering that these are the latest studies, it is necessary to investigate the long-term effects SGs on glucose control (Table 4).

Substitution Sucrose with SGs in Drinks

In the research done by Parker et al. (2018) whey protein based beverages were sweetened with five different sweeteners: sucralose, sucrose, fructose, SGs, and monk fruit sweetener. Astringency and metallic taste were present in tested drinks with sucralose, SGs and monk fruit sweetener, while the use of SGs had the longest duration of sweetness. Also, drinks sweetened with SGs had the longest persistence of metallic and harsh in flavor [57].

It has been established that the quantity of SGs added to apricot nectar plays a significant role in its sweetness. According to a study by Reale et al. (2020), there are no appreciable differences between a nectar sample containing 10% sucrose and one containing 0.07% stevia in terms of acceptability. However, boosting the stevia content to 0.14% had a negative impact on acceptance [58].

Table 4. Presentation of the results of stevia in chocolate

Product	*Combination*	*Results*	*Reference*
Dark chocolate sweetened with stevia	Inulin, sorbitol	- Increases the fat and protein content while decreasing the moisture content, - The amount of fat is decreased by increasing the sorbitol concentration, - The proportion of carbs rises as inulin concentration rises	[14, 47, 48]
	Inulin, polydextrose	- Long-chain inulin is the most suitable for the formulation with stevia and polydextrose	
	Acesulfame, monk fruit	- Greater flavor intensity	
	Sucralose	- Increased taste of acidity	
Milk chocolate with stevia	Inulin, erythritol	- A larger percentage of fat and protein, and a lesser percentage of carbohydrates, - Erythritol concentrations that are greater make colors brighter and absorb less moisture, - Inulin lowers the fat content when it is present in greater amounts, - The percentage of carbs rises when inulin concentrations are higher	[47, 49]
	Maltitol, polydextrose, inulin	- A higher sensory rating in terms of overall preference and melting rate	
Chocolate milk with stevia	Inulin	- Rainfall and a drop in viscosity	[50]
White chocolate	Sucralose	- The sensory properties of chocolate are not acceptable	[51]

The sensory profile of peach nectar was considerably impacted by varied Reb A concentrations in stevia extracts, according to Medeiros et al. (2022), with an effect on

the descriptors of bitter flavor and residual bitter taste, which reduced with increasing Reb A concentration [59].

In comparison to drinks sweetened with sucrose, drinks sweetened with stevia showed a substantially larger loss of flavonones and a lot milder loss of vitamin C when stored in a lit environment at room temperature, according to a study by Salar et al. (2020) [60].

Previous studies have shown that SGs in appropriate percentages are a good substitute for sucrose for the production of fruit nectar.

It is necessary to conduct additional studies in order to investigate the replacement of sucrose with SGs in other drinks (Table 5).

Table 5. Presentation of the results of stevia with bulking agent in drink

Combination	*Drink*	*Results*	*Ref.*
SGs Xylitol, monk fruit	Yogurt	- Reduces the feeling of hunger	[24]
Sucralose, sucrose, fructose, SGs, and monk fruit	Whey protein	-Astringency and metallic taste were present in tested drinks with su-cralose, SGs and monk fruit sweetener, use of SGs had the longest duration of sweetness drinks sweetened with SGs had the longest persistence of bitter and metallic taste	[57]
SGs	Apricot nectar	-The acceptability of a nectar sample containing 0.07% stevia and a sample containing 10% sucrose does not differ noticeably -Increasing the stevia concentration to 0.14% resulted in reduced acceptability	[58]
Reb A	Peach nectar	-The sensory profile of peach nectar was significantly impacted by the different Reb A concentrations in stevia extracts, especially the bitter taste and lingering bitter taste descriptors, which reduced with increasing Reb A concentration	[59]

4 Conclusions

Unlike artificial sweeteners, which have negative consequences and may even be carcinogenic, stevia is a natural sweetener that has been used for millennia and has no calories or negative health effects. The two most significant SGs are stevioside and Reb

A. They have no calories and are 200–300 times sweeter than sucrose. In comparison to other sweeteners (such as sucrose and artificial sweeteners), stevia and SGs offer a significant advantage. In recent years, the chocolate industry has employed it frequently. The reason for the popularity of SGs lies in its many positive effects on health, as well as the fact that it is a natural sweetener without energy value, which makes it suitable for consumption by people who care about their health. The results of previous studies have shown numerous positive effects of consumption of SGs, including reduction of postprandial glucose and insulin levels, feeling of satiety, reduction of triglycerides, cholesterol, lowering of systolic and diastolic blood pressure, as well as cytotoxic and antiproliferative effects on various cancer cells. Several studies have demonstrated that adding SGs to food reduces its GI and energy value while maintaining its sensory qualities. However, it should be pointed out that the use of SGs in confectionery products also shows some disadvantages, namely a bitter after-taste, or even a metallic taste. Also, they do not caramelize, which is crucial for confectionary made with flour, thus they do not contribute to the development of color in baked goods. Furthermore, when they are added independently to chocolate or related products they adversely affect the chocolate's structure and viscosity of the chocolate liquor. Therefore, they are usually added in combination with some of the carriers such as polyhydroxyl alcohols, inulin polydextrose and others.

However, additional research on consequences of SGs on individual health is needed in the future.

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Quality of Commercial Blends for *Tarhana*, *Bey's* and *Sarajevska* Soup

Jasmina Tahmaz[1](✉), Irma Velić[2], Sabina Operta[1], Amila Oras[1], Mersiha Alkić Subašić[1], and Enver Karahmet[1]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
j.tahmaz@ppf.unsa.ba

[2] Secondary Vocational Educational and Training School, Azize Šaćirbegović 80, Sarajevo, Bosnia and Herzegovina

Abstract. The most popular traditional soups in Bosnia and Herzegovina are *Tarhana, Bey's* and *Sarajevska*. These traditional soups are commonly prepared at home, and also have great popularity in the restaurants. Industrial production makes it possible to obtain traditional soups in instant powdered form, which allows them to be stored at room temperature.The aim of this study was to investigate different quality parameters of industrial produced traditional soups from Bosnia and Herzegovina. Samples were collected from local industrial producers and analyzed for different physical, chemical and sensory properties.

The results of chemical analysis showed that all samples were in agreement to *Codex Alimentarius* and national Standard for quality of soups, sauces, food seasonings and related products. Bulk density ranged between 0.585 and 0.621 g/ml. Fat content varied between 4.30 and 12.46%. Amount of NaCl ranged between 8.99 and 10.52 g/L. The highest values of viscosity (111.67 mPas) and fat content were detected in *Bey*'s soup, while *Tarhana* had the lowest viscosity (32.33 mPas) and fat content. *Tarhana* had the lowest dispersibility and wettability. In terms of sensory properties, all samples were evaluated as acceptable with scores between 6.20 and 6.80 for overall acceptability. Industrial producers should use traditional ingredients and technologies to improve the quality of traditional soups.

Keywords: dried powdered soup · traditional soup · flowability · physical and chemical properties · sensory properties

1 Introduction

Soups are nutritionally valuable liquid dishes obtained by cooking of basic and additional ingredients and commonly served at the beginning of the meal. Due to their consistency, soups are classified into two groups: clear/thin and cream/thick [1, 2].

Besides of that main classification, the soups can also be classified by main ingredients (meat, vegetable or combined soups), preservation process (dehydrated, condensed/canned, frozen or non-preserved soups) and by the level of consumption readiness (ready-to-eat (completely prepared), semi-prepared and instant soups).

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Commercial or industrial soups reached their popularity after the invention of the canning process in the 19th century. In that period, many producers started to produce industrial canned and condensed soups packed in jars. But nowadays, the most popular way of soup preservation is drying. Dehydration (drying and powdering) is the most common way for preservation in the industrial production of soups. Dehydrated soups are instant products, which can be reconstructed in warm water or boiled for about 5–10 min resulting in a ready-to-eat meal. The main advantage of dried soup production is the possibility to be kept at room temperature for a long time (6–12 months) in closed dark bags without any undesirable change in microbiological, nutritional or sensory quality [1].

The basic raw materials for soup production are meat, poultry, fish, shellfish, as well as vegetables, fruits and mushrooms. Additional raw materials in the production of soups are milk and milk products, eggs and egg products, vegetable and animal fats, starch, cereals and cereal products, noodles, yeast, yeast and protein hydrolysates, monosodium glutamate and other taste enhancers, table salt, sugars, spices and extracts of natural spices [2–4].

The most popular traditional soups in Bosnia and Herzegovina are *Tarhana*, *Bey's (Begova)* and *Sarajevska (eng. Sarajevo's)*. For this reason, industrial producers try to develop recipes and technologies to produce powdered instant soups with characteristics similar to traditional.

During more than 400 years of the Ottoman Empire in Bosnia and Herzegovina, many influences of Turkish/Ottoman cuisine came very fast came in Bosnia and Herzegovina culture. Because of that, Bosnia and Herzegovina cuisine is still under huge influence from Turkish cuisine. Many Bosnia and Herzegovinian traditional dishes originated from the Ottoman Empire. During such a long period of Ottoman Empire and throughout history many of the original Ottoman dishes have undergone various changes and modifications influenced by Bosnian and Balkan culture, habits and climate (availability of raw ingredients). But the original names of the many dishes have persisted till the present days [5, 6].

In the traditional Turkish cuisine there are many variations of *tarhana* soup, made from different ingredients, depending on the region. Turkish *tarhana* noodles are usually prepared by mixing yoghurt, cereals and/or legume flour and different cooked vegetables such as paprika, tomato, onion and seasoned with spices like mint and paprika [7]. *Tarhana* soup is one of the oldest and the most popular traditional soups in Bosnia and Herzegovina. The main ingredient of this soup is dried *tarhana* noodles. *Tarhana* noodles are small irregular spherical shaped noodles, which are produced from fermented *tarhana* dough. Traditional Bosnia and Herzegovina *tarhana* dough is hard dense dough, made from white wheat flour, eggs and fresh chopped tomatoes, or mashed tomato or tomato juice. Prepared dough is left to ferment at room temperature for 3–4 days, and the obtained product is sour *tarhana* dough. After finished fermentation *tarhana* dough is kneaded with flour, crushed and passed through the small holes of kitchen colander and dried at room temperature. Traditionally, Bosnian *tarhana* soup is prepared by cooking of *tarhana* noodles in the meat broth with addition of salt, butter, chopped/diced tomato and spices like grounded black paper, dried paprika powder celery and parsley [8]. Traditional recipe for *tarhana* dough and noodles requires fermentation and because of

that *tarhana* soup has a pleasant sour taste and aroma specific for fermented dough. In industrial conditions, *tarhana* soup is commonly made from ordinary non-fermented pasta formed in spherical shape similar to traditional pasta, but without the fermentation. Sour taste of industrial *tarhana* soup usually comes from the liquid phase with tomato powder as the main ingredient. Because of that, industrial *tarhana* soups cannot have such specific taste and flavor like traditional one.

Bey's (bos. Begova) soup is traditional Bosnian cream soup made from cooked and chopped chicken meat, chicken broth, carrots, cut celery and parsley roots, whole pieces of dried okra, eggs, wheat flour, butter, sour cream and addition of lemon juice [8]. The name *Bey's* soup (bos. *Begova*) originated from the Turkish word "*bey*" (bos. *Beg*) which means gentlemen (or person with high social status) and the name *Bey's* soup could be translated as Gentlemen's soup [6]. In Bosnia and Herzegovina's cuisine, the *Bey's* soup is commonly recognized as a special dish, prepared and served for special guests and special occasions such as celebrations, weddings or religious occasions and holidays as a part of Eid and Ramadan meals [5].

Sarajevska soup (eng. *Sarajevo's soup*) is traditional cream soup typical for Sarajevo region. This soup is traditionally made from diced and stewed veal meat, cooked okra, butter, wheat flour, stewed vegetable mixture (carrot, onion and parsley cut out into small pieces), salt, black pepper and mixture of egg yolk, sour cream and lemon juice, which is added at the end of cooking [8]. The recipe of *Sarajevska soup* is very similar to *Bey*'s, with the main difference in used meat (veal in *Sarajevska* vs. chicken in *Bey's*).

The quality of industrial soups is regulated by *Codex Alimentarius* [3, 9] and Bosnian national Standard for soups, sauces and food seasonings [4]. According to these two standards the amount of the salt (NaCl) has to be less than 12.5 g/l (calculated to ready-to-eat product).

The aim of this study is to research the physical, chemical and sensory quality of commercial dehydrated traditional Bosnia and Herzegovina soups produced in industrial conditions.

2 Material and Methods

Research was done on the three samples of commercial dehydrated soups (*Tarhana - TS, Bey's - BS* and *Sarajevska - SS*) produced by local producer (Vispak, Visoko, Bosnia and Herzegovina) and collected from local market (Table 1). It can be seen from Table 1 that a sample of industrial *Sarajevska* soup contained chicken meat, while the original traditional recipe for *Sarajevska* soup should contain veal meat.

2.1 Amounts of Ingredients

For determination of the main compound amounts, soup samples were separated into following fractions: dried vegetables and meat, noodles and powdered fraction. Samples were divided into two fractions by sieving through diameter holes of 1 and 0.5 mm (Prufsieb ISO 3310–1). Each fraction was weighted and presented as a percentage of total sample weight.

Table 1. List of soup samples with declared data

Code	Commercial name	Description	Ingredients (declared data)
TS	*Tarhana soup*	Dehydrated clear soup with noodles and tomato	Noodles 55% (wheat flour and tomato paste), concentrate 45% (dehydrated tomato min. 25%, potato starch, salt, hydrogenated vegetable fat, monosodium glutamate, parsley, spices, flavor)
BS	*Bey's soup*	Dehydrated cream soup	Maize starch, dehydrated vegetables (carrot, parsley, okra, celery, onion) 20%, dried chicken meat 9%, wheat flour, salt, hydrogenated vegetable fat, monosodium glutamate, flavor, spices, natural color riboflavin
SS	*Sarajevska soup*	Dehydrated cream soup	Dried vegetables (carrot, parsley, celery, cauliflower, onion) min. 15%, chicken meat 10%, table salt, hydrogenated vegetable fat, maize starch, wheat flour, skimmed milk powder, sugar, spices, monosodium glutamate, riboflavin

2.2 Chemical Analysis

Moisture content was determined by drying at 105 °C in the drying oven to constant weight [10]. Measurement of water activity (aw) and pH values were performed using aw meter (Lab Swift aw meter Nowasina, Switzerland) and pH meter (Mettler Toledo). Total ash content was determined by burning of samples in a muffle furnace at 550 °C during 8 h [11] and NaCl by Mohr titration with 0.1M AgNO3 and 5% potassium chromate as indicator [12]. Amount of NaCl was presented as percentages calculated on dehydrated soup powder as well as g/L in ready to eat liquid soups prepared according to declared instructions. Soxhlet extraction with diethyl ether was used for determination of fat content [13]. Amino nitrogen content was determined by titration in presence of formaldehyde. 5 g of sample and 80 ml of distilled water was mixed and left to stay for 3 h with periodical stirring. Then, the samples were filtered into volumetric flasks and filled with distilled water to 100 ml. 10 ml of this extract was titrated with 0.1 M NaOH to pH 8. After titration 10 ml of formaldehyde (40% p.a.) was added and titration was repeated to pH 8. The amino nitrogen content (%) was calculated using following equation: 1.4xV/m, where V is volume of 0.1 M NaOH used for second titration and m is the weight of sample in 10 ml of extract. Total acidity was determined by titration using 0.1 M NaOH and was calculated to citric acid [13].

Energy value (kCal/100 g) of the soup samples was calculated using the following equation [14]:

$$Energy\ value = (Protein + carbohydrates) \times 4.1 + Fat \times 9.3 = [kcal/100g] \quad (1)$$

The sum of total proteins and carbohydrates was calculated by subtracting the sum of the values of moisture, ash, and fat from 100 (per 100 gm) [15]:

$$Sum\ of\ proteins\ and\ carbohydrates(\%) = 100 - (\%moisture + \%fat + \%ash) \quad (2)$$

2.3 Physical Analysis

For determination of viscosity and density of liquid soups prepared to eat, samples were prepared due to instructions from packaging. Density of cooked soup samples (liquid density) was determined at 50 ± 5 °C by the measuring of the weight and volume of the prepared sample in the measuring cylinder. Density [kg/m^3] was calculated as ratio of weight and volume.

Dynamic viscosity [mPas] was measured at rotational rheometer (Myr, VR 3000) at 50 ± 5 °C using R2 spindle and rotation speed of 200 rev/min. Kinematic viscosity [m^2/s] was calculated as ratio of dynamic viscosity and density of ready to eat samples (liquid density).

Bulk density [kg/m^3] was determined as ratio of weight and volume of powder in measuring cylinder. 60 g of sample was put into a 200 ml graduated measuring cylinder. The volume of sample was recorded and bulk density was calculated as ratio of sample weight and volume [16].

Tapped density was determined after determination of bulk density. Soup powder was tapped 100 times (or to constant volume) in a measuring cylinder by beating on a plain surface. After tapping, the new tapped volume was recorded, and tapped density [kg/m^3] was calculated as ratio of sample weight and tapped volume [16, 17].

Determination of wettability [s] was described by Fernandes et al. [16] with some modifications. Amount of 0.5 g of soup powder was put over the plain calm surface of 200 ml of distilled water at 20 °C without agitation. Stopwatch was turned on and the time was recorded after all powder had been wetted.

For the determination of dispersibility, 5 g of powder was put in a 50 ml measuring cylinder and distilled water (t = 20 °C) was added to 50 ml. The mixture of powder and water was vigorously stirred and left to stay for 3 h without agitation. The volume of the liquid phase was measured. Dispersibility [%] was calculated as percentage of liquid phase volume V_{liquid} in total volume V_{total} (50 ml) [18–20].

Angle of repose and Hausner ratio were used as indicators of flowability and cohesiveness. The Hausner ratio was calculated as the ratio of tapped and bulk density [21]. For determination of angle of repose, 50 ml of dehydrated soup sample powder was carefully sloped through a funnel (D = 1 cm) on a plain surface base. The height (h) and diameter (d) of the powder heap were measured. Angle of repose α was calculated as tg α = 2h/d. Flowability and cohesiveness were described and explained by comparison of obtained results to referent values from literature data [22–24].

2.4 Sensory Evaluation

Sensory evaluation was done using a 9-point hedonic scale, where 1 means extremely unacceptable or extremely bad and 9 means extremely acceptable or excellent. Before sensory evaluation, soups were cooked according to declared instructions and served to panellists at recommended serving temperature (45 ± 5 °C). The panellists (n = 20) were semi-trained students of Faculty of Agriculture and Food Sciences, University of Sarajevo, who intended the subject Technology of ready-to-eat food. The acceptability of following properties was evaluated: appearance, consistency, smell, aroma, taste and overall acceptability. Besides acceptability evaluation, panellists also evaluated the intensity of taste attributes such as saltiness, sourness and umami taste.

2.5 Statistical Analysis

All analyses were done in triplicate and results are shown as mean value with standard deviation. Statistical analysis was performed using one-way ANOVA and post-hoc Tukey test ($p \leq 0.05$) using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

3 Results and Discussion

3.1 Amount of Ingredients

Soup samples had a pretty different composition and different amount of analyzed fraction (Table 2). It was noticed that all samples contained a powdered fraction, but only *Tarhana* was clear soup with noodles. Other soups were typical cream soup with a high amount of powdered fraction and lower amount of dried vegetable fraction. The image of fractions is shown in Fig. 1.

Content of powdered fraction in *Tarhana soup (TS)* was significantly ($p \leq 0.05$) lower than in other soups. Only *TS* didn't contain large particles fraction consisted of pieces of dried vegetable and dried meat. *Bey's* soup (BS) contained a significantly ($p \leq 0.05$) higher amount of dried vegetables in comparison to *Sarajevska (SS).* On the other hand, *SS* contained significantly higher amount of powdered fraction than *Bey*'s and *Tarhana.*

Table 2. Amount of the main compounds in soup samples

	Tarhana - TS	*Bey's - BS*	*Sarajevska - SS*
Noodles (%)	55.99 ± 3.06	–	–
Large particles fraction with dried vegetable (%)	–	26.80 ± 2.95^{a}	18.15 ± 0.92^{b}
Powdered fraction (%)	44.01 ± 3.06^{c}	73.20 ± 2.97^{b}	81.85 ± 0.42^{a}

* Different small letters in rows represent statistically significant differences ($p \leq 0.05$) between samples

Large particle fraction was separated from powder by sieving through a sieve with holes diameter of 1 mm. This fraction had dimensions larger than 1 mm, and the powdered fraction had dimensions smaller than 0.5 mm. Particles with dimensions between 0.5 and 1 mm were not found. The image of the fractions is shown in Fig. 1. After observing a large particle fraction, it can be seen that its composition consisted mainly of dried vegetable pieces (mostly carrot, celery and parsley) (Fig. 1). The amount of okra in particular was 4.52% in total sample weight and 19.02% in the fraction of large particles (Fig. 1).

It should be noted that small pieces of dried meat, declared on the packaging (Table 1) could not be recognized or separated from the dried vegetable fraction. For this reason, that fraction is represented as a large particle fraction, composed mainly of dried vegetable pieces and smaller pieces of dried chicken meat (as declared).

Obtained results are in agreement to literature data [25, 26]. Abeysinghe and Illeperuma [25] reported that soup formulation contained 25% of dried vegetables, which was very similar to results *BS* shown in Table 1.

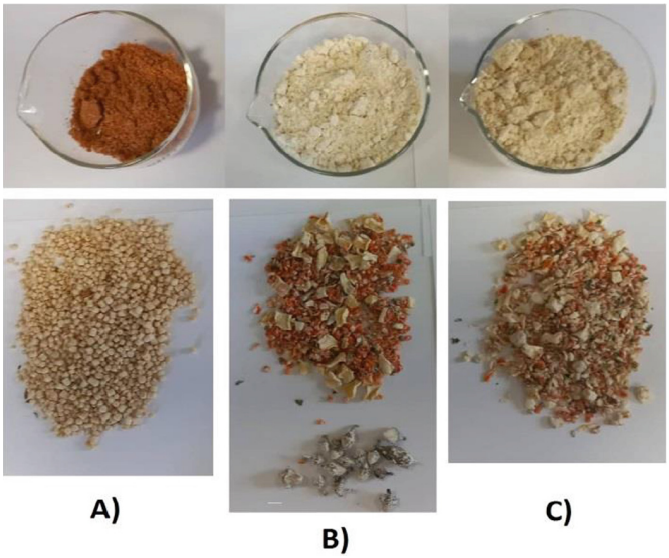

Fig. 1. Images of the dehydrated soup fractions: A) *Tarhana - TS*; B) *Bey's – BS* and C) *Sarajevska* soup *SS*

3.2 Chemical Analysis

Results of chemical analysis are given in Table 3. Chemical quality of dehydrated soups is regulated by *Codex Alimentarius* [3, 9] and the National Standard for soups, sauces, food seasonings and kindred products [4]. According to the official Bosnia and Herzegovinian Standard for soups, sauces, food seasonings and kindred products [4] moisture content in dehydrated soups must not exceed 10%. The maximum permitted amount of NaCl

in processed soups and broths is 12.5 g/L [3, 4, 9] and for amino nitrogen the lowest permitted amount is 210 mg/L for vegetable based soups [4, 9].

Water activity (aw) ranged between 0.335 and 0.434. These values (aw < 0.6) indicate good microbial stability [27, 28]. According to the literature data aw value of soup powders ranges between 0.19–0.58 [29–32]. The most similar values were obtained by Abd-Elhak and Salem [29] for sweet potato soup (0.38–0.47), while Gandhi et al. [32] reported that tomato soup had lower water activity value than vegetable. Moisture content in all samples is lower than 10% which is required by national Standard [4]. *TS* had the highest and *BS* the lowest moisture content, but the differences were not significant ($p \leq 0.05$). Obtained results are in agreement with literature data. Moisture content in different dehydrated soups ranged from 2.70 to 9.84% [33–38]. The most similar values are reported by Niththiya et al. [37] for vegetable instant soup (3.24%) and Verma and Mogra [38] for tomato-mushroom soup (5.16%).

The lowest fat content was obtained in *TS* and the highest in *BS* (Table 3). These results were expected, because tarhana is tomato based soup without meat compounds, while *BS* and *SS* had chicken meat. *TS* had significantly ($p \leq 0.05$) lower fat content in comparison to other two samples. According to different authors, fat content in dried soups can vary in a huge range between 1.16 and 11.96% depending on soup sample type and composition [25, 35, 37, 39]. Thuy et al. [39] reported the most similar values for fat (8.94–11.69%) in instant chicken soup.

Table 3. Chemical properties of soup samples

	Tarhana - TS	*Bey's - BS*	*Sarajevska - SS*
Aw	0.335 ± 0.01^{c}	0.389 ± 0.01^{b}	0.434 ± 0.00^{a}
Moisture (%)	5.10 ± 0.17	3.95 ± 0.12	4.41 ± 0.74
Fat (%)	4.30 ± 0.28^{b}	12.46 ± 1.71^{a}	11.90 ± 0.60^{a}
Ash (%)	20.04 ± 6.21	16.00 ± 1.82	15.92 ± 0.49
NaCl (%)	17.44 ± 1.10	15.70 ± 1.03	14.98 ± 0.90
NaCl (g/L)	10.52 ± 2.30	9.42 ± 0.62	8.99 ± 0.54
Amino-nitrogen (mg/L)	482.58 ± 104.80	563.82 ± 104.03	576.79 ± 196.81
Titrable acidity (%)	1.78 ± 0.12^{a}	0.83 ± 0.08^{b}	0.75 ± 0.13^{b}
pH	5.40 ± 0.01^{c}	6.31 ± 0.06^{a}	6.19 ± 0.00^{b}
Energy (kCal/100g)	329.28 ± 26.35^{b}	392.99 ± 7.65^{a}	388.54 ± 3.95^{a}

* Different small letters in rows represent statistically significant differences ($p \leq 0.05$) between samples

The highest amounts of ash and NaCl were in *TS* and the lowest in *SS*, but the differences were not significant ($p \leq 0.05$). From these results, it could be seen that the main component of the ash was NaCl. Different literature sources reported that ash in dehydrated soups varied from 5.58 to 28.60% [25, 34–37, 40]. The most similar

reported ash amounts were 16.00–28.60% in vegetable chicken soup [40], 16.76% in instant vegetable soup [41] and 20% in vegetable soup [42].

NaCl in analyzed soup samples was in agreement to requirements [3, 4, 9] and literature data reported for similar samples. It was previously reported [26, 31, 33, 35] that NaCl content in dried vegetable soups commonly ranged between 14.70 and 20.00%. According to Janjatović et al. [33] clear soups contained more NaCl than cream soups. Other authors reported lower NaCl content, for example 7.94–12% for vegetable soup powders [25, 32, 37, 43].

Amino-nitrogen content is an indicator of the total amount of free amino acids in samples [13]. Free amino acids and their salts are related to soup taste, especially for desirable umami taste. Presence of protein hydrolysates and monosodium glutamate can increase amino-nitrogen content. According to *Codex Alimentarius* [9] and national standard [4], the lowest required content of amino nitrogen in vegetable soups is 210 mg/L. All samples analyzed in this study had amino nitrogen above the required level. Matijević et al. [44] reported that amino nitrogen in vegetable soup was 680 mg/L, which is slightly higher in comparison to results of this study.

Significantly ($p \leq 0.05$) the lowest pH and the highest total acidity were obtained in *TS*. These results were expected, because the basic ingredient in *TS* is tomato, and this soup also had the most intense sour taste. Tomato soup commonly has a lower pH value in comparison to vegetable soup [31, 43, 45]. Mohajan et al. [43] reported that vegetable soups had pH 6.13–6.17. On the other hand, pH value of tomato soups commonly ranges between 4.00 and 4.62 [31, 45, 46], while original tarhana soup has pH 4.37 [47]. Ansari et al. [36] reported that vegetable soup mix contained 2.50% of citric acid, which was a little bit higher in comparison to results obtained in this study.

As was expected, the energy value of *TS* was significantly ($p \leq 0.05$) lower in comparison to other samples, that's because *TS* is vegetable based soup. Besides that, *Tarhana* soup had also the lowest fat content which caused the lowest energy value. Different studies from literature reported an energy value between 271–401 kcal/100 g for similar soup samples [25, 34, 35, 37, 39].

3.3 Physical Analysis

The results of physical analysis are shown in Table 4.

Dynamic viscosity varied in a very huge range between 32.33 and 111.67 mPas depending on soup consistency. The highest viscosity was noticed in *BS*, and the lowest in *TS*. *Tarhana* soup had significantly ($p \leq 0.05$) lower dynamic and kinematic viscosity in comparison to other samples. After considering that *Tarhana* is clear tomato soup with noodles and has thinner consistency in comparison to cream soups, results for viscosity were expected. Obtained results also were in agreement with literature data, where viscosity of soup can vary in a very huge range (30–390 mPas) depending on composition, temperature and soup type [38, 40, 42, 43, 46, 48–50]. It is not uncommon that tomato soup has lower viscosity than other vegetable cream soups. Viscosity of tomato soup was 40 mPas [45] and of tomato-mushroom soup 47 mPas [38]. These values are very similar to the results obtained for *TS* sample (Table 4). According to literature data viscosity of cream soups commonly varies from 85 to 145 mPas [40, 42, 49, 50]. Also, very similar values for meat and vegetable soups were reported by other

authors, for example: 130–145 mPas for vegetable chicken soup [40], 85–115 mPas for vegetable cream soup [42] and 110 mPas for cereal cream soup [48]. Results for kinematic viscosity followed the same relations as dynamic viscosity. Kinematic and dynamic viscosity had highest values *BS*, and the lowest in *TS*.

The density of prepared ready to eat liquid soups had very similar values for all three samples without significant differences ($p \leq 0.05$). Although *TS* had significantly ($p \leq 0.05$) lower viscosity, its density was slightly higher in comparison to other samples. It is well known that density of liququids depends on composition and temperature. Skotnicka and Ocieczek [49] reported that cream soup had lower density (963 kg/m^3) in comparison to clear soup with pasta (1017 kg/m^2). According to Celik et al. [47] original *tarhana* soup had density in the range 1.04–1.05 g/ml, which is a slightly higher value in comparison to results for *TS* sample. These differences could be explained by different temperatures during testing of density and also by different composition and recipes for *tarhana* soup preparation. Celik et al. [47] measured density at room temperature, while measuring temperature in this study was 50 °C.

Bulk density depends on the size and shape of particles in soup powders. Prevalence of small and uniform size/shape of particles commonly causes higher bulk density. On the other hand, porous ingredients, such as dried vegetables with irregular shapes will decrease the bulk density. *TS* powder had the highest bulk density, probably because of the very high presence of very compact *tarhana* noodles (Fig. 1) and without porous dried vegetable fraction (Table 2). Results for bulk density are in agreement to literature data, where bulk density mostly ranged between 0.43 and 0.84 g/ml [29, 36, 38, 41, 42, 46]. Abd-Elhak and Salem [29] reported that the bulk density of sweet potato soup was 0.54 g/ml.

Table 4. Physical properties of soup samples

	Tarhana - TS	*Bey's - BS*	*Sarajevska - SS*
Dynamic viscosity (mPas) at 50 °C	32.33 ± 3.79^{b}	111.67 ± 11.68^{a}	106.33 ± 1.53^{a}
Kinematic viscosity (cm^2/s) at 50 °C	0.32 ± 0.03^{b}	1.14 ± 0.13^{a}	1.09 ± 0.04^{a}
Liquid soup density (kg/m^3) at 50 °C	996.67 ± 20.82	976.67 ± 5.77	986.67 ± 20.82
Bulk density (kg/m^3)	621.39 ± 17.96	585.53 ± 28.70	599.50 ± 24.26
Tapped density (kg/m^3)	860.67 ± 18.34^{b}	923.91 ± 46.14^{ab}	940.83 ± 22.78^{a}
Hausner ratio	1.39 ± 0.07	1.56 ± 0.10	$1.57 \pm 0{,}09$
Angle of repose (°)	25.62 ± 3.20^{c}	34.13 ± 4.33^{b}	44.69 ± 1.36^{a}
Wettability (s)	585.62 ± 192.95^{a}	16.09 ± 0.25^{b}	24.33 ± 1.13^{b}
Dispersibility (%)	58.62 ± 3.51^{b}	76.50 ± 1.32^{a}	79.00 ± 2.65^{a}

* Different small letters in rows represent statistically significant differences ($p \leq 0.05$) between samples

Values for tapped density were between 860.47 and 940.83 kg/m^2. Cream soup samples (*SS and BS*) had higher tapped density than *TS*. These results were expected because of the higher amount of powdered fraction in cream soups. Small particles of powders could be better compressed than large particles of *tarhana* noodles. Thus, the sample with the highest amount of powdered faction (*SS*) had the highest tapped density. In comparison to *SS*, *BS* had a higher amount of dried vegetables with irregular shape and porous structure (especially large pieces of dried okra) (Table 2, Fig. 1).

Tapped density is an important indicator of powder flowability. Higher differences between tapped and bulk density indicate lower flowability and higher affinity for compression and caking. Hausner ratio and angle of repose are important indicators of powder flowability and cohesiveness. Values of Hausner ratio lower than 1.18 indicate good flowability, while values higher than 1.26 are related to poor flowability. Lower values of angle of repose always mean better flowability. When angle of repose is above 41°, flowability of powder is poor [21, 22]. On the other hand, higher values of Hausner ratio (>1.40) and angle of repose (>45°) indicate high cohesiveness of powder [23, 24].

The values of Hausner ratio and angle of repose in soup samples ranged between 1.39–1.57 and 25.62–44.69° (Table 4). Considering the obtained and referent values of Hausner ratio, flowability of soup samples could be described as poor to passable, while their cohesiveness was intermediate to high. By observing the angle of repose values, the samples had very low to negligible cohesiveness and good flowability [22–24]. From obtained results (Table 4), it can be seen that angle of repose and Hausner ratio did not refer to the same explanations of flowability and cohesiveness character. These opposite results could be explained by the following facts. The Hausner ratio values could serve to explain and classify powder flowability under higher stress or high speed of powder flow, while angle of repose could explain flowability only under low stress and slow flow. Because of that, the Hausner ratio should always be used as a better indicator for flowability description.

This phenomenon is best seen in *TS*. Results showed that *Tarhana* soup had the highest flowability and the lowest cohesiveness, because of the lowest values of Hausner ratio and angle of repose in comparison to other samples. Angle of repose of *TS* shows negligible cohesiveness and good flowability, while Hausner ratio indicates poor flowability and intermediate cohesiveness. These results indicate that slow speed flowability would be good to excellent, while flowability would be poor at fast speeds. The similar observation occurs in *BS* sample. *BS* could have good flowability and negligible cohesiveness in the case of slow flowing (low angle of repose), while under the fast flowing the flowability could be poor and cohesiveness high. Angle of repose of *SS* (44.69°) indicates poor flowability and low-to-intermediate cohesiveness, while Hausner ratio (1.57) indicates very poor flowability and high cohesiveness.

Results for Hausner ratio and angle of repose were in agreement to literature. Bhargavanandha et al. [46] reported that Hausner ratio for tomato soup was 1.3–1.33. Angle of repose for similar soup powders reported by other authors usually ranged between 45 and 57° [42, 46, 51, 52], which indicates poor flowability. Besides, it is possible to notice that tomato soup [46] and other soups consisting of larger size ingredients [51] had lower angle of repose in comparison to cream vegetable soups [42, 52], mostly consisting of powdered fraction.

Dispersibility and wettability are indicators of reconstruction ability. Samples with lower wetting time and higher dispersibility have better reconstruction ability. Dispersibility of soup samples ranged between 58.62% and 79.00% and wettability 16.09–585.62 s (Table 4). *TS* had significantly ($p \leq 0.05$) the lowest dispersibility and the highest wettability. *SS* had the highest dispersibility (Table 4), while the lowest wettability was in *BS*. From the literature data dispersibility of soup powders varied between 69.67 and 82% [41, 43], while wettability varied in a higher range from 1.6 to 230 s, depending on composition and determination method [30, 38, 46, 51].

