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Improvement of stability and carotenoids fraction of virgin olive oils by addition of microalgae *Scenedesmus almeriensis* extracts

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ABSTRACT

Humans are not capable of synthesizing carotenoids *de novo* and thus, their presence in human tissues is entirely of dietary origin. Consumption of essential carotenoids is reduced due to the lower intake of fruits and vegetables. Microalgae are a good source of carotenoids that can be exploited. In the present work, carotenoids rich extracts from *Scenedesmus almeriensis* were added to extra-virgin olive oils at different concentrations (0.1 and 0.21 mg/mL) in order to enhance the consumption of these bioactives.

Extracts brought changes in olive oils color, turning them orange-reddish. Quality of olive oils was improved, since peroxidation was inhibited. Olive oils fatty acids and tocopherols were not affected. β-carotene and lutein contents increase considerably, as well as oxidative stability, improving olive oils shelf-life and nutritional value.

Inclusion of *S. almeriensis* extracts is a good strategy to improve and enhance the consumption of carotenoids, since olive oil consumption is increasing.

1. Introduction

Nowadays fruits and vegetables consumption worldwide is a pressing concern. According to Pomerleau, Lock, Knai, and McKee (2004), in 2000 2.7 million deaths occurred worldwide in 2000 and 1.8% of total global disease burden were attributed to the low fruit and vegetable consumption. Furthermore, low intake of fruits and vegetables is estimated to cause around 14% of gastrointestinal cancer deaths, about 11% of ischaemic heart disease deaths and about 9% of stroke deaths worldwide (World Health organization, 2014). Around the world the intake of fruits and vegetables is not sufficient, with a particular attention to adolescents and children, specially boys and adult males, being this point highlighted by several international organisms and authorities: Organisation for Economic Co-operation and Development (OECD, 2013); European Food Information Council (EUFIC, 2012); Centers for Disease Control and Prevention (CDC, 2013).

With a reduced consumption of fruits and vegetables several bioactive food components are not ingested in a correct dose, having repercussions at human organism (Duyn & Pivonka, 2000). Fruits and vegetable consumption reduce the prevalence of cancer (Block, Patterson, & Subar, 1992) and the risk of cardiovascular diseases (Veer, Jansen, Klerk, & Kok, 2000). Among the most important bioactive components are the carotenoids (Gaziano et al., 1995). Carotenoids are important components of several biological processes in the human organism, having repercussions in human health (Granado, Olmedilla, & Blanco, 2003). The amounts of carotenoids that are found in human tissues are almost exclusively from dietary origin, mainly from fruits and vegetables, or from supplements.

Two of more relevant carotenoids are β-carotene and lutein. β-carotene is a carotenoid that has been used both as a food coloring agent and as a source of vitamin A in animal feed. In addition β-carotene has been used to treat disorders such as asthma and erythropoietic protoporphyria, and to reduce the risk of several cancers as breast and lung cancer in addition to cardiovascular disease (Omenn et al., 1996). It has been also reported that the uptake of β-carotene reduce the risk of age-related macular

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degeneration (AMD) (Teikari et al., 1998). β-carotene can be found in vegetables as carrots, pumpkins and sweet potatoes that colors them orange, although it can be also produced from *Dunaliella* sp., a hypersaline microalgae produced in open raceways.

Lutein is a xantophyll compound with antioxidant activity and recommended to prevent some types of cancer (Nishino, 1998), as well as cardiovascular (Peterson, Dwyer, Jacques, & McCullough, 2012) and retinal diseases (Ozawa et al., 2012). The estimated lutein daily uptake of 1.5 mg day^{-1} by eating fruits and vegetables is not enough to reach the recommended daily uptake of 6 mg day^{-1} (Seddon et al., 1994), then the recommendation to consume lutein supplements. Lutein is commercially produced from marigold (*Tagetes erecta* L.), but the lutein content of marigold flowers is very low, of 0.03% (dry weight; d.w.), whereas microalgae as *Muriellopsis* sp., *Chlorella* sp. and *Scenedesmus* sp. can contain large amounts of this compound, up to 0.90% d.w. (García-González, Moreno, Manzano, Florencio, & Guerrero, 2005; Yen, Sun, & Ma, 2011). The production of lutein from a new lutein-rich microalgae strain, *Scenedesmus almeriensis*, has been already patented.

