Virgin Olive Oil Enriched with Lutein-Zeaxanthin from *Spinacia oleracea*

María Benito de Valle-Prieto¹, Jonathan Delgado-Adámez¹, Ma Victoria Gil², Sara Martillanes¹, Ma Nieves Franco¹ and Daniel Martín-Vertedor^{1*}

¹ Center for Scientific Research and Technology of Extremadura (CICYTEX). Technological Institute of Food and Agriculture (INTAEX). Government of Extremadura. Avda. Adolfo Suárez s/n, 06007 Badajoz, SPAIN

² Organic and Inorganic Chemistry Department, Faculty of Sciences, University of Extremadura, Avda. de Elvas s/n, 06071 Badajoz, SPAIN

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 diet^{3,4}.

The VOO is mostly composed by triacylglycerols rich in the monounsaturated fatty acid, oleic acid. The non-glyceridic 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.

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 deterioration^{6,7}.

Furthermore, the levels of these compounds have also been inversely related with density (opacity) lens and therefore with a lower risk of cataracts⁸. 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 degeneration^{5,6}.

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

* Correspondence to: Daniel Martín-Vertedor, Center for Scientific Research and Technology of Extremadura (CICYTEX). Technological Institute of Food and Agriculture (INTAEX). Government of Extremadura. Avda. Adolfo Suárez s/n, 06007 Badajoz, SPAIN

E-mail: daniel.martin@juntaex.es

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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*¹⁰⁾.

Considering the ingestion of a daily dose of VOO of 23 grams recommended by the U.S. Food and Drug Administration¹¹⁾, 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 *Spinacia 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 Frías¹²⁾. 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 (*Spinacia 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 to Martínez, Muñoz, Alba, and Lanzón¹³⁾. 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 *Spinacia 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 maceration¹⁴⁾. 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 thermobeeper 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

lyophilizer (Virtis Company, Mod. Génesis 25 LL, Hüco-Herlos). The dried extracts obtained were kept in an amber-coloured glass bottles away from the light and at room temperature until analysis. For the extraction, 1 gram of sample was mixed with acetone, shaking for 30 minutes in a magnetic stirrer at room temperature (this process was repeated 4 times) and the solvent removed. The extract obtained was dissolved in 5 mL of methanol and taken to ultrasonic probe to remove solid particles from it. Then, 100 mL of KOH in methanol (20%) were added for lipid saponifications, keeping in amber-coloured glass bottles and shaking overnight. After this time, it was transferred to a decantation funnel with 100 mL of ethyl ether, washed with distilled water and filtered on Na_2SO_4 anhydrous, finally the solvent was removed. The sample dissolved in acetone and filtered through a nylon membrane of 0.20 μm was injected into a liquid chromatograph.

2.4.1.2 Extraction of carotenoid compounds in starting and enriched virgin olive oil

Octadecyl (C18) disposable extraction columns were used for the solid-phase extraction (SPE). The column was firstly conditioned with methanol and then with hexane. The sample of, 1 gram of oil dissolved in 4 mL of hexane, was injected and washed with a further 3 mL of hexane. Lastly, the pigments were eluted with 5 mL of acetone after checking that there were no lipid remains. The sample dissolved in acetone and filtered through a nylon membrane of 0.20 μm was injected into a liquid chromatograph.

Individual carotenoids were determined for analyte detection and quantification was done by external standard method with an UHPLC system (Agilent 1290 series, Madrid, Spain) equipped with a diode array detector (DAD) and with a Kinetex XB-C18 HPLC column (50 mm $\times$ 2.1 mm, 1.7 μm). The temperature of analysis was 28°C. The injected volume was 1.4 μL and the flow was fixed at 0.3 mL $\cdot$ min⁻¹. The separation of carotenoid compounds was achieved by elution in gradient with a mixture of water-acetonitrile (85:15; v/v) and acetonitrile-methanol-ethyl acetate (60:20:20; v/v/v). Carotenoids detection was carried out by a photodiode array at 460 nm. Chromatographic data processing was performed using HP ChemStation software (Rev.B.04.01., Agilent Technologies).

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 $\pm$ SD and analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. The significance level was set at $p < 0.05$.

