Color of extra virgin olive oils enriched with carotenoids from microalgae: influence of ultraviolet exposure and heating

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SUMMARY: A carotenoid-rich extract containing 2.5 mg/mL of lutein and 3.3 mg/mL of β-carotene from the microalga Scenedesmus almeriensis was added to ten extra virgin olive oils from four Spanish cultivars with differing degrees of ripeness, obtaining carotenoid enriched oils with lutein and β-carotene concentrations of 0.082 and 0.11 mg/mL, respectively. Extra virgin olive oils enriched with carotenoids from microalgae were studied by analyzing the effect on color of three different treatments: ultraviolet exposure, microwave heating and immersion bath heating. The methodology was designed to simulate, in controlled laboratory conditions, the effects of household treatments. Spectrophotometric color measurements were then performed to monitor color changes in the enriched and non-enriched extra virgin olive oil samples. Enriched oils are much more chromatic, darker and redder than natural oils. After 55 days UV irradiation, 40 min microwave heating, and 72 hours thermostatic heating, the average color differences for natural/enriched extra virgin olive oils were 98/117, 15/9 and 57/28 CIELAB units, respectively. In general, increasing temperature and ultraviolet exposure produced higher CIELAB color differences in the non-enriched samples. The addition of microalga extracts to extra virgin olive oils was found to induce some color stability and may constitute a future way of increasing the daily intake of beneficial bioactive compounds such as carotenoids.

KEYWORDS: Extra virgin olive oil; Scenedesmus almeriensis; CIELAB system; Carotenoids.

Color de aceites de oliva virgen extra enriquecidos con carotenoides procedentes de microalgas: influencia de la exposición a la radiación ultravioleta y al calentamiento. Añadimos un extracto rico en carotenoides, que contiene 2,5 mg/mL de luteína y 3,3 mg/mL de β-carotenó, procedente de la microalga Scenedesmus almeriensis, a diez aceites de oliva virgen extra de cuatro variedades con diferentes grados de maduración, obteniéndose aceites enriquecidos en carotenoides con concentraciones de luteína y β-carotenó de 0,082 y 0,11 mg/mL respectivamente. Se han estudiado aceites de oliva virgen extra enriquecidos con carotenoides procedentes de microalgas, estudiando el efecto producido sobre el color de los mismos como consecuencia de irradiación ultravioleta, calentamiento en microondas y en baño termostático, reproduciendo en el laboratorio los efectos de los tratamientos domésticos. Se ha determinado el color para monitorizar los cambios de las muestras control y enriquecidas de los diferentes aceites. Los aceites enriquecidos son mucho más cromáticos, oscuros y rojizos que los naturales. Tras 55 días de irradiación UV, 40 minutos de calentamiento por microondas y 72 horas de calentamiento termostático, las diferencias medias de color para los aceites de oliva virgen extra naturales/enriquecidos fueron de 98/117, 15/9 y 57/28 unidades CIELAB, respectivamente. En términos generales, el incremento en la temperatura y la exposición a la radiación ultravioleta produce diferencias de color más grandes en las muestras no enriquecidas. El enriquecimiento de los aceites virgen extra con extractos procedentes de microalgas, induce estabilidad en el color y puede constituir una vía para incrementar la ingesta diaria de compuestos bioactivos beneficiosos como son los carotenoides.

PALABRAS CLAVE: Aceite de oliva virgen extra; Carotenoides; Scenedesmus almeriensis; Sistema CIELAB.


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1. INTRODUCTION

In contrast to the reduced consumption of fruits and vegetables, the production and consumption of premium olive oil has been steadily increasing worldwide in recent years as indicated by the International Olive Oil Council (IOC, 2013; IOC, 2018). Extra virgin olive oil (EVOO) is an important component of the Mediterranean diet, and is obtained exclusively by mechanical methods which conserve nutritional and organoleptic properties (García-Oliveira et al., 2021; Peñalvo et al., 2016). In addition to its unique sensorial attributes, EVOO has been credited with multiple health benefits, such as preventing coronary and degenerative diseases (Borges et al., 2017; García-Oliveira et al., 2021). Unfortunately, olive oil has only a few months’ shelf-life and is easily prone to oxidation and changes in quality. The addition of antioxidants to olive oil, however, may prolong its shelf-life, improve acceptability and enhance its nutritional value (Limón et al., 2015; Murillo-Cruz et al., 2021a; Murillo-Cruz et al., 2021b).

