Croatian Journal of Food Science and Technology
www.ptfos.unios.hr/cjfst/
since 2009

# The effect of natural antioxidants on long-term stability of sweet red pepper seed oil

Jasmina Ranilović[1]*, Tanja Cvetković[1], Irena Vađunec Bajrić[1], Vesna Kadija-Cmrk[1], Tihomir Moslavac[2], Marina Ilakovac Kveder[3†], Dalibor Merunka[3], Jurica Jurec[3], Srećko Valić[3], Stela Jokić[2]

[1]*Podravka Inc., Ante Starčevića 32, 48000 Koprivnica, Croatia*
[2]*Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, Franje Kuhača 18, 31000 Osijek, Croatia*
[3]*Institut Ruđer Bošković, Bijenička Cesta 54, 10000 Zagreb, Croatia*

**ARTICLE INFO**

**TYPE:** Original scientific paper
https://doi.org/10.17508/CJFST.2024.16.2.07

***CORRESPONDENCE**
Jasmina Ranilović
✉ jasmina.ranilovic@podravka.hr

**ARTICLE HISTORY**
Received: October 31, 2024
Accepted: November 25, 2024

**CITATION**
Ranilović, J, Cvetković, T, Vađunec Bajrić, I, Kadija-Cmrk, V, Moslavac, T, Ilakovac Kveder, M, Merunka, D, Jurec, J, Valić, S, Jokić, S. The effect of natural antioxidants on long-term stability of sweet red pepper seed oil. Croatian Journal of Food Science and Technology (2024) 16 (2) xx - xx



**ABSTRACT**
Oils obtained from fruit and vegetable by-products represent a new category of niche oils. Cold-pressed oil from the seeds of sweet peppers of the Podravka and Slavonka varieties is a new, high-quality oil from the vegetable niche oil category, naturally rich in polyunsaturated linoleic fatty acid, γ-tocopherol, and sterols as antioxidants that exhibit positive effects on human health in preventing chronic non-communicable diseases. However, for a broader commercial reach of this oil, preserving its quality over a longer period is of utmost importance. Based on monitoring four parametres (peroxide value, free fatty acids, antioxidative power, and radical potential), pure pepper seed oil did not have adequate quality by the end of the period of 9 month stored at room temperature due to oxidative changes. The addition of natural antioxidants such as green tea extract (0.2%), especially rosemary extract (0.2%) and a blend of rosemary extract (0.2%), and citric acid (0.01%) extended the stability of pure pepper seed oil to a period of one year. Since oxidation affects the organoleptic quality and nutritional composition of the oil, these results are encouraging for niche oil producers, not only because of the expansion of this category of edible oils made from secondary source raw materials (by-products), but also because of the evidence that their special quality will not be compromised within the declared shelf life due to oxidative spoilage.

**KEYWORDS**
sweet pepper seed oil; natural antioxidants; stability; niche oils

**KEY CONTRIBUTION**
Cold-pressed sweet pepper seed oil without added antioxidants is stable for approximately up to 9 months when stored in dark glass bottles and dark place at room temperature.
The addition of a mixture of natural antioxidants, rosemary extract (0.2%) and citric acid (0.01%), to the pepper seed oil significantly slows down oxidative changes compared to the oil

without additives, while the addition of high-oleic sunflower oil in a 1:1 ratio does not.
The results can be useful for all producers of new oils from plant sources that are not typical oilseeds, and whose composition is rich in polyunsaturated fatty acids.

## Introduction

The development of niche vegetable oils such as argan oil, marula oil, moringa oil, and chia seed oil, due to their health benefits, shelf life, and application in various products, represents a significant market opportunity (USD Analytics, 2024). Niche vegetable oils are very positively perceived by European consumers, especially linseed (flaxseed) oil, hemp oil, mustard oil, raspberry seed oil, and sesame oil, due to their composition (tocopherols, sterols, polyphenols, carotenoids, a favourable ratio of mono- and polyunsaturated fatty acids), as well as their sustainable production methods (Czwartkowski et al., 2022a). For the expansion of this category, the authors highlight the opportunity to utilize secondary raw materials (e.g. by-products) for oil production (Czwartkowski et al., 2022b). Pepper cultivation is widespread globally, with China being the largest producer with nearly 16 million tons per year, followed by Mexico (2.3 million), Turkey (2.2 million), and Indonesia (1.8 million) (Panella and Calatyud, 2018). Red horn pepper (*Capsicum annuum* L.) is the most prevalent type of pepper worldwide (Jarret et al., 2013). Besides fresh consumption, peppers are used to prepare various traditional products such as ''ajvar'' (vegan “caviar”, a Balkan spread made from roasted peppers and eggplant), ''pinđur'' (similar to ajvar but with added tomatoes), ''ljutenica'' (a spicy spread made from hot peppers, tomatoes, and spices), as well as preserved side dishes (pepperoncini, peppers in oil) or as a spice (dried and ground). In the production of a kilogram of ajvar, about one kilogram of pepper waste (seeds, stems, seed lodges) is generated, but this waste can be a valuable raw material for various uses (UN Development Programme, 2023).

By cold-pressing of seeds extracted as a secondary raw material during the processing of traditional products from sweet red peppers of the Podravka and Slavonka varieties (*C. annuum* L.), an orange-red oil of very acceptable organoleptic quality was obtained. Due to its high smoke point (230 °C), it can be used in various culinary techniques (Cvetković, 2023; Ranilović et al., 2024a). Polyunsaturated linoleic fatty acid (C18:2) makes up two-thirds of the fatty acids in cold-pressed pepper seed oil, with the remainder consisting of saturated palmitic fatty acid (C16:0; 11.0-11.2%) and monounsaturated oleic fatty acid (C18:1; 9.2-10.3%). The oxidation of oils richer in polyunsaturated fatty acids occurs faster at room temperature, while oils richer in monounsaturated fatty acids oxidize faster at higher temperatures (Porter et al., 1995). γ-Tocopherol was the only isomer found in cold-pressed pepper seed oil (535.30-574.40 mg/kg) (Cvetković, 2023; Ranilović et al., 2024a). Tocopherols are synthesized in plants, and γ-tocopherol is the main form of vitamin E (Wagner et al., 2004). Adequate intake of tocopherols is very important for human health as it prevents cardiovascular diseases, cancer, and age-related cataracts, and only the γ-tocopherol level in plasma serves as a biomarker for cancer and cardiovascular risk. Research shows that most α-tocopherol is synthesized from γ-tocopherol in the intestinal microflora, and the average amount of γ-tocopherol in plasma is lower in Europe than in the USA. Pepper seed oil is rich in sterols (5915.0-6026.7 mg/kg), with β-sitosterol being the most abundant (46.77-46.80%) (Cvetković, 2023). β-Sitosterol forms stigmastadienes during the refining process of vegetable oils, so their presence is proof of oil quality (e.g. pressing and centrifugation in the production of virgin olive oil do not produce measurable amounts of stigmastadienes, i.e. they are present in less

