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Characteristics of cold-pressed walnut (*Juglans regia* L.) oil from Western and Central Serbia

Karakteristike hladno ceđenog orahovog (*Juglans regia* L.) ulja iz Zapadne i Centralne Srbije

Vesna ĐUROVIĆ (✉), Mirjana RADOVANOVIĆ, Dalibor TOMIĆ, Miloš MARJANOVIĆ, Dušan MARKOVIĆ, Leka MANDIĆ

University of Kragujevac, Faculty of Agronomy in Čačak, Cara Dušana 34, Čačak, Serbia

✉ Corresponding author: vesna.djurovic@kg.ac.rs

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ABSTRACT

An ecologically acceptable "green" method of extraction cold-pressing is applied in order to reduce the use of organic solvents and preserve bioactive substances from raw material. Therefore, the work aimed to characterize cold-pressed oils obtained from non-intensively grown walnuts. Walnut samples were collected on the territory of Western and Central Serbia. 12 cold-pressed oils were used for the analysis. The content of water and volatile substances, index of refraction and specific absorbances, content of total phenols and antioxidant activity of the hydrophilic and lipophilic fraction of the oil were examined. Microbiological analyses included: the total number of bacteria, the total number of molds, and the presence of sulphite-reducing clostridia and *Escherichia coli*. In 11 oil samples, the content of water and volatile substances was in accordance with the national Rulebook. The refractive index varies from 1.4725 to 1.4745 for walnut oils. Cold-pressed walnut oils differ in the ranges of specific absorbances (1.08–1.75 at 232 nm and 0.04–0.15 at 270 nm). The content of total phenols in walnut oil was from 3.25 to 5.41 mg GAE/100 mL. The lipophilic fraction of oils showed a significantly higher antioxidant potential compared to the hydrophilic fraction. The results of DPPH• radical scavenging activities the lipophilic fraction was from 17.53% to 39.21%. In all tested walnut oil samples, the total number of bacteria and molds was low. The presence of sulfite-reducing clostridia and *Escherichia coli* was not detected. In addition to the general quality and biological value of the oils, it is important that they are microbiologically safe.

Keywords: refractive index, specific absorbances, phenolic content, antioxidant capacity, microbiological evaluation

SAŽETAK

Ekološki prihvatljiva „zelena" metoda ekstrakcije hladno presovanje se primenjuje u cilju smanjenja upotrebe organskih rastvarača i očuvanja bioaktivnih materija iz sirovine. Stoga je i cilj rada da se okarakterišu hladno ceđena ulja dobijena od neintenzivno gajenih oraha sakupljenih na teritoriji Zapadne i Centralne Srbije. U 12 dobijenih orahovih ulja ispitivan je sadržaj vode i isparljivih materija, indeks refrakcije i specifične apsorbancije, sadržaj ukupnih fenola kao i antioksidativna aktivnost hidrofilne i lipofilne frakcije. Mikrobiološke analize su obuhvatile: ukupan broj bakterija, ukupan broj plesni, prisustvo sulfitoredukujućih klostridija i *Escherichia coli*. U 11 uzoraka ulja sadržaj vode i isparljivih materija bio je u skladu sa Pravilnikom za nerafinisana ulja. Rezultati su pokazali da se indeks refrakcije ulja kretao od 1,4725 do 1,4745. Takođe, među uzorcima su utvrđene varijacije u specifičnim apsorpcijama na 232 i 270 nm (1,08–1,75 na 232 nm i 0,04–0,15 na 270 nm). Sadržaj ukupnih fenola je iznosio od 3,25 do 5,41 mg GAE/100 mL. Lipofilna frakcija ulja pokazala je značajno veći antioksidativni potencijal u poređenju sa hidrofilnom frakcijom. Antioksidativna aktivnost lipofilne frakcije ulja, merena DPPH• metodom, iznosila je od 17,53% do 39,21%. Ukupan broj bakterija i plesni bio je nizak. Prisustvo sulfitoredukujućih klostridija kao i *Escherichia coli* nije detektovano. Pored opšteg kvaliteta i biološke vrednosti ulja, važno je da su ulja mikrobiološki bezbedna.