S. almeriensis can contain up to 1.5% d.w. of lutein together with other carotenoids as β-carotene and can be produced with high productivity in closed tubular photobioreactors, in continuous mode at large scale, thus being a continuous and reliable source of lutein (Fernández-Sevilla, Acién Fernández, & Molina-Grima, 2010; Sánchez et al., 2008a,b).

Contrasting with the reduced consumption of fruits and vegetables is the olive oil consumption. This premium vegetable oil is steadily increasing production and consumption worldwide over the last years (IOC, 2013a,b). Therefore, olive oil could be a good convoy to improve and enhance the intake of carotenoids in a daily basis. In this sense, in the present work, the main goals were to study the effects of the addition of different concentrations of *S. almeriensis* carotenoids rich extracts to extra-virgin olive oils. The olive oils were evaluated for color and quality attributes, composition (fatty acids profile, tocopherols, pigments), and oxidative stability in order to support their efficiency as antioxidants while characterizing the changes induced in the olive oil legislated parameters.

2. Materials and methods

2.1. Production conditions of microalgae S. almeriensis

The microalgae *S. almeriensis* CCAP 276/24 was produced in an industrial size outdoor tubular photobioreactor (3.000 L), in continuous mode at 0.30 L/day dilution rate, on Almería (Spain) at spring. Culture medium used was prepared on freshwater using fertilizers ($NaNO_3$, KH_2PO_4, micronutrients). To avoid contamination the culture medium was ozonised and passed by ultrafiltration up to 0.02 μm. The cultures were performed at pH = 8.0 by on-demand injection of CO_2, and below 30 °C by passing thermostated water through a heat exchanger located inside the reactor. The biomass was daily harvested by centrifugation, then being lyophilised and stored at −18 °C. Lyophilised biomass was used as raw material (Acién, Fernández-Sevilla, Magán, & Molina-Grima, 2012).

2.2. Extraction methods

Lutein is currently separated and purified from marigold flowers by saponification–extraction–recrystallization method (Morita, Watanabe, & Saiki, 2002). A modification of this method has been used to recover carotenoids from lyophilized biomass of *S. almeriensis* (Cerón et al., 2008). First step is a cell disruption process, with alumina in a 1:1 w/w proportion, using a beads mill (Staatlich Berlin Ku 4) at rotation speed of 120 rpm and with beads of 28 mm diameter for 5 min to remove fatty acids soaps. Second step is a saponification performed using 4% w/v KOH with a biomass concentration of 100 g/L for 5 min. Longer alkaline treatments or the use of higher KOH concentrations reduced the yield of the process. Finally, extraction with hexane is performed using a 1:1 ratio hexane to sample volume, a total of three steps being performed to recover more than 90% of carotenoids contained in the processed biomass. In each step a volume of ethanol equal to 1% of the total volume was added for avoiding emulsion (Camacho et al., 2007). Hexane was removed from the extract by high vacuum distillation.

2.3. Olive oils sampling and extract addition

Five commercial extra-virgin olive oils (EVOO's) representative from Jaén region were selected for the present study (from A to E). Three bottles of 750 mL of each olive oil were obtained ($n = 3$). From each bottle, three samples of 50 mL were constituted: control (I – olive oils without extract); olive oil with 0.1 mg of extract per mL of oil (II); and olive oil with 0.21 mg of extract per mL of oil (III). All olive oils were filtrated in the presence of sulfate sodium anhydrous before use. Once prepared, samples were homogenized and kept under 4 °C prior to any analysis.