than 0.01 mg/kg in virgin olive oil) (Dobarganes et al., 1999). The pepper seed oil also contains a certain amount of stigmasterol (8.43-8.65%), which, according to literature sources, has numerous health benefits and anticancer potential (Bakrim et al., 2022). The water content in the oil is 0.1%, and insoluble impurities are 0.01%, proving that cold-pressed pepper seed oil complies with the criteria of the Regulation on Edible Oils and Fats for the category of cold-pressed and virgin oils (Cvetković, 2023). There is also potential for the application of this oil in the cosmetics industry, specifically in sun care products (phytocosmetics), as the natural content of carotenoids and sterols acts as a protective factor (Cvetković et al., 2024). From all the above, it is evident that cold-pressed pepper seed oil is nutritionally of a high-quality, but oxidation of the oil over time is inevitable. By delaying this process in the supply chain, the quality of the oil would be protected. Recently, in the strategy of preserving the stability of similar oils, the addition of natural antioxidants has gained significant attention, as consumers perceive niche oils (with cold-pressed pepper seed oil fitting this category) as minimally processed oils without chemical additives (Czwartkowski et al., 2022a). Various plant extracts (such as rosemary, green tea, sage, and pomegranate extracts), essential oils, as well as phospholipids, some amino acids, protein hydrolysates, and inorganic salts can be used as natural antioxidants (Horvat, 2010; Prce, 2014). Commercial extracts of these plants are obtained by extracting plant parts with organic solvents (Altemimi et al., 2017). Rosemary extract is listed as a food additive under E392. At a concentration of 0.3%, rosemary extract has proven to be the best natural antioxidant in cold-pressed camelina oil (*Camelina sativa*), hazelnut oil (*Corylus avellana* L.), and in cold-pressed pumpkin seed oil at a slightly lower concentration of 0.2% (Prce, 2014; Baotić, 2015). Interestingly, in cold-pressed chia seed oil (*Salvia hispanica* L.), rosemary extract protects the oil from oxidative spoilage better at a concentration of 0.1 than at 0.3% (Lijić, 2014). Pomegranate extract added at concentrations of 0.1 and 0.2% and green tea extract added at concentrations up to 0.1% do not protect cold-pressed pumpkin seed oil from oxidation (Moslavac et al., 2014). In cold-pressed apricot kernel oil (*Prunus armeniaca* L.), the highest efficiency in protecting against oxidative spoilage is achieved by adding green tea extract at concentrations of 0.1 and 0.2% and thyme essential oil at a concentration of 0.05%. Adding green tea extract and thyme essential oil to cold-pressed apricot kernel oil provides more effective protection than adding rosemary extract at concentrations of 0.1 and 0.2% (Kostelac, 2014). Rosemary extract type StabilEnhance OSR, alone and in combination with citric acid, increases the protection of hemp oil from oxidative spoilage (Kelnerić, 2019). The best sustainability of cold-pressed milk thistle oil (*Sylibum marianum* L. Gaertn) is shown by rosemary extract, type StabilEnhance at a concentration of 0.25%, while α-tocopherol and a mixture of tocopherols do not show protection against oxidation (Furundžija, 2020). In cold-pressed plum kernel oil (*Prunus domestica* L.), natural antioxidants such as green tea extract (*Camellia sinensis* L.), rosemary extract, and savory essential oil (*Satureja montana* L.) have a better impact on the stability and sustainability of the oil than the synthetic antioxidant octyl gallate (Moslavac et al., 2018). Citric acid (E330) is a food additive that, depending on the concentration added to food, can also be an antioxidant, acting synergistically with other antioxidants in food (Cvetković, 2023).

Interesting results were presented by Pérez-Gálvez and authors when they added pepper seed oil (*C. annuum* L.) and high-oleic sunflower oil (in various proportions) to ground, dried pepper fruit, aiming to protect the pepper pigment over a longer period of time (Pérez-Gálvez et al., 2000). The addition of pepper seed oil surrounded and impregnated the pepper fruit pigment, slowing down pigment degradation, while the addition of high-oleic sunflower oil further extended the shelf life due to the naturally present antioxidant tocopherol (Pérez-Gálvez et al., 2000). High-oleic sunflower oil has a high content of monounsaturated oleic fatty acid (18:1) (75-90.7%), significantly less polyunsaturated linoleic

fatty acid (C18:2) (2.1-17%), and palmitic fatty acid (C16:0) (2.6-5.0%) (FAO WHO Codex Alimentarius 210-1999). Compared to cold-pressed sweet pepper seed oil, high-oleic sunflower oil has a negligible amount of γ-tocopherol (3-30 mg/kg), but it contains α-tocopherol (400-1090 mg/kg) and much less β-tocopherol (10-35 mg/kg). High-oleic sunflower oil is rich in sterols (1700-5200 mg/kg), similar to pepper seed oil.

Considering the results of previous research, the aim of this study was to examine the impact of adding selected natural antioxidants (rosemary extract, citric acid, and green tea with high polyphenol content) and cold-pressed high-oleic sunflower oil on the stability of innovative, cold-pressed sweet pepper seed oil (*C. annuum* L.) during storage at room temperature over a period of one year.

## Materials and methods

*Blends of pepper seeds oil*

For the experiment, cold-pressed sweet pepper seed oil from the Podravka and Slavonka varieties was used, made from seeds of peppers (*C. annuum L.)* harvested in 2021 from the area of Northwestern Croatia (Međimurje County, Varaždin County, Koprivnica-Križevci County, Virovitica-Podravina County). The pepper seeds were separated from by-products during industrial pepper processing and were then subjected to cold pressing on a laboratory screw press to obtain oil, as described earlier (Ranilović et al., 2024a). Cold-pressed pepper seed oil served as the control (Oil 1) and the base for adding natural antioxidants (Oil 3-5) and for making a mixture with HOSO (Oil 2) (Table 1).

**Table 1**. Concentration of natural antioxidative components added to the cold-pressed pepper seed oil (PSO)

| Antioxidative components (AC) | Concentration of AC added to PSO | Samples |
|---|---|---|
| **No added (control)** | - | Oil 1 |
| **High-oleic sunflower oil (cold-pressed) HOSO** | 1:1* | Oil 2 |
| **Rosemary extract** | 0.20% | Oil 3 |
| **Rosemary extract and Citric acid** | 0.20% + 0.01% | Oil 4 |
| **Green tea extract (95% polyphenols content)** | 0.20 % | Oil 5 |

**Concentration proportion HOSO:PSO (pepper seed oil)*

According to previously published results, cold-pressed pepper seed oil had an average peroxide number of 2.2 mmol $O_2$/kg and free fatty acids of 0.2%, measured over two pepper harvest seasons (Ranilović et al., 2024a). Cold-pressed high-oleic sunflower oil (BB Oil d.o.o., Croatia) had a peroxide number of 0.33 mmol $O_2$/kg and free fatty acids of 0.62%, according to the manufacturer's specifications. Other additives were also commercially obtained: dry rosemary (*Rosmarinus officinalis* L.) extract (>92%) (Naturex SA, France), anhydrous citric acid ((RZBC (Juxian), Co., LTD., China)), and dried Green Tea Extract (95% Polyphenols content) (Pfannenschmidt GmbH, Germany). For all analyses, samples of oil with added antioxidants (Oil 2-5) as well as control (Oil 1) were prepared and packed in dark glass bottles (500 mL), stored at room temperature (25 °C) in a dark place in the Corporate Product Development laboratory, Podravka d.d., Croatia. The analytical monitoring period for oil stability lasted from August 2021 to August 2022 (one year), with analyses conducted at the end of each of the four quarters. Physical-chemical analyses (peroxide value, free fatty acids) were performed by Eurofins Croatiakontrola d.o.o., Croatia, and measurements of antioxidative power and radical potential were conducted in the Magnetic Resonance Laboratory at the Institute of Physical Chemistry, "Ruđer Bošković", Croatia.

*Pepper seeds flour*

For the analysis of antioxidant power (AP), pepper seeds were ground into flour using a regular coffee grinder.

*Analysis*

*Peroxide value (PV)*

Oil oxidation is an inevitable process that occurs due to the gradual spoilage of oil, caused by chemical processes. The peroxide number indicates the amount of hydroperoxides, the main triggers of autoxidation in oil. The lower the peroxide number, the longer the oil will last if properly stored. The peroxide value (PV) was determined titrimetrically (ISO method ISO 3960:2017). The method is previously described in detail (Cvetković, 2023).

*Free fatty acids (FFA)*

Free fatty acids (FFA) are formed by the process of separating from the triglyceride molecule through the action of the enzyme lipase (hydrolytic spoilage). FFA were determined titrimetrically (ISO method 660:2010). The method is previously described in detail (Cvetković, 2023).

