The Effect of Antioxidants on the Quality and Stability of Olive Oil

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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).

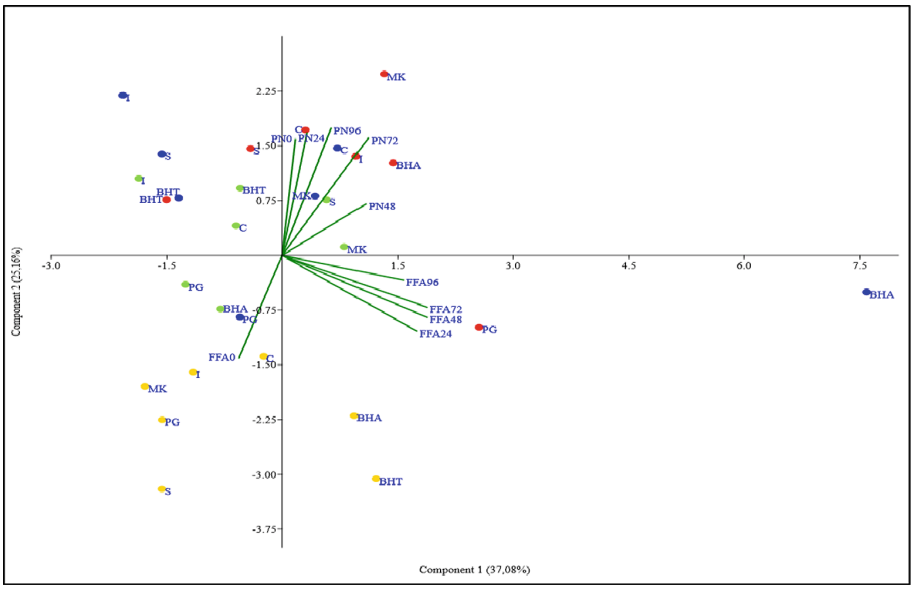

Fig. 1. The plot of principal component analysis of the sustainability parameters (M1-blue, M2-yellow, M3-red, M4-green; M1, M2, M3 and M4 - samples of extra virgin olive oil)

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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