Ključne reči: indeks refrakcije, specifične apsorbance, ukupni fenoli, antioksidativni kapacitet, mikrobiološka evaluacija

INTRODUCTION

In recent decades, health-safe food production has been increasing around the world, and Serbia is also following that trend (Golijan et al., 2017). Less developed rural areas have a special potential in this sense, due to the preserved environment (Radovanović et al., 2022). Cold pressing of the oil makes the initial chemical composition be kept mostly unchanged, due to the low process temperature, which does not exceed 50 °C. Cold pressing is an environmentally friendly method, in contrast to extraction, which requires the use of organic solvents. Cold pressing does not require heat or chemical treatment, so it does not destroy the useful properties of the oil.

The walnut kernel contains about 52 – 75% oil depending on the variety, cultivation and irrigation of walnut trees (Slatnar et al., 2015). One of the uses of walnut kernels is for the production of edible oil, which not only has positive sensory properties for consumers but is a rich source of nutrients. Also, they are very important for human health due to the profile of fatty acids (omega-6 and omega-3 fatty acids) and non-nutritive components with antioxidant capacity. The phenolic compounds contained are significant constituents due to their ability to scavenge free radicals (Slatnar et al., 2015).

Obtaining oil without thermal and chemical treatment enables the preservation of bioactive substances, but on the other hand, also the survival of microorganisms from the seeds and/or production lines (Drewnowska and Swiecicka, 2021). Today, the nutritional composition of cold-pressed oils originating from the seeds of various plants is intensively researched and known (Chew, 2020), but the microbiological quality of the oil is less known. Microorganisms play a significant role in the determination of the shelf lives of food products, and they are responsible for food spoilage (Đurović et al., 2021). The microbiological quality is a very important issue because various pathogenic bacteria, such as *Listeria monocytogenes*, *Salmonella* spp., Shiga-toxin-producing *Escherichia coli*, and yeasts can persist in these products (Zullo et al., 2018). It is recommended to consume unrefined oils without any thermal treatment, but there is the potential risk of introducing pathogenic bacteria and their toxins.

Therefore, the aim of this research was a characterization of some optical, phytochemical and microbiological properties of the cold-pressed oils obtained from non-intensively grown walnuts.

MATERIALS AND METHODS

Collection and preparation of samples

Walnut samples were from the 2021 harvest season evenly collected from the less developed rural areas of Central and Western Serbia. The research had several phases: a collection of samples, obtaining of oil by pressing, and analysis of the obtained oils. Before the analysis, the walnut kernels, manually shelled from the endocarp, were ground and then stored in closed paper bags at ambient temperature. Whole seeds were ground in a blender before pressing. Part of each sample was saved as a bank of genetic material. Twelve walnut oil samples (W1–W12) were analyzed.

Cold-pressed oils

The oil was obtained by pressing grounded walnut kernels on a screw press OP650W (Gorenje, Slovenia; Figure 1). The temperature during pressing did not exceed 50 °C.

