Virgin Olive Oil Enriched with Lutein-Zeaxanthin from Spinacia oleracea

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Abstract: The aim of this work consists of developing a technological process for elaborating a virgin olive oil enriched in lutein-zeaxanthin extracted from spinach, studying different parameters like temperature, time of extraction and different ratios (spinach-oil). It was observed that the amount of carotenoids extracted increased up to a maximum after 24 hours and decreased as the maceration time progressed up to 60 hours, resulting of biological degradation. It was also observed that as more spinach we added, as more lutein-zeaxanthin in the enriched virgin olive oil was obtained. The best results were obtained after 24 hours by using a 75:25 ratio at 30°C. Values of oxidative stability decreased drastically, as well as other parameters such as acidity; peroxides index and Ks were modified when the enriched virgin olive oil was subjected to 45°C for 24 hours of maceration. Thus, the present procedure constitutes a way to achieve an increase in the daily intake of beneficial compounds.

Key words: enriched virgin olive oil, lutein-zeaxanthin, spinach, carotenoids

1 INTRODUCTION

Mediterranean diet has long been known for contributing to a healthy life and preventing diseases¹. Virgin olive oil (VOO) is one of the principal ingredients of this diet². Numerous studies have demonstrated that it has relevant properties that convey important benefits for individual health, when taken as part of a habitual diet³-⁴.

The VOO is mostly composed by triacylglycerols rich in the monounsaturated fatty acid, oleic acid. The non-glyceride constituents of VOO, which comprise approximately 0.5% to 1.0%, include different families of compounds like polyphenols, tocopherols and carotenoids, among others. In spite of its low concentration, these constituents have many benefits and provide many of their properties to the olive oil. Lutein (3,3’-dihydroxy-α-carotene), is one of the carotenoids most widely distributed in frequently consumed fruits and vegetables, and together with zeaxanthin (3,3’-dihydroxy-β-carotene), is selectively accumulated in different parts of the human eye⁵. Lutein and zeaxanthin are commonly referred to as macular pigments (MP)⁶,⁷.

Epidemiological studies have shown an inverse association between high levels in blood of these carotenoids and senile macular degeneration risk. In fact, lutein and zeaxanthin can prevent oxidative damage induced by light in retina and thus, protect against age-associated deterioration⁸. Furthermore, the levels of these compounds have also been inversely related with density (opacity) lens and therefore with a lower risk of cataracts⁹. There are few effective treatments for age-related macular degeneration. The treatment options available nowadays for this disease consist in slowing down the angiogenic process of Age-related macular degeneration by either laser or photodynamic therapies. Laser treatment carries the possibility of damaging the remaining vision. Photodynamic therapy is a FDA approved and commonly performed therapy, but certainly not a definitive treatment. The lack of standard therapies for Age-related macular degeneration, in vitro studies and epidemiological survey, indicate that nutritional intervention using antioxidants and vitamins to enhance vision health may provide an effective way to prevent or treat Age-related macular degeneration⁶,⁷,⁹.

A wide range of by-products from the food industry have been studied for the purpose to increase the health properties of other food. Recently, several studies have investigated the enrichment by co-grinding process of olive oils with a lycopene obtained from skins and seeds of tomato⁹ due
to the powerful antioxidants properties. Furthermore, other studies have increased the amount of lutein and zeaxanthin in virgin olive oil from lyophilized biomass from *Scenedesmus almeriensis*\(^{30}\).

Considering the ingestion of a daily dose of VOO of 23 grams recommended by the U.S. Food and Drug Administration\(^{11}\), the daily ingestion of minority compounds presented in this matrix, like carotenoids, is very low in relation to other sources. In this sense, VOO enriched with carotenoids has a promising future that is supported by the current interest in natural compounds from plant sources. Special sources of these compounds are by-products from spinach (*Spinacea oleracea*).