One-way analysis of variance (ANOVA) and Scheffe's multiple comparison test ($p<0.05$) were used to examine differences in scores between groups. All analyses were calculated using SPSS statistical software (version 25.0, 2017; SPSS Inc., Chicago, IL, USA).

*Antioxidative power (AP)*

Antioxidant power (AP) was measured by electron spin resonance (ESR) spectroscopy, a technique that enables the direct detection of free radicals in the sample. To measure the AP of oils (Oils 1-5), a solution of oil in ethanol with a concentration of 50 mg/mL was prepared. The oils were mixed with ethanol on a Vortex mixer for 3 min, and then the solutions were centrifuged, during which the oils were precipitated and the alcoholic extract of the oil was separated. By diluting the extract with ethanol, three different oil concentrations were obtained, which were used for ESR measurements. The alcoholic extract of the pepper seed flour was prepared so that the concentration in the ethanol solution was 100 mg/mL. It was then mixed on a Vortex mixer for 3 min. The solution was centrifuged and the extract was separated. By diluting the extract with ethanol, the desired concentrations for ESR measurements were obtained. The ESR measurements were performed at room temperature on a Bruker Elexsys E580 spectrometer. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used as a scavenging object. The spectroscopic parametres were microwave frequency of 9.3 GHz, power attenuation of 20 mW, magnetic field modulation amplitude of 1.0 G, and modulation frequency of 100 kHz. Spectra were recorded around the central field of 3430 G, with a sweep range of 100 G and sweep time of 20.97 s immediately after bringing the DPPH solution into contact with sample extracts. Spectral analysis was carried out with spectrometer build-in analysis software by measuring the intensity of the central line (peak-to-peak amplitude), which is proportional to the number of spins in the sample. All measurements were performed using the same procedure; the samples (pepper seed flour; Oils 1-5) were put into the capillary which was then placed in an ESR tube. The tube was immediately put in the resonator and measured for 30 min using the given parametres. At the beginning of each analysis, a control signal was measured first, i.e., the DPPH signal in a solution with a concentration of 0.10 mmol/L prepared by diluting the original DPPH solution with ethanol in a 1:1 ratio. After that, the sample extract was added to the DPPH solution also in a 1:1 ratio, to obtain the same DPPH concentration. The reaction time starts with the addition of the sample to the DPPH solution (t = 0). The solution was then homogenized by mixing on a Vortex mixer for 3 s. The first measurement was started 1 min after the sample was put into

contact with the DPPH solution, and the intensity of the signal was monitored after 2, 3, 5, 8, 12, 18, 24, and 30 min. The obtained values were normalized to the value of the central line intensity of the DPPH control sample. Experimental data were analyzed on a personal computer. Mean values for tr, wc, and AP were calculated considering the statistical weight of each series of measurements (calculated using Excel Microsoft 365, 2020, Microsoft Corporation, USA).

AP, expressed in AU units, where 1 AU corresponds to an AP of the solution of vitamin C with a concentration of 1 ppm, was calculated using the following equation:

$$AP = \frac{RA \times N_{spins}}{w_c \times t_r}$$

where RA is the reduction amplitude constant ($1/e^2$), $N_{spins}$ is the number of reduced radicals characterized by free electrons (spins) from DPPH, $w_c$ is the characteristic weight of the antioxidant product expressed in mg/mL, and $t_r$ is the reduction time expressed in min, defined by the following equation:

$$t_r = \frac{-\ln(1 - 1/e)}{k_{wn}}$$

where $k_{wn}$ is the monoexponential decay reaction constant, and $w_c$ corresponds to the antioxidant capacity and is expressed as:

$$w_c = \frac{-\ln(1 - 1/e)}{\mathrm{k_{tr}}}$$

where $k_{tr}$ is the monoexponential decay rate constant. The relative intensity ($I$) is calculated as:

$$I = \frac{I_t}{I_0} \times 100\ \%$$

where $I_t$ represents the intensity of the spectrum that was recorded after the addition of the sample, while $I_0$ represents the intensity of the spectrum of the control sample.

*Radical potential (RP)*

UV radiation of unsaturated fatty acids generates free radicals. The resulting peroxide lipid radicals are neutralized by PCA whose signal loss is monitored depending on the time of UV treatment of the sample. A solution of PCA with a concentration of 0.02 mmol/L was prepared for RP measurements. The procedure was following: 0.500 mL of PCA solution was added to the same volume of oil in a quartz cuvette; so that the final concentration of PCA was 0.01 mmol/L. The sample was then mixed on a Vortex for 30 s and a capillary filled with this mixture was placed in an ESR tube to register the initial signal (t = 0). The sample in the cuvette was then placed in the UV reactor and the time of its exposure to UV radiation was measured. UV treatment of samples was carried out at a wavelength of 352 nm in a UV photo-reactor LUZCHEM LZC-4V, equipped with 14 lamps, each with a power of 8 W, which makes a total power of 112 W. In order to examine the influence of the duration of UV treatment on the intensity of the PCA signal, the capillaries were filled with the treated oil mixture after 10, 20, and 30 min of radiation, and 60 s after placing the sample in the capillary, the ESR signal was recorded for each treatment time (t). In each quarter, three series of RP measurements (0, 10, 20, 30 min) were made per sample. A PCA solution with a concentration of 0.01 mmol/L was added to the seed samples, where the mass of the seeds was equal to the mass of the oil (0.4510 g), and the volume of the PCA solution was 1.00 mL. Mixing of the sample with the PCA solution, UV treatment, and recording of the ESR signal were performed using the same procedure as for the oil. These facts made it possible to directly

compare the results obtained for oils and seeds. Experimental ESR parametres for RP measurements were the same as for AP measurements. At the beginning of the analysis of each oil and seed sample, the initial PCA signal $I_0$ was measured (before UV treatment, t = 0). During the irradiation of the samples, the intensities (I) of the signal were monitored as a function of the time passed since the beginning of the exposure of the samples to UV radiation. Signal intensity values (I) are normalized to the initial signal intensity ($I_0$). Since the initial intensity is $I=I_0$, it follows that $I/I_0=1$ for t=0.

## Results and discussion

*Peroxide value and free fatty acid*

The highest PV values at the end of the monitoring period (12 months) were expectedly found in cold-pressed pepper seed oil, Oil 1 (11.10 mmol $O_2$/kg), followed by the mixture of pepper seed oil and high-oleic sunflower oil (Oil 2) (11.00 mmol $O_2$/kg). In contrast, pepper seed oil with added rosemary extract (Oil 3) and oil with added rosemary extract and citric acid (Oil 4) had the lowest PV values (2.30 mmol $O_2$/kg), which is more than 5-fold lower than the standard pepper seed oil (Oil 1) (Figure 1). ANOVA Sheffe's post-hoc test did not show a statistically significant difference between oils (data not shown).

Although the average PV levels for all oil samples are below the limit set by regulations for cold-pressed and virgin oils (max. 7 mmol $O_2$/kg) (Regulation on Edible Oils and Fats, NN 11/2019, 117/2021), cold-pressed pepper seed oil would not meet the regulation near the 9-month storage mark. The highest FFA content from the beginning to the end of the measurement was shown by Oil 2 (Figure 2), and Sheffe's post-hoc test additionally showed that this oil was significantly different from all other oils (Oil 1,3,4,5) (Figure 3). The measured properties of all examined oils (PV, FFA) at the end of the 12-month testing period comply with the regulations for cold-pressed and virgin oils, even for extra virgin olive oil (Regulation on Edible Oils and Fats, NN 11/2019, 117/2021; Regulation 1308/2013). The stabilization of cold-pressed pepper seed oil with the addition of natural antioxidants such as green tea, rosemary, and citric acid prevented fat oxidation and extended the oil's shelf life, confirming the results of previous studies (Nain et al., 2021; Blasi and Cossignani, 2020).