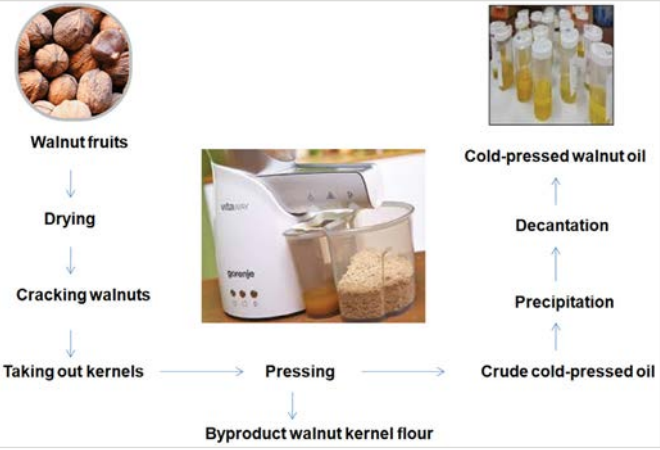

Figure 1. Obtaining cold-pressed oils from walnut kernels

Collected oils were stored at 4 °C overnight to precipitate the sediment. Oils were separated by decantation. During pressing, oil cake remains as a by-product.

Determination of moisture and volatile matter

The content of moisture and volatile matter is an important parameter of oil quality and is necessary as a basic initial characterization of samples. Determination was made according to the standard method (ISO 662, 1980; Dimić and Turkulov, 2000) with modifications of the sample weight. About 3±0.001 g (instead of 5 to 10 g) of the sample was measured and dried in an oven at 103±2 °C to constant weight. The result is expressed as the content of moisture and volatile matter in % (w/w).

Characteristics of walnut oils

The characterization of the obtained oil was performed by determining the optical properties: measuring the refractive index (RI), the specific absorbance of the oils and recording characteristic spectra in the UV/Vis part of the spectra. The RI of walnut oil samples were measured by the standard method (ISO 6320,1995; Dimić and Turkulov, 2000). The samples were prepared by previous drying at 103±2 °C. Abbe refractometer, (A. KRÜSS, Germany), with a range of 1.30–1.70 ± 0.0001 and monochromatic Na light (589,6 nm) was used for analysis.

All samples were measured twice at 23 °C and there were no differences between the two measurements. Specific absorbance measurements were performed on a UV–visible spectrophotometer (Cary 3000, Agilent, USA) using a standard method (ISO 3656, 1989; Dimić and Turkulov, 2000). Absorbances were measured at 232 nm and 270 nm against n-hexane (HPLC grade, Carlo Erba, France) as a blank, using a 10-mm quartz cuvette. The sample weight (about 0.1 g±0.001 g) was suitably diluted in a volumetric flask of 25 mL with n-hexane, thoroughly mixed, and measured. Results are expressed as specific absorbance for 1% solution (g/100 mL) using a 1 cm cuvette at defined wavelengths.

To measure the spectra, the oils were dissolved in n-hexane (HPLC grade, Carlo Erba, France) in a ratio of 1:9. Measurement of spectra was performed on a UV/Vis spectrophotometer (Cary 3000, Agilent, USA) from 200 to 800 nm with data interval 1 nm and scan rate 600 nm/min. Cary Win UV software was used.

Antioxidant properties and total phenolic content

To determine the antioxidant capacity and total phenolic content, the samples were prepared as follows. To separate the hydrophilic (HF) and lipophilic fractions (LF) 1000 µL of oil was mixed with 1000 µL of methanol (80%), and then centrifuged (5000 r/min, 10 min) to separate fractions. The hydrophilic fraction was used for the determination of the content of total phenols, while the antioxidant activity was determined in both the hydrophilic and lipophilic fractions.

The total phenolic content (TPC) was determined using a modified Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). A 40 µL of sample (hydrophilic fraction of oils) or gallic acid standard solution was mixed with 3.16 mL of distilled water whereupon 200 µL of Folin-Ciocalteu reagent was added. 600 µL of 20% Na_2CO_3 solution was added after 8 min. After 2 hours of incubation at room temperature, absorbance was measured at 760 nm. The results were expressed as milligrams of gallic acid equivalents per 100 mL (mg GAE/100 mL).

To evaluate the antioxidant activity of the oils, spectrophotometric analysis was performed using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The DPPH assay was used to determine the antioxidant activity in hydrophilic and lipophilic fractions. 3.9 mL DPPH solution ($6 \cdot 10^{-5}$M) with aliquot 0.1 mL of sample was mixed and kept in the dark for 30 minutes. The absorbance was measured at 515 nm, with distilled water as a reference. Results were expressed as a percentage of inhibition of the DPPH radical.