There is currently a growing interest in the relationships between food and health, well-being, and quality of life. Fruit and vegetable consumption is a pressing concern, and it is known that insufficient intake of these foods causes around 14% of gastrointestinal cancer deaths, about 11% of ischemic heart disease deaths and about 9% of stroke deaths worldwide (World Health Organization, 2014). Consequently, the decrease in the consumption of fruits and vegetables implies that several bioactive food components are not being ingested in sufficient amounts, with negative repercussions on human health. Carotenoids are a group of pigments that are widely extended in nature, but, unfortunately, the amounts of carotenoids found in human tissues are almost exclusively of dietary origin, mainly from fruits, vegetables and from other products like algae (Rodriguez-Concepcion et al., 2018). Carotenoids can be classified into two categories on the basis of their functional groups: xanthophylls (containing oxygen); and carotenes (without oxygen). Xanthophylls include lutein and zeaxanthin, while carotenes include α-carotene, β-carotene and lycopene. Regulation aspects about carotenoids have been developed by the European Commission through the European Food Safety Agency (EFSA, 2012). In addition, there is much evidence that carotenoids (and their derived products and metabolites) may be involved in health-promoting biological activity in humans (Meléndez-Martínez, 2019; Meléndez-Martínez et al., 2020; Van Hoang et al., 2018).

Foods fortified with β-carotene have been used to prevent vitamin A deficiency in pre-school children and pregnant women, although this particular carotenoid is liposoluble and weakly dispersed in foods (Turner et al., 2013). In addition, β-carotene has antioxidant properties that suppress oxygen or free radicals, reducing the risk of chronic diseases, inflammation and cancer (Syamila et al., 2019). It can be found in vegetables such as carrots, pumpkins and sweet potatoes and colors them orange, as well as in purple carrots and purple tomatoes, which also contain anthocyanins (Limón et al., 2015). Lutein, on the other hand, is a xanthophyll compound with an antioxidant activity recommended for preventing some types of cancer and cardiovascular diseases. It also has a protective effect with regard to age-related eye diseases and contributes to maintaining cognition (Ozawa et al., 2012). Lutein is commercially produced from marigold (Tagetes erecta), although the lutein content of marigold flowers is very low, whereas microalgae such as Chlorella and Scenedesmus almeriensis can contain large amounts of this compound (Limón et al., 2015). Specifically, Scenedesmus almeriensis contains up to 1.5% d.w. of lutein, together with other carotenoids such as β-carotene, and can be efficiently produced in closed tubular photobioreactors in continuous mode and on a large scale. Currently, the use of microalgae as a source of carotenoids is a research line in continuous expansion (Meléndez-Martínez et al., 2020). As for the bioaccessibility of carotenoids, several studies have examined potential dietary and host-related factors, and have concluded that dietary lipids can significantly enhance carotenoid absorption by fostering micellization (Iddir et al., 2019).

Color is one of the most important properties in foods for its relationship to chemical and nutritional composition as well as its strong influence on consumer emotions and preferences. Various scales have been proposed for specifying the color of EVOOs in connection with the standard colorimetric system of the International Commission on Illumination (CIE) (Gutiérrez and Gutiérrez, 1986; Moyano et al., 1999; Salmerón et al., 2012; Yu et al., 2020). Attention has also been given to the color emotions aroused by natural antioxidant-enriched virgin olive oils in both the Spanish and Japanese populations (Montoya et al., 2018; Limón, 2017).
According to the definition of the European Union Commission, an extra virgin olive oil must be extracted “only from olives with a superior quality, cannot undergo any treatment other than washing the fruits, and decanting, centrifuging and filtering the extracted olive oil”. Therefore, from a legal point of view, extra virgin olive oil (EVOO) enriched in carotenoids cannot be called “extra virgin olive oil”, and should be named as olive oil that has been processed with vegetables, algae, etc (Issaoui et al., 2016). For instance, currently it would also be possible to use the designation “olive oil enriched in carotenoids”. Currently, efforts are being made for the approval and registration of Scenedesmus almeriensis as a component of functional foods. In the future this may be an additional value to the well-known qualities of natural EVOOs.