The lowest dispersibility of *Tarhana* soup could be explained by the high presence of noodles which absorb a lot of water by swelling. Swelling of the noodles consequently decreased the volume of liquid phase and noodles with absorbed water settled down. Wetting time for *TS* was significantly ($p \leq 0.05$) longer than for other samples. Long wetting time of *TS* could be related to its composition. *TS* contained 44.00% of powder fraction with tomato powder as dominant compound (Table 1). Such a long wetting time of *TS* probably could be explained by the fact that tomatoes contain hydrophobic compounds like lycopene and beta carotene. Lycopene is apolar compound with high hydrophobic character [53–55]. The hydrophobic character of lycopene could increase wettability of *TS* and make it difficult to be wetted. Because of that, the presence of lycopene is probably the main reason for such a long wetting time. This theory could also be supported by results reported by Bhargavanandha et al. [46], where it was found that higher concentration of tomato powder in soup composition induced longer wetting time of tomato soup.

3.4 Sensory Evaluation

Results of sensory evaluation and images of ready to eat soup samples are shown in Fig. 2 and 3.

TS had the highest scores for smell and aroma, but the lowest scores for consistency and overall acceptability. *BS* had the highest scores for appearance, consistency and overall acceptability and the lowest scores for smell, aroma and taste. The scores for *SS* were mostly between *BS* and *TS*. *SS* had slightly the best evaluated taste and slightly the lowest scores for appearance. The scores for acceptability of each sensory property varied in following ranges: 5.83–6.3 for appearance, 5.50–6.60 for consistency, 4.92–6.42 for smell, 6.00–6.90 for aroma, 6.10–6.42 for taste and 6.20–6.80 for overall acceptability. All acceptability scores ranged between 4.92 and 6.90, but there were no statistically significant ($p \leq 0.05$) differences in the scores for acceptability of any sensory attribute.

The highest scores for appearance and consistency were noticed in *BS* probably because of the presence of clearly visible large particles of dried vegetables (such as carrot and okra) in the thick liquid soup base (Fig. 1 and 3). This soup also had a slightly higher score for overall acceptability in comparison to other samples. It is important to be mention that *TS* had significantly ($p \leq 0.05$) lower viscosity than other soup samples, and because of that its consistency was the thinnest. That thin consistency was evaluated as the most undesirable. Results showed that the panelists mostly preferred thicker consistency of soups and suggested that consistency and appearance played a very important role in sensory evaluation of soups. The highest scores for aroma and smell were noticed in *TS*, probably because of the fresh smell of tomatoes. *SS* had the

highest scores for taste. It is also interesting that *SS* had the lowest NaCl and the highest amino nitrogen content (Table 2).

There were no samples evaluated as unacceptable for overall acceptability as well as for any particular sensorial attribute. Although *TS* and *SS* had higher scores for taste, aroma and smell in comparison to *BS*, the highest score for overall acceptability was obtained in *BS* (6.80 – moderately acceptable to acceptable) and the lowest in *TS* (6.20 – moderately acceptable). These results probably suggest that panelists gave more importance to consistency than to taste or aroma. However, the differences between evaluated samples were not significant ($p \leq 0.05$) nor for any sensory attribute nor for overall acceptability.

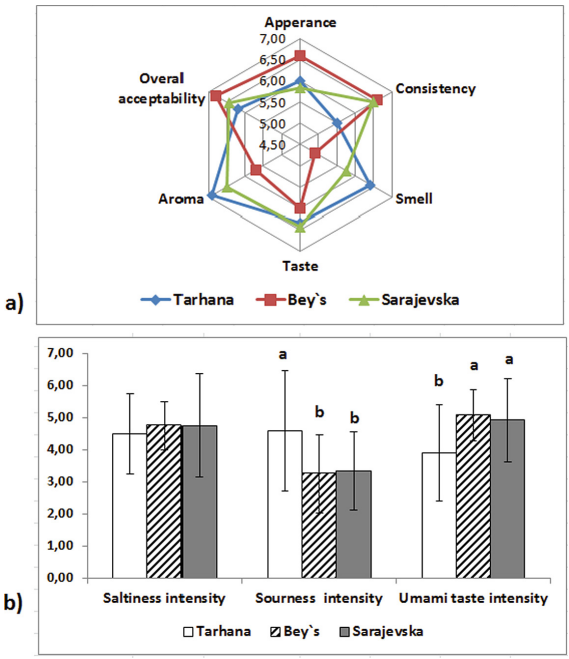

Fig. 2. Results of sensory evaluation of soups: a) acceptability of sensory properties and b) evaluation of intensity of saltiness, sourness ad umami taste

The intensity of salty taste (Fig. 2) was evaluated as moderately salty with scores between 4.5 and 4.75. *TS* had the lowest intensity of saltiness, although NaCl content was the highest in this soup (Table 3). This could be explained by the fact that *TS* had the most intense sour taste, which probably masked all other tastes. Because of that, the real saltiness of *TS* could not be recognized by panelists. From the results of sensory evaluation, it is seen that *TS* had significantly ($p \leq 0.05$) the highest intensity of sourness and the lowest intensity of umami taste.

It was interesting to observe that panelists managed to recognize significantly ($p \leq 0.05$) higher intensity of umami taste in *BS* and *SS* samples. It has to be mentioned that these two soups had higher amounts of amino nitrogen content in comparison to *TS*

(Table 3). Amino nitrogen is related to umami taste in many ready to eat products and it could be recognized as a main carrier of umami taste. Because of that, it was very interesting that panelists were able to recognize more intense umami taste in the samples with higher amounts of amino nitrogen. Sour taste intensity was evaluated with scores 3.25–4.58 (weakly expressed/slightly sour – normal/moderately sour), while umami taste was evaluated as moderately slightly expressed (3.90) in *TS* to moderate intense in *BS* and *SS* soup (4.92 and 5.08).

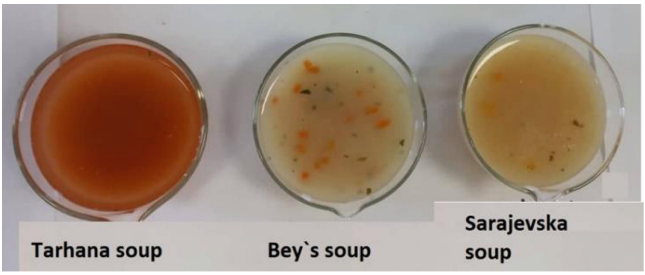

Fig. 3. Images of cooked soup samples prepared for sensorial evaluation

In the beginning of this study, it was expected that soups would be evaluated with higher scores than obtained. Results showed that analyzed samples were not bad in taste, aroma and consistency (the main sensory attributes), but also these samples were not evaluated as very good or excellent. Obtained scores never exceed 6.90 (aroma of *TS*), which was the lowest limit (6.90~7.00) to evaluate it as acceptable or good. The scores for overall acceptability did not exceed 6.80. These unexpected results can be explained by the fact that panelists probably had higher expectations of traditional soups. They probably had habits to consume these traditional soups produced by traditional ways at home or restaurants, and such evaluation was the result of comparisons between analyzed samples and soups produced using real traditional ways and recipes. Comparing industrial and traditional technologies for these soups described by literature [8], it becomes completely clear that these two technologies differ in many aspects, like used raw materials and production procedures.

For example real traditional *tarhana* soup is traditionally made from sour and fermented *tarhana* dough [8], which gives a specific sour fresh taste and aroma of traditionally prepared soup. On the other hand, the sample of commercial *TS* was produced from ordinary non-fermented dough and noodles (with addition of tomato paste in dough for sour taste, but without typical aroma related to fermented dough). Besides, industrial *tarhana* noodles also had different shape in comparison to traditional one. Commercial *tarhana* noodles have uniform size and shape (Fig. 1), unlike the traditional, which are typically in the form of crumbs or pieces of uneven size and shapes. For these reasons, consistency, appearance and overall acceptability for *TS* were evaluated with pretty low scores.

The lowest differences between industrial and traditional recipes were noticed in *BS*. Probably, because of that *BS* had the highest scores for appearance and overall acceptability. But on the other hand, *BS* obtained the lowest scores for aroma, taste and

smell, possibly because of taste enhancers, which were used during industrial production (Table 1) and which masked other typical tastes and flavors of traditional *BS*.

Very large differences between traditional and industrial recipes and technologies were recognized in *SS*. This soup traditionally is made from stewed veal meat and stewed vegetables [8]. Industrial/commercial *SS* was produced from cooked and dried chicken meat and dried but not stewed vegetables (Table 1). However, *SS* obtained the highest scores for taste and aroma, possibly because of the high amount of amino nitrogen (Table 2) and highly recognized umami taste (Fig. 2). Also unlike traditional, industrial *Sarajevska* soup did not contain okra, which is typical for traditional one. Because of that, *SS* had the lowest scores for appearance. Absence of stewed veal and okra probably caused the lowest scores of aroma and appearance of *SS*. However, it is interesting to notice, that slightly the best evaluated taste of *SS*, can be related to the lowest NaCl and high amino nitrogen content (Table 2).Also, it can be seen that *BS* had larger particles of dried vegetable than *SS* (Fig. 1), what was typical characteristic of traditional recipes [8].

Obtained results are in agreement with literature data, which reported that scores of sensory evaluation for similar soup samples ranged between 5.2 and 8.5 [35, 38, 41–43, 47, 51]. Many authors [35, 36, 38, 41, 47] reported that experimental or nontraditional soup samples had slightly lower sensory scores in comparison to control or traditional samples. Celik et al. [47] reported that experimental *tarhana* soup with addition of wheat bran had slightly lower scores than traditional, while Verma and Mogra [38], Ssepuuya et al. [41] and Ansari et al. [36] reported that control soup samples were better evaluated for most sensory attributes in comparison to experimental ones.

4 Conclusion

Physical, chemical and sensory characteristics of analyzed soup samples were in agreement to official standards and literature data. All soup samples contained NaCl and moisture under permitted limits (<12.5 g/L and <10%).

TS had the lowest amount of powdered fraction, pH, fat, amino nitrogen, energy value, tapped density, dispersibility, viscosity, thinnest consistency and the lowest scores for consistency. *BS* had the lowest bulk density and the highest values for vegetable content, fat, energy value, pH, viscosity, thickest consistency and the highest scores for appearance, consistency and overall acceptability. *SS* had the highest values of tapped density, Hausner ratio, angle of repose, dispersibility, pH value and content of amino nitrogen, while the lowest values for acidity, NaCl and ash content.

Due to the results for wettability and dispersibility it can be concluded that *SS* had the best, while *TS* had the weakest reconstruction ability. It was supposed that presence of lycopene from tomato powder in *TS* caused the lowest wettability.

Samples with a higher amount of powdered fraction had higher cohesiveness and lower flowability. The Hausner ratio was recognized as a better flowability indicator in comparison to angle of repose.

There were no samples evaluated as unacceptable for overall acceptability as well as for any particular sensorial attribute. Also, no sample was evaluated as excellent or extremely acceptable. The highest score for overall acceptability was 6.80. Panelists

probably had higher expectations of traditional soups. Results of sensory evaluation indicated that appearance, consistency and taste were recognized as the most important sensory attributes. The panelist preferred thicker soup consistency.

When comparing industrial and traditional technologies for these soups, it is clear that these technologies differ very much as well in ingredients as well as in process procedures. Industrial producers of traditional soups should use traditional ingredients and develop technologies more similar to traditional with the aim to achieve better quality of traditional soups produced in industry. Commercially produced traditional soups need to have characteristics more similar to original traditional soups.

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Role of Sensory Evaluation and Quality in Consumers' Acceptance of Smoked Meat Products

Jasna Djinovic-Stojanovic[1(✉)], Milan Ristic[2], and Klaus Troeger[2]

[1] Institute of Meat Hygiene and Technology, Belgrade, Serbia
jasna.djinovic@inmes.rs

[2] BAFF, Former Federal Centre for Meat Research (retired researchers), Kulmbach, Germany

Abstract. This paper reviews the most important trends and developments in meat sensory analysis in 2022 according to German Agricultural Society (DLG). The document "DLG Trend Monitor - Food Sensors 2022" built on results from 2009 to 2019, but also focused on other current aspects and development trends of sensory activities in food production in connection with the corona pandemic.

On the other hand, selected results concerning the sensory properties and quality of smoked meat products from Serbia and Montenegro are presented. This research is the result of long and successful collaboration between the Institute of Meat Hygiene and Technology, Belgrade, Serbia and the Federal Centre for Meat Research, Kulmbach, Germany (now the Max Rubner-Institute). Sensory evaluation of the products was conducted according to the DLG 5-point-testing-scheme by DLG-trained experts, in Kulmbach, Germany. The DLG 5-point-scheme is a descriptive sensory analysis method with scales, and points are given on the basis of assessment by experts. Analysis included visual (appearance/exterior), haptic (consistence/texture), olfactory (odor) and gustative (taste) criteria of the meat products. Meat products that pass the DLG tests receive a "DLG award winner" medal in Gold, Silver or Bronze. DLG medals are ambassadors for good taste and high-quality foods.

The quality of traditional smoked meat products was tested (from 2009 to 2019) by measuring different physico-chemical parameters (pH, water activity, sodium chloride, nitrite, and nitrate). Content of polycyclic aromatic hydrocarbons (PAHs) were determined by applying HRGC/MS/MS The chosen parameters are presented in this paper.

Keywords: Sensory evaluation · Consumer acceptance · Traditional smoked meat products

1 Developments and Trends in Food Sensory Analysis

1.1 "DLG Trend Monitor" - 2022

Meat and meat products are of high nutritional value, but some of their sensory properties, such as high amount of salts, fat, and smoke, can have negative effects on consumers' attitudes toward meat product quality. Hence, to understand consumers' acceptability of

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different types of smoked meat products and to identify the sensory characteristics that drive consumers' acceptability, sensory analysis is necessary.

According to the German Agricultural Society (DLG), the most important trends in meat sensory analysis in 2022 related to study design and participant profile [1]. Sensory evaluation experts and managers from German-speaking countries were focused on practical sensory experts from the areas of food supply and processing. They concluded that there are the wide range of possible applications and great interest in the practical use of sensory methods. The importance of sensory evaluation of different foods was reflected in the time spent on sensory tasks in daily work in the companies that were involved. It was shown that the majority of respondents (60.5%) still spend "less than 1 h" or "1–2 h a day" (20.3%) on sensory analysis. Sensory technology was located in different areas of the companies, such as "quality assurance" (69.1%), "product development" (57.6%), "market research" (15.3%), and "sensory department" (14.1%). The analytical methods in sensory technology can be divided into difference tests and descriptive tests. "Triangle tests" (66.7%), "rank order tests" (50.1%) and "pair comparisons" (48.1%) were the most dominant difference tests. In the descriptive tests that are currently used by the companies, the "simple descriptive exam" (72.3%) and "descriptive exam with subsequent evaluation" (67.8%) were the most commonly mentioned methods [1].

2 Traditional Smoked Meat Products from Serbia

2.1 Sensory Evaluation and Quality

Consumer evaluation of meat and meat products is becoming a critical issue for the meat industry because it has a direct influence on profitability [2]. Since attitude plays a crucial role in consumer decisions, it has become important to understand Serbian consumers' attitudes toward traditional smoked meat products, in order to market them effectively [3, 4]. In this section, selected results concerning sensory properties and quality of smoked meat products from Serbia and Montenegro, obtained from 2005 to 2019, are presented. During the 81st International Agricultural Fair in Novi Sad (2014, Serbia), Serbian consumers evaluated salami, sausage and chicken salami concerning their taste, salt content and smoke balance (Table 1) [5].

The highest percentage of consumers (55.3–88.2%) evaluated traditional smoked meat products as products with satisfactory taste, balanced salt content and appropriate smoke level. Results of statistical analyses showed that there was a significant difference ($p < 0.01$) between consumers' frequency of answers (satisfactory, average, non-satisfactory). Some consumers evaluated some meat products as too salty, while other products had too strong a smoke characteristic [5]. Literature data [6] indicate that traditional smoked meat and meat products with strong smoke contain phenols, antioxidant constituents that protect fat in meat products from oxidative degradation process and preserve the taste.

During 2015, sensory evaluation of five meat products from Zlatiborac Meat Company (beef salami, pork salami, chicken cajna sausage, homemade salami, and chicken salami) was performed in three large food store chains in Belgrade (Delhaize, DIS, and Mercator-S). For this purpose, sensory evaluation was conducted according to DLG quality testing [7] in which 1,157 randomly chosen persons participated. Consumers were

Table 1. Results of consumers' satisfactory with sensory quality of meat products. (n = 85, rating in %). [5]

Taste % (n)				Salt content % (n)			Smoke (n)		
#	I*	II*	III*	I	II	III	I	II	III
3	55.3 (47)**	63.5 (54)	69.4 (59)	83.5 (71)	84.7 (72)	88.2 (75)	76.5 (65)	82.4 (70)	74.1 (63)
2	31.8 (27)	31.7 (27)	29.4 (25)	15.3 (13)	5.9 (5)	5.9 (5)	16.5 (14)	15.3 (13)	20. 0 (17)
1	12.9 (11)	3.5 (3)	1.2 (1)	2.4 (2)	9.4 (8)	5.9 (5)	7.1 (6)	3.5 (3)	5.9 (5)

* I - Salami (100% beef meat); II - Sausage (20% beef meat and 80% pork meat); III - Chicken Salami (72% broiler breast meat). ** - Absolute number of the samples is shown in parentheses. # - rating level: Taste: 3- satisfactory, 2- average, 1- non satisfactory; Salt content: 3- balanced, 2- not salty enough, 1- too salty; Smoke: 3- balanced, 2- too weak, 1- too strong.

asked to answer the following questions: which type of meat has been processed (beef, pork, poultry, mixture), taste (good, satisfactory, non satisfactory), salt content (well-balanced, not salty enough, too salty), and smoke (well-balanced, too weak, too strong). All meat products were unlabeled, so the names of the manufacturers were unknown to the consumers. The most important results of that investigation [8] are presented in this paper.

With respect to taste, broiler salami was rated the best (77%, Fig. 1), as the salt content was considered well-balanced (78%). The smoke flavor was also described as well-balanced (90%) [8].

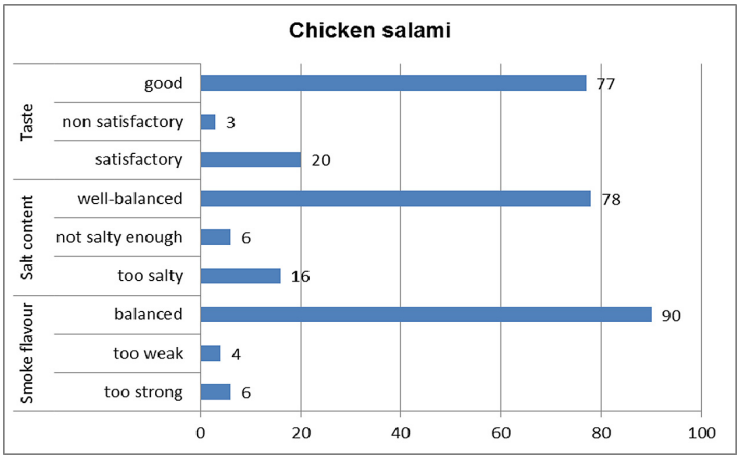


Fig. 1. The results of sensory evaluation of chicken salami (n = 521, [%]) [8]

Beef salami was evaluated similarly to chicken salami (Fig. 2). Most (70%) of the consumers evaluated the taste as good. The salt content of beef salami was acknowledged as being well-balanced (85%), as was the smoke flavor (89%) [8].

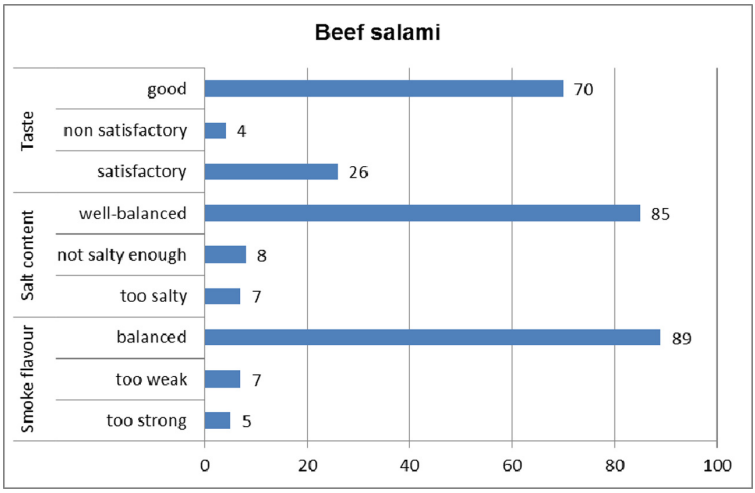


Fig. 2. The results of sensory evaluation of beef salami (n = 521, [%]) [8]

Meat color is an important quality trait in the meat industry sector. It is the most important first impression a consumer has of any meat product. Processed meats can contain high levels of animal fats that have been associated with increased risk of promoting obesity, diabetes and also cancers especially colon cancers [9]. However, reduced fat foods are seen by consumers as having inferior sensory properties than regular fat products. From the consumer point of view, fat content is not a good predictor of meat quality [10].

In 2017, Serbian consumer perceptions of color and fat content in three types of salami (chicken, royal, and beef) were examined [11]. Consumer testing was performed in four large retail stores in Belgrade. A total of 1018 consumers older than 18 years of age participated over six days. For each type of meat product, consumers were asked to express their perception of color and fat. They were asked to consume all types of salami and answer the following questions: (1) Rate the color (the offered answers were: good, too pale, too dark); (2) Rate the fat content (the offered answers were: sufficient, too fatty). The authors prepared a questionnaire as a modified version of the DLG 5-point-scheme [1]. The questionnaire followed with general socio-demographic information related to ages and education levels, including eating behavior. Results, showing consumers' attitudes in relation to education and age, are shown in Tables 2 and 3 [11].

Consumers, from both education levels and in both age groups, evaluated color as good and fat as sufficient, with these categories being chosen by a significantly ($p < 0.05$) higher percentage of consumers in comparison with the other offered answers [11].

The second part, on consumers' sensory acceptance of chicken, royal, and beef salami, were evaluated in 2018 and published the same year [12]. Consumers (n =

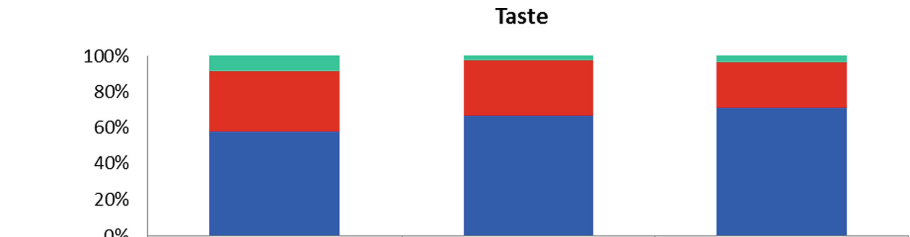
Table 2. The consumers' evaluation of colour and fat content of salami in relation to their level of education [11]

Education	Perception of	Offered answers	Salami		
			Chicken	Royal	Beef
BS-Basic School	Color	*Good*	96.4	**98.8**	72.3
		Too pale	3.6	1.2	–
		Too dark	–	–	27.7
	Fat	*Sufficient*	**98.8**	78.3	95.2
		Fatty	1.2	21.7	4.8
BD-Bachelor Degree	Color	*Good*	96.9	**97.9**	89.8
		Too pale	2.6	1.9	0.7
		Too dark	0.5	0.2	9.5
	Fat	*Sufficient*	90.3	83.6	**96.4**
		Fatty	9.7	16.4	3.6

Table 3. The consumers' evaluation of colour and fat content of salami in relation to their age [11]

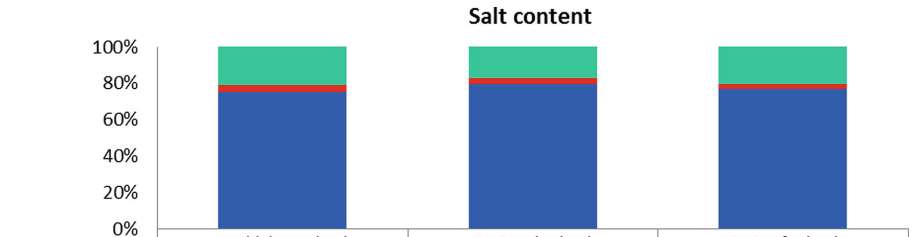
Age (years)	Perception of	Offered answers	**Salami**		
			Chicken	**Royal**	**Beef**
18–29	Color	*Good*	**97.6**	**97.6**	90.2
		Too pale	1.8	2.4	0.6
		Too dark	0.6	–	9.1
	Fat	*Sufficient*	**95.1**	88.4	**95.1**
		Fatty	4.9	11.6	4.9
50–59	Color	*Good*	94.6	**98.2**	87.8
		Too pale	5.0	1.4	–
		Too dark	0.5	0.5	12.2
	Fat	*Sufficient*	88.3	82.4	**94.1**
		Fatty	11.7	17.6	5.9

1031) were asked to evaluate the taste (good, satisfactory, non-satisfactory), salt content (well balanced, not salty enough, too salty) and smoke flavor (balanced, too weak, too strong) of the products during six days (Belgrade, May, 2018) in five retail stores. The questionnaire used was a modified version of the DLG 5-point-scheme. The results of sensory evaluation by consumers of chicken, royal, and beef salami are shown in Figs. 3, 4 and 5 [12].



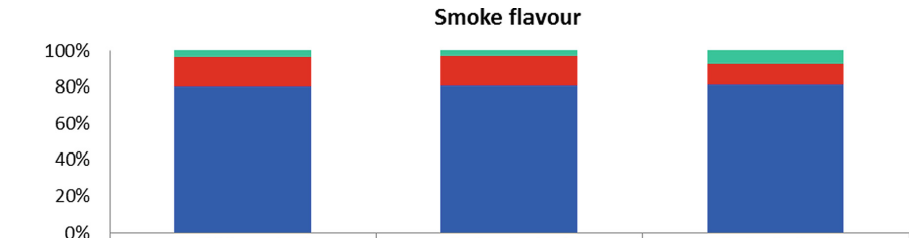
	1- Chicken salami	2 - Royal salami	3 - Beef salami
Not satisfactory	8.2	2.4	3.8
Satisfactory	33.9	31.2	24.7
Good	57.8	66.3	71.5

Fig. 3. The results of sensory evaluation of the taste of three salamis by consumers (n = 1031, [%]). [12]



	1- Chicken salami	2 - Royal salami	3 - Beef salami
Too salty	21.1	17.5	20.3
Not salty enough	3.7	3.3	2.7
Well balanced	75.2	79.2	77.0

Fig. 4. The tesults of sensory evaluation of salt content of three salamis by consumers (n = 1031, [%]) [12]



	1- Chicken salami	2 - Royal salami	3 - Beef salami
Too strong	3.7	2.8	7.7
Too weak	16.3	16.8	11.3
Balanced	80.0	80.4	81.0

Fig. 5. The results of sensory evaluation of the smoke flavor of three salamis by consumers (n = 1031, [%]) [12]

Sensory evaluation of the three types of salami showed that the highest percentages of consumers evaluated the taste, salt content and smoke flavor as positive by choosing the best available answers (Figs. 3, 4 and 5).

Physico-chemical properties of different smoked meat and smoked meat products from Serbia were analyzed in multiple studies [13–15]. Different physico-chemical parameters (pH; water activity (a_w); peroxide value; acid value; and contents of water, protein, fat, ash, sodium chloride, nitrite, and nitrate) were determined [13–15]. Fatty acid composition and content of polycyclic aromatic hydrocarbons of some smoked meat products from Serbia were performed by applying GC/ECD and HRGC/MS/MS [14].

The chosen physico-chemical parameters of beef ham are shown in Table 4 [13]. The pH values were in the standard range for this type of product. The a_w values were between 0.886 and 0.933, and they were low enough to provide a barrier to help protect product safety, e.g. prevent growth pathogenic bacteria [16]. The salt content of ham was between 1.13 and 6.1%. According to the literature data, the average salt content for these products is 4.5% [17]. Contents of nitrate (495 and 680 mg/kg) in two samples were higher than the maximum allowable concentration (MAC), (250 mg/kg), while the nitrite content was in the standard range. Mean content of benzo(a)pyrene (1.7 μg/kg) was below the MAC value (2 μg/kg).

Table 4. Physico-chemical parameters and Benzo(a)pyrene content of raw cured beef [13].

	pH	Water activity (a_w)	NaCl [%]	Nitrite [mg/kg]	Nitrate [mg/kg]	Benzo[a]pyrene [μg/kg]
Mean	5.54	0.903	4.9	13.0	227.5	1.7
Standard deviation	0.17	0.021	1.13	10.8	234.9	0.16
Minimum	5.38	0.886	3.0	1.0	37.0	1.5
Maximum	5.77	0.933	6.1	33.0	680	1.8

3 Conclusion

This review focused on our research papers, from 2005 to 2019, evaluating the role of sensory evaluation and quality in consumers' acceptance of smoked meat products from Serbia. This area of research helps provide important information on consumers' attitudes toward different smoked meat products. The meat industry needs to know which attributes of meat products are considered important by consumers, information that can be obtained from proper sensory evaluation, in order to develop products that meet consumer needs and to promote the products more effectively. Also, this study contributes to display transparency to the public; it made it possible to gather reliable data on the acceptance and attitudes of Serbian consumers toward the different types of traditional smoked meat products that originate from Serbia. Consumers were satisfied

with the color and fat of the analyzed meat products. However, the high salt content and too strong smoke flavor of the meat products were the main deficiencies. The results obtained showed that traditional smoked meat products from Serbia can receive satisfactory sensory evaluations, but from the consumer point of view, their quality should be improved for the (Western) European market.

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Determination of Antioxidant Activity in Parsley, Dill, Coriander and Basil

Lejla Čengić(✉), Zilha Ašimović, and Teofil Gavrić

Faculty of Agriculture and Food Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
l.cengic@ppf.unsa.ba

Abstract. Natural antioxidants are considered to be substances that, in small quantities, can neutralize free radicals. Antioxidant properties are attributed to herbs, which are positively correlated with health effects. Antioxidant activity, as a link between nutrition and disease, finds increasing interest in research. The aim of this assay was to determine the antioxidant activity in herbs and the effect of drying treatment on the antioxidant potential. Plants from the *Apiaceae* family, namely parsley *(Petroselinum crispum*), dill (*Anethum graveolens*), and coriander (*Coriandrum sativum*) and basil (*Ocimum basilicum*) from the *Lamiacea* family, have been selected for this research (fresh, oven drying (45 °C) and air drying). Two different methods for antioxidant activity determination were used: DPPH (2,2-diphenyl-1-picrylhydrazyl), a stable free radical that is decolorized in the presence of antioxidants, and the pFRAP (potassium ferricyanide reducing power) method, based on the reaction between phenolic compounds and pFRAP, resulting with blue complex with absorbance at 700 nm. Fresh samples showed high antioxidant power against the DPPH radical, as follows: basil (90.54%) > dill (82.92%) > coriander (79.06%) > parsley (75.64%). The ability to eliminate DPPH radicals in dried samples varied from 82.83% in air-dried basil to 24.97% in oven-dried coriander. The highest antioxidant activity was recorded in fresh basil (1097.80 ± 17.57 mg GAE/100g dry matter), and the lowest in oven-dried coriander (12.31 ± 0.82 mg GAE/100g dry matter) using the pFRAP method. The values obtained by determining the pFRAP activity of the reduction of Fe^{3+} to Fe^{2+} suggested that herbs contribute with good reducing potential, which can be a useful system for the prevention of oxidation. For all analyzed plants, drying resulted in decrease of antioxidant activity.

Keywords: Antioxidant activity · herbs · DPPH · pFRAP

1 Introduction

Antioxidants have been recognized as substances present in foods in low concentrations, which can delay the oxidation process or inhibit free radical action against cells and tissues [1]. Antioxidative molecules protect cells from oxidative damage caused by reactive oxygen species, reactive nitrogen and reactive chlorine species, which can be related to degenerative diseases [2]. Natural antioxidants, that are present in foods,

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mostly flavonoids, C and E vitamins, and β-carotene, have been lately under growing interest because of their ability to reduce cardiovascular diseases risk, lipid oxidation and free radical action effects [3]. Total antioxidant activity can be a useful tool in the examination and determination of the relationship between antioxidant compounds in foods and diseases caused by oxidative stress [4, 5].

Herbs, due their abundance, are used in medicine, households and, with a special place, in food technology. Herbs are parts of plants with aromatic, bitter or spicy components that are used fresh or dried to enhance dish taste or digestion [6]. Plants from the Apiaceae family, with aromatic characteristics of their essential oils [7], have been traditionally used in folks medicine, due to their high content of phytochemicals and secondary metabolites, like terpenoids, flavonoids, saponins etc. [8]. Numerous plants from the Lamiaceae family, like basil, mint, or rosemary, with high content of essential oils mostly in leaves [9], have been used in the cosmetic industry, culinary [10] as well as in traditional medicine as a remedy for different conditions. Parsley (*Petroselinum crispum*), dill (*Anethum graveolens*), coriander (*Coriandrum sativum*), and basil (*Ocimum basilicum*) have been selected for this research due to their traditional usage as a good source of phytochemicals, thus as a good source of antioxidant activity.

The drying process has been considered essential for herbs, which are sensitive after harvest due to enzymatic actions which can be triggered to metabolic processes like spoilage, bacteria growth etc. [11]. Drying can reduce the amount of water to levels adequate for a safe and longer period of storage. Besides water loss, there are other benefits of drying: reduction of mass and volume of the products, which results in smaller package and storage space [12] as well as enzymatic degradation inhibition which can lead to loss of bioactive compounds. There are different types of drying, but considering the costs of the drying process, air drying, and oven drying are widely used [13].

This research aimed to determine the antioxidative activity in fresh and dried selected herbs using two methods for antioxidative activity determination, DPPH and pFRAP methods, respectively.

2 Materials and Methods

2.1 Materials

For experimental purposes, samples were collected from local herbs producer, in the Kakanj area, Bosnia and Herzegovina. Herbs (leaf) were analyzed immediately after harvest. Parsley (*Petroselinum crispum*), Rialto variety and basil (*Ocimum basilicum*) - Italiano classico were produced in a greenhouse; coriander (*Coriandrum sativum*) and dill (*Anethum graveolens*), under commercial name ELLA, were produced in an open field. Samples were washed with tap and distilled water and the average amount of every herb species was 1.5–2 kg. After the removal of inadequate parts, samples were analyzed. Every herb species was divided into three parts: the first one was analyzed immediately for antioxidant activity, and the other two were subjected to a drying process (air-drying and oven-drying) and antioxidative activity determination afterward.

2.2 Methods

Drying process. For drying of selected herbs, two methods were used. Air-drying of selected herbs was carried out in the well-ventilated dark room at room temperature for a period of seven days. Oven-drying was carried out in a laboratory oven (MEMMERT, Germany) at 45 ± 1 °C for 4 h, with periodic mass measurement. Both drying processes were applied on fresh leaves of selected herbs, with moisture content of 83.82% for parsley, 85.67% for coriander, 84.12% for dill, and 89.21% for basil.

pFRAP antioxidant assay. For the pFRAP method extraction was carried out with 30% ethanol (w/w) as follows:1g of sample was mixed with ethanol, heated to 50 °C and after manual homogenization was transferred to an extraction flask with a round bottom which was heated in water bath with reflux for 1 h. After that, the sample was filtrated in the volumetric flask (50mL) and made up with ethanol to the mark.