3 RESULTS AND DISCUSSION

Lutein-zeaxanthin were identified and quantified by UHPLC using their corresponding external standards calibration curves. The analytical parameters of calibration used in the UHPLC characterization are shown in Table 1. Limit of detection (LOD)¹⁵⁾ and limit of quantification (LOQ) were 0.16 and 0.54 mg/kg, respectively. The linearity range of the analytical procedure was 99.46% for the lutein compound. Seven different concentrations of the lutein standard solutions were used injecting them three times. The calibration curve, obtained as a function of the integrated peak area, was linear over the studied range, with determination coefficients (r^2) > 0.99 for this compound.

Firstly, Gandul-Rojas, Roca, and Minguez-Mosquera¹⁶⁾ described that the majority pigment in VOO is lutein, followed by β -carotene. Thus, the content of lutein observed in our starting VOO was 2.32 ± 0.014 mg/kg, a relatively low value. These results are very similar to those reported by Maia, Casal, and Oliveira¹⁷⁾, who analyzed the content of lutein in extra VOO of twenty-five trade Portuguese oils, founding an average of 2.40 ± 0.62 mg of lutein/kg oil.

Using both starting materials, oil and spinach, and taking into account that lutein and zeaxanthin easily dissolve in a lipidic matrix such as VOO, we explored the possibility of developing a functional food.

In general terms, the oils produced showed values (acidity $< 0.8\%$; peroxide index < 20 meq O_2 /kg; $K_{270} < 0.22$; $K_{232} < 2.5$) within the "extra virgin" category, as stated by Regulation EEC 2568/1991 and modifications (Table 2). On the other hand, the raw spinach exhibited a content in lutein-zeaxanthin of 45.03 ± 0.29 mg/kg. In this sense, Azevedo-Meleiro, and Rodriguez-Amaya¹⁸⁾ studied the total content of these carotenoids in different varieties of spinach in two stage of maturation. The results obtained in ripe stage of maturation were very similar to those found in our study. However, for spinach collected in earlier maturation stage, the values found for these researchers were 1.5 times greater than those obtained in the samples used to enrich this oil.

The content of lutein-zeaxanthin in VOO enriched with by-product from spinach, using different proportions and

Table 1 Analytical figures of merit.

Parameters	Lutein
Linear regression	$y = 27.28 \times - 30.35$
Determination coefficient (r^2)	0.995
% Linearity	98.45
Sy/x	37.77
LOD (mg/Kg) ^a	1.08
LOQ (mg/Kg) ^b	3.60

^a Limit of detection, Long and Winefordner method

^b Limit of quantification, from $\text{LOD} \times 3.33$

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.

	24 hours extraction time, 75:25 ratio		
	25°C	30°C	45°C
Acidity	0.37 ± 0.07 a	0.53 ± 0.04 b	0.93 ± 0.05 c
Peroxide Index	9.38 ± 0.21 a	14.98 ± 0.17 b	24.3 ± 0.39 c
K ₂₇₀	0.17 ± 0.04 ns	0.19 ± 0.01	0.22 ± 0.01
K ₂₃₂	2.12 ± 0.22 ns	2.32 ± 0.51	2.58 ± 0.62

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.

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

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 tem-

Table 4 Lutein-zeaxanthin and oxidative stability in extra virgin olive oil at different maceration temperatures. Results are expressed as mean ± SD of three sample replicates. Different small letters in the same column indicate significant statistical differences (Duncan's Test, $p < 0.05$) among maceration temperatures.

	Lutein (mg/kg)	Oxidative Stability (hours)
25°C	51.75 ± 6.26b	19.78 ± 1.33b
30°C	53.17 ± 7.64b	18.49 ± 1.06b
45°C	79.33 ± 4.84c	5.55 ± 6.89a
EVOO	2.32 ± 0.01a	49.29 ± 1.91c

EVOO: Extra virgin olive oil

perature and discard those that would not be appropriate for its possible impact on the quality of enriched VOO, the effect of this parameter on the extraction of lutein-zeaxanthin in the EVOO was also studied (Table 4). As described above, the maximum amount of carotenoids was obtained within 24 hours of maceration and using a 75:25 ratio; thus, under these conditions the study was conducted at 25, 30 and 45°C to find out which of those temperatures are the

best to increase the amount of carotenoids in the oil but without losing the quality of the final potential functional product¹⁹⁾. 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 of 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 K_{270} , 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 grams¹¹⁾. 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.

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