In summary, we feel that EVOOs with carotenoid-rich extracts from the microalga Scenedesmus almeriensis (henceforth referred to as “enriched EVOOs”, as opposed to “natural EVOOs”) could be a good convey for enhancing the daily intake of carotenoids. However, the color of natural and enriched EVOOs may vary, and this may influence consumer preferences. The main objective of this study was to evaluate whether the enrichment of olive oil with carotenoids extracted from microalgae produces changes in its color and protects its color during UV exposure and heating.

2. MATERIALS AND METHODS

2.1. Production of Scenedesmus almeriensis microalgae

Scenedesmus almeriensis microalgae (CCAP 276/24) were produced at the Chemical Engineering Department of the University of Almería (Spain). The culture medium used was prepared in freshwater using fertilizers (NaNO₃, KH₂PO₄ and micronutrients). The cultures were performed at pH=8.0 by on-demand injection of CO₂ and below 30 ºC, by passing thermostated water through a heat exchanger located inside the reactor. The biomass was harvested daily by centrifugation, then lyophilized and stored at -18 ºC (Acién et al., 2012). This lyophilized biomass was used as raw material.

2.2. Extraction of carotenoids

The carotenoid extract was obtained by following a specific methodology for recovering these compounds from the lyophilized Scenedesmus almeriensis biomass. The first step was a cell disruption process with alumina in a 1:1 w/w proportion, using a mill with beads of 28 mm in diameter and a rotation speed of 120 rpm for 5 min to remove fatty acid soaps. The second step was saponification, using an aqueous solution with KOH and biomass concentrations of 40 g/L and 100 g/L, respectively, for 5 min. Finally, extraction was performed using a 1:1 ratio of hexane to sample volume. By this 3-step method it was possible to recover more than 90% of the carotenoids contained in the processed biomass (Cerón et al., 2008). At each step a volume of ethanol equal to 1% of the total volume was added to avoid emulsion. Hexane was removed from the extract by high vacuum distillation. The carotenoid extract produced was a concentrated oily solution containing lutein and β-carotene (2.5 mg of lutein/mL and 3.3 mg of β-carotene/mL, respectively). Regarding carotenoid quantification in the extract, it was performed using a methodology based on HPLC chromatography. A C18 reverse reversed-phase chromatographic column was used (250 mm x 2.5 mm) (Phenomenex Luna C18 column). The analysis was carried out at 23 ºC with a linear gradient for 30 minutes. The mobile phase was water-methanol (80%) with 0.05% triethylamine and 20% ethyl acetate (with 0.05% triethylamine) with a flow rate of 1 mL/min. From the chromatogram obtained, analyzing the peaks corresponding to the different carotenoids, the amounts of these compounds were obtained (Limón, 2017). Using this methodology, it was found that the composition of the carotenoids extract was β-carotene (56%), lutein (43%) and other undefined carotenoids (1%).

2.3. Addition of the carotenoids extract

Figure 1 shows CIELAB color differences produced in a Picual October EVOO during an enrichment process in which different lutein concentrations were achieved by adding an increasing volume of carotenoid extract (2.5 mg of lutein/mL and 3.3 mg of β-carotene/mL). As expected, parallel to this increase in carotenoid concentration, the color differences also increased. With the first addition of carotenoid extract (0.1 mL) a lutein concentration of 0.02 mg/mL was achieved, producing a significant color change from that of the original oil (∆E*ab,10 =14.7). Successive additions of extract produced an
almost linear growth of the CIELAB color difference (see Figure 1). It is clear that the amount of carotenoid extract added increased color changes in natural EVOOs.

With reference to lutein, it is important to note that the concentration of this carotenoid chosen for this study was 0.082 mg/mL of oil because average olive oil intake (person/day) was estimated at 36 mL/day (data for Spain from IOC, 2018), and thus our enriched EVOOs should provide each consumer with 3 mg of lutein/day. With regard to β-carotene, 4 mg would be ingested from our enriched EVOOs (0.11 mg β-carotene/mL of oil). These values should be 50 and 27% of the recommended intake, respectively, which is 6 and 15 mg/day for lutein and β-carotene, respectively (Meléndez-Martínez, 2019; EFSA 2012). On the other hand, it is necessary to point out that there are also carotenoids which are naturally present in olive oil (5-100 mg/kg) and they can also be incorporated with other foods through the diet (Cichelli and Pertesana, 2004). Obviously, it should be possible to use more concentrated EVOOs to increment carotenoid intake, but it should be remembered that the cost of the carotenoid extract is very high. Therefore, using enriched EVOOs with a concentration of 0.192 mg carotenoids/mL of oil (0.082 mg lutein/mL + 0.11 mg β-carotene/mL) would be acceptable for providing an appropriate dose at a reasonable price. Regarding lutein, this concentration is shown in Figure 1 by an arrow (0.082 mg lutein/mL of oil).