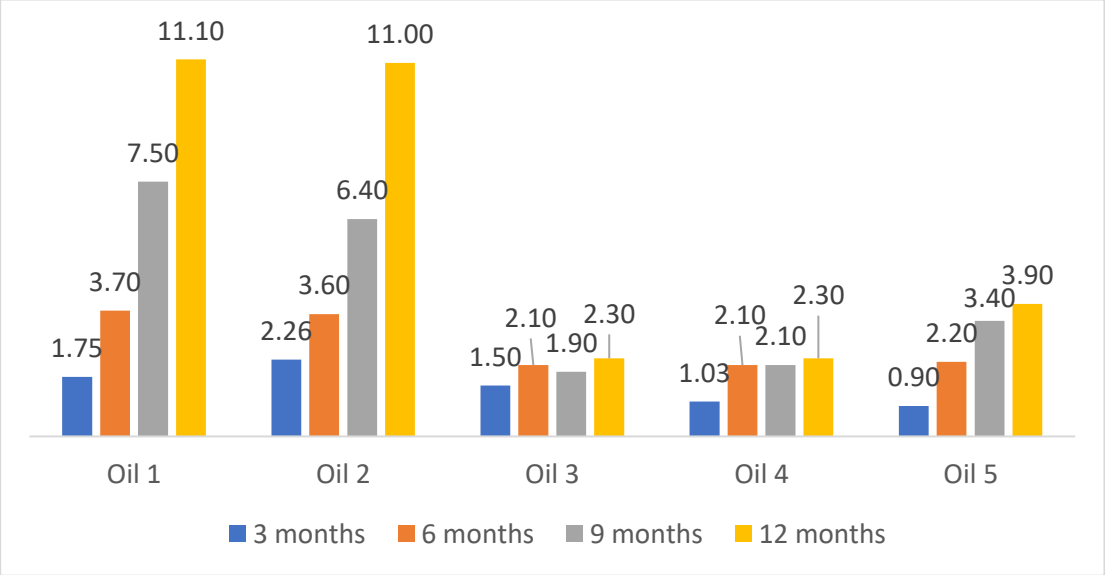



**Figure 1.** Monitoring of peroxide number (mmol $O_2$/ kg) for 12 month period at 25 °C

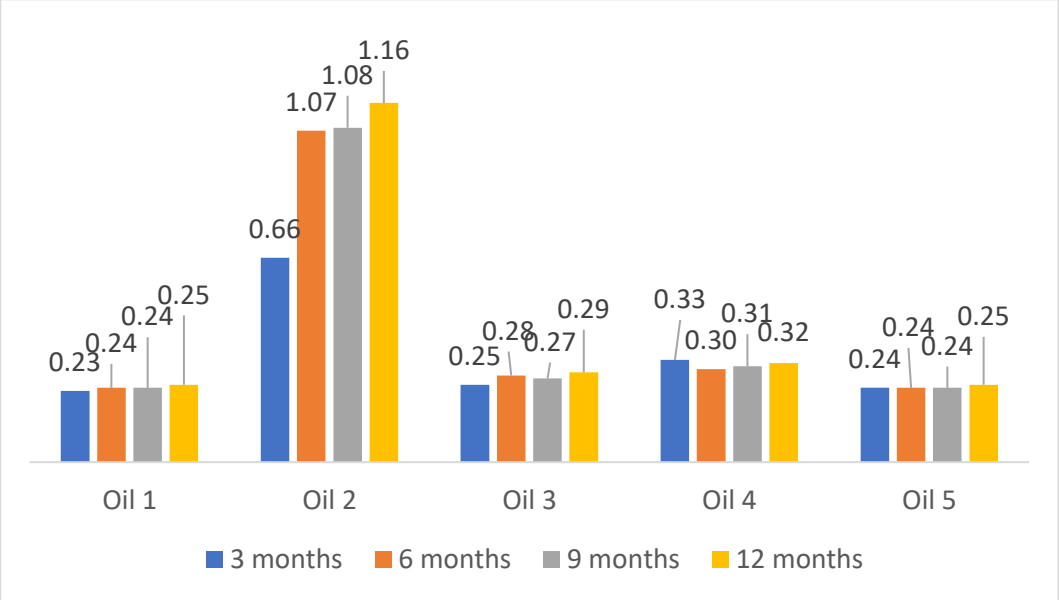
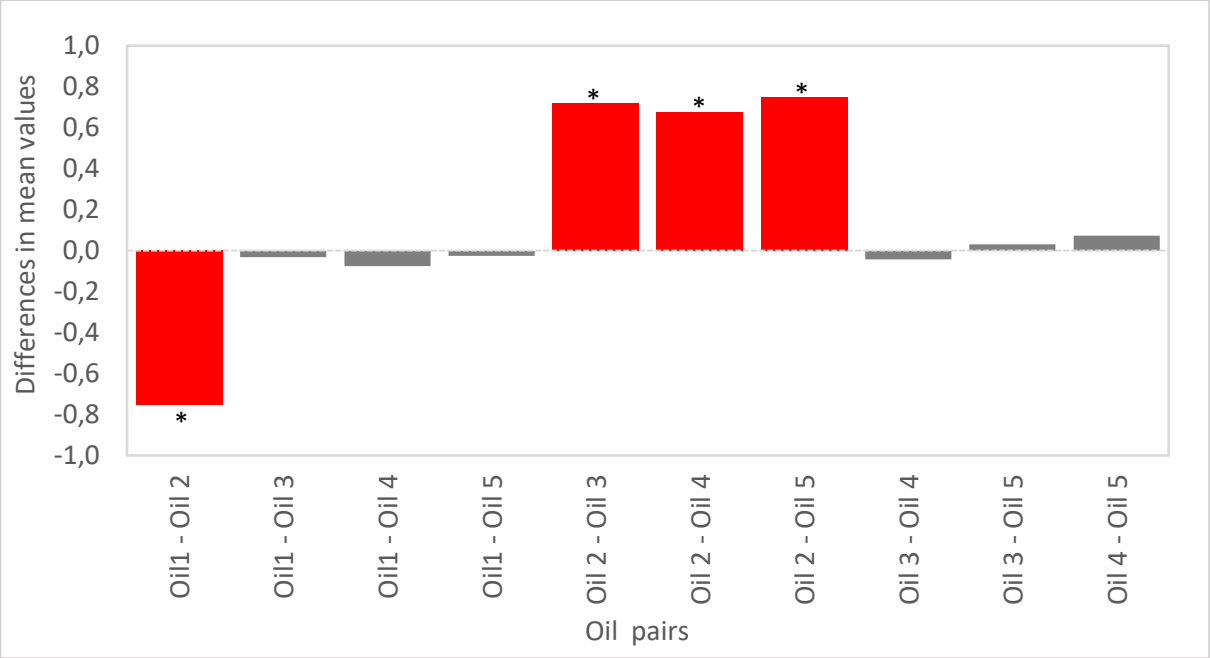



**Figure 2.** Monitoring of free fatty acids (%) for 12 month period at 25 °C




**significantly different within one-way ANOVA using post hoc Scheffe's multiple comparison test (p<0.05)*

**Figure 3**. Differences in free fatty acids mean values between oils

An important fact proven in previous studies should not be overlooked: cold-pressed pepper seed oil is naturally rich in γ-tocopherol (535.30 – 574.40 mg/kg) (Ranilović et al., 2024; Cvetković 2023; Cvetković et al., 2022), which, in combination with added natural extracts and additives, contributes to the stabilization of the oil over 12 months.

Fadda et al. (2022) reported in a review that olive oil, which is naturally rich in tocopherols and polyphenols, is more resistant to oxidation. In strategies that contribute to the oxidative stability of cold-pressed oils, Grosshagauer et al. (2019) indicated that, in addition to the positive impact of

polyphenolic components and natural antioxidants, the seed-toasting process is even more important. Toasting, due to Maillard reactions, creates special products called ALEs (advanced lipid oxidation end products), which have strong antioxidant activity.