Antioxidant activity was determined with pFRAP method according to Meng et al. (2011) [14]. The method is based on the reaction between phenolic compounds with K_3[Fe $(CN)_6$]; a blue complex is created with absorption at 700 nm [14]. Briefly, 1 mL of extract was transferred to the test tube, 1 mL of K_3[Fe $(CN)_6$] was added, and after 5 min 1 mL of $FeCl_3$ and 7 mL of distilled water were added. Test tube content was then subjected to absorbance measurement at 700 nm. The calibration curve was made with gallic acid as standard (0–100 mgl^{-1}) and the results were expressed as gallic acids equivalents GAE/100 g dw.

DPPH antioxidant assay. Antioxidant activity in selected herbs was determined with DPPH (1,1-dipheyl-2-pycrylhydrazile) free radical, which was discolored when interacted with antioxidant molecules [15]. Color of DPPH free radical changes from purple to yellow in the reduction process. The stock solution of DPPH free radical was made by dissolving DPPH in methanol (400μM) and the solution was kept in a refrigerator for 2 h for stabilization. Briefly, 100 μL of the extract was mixed with 2 mL of DPPH solution whose absorbance was read at 517 nm and left for 30 min in the dark. The purple color of the DPPH solution was changed to yellow due to the transfer of electrons from the antioxidative compound to the DPPH molecule. The decrease in DPPH absorbance and antioxidative activity of antioxidative molecules were calculated following equation:

$$AA(\%) = \{(Ao - As)/Ao\}x100$$

Trolox (6-hydroxy-2,5,8,8-tetramethylchroman-2-carboxylcc acid) was used as a standard antioxidant molecule. The amount of the antioxidant as the Trolox equivalent antioxidative capacity was calculated from calibration curve (5–250 ppm) of Trolox and its inhibitory effect (percentage) against DPPH free radical, using linear regression equation ($y = 0.348 + 6.7817$, $R^2 = 0.9998$) [16].

2.3 Statistical Analysis

Obtained data were expressed as mean ± standard deviation (SD) ($n = 3$) and statistically analyzed by ANOVA (Statistical Analysis Software) in order to determine differences depending of drying process. Least significant difference ($p < 0.05$) was used to means comparison.

3 Results and Discussion

Free radicals, which can be regenerated by different mechanisms, can be related to chronic diseases and a faster aging process [17]. Antioxidant activity assay showed that selected herbs exhibited very good antioxidative characteristics. Two different methods were used for determination, DPPH and pFRAP, respectively. Examination of the ability to scavenge free radicals by antioxidative molecules in parsley, dill, coriander, and basil resulted in high antioxidative power, shown in Table 1. Results are expressed as a percentage of inhibition, as well as the Trolox equivalents for DPPH assay, and gallic acid equivalents for pFRAP assay. Figure 1 and Fig. 2 show influence of drying process on the antioxidative activity. Antioxidant activity of Trolox and samples were tested for the same period (30 min), and concentration of antioxidative molecules in herbs extract, as well of Trolox, and its inhibitory effect against DPPH free radical was found. The scavenging effect against DPPH radical in extracts was calculated as Trolox equivalents antioxidant capacity from calibration curve.

Fresh samples showed high antioxidative activity against DPPH free radicals, as follows: basil (90.54%) > dill (82.92%) > coriander (79.06%) > parsley (75.64%). Similar studies showed antioxidative activity in basil leaves against DPPH 94.6% and parsley leaves 30,4% [18] which can be related to our results. High antioxidant activity in basil leaves from 69.33%-89.22% can be attributed to high levels of methyl eugenol found in basil [19, 20]. DPPH activity in methanolic extract ranged between 19.9% and 28.3% whilst water extract showed 13.9%-18.8% [21]. DPPH molecule is soluble in organic solvents, which can be a limitation for hydrophilic antioxidants determination, as well as the reaction between DPPH radical and antioxidative molecule which depends on the chemical structure of antioxidants [22].

Results are expressed as mean ± SD; values with different letters (a, b, c) within the same column showed significant differences at $p < 0.05$; TEAC-Trolox antioxidative capacity equivalents.

Variations in results can be explained as a consequence of different solvents used for extraction, like ethanol, water, or methanol, which is found to be the most effective for antioxidants extractions. The difference in extraction solution polarity can be a possible explanation for different inhibition process within the same herbal species [23]. Dried samples generally showed lower antioxidative activity. For oven-dried samples, basil showed the highest value, and dill extract showed the lowest antioxidant activity (62.23% and 24.97%, respectively). Air dried samples exhibited a higher percentage of inhibition against DPPH radical compared with oven-dried samples. The ability of DPPH radical elimination varied from 82.83% in air dried basil to 24.97% in oven-dried coriander. There was a significant difference ($p < 0.05$) between fresh and dried samples of selected herbs. The drying process resulted with a decrease in antioxidant activity. The decrease of free radical inhibition of air-dried samples can be attributed to enzymatic activity and degradation during a long period of drying. Studies on lemon, oregano, and mint showed different values for antioxidant activity, from significant increase to significant decrease [24]. For every herb analyzed, a decrease in antioxidant activity was recorded. Decrease varied from 43%-68% in parsley, coriander, and dill dried samples compared to fresh samples and from 9.17%-31.4% in basil. Generally, it was noticed the highest

Table 1. Antioxidant activity in parsley, dill, coriander and basil

Herbs	Treatment	pFRAP	DPPH	mg Trolox /g dw
		mg GAE/100g dw	(% inhibition)	DPPH (TEAC)
	Fresh	175.98 ± 6,39[a]	75.64 ± 1.49[a]	128.39 ± 2.79[a]
Parsley	Air-dried	24.08 ± 1.23[b]	43.45 ± 1.23[b]	68.38 ± 2.29[b]
	Oven-dried 45 °C	16.23 ± 0.06[c]	34.46 ± 0.66[c]	51.61 ± 1.23[c]
	Fresh	405.26 ± 5.28[a]	82.92 ± 0.36[a]	154.15 ± 0.72[a]
Dill	Air-dried	44.29 ± 3.11[b]	43.45 ± 1.23[b]	49.51 ± 0.98[b]
	Oven-dried 45 °C	23.82 ± 1.38[c]	34.46 ± 0.66[c]	111.73 ± 0.51[c]
	Fresh	153.67 ± 2,54[a]	79.06 ± 0.45[a]	146.34 ± 0.91[a]
Coriander	Air-dried	26.38 ± 1.23[b]	45.36 ± 0.46[b]	78.10 ± 0.94[b]
	Oven-dried 45 °C	12.31 ± 0.82[c]	24.97 ± 0.41[c]	32.28 ± 0.86[c]
	Fresh	1097.8 ± 17.57[a]	90.54 ± 0.52[a]	243.00 ± 0.41[a]
Basil	Air-dried	63.40 ± 0.29[b]	82.23 ± 0.59[b]	218.31 ± 1.70[b]
	Oven-dried 45 °C	44.29 ± 3.11[c]	62.11 ± 0.63[c]	80.09 ± 1.91[c]

reduction of antioxidant activity (68%) in oven-dried coriander samples, whilst air-dried basil showed the lowest decrease in DPPH radical inhibition.

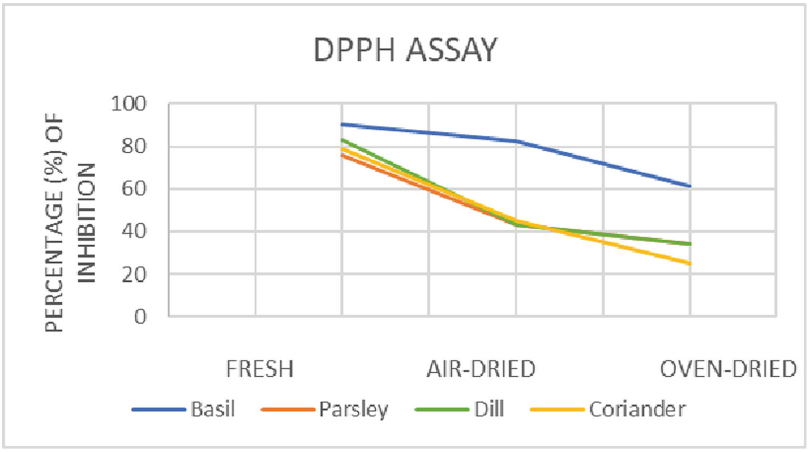

Fig. 1. Influence of drying process on level of inhibition DPPH free radical in selected herbs extracts

pFRAP assay had several advantages for antioxidative activity determination, such as fast, simple and low-cost. pFRAP assay showed the highest antioxidative activity in fresh basil samples 1097.80 ± 17.57 mg GAE/100g dw, and the lowest in oven-dried coriander

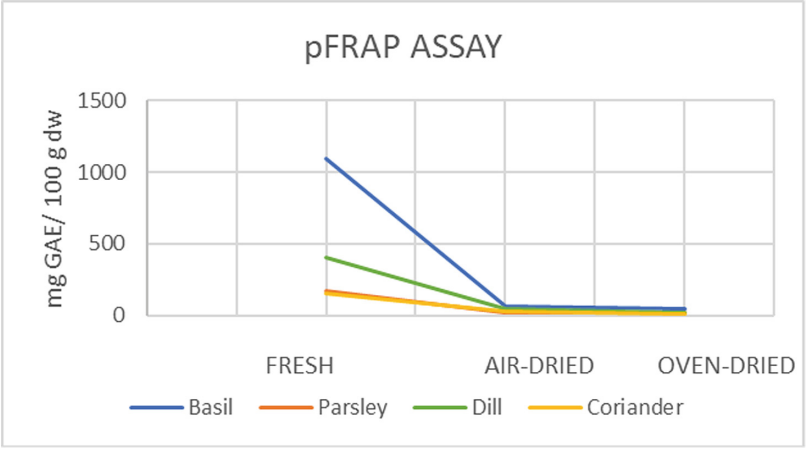

Fig. 2. Influence of drying process on the ability of Fe3+ to Fe2+ reduction in selected herbs extracts

12.31 ± 0.82 mg GAE/100g dw. Results obtained with pFRAP assay had the same trend as DPPH assay, which suggests that herbs exhibit good excellent reduction potential and can be used as a useful system for oxidation prevention. Ašimović et al. [25] showed similar results. Based on the previous studies, it can be said that antioxidant activity is in relation to phytochemical compounds content [26], such as phenolic like ruthin, quercetin, and chlorogenic acids [27]. When it comes to pFRAP activity assays, one of the most important characteristics is the ability of the molecule that poses antioxidative properties to chelate Fe^{2+}. Water extracts of herbs showed 47% chelation ability for Fe^{2+} ions [28]. Different data can be explained as a result of the loss of antioxidative molecules depending on the stage of development at the time of analysis, as the number of phytochemicals is higher in leaves compared with root or stem [29]. A decrease of antioxidative potential is attributed to heat treatments which can lead to the degradation of phytochemicals, enzymatic degradation of phenolic compounds as well as loss of enzymes [30, 31].

4 Conclusion

This research showed that herbs can be a potent source of natural antioxidants, with a focus on basil, dill, coriander, and parsley. Based on obtained data, basil was the strongest and the most potent herb among used herbs that can provide high antioxidant potential. However, high antioxidative activity categorizes these herbs as food with beneficial properties for human health. For all analyzed plants, drying resulted in decrease of antioxidant activity. Hence, to preserve bioactive nutrients, proper care and storage are necessary. Further research should be focused on adequate drying techniques that could better preserve bioactive compounds with antioxidative properties.

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The Chemistry of Fermented and Pickled Food

Amina Stambolić[1(✉)], Selma Burović[2], Semira Galijašević[2], and Enisa Omanović-Mikličanin[1]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja Od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
a.stambolic@ppf.unsa.ba

[2] Sarajevo Medical School, University Sarajevo School of Science and Technology, Hrasnička Cesta 3a, Sarajevo, Bosnia and Herzegovina
selma.burovic@ssst.edu.ba

Abstract. Fermentation, as one of the primary metabolic pathways for energy extraction, has been utilised since antiquity as a means to preserve different types of food. In the most basic of definitions, it includes the breakdown of carbohydrates due to microbial activity. Fermented food has found its place in traditional cuisines all over the world and is consumed by many people on a daily basis.

Pickling, another way to ensure consumption and preservation of mostly vegetables even out of season, can be done in one of two ways: by anaerobic fermentation in brine or by immersion in vinegar.

Both fermentation and pickling change the chemical composition of the food. This review aims to showcase what happens during these food preservation processes on a molecular level and discuss the possible health benefits as well as detriments that come with consumption of food processed this way.

Keywords: fermentation · pickling · preserved food · food chemistry

1 Introduction

Fermentation, by one of its many definitions, is the breakdown of carbohydrates due to microbial activity in anaerobic conditions. Biochemically, fermentation is an anaerobic catabolism of an organic compound in which the compound is used as both an electron donor and an electron acceptor. [1] Primary products of fermentation are alcohols and organic acids. From the standpoint of food processing, the term fermentation implies that the microbial activity is a desired process. Early historical evidence of fermentation being used in food processing is linked to the production of alcoholic beverages such as beer and wine.

Aside from alcohol production, fermentation is also being used as a means to preserve easily perishable food such as fresh fruits and vegetables as well as different kinds of meat and fish. Food preservation via fermentation is achieved through two occurring processes: the production of organic acids and the production of carbon dioxide. The acids lower the system pH making it inhabitable for other pathogenic bacteria and the

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formed CO_2 flushes out the oxygen making it impossible for aerobic microorganisms to thrive.

Fermentation is also widely used to improve the flavor and aroma of food products through various aromatic compounds that are formed during the process.

Considering the complex matrix food represents, possible side reactions and interactions between fermentation products and other food molecules need to be discussed as well.

2 Chemistry of Carbohydrate Breakdown

Sugar is the fuel which most organisms use to extract energy necessary to sustain vital functions. The energy extraction is carried out through energetic pathways of the metabolism. [2] Naturally occurring carbohydrates in food provide the source of sugars the body needs. The Embden–Meyerhof–Parnas pathway, commonly known as glycolysis is a common pathway for the catabolism of sugars. [3] Glycolysis proceeds in two phases: the preparatory phase and the "pay-off" phase. The processes in the preparation phase demand adenosine triphosphate (ATP). Initially, ATP phosphorylates glucose to produce glucose-6-phosphate. Glucose-6-phosphate is isomerized to fructose-6-phosphate, and then converted into fructose-1,6-biphosphate through phosphorylation. Two ATP molecules are used leading up to that step. Aldolase then breaks down fructose 1,6-bisphosphate into two molecules with three carbons each, glyceraldehyde 3-phosphate and its isomer dihydroxyacetone phosphate. [4].

In the second stage, termed as pay-off phase, glyceraldehyde-3-phosphate is combined with an inorganic phosphate unit to produce 1, 3-bisphosphoglycerate, and glyceraldehyde-3-phosphate dehydrogenase reduces NAD + to NADH (nicotinamide adenine dinucleotide). Given that glucose produces two molecules of glyceraldehyde-3-phosphate, this redox process takes place twice. Following that, each molecule of 1, 3-bisphosphoglycerate is converted into 3-phosphoglycerate, which results in the synthesis of four molecules of ATP. Phosphoenolpyruvate is then transformed into pyruvate. Therefore, the net energy yield of glycolysis is two ATP molecules for every glucose molecule. [1].

To produce energy from sugars, microorganisms use two processes: respiration and fermentation. Both processes begin with glycolysis but proceed along distinct pathways after that. Under aerobic conditions, respiration, which can convert pyruvate to carbon dioxide (CO_2) and water with significant amounts of ATP, typically occurs in conjunction with glycolysis. Under anaerobic circumstances, pyruvate is fermented to produce a variety of fermentation products, many of which are important for the industry. [3].

3 Fermentation

During fermentation, the pyruvate produced by glycolysis is converted to various fermentation end products. The type of the end product is determined by the microorganism present. [5].

3.1 Yeast Fermentation

Yeasts are eukaryotic microorganisms that primarily inhabit water, soil, air, and the surfaces of plants and fruits, among many other diverse habitats. Basic nutrients including fermentable carbohydrates, amino acids, vitamins, minerals, and oxygen enable their development. Saccharomyces and non-Saccharomyces are the two main categories of yeasts according to technical convention. Due to its good fermentative capacity, quick growth, and ease of adaptation, S. cerevisiae is the species that has been studied the most and is also the one that is used the most in the fermentation of wines and beers. They have shown resistance to high levels of SO_2 which isn't typical for the majority of non-*Saccharomyces* strains. [6].

Non-*Saccharomyces* are a class of microorganisms that are utilized in a variety of fermentation processes because of their considerable metabolic diversity, which enables the synthesis of many final products. [7] When spontaneous fermentation initially begins, these yeasts predominate until the ethanol concentration reaches 4 to 5% v/v. Their development is then inhibited by the alcohol and the depletion of dissolved oxygen. The fermentation is finished when the most ethanol-resistant yeasts, *Saccharomyces*, prevail and take over. [8, 9].

Even though most non-*Saccharomyces* yeasts show some technological disadvantages compared to *S. cerevisiae* such as lower fermentative power and production of ethanol, non-*Saccharomyces* yeasts possess characteristics that *S. cerevisiae* doesn't exhibit, for instance, production of high levels of aromatic compounds such as esters, higher alcohols and fatty acids which contribute to the taste of the final product. [10].

Among the most studied non-*Saccharomyces* yeasts that reached special importance for researchers include *Candida, Kloeckera, Hanseniaspora, Brettanomyces, Pichia, Lanchacea* and *Kluyveromyces*.

Among all the food fermented due to yeast activity, the most widely consumed are beer, wine, bread, chocolate and coffee. A traditional yeast fermented drink called *boza* is made and consumed in the Balkans and Turkey.

Chemistry of Yeast Fermentation

The most significant chemical created by yeast is ethanol, which is used in many industrial fermentation processes. Furthermore, the initial interest in beverage fermentation was prompted by the synthesis of this main metabolite.

An important regulatory point in metabolism, the divergence of pyruvate following glycolysis has become a focus of biochemical and industrial research. At this stage, pyruvate can go in one of two basic directions: fermentation or respiration. This depends on the presence of oxygen in the majority of eukaryotes. Pyruvate dehydrogenase will convert pyruvate to acetyl-coA under aerobic conditions, and the two compounds will then move toward the citric acid cycle. Pyruvate is directed toward fermentation when anaerobic (fermentative) conditions are present. [11].

Pyruvate is transformed into ethanol in two steps. Pyruvate decarboxylase (PDC) first converts pyruvate to acetaldehyde, releasing carbon dioxide as a waste product. The *S. cerevisiae* genome contains three identified PDC enzymes that serve as a metabolic junction between fermentation and respiration. PDCs can remove extra pyruvate from the

pathway and shift it towards ethanol production in direct competition with pyruvate dehydrogenase. An alcohol dehydrogenase (ADH) then converts acetaldehyde into ethanol. This type of oxidoreductase can catalyze the reversible interconversion of alcohols and the corresponding aldehydes or ketones. [11] (Fig. 1).

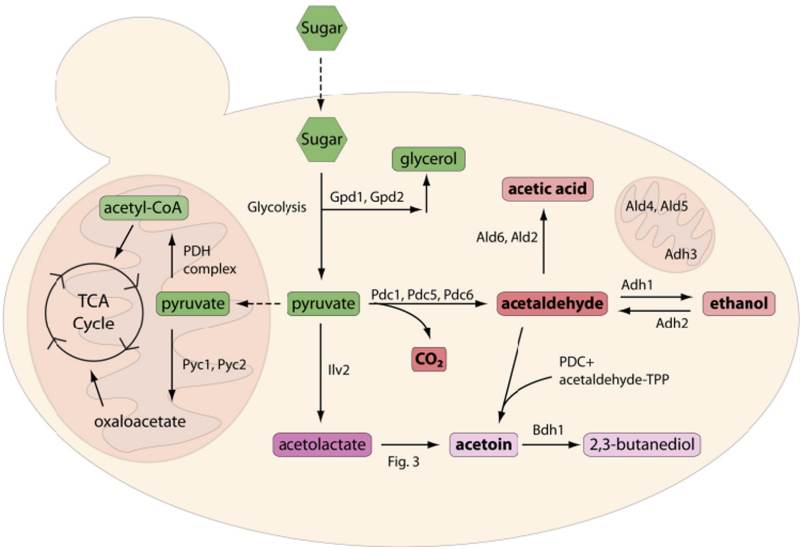

Fig. 1. Production of ethanol, acetaldehyde, acetic acid, and CO_2. Fermentable carbons are assimilated from the medium and converted to glycerol or pyruvate via glycolysis. Pyruvate can be shuttled towards the TCA cycle and respiration (left) or towards alcoholic fermentation (right). [11]

Alcohol produced by yeast-fermentation can further interact with urea usually present due to metabolic breakdown of arginine or citrulline in the presence of yeast. This interaction gives rise to ethyl carbamate, also known as urethane, which is a known carcinogen. Urethane has been found in almost all yeast fermented alcoholic beverages and bread at concentrations of 2 μg/kg. [12] (Fig. 2).

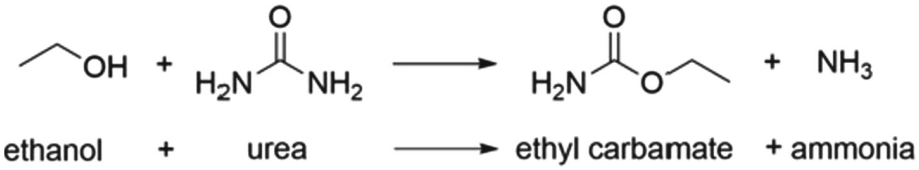

Fig. 2. The synthesis of ethyl carbamate in fermented food

Vicinal diketones can be produced during fermentation through non-enzymatic decarboxylation of intermediates in the anabolic pathways of valine and isoleucine. Vicinal diketones can provide a pleasant nutty, toasty and toffeelike flavor in fermented foods

and beverages, most notably beer, wine and dairy products. However, they are considered off-flavors when present in high concentrations, changing their sensory perception to 'buttery' or 'rancid'. [11] (Fig. 3).

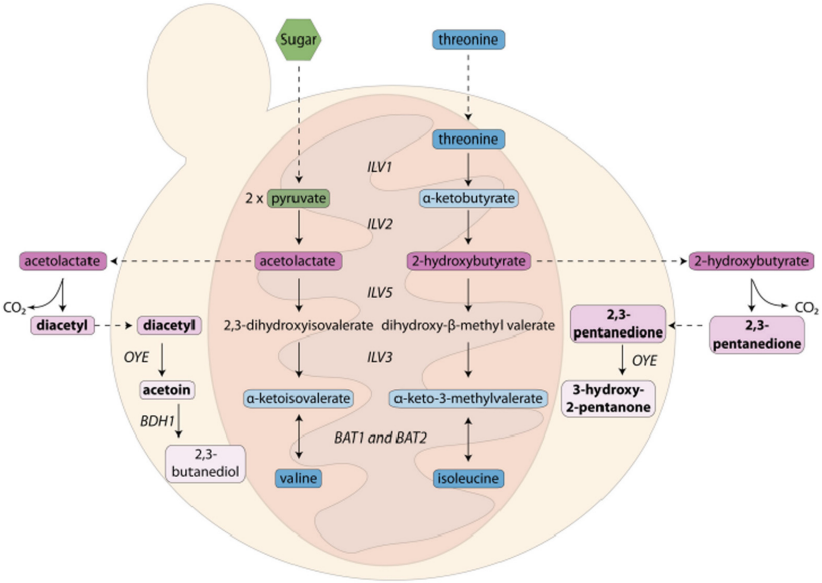

Fig. 3. Metabolic production of vicinal diketones [11]

The Ehrlich route modifies assimilated amino acids, a major source of nitrogen in many fermentation processes, in three stages. Amino acids are generally deaminated, decarboxylated, and then reduced to the corresponding alcohol derivatives.

Despite having a higher sensory threshold than their corresponding acetate esters—which can vary by several orders of magnitude—higher alcohols might nevertheless have a desired impact on a product's flavor. The major fusel alcohols found in alcoholic beverages are 1-propanol (alcoholic aroma), 1-butanol (alcoholic), isobutanol (alcoholic), 2-phenylethanol (roses, flowery) and isoamyl alcohol (banana, fruity). [11] (Fig. 4).

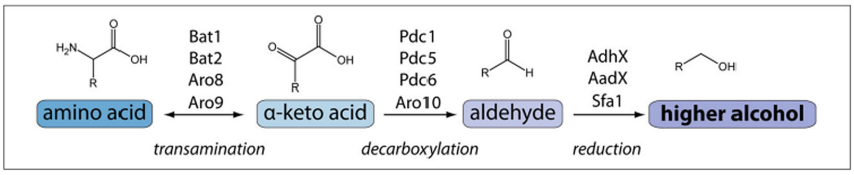

Fig. 4. Metabolic production of higher alcohols [11]

Esters are formed by a condensation reaction between acetyl/acyl-CoA and an alcohol. Ester synthesis is carried out by alcohol-O-acetyl (or acyl)- transferases (AATases).

Esters are widely acknowledged as some of the most significant flavor and aroma contributors to alcoholic beverages, giving the beverage notes of fruit and flowers. In the perception of many esters, a combination of compounds will either highlight or hide the existence of additional compounds. The necessity of balance in the creation of aroma compounds is highlighted by the fact that an excess of esters frequently leads to an unappealing product. [11].

The catabolism or anabolism of sulfur-containing amino acids methionine and cysteine generates all sulfur compounds generated by yeast. Although these amino acids are present in both natural and industrial environments in very low proportions, yeasts also need to ingest inorganic sulfur through the sulfate reduction pathway. Sulfates are sequentially reduced to sulfide which can combine with a nitrogen source (O-acetylserine or O-acetyl-homoserine) to form cysteine and subsequently, methionine. [11] Bacteria have been more widely studied in regard to sulfur production since the negative odors are generally associated with spoilage or desired aromas in specific types of cheese which utilize lactic acid bacteria. [13] Sulfur compounds are most relevant in beer, wine and cheesemaking industries. Contrary to fusel alcohols or esters, some sulfur compounds are categorized as having good scents while others have unpleasant aromas. For instance, hydrogen sulfide (H_2S) is the source of the traditional "rotten-egg" smell typically associated with sulfur, but furfurylthiol has a roasted coffee aroma. Interestingly, the perception of these compounds is highly context specific. While dimethyl sulfide typically smells of cabbage, it can give the desired aroma notes to lager beers and whiskey [11].

In beverage production, pre-treating different lignin polymers of plant cell walls is a standard technique. The bioprocessing of these polymers prior to the fermentation process releases a variety of furans, carboxylic acids and phenolic compounds which can greatly inhibit microbial growth. Many microbial species including *S. cerevisiae* convert these compounds into derivates less toxic to the yeast. Several of the hydroxycinnamic acids, such as cinnamic acid (phenylacrylic acid), caffeic acid, ferulic acid and *p*-coumaric acid, can be decarboxylated to less toxic phenolic compounds. [11].

3.2 Lactic Acid Bacteria Fermentation

Lactic acid bacteria (LAB) are Gram positive procaryotes that are acid tolerant and non-respiring. Those two characteristics are essential for their use in food processing. They form lactic acid as the major end product from the fermentation of carbohydrates. The produced lactic acid may be in the form of L (+) or D (−) stereoisomers or a mixture of both. [14].

LAB include the genera of *Lactobacillus* (*Lb*), *Carnobacterium*, *Leuconostoc* (*Leu*), *Oenococcus*, *Streptococcus*, *Lactococcus* (*Lc*), *Enterococcus*, *Vagococcus*, *Pediococcus* (*Pe*), *Aerococcus*, *Tetragenococcus*, and *Weissella* [15].

Food processed using LAB is commonplace in cuisines across the globe with some of the most popular ones being: yoghurt, kimchi, sauerkraut, kefir, kumis, fish sauce, and a wide variety of fermented dairy and meat products.

Chemistry of Lactic Acid Bacteria Fermentation

According to their capacity to ferment sugars, LAB are categorized as homofermentative

and heterofermentative from a biochemical standpoint. Lactic acid is the sole fermentation product produced by the homofermentatives. The heterofermentatives, on the other hand, produce a wide range of fermentation end products, primarily ethanol, CO_2, as well as acetic acid, acetaldehyde, diacetyl, and acetoin in addition to lactic acid. [16] (Fig. 5).

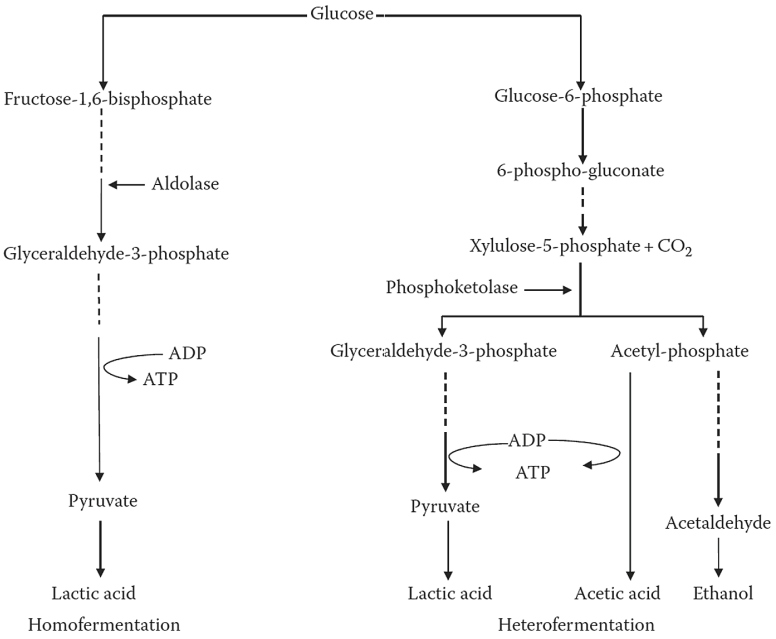

Fig. 5. Difference between homofermentation and heterofermentation [4]

The reason for the difference in end products for homo- and heterofermentation is the presence of aldolase in homofermentatives which ultimately gives rise to two molecules of lactic acid per one glucose molecule and twice the amount of energy compared to heterofermentatives. Homofermentative LAB include *Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, and some species of *Lactobacillus* such as *Lb. Delbrueckii* subsp. *Delbrueckii*, *Lb. Delbrueckii* subsp. *Lactis*, *Lb. Delbrueckii* subsp. *Bulgaricus*, *Lb. Acidophilus*, *Lb. Helveticus*, and *Lb. Salivarius* [4].

Heterofermentative LAB such as *Leuconostoc*, *Oenococcus*, and some *Lactobacillus* such as *Lb. Brevis*, *Lb. Buchneri*, *Lb. Fermentum*, and *Lb. Reuteri* use the pentose phosphate pathway and produce especially CO_2 and ethanol along with lactic acid. [14].

They are unable to convert fructose 1,6-bisphosphate into triose phosphate since they lack aldolase. But they oxidize glucose 6-phosphate to 6-phosphogluconate. Then, 6-phosphogluconate is decarboxylated to pentose phosphate, and CO_2 is released. The vital enzyme phosphoketolase transforms the created pentose phosphate into triose phosphate and acetyl phosphate. Acetyl phosphate is used as an electron acceptor and is reduced by NADH to ethanol. [1].

Heterofermentative LAB produce more flavor and aroma compounds such as acetaldehyde and diacetyl when they are compared to homofermentative LAB.

Some *Lactobacilli* such as *Lb. Casei*, *Lb. Curvatus*, *Lb. Plantarum*, and *Lb. Sakei* behave as facultatively heterofermentative depending on the presence of pentose sugars and gluconate due to having both aldolase and phosphoketolase enzymes. [17].

Most of the formation routes of aroma compounds in LAB rely on the presence of functional metabolic pathways, rather than single-enzyme conversions. During the fermentation of food raw materials, both intact and lysed LAB cells contribute to the process of aroma formation. The cytoplasmic enzymes in the matrices of the fermentable food materials are released as a result of cell lysis. Many of these released enzymes continue to function outside of the cell and will keep converting food matrix substrates. The primary food matrix substrates for the cytoplasmic enzymes released by fermenting LAB, which may have an effect on the aroma profile of the fermented food product, are proteins, oligopeptides, lipids, fats, and fatty acids. [18] It has been demonstrated that lysis of LAB in dairy starters plays an essential role in the process of cheese maturation, mainly because of the release of intracellular peptidases acting on the oligopeptides derived from partially hydrolyzed caseins. [19].

The production of specific flavor profiles depends strongly on the conversion of amino acids into various alcohols, aldehydes, acids, esters and sulfur compounds. As the formation of aromatic compounds is highly dependent on the enzymes present in various strains of LAB, comparative genomics analyses have been undertaken with the goal of producing starter cultures with predictable flavor-forming metabolic pathways. [18].

Citrate can be found in fermentable food products such as fruit, vegetables, and bovine milk. Citrate is broken down by microbes into substances like diacetyl, acetoin, butanediol, and acetaldehyde, all of which can significantly alter the scent of fermented food products. *Leuconostoc mesenteroides* and *L. lactis* are two of the few LAB species that can use citrate. Using citrate results in increased pyruvate intracellular pools and a variety of metabolites produced. Pyruvate is then dissipated, resulting in the creation of C4 aroma molecules such diacetyl, acetoin, and 2,3-butanediol. [18].

Interestingly, citrate metabolism was also shown recently to be correlated with the production of aroma compounds from amino acids. The amino acid transamination is combined with the glutamate conversion of α-ketoglutarate, which gives fermented foods like fish sauce their umami flavor. The conversion of threonine, a special aroma precursor, into aroma molecules depends on both transamination pathways and other metabolic processes. Yogurt starter cultures use an enzyme with threonine aldolase (TA) activity, a member of the lyase enzyme family, to directly convert threonine into acetaldehyde and glycine. As the starter cultures used to make yogurt do not include alcohol dehydrogenase, the enzyme that transforms acetaldehyde into ethanol, acetaldehyde is thought to be the predominant fragrance component in yogurt. [20].

4 Pickling

Depending on the season and what's available, many different kinds of fruit and vegetables are pickled. Although pickling is used more for the preservation of vegetables and fruits, there are several examples where this type of preservation is also used for the

preservation of meat and meat products. Pickling can be done in two ways: anaerobic fermentation in brine and immersion in vinegar. Although there are two ways of pickling, anaerobic fermentation is much more often followed by immersion in vinegar with the addition of antimicrobial herbs and spices, which further increase the taste and preserve the nutritional value of the food.

Depending on the nature of the fresh vegetable and the desired outcome, vegetables can be treated in a different way prior to fermentation. Washing, chopping, peeling, blanching, and exposure to NaOH solution are some examples of prebrining treatments. Acid content, pH value, salt concentration, temperature, natural inhibitory chemicals found in vegetables, chemical additions, sugar content, redox potential, and nutrient availability in the brine are all factors that influence fermentation.

4.1 Anaerobic Fermentation in Brine

Brining can be done in two ways: dry brining or wet brining. Dry brining includes coating the meat in coarse dry salt and allowing it to rest for several hours. Wet brining includes immersion of foods in a solution of sodium chloride with a concentration of 3–6% by weight, sometimes higher concentrations of sodium chloride solutions (up to 20%) are also used depending on the type of food.[21] Brine can also include addition of sugars, spices and flavorings.[21, 22] This process can take place from couple of hours to days. Salting reduces the water activity which in return reduces the growth of microorganisms.[23] In order to preserve fermented vegetables, salt, particularly NaCl, plays four key roles: (a) it affects the type and intensity of microbial activity; (b) it prevents vegetable tissue from becoming too soft; (c) it determines the flavor of the finished product; and (d) it aids in rupturing fruit membranes, allowing the diffusion of different ingredients into the cover brine solutions used by microbes for growth and metabolic activities.[24] Commercial processors use fermentation in NaCI brines to temporarily preserve around 40% of the crop. Fermentable carbohydrates are transformed into lactic and acetic acids, ethanol, CO_2, and traces of other chompounds by naturally occurring lactic acid bacteria and yeast during this process. For fermentation, which can take 10 to 21 days, open-top wood, fiberglass, or polyethylene tanks up to 76 m^3 in capacity are typically used. Storage times (in the same tank) are typically less than a year, but they can occasionally be longer. The tanks are kept outside so that sunlight's UV rays can hit the brine surface and prevent the growth of mold, yeast, and other oxidative microorganisms. Also, closed containers have been developed for this purpose, which avoids contamination of brine and dilution of NaCl and thus the consumption of NaCl for fermentation.[25].