2.4. Physical and quality parameters evaluated

2.4.1. Color determination

Color of olive oils with and without extract addition was measured with a Konica Minolta model CR-400 colorimeter. Color differences (ΔE) between control samples considered as standard (olive oils without extract) and oils with extract addition were calculated from the determined monochromatic variables L^*, a^*, and b^* obtained from CIELAB method, as well as yellowness index (YI) as described by Zamora, Olmo, Navarro, and Hidalgo (2004):

$$\Delta E = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right]^{1/2}$$

$$\mathrm{YI} = \frac{142.86 \times b^*}{L^*}$$

L^* is a measure of luminance or lightness component, which ranges from 0 to 100 (black to white); a^* ranges from negative to positive (green to red); and b^* also ranges from negative to positive (blue to yellow).

2.4.2. Quality parameters determination

Free acidity (FA), peroxide value (PV) and coefficients of specific extinction at 232 and 270 nm (K_{232} and K_{270}) were determined according to European Union standard methods (Annexes II and IX in European Community Regulation EEC/2568/91 from 11th July).

2.5. p-Anisidine value determination

Unsaturated aldehydes, secondary oxidation products, were estimated by the anisidine value (*p*-AV), as detailed in ISO 6885 (2006). This measurement is based on the absorbance increase per g of oil, measured at 350 nm (Genesys 10UV), of an olive oil solution in iso-octane, before and after reaction with *p*-anisidine reagent in the dark.

2.6. Oxidative stability

The oxidative stability was estimated by measuring the oxidation induction time, on a Rancimat 743 apparatus (Metrohm CH, Switzerland). Filtered, cleaned, dried air (20 L/h) was bubbled

through the oil (3.00 g) heated at 120 ± 1.6 °C, with the volatile compounds being collected in water, and the increasing water conductivity continuously measured. The time taken to reach the conductivity inflection was recorded.

2.7. Fatty acids composition

Fatty acids were evaluated as their methyl esters after cold alkaline transesterification with methanolic potassium hydroxide solution (European Community Regulation EEC/2568/91 from 11th July) and extraction with n-heptane. The fatty acid profile was determined with a Chrompack CP 9001 chromatograph equipped with a split–splitless injector, a FID detector, an autosampler Chrompack CP-9050 and a 50 m × 0.25 mm i.d. fused silica capillary column coated with a 0.19 μ film of CP-Sil 88 (Varian). Helium was used as carrier gas at an internal pressure of 110 kPa. The temperatures of the detector and injector were 250 and 230 °C, respectively. The oven temperature was programmed at 120 °C during the first 3 min with an increase of 4 °C/min until 220 °C. The split ratio was 1:50 and the injected volume was of 1 μL. The results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area eluting between myristic and lignoceric methyl esters. A control sample (olive oil 47118, Supelco) and a fatty acids methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification and calibration purposes (Sigma, Spain).

2.8. Tocopherols composition

Tocols were evaluated following the international standard ISO 9936 (2006), with some modifications as described by Malheiro et al. (2013a). Tocopherols standards (α, β, γ and δ) were purchase from Calbiochem (La Jolla, San Diego, CA) and Sigma (Spain), while the internal standard 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA). An accurate amount of filtered olive oil or *S. almeriensis* extract were blended with an appropriate amount of internal standard solution (tocol) in a 1.5 mL volume of *n*-hexane and homogenized by stirring. Sample preparation was conducted in dark and tubes containing the samples were always wrapped in aluminum foil. The mixture was centrifuged for 5 min at 13,000 rpm and the supernatant analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with a Jasco LC – NetII/ADC data unit, a PU-1580 Intelligent Pump, a LG-1580-04 Quaternary Gradient Unit, a DG-1580-54 Four Line Degasser and an FP-920 fluorescence detector (λ_{exc} = 290 nm and λ_{em} = 330 nm). The chromatographic separation was achieved on a Supelcosil™ LC-SI column (3 μm) 75 × 3.0 mm (Supelco, Bellefonte, PA), operating at constant room temperature (23 °C). A mixture of *n*-hexane and 1,4-dioxane (97.5:2.5) was used as eluent, at a flow rate of 0.7 mL/min. Data were analyzed with the ChromNAV Control center – JASCO Chromatography Data Station (Japan). The compounds were identified by chromatographic comparisons with authentic standards, by co-elution and by their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response.