Carotenoids are present in olive oils in a range of 5-100 mg/kg and their lutein content usually ranges from 2-8 mg/kg (Cichelli and Pertesana, 2004). Thus, our enriched EVOOs, which contain 0.082 mg lutein/mL of oil, are 10-40 times more concentrated in this kind of carotenoid than natural EVOOs. Concerning β-carotene, this compound content ranges from 1-4 mg/kg, with our enriched oils (0.11 mg β-carotene/mL of oil) being 28-110 times more concentrated in this carotenoid (Limón et al., 2015). Here the values expressed in mg/mL were obtained assuming a density of 0.918 mg/mL for EVOOs. Because of the minor amount of carotenoids contained in the control (non-enriched) oils, the total amount of carotenoids in the enriched oils may be considered as the one from the added extract.

2.4. Extra-virgin olive oils

Four cultivars of representative EVOOs from northeastern Jaén (a province in Andalusia, Spain) were selected for the present study: Picual, Arbequina, Royal, and Frantoio. The EVOO samples were provided by the Castillo de Canena Olive Juice Company, from the same crop season (2018/2019) but harvested in different months (October-January), which implies olives with different ripening degrees. All EVOOs were initially filtrated in the presence of anhydrous sodium sulphate. After filtration, samples were homogenized and stored at 4 °C in the dark, using amber glass bottles without head space until analysis. For each type of oil, two 50-mL samples were constituted: natural EVOO (without the carotenoid extract); and enriched EVOO (with 0.082 mg of lutein per mL of oil and 0.11 mg β-carotene per mL of oil).

Concerning the quantification of the carotenoid content in the oil after the enrichment process, it is important to highlight that the extract added to the oils dissolved perfectly. We can assume that the concentration of carotenoids in the oils corresponds to the total amount of carotenoids from the extract which was contained in the volume of extract used for enrichment. The corresponding extract volume was added at room temperature to the oil sample and manually stirred until it was completely dissolved, waiting overnight to ensure adequate stabilization of the enriched samples.
2.5. Color measurement

The colors of both the natural and enriched EVOOs were measured with a CM-5 Konica Minolta spectrophotometer using appropriate 14-mL cuvettes with 10-mm path lengths. In this way, the CIELAB coordinates \((L^*_{10}, a^*_{10} \text{ and } b^*_{10})\) of the samples were measured by the spectrophotometer, after proper calibration, assuming the CIE D65 illuminant and CIE 1964 standard colorimetric observer. \(L^*_{10}\) is a measure of lightness, usually ranging from 0 to 100 (corresponding to black and white, respectively). The \(a^*_{10}\) coordinate ranges from negative (green) to positive (red) values, while the \(b^*_{10}\) coordinate also ranges from negative (blue) to positive (yellow) values. Color features were obtained as the average of three measurements performed on each sample. In the current paper CIELAB cylindrical coordinates (lightness \(L^*_{10}\), chroma \(C^*_{ab,10}\), and hue-angle \(h_{ab,10}\)) computed by conventional equations from the International Commission on Illumination (CIE, 2018) were used, as these are more intuitive than CIELAB Cartesian coordinates \((L^*_{10}, a^*_{10}, b^*_{10})\). The intensity of color is related to \(C^*_{ab,10}\), which has a null value for achromatic colors (i.e., white, black, and gray). The color attribute denominated as hue is related to hue-angle, \(h_{ab,10}\), which has values of 0, 90, 180, and 270° for red, yellow, green, and blue samples, respectively.