The Chemical Changes During Anaerobic Fermentation

Morcos and El- Husseini investigated the chemical changes that occur during anaerobic pickling of cucumber and carrots in fresh brine containing 12% salt (1976). Cucumbers took 9 days to pickle completely, whereas carrots took 15 days. During the early stages of fermentation, total titratable acidity (TTA) increased progressively in the brine solution. On the 15th day, a pH of 3.0 was achieved. The acidity then proceeded to drop, eventually reaching a pH of 3.4 at the end of the fermentation time (27 days). The brine's salt concentration initially at 12%, then decreased to 10.5% after 1 day, and finally to 7.8%

on the 15th day due to osmosis. The salt content remained constant after that. During the first six days of pickling, the concentration of reducing sugars in the brine increased (0.05% on the first day and 0.51% on the sixth). This is consistent with the observed glucose loss from carrot tissue. The amount of reducing sugars gradually dropped as a result of microbial action, reaching 0.024% at the end of the fermentation.

TTA and sugar reduction were higher when giant carrots were used than when small carrots were used. Cucumber TTA grew to 0.86% by the 11th day after fermentation with a used brine starter, then steadily dropped due to acid breakdown by nonlactic acid bacteria.

4.2 Immersion in Vinegar

Vegetables are rinsed to eliminate excess salt before being packaged in a variety of containers (plastic pails, pouches, or jars) with appropriate cover liquor prior to sale. In addition to leftover lactic acid, the cover liquor generally contains acetic acid and spices. The most common acids are lactic and acetic acids, but other organic acids such as succinic, malic, and propionic acids can also be generated in small amounts. Ethanol, mannitol, CO_2, and other chemicals are created in varying proportions as a result of heterofermentative LAB metabolism. Acetic acid is often used as an acidulant in concentrations ranging from 0.5 to 2% Vinegar's preservation activity is related to its acetic acid component, but it is not merely a pH impact. The unionized, lipophilic molecule is related with activity because it can enter the cell membrane, interrupting membrane transport processes and dissociating within the cell to increase acidity and produce lethal quantities of the anion. Aside from being a preservative, acetic acid is also a flavour and acidulant. Acetic acid is utilized in food preservation in two forms: 5–10% vinegar and 25–80% aqueous synthetic acetic acid solutions. [26].

Antinutritional factors in foods are responsible for the negative consequences associated with vitamin and micronutrient absorption, which may interfere with the function of specific organs. The majority of these antinutritional agents are found in plant-based meals. Thus, the presence of cyanogenic glycosides, protease inhibitors, lectins, tannins, alkaloids, and saponins in foods may cause adverse consequences in humans if consumed in excess. Certain negative effects may also be caused by the degradation products of these substances.

However, some antinutritional substances and their breakdown products may have health benefits when present in modest levels. The method by which food antinutritional factors exert their antinutritional and positive effects is the same.[27].

5 Combined Fermentation Products

Most of fermented products are fermented using either yeast or bacteria. In recent years the fermented drink *kombucha* became very popular as it boasted various health benefits to the consumer. Kombucha is made by fermenting sweetened black tea using a *Symbiotic Culture of Bacteria and Yeast* commonly known as a SCOBY which forms a biofilm on the surface of the liquid. The yeast component of the SCOBY is usually *S. cerevisiae*, whose task is to convert the sugar from the tea to ethanol. The bacterial component almost

always includes *Gluconacetobacter xylinus* to oxidize yeast-produced alcohols to acetic acid (and other acids) [28].

Kombucha is also brewed in households all across the globe alongside its industrial production. Although there is no conclusive proof about the health benefits of drinking kombucha it is advertised to help promote gut health as the bacteria used in its production is said to be probiotic. [29] (Fig. 6).

Fig. 6. Mature kombucha with the SCOBY on the surface. The SCOBY forms on the surface because the acetic acid bacteria in it is aerobic and migrates to the surface in search for oxygen. [30] (Picture taken from Wikipedia; Original Author: Mgarten https://creativecommons.org/licenses/by-sa/3.0/legalcode)

6 Impact of Fermentation on the Nutritional Value of Food

Along with the formation of alcohol, the primary fermentation product, and other compounds which contribute to the overall aroma and sensory experience of a product, changes in the chemical composition of the raw material can greatly impact the nutrition and health of the consumer.

Although there are conflicting reports, the majority of researchers have found that in general the fermentation process reduced most of the basic nutrients in vegetables namely crude ash, crude protein, and fiber content. Nutrient losses during fermentation are related to meeting the lactic acid bacteria's growing nutritional needs as well as their metabolic needs when the fermented plant material serves as a medium for these microorganisms. The degree of protein's digestibility has been found to be greater in fermented food despite their reduced protein content. Pro-teases created by microorganisms during fermentation partially break down and release certain proteins, raising the concentrations of peptides and free amino acids. The bioaccessibility of cystine, histidine, and asparagine has been found to be higher. Also, there have been reports of decreased levels of anti-nutritional substances that promote protein cross-linking (such as phenolic and tannin compounds) and block digestive enzymes (such as trypsin and chymotrypsin inhibitors) [31].

Phytic acid which is abundant in legumes, pseudocereals, pollen and nuts (1–5% w/w) is an important compound which plants use to store phosphorus. However, phytic acid is deprotonated to the phytate ion at physiological pH which gives it a negative charge and therefore a strong ability to chelate minerals and other trace elements. The formed complexes are highly stable, insoluble, and difficult to digest which greatly diminishes their bioavailability. Along with its ability to chelate metal ions phytic acid can also form complexes with proteins and resist their proteolysis. This interaction is highly dependent on the pH but in general, the presence of phytic acid negatively impacts the digestibility of protein. [32] Phytases are naturally occuring enzymes that are usually present in phytic acid containing grains and legumes. Their activation recquires a pH between 5 and 6. Considering the lactic acid produced during fermentation lowers the pH of the system significantly, studies have shown that the environment achieved during fermentation provides the necessary conditions for the enzymatic degradation of phytic acid and increased bioaccessibility of minerals and other micronutrients. [33].

Very high concentrations of sodium were found in fermented vegetables, which was linked to the traditional fermentation method's use of salt. The consumption of fermented vegetables by humans is restricted due to their high salt content. On the other hand, salt acts as a flavor enhancer and improves the palatability of certain food products. In comparison to the raw material, the fermented vegetables had lower amounts of the majority of macroelements. Decrease in amounts of heavy metals like Cu, Pb, and Cd—which are generally challenging to remove from plant material by culinary processing—was one of the positive effects of vegetable fermentation. The most likely cause of this occurrence is metal adhering to the cell walls of fermentation-producing bacteria and fungi. [31].

Researchers have reported variable effects of fermentation of vegetables on the level of vitamin A and β-carotene. They emphasize that changes in the β-carotene content during fermentation are highly variable, depending on the plant material and fermentation conditions. Some authors report an increase in amounts of volatile β-carotene derivates e.g. β-ionone, β-cyclocitral, α-ionone, and β-damascenone, which gradually increased during the fermentation process. β-damascenon is characterized by a fruity and floral aroma, which significantly improves the flavor and aroma of fermented vegetables. An almost 70% increase in the carotene content in fermented tomatoes was reported by Bartkiene et al. Some researchers hypothesize that the increase in carotene levels can be explained by structural changes in the plant material during fermentation making extraction more efficient. [34].

In meals generated from plants that have undergone lactic acid fermentation, varying levels of antioxidant activity have been noted. This is likely due to the release of bioactive molecules that have conjugate phytochemicals like phenols. However, lactobacilli's metabolism of phenolic compounds during food fermentation is still not well understood. [35] The levels of these compounds in fermented vegetables are most often reduced, which is also accompanied by a decline in the content of flavonoids. In Syzygium cumini L. fruit juice fermented by Lb. Paracasei strain HII01, Sirilun et al. reported an increase in the total phenolic content and the antioxidant activity, and Bujna et al. noticed an increase in the amount of phenols and antioxidant activity in apricot juice. Vegetable phenolic compounds have a well-known functional value, but how well they are

absorbed in the digestive tract affects their beneficial effect on human health. Esterases, which hydrolyze chlorogenic acid to release caffeic acid, are not present in human or animal tissues or biological fluids. Only the gut microbiome does this activity. Chlorogenic acid is not as effectively absorbed in the stomach and small intestine as free caffeic acid. Dihydrocaffeic acid exhibits high bioaccessibility and better antioxidant properties than its precursor, i.e. caffeic acid. Therefore, through bacterial bioconversion, fermented vegetables are enriched with phenolic derivatives with increased bioaccessibility to humans and animals. [36].

7 Conclusion

In recent years, more so with the era of the COVID-19 pandemic, people have sought out ways to improve their health and be more mindful of their nutrition. A well balanced and varied diet can supply the human body with the necessary fuel, cell building blocks as well as additional supplements that help it fight off free radicals and external stressors.

As we have showcased throughout this paper, fermentation is an effective way to preserve easily perishable food as well as enrich it with flavor and aroma not present in the raw materials. The reason for it lies in the complexity of food as a matrix and all the different ways the components can interact. Although the microorganisms without which fermentation cannot occur deplete the raw materials of some nutrients to sustain themselves, they also give back in the way they eliminate anti-nutrients and make vital trace elements more bioavailable.

After summarizing all of the possible pros and cons, one can draw the conclusion that fermented and pickled food is a beneficial addition to a person's diet as it allows consummation of food for a longer period of time and out of season as well as providing a source of nutrients and supplements not present in the unfermented raw material.

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Encapsulation and Its Possible Application in the Technology of Ready-Made and Ready to Eat Food - A Review

Jasmina Tahmaz(✉)

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
j.tahmaz@ppf.unsa.ba

Abstract. Encapsulation is the process of entrapping or packaging active compounds into the structure of coating or carrier material. Each capsule contains two main materials: 1) carrier/matrix or coating material and 2) encapsulated active compound. The main purpose of encapsulation is protection of the active compound. Commonly used coating materials are different hydrocolloids (polysaccharides or proteins) like starch, cellulose, alginate, carrageenan, casein, gelatine etc. Encapsulation is widely used in the pharmaceutical industry.

Nowadays, encapsulation has an increasing application in food technology, mostly in bakery, functional food and beverages production. Encapsulation in ready-made and ready to eat food is still poorly researched. But in recent times, many examples for encapsulation in ready-made food technology can be found for research and practical purposes.

The common encapsulated compounds used in ready-made food are: essential oils, vitamins, fish oil, probiotics, folic acid and beta carotene. Nanocapsules and microcapsules with encapsulated active compounds can be added to improve nutritional or sensorial quality and shelf life of different ready-made foods like instant soups, mayonnaise, sauces, dressings, meat or fish burgers, tortillas, spicy and seasoning powders and dried mixtures for different dishes etc.

There are two main ways of applying encapsulation in ready-made/ready to eat food. The common way is encapsulation of sensitive active compounds and addition of prepared capsules to the ready-made food product during its production. Another way of application implies encapsulation of liquid sauces and spice extracts into macrospheres and macrocapsules.

Keywords: encapsulation · soups · mayonnaise · sauces and dressings · ready-made/ready to eat food

1 Introduction

Encapsulation is a technique of packing or entrapping active compounds into the structure of coating material. It is mostly used in the pharmaceutical, and recently in the food industry too. Most commonly encapsulated active materials in the food industry are vitamins, minerals, enzymes, probiotics, antioxidants, omega fatty acids, fish oil, essential oils and aromatic substances.

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Encapsulation is a technique that can improve the stability, usability and availability of many bioactive ingredients. The main aim of encapsulation is the formation of stable capsules from natural or synthetic polymer materials with encapsulated active substances. Each capsule consists of two main parts [1]:

- coating (wall material, carrier, shell or encapsulation matrix) and
- active compound (encapsulant, internal phase or capsule core).

The active compound is the material that is encapsulated inside the capsule. The coating (carrier/shell/matrix) is the outside material which coats the encapsulated active substance and protects it from degradation influenced by external factors. The role of the carrier/coating material is to create a boundary layer between the active substance and the environment.

2 The Main Fact About Encapsulation

The largest number of encapsulation techniques is based on dispersing very small particles of the active substance together with the particles of the encapsulation carrier, which is dissolved in the liquid phase [2]. The key factor for successful encapsulation is achieving as large a contact surface as possible between the particles of the coating material and active substance. This is the only way to make it possible to obtain completely coated active compounds. Most encapsulation techniques are based on fine dispersion of the active substance solution in the carrier solution, and this is commonly achieved by following techniques: spraying (or spray drying), slow dosing (drop-by-drop), emulsification, energetic homogenization (e.g. ultrasonic or membrane homogenization), application of high pressure, extrusion, etc. An exception to this rule is the encapsulation by co-extrusion. During co-extrusion the contact of the active substance and the carrier is achieved by flowing through concentric nozzles under high pressure. The active substance passes through the inner nozzle, while the coating material passes through the outer nozzle and wraps around the active substance at the exit of the extruder. Spray drying is the earliest and the most applied encapsulation method in the food industry [1, 3].

2.1 Capsule Types

Considering the structure of the capsule and the distribution of coating around the active substance, there are three basic types of capsules [1, 2]:

1. Aggregate, matrix or microsphere. Matrix type capsules have several separated cores of the active substance embedded within a continuously distributed matrix or carrier. This is the most common type of capsule formation. In the matrix types, active compounds can be found on the capsule surface. Therefore, this is the least efficient way of encapsulation. The example of encapsulation mechanism into matrix capsule is illustrated in Fig. 1.
2. Type of central accumulation. It is the simplest and most obvious form of capsule structure. The core with the encapsulated active compound is spherical in shape, surrounded by a shell or membrane.

3. Multilayer coating type. The coating is distributed around the core in the form of two or more different layers. Such capsules are designed to allow controlled release of the active substance.

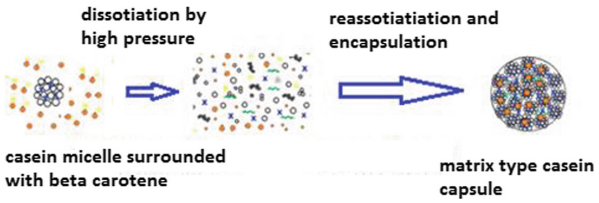

Fig. 1. Illustration of formation of matrix type casein capsule with beta carotene [4]

2.2 Coating Materials

The first step during encapsulation planning is the selection of the coating material. Various natural or synthetic materials can be used as carriers/coating materials [1], but food compatible biopolymers are commonly used, e.g. polysaccharides and other hydrocolloids, proteins, gums, etc. [5]. The choice of carrier depends on different factors such as the nature of the active substance, encapsulation method and the type and purpose of the capsule as the end product. The success of the encapsulation and the physical properties of the capsules mostly depend on the choice of the encapsulation carrier. Edible and harmless biopolymers, which do not react with the active substance, are commonly used as carriers. In ideal cases, the carrier materials should meet the following criteria [1]:

- good rheological properties,
- good dispersibility and solubility in edible solvents (water, oil or alcohol);
- ability to protect encapsulated substance from physical or chemical changes in the contact with external factors (oxidation, heat, pH value, light, humidity),
- ability to protect and retain the encapsulated substance during different processing and storage conditions; and
- to be harmless, compatible with food and approved by the FDA and FSA.

Commonly, it is impossible to find a material that would meet all of the required criteria. Because of that, coatings are often mixed or combined with different modifying agents with the purpose to improve the function of the matrix [6–8]. The encapsulation matrix should have the ability to form a cohesive film that adheres to the particles of the active substance, without reacting with them. In order to improve their properties, the materials used as encapsulation carriers often undergo different physical or chemical modifications [8, 9].

2.3 Benefits of Encapsulation

Protection of the active substance from external/environmental influences, i.e. from degradation influenced by external factors (heat, oxidation, moisture, air, light, chemical

ingredients) is the main reason for encapsulation. Encapsulated compounds can be added to different foods with the aim to enrich them with nutritionally valuable ingredients (vitamins, minerals, omega fatty acids), which are often sensitive and easily decomposed. Encapsulation can also modify the physical characteristics and structure of the original food products, which makes it possible to achieve easier handling, mixing or solvation [5]. E.g. encapsulated liquid substances can be added into solid materials. Addition of encapsulated compounds can improve the texture and rheological properties of the products. By using encapsulation it is also possible to prevent the formation of lumps, reduce hygroscopicity, improve flowability, and modify the consistency of different food products [4, 10, 11]. For example small nanocapsules and microcapsules commonly have a good dispersibility. Because of their improved dispersibility, the small capsules could be added to the products with soft structure like foam drinks and cakes, ice cream blends etc. On the other hand, microcapsules and aggregates commonly have improved hydration ability, higher swelling power and voluminosity. After addition of large capsules into cream soups and sauces, the viscosity could be increased and thicker consistency could be obtained [4].

Another benefit of encapsulation is controlled release of the active substance (gradual, slow or at the exact desired moment). The release of the encapsulated active substance in the product is slower in comparison to non-encapsulated one. For example, by encapsulating the sweetener, a gradual release of the sweet taste can be achieved. By encapsulation it is possible to achieve uniform distribution of the active substance. Encapsulation can dilute the concentration of the active compound, which is very significant in the cases when it is necessary to obtain uniform mixing of very low concentrations of the active substance in a large amount of final product. In some cases the active compounds have undesirable or unpleasant flavor and taste and for that reason those substances should be added in encapsulated form. Encapsulation can mask unwanted or unpleasant taste, smell and aroma (e.g. encapsulated fish oil, bitter substances, essential oils etc.) Besides, encapsulation can prevent unwanted reactions between active compound and ingredients in the products [5, 10, 11].

3 Encapsulation in Ready-Made and Ready to Eat Food

Ready-made food represents a very wide group of food products, including salads, soups, sauces, omelets, pies, sandwiches, snack products, seasonings, spice blends and ready-made meals (main and side dishes). Many of these products are produced by intensive thermal culinary processing methods such as frying, baking and boiling. Prepared ready to eat food can be preserved by different techniques like dehydration, freezing and thermal canning (pasteurization and sterilization). Many of these processes have a destructive effect for sensitive bioactive substances and aromas. Because of that encapsulation could be a promising method to protect these sensitive substances during thermal processes.

On the other hand, as a part of ready to eat foods, there are some products which don't require heating treatments (salads, mayonnaise and related products, sauces and dressings). Encapsulated bioactive compounds could be added to these products with the aim of fortification and nutritional quality enhancement. The common characteristic of these products is low pH value, mostly under 4.5. Some bioactive compounds like probiotics

and enzymes are sensitive in the condition with low pH value. Encapsulation can protect them from degradation and protein denaturation under low pH value. On the other hand, in some cases addition of encapsulated ingredients can modify the consistency and the texture (by increased hydration and voluminosity) of the final product.

In the ready-made food products, encapsulated active compounds could have the most promising application in the technology of mayonnaise, sauces, dehydrated mixtures for soups, sauces and seasonings, minced meat products, breaded and fried products (e.g. fish, chicken and meat burgers) etc. Encapsulated omega fatty acids, vitamins, probiotics, additives, antioxidants, colors, essential oils and flavors could be used in ready-made food technology with the aim to enhance nutritional value of final products.

Ready-made foods, that normally don't contain omega fatty acids (dehydrated soups, breaded chicken products, mayonnaise and other products), can be enriched by addition of encapsulated omega fatty acids. The stability of encapsulated omega fatty acids in frozen chicken nuggets was extremely high and commonly changes in sensory properties didn't occur during the freeze storage [12]. Ready-made food can also be enriched with encapsulated oils. According to Rubillar et al. [13] dehydrated cream soup with encapsulated fish oil had acceptable sensory properties, because the encapsulation masks the undesirable smell and aroma of fish oil. Microencapsulated olive oil or a mixture of olive oil and lemon juice can be added to instant salad dressings [14].

Instead of final product fortification, encapsulation can provide modification of the product texture since obtained microcapsules have typical and improved physical properties such as dispersibility, wettability, hydration, voluminosity, swelling power etc. Generally, smaller capsules have better dispersibility, solubility and wettability, while larger microcapsules (as casein aggregates) have higher swelling power, voluminosity, water binding capacity and higher viscosity. It was suggested that small capsules of casein with beta carotene obtained by high hydrostatic pressure probably could be added to products which need to have better dispersibility. On the other hand, large microcapsules could be added to the products which should have thicker consistency, like cream soups, sauces and gravies [4].

Examples of application of encapsulated compounds in different types of ready-made and ready to eat food are given in Tables 1, 2, 3 and 4.

3.1 Soups

The examples of possible applications of encapsulated compounds in soups are given in Table 1.

Microencapsulated linseed oil (rich in omega 3 fatty acids) was added to a soup powder with the aim to fortify it with omega 3 fatty acids. Prepared soup samples with encapsulated linseed oil had good sensory properties and the shelf life of the soup was about 8 months [13].

Hastarini et al. [15] encapsulated catfish oil and the obtained microcapsules were added to the instant mushroom cream soup. The aim of encapsulation was to provide enrichment of instant soup with unsaturated omega 3 fatty acids from fish oil without a negative effect on taste, smell, aroma and other sensory properties. Since fish oil has a specific, intense and unpleasant smell, it is suggested to be added in encapsulated form to avoid influence on product taste. Commonly mushroom cream soup doesn't

Table 1. Examples of encapsulation applied in preparation of different soups

Soup	Active compound/	Coating material	Encapsulation method	Achieved effect
Dehydrated cream soup	Linseed oil	Gum arabic and maltodextrin	Spray drying	Improved oxidative stability of omega 3 fatty acids [13]
Mushroom cream soup	Catfish oil	Maltodextrin	Spray dying	Fortification with omega 3 fatty acids without negative effect on sensory properties, masking of fish oil taste [15]
Instant soup mix	Fish oil	Skimmed milk powder	Emulsification and spray drying	Improved oxidative stability of omega 3 fatty acids, acceptable sensory properties, masking fish oil taste [16]
Commercial soup	*Zataria multiflora* essential oil	*S. cerevisiae*	Freeze drying	Enhanced antimicrobial effect against phatogenic bacteria, masked essential oil flavor, acceptable sensory properties [17]
Bone broth powder	Bone broth extract	Maltodextrin and whey protein isolates	Emulsification and spray drying	Good rheological and sensory properties [18]
Chicken soup	Garlic extract	Maltodextrin	Oven and freeze drying	Improved antioxidative properties [19]

contain omega-3-fatty acids, hence the interest to enrich it with encapsulated catfish oil. With the addition of catfish oil microcapsules, the soup powder had modified physical properties. Water absorption index and water solubility were decreased, but the lightness was increased. Moisture content decreased and fat content increased.

Maryam et al. [17] added encapsulated essential oil of *Zataria multiflora* to chicken commercial soup powder and obtained the following results. *Zataria multiflora* essential oil showed high antioxidative and antimicrobial properties. Commercial soup with addition of encapsulated oil had lower microbial growth for *E. coli* and *Listeria monocytogenes*. Since the essential oils have a very strong taste and flavor, it is suggested that they should be added to the product in encapsulated form to avoid the influences to sensory properties. The commercial chicken soup samples with encapsulated essential oil had acceptable sensory properties with slightly higher scores in comparison to the control sample with free essential oil.

Beef bone broth can be encapsulated by spray drying into maltodextrin and whey protein isolates. The obtained encapsulated and spray dried bone broth was freeze-stored at −18 °C for 3 months and tested for storage stability. During the storage period pH value and browning index increased. Bone broth microcapsules loaded with whey protein had lower changes in color, while maltodextrin loaded microcapsules showed better solubility, wettability and dispersibility [18].

Barido et al. [19] encapsulated black garlic extract, which was added to Korean ginseng chicken soup with the aim to improve antioxidative activity. The soup was prepared with chicken broth, chicken meat and black garlic extract encapsulated into maltodextrin microcapsules. Chicken soup with encapsulated black garlic extract had improved antioxidative activity, significantly higher phenolic content and higher presence of yellow and red color in comparison to samples with free garlic extract and control (without garlic extract).

3.2 Burgers and Similar Products

Different encapsulated compounds can be added to fish and meat burgers (Table 2).

Baron-Yusty et al. [20] encapsulated extra virgin olive oil and added it to the breadcrumbs which were used for breading of chicken nuggets. The main purpose of encapsulation was to produce healthier chicken nuggets enriched with encapsulated olive oil, which were baked without a deep fat frying process. Fat and acrylamide content in olive oil loaded fish burgers decreased by 88% and 55% in comparison to control sample (traditionally deep fried) without negative changes in the sensory properties.

Raeisi et al. [21] encapsulated fish oil and garlic essential oil extract into chitosan microcapsules and investigated shelf life, chemical and sensory properties during 20 days cold storage of chicken nuggets. Results showed that addition of encapsulated fish oil and garlic essential oil extract significantly prevented spoilage and extended shelf life of chicken nuggets. Encapsulated active compounds prevented and delayed lipid oxidation process and inhibited microbial growth. Chicken nuggets with encapsulated compounds had improved sensory properties, because the encapsulation avoided release of undesirable smell and taste of fish oil and garlic extract.

Encapsulated safflower (*Carthamus tinctorius*) oil enriched with acai (*Euterpe oleracea*) extract was used as fat substitute in the experimental preparation of beef burgers. Grilled beef burgers with encapsulated compounds had lower hardness and cooking weight loss, better chewiness, unchanged color and extended shelf life in comparison to control [23].

Table 2. Examples of encapsulation applied in burgers and nuggets preparation

Food product	Active compound	Coating material	Encapsulation method	Achieved effect
Frozen chicken nuggets	Fish oil	Lecithin-chitosan and maltodextrin	Multilayer coating	Protected against lipid and protein oxidation, good sensory quality, higher juiciness and saltiness [12]
Chicken nuggets breadcrumbs	Extra virgin olive oil	α-Cyclodextrin	Molecular inclusion	Reduced fat and acrylamide content, lower microbial growth, longer shelf life, good sensory properties [20]
Chicken nuggets	Fish oil and garlic essential oil	Chitosan	Rotor-stator homogenization and freeze drying	Prevention of lipid oxidation and microbial spoilage, good sensory quality [21]
Beef burger	*Thymus vulgaris* essential oil	Chitosan	o/w emulsification and ionic gelation	Improved antimicrobial stability and sensory properties, inhibition of discoloration [22]
Beef burger	Safflower oil enriched with acai extract	*Amorphophallus konjac* and Na-alginate	Hydrogel emulsion technique	Extended shelf life [23]
Fish burger	Propolis	Gum arabic and modified starch	Spray drying	Improved texture and sensory quality, especially softness, taste and juiciness [24, 25]
Fish burger	Lemon essential oil	Chitosan-modified starch	Emulsification	Reduced oxidation during cold storage and increased shelf life [26]

The common maximal shelf life of fresh beef burger patties is three days at 4 °C. After addition of prickly pear (*Opuntia* spp.) extract encapsulated in alginate beads the shelf life of patty can be extended up to 8 days with minimal changes in sensory properties. Burger patty samples with encapsulated prickly pear extract had lower amounts of mesophilic bacteria, enterobacteriaceae and *Pseudomonas* [27].

Reza et al. [28] reported that fish burger prepared with encapsulated *Ziziphora clinopodioides* essential oil and nisin had improved sensory properties and shelf life during a 20-day-storage period in comparison to control. *Ziziphora clinopodioides* is an edible endemic specie from Iran reach in the phenolic compounds with high antioxidative activity. Encapsulation prevents and slows down the oxidation of phenolic compounds from essential oil.

Fish burgers produced with the addition of nanocapsules of lemon essential oil encapsulated in chitosan/starch mixture had extended shelf life and better quality during 18 days of cold storage. Nanocapsules with essential oil improved antioxidative properties and inhibited lipid oxidation of fish burger during storage. Fish burger with encapsulated lemon oil had better overall acceptability in comparison to control [26].

The addition of microencapsulated propolis can increase the antioxidant activity and improve the sensory properties of fish burgers made from sea bream (*Sparus aurata*). Propolis is a biologically valuable product rich in antioxidants, and because of that there is a great interest to be added in different foods. However, the negative trait of propolis is its very strong, sharp and specific smell. Because of this, it is recommended that propolis should be dosed in an encapsulated form, in which it doesn't affect the sensory properties of the final food product [24, 25].

Antioxidant properties of fish burger can be improved by addition of microcapsules with encapsulated vitamin C. The content of vitamin C in the burger decreases slightly after 60 days of storage and remains about 89% from initial amount. The appearance of the fried fish burger with the addition of encapsulated and free vitamin C was very similar [29].

Table 3. Examples of application of encapsulated compounds in tortillas

Active compound	Coating material	Encapsulation method	Achieved effect
Beta vulgaris juice	Gum arabic	Spray drying	Better stability of antioxidants, acceptable sensory properties [30]
Folic acid and ferrous fumarate	Acetiled maize starch	Extrusion and spray drying	Improved stability during cold storage without negative effect on texture, color and taste [31]

3.3 Tortillas

Tortillas with encapsulated beetroot juice (Table 3) showed about 70% inhibited free radicals and acceptable sensory properties [30].

Microcapsules of iron (II) fumarate and folic acid (Table 3) can be added to nixtamalized corn flour for production of corn tortillas. Addition of microcapsules didn't cause the negative change in sensory properties such as color, texture, taste and flavor. Sensory properties of tortillas enriched with encapsulated compounds were very similar to control samples. The main reason for encapsulation was fortification of tortillas with folic acid and iron, the micronutrients which aren't contained in corn flour in high amounts. Reported results showed that encapsulation provided good retention of encapsulated folic acid and iron (II) fumarate [31].

Addition of encapsulated fumaric acid can extend the shelf life of industrially produced tortillas and slow down the development of molds. It has been proven that tortillas produced with encapsulated fumaric acid have a shelf life of 45 days, while the molds appeared in tortillas with free fumaric acid after 10 days of storage. The main problem with keeping packaged corn tortillas is the sticking of their sheets. In addition to increasing shelf life and preventing the development of molds in tortillas, encapsulated fumaric acid has been proven to reduce the sticking of packed tortillas [32].

3.4 Mayonnaise, Sauces and Dressings

Examples of encapsulated compounds application in mayonnaise and sauces are given in Table 4.

Mayonnaise can be enriched with encapsulated probiotics, omega fatty acids, fish oil and vegetable oils, essential oils, polyphenols, flavonoids, vitamin D, beta carotene and other antioxidants. Due to the presence of acetic acid, mayonnaise has a low pH value (3.6–4.6), which represents an unsuitable environment for probiotics. It is well known that denaturation of proteins occurs in an acidic environment. Free bifidobacteria are very sensitive at pH < 5. Encapsulated bifidobacteria (*B. bifidum* and *B. infantis*) in mayonnaise can survive even after 12 weeks, while free bifidobacteria in mayonnaise drops sharply in the first two weeks. Sensory properties of mayonnaise with encapsulated probiotics are assessed as better than mayonnaise with free probiotics [27, 33, 34].

Encapsulated probiotic bacteria *L. acidophilus* added to mayonnaise sauce had survived longer in comparison to free bacteria. The *L. acidophilus* cells are very sensitive under different factors during processing, and because of that encapsulation could serve as a promising method to protect probiotics and to improve the probiotic activity of the yogurt sauce. Besides the improved probiotic activity, yogurt sauce with encapsulated probiotics had better sensory properties [33]. Encapsulated bacteria *L. acidophilus* and *L. casei* reduce the overall acidity of the mayonnaise-based sauce without significant changes in consistency and rheological properties. After 90 days of refrigerator storage, encapsulated probiotic cells in sauce can remain preserved in an amount corresponding to the probiotic therapeutic minimum [34].

Shaygannia et al. [35] encapsulated phenolic compounds from lemon waste into the coating of basil seed gum and Persian gum. Mayonnaise samples with encapsulated compounds had higher antioxidative activity, delayed spoilage, extended shelf life and acceptable sensory properties.

Besides the probiotics and essential oils, mayonnaise can be enriched with encapsulated oils as a source of polyunsaturated fatty acids. Rojas et al. [36] found that appearance of control mayonnaise sample (without encapsulated oils) was very similar to mayonnaise with addition of chia (*Salvia hispanica*), pumpkin (*Cucurbita pepo*), and baru (*Dipteryx alata*) seed oils encapsulated in a Na-caseinate carrier. Mayonnaise samples with encapsulated oils had almost the same consistency (slightly firmer) as the control mayonnaise. The panelists involved in sensory evaluation mostly could not recognize the differences. All of these oils contain high amounts of polyunsaturated fatty acids. The encapsulation protected oils from oxidation and rancidity, and the obtained mayonnaise could be recognized as a functional product.

Table 4. Examples of application of encapsulated compounds in mayonnaise and sauces

Sauce type	Active compound	Coating material	Encapsulation method	Achieved effect
Mayonnaise	*L. acidophylus*	Ca-alginate and resistant starch	Emulsification	Better survival of probiotics, improved sensory properties [33]
Mayonnaise	*L. acidophilus, L. casei/*	Ca-alginate, resistant starch	Emulsion technique	Better probiotic stability, lower acidity and better sensory properties [34]
Mayonnaise	Phenolic compounds from lemon waste	Persian and basil seed gums	Freeze drying	Enhanced antioxidant and antimicrobial activity, better storage stability [35]
Mayonnaise	Walnut oil	Pectin and maltodextrin	Ultrasound emulsification	Improved shelf life, lower peroxide number and lighter color [37]
Mayonnaise	*L. casei, B. bifidum*	Resistant starch and Ca-alginate	Emulsification	Enhanced survival of probiotics [38]
Salad dressing	Red onion skin antocyanins	Soy protein isolate, apple pectin, Na-carboxymethyl cellulose	Gelation and freeze drying	Better storage stability and antocyanins protection [39]
Yogurt sauce	*L. paracasei*	Resistant starch and Na-alginate	Emulsion technique	Increased survival of probiotics without negative effects on color, consistency, taste and acidity [40]
Cheese sauce	Fig leaves extract *and L. helveticus*	Skimmed milk and sodium alginate	Freeze drying	Improved shelf life and stability against yeasts and molds, better melting and sensory properties [41]
Soy sauce	*Z. rouxii, T. halophylus*	Alginate	W1/O/W2 emulsification	Enhanced soy sauce aroma development during moromi fermentation [42]

Encapsulated *L. paracasei* with resistant starch and Na alginate were added to yogurt sauce. In comparison to control, yogurt sauce prepared with encapsulated probiotic bacteria had higher viscosity, better sensory properties and better survival of probiotics during 30 days of storage [40].

El-Sayed et al. [41] prepared the cheese sauce with encapsulated fig (*Ficus carica*) leaf extract. Fig leaf extract is rich in phenolic compounds and has a strong antioxidative and antimicrobial activity. Encapsulation protected phenolic compounds from oxidative

degradation and enriched cheese sauce had improved antioxidative and antimicrobial properties during 50 days of storage.

Addition of encapsulated *Z. rouxii, T. halophylus* in production of soy sauce can improve formation of aromatic compounds during fermentation of moromi [42].

Olive oil and lemon juice based salad dressing was encapsulated by freeze drying into different coating materials (maltodextrin, alginate, gum arabic and carboxymethyl cellulose) by spray dying. This technology allowed the production of encapsulated salad dressing in dried powdered form which can be stored at room temperature. Microcapsules had rounded shapes and could be easily reconstructed [14].

3.5 Macrocapsules with Encapsulated Sauces and Spice Extracts

Besides micro and nanocapsules, nowadays it is a very popular trend related to the production of macrocapsules with encapsulated liquid sauces, spicy extract, soy sauce, balsamic vinegar, liquid spice mixtures of spice extracts in olive oil, etc. Many of these products are produced commercially and can be found on the market in some countries.