2.9. Pigments composition

The extracts were prepared in accordance with Achir, Randrianatoandro, Bohuon, Laffargue, and Avallone (2010). Briefly, olive oil samples (200 mg) were added with an appropriate amount of internal standard β-apo-carotenal (Sigma–Aldrich), mixed with 2 mL acetone, vortexed for 10 s, and left overnight at −20 °C for triacylglycerol crystallization. Triacylglycerols were separated by rapid sampling followed by centrifugation at 13,000 rpm. These steps were omitted in the *S. almeriensis* extract analysis, being directly diluted in acetone after internal standard addition. The extract was directly injected into the HPLC column, a Phenomenex Luna C18 (250 × 3.5 mm internal diameter) at 23 °C and eluted with a 30-min linear gradient from 80% aqueous methanol (v/v) containing 0.05% triethylamine and 20% ethyl acetate (containing 0.05% triethylamine) at 1 mL/min. The analysis was performed on the same HPLC equipment described for the tocols, except for the use of a DAD detector (JASCO MD-2015-Plus, Japan). Lutein and β-carotene were obtained from Sigma. Calibration curves were constructed at 440 nm for lutein and β-carotene against the internal standard.

2.10. Statistical analysis

2.10.1. Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov–Smirnov with Lilliefors correction (if $n > 50$) or the Shapiro–Wilk's test (if $n < 50$), and the Levene's tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the effect of the addition of different concentrations of *S. almeriensis* extract in EVOO's quality (FA, PV, *p*-AV, TOTOX, K_{232}, K_{270}, and ΔK), color parameters (a^*, b^* L^*, ΔE and YI), fatty acids profile, tocopherols composition (α-, β-, γ-, and total tocopherols), oxidative stability, and pigments composition (lutein and β-carotene). If a statistical significant effect was found, means were compared using Tukey's honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

2.10.2. Principal component analysis (PCA)

Principal components analysis (PCA) was applied for reducing the number of variables in the olive oils enriched with different concentration of *S. almeriensis* extract (4 variable corresponding to color parameters – L^*, a^*, b^*, and YI; 5 variables corresponding to the quality parameters – FA, PV, K_{232}, K_{270} and ΔK; *p*-AV, TOTOX index; 4 variables corresponding to tocopherols, including total vitamin E content (total tocopherols); oxidative stability; lutein and β-carotene contents; with a total of 18 variables) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the addition of different concentrations of *S. almeriensis* to EVOO's. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

2.10.3. Linear discriminant Analysis (LDA)

A linear discriminant analysis (LDA) was used as a supervised learning technique to classify the EVOO's with different concentrations of *S. almeriensis* extract added according to their color and quality parameters, TOTOX, *p*-AV, tocopherols content, fatty acids profile, pigments content, and oxidative stability (32 variables overall). A stepwise technique, using the Wilk's lambda method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable to be included, it is verified

whether all variables previously selected remain significant. With this approach, it is possible to identify the significant variables among all variables in study. To verify which canonical discriminant functions were significant, the Wilks' Lambda test was applied. To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance. Moreover, the sensibility and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group. Sensibility was calculated by dividing the number of samples of a specific group correctly classified by the total number of samples belonging to that specific group. Specificity was calculated by dividing the number of samples of a specific group classified as belonging to that group by the total number of samples of any group classified as belonging to that specific group. The LDA was performed by using the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