Microbiological evaluation of oil

The number of aerobic mesophilic bacteria and molds, the presence of sulfite-reducing bacteria and *Escherichia coli* were determined. Enumeration of aerobic bacteria and molds was determined using the standard microbiological plating method (ISO methods 4833 and

21527-1). Nutrient Agar was used for the total number of aerobic mesophilic bacteria; Sabouraud Dextrose Agar for cultivation and isolation of molds and Endo agar for *E. coli*. For the determination of the presence of sulphite-reducing clostridia, test tubes with 1 mL of basic dilution were heated in a water bath for 10 min at 80 °C, and then the Sulphite Agar was poured into the tubes (Đurović et al., 2022).

Statistical analysis

Experimental data in this work were statistically analyzed by one-way ANOVA followed by the least significant test which was used to detect significant differences among the means. The level of significance was assigned at $P < 0.05$ for the content of phenolic and antioxidative activity. The statistical analyses were performed using the program STATISTICA 12 (StatSoft, Inc. 2012).

RESULTS AND DISCUSSION

Moisture content and volatile materials in the oil

The moisture and volatile matter content in walnut oil ranged from 0.007–0.07% (w/w), except for one sample with a significantly higher value (0.24%; Figure 2). Only one walnut oil sample had a higher content than the maximum value (0.2%) defined for unrefined edible oils (Codex Alimentarius Commission, 1999) and in the domestic legislation (Rulebook, 23/2006; 43/2013).

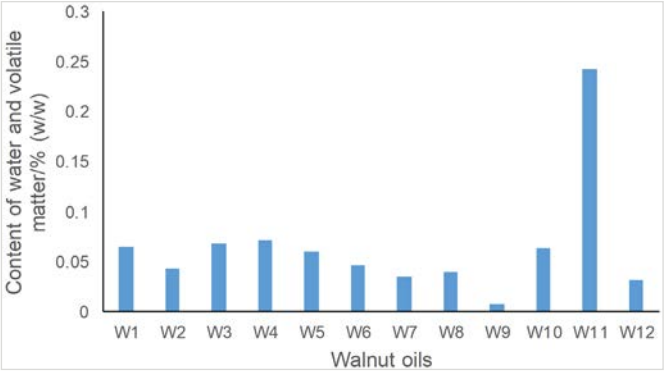

Figure 2. Content of moisture and volatile matter in cold pressed walnut oils

Higher moisture content in oil samples can cause hydrolytic changes: breakdown of triacylglycerols and release of fatty acids, which could cause a decline in the quality of oil samples and less sustainability. The analyzed values, in addition to the moisture, also include the volatile matters. Volatile matters are naturally present in oils or as a result of non-enzymatic oxidative changes - autoxidation of unsaturated fatty acids and give the perception of rancidity. Volatile compounds of the kernel are a good indicator of walnut oxidation during storage (Grilom and Wangm, 2021). The proportions of walnut oil volatile matter depend on the cultivar (Ojeda-Amador et al., 2018).

Optical characterization

The walnut oils were almost identical in color with only slight variations in the shade of golden yellow. It must be kept in mind that addition to the botanical species cultivar and geographical origin can also play a decisive role in the variability of the color of cold-pressed oils (Turrini et al., 2021).

The oil samples were grouped according to the values of the RI (Table 1). The walnut oil samples had RI from 1.4725 to 1.4745, with a different distribution of the number of samples per group. The RI differed depending on the type of cultivar walnuts (Gharibzahedi et al., 2014).