To further support the functional value of VOO and increasing the value of by-products generated in the food industry, the general objective of this research was to evaluate strategies for the development of a VOO enriched with lutein and zeaxanthin. The enrichment with both xanthophylls, extracted from *Spinacea oleracea*, increase and standardize the necessary dose in the diet of these macular pigments, without the drawback of a high caloric intake. As spinach presents high content of this family of compounds, in a second step and following diverse procedures, different enriched VOO were prepared with the spinach leaves. Finally, the effect of the extraction temperature on carotenoids enrichment was evaluated.

### 2 MATERIAL AND METHODS

#### 2.1 Plant materials

The study was carried out in the Technological Institute of Food and Agriculture of Extremadura (INTAEX); for this purpose, the olive fruits were picked up from the experimental olive orchard (*Olea europaea* L.) maintained by the Researcher Center "Finca La Orden-Valdesequera" (Badajoz, Spain) within the limits of the olive-growing area "Tierra de Barros". The olive fruit samplings of *Arbequina* cultivar were carried out in the morning, taking samples randomly under perfect sanitary conditions. Three samples of 10 kg of olive fruits were taken, in different parts of the central area of the olive tree. The fruit samples were collected at spotted stage of maturation, following the criteria for commercial harvesting, using the subjective evaluation of colour of the skin and flesh as proposed by Uceda and Frías\(^{12}\). After harvesting, all samples were immediately transported to the INTAEX laboratory in ventilated storage trays to avoid compositional changes.

At the same time, spinach leaves (*Spinacea oleracea*) were picked up from a freezing industry as by-product. In the same way, all samples were immediately transported to INTAEX laboratory in ventilated storage trays to avoid compositional changes. The spinach leaves were vacuum-packed (Gustav Müller VS 100, Germany) in plastic bags and quickly stored at frozen conditions \((-80°C\). Some samples were freeze-dried and they were stored away from the light in amber-coloured glass bottles at room temperature until analysis.

#### 2.2 Oil extraction

Oil extraction of the three samples was carried out within 24 hours from harvesting in similar industrial extraction conditions using an Abencor analyzer (MC2 Ingeniería systems, Sevilla, Spain) according Martinez, Muñoz, Alba, and Lanzón\(^{13}\). Olives were crushed with a hammer mill and slowly mixed for 30 min at 25°C. The paste obtained was centrifuged at 1438 g over 3 min. The oil was separated by decantation and was stored away from the light in amber-coloured glass bottles at 4°C until analysis (within 1 month). This VOO was the matrix used for the enrichment with carotenoid compounds.

#### 2.3 Lutein-zeaxanthin enrichment of virgin olive oil

The search for an extraction method to enrich virgin olive oil with lutein-zeaxanthin from *Spinacea oleracea*, improving its health properties and maintaining its appreciated organoleptic quality, led us to develop a novel solvent-free method based on the use of moderate temperature and stirring-assisted maceration\(^{14}\). Therefore, several trials were carried out testing different variables for extraction in order to optimize the conditions of the process leading to an oil with the highest concentration of lutein and zeaxanthin. That included various combinations of processing, such as proportions of oil and spinach, time and temperature of maceration. Firstly, extraction curves with different ratios (spinach:oil; w:w) 75:25, 50:50, 25:75 and times 0, 12, 24, 36, 48, and 60 hours at 30°C were registered. Once elected both suitable parameters, the extraction temperature was evaluated at 25, 30, and 45°C.

In order to enrich the VOO previously obtained, the spinach leaves were weighted and boiled for 30 seconds to disable polyphenoloxidase enzyme, pouring them immediately on ice-water for 30 seconds. The spinach leaves were drained and mixed with VOO using a commercial thermobate at top speed for 35 seconds to obtain a homogeneous mixture. Finally, bioactive compounds were extracted from crushed samples at the temperature selected during 0, 12, 24, 48, and 60 hours in a shaking bath. The mixture was centrifuged at 21036 g to remove solid particles from it. The different enriched oils were stored away from the light in amber-coloured glass bottles at 4°C until analysis.