3. Results and discussion

3.1. Color parameters

The color of the EVOO's was evaluated to assess the addition of *S. almeriensis* in their appearance, since olive oils visual aspect is an important factor for consumers' evaluation and preference (Gómez-Caravaca et al., 2007). In Table 1 are presented the CIELAB main axes, L^*, a^* and b^* values obtained from EVOO's with different concentrations of *S. almeriensis* extract. In the five olive oils studied its clear the impact of the addition of the extracts. Values of a^* decrease with the concentration of extract added in similar way in the five olive oils, with significant differences observed (Table 1). This means that green coloration, typical of virgin olive oils, pass to a more yellow-orange coloration (Fig. 1). Regarding b^* value its observed an increase in this parameter, mainly with the addition of 0.1 mg of extract per mL of olive oil, since olive oils become more yellow with the addition of *S. almeriensis* extract (Table 1 and Fig. 1). The luminosity (L^*) of olive oils decrease significantly in all samples, being observed a direct proportion between the concentration of extract and the luminosity observed, which turn olive oils more darker than control olive oils (Fig. 1).

The results obtained in a^*, b^* and L^* showed that *S. almeriensis* extract addition to olive oils brought significant changes in their color, observations corroborated by ΔE parameter (color differences). In Fig. 1 are reported the color differences (Fig. 1A) between olive oils without and with *S. almeriensis* extract and the yellowness index (YI) (Fig. 1B). ΔE values are higher with the amount of extract added to olive oils, which means that color differences with extract increase (Fig. 1). YI increases significantly with extract addition, however, no differences were observed between the addition of 0.1 and 0.21 mg/mL.

The addition of *S. almeriensis* extracts is clearly seen, even at naked eye (Fig. 1). The color adopted by the olive oils is related to extract composition, mainly carotenoids (Cerón et al., 2008), as properly discussed in Section 3.5.

3.2. Quality parameters

The quality of the olive oils was assessed to verify if the addition of *S. almeriensis* extracts cause quality changes. The global observations clearly highlight that no quality degradation is undertaken by extract addition (Table 1). Regarding FA values in four of the five olive oils studied no significant changes were observed, and in one olive oil (olive oil B), extract addition reduce the levels of free fatty acids in the olive oils, but without significant changes between olive oils with extract. Such results are positive, since the addition of extracts does not trigger hydrolytic reactions in the olive oils, maintaining the levels of free fatty acids.

The possible occurrence of oxidative processes in the EVOO's was monitored by several quality parameters (PV, K_{232}, K_{270}, ΔK, and *p*-AV) reported in Table 1. The peroxidation of the EVOO's is reduced by *S. almeriensis* extracts, since in all olive oils, except olive oil C, a significant decrease in PV was observed. Therefore the formation of primary oxidation products (mainly hydroperoxides) (Laguerre, Lecomte, & Villeneuve, 2007) is inhibited, acting the *S. almeriensis* extracts as antioxidants. Specific extinction coefficients at 232 and 270 nm (K_{232} and K_{270} respectively) also corroborate

Table 1
Quality parameters (FA – free acidity; PV – peroxide value), *p*-anisidine value (*p*-AV), TOTOX index, and color parameters (a^*, b^* and L^*) of EVOO's without (I) and with *S. almeriensis* extract (II = 0.1 mg/mL; III = 0.21 mg/mL).