Table 1. The refractive index of cold-pressed oils from different walnut samples

Cold-pressed walnut oil	Samples RI (23 °C)
W10	1.4725
W7, W9	1.4730
W8, W12	1.4735
W1, W2, W3, W5, W11	1.4740
W4, W6	1.4745

RI: Refractive Index

Considering that the oils mostly consist of triacylglycerols (98%), it is the fatty acid composition, their molecular weight, chain length, and degree of unsaturation of fatty acids that affect the value of the RI. Unsaturated fatty

acids have higher RI than saturated ones, with an increase in the number of double bonds in fatty acids.

Cold-pressed walnut oils differ in the ranges of specific absorbances (Figure 3). One walnut oil sample gave cloudy mixtures with hexane and could not be analyzed spectrophotometrically. Walnut oils show specific absorbances in the range of 1.08–1.75 at 232 nm and 0.04–0.15 at 270 nm (Figure 3). This is in agreement with the results of the other authors. Specific absorbances in the 8 tested walnut cultivars ranged from 1.04 –1.21 at 232 nm and ranged from 0.05–0.07 at 270 nm (Martínez and Maestri, 2008). For three walnut cultivars ranged from 1.05–1.12 at 232 nm and 0.05–0.06 at 270 nm (Gharibzahedi et al., 2014). Grilom and Wangm (2021) showed values of specific absorbances for Chandler at 0.95/0.07 and 1.13/0.13 for Howard at 232/268 nm, respectively. Ampofo et al. (2022) measured 1.06/0.09 for Chandler walnut and 0.9/0.09 Howard oil at 232/268 nm, respectively.

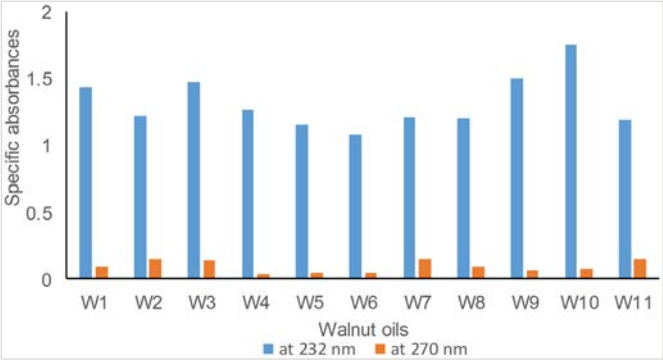

Figure 3. Specific absorbances of cold-pressed walnut oils

The specific absorptions indicate possible oxidative changes: absorption at 232 nm gives insight into the presence of primary oxidation products, and absorption at 270 nm gives insight into possible secondary products of oil oxidation. The values of these parameters of the tested oils are similar to the literature data. Better oxidative stability of walnut kernels and their oils with lower specific absorption can be assumed. It is important to note that walnut oils are characterized by low values of specific absorption, which is confirmed by the cited literature data, and which is not caused by oxidative changes.

In this spectral band, there are important bioactive substances: juglone (245 nm), tocopherol (295 nm) and retinol (325 nm) (Popovici and Deseatnicova, 2013). Probably, these compounds can play a protective role in the oxidation process of walnut oil.

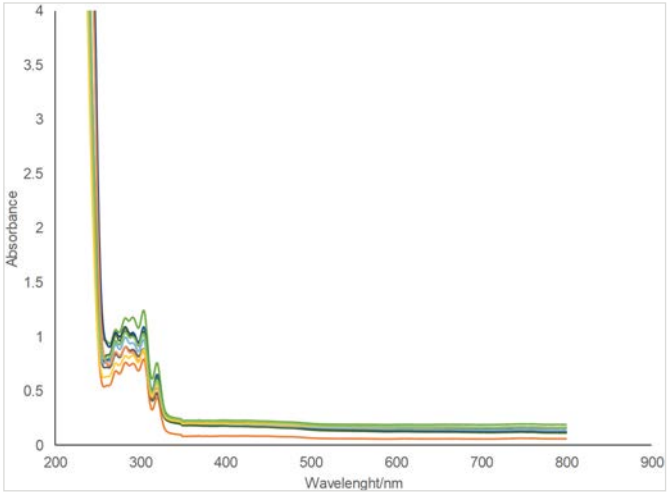

Figure 4. UV-Vis spectra of cold-pressed walnut oils; mixture oil: n-hexane (1:9)

