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COMPARATIVE ASSESSMENT OF SPICE MIXTURES ON THE OXIDATIVE STABILITY OF WALNUT OIL DURING STORAGE

Diana MOIGRADEAN[1], Andreea GHITULESCU[1], Mariana-Atena POIANA[1], Daniela STOIN[1], Liana ALDA[1], Simion ALDA[2], Corina MEGYESI[1], Carmen Daniela PETCU[3], Laura RADULESCU[1], Despina BORDEAN[1]

[1]University of Life Sciences "King Mihai I" from Timisoara, Faculty of Food Engineering, 119 Calea Aradului Street, 300645, Timisoara, Romania
[2]University of Life Sciences "King Mihai I" from Timisoara, Faculty of Engineering and Applied Technologies, 119 Calea Aradului Street, 300645, Timisoara, Romania
[3]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, District 5, 050097, Bucharest, Romania

Corresponding author email: marianapoiana@usvt.ro

Abstract

This study investigated the impact of supplementing walnut oil with spice mixtures on its oxidative stability during six months of storage at 20°C. Three spice mixtures were incorporated into the oil: (1) garlic (Allium sativum) combined with black pepper (Piper nigrum); (2) pistachio (Pistacia vera), pine seed (Pinus pinea), and black pepper; and (3) cinnamon (Cinnamomum zeylanicum) together with vanilla (Vanilla planifolia). Oxidative stability was assessed using peroxide value (PV) for primary oxidation and para-anisidine value (p-AV) for secondary oxidation. In addition, the TOTOX value provides an overall measure of oxidation, reflecting cumulative formation of primary and secondary products, calculated by combining PV and pAV. The enhancement of walnut oil oxidative stability by spice mixtures was directly related to their antioxidant content, with higher levels providing greater protection. Among the mixtures tested, the pistachio, pine seed, and black pepper combination showed the strongest protective effect, delaying lipid oxidation and improving oil stability. These findings demonstrate the potential of spice mixtures as functional additives with antioxidant properties to extend the shelf life and quality of walnut oil.

Key words*: antioxidant compounds, oxidative stability, primary and secondary oxidation, spice mixtures, walnut oil.*

INTRODUCTION

The walnut (*Juglans regia* L.) is the seed of a drupe and is commonly consumed as a nut in various culinary applications. Oil produced by cold pressing walnut kernels at approximately 60°C is appreciated both for its distinctive walnut aroma and for its valuable nutraceutical profile (Anwar et al., 2020).

Because it contains a large proportion of unsaturated fatty acids, walnut oil is highly vulnerable to oxidative processes. Linoleic acid is the dominant component of its fatty acid profile, followed by oleic and linolenic acids. The oil also provides considerable amounts of vitamin E (tocopherols), which contributes to slowing oxidative deterioration (Leahu et al., 2016). In addition to enhancing oxidative stability, naturally occurring antioxidants and their derivatives offer notable health benefits to consumers of vegetable oils (Mishra et al., 2021).

Even with the presence of antioxidants such as polyphenols, vitamin E, and sterols, walnut oil continues to face significant limitations in effectively resisting oxidation.

There are three main categories of oil oxidation: enzymatic oxidation, auto-oxidation and photo-oxidation.

The process of auto-oxidation can be delayed by the addition of antioxidants. Today's, the edible oil and oil-containing products industry primarily utilizes two types of antioxidants, natural and synthetic, to ensure product freshness and stability. The following synthetic antioxidants are well-known: BHA (butylhydroxyanisole), BHT (dibutylhydroxytoluene) and TB-HQ (tert-butylhydroquinone) (Jin et al., 2023).

Natural antioxidants occurring in fruits, vegetables, peels, seeds and various spices have demonstrated strong effectiveness in limiting hydrolysis, polymerization and the development of secondary oxidation compounds (Yildiz et al., 2025). At the same time, the growing consumer preference for "natural" ingredients has encouraged the edible oil industry to increasingly rely on plant-derived antioxidant sources. Over the past two decades, numerous studies have examined the incurporation of pure antioxidants, antioxidant blends and plant extracts into bulk edible oils. These compounds, including tocopherols, flavonoids, phenolic acids, polyphenols, carotenoids, lignans and ascorbate derivatives, have been associated with anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities (Mishra et al., 2021).