#### 2.4 Analytical methods

##### 2.4.1 Carotenoid compounds determination

##### 2.4.1.1 Extraction of carotenoid compounds from *Spinacea oleracea*

Previously, samples of *Spinacea oleracea* were stored at frozen conditions \((-80°C\) until being freeze-dried in a
Virgin Olive Oil Enriched with Lutein-Zeaxanthin from Spinacia oleracea


2.5 Statistical analysis

For statistical studies SPSS 17.0 software was used (SPSS Inc. Chicago, IL, USA). All analyses were done in quintuple except when is expressly referred. Data were expressed as means ± SD and analyzed using a one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test. The significance level was set at p<0.05.

Table 1 Analytical figures of merit.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lutein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear regression</td>
<td>y = 27.28x - 30.35</td>
</tr>
<tr>
<td>Determination coefficient (r^2)</td>
<td>0.995</td>
</tr>
<tr>
<td>% Linearity</td>
<td>98.45</td>
</tr>
<tr>
<td>Sy/x</td>
<td>37.77</td>
</tr>
<tr>
<td>LOD (mg/Kg)^a</td>
<td>1.08</td>
</tr>
<tr>
<td>LOQ (mg/Kg)^b</td>
<td>3.60</td>
</tr>
</tbody>
</table>

^a Limit of detection, Long and Winefordner method
^b Limit of quantification, from LOD × 3.33
Table 2  Acidity(%) , peroxide index (meqO₂/kg), K₂₇₀ and K₂₃₂ of virgin olive oil enriched in lutein-zeaxanthin macerated at different temperatures. Results are expressed as mean ± SD of three sample replicates. Different small letters in the same row indicate significant statistical differences (Duncan’s Test, p < 0.05) among maceration temperatures.

<table>
<thead>
<tr>
<th>Extraction Time (hours)</th>
<th>25°C</th>
<th>30°C</th>
<th>45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidity</td>
<td>0.37 ± 0.07 a</td>
<td>0.53 ± 0.04 b</td>
<td>0.93 ± 0.05 c</td>
</tr>
<tr>
<td>Peroxide Index</td>
<td>9.38 ± 0.21 a</td>
<td>14.98 ± 0.17 b</td>
<td>24.3 ± 0.39 c</td>
</tr>
<tr>
<td>K₂₇₀</td>
<td>0.17 ± 0.04 ns</td>
<td>0.19 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>K₂₃₂</td>
<td>2.12 ± 0.22 ns</td>
<td>2.32 ± 0.51</td>
<td>2.58 ± 0.62</td>
</tr>
</tbody>
</table>

Table 3  Lutein-zeaxanthin in enriched virgin olive oil (mg/kg) at different ratios at 30°C during 60 hours maceration. Results are expressed as mean ± SD of three sample replicates. Different small letters in the same row indicate significant statistical differences (Duncan’s Test, p < 0.05) among maceration times. Different capital letters in the same column indicate significant statistical differences (Duncan’s Test, p < 0.05) among different ratios.

<table>
<thead>
<tr>
<th>spinach:oil ratios</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>75:25</td>
<td>31.98 ± 1.00b C</td>
<td>36.92 ± 9.42bc B</td>
<td>50.53 ± 6.18d C</td>
<td>42.33 ± 2.13c C</td>
<td>40.84 ± 4.34c B</td>
<td>15.94 ± 4.04a B</td>
</tr>
<tr>
<td>50:50</td>
<td>11.02 ± 0.35cd B</td>
<td>11.71 ± 0.43d A</td>
<td>12.80 ± 0.61e B</td>
<td>10.29 ± 0.35d B</td>
<td>9.61 ± 0.54b A</td>
<td>8.40 ± 0.16a A</td>
</tr>
<tr>
<td>25:75</td>
<td>6.75 ± 0.53ab A</td>
<td>6.88 ± 0.10ab A</td>
<td>7.26 ± 0.13b A</td>
<td>6.78 ± 0.06ab A</td>
<td>6.68 ± 0.42a A</td>
<td>6.36 ± 0.15a A</td>
</tr>
</tbody>
</table>

times at 30°C is shown in Table 3. Thus, it was observed that the content of carotenoids in enriched VOO increased when the proportion of spinach with respect oil was increased. The maximum values of lutein-zeaxanthin were obtained by using a 75:25 ratio and 24 hours of maceration. The three studied ratios showed exponential increasing extraction curves reaching a maximum to 24 hours from which was stabilized and began to decrease exponentially.