Total phenolic content and antioxidant activity of walnut oil

The content of total phenols in the analyzed walnut oil samples (Table 3) ranged from 3.25 (W9) to 5.41 mg GAE/100 mL (W4). It can be observed that there were differences in the content of total phenols among the samples. The highest content was recorded in sample W4 (5.41 mg GAE/100 mL) which is significantly higher than sample W9 (3.25 mg GAE/100 mL), even by 60%. These differences could be explained by the influence of the genotype and environmental conditions on the biosynthesis and accumulation of phenolic compounds. The chemical composition of phytoconstituents differs depending on geographical location, meteorological conditions and soil type. The total content of phenolic compounds in the oil depends on many factors, the cultivar, the climatic and cultivation conditions, methods of obtaining oil, packaging or storage (Boskou et al., 2005; Kulaitienė et al., 2017).

Table 2. Total phenolic content and antioxidative activity of the cold-pressed walnut (*Juglans regia* L.) oils

Walnut sample	Total phenolic content mg GAE/100 mL	DPPH % inhibition LF	DPPH % inhibition HF	Ratio LF/HF
W1	4.14± 0.15^{bcd}	23.96± 1.94^{c}	3.06±010^{d}	7.95
W2	4.46 ±0.56^{abc}	24.38± 1.81^{c}	3.8± 0.22^{d}	6.39
W3	4.46± 0.81^{abc}	28.67 ±3.39^{b}	3.69± 0.22^{d}	7.82
W4	5.41±0.21^{a}	24.92± 0.49^{c}	3.39 ±0.18^{d}	7.34
W5	4.93±0.07^{ab}	39.21±5.44^{a}	4.43± 0.34^{c}	8.86
W6	4.56± 0.43^{ab}	31.96 ±1.82^{b}	9.91 ±0.31^{a}	3.21
W7	4.31±1.39^{a-d}	24.07±0.71^{c}	3.47± 0.40^{d}	6.93
W8	4.46 ±0.13^{abc}	17.53 ±1.67^{d}	3.45±0.03^{d}	5.08
W9	3.26 ±0.18^{d}	24.22± 8.36^{c}	3.79± 0.39^{d}	6.38
W10	3.41 ±0.20^{d}	18.07 ±3.56^{d}	3.36± 0.06^{d}	5.38
W11	3.84 ±0.34b^{cd}	23.07 ±0.53^{cd}	3.21 ±0.02^{d}	7.18
W12	4.65± 0.88^{ab}	21.79 ±1.83^{cd}	4.73 ±1.51^{b}	4.61

Hydrophilic (HF) and lipophilic fractions (LF)
Results are reported as mean value ± standard deviation of three replicate analyses (n = 3)
$^{a-d}$ Means of triplicate determination
Means with the same superscripts within the column are not significantly different ($P > 0.05$)
Means without the same superscripts within each column are significantly different ($P < 0.05$)

The antioxidant activity measured by the DPPH method and expressed in % inhibition in the lipophilic fraction ranged from 17.53% (W8) to 39.21% (W5), and in the hydrophilic fraction from 3.015% (W1) to 9.97% (W6). The ratio LF/HF was from 3.20 to 8.85. Prescha et al. (2014) reported the ratio of antioxidant activity of lipophilic and hydrophilic fractions of walnut oil from 1.70 to 7.34. The lipophilic components in which the oils are rich give a significantly greater contribution to the antioxidant activity in walnut oil. This is in agreement with other authors who state significant amounts of lipophilic antioxidants (tocopherols) than hydrophilic ones (phenolic compounds) present in oils (Espin et al., 2000; Prescha et al., 2014).