Color change was quantified using the CIELAB color-difference \((\Delta E^*_{ab,10})\), defined as follows:

\[
\Delta E^*_{ab,10} = \left[ (\Delta L^*_{10})^2 + (\Delta a^*_{10})^2 + (\Delta b^*_{10})^2 \right]^{1/2} = \left[ (\Delta L^*_{10})^2 + (\Delta C^*_{ab,10})^2 + (\Delta H^*_{ab,10})^2 \right]^{1/2}
\]

The total CIELAB color differences \(\Delta E^*_{ab,10}\) can be split into lightness, chroma, and hue differences, with the corresponding percentages defined as follows:

\[
\%\Delta L^*_{10} = 100 \left( \frac{\Delta L^*_{10}}{\Delta E^*_{ab,10}} \right)^2 \\
\%\Delta C^*_{ab,10} = 100 \left( \frac{\Delta C^*_{ab,10}}{\Delta E^*_{ab,10}} \right)^2 \\
\%\Delta H^*_{ab,10} = 100 \left( \frac{\Delta H^*_{ab,10}}{\Delta E^*_{ab,10}} \right)^2 \\
\%\Delta L^*_{10} + \%\Delta C^*_{ab,10} + \%\Delta H^*_{ab,10} = 100
\]
typically applied in stability studies involving fatty food matrices (Rancimat methodology).

3. RESULTS AND DISCUSSION

3.1. Initial color evaluations

It is well known that consumers judge foods and beverages according to external appearance, and color plays a key role in their final preference (Cavallo and Piqueras-Fiszman, 2017). Consequently, to characterize the natural and enriched EVOOs we studied, their colors were measured. The results are shown in Table 1. From the Wilcoxon nonparametric test at the 0.05 level, it was determined that the differences in average values for \( L^{*}_{10} \), \( C^{*}_{ab,10} \), and \( h_{ab,10} \) for the natural and enriched EVOOs in Table 1 are statistically significant.

Figure 2 shows color shifts from natural to enriched EVOOs in two CIELAB planes, \( C^{*}_{ab,10}-L^{*}_{10} \) and \( h_{ab,10}-L^{*}_{10} \). As can be seen in this figure, the addition of carotenoid extract always increased chroma (with the exception of sample 9) and decreased both lightness and hue-angle in natural EVOOs. Enriched EVOOs were therefore more chromatic, darker, and redder than natural EVOOs, which agrees with the visual perception of our samples by untrained observers with normal color vision. The highest shift produced in the samples was in chroma (Figure 2, top), as expected from the high color intensity of the added carotenoid extract. Specifically, the greatest variation in chroma was found for oil sample number 3 with initial and final chroma values of 75.9 and 134.7, respectively, and the lowest chroma variation was found for oil sample number 1 with initial and final chroma values of 121.8 and 124.2, respectively.

Figure 3 shows the total color differences and their three components (lightness, chroma and hue) in CIELAB space, as a consequence of adding the carotenoid extract to the 10 natural EVOOs. It can

TABLE 1. CIELAB cylindrical coordinates (lightness \( L^{*}_{10} \), chroma \( C^{*}_{ab,10} \), and hue-angle \( h_{ab,10} \)) of natural and enriched EVOOs. The enriched EVOOs were produced by adding carotenoid extract from the microalga *Scenedesmus almeriensis* (0.008 mg lutein/mL of oil, 0.11 mg β-carotene/mL of oil). Results are expressed as mean ± standard deviation of three sample replicates.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Variety</th>
<th>Harvest Month</th>
<th>Natural EVOOs</th>
<th>Enriched EVOOs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( L^{*}_{10} )</td>
<td>( C^{*}_{ab,10} )</td>
</tr>
<tr>
<td>1</td>
<td>Picual</td>
<td>October</td>
<td>80.4±1.8</td>
<td>121.8±1.6</td>
</tr>
<tr>
<td>2</td>
<td>Picual</td>
<td>November</td>
<td>84.4±1.1</td>
<td>117.5±0.3</td>
</tr>
<tr>
<td>3</td>
<td>Picual</td>
<td>December</td>
<td>93.0±0.3</td>
<td>75.9±1.5</td>
</tr>
<tr>
<td>4</td>
<td>Picual</td>
<td>January</td>
<td>89.6±0.2</td>
<td>104.0±1.1</td>
</tr>
<tr>
<td>5</td>
<td>Arbequina</td>
<td>October</td>
<td>84.5±0.8</td>
<td>118.6±0.6</td>
</tr>
<tr>
<td>6</td>
<td>Arbequina</td>
<td>November</td>
<td>90.5±0.4</td>
<td>93.4±1.7</td>
</tr>
<tr>
<td>7</td>
<td>Arbequina</td>
<td>December</td>
<td>92.0±0.3</td>
<td>82.2±1.5</td>
</tr>
<tr>
<td>8</td>
<td>Arbequina</td>
<td>January</td>
<td>88.5±0.2</td>
<td>98.7±1.6</td>
</tr>
<tr>
<td>9</td>
<td>Royal</td>
<td>October</td>
<td>70.8±1.6</td>
<td>115.2±1.4</td>
</tr>
<tr>
<td>10</td>
<td>Frantoio</td>
<td>October</td>
<td>87.0±0.5</td>
<td>110.2±1.5</td>
</tr>
</tbody>
</table>
3.2. Color change during UV-light exposure