However, if we calculate the percentages of extracted carotenoids, both related to spinach and oil, we observe that in the optimum extraction time of 24 hours the amount of lutein-zeaxanthin extracted from spinach was 35.7, 23.3, and 32.9% in 75:25, 50:50, and 25:75 ratios, respectively. Therefore, the percentages of extraction were not very high, noting that the highest extraction rate was observed when the greater amount of oil was added.

An important effect of saturation in the oil matrix was observed when ratio spinach - olive oil was increased, making the extraction process more difficult. In this sense, when the proportion of spinach was increased with respect to olive oil until reaching a 90:10 ratio, the extraction yields did not improve significantly, since it produced an important effect of saturation, resulting in the loss of original fluidity of oil.

Moreover, in order to obtain the optimum working temper-
best to increase the amount of carotenoids in the oil but without losing the quality of the final potential functional product[9]. The concentration of carotenoids extracted at 25 and 30°C showed to be approximate. However, significant differences in the extraction of these compounds were found at 45°C, being the extracted amount greater than the observed with the other two temperatures tested. Nevertheless, the use of high temperatures and times of maceration contributes to a worsening of the quality of the enriched olive oil. In this sense, the acidity increased when the maceration temperature was increased, resulting in free fatty acids in oil expressed as percentage of oleic acid (Table 2). Similarly, peroxide index that represents the degree of oxidation of the oil was affected. Finally, the absorbance measurement in the ultraviolet was also altered by the increase in the temperature. Therefore, when oils were macerated at 45°C for 24 hours, oxidation reactions took place in VOO and a loss of quality was observed; in fact, all analytical parameters, except K270, were found above the maximum levels established by the Regulation (EEC)2568/91 for maximum commercial-grade olive oil: “Extra Virgin Olive Oil” (Table 2); also, the values of oxidative stability decreased drastically (Table 4). Thus, we can conclude that this oil is very sensitive to rancidity. Therefore, it would be necessary to commercialise it in topaz bottles and keep it away from any source of heat, even without any head space in the bottle.

There is currently not Recommended Dietary Allowance (RDA) or Recommended Daily Intake (RDI) for lutein and zeaxanthin but some experts say you should ingest at least 4-6 milligrams (mg) of these carotenoids per day for beneficial effects[20, 21]. Thus, in this study we managed to obtain 50.5 mg/kg in olive oil enriched from spinach (24 hour; 75:25 ratio) in addition to the recommended daily amount of virgin olive oil of 23 grames[13]. With this enriched VOO it is possible to consume a daily amount of both carotenoids of 1.16 mg. Consequently, the design of this functional food is suitable for being consumed in a daily diet.

With this method of extraction, an enriched virgin olive oil with lutein and zeaxanthin was obtained as potential functional food. We should take into account that this new product was obtained from natural by-product from the industry which has high antioxidant activity and with health properties. These results are similar to those obtained by Bendini and cols.[9], with oil enriched with lycopene. Furthermore, Granado-Lorencio and cols.[10], indicated that a high sources of lutein and zeaxanthin was extracted by lyophilized biomass of S. almeriensis. However, this microalgae extract had low bioavailability of these compounds. In this sense, the use of oil matrix could contribute to increase the bio-accessibility of carotenoids in the organism.

4 CONCLUSIONS
In this work, a functional food suitable to follow a balanced diet was prepared making a healthy product, such as virgin olive oil, into other more beneficial for health enriched in lutein-zeaxanthin. This could help in reducing and preventing the incidence of certain chronic diseases such as macular degeneration. Additionally, a by-product of the agricultural sector was employed, with the consequent use and revalorisation in agro-food industries. Moreover, the present procedure constitutes a way to achieve an increase in the daily intake of beneficial compounds and an attractive tool for future industrial implementations.

REFERENCES
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