These products are mostly alginate macrocapsules with encapsulated liquid spice extracts stabilized with addition of $CaCl_2$ (E 509). $CaCl_2$ enables the creation of a more rigid and compact structure of alginate coating. The mentioned products have a minimal shelf life of one month after opening the container and must be stored in a refrigerator at temperatures below 6 °C. Due to the appearance of these microcapsules, this product is sold under the commercial name "*Caviaroli*". Encapsulated soy sauce in the form of alginate macrocapsules is also commercially available (brand *Caviaroli*), and it is recommended to be served with fresh or cooked vegetables and raw fish dishes such as sushi [43, 44].

De Farias and Norena [45] encapsulated light soy sauce inside Na alginate macrocapsules. Soy sauce was mixed with different concentrations of xanthan gum before encapsulation. Xanthan gum was used for the same reason as $CaCl_2$ (E 509) in "*Caviaroli*" macrocapsules to create a firmer structure of the encapsulated liquid. Encapsulation was performed by ultrasonic homogenization of soy sauce with xanthan gum and Ca-lactate and freezing it (at −60 °C, 30 min). Before freezing, the homogenized mixture was dosed into acrylic molds with holes of size 5 cm. The frozen balls of soy sauce were coated by immersion in Na alginate solution (for 15 min at room temperature) and with constant stirring at a speed of 200 rpm. Capsules with a higher concentration of xanthan gum had a darker color and a more fibrous structure.

Balsamic vinegar can be encapsulated into macrocapsules of gellan gum. It is believed that gellan gum coating has better properties than Na alginate, related to its ability to form attractive smooth surfaces and allow better aroma release [46].

Different ways of serving and adding to ready-made food could be suggested, such as e.g. addition to the tomato salad, serving on the ready to eat meat surface or could be simply mixed to liquid ready to eat food such as soups and sauces.

4 Conclusion

Encapsulation can be assessed as a process with promising application in technology of ready to eat food. Encapsulated active compounds could be added to different ready-made food for the purpose of fortification. As encapsulation is a relatively new process for applying in the food industry, its benefits and commercialization have not been completely researched. In recent times, the possibilities of applying encapsulation in food technology have been increasingly explored, especially in the area of ready to eat food products such as sauces, soups, burgers and similar products. The most publications related to encapsulation applying in ready to eat food dated to the period from 2013th to 2022nd. A significant number of papers, closely related to this topic, is published in a very short time, more precisely in the last 5 years (about 20 papers from 2018 to 2022).

In the preparation of instant soups, the mostly used encapsulated compounds are: plant extract with high antioxidative properties, oils rich in omega 3 fatty acids (fish oil, linseed oil) and different essential oils with high antioxidative and antimicrobial properties. Encapsulated compounds are mostly added to mayonnaise, sauces and dressings are: probiotic bacteria, omega-3 fatty acids, fish oil and essential oils. The commonly used encapsulation technique in instant soup production is spray drying, while in mayonnaise and sauce production the most common encapsulation technique is emulsification.

Although encapsulation is a promising process with numerous advantages and benefits, it is still not used enough in the food industry. The main reasons for weak use of encapsulation in the industry of ready-made food are: high costs and complications related to high-scale industrial application, as well as poorly explored benefits and possibilities. After summarizing all encapsulation's advantages and disadvantages, it becomes clear that encapsulation will continue to develop with the aim of being applied to ready-made food as well as other food industries. Encapsulation techniques which are easy to apply and for which industrial equipment already exists (or is not expensive) would have the greatest perspective for more significant application in the ready-made food industry. Examples of such techniques are spray drying, freeze drying (lyophilization) and co-extrusion. For example, encapsulation by spray drying and freeze drying could be used in the industry of powdered ready to eat food such as instant soups, powdered seasonings mixtures, dehydrated mashed potatoes and dehydrated ready to eat meals. Co-extrusion is already used for industrial production of capsules with encapsulated sauces, balsamic vinegar or spice extracts.

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Green Synthesis of Silver Nanoparticles from *Mentha Piperita* Extract and Their Antimicrobial Activity

Asima Buljina, Amina Stambolić(✉), Saud Hamidović, and Enisa Mikličanin-Omanović

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
{asima.buljina,a.stambolic}@ppf.unsa.ba

Abstract. Recently, we are increasingly growing interest in nanotechnology, which has developed over the years and based on which research has been conducted in various fields, from medicine to the food industry.

Green synthesis involves the use of an acceptable solvent, an environmentally friendly reducing agent and the selection of non-toxic substances to stabilize the synthesized nanoparticles.

Increasing interest is related to silver nanoparticles and their antimicrobial activity.

This work presents the results of the synthesis of silver nanoparticles from extract of *Mentha piperita* and their antimicrobial activity towards the bacteria Escherichia coli and Salmonella. The extract of Mentha piperita was used because of her wide pharmacy application, including the shampoo and fragnance industry to the confectionary and food industry, as well as her medicinal effect on the human body.

Keywords: silver nanoparticles · antimicrobial activity · Mentha piperita

1 Introduction

Concern about the harmful effects of pathogenic bacteria, is growing every day. That is the driving force behind researches which are related to the suppression of those bacteria. Because of the negative effects of bacteria like *Escherichia Coli* and *Salmonella sp.*, the need for antibiotics is increasing. This research on silver nanoparticles was carried out in order to find new ways of suppression of those types of bacteria. Silver nanoparticles have antibiotic properties, so they can be used to make new types of bactericidal agents. Because of antibiotic properties, silver nanoparticles can stop cell growth or cause cell death. They have the ability to bind to the bacterial cell wall and then penetrate into the cell. In this way they make structural changes in the cell membrane, such as cell membrane permeability or cell death. Silver nanoparticles accumulate on the cell surface by creating „holes“ on the cell surface [1] They also can generate free radicals in interaction with bacteria, so the free radicals have the ability to damage the

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cell membrane and make it porous, which can ultimately lead to cell death [2] Silver nanoparticles have pronounced antibacterial, antifungal, antiviral and anti-inflammatory properties, so in the combination with antibiotics such as penicillin G, amoxicillin, erythromycin, clindamycin or vancomycin, they enhance the effect of these antibiotics in the treatment of infections caused by resistant *Staphylococcus aureus* and *Escherichia coli* bacteria [3].

Bacteria that have been used to synthesize silver nanoparticles are given in Table 1.

Table 1. Silver nanoparticle synthesizing bacteria and the size of the resulting nanopraticles

S. no	Bacteria	Size (nm)	Author (year)
1	*P. stutzeri AG259*	200	(Tanja et al., 1999)
2	*Bacillus megaterium*	46,9	(Fu et al., 1999)
3	*Plectonema borianum*	1–200	(Lengke et al., 2007)
4	*Enterobacter cloacae*	50–100	(Minaeian et al., 2008)
5	*Escherichia coli*	5–25	(El-Shanshoury et al., 2011)
6	*B. licheniformis*	50	(Kalimuthu et al., 2008)
7	*Lactobacillus fermentum*	11,2	(Sintubin et al., 2009)
8	*Klebsiella pneumonja*	50	(Mokhtari et al., 2009)
9	*Proteus mirabilis*	10–20	(Samadi et al., 2009)
10	*Brevibacterium casei*	50	(Kalishwaralal et al., 2010)

The sience that studies the properties of nanoparticles is called nanoscience. Nanoscience has significantly evolved in the last few years, from science that was exclusively developed in the laboratory to its application in technology, like nanotechnology [4]. The term nanotechnology comes from the Greek word “nanos” which means “dwarf”, and denotes a unit which is a billion times smaller than the base unit [5]. The other definition of “nanotechnology” includes the research and development of technologies and new properties of structures with dimensions on the nanoscale [6, 7].

Nanotechnology implies the application of science and technology which means design, synthesis and manipulation of structures in the range from 1 to a 100 nm. It has been implemented in almost all areas of human activity. Besides, nanotechnology has a major role in alternative medicine for the production of nanominerals, like nanosilver or nanogold as they are natural antibiotics. Nanotechnology also means application of nanomaterials. Nanomaterials are made of nanoparticles and they are used from the cosmetic to the food industry, especially in the industry improvement, food packaging, increasing the shelf life and bioavailability of different food.

There is the concept of „green synthesis“ which is based on the principle of green chemistry. Green synthesis was developet due to concern about environmental protection and contribution to sustainable development. The principles of green chemistry refer to the prevention of waste generation, finding new solutions for obtaining ecologically

harmless products, which also means "not toxic to the environment and not toxic to people" products [8] and finding new "environmentally non toxic" materials [9].

Because of that, we can say that green synthesis represents environmentally friendly synthesis. President of the American Chemical Saciety, Daryle Busch, said "Green chemistry represents the pillars that hold up our sustrainable future. It is imperative to teach the value of green chemistry to tomorrow's chemicts" [10].

This paper describes the green synthesis of silver nanoparticles from *Mentha piperita* extract, because of its antimibrobial activity and possibility of getting silver nanoparticles. Effect of silver nanoparticles synthesized like this on the growth of bacteria like *Escherichia coli* and *Salmonella sp.* was studied.

2 Method and Materials

Green synthesis of silver nanoparticles from *Mentha piperita* extract was conducted in the laboratory for chemistry and biochemistry in the University of Sarajevo, Faculty of Agriculture and Food Sciences.

2.1 Plant Extract Preparation and Silver Nanoparticles Synthesis

The herbal extract of *Mentha piperita* was prepared using the dried plant material acquired through commercial sources. The aqueous extract was acquired by boiling 5 g of the dried herb in 50 ml of MiliQ water for 30 min and filtering using blue ribbon filter paper. Three samples of silver nanoparticles were synthesized by mixing aliquots of *Mentha piperita* extract (150 μl, 250 μl, and 350 μl) and 10 ml of 0.1 mM $AgNO_3$ solution. The reaction mixtures were stirred using a magnetic rod for 2 h and incubated at room temperature for 24 h. Silver nanoparticle synthesis was confirmed using UV/Vis spectroscopy. The spectra were collected in the range from 300 nm to 600 nm against MiliQ water as a blank.

2.2 Anti-microbial Activity of Silver Nanoparticles

Anti-microbial activity of the synthesized nanoparticles was determined against pure bacterial cultures of *Escherichia coli* and *Salmonella sp.* on a Mac Conkey agar nutrient medium. The nutrient medium was prepared according to the instructions on the packaging. All the dishes and utensils were sterilized at 120 °C beforehand in an autoclave for 15 min at 1,5 atm.

For nutrient medium preparation, 10,4 g of Mac Conkey agar was added to 200 ml of distilled water and homogenized. After that, it was heated in the microwave until boiling. The nutrient medium was poured into sterilized Petri dishes and left to solidify at room temperature.

2.3 Inoculum Preparation

Preparing the inoculum was carried out according to the EUCAST (The European Comittee on Antimicrobial Susceptibility Testing, 2017). The microorganisms were transferred

from their growth plate to a test tube containing 5 ml of saline solution using a sterilized inoculation loop to achieve a homogenous turbidity of 0,5 McFarland standard and concentration of microbial cells of 1x108 CFU/ml.

The prepared inoculum were incubated at 37 °C for 24 h. The bacteria were then smeared using a swab across the surface of the agar for antimicrobial activity testing.

2.4 Disc Difusion Method

For antimicrobial activity assesment of the prepared nanoparticles, the disc difusion method, also known as the Kirby-Bauer method, was used. The method consists of placing a disk impregnated with the sample being tested on the agar inoculated with bacteria [11] The agar is then incubated for 16 do 24 h at 37 °C. If there is an inhibitory effect on the growth of microorganisms it manifests itself as a transparent disc formed around the discs [12].

Bactera sensitivity was determined by measuring the diameter of the inhibition zone using a ruler. Results are usually expressed in millimeters.

Based on the measured diameters of the inhibition zone, the examined species are classified into the following categories:

S – sensitive - probability of therapy success is high after using the usual doses of antibiotics which were given in the usual way;

I - intermediate (moderately) sensitive - possible therapy success if the antibiotic was given in the maximum concentrations and by parenteral route;

R – resistant - never used in the therapy; no matter of dose, therapy will be probably unsuccessful.

3 Results and Discussion

3.1 Nanoparticle Characterization

The synthesized silver nanoparticles were characterized by UV/Vis spectroscopy using a Varian Cary 1E Spectrometer. The spectra were collected in the range from 300 to 600 nm. Previous research literature places the wavelength of maximum absorption for silver nanoparticles in the range from 410 to 450 nm [13] (Fig. 1).

The λ_{max} of the recorded samples was 414 nm which in accordance with literary data and serves as proof of silver nanoparticles being synthesized. The highest peak corresponding to the highest concentration of nanoparticles was recorded in the sample treated with 350 μl of mint extract (purple graph line). The lowest concentration of nanoparticles was discovered in the sample treated with 150 μl of plant extract (blue graph line).

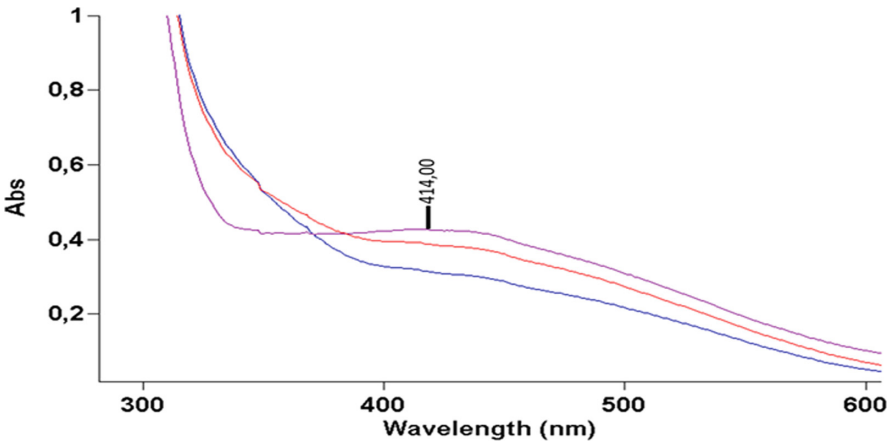

Fig. 1. Recorded spectra of three resulting samples with the $\lambda_{max} = 414$ nm

3.2 Antimicrobial Activity

According to EUCAST standards, a comparative analysis of the results was conducted in comparison to standard values of antibiotics Trimethoprim in combination with Sulphomethoxazole for *E. coli* and *Salmonella sp* (Table 2).

Table 2. Diameter of the inhibition zone for *Escherichia coli i Salmonella sp.*

Inhibition zone diameter (mm)			
Escherichia coli; Salmonella sp.	S	I	R
Trimethoprim + sulphamethoxazole	≥16	11–15	≤10

The following tables are showing the results of measured diameters of inhibition zones of samples acquired with different volumes of plant extract (150 μl, 250 μl, 350 μl) for bacteria *Escherichia coli* (Table 3).

Table 3. Measured diameters of inhibition zones for silver nanoparticles from 150 μl of Mentha *piperita* extract

Silver nanoparticles acquired from 150 μl of *Mentha piperita* extract	Diameter of inhibition zone for *Escherichia coli* (mm)
Disc 1	12
Disc 2	13
Disc 3	11
Disc 4	12
Disc 5	11
Disc 6	12
Average value	11,8

The average value of measured diameters of inhibition zones is 11,8 mm which means that bacteria *Escherichia coli* is intermediately sensitive to silver nanoparticles acquired from 150 μl of Mentha *piperita* extract, because the average value of 11,8 mm is in the rank of value of intermediate sensitive which is 11–15 (Table 4).

Table 4. Measured diameters of inhibition zones for silver nanoparticles from 250 μl of Mentha *piperita* extract

Silver nanoparticles acquired from 250 μl of *Mentha piperita* extract	Diameter of inhibition zone for *Escherichia coli* (mm)
Disc 1	14
Disc 2	12
Disc 3	11
Disc 4	13
Disc 5	10
Disc 6	11
Average value	11,8

According the average value of diameter of inhibition zone, *Escherichia coli* is intermediately sensitive to silver nanoparticles acquired from 250 μl of Mentha *piperita* extract, because the average value of 11,8 mm is in the rank of value of intermediate sensitive which is 11–15 (Table 5).

Table 5. Measured diameters of inhibition zones for silver nanoparticles from 350 μl of Mentha *piperita* extract

Silver nanoparticles acquired from 350 μl of *Mentha piperita* extract	Diameter of inhibition zone for *Escherichia coli* (mm)
Disc 1	10
Disc 2	10
Disc 3	11
Disc 4	12
Disc 5	10
Disc 6	12
Average value	10,8

The average value for silver nanoparticles acquired from 350 μl of *Mentha piperita* extract means that *Escherichia coli* is intermediate sensitive because the value of 10,8 mm is closer to rank of value of intermediate sensitive which is 11–15 (Fig. 2).

Fig. 2. The zone of inhibition of Escherichia *coli* for silver nanoparticles

The following tables are showing the results of measured diameters of inhibition zones of samples acquired with different volumes of plant extract (150 μl, 250 μl, 350 μl) for bacteria *Salmonella sp* (Table 6).

Table 6. Measured diameters of inhibition zones for silver nanoparticles from 150 μl of Mentha *piperita* extract

Silver nanoparticles acquired from 150 μl of *Mentha piperita* extract	Diameter of inhibition zone for *Salmonella sp.* (mm)
Disc 1	8
Disc 2	10
Disc 3	9
Disc 4	7
Disc 5	9
Disc 6	7
Average value	8,3

The average value of 8,3 mm means that *Salmonella sp.* is resistant to silver nanparticles from volume solution of 150 μl of mint extract (Table 7).

Table 7. Measured diameters of inhibition zones for silver nanoparticles from 250 μl of Mentha *piperita* extract

Silver nanoparticles acquired from 250 μl of *Mentha piperita* extract	Diameter of inhibition zone for *Salmonella sp.* (mm)
Disc 1	10
Disc 2	9
Disc 3	8
Disc 4	11
Disc 5	11
Disc 6	8
Average value	9,5

The inhibition zone of nanoparticles acquired from 250 μl of mint extract recorded an average value of 9,5 mm and it can be said that *Salmonella sp.* is resistant to the silver nanoparticles (Table 8).

Table 8. Measured diameters of inhibition zones for silver nanoparticles from 350 μl of Mentha *piperita* extract

Silver nanoparticles acquired from 350 μl of *Mentha piperita* extract	Diameter of inhibition zone for *Salmonella sp.* (mm)
Disc 1	9
Disc 2	10
Disc 3	9
Disc 4	7
Disc 5	9
Disc 6	7
Average value	8,5

The average value of 8,5 mm means that *Salmonella sp.* is resistant to the silver nanoparticles from *Mentha piperita* extract (Fig. 3).

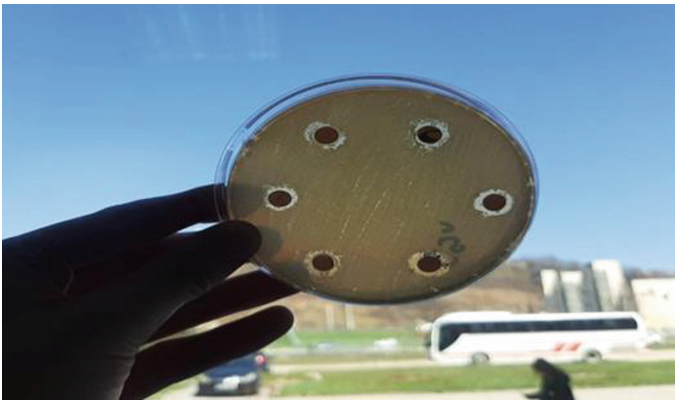

Fig. 3. The zone of inhibition for bacteria *Salmonella sp.* for silver nanoparticles

4 Conclusions

This work has shown the green synthesis of silver nanoparticles from *Mentha piperita* extract whose presence was proven by UV-VIS spectrometry with an absorbance peak wavelength in the range 410 to 450 nm. Their antimicrobial activity was also tested which led us to the following conclusions:

- Maximum of absorbance peak for silver nanoparticles is 414 nm which means that is in the range from 410 to 450 nm, which is the range of silver nanoparticles, which is a confirmation of silver nanoparticles' successful synthesis

- Comparing the average value of inhibition zone diameters with average value of antibiotics Trimethoprim + sulphomethoxazole, it is established that Ag nanoparticles are intermediately successful in inhibiting the growth of *Escherichia coli,* whereas they have been shown to be unsuccessful in suppressing the growth of *Salmonella sp.*
- The strongest antimicrobial effect was shown for volumes of plant extracts used being 150 μl and 250 μl on the bacteria *Escherichia coli* and the least inhibitory action for the sample where 350 μl of the sample were used. This finding implies that the antimicrobial effect is not in direct correlation with the concentration of nanoparticles. The diameter of the inhibition zone for *Salmonella sp.* Shows that the strongest antimicrobial effect was for the sample treated with 250 μl of extract and the weakest for the sample treated with 150 μl of extract.

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Quality and Technology of White Sheep's Cheese from Bjelašnica

Adela Milišić, Zlatan Sarić(✉), Tarik Dizdarević, and Svjetlana Sakić-Dizdarević

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne, 71000 Sarajevo, Bosnia and Herzegovina
z.saric@ppf.unsa.ba

Abstract. The sheep's cheese produced on the Bjelašnica mountain belongs to the group of white brine cheeses, which are predominantly produced in the countries of the Mediterranean and the Balkan Peninsula. For production, the milk of sheep of the autochthonous Pramenka breed, Dubski strain, was used, without the addition of starter cultures. The main task of this study was to research the technological process of white sheep's cheese production and to determine the quality of sheep's milk and cheese based on the results of the analysis. By examining the physico-chemical composition and hygienic conditions of 7 milk samples, it was determined that all samples meet the quality requirements. The average values of the chemical composition of sheep's milk were (%): dry matter 19.88, fat 8.15, proteins 6.44, lactose 3.90, fat-free dry matter 11.63. Based on the examination of 7 samples of mature sheep's cheese, the average values of the chemical composition were (%): dry matter 45.60, fat 26.86, proteins 15.63, salt 4.57. Sensory analysis of the sheep's cheese from Bjelašnica revealed that 43% of the evaluated samples were of the extra class, 43% of the first class cheeses, while 14% of the second class cheeses were included.

Keywords: sheep's milk cheese · quality · technology

1 Introduction

Of at least 500 varieties of cheeses produced in the world [1], a special variety of "cheeses with a high percentage of salt" stands out, which includes white cheeses in brine [2]. White brine cheeses are ripened and stored in brine from production to consumption. They are skinless, with a pure sour-salty taste that becomes more piquant as they ripen. The white color of these cheeses comes from sheep's, goat's or buffalo's milk, from which they are most often produced, but over time more of these varieties are also produced from cow's milk [3]. Simple technology, a relatively short ripening period and the fact that they do not require special equipment and devices for production characterize the cheeses of this group. All of this influenced the development of the technology of white brine cheeses in Bosnia and Herzegovina and it has been maintained up to today in difficult mountain conditions [4]. The most famous Bosnian representative of this group of cheeses is the Vlašić cheese, which has achieved significant recognition within

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but also outside the borders of the Balkan region [5]. Although the mountain Vlašić represents the center of production, the technology of this type of cheese is spread over almost the entire territory of Bosnia and Herzegovina. On the Bjelašnica mountain, the production of Vlašić type white cheese started a few years ago. This cheese is made from raw sheep's milk. While for the technology and chemical composition of Vlašić cheese, there are numerous data in the literature [4, 6–17] white sheep's cheese from Bjelašnica has not been studied so far.

Sheep's milk is white, opaque in color, has a pleasant taste and is odorless if the milking conditions are good, but it absorbs all the smells of the environment [18]. Sheep's milk is usually used for processing into various dairy products, while its fresh consumption is insignificant [19]. The main use of sheep's milk is for the production of traditional cheeses, and sometimes also for the production of sour milk [19, 20]. Very famous cheeses are produced from sheep's milk in the world, such as: Roquefort, Fiore Sardo, Pecorino Romano, Castellano, Fetta, Halloumi, and in Bosnia and Herzegovina Vlašić cheese [21]. Sheep's milk is about 50% richer in dry matter than cow's milk. This is primarily due to the higher content of fat and protein. The ratio of individual components is not like that of cow's milk. There is a higher proportion of fat, followed by protein. Due to the higher content of dry matter, especially fat and protein, this milk is very suitable for processing into cheeses, so double the yield is achieved. It is also suitable for the production of sour milk, because it is a product of excellent solid consistency [4]. According to the Rulebook on the quality of fresh, raw milk [22] sheep's milk must contain a minimum of 4.00% milk fat, a minimum of 3.80% protein and 9.50% non-fat dry matter. The titration acidity of sheep's milk varies between 7.0–9.5°SH. The higher titration acidity compared to cow's milk is a consequence of the higher content of casein and salts with a buffering effect [4]. Comparing the physical properties of cow's, goat's and sheep's milk, it can be said that the density of sheep's milk (1.0347–1.0384) is higher compared to goat's and cow's milk which are comparable (goat's milk 1.0290–1.0390, and cow's 1.0231–1.0398 g/cm^3). The content of lactic acid (0.22–0.25%) is higher than that of goat (0.14–0.23%) and cow's milk (0.15–0.18%). The freezing point (−0.570°C) is lower compared to goat's milk (0.540–0.573 °C) and cow's milk (0.530–0.570 °C), while the pH value of all three types of milk is at an approximate level (sheep's 6.51–6.85; goat's 6.50–6.80 and cow's milk 6.65–6.71). Regarding the pH value, it can only be said that the range of variation is narrower in cow's milk [23–26]. Sheep's milk is characterized by a higher count of microorganisms in 1 ml compared to cow's milk, because it is very difficult to achieve such a high level of hygiene during production as in the production of cow's milk. This can also affect the faster increase in acidity. It is certain that in the production of autochthonous cheeses, the most important role plays the natural microflora of sheep's milk, which creates a specific aroma during their ripening [4]. The aim of the work is to determine the quality and the technological process of the production of white sheep's cheese from Bjelašnica, all with the aim of achieving the unique features of this cheese and its recognition on the market and literature.

2 Materials and Methods

The production of sheep's cheese on the mountain Bjelašnica is studied in this work. The time and temperatures of production phases were monitored. On that occasion, sheep's milk and cheese were sampled. A total of 7 samples of sheep's milk and 7 samples of mature cheese aged 100 days were collected.

All milk samples were subjected to microbiological and physical-chemical testing. The following analyzes were performed on the milk: content of dry matter, fat, protein, lactose, non-fat dry matter by FTIR spectrophotometry, BAS ISO 9622:2015 [27] on the MilkoScan FT6000. The total count of bacteria/ml of milk was determined using the flow cytometry method by EN ISO 21187:2021 [28] on the BactoScan TM FC 50H.

Physical and chemical analyzes were performed on the cheese samples. The dry matter of all cheese samples was determined, using the method of drying in an oven (Heraeus) at 102 + 2 °C according to Regulations on sampling methods and methods of chemical and physical analysis of milk and milk products [29]. The fat content in cheese was determined by the Van Gulik method (ISO 3433:2008) [30]. To determine the pH value of the cheese, a Methrom 632 pH meter was used, with a stabbing electrode for cheese WTW – SenTix Sp. The pH value of all samples was determined in three different places (top of the slice, middle of the slice and center of the section). The final pH value is the average of these three measurements. The concentration of salt in cheese was determined by the standard "Norwegian method" potentiometrically using "MK II chloride Analyzer 926, Sherwood". The fat on dry basis (FDB) and the moisture on fat-free basis (MFFB) were determined by calculation [2, 31]. The protein content was determined using the Kjeldahl method according to ISO 8968:2004 [32] by determining the protein content through the N (nitrogen) content. The method is carried out in three phases: 1. Combustion of samples (using the apparatus Büchi Digest system K - 437), 2. Distillation (distillation apparatus Büchi, KjelFlex, K - 360) and 3. Titration (titration apparatus Methrom Herisau, Titrator E – 526 with dispenser 665). The protein content was obtained by multiplying the amount of TN by a factor of 6.38.

Sensory evaluation was performed using two cheese grading systems. The first sensory evaluation was done by the consumer group, and the second sensory evaluation was done by a panel composed of six expert evaluators. Cheeses for evaluation were coded from 1 to 7 (S1–S7). The first sensory evaluation was done according to the hedonic scale method. It is used to measure the consumer's acceptance of a product, i.e. whether one likes or dislikes a certain food. The scale consists of grades from 1 to 5, which represent the degree of acceptability, where grade 5 is the best degree of acceptability. All the presented ratings represent the average values of the ratings of all lay evaluators (36). For the second sensory evaluation, a 20-point scoring system was used, whereby different characteristics were evaluated (appearance, color, consistency, cut, smell and taste). Each of the characteristics was evaluated and the maximum number of points for individual characteristics was: external appearance-2, color-1, consistency-2, cut-3, smell-2 and taste-10 points. Based on this assessment, the cheeses were classified into quality classes. All values represent the average ratings of all evaluators.

The results of milk and cheese analysis and the sensory evaluation of the cheese were statistically processed using descriptive statistics (MS Excel).

3 Results and Discussion

The production of white sheep's cheese on the Bjelašnica mountain is seasonal, and usually starts in mid-May and lasts until the beginning of October. During this period, the sheeps are fed exclusively on the pastures of the Bjelašnica mountain, which gives the milk and thus the cheese specific features. For the production of cheese, only the milk of sheep of the autochthonous Pramenka breed, Dubski strain, is used.

3.1 Technology of White Brined Cheese from Bjelašnica

The technology of white sheep's cheese produced in the area of the Bjelašnica mountain, consists of several basic stages (Fig. 1, Table 1). Rennet is added into raw sheep's milk immediately after milking. Liquid rennet "Vlašić HA-LA" from Bosnahem-Travnik, strength 1:10,000, is used for coagulation. The curdling time depends on a number of factors, especially the strength of the rennet and the temperature of the milk, and lasts from 90 to 135 min. The temperature of milk during curdling is from 28 to 30 °C. The temperature of milk before during renneting has significant oscillations, which is influenced by external climatic factors on the day of production. On days when the outside temperatures were low, the milk was heated to a temperature of 28 to 30 °C before renneting, and coagulation was performed in containers placed near the heat source. A measured amount of rennet is poured into the milk while stirring, in order to distribute it equally in whole milk quantity. After adding the rennet, the milk is left to stand until coagulation is complete. The end of the curdling phase is determined by the firmness of the curd. When the curd reaches a certain firmness, it is cut into cubes of 9 to 10 cm in size and left to rest until the whey begins to separate. The whey should have a green-yellow color. If the whey is whitish, it means that the rennet process is not complete. In this case, the cut curd is left to stand until it becomes firm enough. When a good quality curd is obtained, it is immediately strained. The draining is done in cloth bags. Preparation of bags for squeezing is done during the milk coagulation. The curd is carefully transferred to the bags with a large ladle, without breaking it. Transferring the curds to the bags proceeds slowly, so that the whey flows evenly and no whey nests remain. The cheese is drained without pressure, under its own weight. While placing the curds in the bags, the cheesemaker occasionally helps the whey to swell by gently lifting the sides of the cheese bags alternately to prevent the formation of a crust on the cheese, because they would prevent the whey from flowing out. The extraction of whey is monitored all the time, until the moment when there is no residual whey on the surface. The cheese squeezed in this way stands for a while so that the remaining whey is separated without any pressure. Shaping the round form of the cheese curd is achieved by tying the bag.

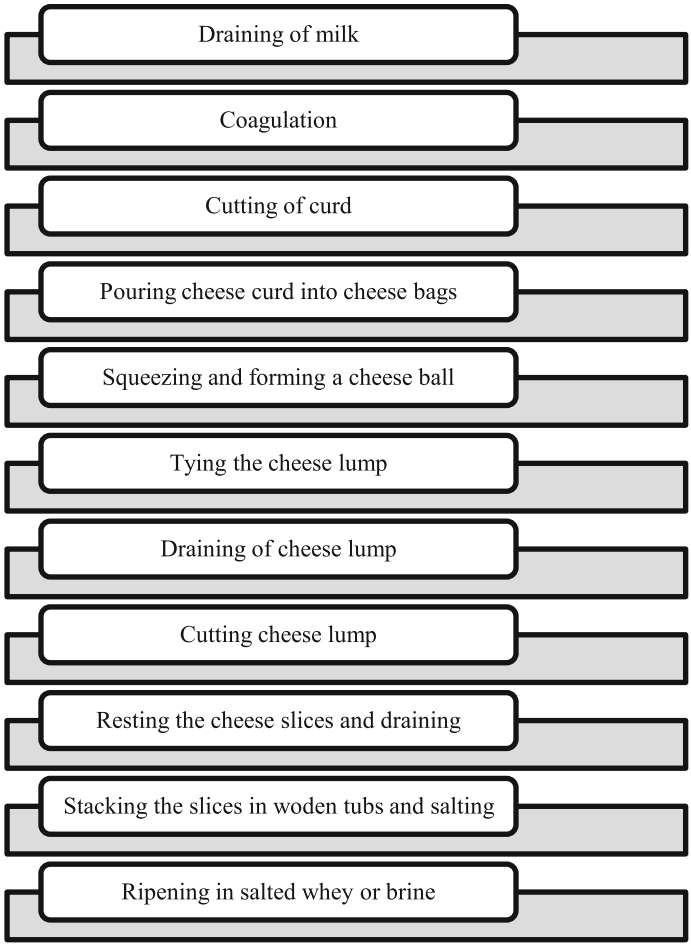

Fig. 1. Basic technological procedure of Bjelašnica white sheep's cheese production

The sqeezing continues for the next 7 to 9 h, which depends on the temperature and firmness of the curds. During warm weather, the length of squeezing is shorter, and the curd is more tender. After the squeezing is finished, the cheese bags are carefully removed from the carrier and the cheese lump is carefully removed. The cheese lump is first cut in half, and each half into 2 equal parts, then the ends of each slice are cut. After cutting the cheese curd the cheese slices are left to release the rest of the whey. On the cheese table, slices stand for 30 to 60 min, which depends on the intensity of the previous squeezing. After standing on the table, the cheese slices are placed in wooden tubs. Stacking the cheese slices and salting them in the vats is done so that the rounded sides of the cheese slices face the inside of the vat, while the flat surface faces the outer part. Pieces of cheese obtained by cutting the edges of the slices are used to fill the free space between the stacked slices. In this way, in addition to filling the free space, the slices are also prevented from sticking together. Thus, it is easier to remove the cheese

slices after ripening. Each complex layer is salted separately. The amount of salt depends on the size of the wooden tub and ranges from 40 to 105 g of salt per row. The filled tub is left without load for two days, so that the salt penetrates into the cheese. Then a wooden lid is placed on the surface of the cheese and weighed down, usually with stones, for pressing. If there is not enough separated whey in the tub, water or whey containing 15% salt should be added. The cheese is cured during standing. If mold appears on the surface, the upper layers of cheese and the upper part of the tub are washed with salt water. The size of wooden tubs depends on the volume of production and market demand. Tubs of 5, 10, 20, 40 and 50 kg can be used. The dimensions of the tub also depend on the amount of cheese. Before transporting the cheese from the mountain, the wood (lid) is tightly closed and the whey is poured out.

Table 1. Technological parameters in Bjelašnica white brined sheep cheese manufacture

Indicators	
Productions (number)	7
Temperature during curdling (^{0}C)	
- room for cheesemaking - curdling	19–22 28–30
Duration of technological steps	
- curdling (minutes) - cutting of curd and separation of whey (minutes) - squeezing (hours) - cutting and resting of cheese slices (minutes)	90–135 9–15 7–9 30–60
Technological characteristics	
- cutting of curd into cubes (cm) - color of separated whey - the appearance and consistency of the curd	9–10 green-yellow solid, with a sharp break
Method of squeezing	
- putting the curds in cheese cloths and helping to strain - shaping of lump	until the formation of lump tying the bag
Salting of cheese	
- while stacking in the tube, layer by layer (g)	40–105
Cheese ripening	
- without whey or brine (aerobic) - within whey or brine (anaerobic) - temperature of ripening (°C)	2 days 30 days and longer 14–15
Yield and liters of milk per kg of cheese	
- liters of milk per 1 kg of cheese - from 100 L of milk	4,2 L of milk 23,58 kg of cheese

Cheese ripening lasts at least 1 to 2 months, at a temperature of 14 to 15 °C. The cheese is watched during ripening and storage. A grey-white layer is created on the surface of the brine by the action of microorganisms. At the beginning, this phenomenon is much more intense than in the further course of ripening. Thus, in the beginning the cheese is washed and cleaned twice a week, and later every 10 to 15 days. If the cheese is not cared for regularly, the protein and fat break down faster, so it loses its quality. After ripening, the cheese is stored in a warehouse. The basement is used as a warehouse, where the temperature is low even during the summer. By comparing all production parameters with the technology of Vlašić cheese, it can be said that the production of white soft cheese from Bjelašnica is very similar to the technology of Vlašić cheese.