The addition of 0, 100, 200, 500, and 1000 ppm (mg/kg) of oxidized α-, γ-, and δ-tocopherols to refined soybean oil (without peroxides, chlorophyll, free fatty acids, tocopherols, and phospholipids, colour) over a period of 7 days at a temperature of 55 °C showed that with an increase in concentrations of oxidized tocopherols, the PV increased, and headspace oxygen decreased (Jung and Min, 1992). This means that tocopherols act pro-oxidatively at higher concentrations, with oxidized α-tocopherol showing the greatest pro-oxidative effect, followed by γ- and δ-tocopherols to a lesser extent. Older literature data indicate that the optimal concentration of tocopherols is 400-600 ppm, which acts antioxidatively, positively affecting the oxidative stability of soybean oil, while concentrations of 1500 ppm act pro-oxidatively during storage (Frankel et al., 1959; Cillard et al., 1980).

Naturally present α- and γ-tocopherols in rapeseed oil led to mild oxidation over 28 days at a temperature of 40 °C (Lampi et al., 1999). The initial PV ranged from 0.2 meq/kg (0.1 mmol $O_2$/kg) to 2.1 meq/kg (1.05 mmol $O_2$/kg) at the end of the experiment, meaning that both tocopherols are stable under these conditions and can successfully slow down oil oxidation. This study also confirmed earlier evidence that α-tocopherol acts antioxidatively at low concentrations, while γ-tocopherols do so at higher concentrations, which is explained by the greater ability of α-tocopherol to donate hydrogen to free radicals, but also its consumption in other reactions is not fully explained.

Fuster et al. (1999) investigated the effect of concentrations of 1-2000 mg/kg α- and γ-tocopherols on the stability of refined sunflower oil (without anti- and pro-oxidative components; rich in highly oxidizable linoleic acid) with the addition of $FeSO_4$ (ferrous sulfate) at 55 °C over 7 days, showing that α- and γ-tocopherols can potentially act synergistically at concentrations below 200 mg/kg, but not when present in higher concentrations. α-tocopherol was a better antioxidant than γ-tocopherol at concentrations ≤40 ppm, but a poorer antioxidant at concentrations >200 ppm.

The synergistic effect of antioxidants can be mathematically expressed as the ratio of the experimental and theoretical values of individual antioxidants, and if this ratio is greater than 1, a synergistic effect occurs, if it is equal to 1 an additive effect is present, and if it is less than 1, the effect is antagonistic (Tang et al., 2022). This can be due to various mechanisms such as regeneration, sacrificial oxidation (protecting each other by free radical scavenging), or others (Becker et al., 2004).

By measuring antioxidant capacity Neunert et al. (2015), recorded the greatest synergistic effect of ferulic acid (a phenolic acid with high antioxidant activity) and α-tocopherol in liposomal systems, and antagonistic in mixtures of chlorogenic acid and α-tocopherol. They explained this by the fact that the weakly polar ferulic acid is located deeper in the cell membrane, allowing it to react with free radicals closer to the membrane surface, thus protecting α-tocopherol through sacrificial oxidation or regeneration mechanisms.

In another study, results showed that the ratio of binary antioxidants is important for the synergistic or antagonistic effect. The combination of rosemary extract and tea polyphenol palmitate in a 5:3 ratio showed the greatest synergistic effect on free radical scavenging DPPH and ABTS activity in sunflower oil, even 1.2-fold greater than their individual theoretical sum of values, while the 1:1 ratio showed an antagonistic effect (Wang et al., 2020).

In addition to the ratio of binary blends of antioxidants, their interaction type in the oil base depends on the proportion and length of saturated triglycerides (Tang et al., 2022). Mixtures of α-tocopherol with γ-oryzanol and α-tocopherol with phytosterol (β-sitosterol [75%], campesterol [15%], and stigmasterol [5%]) showed antagonistic effects in rice bran oil (long-chain unsaturated triglycerides) and synergistic

effects in refined coconut oil (predominantly medium-chain saturated triglycerides), indicating that the same combinations of antioxidants behave differently in oil bases with different triglyceride compositions (Tang et al., 2022).

Although this study did not determine the experimental values of individual antioxidants at the beginning and end of the monitoring period (12 months) to calculate the synergistic effect of antioxidants, based on the measured values of PV and FFA, it could be concluded that a synergistic effect is present between the naturally occurring antioxidant γ-tocopherol in pepper seed oil (PSO) (initial content 535.30-574.40 mg/kg) and added natural antioxidants (rosemary extract, rosemary extract, and citric acid, and green tea extract rich in polyphenols) at concentrations of 0.2% (2000 ppm or mg/kg), even at the end of the 12-month period. The background of this effect in this study cannot be explained, but the confirmed strong antioxidant effect may be of interest to producers in protecting the stability of vegetable oils rich in polyunsaturated fatty acids, which are particularly sensitive to oxidation at room temperatures.

In the mixture of PSO and HOSO (Oil 2), naturally occurring tocopherols at concentrations higher than 400 ppm (α-tocopherol in HOSO and γ-tocopherol in PSO) acted pro-oxidatively on the stability of the oil, which was evident within the first 3 months of oil storage, and this only increased by the end, suggesting that this oil mixture would not be organoleptically acceptable by the end of that period.

*Antioxidative power*

Pepper seeds flour sample stored at a temperature of 4 °C during both quarters had twice the AP of seed flour stored at room temperature (60 *vs*. 29-31 AU), and it remained unchanged until the end of the 6th month (Figure 4).

In Figure 4, the AP of the oil samples, measured successively at the end of each quarter over a 12-month period, is shown. Pepper seed oil with the addition of 0.2% rosemary extract (Oil 3) showed constant AP throughout the period without significant fluctuations (155-164 AU). Slightly lower AP was shown by the oil with added 0.2% rosemary extract and 0.01% citric acid (Oil 4), while all other oils had much lower AP (below 100 AU), with the lowest being the mixture of pepper seed oil and high-oleic sunflower oil (Oil 2).

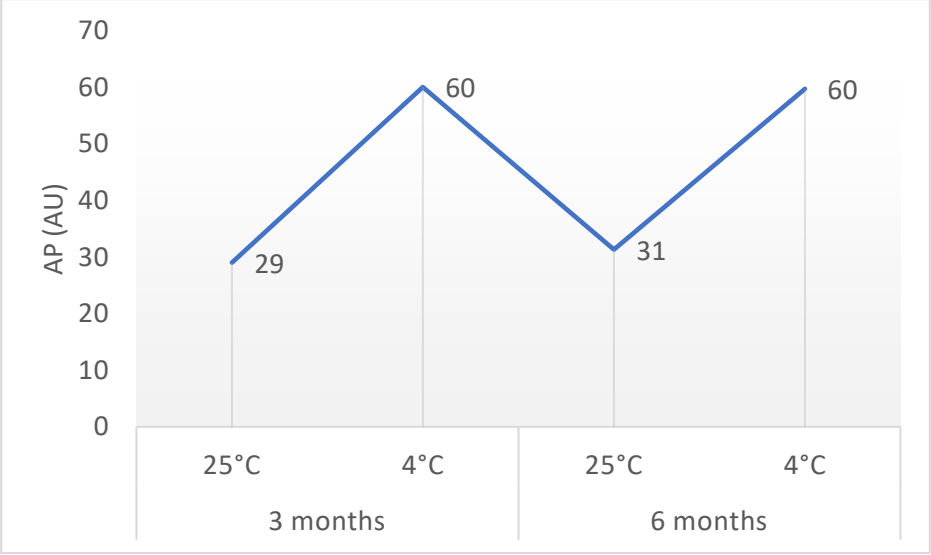



**Figure 4.** Monitoring of an antioxidative power of pepper seeds flour during storage 6 months period at 25 and 4 °C

Pepper seed oils stabilized with antioxidants such as rosemary extract (Oil 3,) or a combination of rosemary extract and citric acid (Oil 4) showed approximately 2-fold higher antioxidant power when compared to pure oil (Oil 1), which is also found in the literature (Jelić, 2022). While the addition of high-oleic sunflower oil to cold-pressed pepper seed oil (Oil 2) ratio did not significantly affect PV and FFA, it had the opposite effect on AP, reducing (nullifying) the AP of cold-pressed pepper seed oil (Oil 1) throughout the year. Athanasiadis et al. (2023) found that the addition of a mixture of α- and δ-tocopherol in a 7:1 ratio has a synergistic antioxidant effect on the stabilization of sunflower oil and olive pomace oil, but significantly reduces the ratio between omega-6 and omega-3 fatty acids.