Spices also contain high levels of phenolic compounds and are used in food products to enhance their flavour profile, as well as to provide beneficial bioactive components, thereby increasing their value as functional foods (Somogyi et al., 2015). Except for pine seed (*Pinus pinea*), all spices included in this study are well known to the consumers in our country: garlic (*Allium sativum*), black pepper (*Piper nigrum*), pistachio (*Pistacia vera*), cinnamon (*Cinnamomum zeylanicum*) and vanilla (*Vanilla planifolia*). The genus *Pinus L.* represents the largest and most diverse group within the Pinaceae family, primarily grows throughout the Northern hemisphere. While pine-based products are common globally, they are rarely used as food ingredients. However, research highlights the significant therapeutic benefits of pine preparations for human health (Dziedzinski et al., 2021).

This study examined the improvement of oxidative stability in cold-pressed walnut oil enriched with different dried spices (garlic, black pepper, pistachio, pine seed, cinnamon, and vanilla) during six months of storage at ambient temperature. The progression of lipid oxidation was assessed by determining the peroxide value, the p-anisidine value and the total oxidation value, referred to respectively as PV, p-AV and TOTOX.

MATERIALS AND METHODS

Preparation of walnut oil samples

Walnut oil produced in Romania is commercially available in supermarkets across Timisoara, and this product served as the base material for preparing the spice-enriched walnut oil samples. Three mixtures were added to oil: garlic and black pepper, noted WOS1; pistachio, pine seed and black pepper - WOS2; cinnamon and vanilla - WOS3. Walnut oil was the control sample (C) used for this study. The dry spice mixtures were incorporated into the oil at a proportion of 5:100 (w/v), and the samples were monitored monthly over a six-month storage period at 20°C. Oxidative stability was assessed by determining the peroxide value, the p-anisidine value and the total oxidation value, which together reflect the progression of primary and secondary lipid oxidation.

Chemical analysis

The extraction of bioactive compounds from the oil was carried out by mixing 2 mL of each walnut oil sample with 20 mL of a 70% (v/v) ethanol solution, followed by a 30-minutes rest at room temperature. The mixture was then centrifuged at 5000 rpm for 10 minutes using a Hettich EBA 200 centrifuge, after which the resulting supernatant was collected and used for determining the total antioxidant capacity (TAC), which reflects the sample's overall reducing power, and the total phenolic content (TPC), representing the concentration of phenolic compounds responsible for antioxidant activity.

The total antioxidant capacity was assessed using the FRAP method, which measures the ability of the sample to convert ferric ions into their ferrous form, following the procedure originally introduced by Benzie and Strain (1996). To prepare the working solution, three components were freshly combined: a 10 mM TPTZ solution prepared in 40 mM hydrochloric acid, a 20 mM ferric chloride solution and a 300 mM sodium acetate buffer adjusted to pH 3.6, mixed in a 1:1:10 proportion. Each supernatant sample was diluted tenfold with distilled water, after which 2.5 mL of the FRAP reagent was added.

The reaction mixture was left to stand for 30 minutes at room temperature to allow full color development. Absorbance was then recorded at 593 nm using a SPECORD 205 UV-VIS spectrophotometer (Analytik Jena). The antioxidant capacity was expressed as millimoles of Fe^{2+} per gram of sample.

The total phenolic content was determined using the Folin-Ciocalteu method described by Singleton et al. (1999).

For this analysis, 0.5 mL of each supernatant sample, previously diluted tenfold with distilled water, was combined with 2.5 mL of the diluted Folin-Ciocalteu reagent and 2.0 mL of a sodium carbonate solution at a concentration of 7.5 percent. The mixture was kept at room temperature for 120 minutes to allow the reaction to proceed. Absorbance was then recorded at 750 nm. Gallic acid was used to generate the calibration curve, and the results were expressed as milligrams of gallic acid equivalents per gram of sample, as reported by Gao et al. (2022).

The peroxide value was determined using the classical iodometric procedure commonly applied for evaluating the primary oxidation products formed in edible oils, as described by Ampofo et al. (2022). The results were expressed as milliequivalents of active oxygen per kilogram of oil.