Olive oil	Colour parameters			Quality parameters					*p*-AV	TOTOX
	a^*	b^*	L^*	FA (%)	PV (mEq. O_2/kg)	K_{232}	K_{270}	ΔK		
A										
I	−11.77 ± 0.29a	81.95 ± 0.81a	73.95 ± 0.52c	0.3 ± 0.04a	9 ± 1b	0.86 ± 0.15a	0.15 ± 0.02a	−0.003 ± 0.002a	17 ± 1a	35 ± 2a
II	−2.33 ± 0.42b	86.01 ± 1.32b	70.70 ± 0.85b	0.3 ± 0.05a	6 ± 1a	0.83 ± 0.09a	0.15 ± 0.01a	−0.003 ± 0.001a	16 ± 0a	28 ± 3a
III	3.92 ± 0.51c	82.65 ± 0.96a	68.24 ± 0.61a	0.3 ± 0.04a	7 ± 2a	0.94 ± 0.12a	0.19 ± 0.03b	−0.001 ± 0.002a	16 ± 1a	30 ± 4a
B										
I	−12.77 ± 0.27a	81.41 ± 1.46a	75.60 ± 1.01c	0.2 ± 0.00a	9 ± 1b	0.94 ± 0.17a	0.13 ± 0.02a	−0.002 ± 0.005a	12 ± 0a	30 ± 2b
II	−3.08 ± 0.45b	86.39 ± 2.23c	70.96 ± 1.38b	0.2 ± 0.00a	6 ± 1a	0.86 ± 0.18a	0.12 ± 0.01a	−0.005 ± 0.002a	13 ± 1a	24 ± 2a
III	3.35 ± 0.36c	83.89 ± 1.49b	69.01 ± 0.89a	0.2 ± 0.00a	6 ± 0a	1.17 ± 0.55a	0.16 ± 0.01b	−0.003 ± 0.004a	13 ± 1 a	25 ± 1a
C										
I	−13.13 ± 0.76a	73.24 ± 2.39a	78.53 ± 0.85c	0.3 ± 0.00a	9 ± 0a	0.84 ± 0.07a	0.18 ± 0.02a	−0.001 ± 0.003a	13 ± 1a	32 ± 0b
II	−1.28 ± 1.10b	89.40 ± 1.56b	72.93 ± 1.01b	0.3 ± 0.00a	8 ± 0a	0.88 ± 0.03a,b	0.21 ± 0.02a,b	−0.007 ± 0.008a	14 ± 1a	31 ± 0b
III	5.42 ± 1.09c	88.15 ± 0.56c	71.58 ± 0.34a	0.3 ± 0.00a	8 ± 1a	0.92 ± 0.04b	0.24 ± 0.03b	−0.003 ± 0.002a	13 ± 1a	30 ± 1a
D										
I	−11.77 ± 0.37a	82.34 ± 1.08a	74.85 ± 0.82c	0.2 ± 0.00a	9 ± 0b	0.82 ± 0.05a	0.13 ± 0.01a	−0.003 ± 0.001a	15 ± 1a	33 ± 1b
II	−3.48 ± 0.21 b	85.87 ± 2.02b	70.96 ± 1.01b	0.2 ± 0.00 a	6 ± 1a	0.84 ± 0.04a	0.15 ± 0.02a,b	−0.004 ± 0.002a	16 ± 1a	27 ± 2a
III	2.36 ± 0.46c	85.17 ± 1.23c	69.87 ± 0.76a	0.2 ± 0.04a	6 ± 1a	0.81 ± 0.05a	0.16 ± 0.01b	−0.002 ± 0.001a	16 ± 1a	28 ± 1a
E										
I	−14.15 ± 0.04a	71.48 ± 2.12a	81.34 ± 0.61c	0.3 ± 0.00a	8 ± 1b	1.00 ± 0.07a	0.13 ± 0.02a	−0.003 ± 0.001a	14 ± 1a	29 ± 1a
II	−5.38 ± 0.20b	95.20 ± 0.38c	77.49 ± 0.26b	0.3 ± 0.05a	5 ± 1a	0.93 ± 0.08a	0.13 ± 0.02a	−0.009 ± 0.017a	14 ± 1a	24 ± 2b
III	0.85 ± 0.83c	93.58 ± 0.81b	75.13 ± 0.58a	0.3 ± 0.00a	5 ± 1a	1.01 ± 0.03a	0.16 ± 0.01b	−0.002 ± 0.002a	14 ± 1a	24 ± 1b