Comparing the AP of pepper seeds (Figure 4) and the AP of pepper seed oils (Figure 5), it can be seen that all oils have 2-fold or higher AP than the seeds. During the cold-pressing process, mechanical pressure on the seeds, oil extraction, and the release of antioxidants such as vitamin E (tocopherols) and polyphenolic components occur, which increase the oxidative stability of the oil (Grosshagauer et al., 2019). This is confirmed by the results of this measurement and comparisons between seeds and oil.

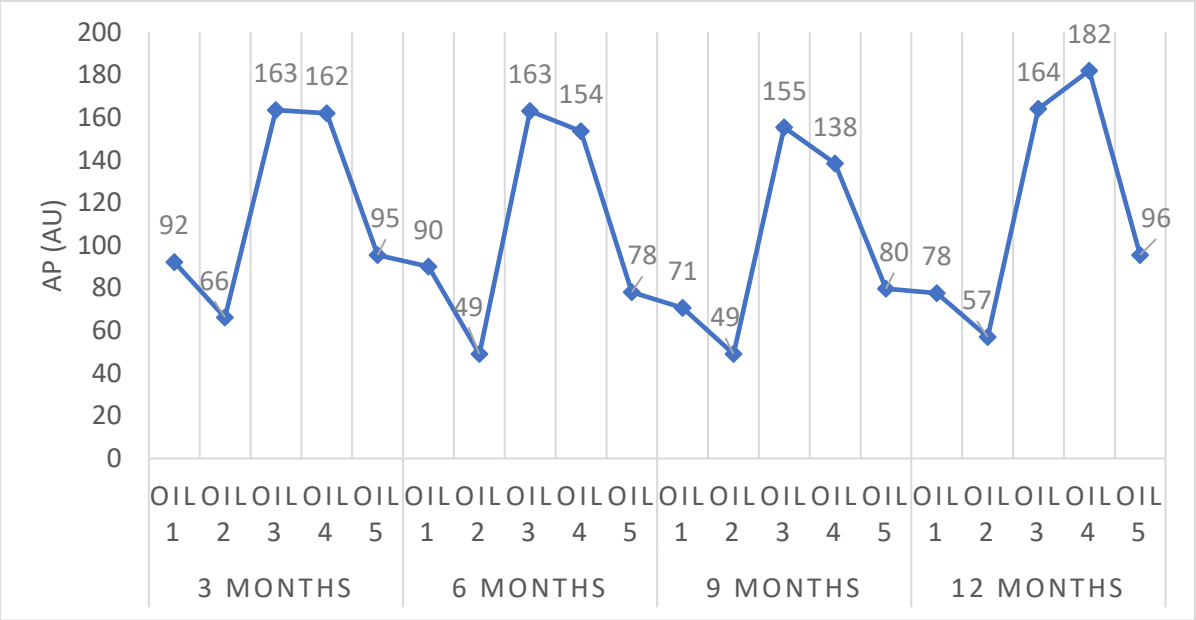



**Figure 5.** Monitoring of antioxidative power in different variations of pepper seed oil during 12 month period at 25 °C

*Radical potential*

The analysis of RP provides information on the stability of the tested samples in relation to UV radiation. The decrease in the ESR signal of the nitroxide radical PCA indicates the formation of radicals in the oil samples subjected to UV radiation. A greater decrease in the PCA signal means lower sample stability. It was observed that the signal decrease is greater the longer the exposure time (20, 30 min) of the samples to radiation (Figure 5). The signal decrease in the mixture of pepper seed oil and high-oleic sunflower oil (Oil 2) is somewhat weaker compared to other oil samples, especially for shorter exposure times (10 min). This means that the concentration of radicals formed by radiation under the given conditions is lower in the Oil 2 sample compared to other samples.

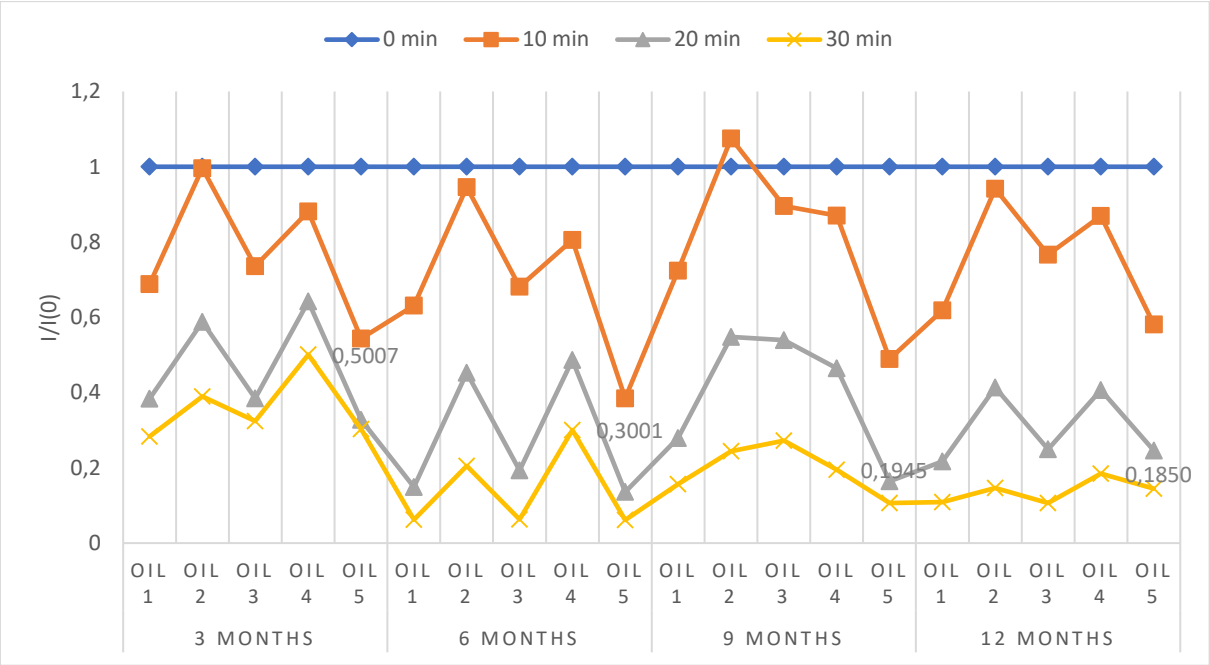



**Figure 5.** Monitoring of average radical potential (0-30 min) measuring of different pepper seed oils during 12 months period storage at 25 °C

Considering the results obtained after UV treatment of oil samples for 30 min, considering all measurement series, Oil 4 - pepper seed oil with rosemary extract (0.2%) and citric acid (0.01%) showed the greatest stability. This sample is followed by Oil 2, then Oil 3, and Oil 5, while Oil 1 was the least stable. The impact of storage time on the RP analysis results indicates a slight decrease in sample stability over time, which is somewhat more pronounced in Oil 3 - pepper seed oil with rosemary extract (0.2%) compared to other samples. It can be inferred that with longer storage at room temperature, the oil samples gradually lose stability.

The results of AP and RP measurements cannot be directly compared, because RP measurements exclusively monitor the stability of the sample in relation to UV radiation, and these results must be viewed considering the newly formed radicals (formed by UV radiation) and only those that react with PCA. In contrast, AP measurements are based on the direct interaction of antioxidant components contained in the sample (soluble in alcohol) with DPPH, where these components most often act as proton donors and do not necessarily have a radical structure.