The p-anisidine value was determined to evaluate the secondary oxidation compounds present in the oil. The method is based on the reaction between aldehydes formed during lipid degradation and the amine group of p-anisidine, which produces a colored Schiff base with maximum absorbance at 350 nm. Isooctane served as the reference solution. For the analysis, 2 g of each walnut oil sample were dissolved in 25 mL of isooctane, and the absorbance was recorded at 350 nm. A portion of this solution was then mixed with a p-anisidine reagent prepared in glacial acetic acid, and after a reaction time of 10 minutes, the absorbance was measured again at the same wavelength. The p-anisidine value was calculated following the approach described by Gharby et al. (2021).

The total oxidation value (TOTOX) was determined following the method described by Gharby et al. (2021).

This index was obtained by combining the contribution of primary and secondary oxidation products, being calculated as twice the peroxide value added to the p-anisidine value.

All analyses were performed in triplicate to ensure the reliability of the results. All chemicals and solvents used throughout the experiments were of analytical grade.

Statistical analysis

Origin 9.0 software was used to conduct simple linear regression, correlating storage time with the PV, p-AV and TOTOX values of walnut oils samples with spice mixtures.

Principal Components Analysis (PCA) is a technique for reducing data complexity while preserving its essential features and patterns, without losing significant statistical information (Zakaria, 2025).

RESULTS AND DISCUSSIONS

The evolution of the total antioxidant capacity during storage was monitored for all samples (C, WOS1, WOS2 and WOS3), and the corresponding results are illustrated in Figure 1. The figure includes both the experimental TAC values recorded at each sampling point and the linear regression lines fitted for each sample, which provide a clearer representation of the overall trend over time.

Figure 2 illustrates how the total phenolic content changed during the storage period for the four walnut oil variants (C, WOS1, WOS2 and WOS3).

In addition to the experimental values recorded at each sampling point, the figure also includes the fitted regression lines for each sample, which help visualize the overall trends and the way phenolic compounds evolved over time.

The regression analysis describing the temporal evolution of the WOS3 sample showed R^2 values of 0.9694 for TAC and 0.9304 for TPC. Adding dry spices to walnut oil has been shown to enrich its bioactive compound profile.

In contrast, the WOS2 sample showed lower correlations over time, with R^2 values of 0.3723 for TAC and 0.4432 for TPC.

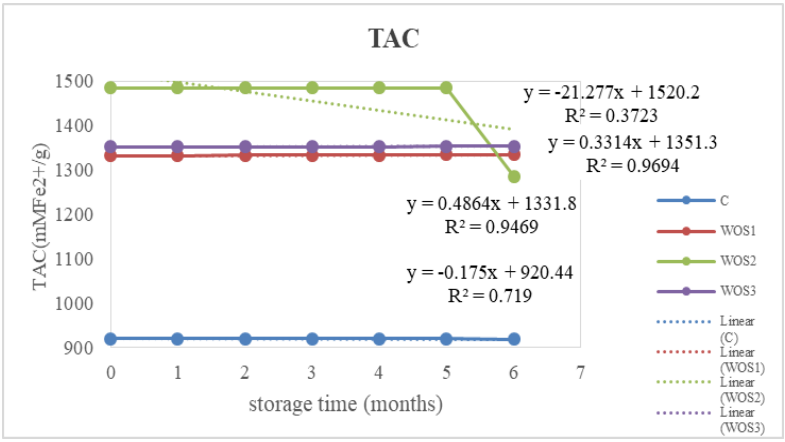


Figure 1. Evolution of the total antioxidant capacity of walnut oil samples during storage, together with the fitted linear regression lines

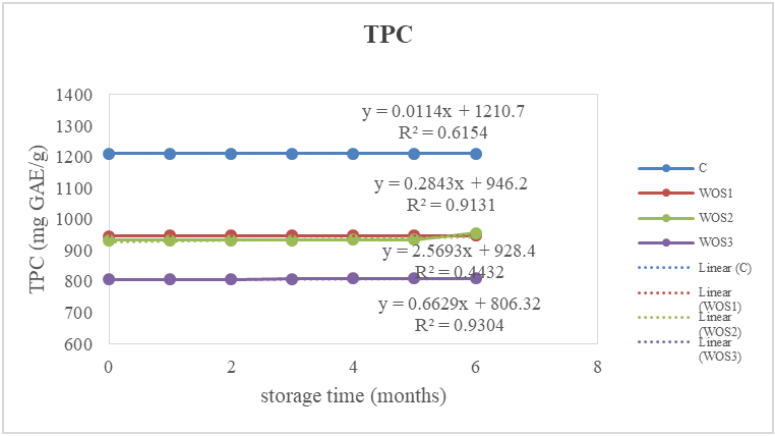


Figure 2. Evolution of the total phenolic content of walnut oil samples during storage, together with the fitted linear regression lines