Walnut is a source of vitamin structures such as alpha-tocopherol and delta-tocopherol, vitamin D-3, retinol (vitamin A), folate, ergosterol, β-sitosterol and stigmasterol (Aydin et al., 2015). These components contribute to the antioxidant activity of walnuts and walnut oil.

Microbiological evaluation

The total number of bacteria in walnut oil was not higher than 100 cfu/mL in any of the tested oil samples (Table 4). In all 12 tested walnut oil samples, the total number of molds was lower than the limit values prescribed in the "Guide to the Application of microbiological criteria for Food" (Ministry of Agriculture, Forestry and Water Supply Trade, 2011). *Escherichia coli* and sulfite-reduction clostridia were not detected in any sample of walnut oil.

Significantly, the results of the microbiological analysis showed that the microbiological quality of the oil is good, the number of bacteria and molds is very low and in accordance with the Guide (Ministry of Agriculture, Forestry and Water Supply Trade (2011) and the presence of *E. coli* was also not detected as well as sulfite-reducing bacteria.

Microorganisms can be found in the final product from different sources. During growth, crops are often exposed

Table 3. Microbial evaluation of walnut oil

Sample of walnut oil	Bacteria	Molds	*E. coli*	Sulphite-reducing clostridia
W1	10–100	1	nd	nd
W2	<10	1	nd	nd
W3	<10	nd	nd	nd
W4	<10	>10	nd	nd
W5	10–100	5	nd	nd
W6	10–100	<10	nd	nd
W7	<10	2	nd	nd
W8	10–100	1	nd	nd
W9	<10	nd	nd	nd
W10	<10	nd	nd	nd
W11	10–100	nd	nd	nd
W12	<10	12	nd	nd

nd - non detected

to a wide range of microbial contamination from many sources, such as manure, wild birds and animals, especially soil and irrigation water contaminated with industrial and domestic wastes (Mapanda et al., 2005; Doyle and Erickson, 2008). *E. coli* (the family Enterobacteriaceae) as indicative organisms for fecal contamination/poor sanitary conditions (Ike et al., 2020), can be found in water, food products, plants, soil, animals and human. Microbial contamination of fruits and vegetables is directly linked to hygienic practices during their production, harvesting, postharvest handling, processing and distribution of the product (Heaton and Jones, 2008). Drewnowska and Swiecicka (2021) reported the presence of several spore-forming bacilli from Bacillus sp., as well as the dominance of cyanobacteria and/or proteobacteria in samples of cold-pressed oil, which is related to plant cultures. Although microbiological analyzes showed the obtained walnut oil was of good quality within the examined microbiological parameters, it is important to emphasize the importance of good hygiene practices in all stages of production to avoid contamination of the final product with pathogenic bacteria.

CONCLUSION

Cold-pressed walnut oils were produced at temperatures lower than 50 °C. The tested parameters of cold-pressed walnut oils have the expected values and in accordance with international and domestic legislation, with the exception of one sample with a higher content of moisture and volatile matter. The UV-Vis spectra of cold-pressed walnut oils are a reliable parameter of oil identification. In eleven genotypes, no differences were observed in the shape of the spectrum of cold-pressed walnut oils. The content of total phenols in walnut oil is from 3.25 to 5.41 mg GAE/100 mL. The lipophilic fraction of walnut oils showed a significantly higher antioxidant potential compared to the hydrophilic fraction. The presence of sulfite-reducing bacteria and *E. coli* was not detected in walnut oil. The results of the microbiological analyses show that good manufacturing practice was applied. The importance of production and hygiene practices at all levels of the production/processing chain in order to prevent and reduce microbial contamination of the final product. Drying, storage, and packaging conditions, along with decontamination techniques,

equipment disinfection, and compliance with hygiene control protocols are the main guidelines for minimizing the risk of cross-contamination.

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