From these results, it can be concluded that Oil 4 - pepper seed oil with rosemary extract (0.2%) and citric acid (0.01%) showed the most consistent quality over the one-year period. This study confirmed previous findings that the addition of citric acid as a synergist showed about a 2-fold increase in the stability of virgin oil, i.e., its overall antioxidant capacity (154-164 AP), which was certainly influenced by the natural composition of pepper seed oil (γ-tocopherol, linoleic fatty acid, polyphenols).

The proof of the stability of this combination of oil and natural antioxidants against UV radiation (part of the electromagnetic radiation of sunlight) and the formation of free radicals are also important for oil storage.

## Conclusions

These results complete the characterization of cold-pressed sweet pepper seed oil from the Podravka and Slavonka varieties (Oil 1), particularly in the important aspect of its storage at room temperature over one year. Despite the presence of inherent natural antioxidants (γ-tocopherol, sterols, carotenoids), oils rich in essential fatty acids like this one are susceptible to oxidative spoilage over long storage periods, leading to changes in organoleptic properties. Cold-pressed pepper seed oil without added antioxidants (Oil 1) would meet the requirements of the Regulation on Edible Oils and Fats up to 9 months of storage (more likely around 8 months, but measurements were not made). However, the addition of natural antioxidants - rosemary extract (0.2%), especially the combination of rosemary extract (0.2%) and citric acid (0.01%) - significantly positively affected minimal oxidative changes, as the PV at the end of the year was only slightly higher than in the first quarter. Green tea extract also significantly contributed to the stabilization of this oil, but somewhat less than rosemary extract. Although organoleptic tests were not conducted, it is assumed that the addition of these natural antioxidants in the mentioned concentrations would not significantly impair the desirability of the oil for consumption, as no oxidative changes were recorded that would lead to changes in colour, taste, and smell. To explain the effect of high-oleic sunflower oil on the stability of pepper seed oil, future research should include the analysis of the α-, γ-tocopherols, sterols, fatty acid composition, and carotenoids of the mixtures at the beginning and end of the shelf-life monitoring period.

***Author Contributions:*** J.R. conceptualized and wrote the manuscript. T.C., I.V.B., and V.K.C. performed statistical analysis and reviewed the manuscript. T.M. advised about natural antioxidants and reviewed the manuscript. M.I.K., D.M., J.J., and S.V. performed the measurement (AP, RP) analysis and discussion. S.J. reviewed the manuscript.

***Funding:*** This research was part of project KK.01.2.1.02.0069, titled “Development of innovative products from by-products during vegetable processing.” The project was co-funded by the Croatian Operational Program “Competitiveness and Cohesion 2014-2020,” under the call for “Strengthening the economy by applying research and innovation” (KK.01.2.1.02).

***Acknowledgments:*** We express our gratitude to the Croatian Ministry of Economy and Sustainable Development, the European Union Structural and Investment Funds, and Podravka Inc. for co-funding this research within the project KK.01.2.1.02.0069.

***Conflicts of Interest*:** The authors declare no conflict of interest.

## References

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G., Lightfoot, D.A. (2017): Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants* 6(42).https:// doi:10.3390/plants6040042

Athanasiadis, V., Chatzimitakos, T., Kalompatsios D., Palaiogiannis, D., Makrygiannis, I., Bozinou, E., Lalas, S.I. (2023): Evaluation of the Efficacy and Synergistic Effect of α- and β-Tocopherol as Natural Antioxidants in the Stabilization of Sunflower Oil and Olive Pomace Oil during Storage Conditions. *International Journal of Molecular Sciences* 24, 1113. https://doi.org/10.3390/ijms24021113

Bakrim, S., Benkhaira, N., Bourais, I., Benali, T., Lee, L.H., El Omari, N., Sheikh, R.A., Goh, K.W., Ming, L.C., Bouyahya, A. (2022): Health Benefits and Pharmacological Properties of Stigmasterol. *Antioxidants* 11, 1912. https://doi.org/10.3390/antiox11101912

Baotić, M. (2015): Proizvodnja i održivost hladno prešanog lješnjakovog ulja. Diplomski rad, Prehrambeno-tehnološki fakultet, Osijek, Sveučilište J.J. Strossmayera Osijek.

Becker, E.M., Nissen, N.L., Skibsted, L.H (2004): Antioxidant evaluation protocols: food quality and health effects. *European Food Research and Technology* 219, 561–571. https://doi.org/10.1007/s00217-004-1012-4

Blasi, F. and Cossignani, L. (2020): An Overview of Natural Extracts with Antioxidant Activity for the Improvement of the Oxidative Stability and Shelf Life of Edible Oils. *Processe*s 8, 956. https://doi:10.3390/pr8080956

Cillard, J. and Cillard, P. (1980): Behavior of a-, y-, and 8- tocopherols with linoleic acid in aqueous media. *Journal of the American Oil Chemists' Society* 57, 39-42. https://doi.org/10.1007/BF02675523

Cvetković, T. (2023): Dobivanje i karakterizacija ulja sjemenki hrvatskih sorti paprike *Capsicum annuum* L. te njegova primjena u prehrambenoj industriji i gastronomiji. Doktorski rad. Prehrambeno-tehnološki fakultet Osijek, Sveučilište J. J. Strossmayera Osijek, Osijek.

Cvetković, T., Ranilović, J. Jokić, S. (2024): Potencijal primjene ulja sjemenki paprike u kozmetičkoj industriji (Potential application of pepper seed oil in the cosmetic industry). In: Neke mogućnosti iskorištenja nusproizvoda prehrambene industrije, Šubarić, D., Jašić, M., Jokić, S. (ed.), Sveučilište J.J. Strossmayera u Osijeku, Prehrambeno-tehnološki fakultet Osijek, pp. 163-176.

Czwartkowski, K., Wierzbic, A., Golimowski, W. (2022a): Awareness and Expectations of Polish Consumers Regarding Edible Niche Oils as a Food Product. *Sustainability* 14, 14239. https://doi.org/10.3390/su142114239

Czwartkowski, K., Wierzbic, A., Golimowski, W. (2022b): Quality, Key Production Factors, and Consumption Volume of Niche Edible Oils Marketed in the European Union. *Sustainability* 14, 1846. https://doi.org/10.3390/su14031846

Dobarganes, M.C., Cert, A., Deiffenbacher, A. (1999): The deterimination of stigmastadienes in vegetable oils. *Pure and Application Chemistry* 71 (2). 349-359. https://doi.org/10.1351/pac199971020349

Fadda, A., Sanna, D., Sakar, E.H., Gharby, S., Mulas, M., Medda, S., Yesilcubuk, N.S., Karaca, A.C., Gozukirmizi, C.K., Lucarini, M., Lombardi-Boccia, G., Diaconeasa, Z., Durazzo, A. (2022): Innovative and Sustainable Technologies to Enhance the Oxidative Stability of Vegetable Oils. *Sustainability* 14, 849. https://doi.org/10.3390/su14020849

FAO-WHO: Codex Alimentarius for Fats and Oils from Vegetable Sources, Section 2 (Codex-Stan 210-1999)

Frankel, E.N., Evans, C.D. Cooney, P.M. (1959): Tocopherol oxidation in natural fats. *Journal of Agricultural and Food Chemistry* 7, 438-40. https://doi.org/10.1021/jf60100a013

Furundžija, S. (2020): Stabilizacija ulja sikavice s prirodnim i sintetskim antioksidansima. Diplomski rad, Prehrambeno-tehnološki fakultet, Osijek, Sveučilište J.J. Strossmayera Osijek.