The oxidative stability of oils is commonly used to estimate their shelf life, defined as the time required for the peroxide value to reach 10 mEq O_2/kg (Moslovac et al., 2023). Previous studies (Leahu et al., 2016; Masoodi et al., 2025) indicate that fresh walnut oil typically presents very low peroxide values, ranging from 0.4 to 0.83 mEq O_2/kg, which increase during storage, particularly over several months. According to the American Oil Chemists' Society standard methods (1997), the peroxide value of fresh oils should remain below 10 mEq O_2/kg. In contrast, oils with a p-anisidine value exceeding 30 mEq O_2/kg are considered rancid (Rebufa et al., 2022). Based on peroxide value measurements, Nosenko et al. (2019) reported that walnut oil maintains stability for approximately 56 days (induction period) and reaches the end of its shelf life after around 90 days.

In Figure 3, the evolution of the peroxide value of walnut oil samples during storage is shown together with the corresponding linear regression lines. After six months of storage (180 days), the walnut oil in our study reached a peroxide value of 2.5 mEq O_2/kg, indicating that the oil maintained a low level of primary oxidation throughout the storage period. This behavior is consistent with previous findings showing that the oxidative stability of walnut oil is influenced by its phenolic and tocopherol content, which varies among different walnut varieties (Rebufa et al., 2022; Moslovac et al., 2023).

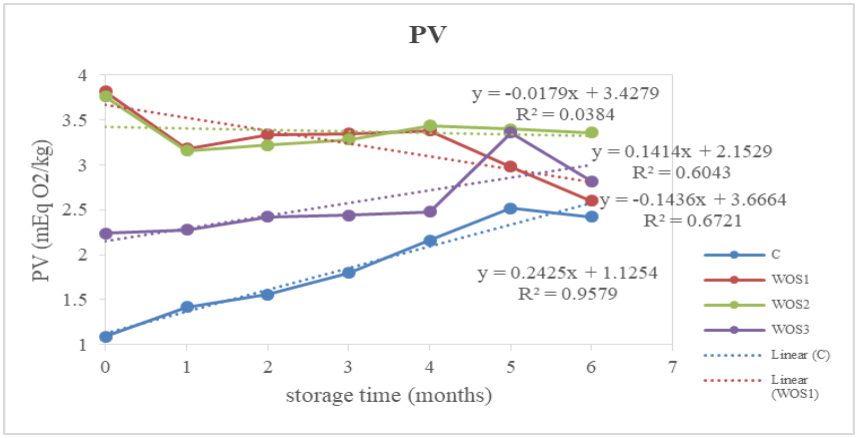


Figure 3. Evolution of the peroxide value of walnut oil samples during storage, together with the fitted linear regression lines

Figure 4 illustrates the evolution of the p-anisidine value of the walnut oil samples during storage, together with the fitted linear regression lines. With an R^2 value of 0.9632, the WOS2 sample shows that the linear model provides a good fit for the temporal evolution of p-AV. After six months of storage, the p-AV reached 1.01 for WOS1, a lower value compared to WOS3, which recorded 1.44

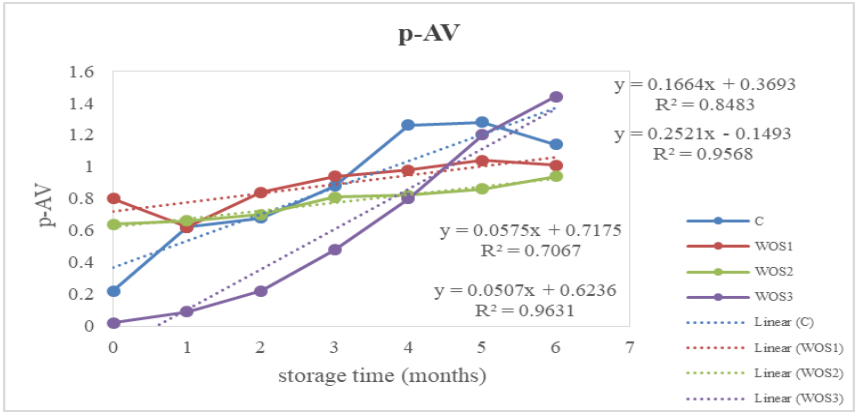


Figure 4. Evolution of the para-anisidine value of walnut oil samples during storage, together with the fitted linear regression lines