3.2 Chemical, Physical and Hygienic Quality of Sheep's Cheese Milk

Table 2 lists the physical and chemical characteristics of sheep's milk samples for the production of white brine cheese from Bjelašnica.

Table 2. Physico-chemical properties of sheep's cheese milk

	Fat (%)	Proteins (%)	Lactose (%)	Density (g/cm^3)	DM[a] (%)	NFDM[b] (%)
Mean value	8.15	6.44	3.90	1.037	19.88	11.63
St. Dev.	0.43	0.33	0.14	0.00	0.64	0.24
Coef. Var. (%)	5.27	5.15	3.58	0.11	3.23	2.04
Min	7.56	6.16	3.59	1.036	19.16	11.38
Max	8.91	7.13	4.01	1.039	21.11	12.06

[a] – Dry matter; [b] – Non-fat dry matter

Sheep's milk for the production of white brine cheese from Bjelašnica was high in fat and protein content but low in lactose one. The mean value of fat content for all seven samples was 8.15%. The deviation of the individual milk fat percentages of the samples from the average was 5.27% due to small variations. For protein content, the average value for all analyzed samples was 6.44%. When individual protein content values were observed in all milk samples, smaller variations in values were noticeable, so the coefficient of variation was 5.15%. For lactose content, the average value for all milk samples was 3.90%. The coefficient of variation was 3.58% due to smaller deviations of the individual percentages of lactose in the samples from the average value. The smallest coefficient of variation of 0.11%, and at the same time the smallest variations, were in the case of density in milk samples. The mean value for the density was 1.037%. The average value of dry matter content was 19.88%. The calculated coefficient of variation was 3.23% due to smaller deviations of individual percentages of dry matter from the average. The average value of the non-fat dry matter content was 11.63%. There is no data on the composition of sheep's milk for the production of white brine

cheese from Bjelašnica, but the sheep's milk for the production of Vlašić cheese had the following composition [13]: fat 9.31%, proteins 5.98%, dry matter 18.66%, non-fat dry matter 9.35%, mineral components 0.93% and density 1.037 g/cm^3. It is known that the quality of cheese strongly depends on the hygienic quality of milk. Therefore, the count of microorganisms/ml of sheep's milk for the production of white brine cheese from Bjelašnica was analyzed (Table 3).

Table 3. Count of microorganisms/ml of sheep's milk

Mean value	16,000.00
St. Dev.	6,271.63
Coef. Var. (%)	39.20
Min	7,000.00
Max	27,000.00

The hygienic quality of sheep's milk for cheese production was high with an extremely low count of microorganisms/ml of milk. This is somewhat surprising considering that milking of sheep is done by hand. It is obvious that the farmers take care of hygiene during milking, and it is also certain that the mountain climatic conditions affected the good microbiological quality of the milk. The average number of CFU/ml in the milk samples was extremely low, 16,000.00 CFU/ml.

3.3 Chemical Composition and pH Value of White Brine sheep's Cheese from Bjelašnica

Sheep cheese technology is based on the use of raw sheep's milk, which is not standardized for fat content. The values of chemical composition and pH values of 7 tested cheese samples after 100 days of ripening are shown in Table 4.

The average dry matter content was 45.40%, which is typical for soft cheeses. Variations were low, which indicates the uniformity of the chemical composition of milk for cheese production and technology. The average percentage of fat content was 26.86%, and for proteins content 15.63%. Slightly larger deviations of individual values from the average related to the content of dry matter were recorded in the percentage of fat and protein. The salt content was typical for this variety of cheese (4.57%), while according to the average FDB content, it can be classified into the categories of extra-fat cheeses and full-fat cheeses. According to the proportion of MFFB, this cheese belongs to the category of soft cheeses. The pH value of all cheese samples was below 5.00, which shows that the production and ripening processes of this cheese were proceeding normally. The content of basic chemical components in mature sheep's milk Vlašić cheese varied in the following limits: dry matter 48.91–50.24%; fat 22.43–27.42%; FDB 46.09–54.80%; proteins 20.10–21.84%; salt 3.77–5.44% and lactic acid 0.557–0.650% [7]. It can be noticed that the ratio of fat and protein is different in Bjelašnica cheese compared to Vlašić cheese.

Table 4. Chemical composition and pH value of sheep's cheese from Bjelašnica

	Dry matter (%)	Fat (%)	Proteins (%)	Salt (%)	pH	FDB[a] (%)	MFFB[b] (%)
Mean value	45.40	26.86	15.63	4.57	4.62	60.24	75.15
St. Dev.	2.25	3.04	1.09	0.30	0.16	6.02	3.03
Coef. Var. (%)	4.97	11.31	7.00	6.54	3.50	10.00	4.03
Min	42.11	23.00	14.42	4.03	4.48	53.70	69.70
Max	48.42	31.50	17.28	4.96	4.90	67.64	78.77

[a] – Fat on dry basis; [b] – Moisture on fat-free basis

3.4 Sensory Evaluation of White Brined Cheese from Bjelašnica

All cheese samples were evaluated by the consumers as medium likeable, with an average rating of 2.28 (Fig. 2).

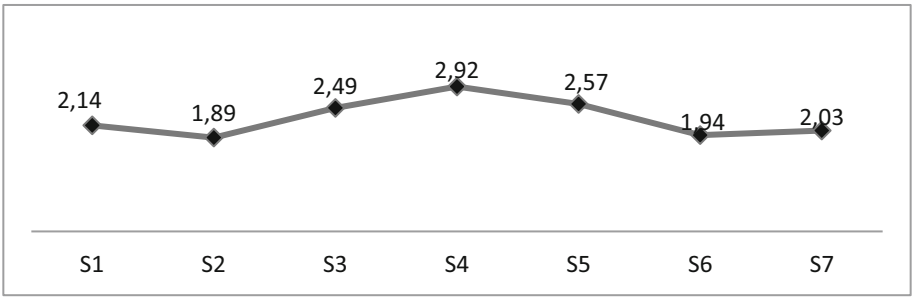

Fig. 2. Consumers sensory evaluation of cheese

In contrast to the assessment of likability, which was carried out by a group of lay evaluators, the expert panel gave the cheeses high marks, a total of 15.75 to 19.00, an average of 17.25. According to the total number of points, three cheeses were in the extra class, two in the first class, and only one was classified in the second class. This shows that these cheeses have good sensory properties, typical for this type of cheese (Table 5 and Fig. 3).

Table 5. Expert sensory evaluation of cheese

	Mean value	St. Dev.	Coef. Var. (%)	Min.	Max.
Outer appearance	1.76	0.18	10.18	1.40	2.00
Color	0.96	0.09	9.80	0.75	1.00
Consistency	1.54	0.33	21.12	1.00	1.90
Cut	2.35	0.49	20.66	1.70	2.90
Odour	1.84	0.14	7.82	1.60	2.00
Taste	8.80	0.57	6.46	8.20	9.60
Total	17.25	1.70	2.88	15.75	19.00

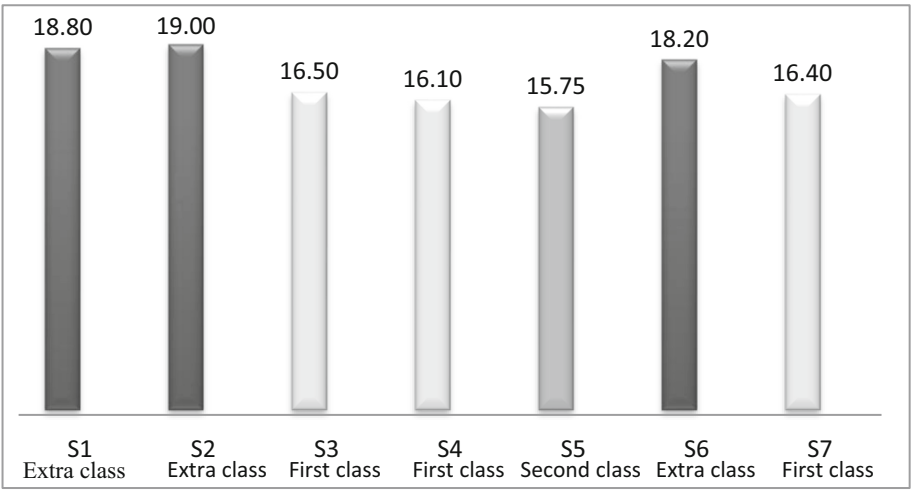

Fig. 3. Grading of cheese into the quality classes according to sensory evaluation

4 Conclusions

In this paper, the research was carried out on white brine sheep's cheese produced on the Bjelašnica mountain. It can be stated that the technology of this cheese is typical for the group of white brine cheeses. It is very similar to the technology of Vlašić cheese, which is the most important representative of this group of cheeses in Bosnia and Herzegovina. Sheep's milk for cheese production had a usual chemical composition and high hygienic quality with a low count of microorganisms. The chemical composition of the cheese and the low pH value characterize this cheese as a white brine cheese. According to the FDB content, this cheese can be classified into the categories of extra-fat and full-fat cheeses. According to the proportion of MFFB, this cheese belongs to the category of soft cheeses. The cheese samples were evaluated by the consumer group as moderately appealing, but the sensory evaluation of the expert panel placed them in the categories of extra and first class. Only one cheese sample was in the second class. It can be concluded that the

white brine cheese from Bjelašnica is produced from good quality sheep's milk and it is a typical representative of white cheeses in brine in terms of chemical composition, pH value and sensory properties. It was highly sensory rated by the expert panel.

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Nanotoxicology in Food Technology

Maid Ćibo(✉), Adis Šator, Amina Stambolić, and Enisa Omanović-Mikličanin

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
maid_cibo@hotmail.com

Abstract. In addition to the benefits of nanotechnology, several experts and researchers have raised concerns about the potential toxicity of nano-food. The study of interactions between nanostructures and biological systems is the focus of the specialized field of toxicology known as nanotoxicology, which also emphasizes the relationship between the physicochemical characteristics of nanoparticles and their toxicological consequences. For instance, it is becoming clear that there will be toxicological consequences of persistent exposure to heavy metals, asbestos, silicate surfaces, or autoimmune responses caused by silicate surfaces (causing genotoxicity, neurodegenerative problems, and liver disease). Scientists and researchers believed that new nanomaterials might exhibit some toxicological effects similar to those previously mentioned because of how similar these elements are to nanostructures (such as carbon nanotubes with asbestos, silicon nanomaterials with silicon dust, and quantum dots containing heavy metals). An increasing concern is becoming legitimate, because of as many scientific papers have proven that nanoparticles at the nanometer level show different properties compared to particles in the macro and micro dimensions. Well, due to their size, nanoparticles can enter by ingestion, inhalation, transdermally or intravenously into cells, tissues, and other organs, which can cause adverse effects on human health, such as brain diseases, heart diseases, gastrointestinal tract disease, lungs disease, lymphatic and circulatory systems disease.

Keywords: Nanotoxicology · Nano-food · Toxicity of nanoparticles · Effects on human health

1 Introduction

A special branch of toxicology deals with the study of the interaction of nanostructures with biological systems, emphasizing the connection between the physicochemical properties of nanoparticles and their toxicological effects. That is nanotoxicology deals with the study of the toxicity of nanomaterials. Nanostructures, like everything else in the world, have their drawbacks. Namely, the new area that has attracted everyone's interest in the last decade (nanotechnology), in addition to the great advantages it has brought in various fields, including food engineering, has also brought a question mark about the impact on human health, as well as the environment. When talking about new technologies related to the production and processing of food such as nanotechnology, it

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is admitted that there is always apprehension, especially when it comes to such small particles that are at the nanometer level, especially since it is food. Well, today, very often in many media can be heard more and more about how harmful nano-food is for human health. In this work it will be explained how nanoparticles, nanocomposites, and nanosensors created are justified for use in food, that is, how and in what way they can affect human health [1, 2].

Nanostructures are much more complex than other products, such as those in micro or macro size, so because they can be deposited in air, water, and soil, but also in food, as well as in humans, their effect must be thoroughly investigated. For example, it is known that there are proven toxicological effects if people are chronically exposed to silicate surfaces (causing autoimmune reactions), asbestos (causing pleural cancer), or heavy metals (causing genotoxicity, neurodegenerative problems, and liver disease). It is the great similarity of these elements with nanostructures (such as carbon nanotubes with asbestos, silicon nanomaterials with silicon dust, and quantum dots containing heavy metals) that led many scientists and researchers to think that new nanomaterials could exhibit some toxicological effects like those previously mentioned [3, 8, 11–13].

1.1 What Does the Toxicity of Nanomaterials Depend on?

In order to know how much toxicity nanomaterials have, it is first necessary to answer the question, what are the main factors that are directly involved in their toxicity?

According to many types of research and findings, toxicity does not depend only on one factor, but on many factors that contribute to toxicity, such as: the size of nanomaterials (their small size means better communication with biological systems); the form of nanomaterials; chemical composition; charge; aggregations; self-assembly capacity; conjugation capacity (strong or weak bonds) with other components such as proteins, lipids, etc.; catalytic properties or combinations with other biological components [3, 11–13]. These factors affecting the toxicity of nanomaterials are shown in Fig. 1.

In addition to these factors, toxicity also depends on the biodegradability of nanomaterials, tissue localization, their speed of removal, and what is very important, whether the materials used are biodegradable or non-degradable [3, 8, 11, 12].

Due to all of the above, great attention is focused on the toxicology of nanoparticles and the possible diseases they cause, because, according to many studies, nanoparticles are more toxic than larger particles of identical chemical composition [3, 12].

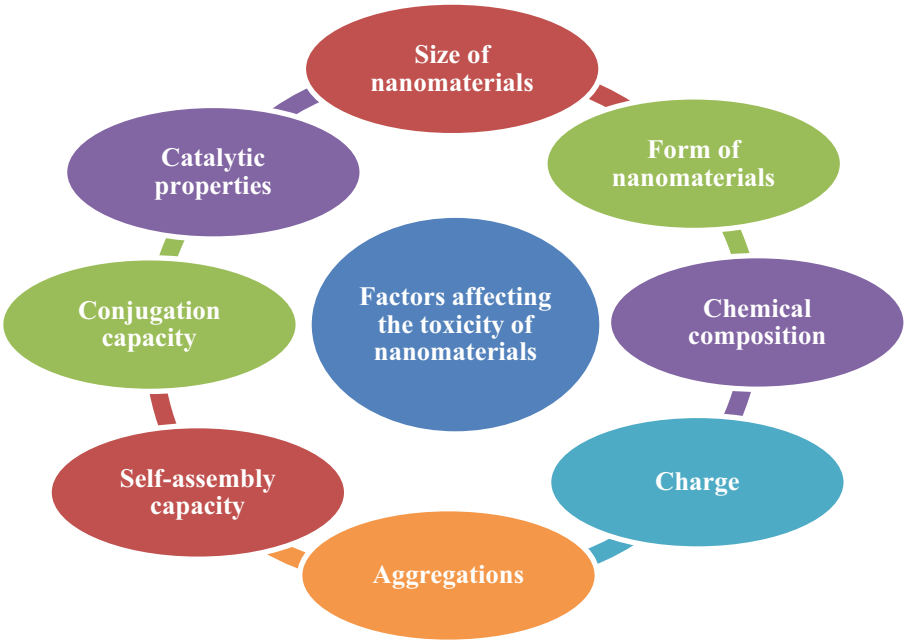

Fig. 1. Factors affecting the toxicity of nanomaterials [3, 8, 11–13]

2 Determining the Activity of Nanomaterials in Food

Due to the fact that the size of nanomaterials is in the range of 1–100 nm, such materials show new, unique properties compared to other materials. Before determining the toxicity of nanomaterials and their impact on human health, it is necessary to determine the activity of these nanomaterials, which was previously mentioned in the text, such as chemical composition, surface structure, solubility, shape, and aggregation, which will be explained in more detail below [7, 8, 11, 16].

2.1 Factors Contributing to the Toxicity of Nanomaterials

One of the important factors for the unique properties of nanoparticles is their size. Precisely, because of the size of 1–100 nm, the surface areas are significantly larger than those in bulk. For example, lung inflammation, according to much evidence, can be caused by the size, surface area, and surface properties of nanoparticles. Nanoparticles can accumulate in the alveoli of the lungs, and then they can be transferred to distant organs such as the liver, kidneys, spleen, and brain. Research has shown that, for example, ultra-fine particles of TiO_2, compared to those fine particles of TiO_2, cause a higher percentage of lung cancer, which in this case would mean that the toxicity depends on the size of the particles. However, research was recently done and an interesting result was found that particles like soot and TiO_2 in a concentration of 20 $\mu g/cm^2$ become toxic to kidney cells, but are safe in vivo. This means that, in order to provide useful data for

nanoparticle toxicity testing, an in vitro cell system should be developed. In addition to all these negative effects, there are also some advantages of nanomaterials, such as TiO_2, which the immune system can recognize as "danger signals". To be more specific, the immune system of the human body has developed to recognize and get rid of potentially dangerous items, such as foreign invaders like viruses, bacteria, and other diseases. TiO_2 nanoparticles can set off a chain of immunological reactions when they enter the body because the body interprets them as "danger signals." During the recognition process, immune cells that are involved in phagocytosis, such as macrophages, may become activated (the process of engulfing and destroying foreign particles). This shows that TiO_2 nanoparticles can be a very important addition to the immune system. Also, there are research results that have shown that Crohn's disease would become more serious if the dose of microscale TiO_2 in the diet for patients was reduced [7, 8, 11–13, 15].

2.2 Chemical Composition

Purity, electronic properties, and crystallinity are some of the critical parameters for a nanomaterial. During the preparation of some materials such as carbon nanowires, there are usually some residues or impurities. Undoubtedly, these impurities can cause serious health problems and make it difficult to understand the true toxicity of carbon nanotubes. For example, single-walled carbon nanotubes usually contain a higher amount of iron, which can be a catalyst for oxidative stress, which would mean that carbon nanotubes containing iron are more toxic than those without iron [8, 9, 11].

2.3 Surface Structure

The surface structure of nanoparticles includes surface reactivity, inorganic or organic coatings, surface groups, etc. After modification with these compounds, the biocompatibility, dispersion, or activity of nanoparticles will be significantly improved. For example, in a comparison of nanoparticles alone and iron dioxide nanoparticles, iron dioxide nanoparticles showed no cell toxicity when coated with polyethylene glycol. And in the meantime, the surface charge of the nanoparticles changes with the changes themselves, so the activity and toxicity will also change. Auffan et al. (2009) examined the influence of the chemical stability of metal nanoparticles on cellular toxicity. The conclusion was that chemically stable metal nanoparticles have no cellular toxicity, while nanoparticles that can be oxidized, reduced, and dissolved showed cytotoxicity and even genotoxicity for cellular organisms. This means that the surface structure can play a very important role in the toxicity of some specific nanomaterials [10–13].

2.4 Aggregation

Functions are determined by the structure and characteristics of materials such as proteins, which means that to know the function of a material completely, it should be known all its structures and characteristics [8, 11–15].

As is known, nanotoxicological research is carried out by in vitro and in vivo experiments, but the detailed changes of nanomaterials after their entry into cells are still not known. For example, it is not clear how aggregation affects toxicity.

Nanoparticles in the dry state can be in two forms, namely aggregated (strong connections between primary particles) and agglomerated (which have weaker van der Waals forces). Thus, agglomerated nanoparticles can be separated by overcoming weaker attractive forces when dispersed in a solution. What is important to know is that toxicity cannot be determined by just one element, but by many that influence toxicity, and among them is the pH value, which in the medium plays an important role in the influence of lung toxicity with TiO_2 nanoparticles [11–14].

2.5 Solubility

It has been shown that the solubility of nanoparticles has a great influence on toxicity. Pott et al. (1998) showed the results of research that hydrophilic ultra-fine TiO_2 particles are very toxic and more lethal to rats than hydrophobic ultra-fine TiO_2 particles. Thus, research has shown that solubility has a great influence on the cytotoxicity of nanoparticles. When exposed to poorly soluble nanoparticles such as ZnO (zinc oxide) nanoparticles at concentrations above 15 ppm, all 3T3 cells (cells derived from Swiss albino mouse embryonic tissue) died. However, the cells did not die completely even at high concentrations (30 ppm) of insoluble nanoparticles. However, there are also data showing that solubility may not be a determinant of toxicity [11–15, 21, 31].

Among other things, all of the above, are only indicators that many factors influence the activity and toxicity of nanomaterials. It must be known what dose and at what point it becomes toxic, that is, it must be fully understood and evaluated the positive and negative effects of nanomaterials to fully utilize them [8].

3 Toxicology of Nanostructures in Food and Impact on Human Health

Human exposure to nanomaterials is increasing as the intensive use of nanotechnology increases. People who work with nanomaterials during production are exposed, while consumers are exposed during the use of products obtained from nanomaterials. By releasing nanomaterials into the environment (soil, water, air), it is to be expected that they can also enter the human body. What worries scientists and experts, as well as researchers, is the reactivity of nanoparticles when they are used in the food chain, as well as their size, considering that as such they can reach those parts of the body where larger particles cannot reach. Precisely in this way, nanomaterials can threaten the immune system in humans, as well as cause health problems that are not negligible at all, on the contrary [16–20].

3.1 Toxicokinetics

Regardless of the way of entry (oral, inhalation, transdermal, or intravenous), nanomaterials can cause the same toxicological effects on health, such as genotoxicity, cytotoxicity, as well as organ toxicity [16–18]. The way, that is, a potential path of entry of nanoparticles is shown in Fig. 2.

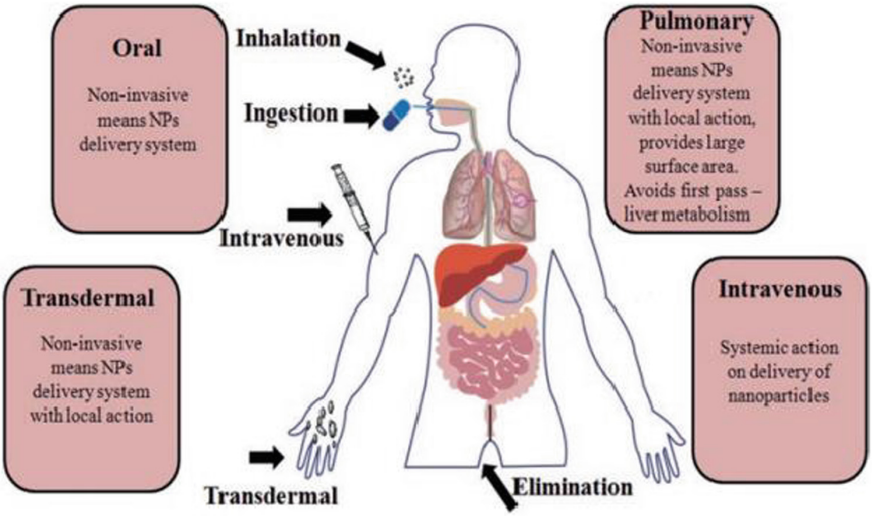

Fig. 2. Presentation of the way nanoparticles enter the human body [31]

Nano-food is food that implies the use of nanoparticles in any phase of food production, that is, in which nanomaterials are used throughout the entire food supply chain, but there is not much information about the interaction of food and nanoparticles in the human body. That is, there is not much data from studies on the mechanisms of toxicity of nanomaterials that are present in food. The use of nanoparticles, which can be intensive and extensive in food production, can result in the accumulation in food, which can lead to local and systemic health problems that can be of a more serious nature. The presence of nanoparticles in food can be intentionally used additives, i.e. food ingredients, or unintentionally, i.e. when nanoparticles unintentionally from packaging or nanosensors can pass into food, including the use of nano-agrochemicals. The toxicity and final fate of nanoparticles are based on their concentration and physicochemical characteristics, regardless of how these nanoparticles entered the food [16–25, 31–33]. There is a great concern when it comes to the presence of nanoparticles in food, so this concern can be classified into three groups, as shown in Fig. 3.

An increasing concern is also related to the toxicity of various nano-additives that are added to food and beverages in order to improve taste, smell, color, texture, and nutritional value. As mentioned previously, there are already a large number of food additives on the market, the effectiveness, and toxicity of which have not been proven. There is also an example when talking about nano-encapsulated particles that have great advantages, however, due to their absorption, distribution, metabolism, and excretion, they behave differently when they enter the body and make direct contact with numerous organs. It has also been proven that the higher absorption of nano-additives can increase health problems. Considering that the safety of additives is still questionable, much more detailed research and risk assessment must be done [16–20, 25–30]. The level of exposure to nanoparticles in food depends on two factors, which are shown in Fig. 4.

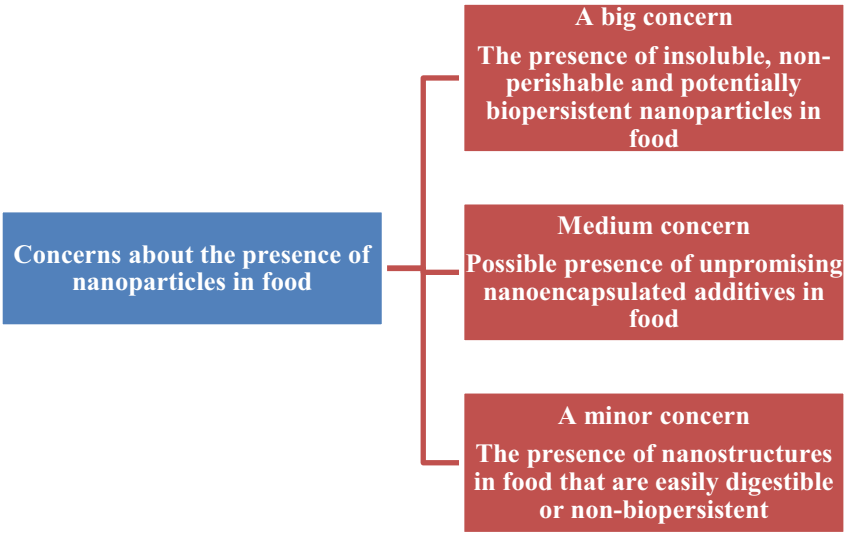

Fig. 3. Classification of concerns regarding the presence of nanoparticles in food [16–19, 24–27]

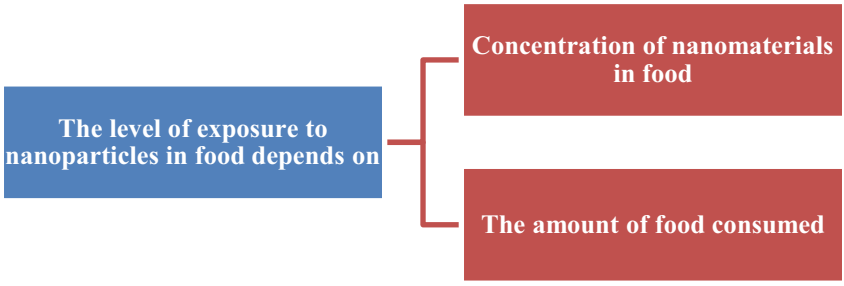

Fig. 4. Factors on which the level of exposure to nanoparticles in food depends [16–20, 25–30]

There are many more ways in which nanoparticles can enter the human body, and some of them are, for example, nano-packaging that is edible and that after metabolism can be excreted in the gastrointestinal tract, but about that, as well as nano-coatings used for some food products, which are consumed for a long time, there is no scientific evidence of their toxicity [11–13, 16–31].

In addition to this, the use of nanosensors has brought, it seems, a completely new system by which nano-food would be produced, processed, stored, distributed, or sold. However, some fragments of nanosensors, such as metals and metal oxides, which were previously mentioned are used as part of intelligent packaging, can be unintentionally transferred into food and thus affect its safety. All this, in fact, represents some undesirable changes in food, which is against the current EU laws, which state that materials that come into direct contact with food should not cause reactions that can lead to some changes in it. A big problem is that there is still no such analytical technique that could

investigate the impact of nanoparticles in food on human organs, that is, there is no such analysis that could investigate such small particles, such as those at the cellular or molecular level. Thus, for example, there is a big problem with nano-packaging, which represents the greatest potential for application in the food industry, from which nanoparticles can migrate into food and thus reach the body and cause health consequences, and for this reason, it is recommended to first test for migration, regulate and only then approve, depending on the case by case [16–30, 34].

Figure 2 shows the toxicokinetics of nanoparticles, while Fig. 5 shows a schematic representation of the human body with routes of exposure to nanoparticles, affected organs, and related diseases from epidemiological in vivo and in vitro studies. Ultimately, it can be said that the main way nanoparticles enter the human body is through the consumption of nano-food and drinks. Furthermore, after consumption, nanoparticles travel through the gastrointestinal tract. Due to various chemical reactions, such as agglomeration, aggregation, adsorption, or binding with other food ingredients, and their interaction with digestive enzymes, the properties of nanoparticles can change during the entire journey through the gastrointestinal tract. Due to possible accumulation within body tissues, but also the possibility of passing biological barriers, nanoparticles can change their composition and function. What still represents a potential risk for consumers is the consumption of products that may contain nanomaterials, mainly animal ones, for the reason that the application of nanomedicine in animal production may result in the transfer of possible residues of veterinary drugs further into food products that consumers, as previously mentioned, they consume and thus enter their body. Further, nanoparticles that enter the gastrointestinal tract can cause some diseases such as colon cancer and Crohn's disease [16–30].

From the gastrointestinal tract into the bloodstream, up to the liver, spleen, brain, reproductive system, and other organs, according to several studies, nanoparticles of silver, carbon, and titanium dioxide have been shown to be able to move. However, the mode of action, metabolism, and elimination of nanoparticles through the gastrointestinal tract is still largely unexplored, so data on the risk are also incomplete. It is precisely for this reason that there is limited data on the risk of nanomaterials that are taken in orally, that the assessment itself, as well as the recommendation of an acceptable daily intake of nanoparticles, is difficult. In environments where nanomaterials are produced, nanoparticles can be inhaled and thus enter the body [16–30].

For example, workers working in the agricultural and food sector who are directly involved in the production, handling, and use of nanomaterials are constantly exposed to them. Regardless of whether it is long-term or short-term exposure to nanomaterials, they can be absorbed into the skin via sweat ducts, hair follicles, etc. It is important to mention here that education of employers and employees about nanomaterials is required, as well as the application of safety practices, such as maintaining hygiene, and use of protective equipment (masks, glasses, gloves) in order to reduce the risk of nanoparticles and protect the health of those who handle nanomaterials, given that there is no legal framework that could protect the health of employees in the workplace. Almost the largest number of studies related to exposure to the inhalation of nanoparticles have shown that inhaled nanoparticles are associated with numerous diseases. Some of the factors that influence the retention and deposition of nanoparticles in different parts of the lungs are size,

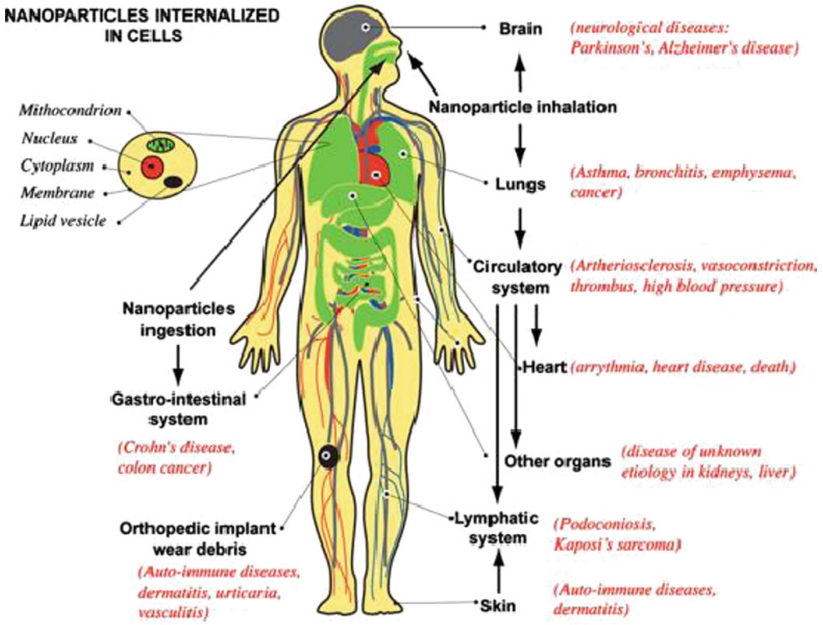

Fig. 5. Schematic representation of the human body with routes of exposure to nanoparticles, affected organs, and associated diseases from epidemiological in vivo and in vitro studies [23]

the dose of nanoparticles, anatomy of the respiratory tract, time of exposure, but also method of inhalation. Research has shown that smaller nanoparticles compared to larger ones cause more serious lung diseases. Furthermore, nanoparticles that are inhaled can also reach other organs via blood and lymph and thus can cause health problems. It is through the respiratory flow and blood flow that nanoparticles penetrate the heart, lymph nodes, spleen, bone marrow, etc., which can lead to their potentially different functioning and various diseases. Some diseases that can be a consequence of inhalation of nanoparticles are lung diseases such as asthma, bronchitis, emphysema, lung cancer, neurodegenerative diseases (Parkin's and Alzheimer's diseases), while diseases such as heart disease, arrhythmia, arteriosclerosis, autoimmune diseases can occur if the nanomaterial enters the circulatory system. It has been proven that nanoparticles of silicon dioxide, and titanium dioxide, as well as ultra-fine particles of soot and diesel particles, have toxicological effects. But research has also proven that other nanoparticles in the body can be distributed in such a way that they cause and provoke some diseases. However, today there is a lot of research on silver nanoparticles because they are the most used of all nanomaterials, and it has been proven that these nanoparticles can reach the systemic circulation because they can pass physiological barriers and thus achieve communication with plasma proteins and other blood components or be further transmitted in other organs such as the liver, kidneys, lungs, heart, brain, etc. Some nanomaterials, according to research, can exhibit genotoxic, cytotoxic, and carcinogenic effects, for example, zinc oxide nanomaterials are cytotoxic to liver cells and can damage DNA [16, 23, 34].

It is not only zinc oxide nanomaterials that exhibit toxic effects, so fullerenes, quantum dots, and metal and metal oxide nanoparticles can cause changes in chromosome fragmentation, gene precision, as well as DNA chain breakage if they enter cells, tissues, and organelles [16]. Some of the possible risks brought by nanomaterials used in food, such as silver, titanium dioxide, zinc oxide, silicon dioxide, carbon, iron oxide, etc., are shown in Table 1.