Fuster, M.D., Lampi, A.-M., Hopia, A, Kamal-Eldin, A. (1998): Effects of α- and γ-Tocopherols on the Autooxidation of Purified Sunflower Triacylglycerols. *Lipids* 33, 715-722. https://doi.org/10.1007/s11745-998-0261-3

Grosshagauer, S., Steinschaden, R., Pignitter, M. (2019): Strategies to increase the oxidative stability of cold pressed oils. *LWT Food Science and Technology* 106, 72-77. https://doi.org/10.1016/j.lwt.2019.02.046

Horvat I. (2010): Antioksidansi u prehrani. Preddiplomski rad, Prehrambeno-biotehnolški fakultet, Zagreb, Sveučilište Zagreb.

Jarret, R.L., Levy, I.J., Potter, T.L., Sermak, S.C. (2013): Seed oil and fatty acid composition in *Capsicum* spp. *Journal of Food Composition and Analysis* 30, 102-108. https://doi.org/10.1016/j.jfca.2013.02.005

Jelić, L. (2022): Utjecaj dodatka antioksidansa na održivost mješavine konopljinog i suncokretovog ulja. Diplomski rad. Prehrambeno-tehnološki fakultet Osijek, Sveučilište J. J. Strossmayera Osijek.

Jung, M.Y., Min, D.B. (1992): Effects of oxidized α-, γ-, and δ- tocopherols on the oxidative stability of purified soybean oil. *Food Chemistry* 45, 183-187. https://doi.org/10.1016/0308-8146(92)90112-F

Kelnerić, L. (2019): Utjecaj mikrovalnog zagrijavanja i antioksidansa na održivost ulja konoplje sorte finola, završni rad, Veleučilište u Požegi.

Kostelac, I. (2014): Proizvodnja i stabilizacija hladno prešanog ulja iz koštice marelice. Diplomski rad, Prehrambeno-tehnološki fakultet, Osijek, Sveučilište J.J. Strossmayera Osijek.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A., Vieno, P. (1999): Antioxidant Activities of α- and γ-Tocopherols in the Oxidation of Rapeseed Oil Triacylglycerols. *Journal of the America Oil Chemists' Society* 76, 6, 749-755. https://doi.org/10.1007/s11746-999-0171-7

Lijić, M. (2014): Proizvodnja i stabilizacija hladno prešanog ulja chie (*Salvia hispanica* L.). Diplomski rad, Prehrambeno-tehnološki fakultet, Osijek, Sveučilište J.J. Strossmayera Osijek.

Moslavac, T., Jokić, S., Pozderović, A., Pichler, A., Škof, B. (2014): Proizvodnja i stabilizacija hladno prešanog bučinog ulja, znanstveni rad, *Glasnik Zaštite Bilja* 37(6, 70-79). https://hrcak.srce.hr/file/239607

Moslavac, T., Jokić, S., Šubarić, D., Cikoš, A.M., Lončarić, M. (2018): Proizvodnja i stabilizacija hladno prešanog ulja koštice šljive, Hranom do zdravlja: zbornik radova s 10. međunarodnog znanstvenostručnog skupa, pp. 203 – 214, rad u zborniku.

Nain, A.W., Berdal, G., Thao, P.T.P, Mignolet, E., Buchet, M., Page, Larondelle, Y. (2021): Green Tea Extract Enhances the Oxidative Stability of DHA-Rich Oil. *Antioxidants* 10, 982. https://doi.org/10.3390/antiox10060982

Neunert, G., Górnas, P., Dwiecki, K., Siger, A., Polewski, K. (2015): Synergistic and antagonistic effects between alpha-tocopherol and phenolic acids in liposome system: spectroscopic study. *European Food Research and Technology* 241, 749-757. https://doi.org/10.1007/s00217-015-2500-4

Ottaviani, M.F., Spallaci, M., Cangiotti, M., Bacchiocca, M., Ninfali, P. (2001): Electron Paramagnetic Resonance Investigations of Free Radicals in Extra Virgin Olive Oils. *Journal of Agricultural and Food Chemistry* 49 (8), 3691-3696. https://doi.org/10.1021/jf001203

Panella, C., Calatayud, A. (2018): Chapter 7 - Pepper Crop under Climate Change: Grafting as an Environmental Friendly Strategy. In: *Climate Resilient Agriculture: Strategies and Perspectives* IntechOpen., Rao, C.S., Shanker, A.K., Shanker, C. (ed.), *InTech, Croatia, pp.* 129-154. https://doi.org/10.5772/intechopen.72361

Pérez-Gálvez, A., Garrido-Fernández, J., Mínguez-Mosquera, M.I. (2000): Effect of High-Oleic Sunflower Seed on the Carotenoid Stability of Ground Pepper. *Journal of the American Oil Chemists' Society* 77(1), 79-83. https://doi.org/10.1007/s11746-000-0012-x

Porter, N.A., Caldwell, S.E., Mills K.A. (1995): Mechanisms of Free Radical Oxidation of Unsaturated Lipids. *Lipids* 30: 277–290 (1995). https://doi: 10.1007/BF02536034

Prce, N. (2014): Utjecaj prešanja i prirodnih antioksidanasa na iskorištenje i oksidacijsku stabilnost Camelina sativa ulja. Diplomski rad, Agronomski i prehrambeno-tehnološki fakultet Sveučilišta u Mostaru.

Ranilović, J., Cvetković, T., Kušević, T., Gajari, D., Tomić-Obrdalj, H., Aladić, K., Jokić, S. (2024a): The impact of smoke point and aroma profile of sweet red pepper seed oil on culinary application and consumer acceptance. *Croatian Journal of Food Science and Technology*, 16 (1), 173-187. https://doi.org/10.17508/CJFST.2024.16.1.12

Ranilović, J., Cvetković, T., Jokić, S., Panjičko, M. (2024b): Studija slučaja: od otpada iz proizvodnje ajvara do novih visokovrijednih proizvoda (Case study: From ajvar production by-products to new highly-valuable products). In: Neke mogućnosti iskorištenja nusproizvoda prehrambene industriji, Sveučilište J.J. Strossmayera Osijek Prehrembeno-tehnološki fakultet Osijej, Šubarić, D., Jašić, M., Jokić, S. (ed.), Osijek, Hrvatska, pp. 149-162.

Regulation on Edible Oils and Fats, NN 11/2019, 117/2021; Regulation 1308/2013

Tang, L., Liu, R., Xu, Y., Zhang, X., Liu, R., Chang, M., Wang, X. (2022): Synergistic and antagonistic interactions of α-tocopherol, γ-oryzanol andphytosterol in refined coconut oil. *LWT – Food Science and Technology* 154,112789. https://doi.org/10.1016/j.lwt.2021.112789

UN Development Programme (2023). Cosmetics made of Leftover Ajvar Peppers. https://www.undp.org/serbia/stories/cosmetics-made-leftover-ajvar-peppers. Accessed August 26, 2024.

REGULATION (EU) No 1308/2013 OF THE EUROPEAN PARLIAMENT AND OTH THE COUNCIL of 17 December 2013 establishing a common organisation of the markets in agricultural products and repealing Council Regulations (EEC) No 922/72, (EEC) No 234/79, (EC) No 1037/2001 and (EC) No 1234/2007, Official Journal of EU, L 347/671

USD Analytics (2024): Vegetable Oil Market Size, Share, Trends, Growth Outlook and Opportunities to 2030-by Type (Palm oil and palm kernel oil, Soybean oil, Canola oil, Sunflower oil, Olive oil, Others), Application (Food, Feed, Industrial), and Companies Report. https://www.usdanalytics.com/industry-reports/vegetable-oil-market. Accessed August 2, 2024.

Wagner, K-H., Kamal-Eldin, A., Elmadfa, I. (2004): Gamma-Tocopherol – An Underestimated Vitamin? *Annals of Clinical Nutrition and Metabolism* 48, 169-188. https://doi.10.1159/000079555

Wang, K., Zheng, Z., Liu, C., Wang, Y., Li, J., Liu, Y. (2020): Identification and quantification of synergetic antioxidants and their application in sunflower oil. *LWT – Food Science and Technology* 118, 108726. https://doi.org/10.1016/j.lwt.2019.108726