Figure 5 presents the evolution of the TOTOX value of the oil samples during storage, together with the fitted linear regression lines. The TOTOX value is an empirical indicator that reflects the overall oxidation state of an oil, obtained by combining two oxidation parameters: PV (specific to primary oxidation) and p-AV (specific to secondary oxidation). A lower TOTOX value is associated with better oil quality (Popovici & Deseatnicova, 2013).

The findings obtained from the oxidative stability indices showed strong consistency, indicating that all parameters related to lipid oxidation responded similarly to the oxidative changes occurring during storage.

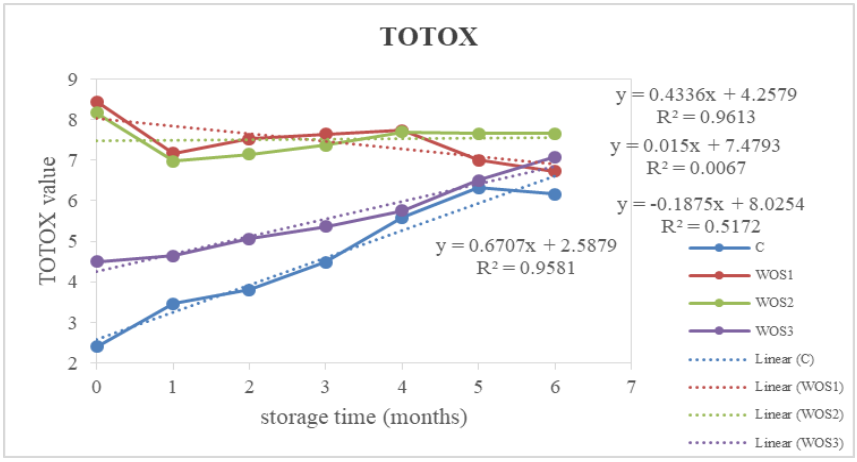


Figure 5. Evolution of the TOTOX value of walnut oil samples during storage, together with the fitted linear regression lines

This coherence highlights the protective effect of the spice mixtures incorporated into the walnut oils, which acted as natural antioxidants and contributed to slowing down both primary and secondary oxidation processes. Figure 6 shows the PCA biplot of antioxidant parameters (TAC, TPC) and oxidative markers (PV, p-AV, TOTOX).