Table 1. Potentially harmful effects of nanomaterials on human health [8, 16–30]

Nanomaterial	Possible risks
Silver	May damage human cells, toxic to cells derived from the brain, lungs, skin, liver, vascular system, and reproductive organs
	Can cause DNA damage and chromosomal aberrations
	It can be translocated by the bloodstream and thus cause destruction of the blood-brain barrier and degeneration of neurons
	May alter mitochondrial function by producing reactive oxygen species
	Enlargement of the membrane
	Permeability and increased generation of reactive oxygen species
Titanium dioxide	Causes tumor changes - turns benign into malignant tumor cells by creating reactive oxygen species
	Oral consumption can lead to various diseases such as gastritis, colitis, and Crohn's disease
	Causes thrombosis
	Causes liver damage
	Inhaling insoluble particles of titanium dioxide can cause pneumonia
Zinc oxide	Causes toxicity of the digestive system
	Causes cytotoxicity and DNA damage
	Causes damage to liver and kidney cells
Silicon dioxide	Effects on the gastrointestinal system
	Causes pulmonary inflammations, granulomas, and fibrosis
Cobalt oxide	Can cause cardiomyopathy, hearing, and vision impairment
	Increases the risk of lung cancer
Carbon	Entering the human body through the skin, it interferes with the lymphatic system and lymph nodes, which results in various health conditions
	Causes pneumonia and vascular diseases
	Causes thrombosis
Tin dioxide	There is no study related to oral intake of tin dioxide nanoparticles
Iron oxide	Causes cytotoxicity and DNA damage

Finally, the removal or elimination of nanoparticles from the human body is very important. Data from the research show that the size and chemical composition of nanoparticles affects their excretion. Excretion/elimination of nanoparticles from the body can be carried out through the gastrointestinal tract or kidneys. It is also important to know that smaller nanoparticles can be excreted through the kidneys, while larger nanoparticles increase the risk of toxicity because such particles stay longer in the kidneys [16–30].

It can be concluded that the number of studies on the toxicity of nanomaterials, and their assessment, and characterization must be increased to obtain much more data and information about nanomaterials, and in addition, it is necessary to create and develop a regulatory framework. Also, the popularity that nanotechnology has experienced in the past years is becoming similar to what happened to genetic engineering, after which there was great opposition and resistance due to the risks it carries. For this reason, it is necessary to do much more research, both at the academic and industrial level, in order to know much more detail about the possibilities, but also the risks, that nanotechnology brings. Among other things, due to the aforementioned toxicological effects of nanomaterials, green nanotechnology has emerged as a new solution. The new, so-called 'Green approach' implies alternative, more environmentally friendly methods of synthesis of nanoparticles. In such methods, it is not necessary to use high pressure, energy, temperature, as well as toxic chemicals, so, for example, nanoparticles obtained by green synthesis using plants and their extracts also show antimicrobial properties. Numerous studies support this, and one such study is the green synthesis of silver nanoparticles using apple extract and its antimicrobial properties, where a great potential for inhibiting the growth of bacteria such as Escherichia coli and Salmonella spp was shown. This means that a solution always exists, and in order to find it, great attention should be paid to the study and research of nanotechnology, a technology that represents a new technological revolution and a possible major turning point in human history [32, 35–38].

4 Conclusion

In addition to all the advantages that it has brought, nanotechnology, on the other hand, has caused a lot of controversies, and different opinions of experts about the safety and toxicity of nano-food. Nanotoxicology is a science that studies the toxicology of nanomaterials.

Many studies have confirmed that some nanoparticles used in food, such as nanoparticles of silver, titanium dioxide, zinc oxide, silicon dioxide, carbon, and iron oxide, can cause serious consequences for human health. Regardless of the way of entering the human body (intravenous, transdermal, by inhalation, ingestion), they can cause serious diseases such as lung diseases, such as asthma, bronchitis, and even lung cancer, then diseases of the circulatory system (atherosclerosis, thrombosis, etc.), then heart diseases (arrhythmia), skin diseases (dermatitis, autoimmune diseases), diseases of the lymphatic system (podoconiosis, Kaposi's sarcoma), and diseases of the gastrointestinal system (Crohn's disease, bowel cancer), but also brain diseases (Parkinson's disease, Alzheimer's disease), as well as many other diseases. Therefore, it is necessary to carry

out many more analyses in the field of toxicology of nanomaterials, because according to research so far, it can be said that the action, metabolism, and function of nanomaterials in food are still unknown.

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Phytomelatonin: Recent Advances – Systematic Review

Semira Galijašević[1](✉), Selma Burović[1], Amina Stambolić[2], and Enisa Omanović –Mikličanin[2]

[1] Sarajevo Medical School, University Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina
semira.galijasevic@ssst.edu.ba

[2] Faculty of Agriculture and Food Sciences, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

Abstract. Human hormone melatonin (N-acetyl-5-metoxy tryptamine), is a tryptophan metabolite synthesized in pineal gland. Melatonin is shown to control and a modulate circadian rhythms, seasonal reproduction, sleep regulation, retinal physiology. Besides its physiological role, the role of melatonin as a strong antioxidant that can subdue inflammatory pathways and scavenge free radicals has been proven in a numerous studies. Antioxidative ability of melatonin is based on its role as a scavenger of reactive oxygen species including hydroxyl radical, superoxide ion, peroxy radicals, singlet oxygen, nitric oxide, peroxynitrate and its metabolites. Antioxidative mechanism can proceed through several mechanistic pathways including hydrogen atom transfer, electron transfer and formation of radical adducts. Only much later melatonin has been detected in a different plant species. High concentrations of melatonin have been found in a different medicinal plant. Amounts of melatonin in these plant tissues amounted to several micrograms per gram of tissue (amounts more than those found in blood).

Daytime melatonin concentrations are related to light intensity to which the plants are exposed. Meanwhile, it has been shown in various plants that contain large amounts of melatonin to respond to intense light, especially UV, by increasing of methoxyl indole. This suggests a photoprotective role, which it already is presumably because of the numerous photoreactions to which melatonin is subject and ability melatonin to neutralize singlet oxygen and free radicals caused by UV rays. Recent research shows that melatonin concentrations differ not only between plants species but also among varieties of the same species, and to a considerable extent about the degree of growth, location, specific plant organ, as well as the time and season of harvest. At the current level of understanding, the presence of melatonin in plants is, in most cases, accepted although there are still many unanswered questions. Thus, we performed systematic review of a literature according to PRISMA protocol to analyze the origin, content in various plant species, detection and therapeutic potentials of phytomelatonin.

Keywords: Melatonin · antioxidant · plants · phytomelatonin · plant hormone

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1 Introduction

Melatonin (N-acetyl-5-methoxytryptamine). Indoleamine is ubiquitous neurohormone synthesized from the amino acid tryptophan and secreted by the pineal gland in the brain [1–3]. It is involved in a numerous aspect of biological and physiological regulation including circadian entrainment, blood pressure regulation, oncogenesis, retinal physiology, seasonal reproduction, ovarian physiology, immunity and inflammation inhibition [4–8] (Fig. 1).

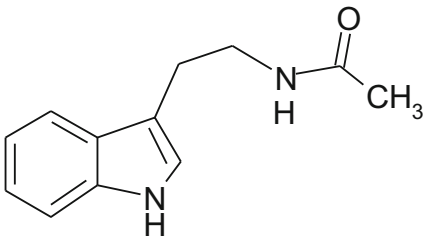

Fig. 1. N-acetyl-5-methoxytryptamine

Its synthesis and release are stimulated by darkness and suppressed by light so the melatonin is known as a hormone of darkness [9–11]. On average, the pineal gland produces between 0.1 and 0.9 μg of melatonin per day, but melatonin synthesis and circulating melatonin are elevated at night. In addition, it has a significant role in promoting immunomodulatory and cell protection properties in animals and humans. The property of melatonin to actively react with free radicals distinguishes melatonin from classical hormones [12]. As part of its antioxidant activities, it binds reactive oxygen species more efficiently than vitamin E and glutathione, regulates the activity of antioxidant enzymes such as peroxidases, glutathione reductases, superoxide dismutases and catalases [13]. Melatonin has been proposed to scavenge reactive oxygen and nitrogen species (ROS and RNS respecitively) in mitochondria, leading to improved mitochondrial respiration and increased adenosine triphosphate (ATP) synthesis during stress, which in turn may prevent the apoptotic cascade [14]. Melatonin can then be seen as an intermediate regulator of programmed cell death. At the genomic level, melatonin regulates gene expression by acting on antioxidant activity of enzymes and cellular mRNA levels of enzymes under conditions of basal and elevated stress [15]. Research studies on melatonin and its role in human physiology are numerous. In correlation with its beneficial effects, supplements and melatonin-based drugs are currently in use for a treatment of sleep disorders and jet-lag. After detection of melatonin in humans and animals, researchers discovered melatonin in algae and confirmed a circadian rhythm that persists even when organisms are kept in constant darkness [16]. That discovery posed a question about melatonin existence and physiological role in plants. Initial research that attempted to measured concentrations of melatonin in plant encountered a difficulty to extract and measure exact concentrations of melatonin. Only in 1997, melatonin concentrations in some medicinal plants were measured and reported [17]. Melatonin was discovered in organisms such as bacteria, mono- and multicellular algae, invertebrates, vertebrates, but lastly in some

plants [18–20]. In plants, chloroplast and mitochondria are organs where melatonin is produced. Several metabolic pathways of melatonin synthesis were discovered; classic pathway of melatonin synthesis via tryptophan is presented in Fig. 2. and is considered the main pathway in normal growth condition [21].

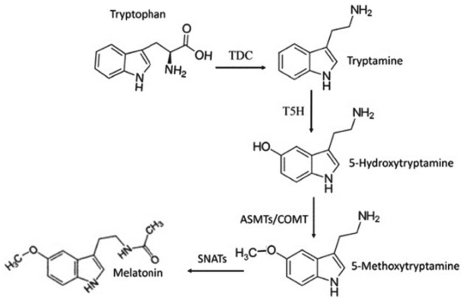

Fig. 2. Classical pathway of synthesis of melatonin in a plants (other pathway includes shikimic acid via tryptophan), (TDC,tryptophan decarboxylase T5H, tryptamine 5-hydroxylase; SNATs, several different serotonin N-acetyltransferases; ASMTs, several different N-acetylserotonin-O-methyltransferases, COMT, caffeic acid O-methyltransferase)

Various extraction and detection methods have been used and melatonin has been independently reported to be present in many plants and plant products. Plants/plant products investigated were tobacco, rice, maize, wheat, sour cherries and banana, coffee beans among others [22]. High concentrations of melatonin have been found in the medicinal plants of chrysanthemum, *Tanacetum parthenium*, and St. John's wort, *Hypericum perforatum* [17]. The amounts of melatonin in these plant tissues were several micrograms per gram of tissue (amounts much higher than those found in blood). Meanwhile, various plants containing high amounts of melatonin have been shown to respond to intense light, especially UV, by increasing methoxyl indole [22]. As shown in subsequent research, melatonin concentrations differ not only among plant species but also among varieties of the same species, and depend on the degree of growth, location, specific plant organ, exposure to the environmental stress as well as the time and season of harvest [17, 23].

The measured level of melatonin in a different plant so far varied from 2–5000 μg/g of dry matter [24]. Interestingly, levels of melatonin measured in vertebrate blood and tissue are usually in pg/ml and pg/g protein. Exact concentration of melatonin either in the blood or in a different plant tissues is difficult to estimate. It highly depends on a metabolic process, diet, total antioxidant status and day/night cycle in humans as well as a total pathophysiological profile. In plants, levels are affected by genotype, stage of development, plant tissue, and significantly by environmental factors such as salinity, cold, heat stress, ultraviolent radiation, heavy metals and chemical stress [25].

In addition, a number of extraction methods and measurement of concentrations have been used in a research studies without exact validation in terms of sensitivity

due to the absence of standard values and specificity and metabolic stability of melatonin molecule. Effect of consummation of edible plants on blood levels of melatonin have been measured, but again this data is relative considering above mentioned factor and fast catabolism of melatonin in humans. So far, research shows that melatonin in plants influence seed germination, crop growth, fruit ripening, postharvest preservation. It has a role as a stress mediator, growth regulator, and antimicrobial agent [26]. As an antioxidant, melatonin is directly involved in a redox network that controls a biochemical, cellular and physiological processes. More research is needed to detect and define biosynthesis, catabolism, transport, receptor(s), and physiologi-cal effects of phytomelatonin that is considered endogenous melatonin. This system-atic review will collect and analyze a published researched about endogenous mela-tonin in a plants and analyses method of quantification of melatonin and deter-mined concentrations.

2 Methodology

This systematic review was conducted in accordance with the established guidelines from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement [27], to the best of our ability.

2.1 Procedure and Search Strategy

All scientific papers were found by searching the PubMed database. Literature search was performed during month of October, 2022. All studies in this review were exclusively found through a thorough search strategy on PubMed. Publication time limitations were not set. Scientific articles and reviews (except those fulfilling the exclusion criteria) published on PubMed, which included studies about melatonin detection in plants were included in the systemic review. Scientific literature, which were scientific articles on melatonin without specific definition of melatonin as a phytomelatonin, articles on experimental modification in *Arabidosa thaliana* as a research model plant, studies on genetic modifications in a different plant in regards to melatonin levels, studies focusing on melatonin use as a supplement, and all studies about the role of melatonin in humans and animals were excluded. Systematic reviews describing mainly physiology of melatonin in a plants were excluded too. Studies that were eligible for inclusion if the following applied: the study in question examine determine concentrations of melatonin in a different plant with published detection method. Studies that analyzed plant mixture or did not have the method precisely defined were excluded. Only studies that are in the English language were considered. Pre-prints or other types of studies that are not peer-reviewed were also excluded from this review. Initially, literature search was performed in a period from 2012. to 2022. However, results did not return a complete data on a basic of search terms except several reviews. Thus, search was expanded without time limitation. Initial search strategy was initiated with the following search term: melatonin and plants without time limitation with the search words only in title/abstract. Return search result showed 796 articles. After manual screening of a selected publications, it was apparent that search terms were mentioned in a number of abstracts that did not directly correlate to a topic defined in inclusion criteria. Surprisingly, many of the publications mentioned melatonin the plants but the research deviated the significantly from

the keywords. Several different search were attempt to select a research papers focusing on phytomelatonin concentration with a described method of quantification but in a last ten years. New modified search was performed with the following search term: melatonin and plants and quantification.

2.2 Selection Process

Following the search query and after addition of search word quantification, each search result (n = 19) was manually screened by one individual without any automation tools. For the first screening process, the title of each result was exclusively considered. If the title was apparently irrelevant to the objectives of this review, it was disregarded and did not proceed to the second screening step. Following title screening, the number of potential studies decreased (n = 15). Most results were filtered in the first two screening steps by going through the title and abstract, respectively. At last, a few (n = 6) articles were excluded after a full-text analysis. Finally, eleven full text articles are excepted for the analysis in this review. After full article screening, manual search was performed again since based on our previous knowledge, selected articles by a keyword did not represent appropriate data. Articles are searched by title and abstract with data correctly represented in an abstract. Number of studies, in fact mentioned key words of our search, but full text article was related to the wide topics on melatonin.

2.3 Data Collection Process

All data was retrieved by the author(s) independently and manually. For each selected study, we obtained the full text publications and extracted the following data into a spreadsheet: author, year of publication, research design, key ideas of research, table with plant names, melatonin concentrations and detection method.

2.4 Study Risk of Bias Assessment

Each study was thoroughly read at least once by two reviewers, without any automation tools used in the process. In order to Human error would therefore be a potential factor; however, several steps and methods were practiced in order to minimize errors when extracting data. These include double-checking the data and using formulas to notice considerable deviations of data that could imply a faulty datapoint. One reviewer assessed all studies and extracted the data with supervision. Each study may have had its respective biases, which were noted and up for discussion.

3 Discussion

After intensive research of melatonin in humans and animals, and conformation of melatonin as a strong antioxidant, a number of studies focused on detection, quantification and biosynthesis of melatonin in a plants [28]. In addition, the role of melatonin in mediating abiotic and biotic stress is intensively researched in last decades [29]. The difficulty of detection of melatonin in plants can be explained by a number of factors that are affecting the determination of an exact concentration.

Melatonin levels depend on genetic traits of the plant, the photoperiod, plant growth stage, and abiotic stressors such as temperature, drought, ultraviolet light exposure, and agrochemicals. Resent research discovered ability of plants melatonin to interact with heavy metals that plants have been exposed to and control transport of the metal ions across organs in the plant [25].

Concentration of melatonin in a tested plant species range from 0.001 ng/g DW to 5800 ng/DW. Some of the traditional herbal medicine such as Chinese licorice showed significantly high values as 34000 ng/g [28].

Roots, shoots, leaves, seeds, flowers and fruits, have been used for the detection of melatonin but the highest concentrations were detected in reproductive organs, particularly in seeds. Manchester et al. [18] evaluated melatonin concentrations in the seeds of 15 different plants and all had high indole levels. Their values varied from 2–200 μg/g dry matter with the highest concentrations in white and black mustard seeds Interestingly that the highest level of melatonin measured in all plant organs so far was in pistachios (Pistacia vera), where reported values ranged from several mg/ml methanol extract [30].

Proposed explanation for such high values of melatonin was necessity of the plant to overcome harsh growing conditions. Number of other studies confirmed that various plants with high melatonin concentration have an increased capacity of stress tolerance. In order to analyze a literature, it is important to distinguish between exogenous melatonin (one that was used to treat different plant organs, or soil and endogenous one that is produced by a plant itself).

Determination of a melatonin concentrations in a samples us affected by a type of the sample and actual concentration that can be as low as low ng values. Any loss during sample preparation and purification will return a high error. Type of plant and organ, grow conditions, soil composition, water availability, heavy metal presence all are significantly changing melatonin concentration. When starting from raw material necessary steps for the melatonin determination involve extraction from raw material, purification of obtained extract and quantification with a selected method. In Table 1 are presented standard methods utilized for the extraction of melatonin.

Table 1. Standard methods utilized for the extraction of melatonin [31]

Solvents: Methanol 10–50%, acetone 89%; 1 M tris-HCl, 0,4 M perchloric acid, 0,1 M EDTA, 0,05% $Na_2S_2O_5$, 10 M ascorbic acid; 50 mM phosphate buffer, pH 7,4, chloroform;
Freezing or drying: fresh frozen, dry
Grinding: M or LM
Mixing: 3 min to 15 h
Light exposure: dark or dim light
Drying: vacuum, N_2 gas, speed vac. Evaporation
Length of extraction methods: from 14 min to 50 min

Selection of extraction procedures vary significantly even when instrumental methods was the same such as LC-MS [31]. Compared two different sample extraction procedures: a direct-sample extraction (DSE) and a homogenized- sample extraction (HSE) [32]. DSE showed higher recovery rates are obtained both in standard solutions and in plant samples. Extraction methods based on acetone or perchloric acids were tested in a study that attempted to compare quantification methods of melatonin in phototrophic organisms. Results showed no substantial difference in melatonin quantification when compared two extraction methods. In the next step of a sample preparation purification techniques that were used included liquid-liquid extraction, micro extraction by a packed sorbent, solid phase extraction and HPLC purification. These methods were not compared for the same sample and the same method of detection.

In a Table 2. Are presented instrumental methods for the melatonin quantification published in a literature [32].

Table 2. Instrumental methods for the melatonin quantification [32]

Method	Detector
LC-UV, Liquid Chromatography with UV detector	UV
LC-ECD-UV, Liquid Chromatography with electrochemical detector and with UV detector	Electrochemical detector with UV detector
LC-FLD, Liquid Chromatography with fluorescence detector	Fluorescence detector
LC-MS, Liquid Chromatography–mass spectrometry	Mass spectrometry
LC-ECD, Electrochemical (EC) detection (ECD) coupled with Liquid Chromatography	Electrochemical (EC) detection (ECD)
ELISA	
HPLC-MS, High performance liquid chromatography mass spectrometry	Mass spectrometry
HPLC-ECD, Electrochemical (EC) detection (ECD) coupled with HPLC	Electrochemical (EC) detection (ECD)
LC-MS-MS, Liquid Chromatography–mass spectrometry-mass spectrometry	Mass spectrometry
RADIOIMMUNOASSAY	
HPLC-FD-ELISA, High performance liquid chromatography with fluorescence detecor-ELISA	Fluorescence detector
TOF-LC-MS, Time of Flight (TOF) Liquid Chromatography-Mass spectrometry	Mass spectrometry
RIA & GC-MS, Radioimmunoassay, Gas chromatography-Mass spectrometry	Mass spectrometry
Ultra-performance liquid chromatography (UPLC)	Mass spectrometry

In one of the analyzed article [33] authors reviewed LC and LC-MS as the methods of choice for melatonin detection in a complex matrix. They concluded that LC-MS method was highly selective and sensitive when determining concentration of melatonin, especially because concentration of serotonin can be determined at the same time. Authors reviewed published articles about methods used for the determination of melatonin in period from 1992 to 2008.

Accurate determination of melatonin has been attempted using different instrumental techniques. Methods such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RAI) have been used early on but due to the cross-reactivity of antibodies reduced specificity and accuracy was observed. Concentrations of melatonin determined by these methods is still used in literature and reviews, and should be only considered when other data is not available. High performance liquid chromatography (HPLC) was most common and frequent technique used for analysis of melatonin. MS detector improved accuracy and sensitivity compared to other defectors used such as fluorescence detector, electrochemical detector and UV detector.

HPLC with electrochemical detector is more sensitive compared to the fluorescence detector. However even with electrochemical detector, sensitivity of the method is not high. Plants are containing a number of well-known compounds with an oxidative ability such as alkaloid, carotenoid, coumarin, flavonoid, phenolic, and terpenoid group, serotonin that is precursor of melatonin, and enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase. In these cases, extraction of melatonin and purification methods are crucial for the validity of data. Liquid chromatography-mass spectrometry (LC-MC) is one of the better methods, with a high specificity for melatonin. But, at the same time, due to the matrix effect, LC-MC method is not highly specific.

Ultra-performance liquid chromatography (UPLC) coupled with MS detection was recently used for the determination of melatonin in shoot and root of the rice. The limit of detection was 0.03 pg. Method was highly specific and samples were pretreated in one step liquid-liquid extraction [28].

Table 3 lists examples of edible plants, melatonin concentrations in ng/g of dry weight or fresh weight, used method for detection and reference of original publication [34]. The highest concentration was found in a different type of coffee beans of 6800 ng/g. Interestingly, when these high concentrations were first discovered, it was taught that melatonin is product of a chemical reaction that was part of a production process. Subsequent research showed that determined concentration was actual concentration in melatonin and varied between the different coffee species. Methods used for the concentration determination was mostly HPLC with either MS or ECD detectors. Interestingly, Caniato *et al.*, found high melatonin levels in alpine plants that was explained as resistance to abiotic stress these plants are exposed to [33].

In Table 4, presented are the selected concentration of melatonin from the screened literature. Selected plants are showing either the highest or lowest values of melatonin concentration. Interestingly, the lowest values are obtained either by radioimmunoassay or ELISA method that were recently described as a method with a low sensitivity. However, Carsten et al. [34] found out that methods with HPLC purification and quantification by ELISA and HPLC-PD were highly sensitive for melatonin determinations.

Compared to other methods, determination of a concentration of melatonin in coffee beans by LC-MS/MS show highly accurate results compared to other research studies for the same plant but with a different method.

Table 3. Concentrations of melatonin in different plants

Common name	Latin name	Melatonin [ng/g DW](or FW*)	Method	Ref
Coffee robusta	*Coffea canephora Pierr*	5800	HPLC-MS	[35]
Coffee arabica	*Coffea arabina (L.)*	6800	HPLC-MS	[35]
Black pepper	*Piper nigrum (L.)*	1093	HPLC-MS	[36]
Wolf berry (goji)	*Lycium barbarum (L.)*	530	HPLC-MS	[37]
White radish	*Raphanus sativus (L.)*	485	HPLC-MS	[37]
White mustard	*Sinapis alba (L.)*	189	HPLC-ECD	[18]
Black mustards	*Brassica nigra (L.)*	129	HPLC-ECD	[18]
Curcuma	*Curcuma aeruginosa Roxb*	120	HPLC-MS	[37]
Wolf berry	*Lycium barbarum*	103	HPLC-ECD	[18]
Burmese grepe	*Baccaurea ramiflora Lour*	43,2	HPLC-MS	[36]
Fenugreek	*Trigonella foenum-graecum (L.)*	43	HPLC-MS	[18]
Almond	*Prunus amygdalus (Batsch)*	39	HPLC-MS	[18]
Sunflower	*Helianthus annus (L.)*	29	HPLC-MS	[18]
Fennel	*Foeniculum vulgare (Gilib.)*	28	HPLC-MS	[18]
Agati	*Sesbania glandiflora (L.) Desv*	26,3	HPLC-MS	[36]
Bitter melon	*Momordica charantia (L.)*	21,4	HPLC-MS	[36]
Alfalfa	*Medicago sativum (L.)*	16	HPLC-MS	[18]
Green cardamom	*Elettaria cardamomum (White et Maton)*	15	HPLC-MS	[18]
Flax	*Linum usitatissimum (L.)*	12	HPLC-MS	[18]
Linseed (flax)	*Linum usitatissimum (L.)*	12	HPLC-MS	[18]
Java bean	*Senna tora (L.) Roxb*	10,5	HPLC-MS	[36]
Sesban	*Sesbania sesban (L.) Merr*	8,7	HPLC-MS	[36]
Anise	*Pimpinela anisum (L.)*	7	HPLC-MS	[18]
Celery	*Apium graveolens (L.)*	7	HPLC-MS	[18]
Coriander	*Coriandrum sativum (L.)*	7	HPLC-MS	[18]
Poppy	*Papaver somniferum (L.)*	6	HPLC-MS	[18]
Walnut	*Juglans regia (L.)*	3,5	Radioimmunoassay	[38]
Milk thistle	*Silybum marianum (L.)*	2	HPLC-MS	[18]

(*continued*)

Table 3. (*continued*)

Common name	Latin name	Melatonin [ng/g DW](or FW*)	Method	Ref
Sweet cherries	*Prunus avium (L.)*	120*	HPLC-MS	[39]
Tart cherries	*Prunus cerasus (L.)*	19,5*	HPLC-MS	[40]
Grapevine	*Vitis vinifera (L.)*	18*	HPLC-ECD	[41]
Cherry	*Prunus cerasus (L.)*	18*	HPLC-ECD	[40]
Corn	*Zea mays (L)*	14–53*	HPLC-MS	[42]
Cucumber	*Cucumis sativus (L)*	11–80*	HPLC-MS	[42]
Strawberry	*Fragaria x ananassa (Duch.)*	11,3*	LC-MS-MS	[43]
Pomegranate	*Punica granatum (L.)*	5,5*	HPLC-ECD	[44]
Tall fescue	*Festuca arundinmacea*	5,3*	HPLC-ECD	[45]

*corresponds to FW. DW, dry weight; FW, fresh weight

Table 4. Concentration of melatonin in different plants

Common name	Scientific name	Melatonin (ng/g)	Method	Ref
Festival strawbery	*Fragaria ananassa*	11,26	HPLC-MS	[46]
Montmorency tart cherry	*Prunus cerasus L*	13,36	HPLC-EC	[40]
		12,3	HPLC-MS	[47]
Gordal tomato	*Lycopersicon esculentum Mill*	17,1	HPLC-MS	[46]
Sunflower seed	*Helianthus annus*	29	HPLC-ECD	[18]
Almond seed	*Prunus amygdalus*	39	HPLC-ECD	[18]
Green cardamom seed	*Elettaria cardamomum*	15	HPLC-ECD	[18]
Flax seed	*Linum usitatissimum*	12	HPLC-ECD	[18]
Alfalfa seed	*Medicago sativum*	16	HPLC-ECD	[18]
Fenugreek seed	*Trigonella foenum-graecum*	43	HPLC-ECD	[18]
Black mustard seed	*Brassica nigra*	129	HPLC-ECD	[18]
White mustard seed	*Brassica hirta*	189	HPLC-ECD	[18]
Extra virgin olive oil	–	71–119 pg/ml	ELISA	[48]
Coffee	–	5800	LC-MS/MS	[49]
Beetroot		0,002	RIA and GC-MS	[50]
Kiwifruit	*Actinidia chinesis*	0,02	Radioimmunoassay	[45]
Welsh onion	*Asparagus officinalis L*	0,01	Radioimmunoassay	[45]
Banana	*Musa sapientum L*	0,01	HPLC-FD & ELISA	[51]

(*continued*)

Table 4. (*continued*)

Common name	Scientific name	Melatonin (ng/g)	Method	Ref
Strawberry	*Fragaria magna*	0,01	Radioimmunoassay	[45]
Cabernet franc grape (Skin)	*Vitis vinifera L*	0,01	HPLC-FD & ELISA	[52]
Van cherry	*Prunus avium L*	0,01	HPLC-MS	[53]

4 Conclusion

After the screening of available publications that studied melatonin concentration in plants with different instrumental methods, we analyzed a number of studies and available data. Determining melatonin concentration is a difficult task, and comparison of a results in a same plant but in a different study cannot give completely valid results. Concentration and accumulation of a melatonin in a plant heavily depends on environmental factors, and biochemistry of melatonin in the plant under those conditions.

Melatonin is a strong antioxidant and any heavy stress and production of a free radicals in a plant exposed to abiotic stress will increase its concentration for a limited period of time. Future studies should measure melatonin concentration in correlation to environmental factors and soil composition compared to a sample kept under control condition.

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Determination of Methanol Content in Homemade Brandy Using Raman Spectroscopy

Amina Stambolić(✉), Emina Drkić, Sabaheta Keser, Nermina Spaho, and Enisa Omanović-Mikličanin

Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
a.stambolic@ppf.unsa.ba

Abstract. Methanol and ethanol are alcohols naturally produced during fermentation. While similar in physical and chemical properties their toxicity is very different. Small quantities of methanol as low as 10 ml can cause permanent blindness while larger quantities have lethal outcomes. The history of human alcohol consumption is marked with instances of accidental methanol poisoning which led to many lives being lost. Methanol content in alcoholic beverages is highly monitored and regulated by law to prevent accidental poisonings. The method commonly used for methanol content determination is the spectroscopic method using chromotropic acid. This method requires a lengthy process of sample preparation and the use of many chemical reagents which is not the most practical approach, especially if there are many samples to be analysed. Raman spectroscopy is a non-destructive, quick instrumental method that can be used to determine methanol content in alcoholic beverages utilising the characteristic band of methanol noticable in the recorded spectra of samples. Considering the tradition of homemade brandy production and consumption in Bosnia and Herzegovina as well as in neighbouring countries, accidental methanol poisoning is a possible occurence. The goal of this study was to analyse the methanol content in homemade brandy samples which usually do not undergo any inspection before consumption. Fifteen samples of fruit brandy where analysed using both Raman spectroscopy and the chromotropic acid method. Out of these fifteen samples only one of them had a methanol content below the legal limit.

Keywords: Methanol · Alcoholic beverages · Raman spectroscopy

1 Introduction

Brandy, also known as *rakija* or *rakia* in the Balkans, is a very popular strong alcoholic beverage produced by distilling fermented fruit. Although produced industrially and available commercially, there is also a tradition of making rakija at home. Rakija is mostly made from fermented plums in which case it's called *šljivovica,* but can also be made from other types of fruit such as apples, cherries, apricots, quinces and grapes. The

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ethanol content is usually in the range from 40 to 45 vol% but homemade brandy can be even stronger, up to 60 vol% as it is usually made according to the maker's preferences. [1].

During the fermentation process of the chosen fruit, the sugars are converted into ethanol via microbial activity. Since traditional fermentation occurs via spontaneous inoculation from the substrate and processing equipment. Accordingly, fermentation is typically carried out by mixed cultures. Therefore, contamination with bacteria, fungi, and other yeasts could lead to the production of methanol and other products. Additionally, because methanol has a lower boiling point (65 °C) than ethanol (78 °C), it might be concentrated even more during the distillation process. Methanol is not produced from sugar but from pectin. Many strains of bacteria and yeasts possess pectinolytic enzymes such as pectin methyl esterase, pectin lyase, and polygalacturonase which ultimately lead to the production of methanol, an unwanted side product. [2].

2 Methanol and Ethanol

Methanol (CH_3OH) is the simplest aliphatic alcohol with just one C atom. It is usually produced by distilling wood. It's a volatile, flammable, colorless liquid which forms mixtures with water and has a characteristic alcoholic scent. It's widely used as a solvent in chemical labs as well as in industrial synthesis of organic compounds.

Ethanol (C_2H_5OH) is by far the most utilized alcohol to the point where it is synonymous with the term. It is mostly consumed as a component of alcoholic beverages for its intoxicating effect on the human body (Fig. 1).

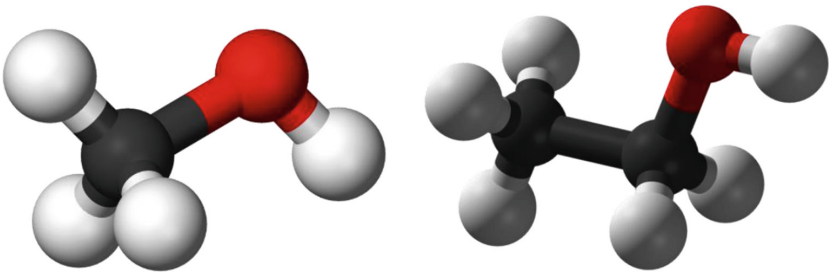

Fig. 1. Ball and stick model of methanol (left) and ethanol (right)

Organoleptically, methanol and ethanol are indistinguishable which is the primary reason for accidental methanol poisoning. Although very similar in physical and chemical properties, methanol and ethanol have different effects on the human body. Where ethanol is toxic only if consumed in large quantities, methanol is classified as moderately to highly toxic to humans. Quantities as low as 10 mL of pure methanol are known to cause permanent blindness by destroying the optic nerve [3].

Reports on the fatal dose of methanol vary with some sources citing 15 mL as potentially fatal [3], while others place the lethal dose in the wide range from 30 to 240 mL [4].

Methanol is toxic by two mechanisms. First, methanol poisoning, like ethanol poisoning, can be fatal due to its CNS depressant properties. Second, it is metabolized to formic acid (formate anion) via formaldehyde in a toxication process that is started by the liver's alcohol dehydrogenase enzyme [5].

Formate is toxic because it inhibits mitochondrial cytochrome c oxidase, causing hypoxia at the cellular level, and metabolic acidosis, among a variety of other metabolic disturbances [6].

3 Methanol in Brandy

As stated previously, methanol is inevitably produced during fermentation of fruit and therefore can be a contaminant in the final product. Brandy produced from fruit richer in pectin will have a higher methanol content compared to fruit brandy made from softer fruit with a lower pectin content. Over the history of brandy production various methods for methanol removal have been developed.

One of the most common ways to prevent methanol contaminating the final product is by discarding the distillate which forms before the system temperature reaches the boiling point of ethanol (78 °C). To allow enough time for all of the methanol to evaporate before the boiling point of ethanol is reached, some producers advise using low quality fuel for the heating of the system. Low quality fuel prevents the production system from reaching higher temperatures too quickly, and consequently the simultaneous boiling of methanol and ethanol. To further ensure methanol removal, it is recommended to discard the first 500–750 mL of distillate per 100 L of brandy produced [7].

Another method for methanol removal is the so called "bottle method" utilized in homemade production. The distillate is let to flow over an open bottle placed in a bigger container. The rationale behind this method is that methanol will sink to the bottom of the bottle because its density is higher than the density of ethanol (0,792 g/mL vs 0,789 g/mL). However, experts advise against the use of this method as the difference in density is not big enough to ensure effective separation of methanol.

Because of its detrimental effects on human health, methanol content in commercially available alcoholic beverages is highly monitored and the legal limits determined by a rulebook [8]. According to the *Rulebook on defining, describing, presenting, marking and protection of geographical indications of strong alcoholic beverages* in Bosnia and Herzegovina, fruit brandy should not contain more than 1000 g of methanol per hectoliter of pure ethanol. The exceptions to this rule are brandy made from plums, pears (excluding the sort *Pyrus communis* L. cv "Williams"), apples, raspberries, blackberries and apricots where the legal limit is 1200g per hectoliter of pure ethanol and brandy made from quinces, juniper berries and the *Pyrus communis* L. cv "Williams" sort of pear with the legal limit of 1350 g of methanol per hectoliter of pure ethanol [8]. The different legal limits are in accordance with the pectin content of different fruit sorts which leads to a higher methanol content.

4 Methanol Determination

4.1 Chromotropic Acid Method

The chromotropic acid method is a spectrophotometric method used to detect and quantify methanol in samples of alcoholic beverages. The method is based on the oxidation of methanol to formaldehyde using potassium permanganate. The resulting formaldehyde forms a purple-colored complex with the chromotropic acid in the presence of concentrated sulfuric acid. The absorbance of the complex is measured at 570 nm and compared to the absorbance of a standard or determined using a calibration curve constructed using a standard series [9].