One of EVOO degradation variables that must be taken into account is exposure to electromagnetic radiation, which promotes changes during olive oil storage until use (Luna et al., 2006). Natural and enriched EVOOs follow similar trends under UV irradiation: increase in lightness ($L^*_{10}$), increase in hue-angle ($h_{ab,10}$), and decrease in chroma ($C^*_{ab,10}$), the last of these presenting the greatest change (about 110 and 100 units for natural and enriched EVOOs, respectively) (Figure 4). It can also be noted that the chroma of enriched EVOOs was nearly constant during the first 15 days of irradiation. Total color change ($\Delta E^*_{ab,10}$) after 55 days of irradiation was also very high (97.7 and 116.7 CIELAB units for natural and enriched, respectively), and during the first 30 days of irradiation it was lower in enriched than in natural EVOOs. It can be noted as well that UV irradiation produces greater color variability in the 10 enriched EVOOs than in the 10 natural EVOOs (i.e. larger error bars in the plot in Figure 4D for $\Delta E^*_{ab,10}$ change).

At the 0.05 level, the Wilcoxon rank test for paired samples indicated that the total CIELAB color differences produced by UV irradiation (Figure 4D) were statistically significant for natural and enriched EVOOs only for the irradiation times below 20 days.

3.3. Color change during microwave heating

Another oil degradation variable to consider in food is temperature, with microwave heating being a common methodology for studying oil stability (Malheiro et al., 2013). As can be seen in Figure 5, microwave heating for 40 min had a very small influence on lightness ($L^*_{10}$) and hue-angle ($h_{ab,10}$), and slightly reduced chroma ($C^*_{ab,10}$) in a nearly linear way for both the enriched and natural oils. It is also observed that the change in chroma was smaller for enriched than for natural EVOOs, without any overlapping of error bars for the 10 tested samples. Specifically, for enriched oils the average chroma changed from 129.5 to 121.6 (i.e. 7.9 CIELAB units) while for natural oils the average chroma changed from 103.6 to 89.6 (i.e. 14.0 CIELAB units). For enriched and natural EVOOs the total color differences after 40 min were high (Figure 5D) in comparison with human-vision color-threshold values (Huang et al., 2015). Regarding foods, it has been reported that for red wines a 50% acceptance percentage resulted from a color difference of 2.8 CIELAB units, using a reference anchor-pair of wine samples with 4.0 CIELAB units (Martínez et al., 2001). For orange juice, it has been stated that untrained assessors can easily distinguish color differences of 2.8 CIELAB units (Fernández-Vázquez et al., 2013). As far as we know, magnitudes of just perceptible or acceptable color differences in EVOOs have not been reported in previous literature. However, we can assume that the minimum difference shown in Figure 3 (i.e. 5.7 CIELAB units) is above the human visual threshold, and we can conclude that any observer with normal color vision will easily perceive the color differences among the 10 natural and enriched oils considered in the current paper.

Average threshold color-difference values in visual experiments were below 1.6 CIELAB units (Melgosa et al., 1992), and in a recent experiment it was reported that such values were even lower, in the range 0.55-1.10 CIELAB units (Huang et al., 2015). Regarding foods, it has been reported that for red wines a 50% acceptance percentage resulted from a color difference of 2.8 CIELAB units, using a reference anchor-pair of wine samples with 4.0 CIELAB units (Martínez et al., 2001). For orange juice, it has been stated that untrained assessors can easily distinguish color differences of 2.8 CIELAB units (Fernández-Vázquez et al., 2013). As far as we know, magnitudes of just perceptible or acceptable color differences in EVOOs have not been reported in previous literature. However, we can assume that the minimum difference shown in Figure 3 (i.e. 5.7 CIELAB units) is above the human visual threshold, and we can conclude that any observer with normal color vision will easily perceive the color differences among the 10 natural and enriched oils considered in the current paper.
natural EVOOs were 8.7 and 14.5 CIELAB units, respectively. At the 0.05 level, the Wilcoxon rank test for paired samples indicated that the total CIELAB color differences produced by microwave heating (Figure 5D) were statistically significant for natural and enriched EVOOs at all measured times above 1 min. Total color differences after 40 min microwave (Figure 5D) heating were 6.7 and 13.4 times lower than those after 55-day UV exposure (Figure 4D) for natural and enriched EVOOs, respectively.