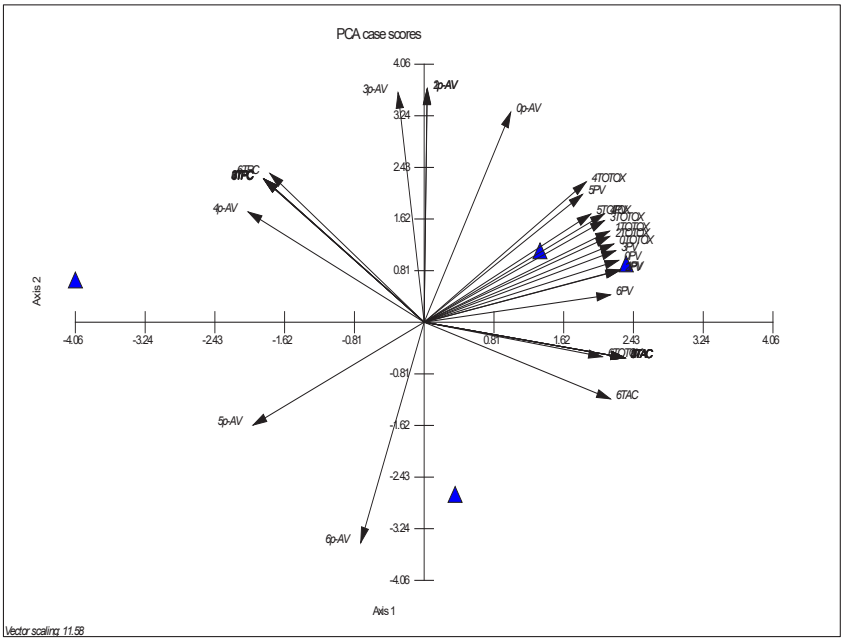


Figure 6. PCA biplot of antioxidant and oxidative markers

Legend: 0TAC- total antioxidant capacity at initial time, 1TAC- total antioxidant capacity after 1 month at storage, 2TAC- total antioxidant capacity after 2 months at storage, 3TAC- total antioxidant capacity after 3 month at storage, 4TAC- total antioxidant capacity after 4 month at storage, 5TAC- total antioxidant capacity after 5 month at storage, 6TAC- total antioxidant capacity after 6 month at storage; idem for TPC, PV, p-AV and TOTOX value.

Principal Components Analysis (PCA) applied to standardized data square-root transformed shows the following: the PCA biplot clearly separated antioxidant parameters (TAC, TPC) from oxidative markers (PV, p-AV, TOTOX),

confirming distinct biochemical responses during storage.

WOS2 demonstrated a delayed oxidative pattern, suggesting a protective effect on lipid stability, while WOS3 exhibited a rapid transition toward the oxidative zone, indicating limited storage suitability. p-AV and TOTOX were identified as the most sensitive predictors of oxidative deterioration (Figure 6).

The heatmap generated from standardized values (Figure 7) illustrates the associations between the oxidative and antioxidant parameters. Based on the heatmap with standardized data, we observe two clear clusters.

Cluster 1: TAC and TPC which show high intensity which is equivalent with strong antioxidant capacity.

Cluster 2: PV, p-AV, TOTOX, which show low intensity which is equivalent with oxidative degradation; WOS2 shows intermediate-high stability, but degradation begins after month 4-5; WOS3 shows the lowest phenolic stability and highest oxidation risk (late months). Control sample (C) remains chemically predictable; is ideal as a kinetic reference.

The heatmap clearly separates functional antioxidant behavior (TAC/TPC) from oxidative stress markers, confirming different metabolic pathways during storage.

The findings from the oxidative stability indices were in good correlation with each other, showcasing the impact of the spice mixtures that were present in the walnut oils about oxidative stability.

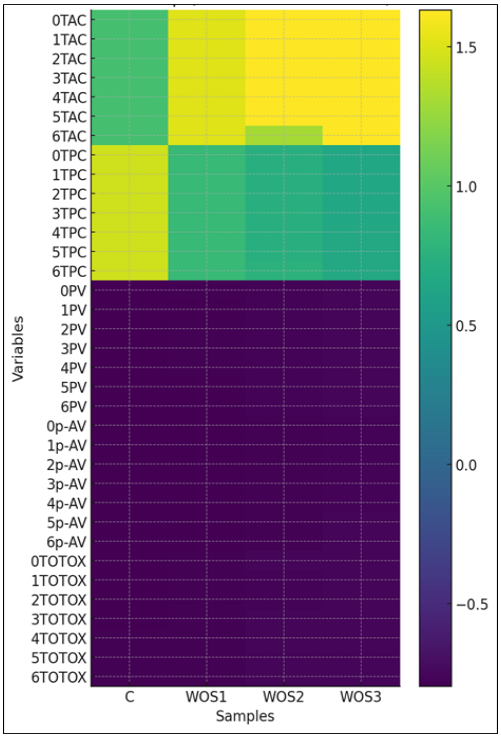


Figure 7. Heatmap based on standardized values

CONCLUSIONS

Our findings provide strong evidence that dried spice mixtures act as effective natural antioxidants in cold-pressed walnut oil. Pistachio, pine seed, and black pepper showed the strongest protective effect, significantly delaying lipid oxidation and improving overall stability. By enhancing resistance to oxidative damage during storage, these natural additives help maintain product quality and may offer additional health benefits. Multivariate analyses supported these results, distinguishing antioxidant parameters from oxidative markers

and identifying p-AV and TOTOX as the most sensitive indicators of deterioration. Overall, this study highlights the potential of spice mixtures as clean-label, functional ingredients capable of extending the shelf life and technological value of walnut oil.

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