4.2 Raman Spectroscopy

Raman spectroscopy is a vibrational spectroscopy method which provides a very specific spectrum for every substance analyzed. The unique position and intensity of bands on a Raman spectrum is regarded as a "fingerprint" of a particular substance [10].

Raman spectroscopy is based on the ability of molecules to absorb or emit energy in the form of photons and change their own energy in this way. When irradiated by monochromatic light, the molecules scatter the light with a change in the wavelength characteristic for that molecule. Raman spectroscopy is non-destructive and contactless so working with very small quantities of a sample is made easier. Another advantage is the ability to acquire spectra in seconds and through transparent packaging like glass and plastic which is convenient for analyses of volatile or toxic liquids [10].

5 Method and Materials

5.1 Samples and Reagents

Samples of homemade fruit brandy were collected from friends and acquaintances. Out of fifteen samples collected, four were plum brandy (S1, S2, S3 and S4), three apple brandy (J1, J2 and J3), two were apricot brandy (K1 and K2), two pear brandy (Kr1 and Kr2), one quince brandy (D1), one grape brandy (L1) and two samples of plum brandy infused with herbs (T1 and T2).

All reagents were acquired through commercial sources and used without further purification. Ethanol and methanol of spectroscopic grade were used for the standard solutions.

5.2 Determination of Methanol Content Using the Chromotropic Acid Method

The ethanol content of the samples was measured using a portable Mettler Toledo densimeter. The samples were then diluted to 2,5 vol% of ethanol using distilled water. A stock solution of methanol is prepared by pipetting 1 mL of pure methanol into a 100 mL flask that is then filled to the mark with a 2,5 vol% solution of ethanol. The stock solution was then used to prepare standard solutions of 0.16, 0.32, and 0.80 vol%/p.e. (pure ethanol). 2,5 mL of the samples and the standards were pipetted into test tubes and

1 mL of sulfuric acid (1:3) was added. To ensure the oxidation of methanol, 1 mL od 1% potassium permanganate was added. After 10 min, the mixture was clarified using a saturated solution of sodium sulfite. 0,5 mL of 2% chromotropic acid is then added to the mixture along with 5 mL of concentrated sulfuric acid.

The treated samples and standards were then incubated for 20 min at 65 °C. After cooling the absorbance was recorded at 570 nm using a Varian Cary 1E UV-Visible Spectrophotometer.

5.3 Determination of Methanol Content Using Raman Spectroscopy

For the acquisition of Raman spectra of the samples no pre-treatment was required. A series of standard solutions of methanol in pure ethanol were made for the calibration curve. The concentrations used were 2.5, 5, 10 and 20 vol%/p.e.

The spectra were recorded using the ANT Flipper with a 785 nm excitation laser. The standards, samples as well as pure ethanol and methanol were poured into amber glass vials which were closed to prevent evaporation. The spectra were acquired in the range from 400 to 2000 cm^{-1} with a 10 000 ms acquisition time. The spectra for each sample were acquired five times.

6 Results and Discussion

The spectra of pure ethanol and pure methanol are given in Fig. 2.

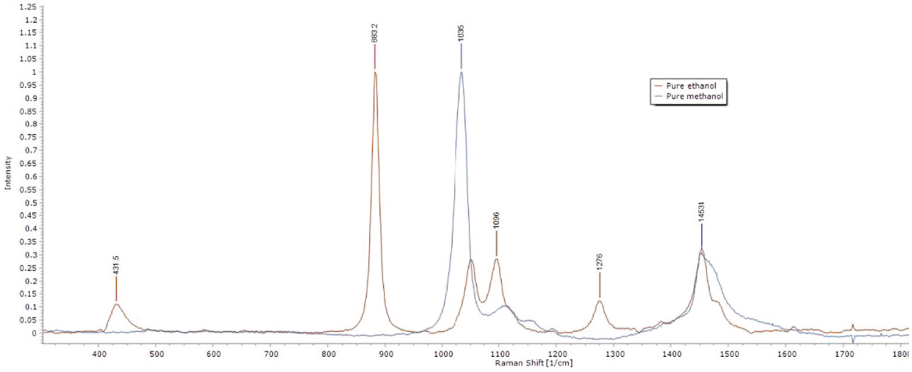

Fig. 2. The Raman spectra of ethanol and methanol

The main peaks important for spectra interpretation and processing are found in the 800–1200 cm^{-1} range. This area of the spectra contains the very intensive peak of methanol found at 1035 cm^{-1} that is assigned to the stretching of the C-O bond, and the C-C stretching of ethanol found here at 883 cm^{-1}. Other characteristic vibrations for ethanol and methanol are given in Table 1.

All spectra were recorded under the same conditions and were subjected to a baseline correction and normalization according to the ethanol band at 883 cm^{-1}. To measure

Table 1. The characteristic peaks in the Raman spectrum of ethanol and methanol

Raman peak (cm^{-1})			
Ethanol			
Measured	Literature	Assignment	Intensity
883	883	C-C stretching	vs
1052	1054	C-O stretching	s
1096	1096	CH_3 rocking	s
1455	1454	CH_3 bending	m
1482	1479	CH_3 bending	vw
Methanol			
1035	1033	C-O stretching	vs
1112	1106	CH_3 rocking	w
1162	1149	CH_3 bending	s
1461	1448	-	s

methanol content in standard solutions and in samples the normalized spectra of ethanol was subtracted from the spectra of the mixtures and the intensity of the peak at 1035 cm^{-1} measured.

According to the concentrations of standard solutions and the peak intensity at 1035 cm^{-1}, a calibration curve has been constructed (Fig. 3).

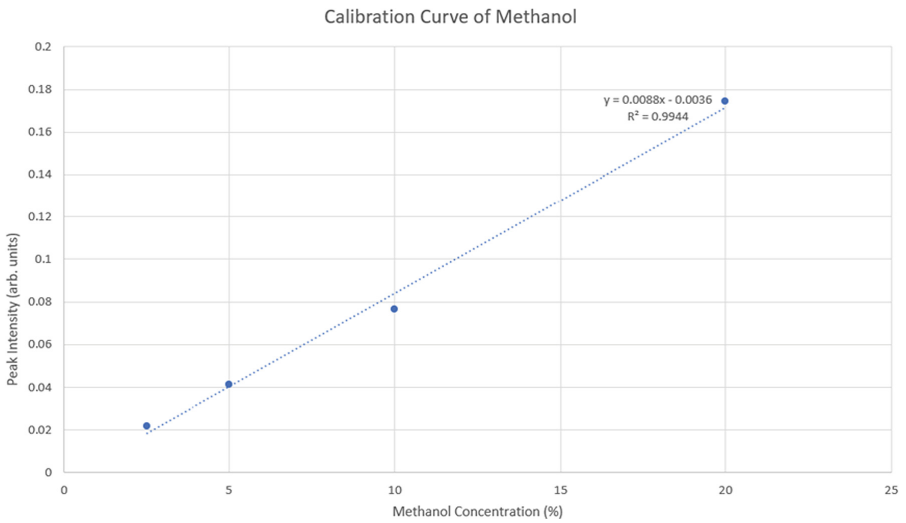

Fig. 3. Calibration curve of methanol

When processing the spectra of the alcohol mixtures no shift in peaks has been detected due to the absence of water. However, the spectra of brandy samples show a significant shift of some bands, primarily the C-O stretching of ethanol which experiences a shift from 1052 cm^{-1} to 1046–7 cm^{-1} (depending on the sample), as well as the C-O stretching of methanol which can be found at 1030 cm^{-1} as opposed to 1035 cm^{-1} in pure methanol. The comparison of the peak positions of a brandy sample and the ones of pure ethanol are given in Fig. 4. The C-C stretching of ethanol also experiences a shift in position from 883 to 879 cm^{-1}. This occurs most likely due to the presence of water molecules in brandy samples which mitigate the formation of hydrogen bonds. Hydrogen bonds can cause the peak position to shift towards lower wave numbers. This is further supported by the fact that the bonds experiencing the biggest changes in position are the ones most affected by the hydrogen bond formation.

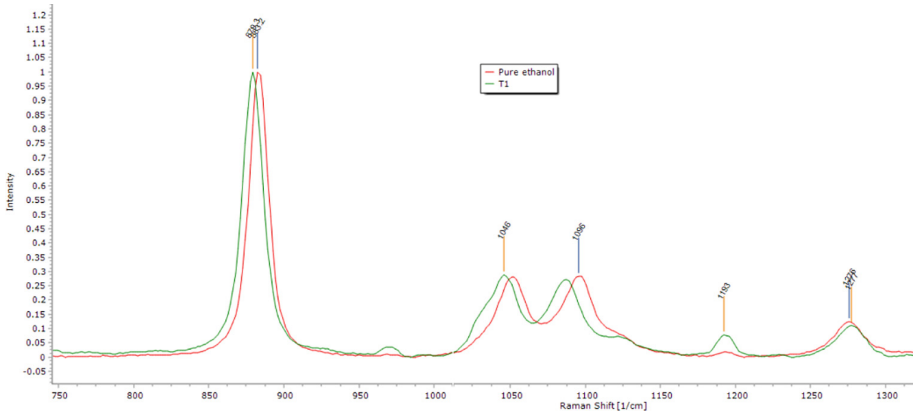

Fig. 4. The difference in peak positions between pure ethanol and a sample of brandy (T1)

The band shift that occurs in aqueous solutions demands the additional step of curve fitting this particular band in the goal of successfully determining the concentration of methanol. The results are displayed as the mean value of five analyses along with the standard deviation.

The absorbance of samples tested using the chromotropic acid was measured at 570 nm and the concentration calculated using the calibration curve equation. The analyses were done in duplicate and the results expressed as the mean value.

The results of both methods are given in Table 2.

Out of all the samples analyzed, only sample J3 (apple brandy) has the methanol content within the legal limits; 1.24% of methanol corresponds to 980 g of methanol per hectoliter of pure ethanol and the legal limit for apple brandy is 1200 g/100 l p.e.

Table 2. Results for the methanol content obtained using the chromotropic acid method and Raman spectroscopy

Sample name	MeOH concentration (Chromotropic acid method) %	MeOH concentration (Raman spetroscopy) %
S1	7.60	7.53 ± 0.13
S2	1.98	3.11 ± 0.09
S3	3.82	4.50 ± 0.11
S4	7.65	6.83 ± 0.08
T1	11.49	10.40 ± 0.15
T2	4.50	4.56 ± 0.16
K1	7.11	5.66 ± 0.08
K2	1.68	1.89 ± 0.09
J1	4.32	5.09 ± 0.30
J2	2.86	2.41 ± 0.05
J3	1.76	1.24 ± 0.04
L1	14.02	12.30 ± 0.14
D1	5.09	5.01 ± 0.29
Kr1	1.88	2.30 ± 0.04
Kr2	1.84	1.93 ± 0.09

7 Conclusions

Compared to the standard method of determining methanol content, Raman spectroscopy offers an alternative which is less time consuming as it doesn't require sample preparation and long-term cost effective as it doesn't require any additional use of chemicals. Based on our results we conclude that Raman spectroscopy is a viable method for methanol content determination and should be further researched and optimized in the hopes of wide spread industrial implementation.

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Economic Analysis of Semi-hard Cheese Production Depending on the Applied Technology

Tarik Dizdarević[1(✉)], Adin Fakić[2], Vedad Falan[1], Nedim Hadžiomerović[3], Mersiha Alkić-Subašić[1], Nermina Đulančić[1], Zlatan Sarić[1], and Svijetlana Sakić-Dizdarević[1]

[1] Faculty of Agriculture and Food Sciences, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina
t.dizdarevic@ppf.unsa.ba
[2] Agrokultura o.d., Ćamila Sijarića 21, 71000 Sarajevo, Bosnia and Herzegovina
[3] Velvet Trade d.o.o., Hasana Merdžanovića 42, 71320 Vogošća, Bosnia and Herzegovina

Abstract. The analysis of milk processing in Bosnia and Herzegovina showed the orientation of the dairy sector towards liquid and fermented products. During 2021, the export of dairy products from BiH amounted to 48 million euros (mainly drinking milk and cream), while the import reached 89 million euros, with the import of cheese (predominantly semi-hard) exceeding half of the stated amount. In order to reduce the market deficit of semi-hard cheeses, the research was based on the analysis of the profitability of investment in the technology of semi-hard type cheeses. Business risk assessment using analytical calculations was done on the basis of the applied technology of *Gouda* and *Saint Paulin*, the duration of technological operations, consumption of the amount of milk per 1 kg of cheeses, and the degree of tempering, while other parameters (rennet, culture starter, energy, etc.) were fixed in the calculations. The results of the analysis of the experimentally produced cheeses showed that the production of 1 kg of *Gouda* cheese requires 10.22 l, while for 1 kg of *St. Paulin* cheese is required 8.76 l of milk. Cheese yield was 9.80 kg (*Gouda*) and 8.38 kg (*St. Paulin*). The average duration of the primary production period for *Gouda* cheese technology was 5.01, and 3.83 h for *St. Paulin's* cheese. This research showed that technology of *St. Paulin* cheese is more economical, and this type of cheese can be recommended for the production.

Keywords: technology of semi-hard cheese · cheese yield · economic aspects

1 Introduction

Historically, animal husbandry and milk processing represent one of the most important agricultural branches in Bosnia and Herzegovina. This sector was almost completely destroyed during the citizen's war (1992.–1995.), but after the war, dairy farming gradually recovered and today it represents a very important part of country economy. Bosnia and Herzegovina have also a long tradition of animal husbandry, as well as production

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of milk and dairy products. In addition, the dairy sector creates also opportunities for business and employment, especially in the rural areas, but not only, since plenty of persons from urban parts are working in dairy business (Sekovska et al. 2015).

The quantity of collected cow's milk was 21.571 tons in 2022nd year and decreased for 6.30% compared to 2021st (Agency for Statistics of the Bosnia and Herzegovina, 2022). Modern milk processing in B&H is based on the production of liquid dairy products (sterilized milk and fermented products). In the structure of dairy products consumption in entity Federation of B&H liquid dairy products make up to 80.80%, cheeses represent an average of about 17.00%, while other dairy products make up only 2.20% (Bajramović et al. 2015). In addition to the enormous import of dairy products in B&H, a vital problem of the dairy sector is the lack of a milk drying plant that could regulate market surpluses in the summer period.

The successful development and the introduction of new dairy products to the market is a very complex process that depends on numerous factors (technology, quality, economy, etc.). Cheese ripening represents one of the most important stages during production and in this phase, moisture evaporates from the inner part of cheese towards the surface. At the same time, complex biochemical processes such as glycolysis, lipolysis and proteolysis take place in the cheese, all together forming the sensory properties of the final product (Fox et al. 2017).

Cheese yield is of exceptional economic importance for cheese producers, because small differences in yield can lead to significant differences in profit, and that yield measurement should become a tool not only for cheese production but also for management (Abd El-Gawad et al. 2011). Everard et al. (2008) classifies the factors affecting cheese yield into 2 main groups: milk composition and technological conditions used during cheese production.

In order to assess the profitability, but also to reduce the risk of the investment in cheese technology is necessary to collect and analyze numerous inputs data such as: price of milk, transport costs, labor, process equipment, energy, rennet, starter cultures, etc. These inputs data from the experimental cheese production were used to calculate the economy using the cost calculation model.

The development of new dairy products is usually done in research and development centers (RDC), and this type of multidisciplinary research is related to large dairy companies because they are time-consuming, technical-technologically demanding, and involve many various areas of professions. Dairy sector in B&H is dominantly owned by multinational companies and the development of new dairy products is mainly based on the technologies transfer from "central" laboratories located in the country of origin, because this companies usually do not have departments for research and development in B&H. To overcome this problem some companies made cooperation with Faculty of Agriculture and Food Sciences University of Sarajevo (FAFS) in order to exchange knowledge and experience, and to overcome everyday problems in dairy production.

The aim of this study was to determine the profitability of semi-hard cheeses production depending on different inputs parameters (applied technology, length of technological process, fat content, cheese yield, method of cheese packaging), in order to reduce the market deficit of semi-hard cheese in B&H.

2 Methods

2.1 Analysis of the Dairy Sector in Bosnia and Herzegovina

Analysis of the dairy sector in Bosnia and Herzegovina was done through the desk research method using official data from Agency for Statistics of the Bosnia and Herzegovina, Foreign Trade Chamber of Bosnia and Herzegovina, company "Milkprocessing d.o.o." and Association of the Dairy Industry of B&H. Authors also used many years of knowledge and experience in dairy and cheese sector.

2.2 Technology of Semi-hard Cheeses

Two technologies of semi-hard type cheeses were selected and used for experimental cheese production: *Gouda* (Kammerlehner, J. 1989) and *Saint Paulin* (Scott et al., 1998). All experimental semi-hard cheeses were produced on a volume of 100 l of milk in a pilot plant (Faculty of Agriculture and Food Sciences-FAFS, University of Sarajevo) equipped with two 200 l vats. Sixteen productions of semi-hard cheeses were made in total (8 *Gouda* and 8 *Saint Paulin*).

Fresh, raw cow milk was purchased from dairy company Milkos d.o.o. (Sarajevo) Milk was standardized (2.80% and 3.20%), pasteurized (65 °C/30 min), cooled (32 °C) and inoculated with mesophilic and thermophilic cultures, after 25 min calcium chloride (15–20 g/100 l) and 2g/100 l lysozyme Afilact (ChrHansen, Denmark) were added. CHY-MAX M rennet (Chr. Hansen, Denmark) was added (1g/100 l), and the milk coagulated for 25 to 35 min.

Major difference between two technologies (*Gouda* and *St. Pauline*) of experimental cheeses was during production phase of washing the cheese curd, whereby for *Gouda* cheeses consisted in 35% of whey draining and adding of 25% of hot water, and for *St. Paulin* cheeses 45% of whey was replaced with same amount of water. After, one part of cheese curds was molded and pressed (0.2–0.3 bar) and other part was self-pressed in perforated cheese molds. All cheeses were salted in brine (° Be = 18.50) for 24 h and coated with Cleriplast S (Clerici Sacco Group, Italy).

After salting cheeses were prepared for ripening using three different packaging methods: pressed and packaged in foil (F), pressed without foil (BF), and self-pressed packaged in foil (SP).

2.3 Sensory Analysis

Sensory analysis was used to determine cheeses quality and it was done after 30 days of ripening. Evaluation was performed by five trained sensory experts, using 20-point scoring system, with maximum score of 20 points as follows: 2 points maximum for appearance, 1 point for color, 2 points for texture, 3 points for cut surface, 2 points for odor and 10 points for taste.

2.4 Cheese Yield

First was calculated the amount of milk required to produce 1 kg of cheese (L) according to the formula:

$$L = \text{kg of milk/kg of cheese}$$

In order to express the quantity of liters of milk in kilograms, result was multiplied with the average density of milk = 1.030 (Dozet, 1970).

Cheese yields were calculated using the formula:

$$(R) = 100/L$$

where (R) = cheese yield and L = amount of milk required to produce 1 kg of cheese.

In order to determine cheese mass loss (K) all experimental cheeses were weighed after salting and after 30 days of ripening. The percentage of cheese mass loss is calculated according to the formula:

$$K = (\text{Difference in weight before and after cheese ripening} \times 100)/\text{Kg of cheese before ripening.}$$

2.5 Analytical Model for Estimating the Economics of Semi-hard Cheese Production

Time is an important economic parameter for cheese technology, and it can be divided in the primary and secondary periods. The primary period of cheese production is considered to be the time from the reception of milk in the dairy until the cheese is placed in the ripening room. While the secondary period is considered the period of ripening and distribution of the cheese.

In this research, the duration of all technological operations in 16 experimental productions was measured, and the average time necessary for the production of *Gouda* and *St. Paulin* cheese. Economic efficiency production of semi-hard cheese was calculated on the results of experimental cheeses production in a dairy pilot plant (FAFS, University of Sarajevo).

The economy of production of experimental cheeses was calculated using the cost calculation model. Depending on the observed parameters, the economic efficiency of the production of experimental cheeses was calculated using a cost calculation model.

Falan and Mujčinović (2022) state that the budget of turnover ratio (TR), total costs (TC), production value (VP), cost price (CP), financial result (FR), and production economy (E) and profitability (P) can be successfully used to make business investment decisions.

The turnover ratio (TR) shows number of turnover (cycles) for cheese production during one business year, i.e. how many times an asset is turned over during one business year or 360 days, and is calculated according to the formula:

$$TR = 360/TO$$

where TO = the number of days of one turnover of an asset (production).

The cost price was calculated on 1000 kg of cheese by dividing a kg of cheese by the total costs with the total yield (CP = TC/Y), and the financial result was calculated based on the difference between the total costs and the production value (FR = VP−TC). Production economy (E) was obtained by dividing the production value and the total costs (E = VP/TC) and it is necessary to be E > 1. The percentage of profitability (R) is calculated on the relationship between the financial result and the value of production (P = FR/VP) and multiplied by a factor of 100. This parameter should give us an answer as to whether some production is profitable/profitable.

3 Results

The results of dairy sector analysis showed a great deficit of cheeses, especially of the semi-hard type, as well as extremely small production and export of these type of cheeses, like *Tilsit* (0.00%), *Edamer* (0.01%) and *Gouda* (0.13%). It has been established that on the B&H dairy market dominated production of low-accumulative products with a short shelf life and enormous import of different types of cheese.

Deregulation of the raw milk market during the summer months (lactation peak) could be partially solved by investing in cheese production.

3.1 Analysis of the Dairy Sector

Major part of Bosnia and Herzegovina presents the economic and socio-economic conditions of the mountainous area and provide for the livestock and milk sector the character of the leading fields of agriculture in the country (Jalić et al. 2022).

The analysis of the dairy products market (2021) in B&H showed that dairy products export was 48 million euros (€), while import was 89 million euros. Among all dairy products imported in B&H the most dominant was cheese (47 million euros), especially semi-hard type (Foreign Trade Chamber of Bosnia and Herzegovina 2022).

Muhamedagić & Begović (2019) stated that the export of dairy products in B&H in 2019th was higher for 6,855 tons compared to imports. But ratio between import and export has a huge disproportion in financial terms, because lower price products such as milk and cream were dominantly exported, while higher price dairy products (cheeses) were imported.

3.2 Technology of Semi-hard Cheese

There are various technological ways to improve rate of ripening of cheese and reduction of costs that affect the sensory properties and quality of cheese (Cocolins et al. 2018).

Table 1 shows average duration period in minutes for each technological operation for experimental semi-hard cheeses (*Gouda* and *St. Paulin*) which starts with pasteurization and ends with coating and packing and it was used to calculate the economic parameter, i.e. the turnover ratio (Ko). By comparing the production length of *Gouda* and *St. Paulin* cheeses it was found out that for production of *St. Paulin* cheese was required shorter period (3.83 h), instead of 5.01 h needed for *Gouda* production. Where operations of adding culture, $CaCl_2$, rennet, cutting the curd, and especially washing cheese curd and drying grains lasted diverse.

Table 1. Average duration period (minutes) for each technological operation for experimental semi-hard cheeses (*Gouda* and *St. Paulin*).

	Gouda	***St. Paulin***
1. Pasteurization	15–35	15–35
2. Cooling	10–20	5–20
3. Adding culture, $CaCl_2$	35–45	25–40
4. Adding rennet (salting of milk)	25–35	20–30
5. Cutting the curd	10–15	5–10
6. Release of whey	5–10	5–10
7. Washing cheese curd	35–40	20–30
8. Drying grains	40–50	15–20
9. Discharge of whey	5–8	5–8
10. Molding	20–30	20–30
11. Prepressing	5–15	5–15
12. I. pressing	25–35	25–35
13. Turning the cheeses	10–20	10–20
14. II. Pressing	50–70	50–70
15. Salting (20h)	5–10	5–10
16. Coating and packing	50–70	50–70
Average	**303,75 min. (5,01 h)**	**229,50 min. (3,83 h)**

3.3 Sensory Analysis

The organoleptic quality of cheese arises as a result of complex changes that occur during production and ripening period. The ripening process plays a crucial role in the development of sensory properties, such as the odor and flavor of the cheese (Weimer, Seefeldt and Dias 1999). The results of the sensory analysis of the experimental cheeses showed exceptional quality, where 81.25% were categorized in the extra class cheese, regardless of the proportion of fat in the cheese-making milk. St. Paulin cheeses had slightly better average score (18.62) compared to the Gouda technology (18.59).

After 30 days of ripening, the sensory profile of the cheeses was formed and gustatory characteristics of the experimental cheeses were: a pleasant, expressed smell with a mild milky-sour taste. The cheeses had pure odor and flavor, specific for this type of cheeses. Düsterhöft et al. (2017) states similar results, where almost all cheeses after 30 days of ripening had fully formed sensory characteristics necessary for placing the cheese on the market.

Shorter ripening period (30 days) needed to form sensory properties in experimental cheeses indicates that semi-hard cheeses could be more competitive in the economic sense compared to some other types of cheese.

3.4 Cheese Yield

The ripening process is very important in the production of cheese with improved and consistent quality with minimum costs and maximum consumer acceptance. The parameters amount of milk required to produce 1 kg of cheese and cheese yield were used as input data to assess the profitability of introducing a new product.

The average cheese yield for *Gouda* technology was 9.80 kg, while amount of milk required for 1 kg of cheese was 10.22 kg. Quantity of milk required for 1 kg of *St. Paulin* cheese was lower (8.76 kg) and the cheese yield amounted 8.38 kg. Differences in cheese yield and amount of milk required for 1 kg of cheese among *Gouda* and *St. Paulin* cheese were important from an economic aspect, but it should be observed together with the cheese weight loss during ripening (Fig. 1).

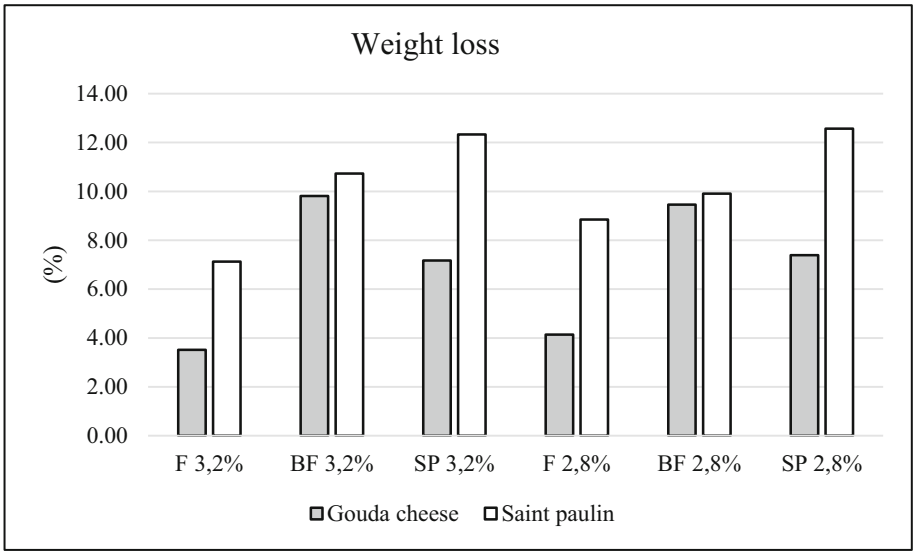

Fig. 1. Average values of weight loss (%) for *Gouda* and *St. Paulin* cheeses depending on packaging method and fat content.

Average percentages of weight loss during 30 days of ripening for *St. Paulin* cheeses was higher compared to *Gouda.* A particularly high percentage of weight loss (of about 12%) was found in *St. Paulin* cheeses, regardless of the fat content in cheese milk (SP 3.2% and SP 2.8%). On the other hand, cheeses that were packed in foil had the lowest percentage of weight loss and it varied from 3.52% (*Gouda*) to 8.85% (*St. Paulin*).

3.5 Analytical Model for Estimating the Economics of Semi-hard Cheese Production

The average time of the primary period for *Gouda* cheese technology was 303.75 min and it was longer compared to the time required for the production of *St. Paulin* cheese

(229.50 min.), the main reason was shorter time of the technological operation of "drying the cheese grain". Results obtained in the technology of semi-hard cheeses were used as input data for calculating the economics. Experimental cheeses produced by self-pressing in both technologies had an identical duration.

The turnover ratio shows how many times receivables from customers are turned over during the year. A higher number of turnovers is considered better because then the company needs fewer financial resources to finance loans, goods and services (Škrtić & Mikić., 2011). Cheese ripening was taken as the secondary period of production and for both technologies was 30 days. The result of the turnover ratio (Ko) analysis of all experimental cheeses was 12, as a result of a shorter ripening period (30 days).

Some sensory characteristics, like a mild odor or flavor, were the result of a shorter ripening period. But total sensory score of the cheeses showed that all cheeses had a satisfactory maturity for placing it on the market. Since milk represents the main material in cheese technology, the economy and profitability of cheese production is determined by the price of milk. In addition to milk, other ingredients were used in cheese technology, such as: starter culture, calcium chloride, rennet, protective coating, salt, detergent, chlorine, etc. (direct costs), and were used to calculate profitability (Table 2).

Table 2. Income, costs and economic indicators of experimental *Gouda* and *St.Paulin* cheese production.

Indicator	*Gouda*		*St. Paulin*	
Yield (kg)	100	1282	100	1380
Production value (8,68 €/kg)	868	1112313	868	1196618
Total Direct costs (€)	5018	643181	4808	663069
Total Indirect costs (€)	3250	416621	3247	447814
Total costs (€)	8268	1059802	8056	1110883
Cost price (€/kg)		8.27		8.05
Financial result (€)	410	52511	621	85734
Economy of production (Coefficient)		1,05		1,08
Profitability (%)		4,72		7,16

Based on the results of experimental cheeses production, including direct and indirect costs, analytical calculation was made depending on the applied technology, fat content and packaging method. The experimental cheese production capacity was calculated at 100 kg and the annual production.

Annual production of *Gouda* and *St. Paulin* cheeses were 128,180 kg and 137,895 kg, while the total value of productions were 1,112,313 € and 1,196,615 € respectively (calculation based on the price of 8,68 €/1 kg of cheese).

Based on analytical calculations, it was determined that the total costs did not overcome the production value. The cost price (CK) calculated on the basis of the ratio of

total costs and volume of production amounted to 8,27 € for 1 kg of *Gouda* cheese, or 8,05 € for *St. Paulin* cheese.

Financial result (FR) was calculated based on the difference between total costs and production value. In the technology of *Gouda* cheese FR was 52511 €, while *St. Paulin* cheese had a better result 102 593,00 € and amounted to 50 081,00 €

The coefficient of economy for the experimental *Gouda* technology was 1,05 and it was lower compared to *St. Pauline* technology which was 1,09.

Šarlija (2009) states that profitability indicators show the financial strength of the company, in fact these indicators measures return on invested capital. The percentage of profitability for *Gouda* and *St. Pauline* cheese was 4,72% and 7,16% respectively. The established profitability of experimental cheeses shows that for every 100,00 € invested in the production of cheese, the profit varies from 2,41 to 3,65 €.

High allocations for VAT (17%) and trade margin (of about 20%) brings production of both cheeses to the limit of economy and profitability.

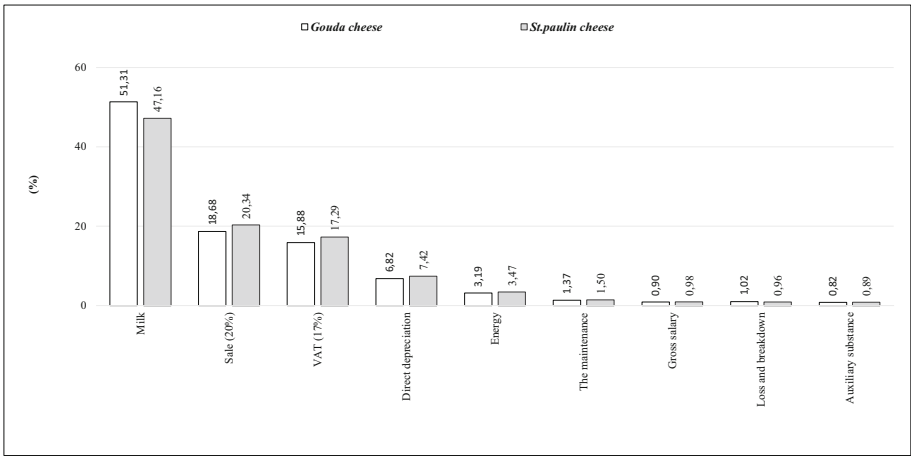

Fig. 2. The percentage share of individual items in the total costs of semi-hard cheeses calculated on 100 kg.

Figure 2 shows the percentage share of individual items in the total costs of production of semi-hard cheeses (*Gouda*, *St Paulin*) and it is calculated on 100 kg. In the total cost structure cost of raw milk have the largest share for both cheeses, 51,31% or 476,71 € (*Gouda*) and 41.39% or 402,43 € (*St Paulin*). Lover cost of raw material for *St. Paulin* cheese was a result of better cheese yield, however during ripening the weight loss for this cheese was higher. The costs of raw material were followed by.

selling costs (20%): 18.68% and 20.34%; indirect cost for VAT (17%): 15.88% and 17.29%, costs for the energy 3.19% and 3.47%, salary 0.90% and 0.98% for *Gouda* and *St. Paulin* cheese respectively. The losses and breakdown for both cheeses were relatively small in calculation and this cost amounted to approx. 1,00%. Other additional materials required in technology, such as rennet, starter cultures, packaging, disinfectants and hygiene goods, etc. account a lower share (~5, 0%) in the cost structure.

A significant item in the total cost of experimental cheese was the allocation for direct depreciation for processing equipment for *Gouda* 6.82% and 7.42% *St. Paulin* cheeses.

The price of the process equipment was 127614,00 €, which, converted to months amounted 633,37 € per months.

According to Milić et al. (2020) the largest share and amount in the costs for production of semi-hard and hard cheeses had raw material (milk), because milk represents the main ingredient of the final products.

In the technology of semi-hard cheeses fat content in raw milk is usually higher than required for this type of cheeses. After standardization of milk fat, the excess of fat represents a side product and can be used for production of various products (cream, sour cream, butter, etc.). In this economic calculation, as an extra profit, this parameter was not taken into account for profitability.

4 Conclusion

Experimentally produced cheeses during this study were predominantly of premium quality, and *St. Paulin* cheeses were rated slightly better (18.62) compared to *Gouda* (18.59).

The impact of packaging on the cheeses quality for both examined technologies showed that self-pressed cheeses packaged in foil (SP) were better compared to the one pressed and packaged in foil (F), and better yet compered to pressed cheeses without foil (BF).

The fat content (2.80% and 3.20%) in milk used for production did not show a significant impact on the sensory quality of the cheeses (*St. Paulin* and *Gouda*).

The results of the analysis of the experimentally produced cheeses showed that the production of 1 kg of *Gouda* cheese requires 10.22 L, while for 1 kg of *St. Paulin* cheese is required 8.76 L of milk. Cheese yield was 9.80 kg (*Gouda*) and 8.38 kg (*St. Paulin*), and degree of tempering was lower for *Gouda* cheeses (6.91%) than for *St. Paulin* (10.13%).

Although this study showed that the coefficient of economy and profitability of both examined technologies were positive, analytical calculation showed that the cost price of *Gouda* cheeses production was higher compared to *St. Paulin*, and this type of cheese can be recommended for the production.

In Bosnia and Herzegovina, the Law on Internal Trade of the Federation of B&H ("Official Gazette of the Federation of Bosnia and Herzegovina", no. 40/2010 and 79/2017) abolished the priority provisions of domestic products on the shelves of shopping centers, which put domestic production in an even more unfavorable position.

Based on import-export analysis of dairy products from and in Bosnia and Herzegovina, it was identified that there is a true opportunity to place on the market semi-hard type cheeses produced in domestic dairies, but difficult business conditions, low technological level can hardly be competitive with imported products that are stimulated for export from their own countries.

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