3.4. Color change during thermostatic immersion bath

The technique of immersion in a thermostatic bath is a practice which is quite common in conventional household food treatments (Nogueira-de-Almeida and de Castro, 2018). As can be seen in Figure 6, this heating method increased lightness and hue-angle in a nearly linear manner, and this is similar for both natural and enriched EVOOs. Chroma, however, decreased much more dramatically for natural than for enriched EVOOs (after 72 hours, chroma change in natural EVOOs was about 2.2 times higher than that of enriched EVOOs). Specifically, for enriched oil the average chroma value changed from 129.5 to 104.3; while for natural oils the average chroma change was from 104.0 to 47.8 CIELAB units. The heating process made EVOOs lighter, less reddish, and considerably less chromatic. In particular, after 72 hours the natural EVOOs came to resemble neutral transparent liquids. Overall, total CIELAB color differences (Figure 4).

**Figure 4.** Color change as a consequence of UV exposure over 55 days for natural (black) and enriched (white) EVOOs. Error bars indicate standard deviations of 3 independent measurements of all EVOOs. Changes in the CIELAB color attributes of lightness \(L^*\) (A), chroma \(C^*\) (B), and hue-angle \(h_{ab}\) (C), as well as total CIELAB color differences with respect to the oils without UV exposure \(\Delta E_{ab}\) (D), are shown.
Figure 5. Color change as a consequence of microwave heating over 40 min for natural (black) and enriched (white) EVOOs. Error bars indicate standard deviations of 3 independent measurements of all EVOOs. Changes in the CIELAB color attributes of lightness $L^*_{ab}$ (A), chroma $C^*_{ab,10}$ (B), and hue-angle $h_{ab,10}$ (C), as well as total CIELAB color differences $\Delta E^*_{ab,10}$ (D), are shown.

Figure 6-D) produced by thermostatic bath heating were 2.0 times lower for enriched than for natural EVOOs. At the 0.05 level, the Wilcoxon rank test for paired samples indicated that the total CIELAB color differences produced by thermostatic immersion bath heating (Figure 6D) were statistically significant for natural and enriched EVOOs at all measured times. Specifically, after 70 h the average total color difference for the enriched and natural EVOOs were 27.9 and 57.1 CIELAB units, respectively.

4. CONCLUSIONS

Spectrophotometric color measurements were made for a set of 10 natural and 10 enriched EVOOs, the latter being obtained by the addition of a carotenoid extract (0.082 mg lutein per mL of oil and 0.11 mg of β-carotene per mL of oil) from the microalga Scenedesmus almeriensis. Enriched EVOOs were much more chromatic, darker, and redder than natural EVOOs, and the average color difference between the two types of EVOOs was high (29.8 CIELAB units). This may affect consumer preferences for EVOOs, although this point was not tested here. UV irradiation made natural and enriched EVOOs lighter, less chromatic, and less reddish. The average color difference after 55 days was very high (97.7 and 116.7 CIELAB units for natural and enriched EVOOs, respectively). However, during the first 20 days of UV irradiation, the color change was statistically significantly lower in enriched than in natural EVOOs. Micro-
wave heating for 40 min did not change lightness or hue-angle, but did decrease the chroma of natural and enriched EVOOs. It could be stated that the chroma difference for natural oil was higher than that of enriched oil after 40 min of microwave heating. After 40 min of microwave heating the total color differences for the enriched and natural EVOOs were 8.7 and 14.5 CIELAB units, respectively. Thermostatic bath heating for 72 hours made EVOOs lighter, less reddish, and considerably less chromatic, and the total CIELAB color difference was higher for natural than for enriched EVOOs (57.1 and 27.9 CIELAB units, respectively). It can be concluded that color change produced by increasing temperature was lower in enriched than in natural EVOOs. These colorimetric analyses may be relevant with regard to the potential future use of enriched EVOOs as functional foods, increasing the daily intake of beneficial bioactive compounds such as carotenoids. In addition, these enriched oils could have a longer half-life, since carotenoids are antioxidants.

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