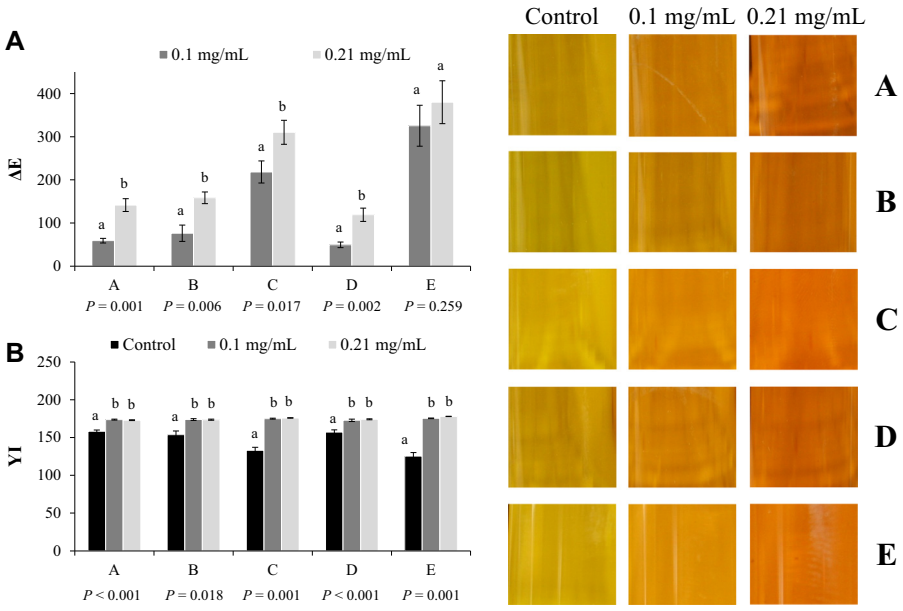

Fig. 1. Color differences (ΔE) (A) and yellowness index (YI) (B) between EVOO's with and without *S. almeriensis* extract. In each olive oil mean values with different letters differ significantly ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that extracts addition does not affect olive oils quality. In K_{232} and ΔK no significant changes were observed in the olive oils, however the addition of extracts to olive oil increase significantly K_{270} values. K_{270} is indicative of the formation of secondary products of oxidation, meanwhile, and by evaluating the values obtained in *p*-AV, we clearly conclude that the addition of extracts does not inflict secondary oxidation in olive oils, and those results obtained in K_{270} could be related with the color conferred to olive oils by the extracts. According to Malheiro, Casal, Ramalhosa, and Pereira (2011) *p*-AV is an empirical determination used to assess advanced oxidative rancidity of vegetable oils. The analytical method is based on the reactiveness of the aldehyde carbonyl bond on the *p*-anisidine amine groups, leading to the formation of Schiff base that absorbs at 350 nm. It allows the estimation of secondary products of oxidation of unsaturated fatty acids, principally dienals and 2-alkenals (Labrinea, Thomaidis, & Georgiou, 2001). Therefore, by studying the data obtained in quality parameters (Table 1) of the EVOO's studied is clear that extracts addition inflicts neither primary (PV and K_{232}) nor secondary (*p*-AV) oxidative reactions. In fact the addition is positive since a lower formation of hydroperoxides is observed and the overall quality of EVOO's increase, as observed in TOTOX value (Table 1). TOTOX value is a useful tool when assessing the oxidative deterioration of vegetable oils, being also reported as the total oxidation value according to ISO 6885 (2006). In the five olive oils analyzed, only in olive oil A no significant decreases in TOTOX value were observed. The remaining olive oils reported lower oxidative deterioration with extracts addition. Lower TOTOX values were reported in comparison to those obtained in Italian EVOO and OO (olive oil) (Cerretani, Bendini, Rodriguez-Estrada, Vittadini, & Chiavaro, 2009).

Regarding all the values obtained in the quality parameters of the olive oils with and without *S. almeriensis* extracts, all oils could be classified as EVOO's according to the European Communitarian legislation (Commission Implementing Regulation (EU), No. 299/2013).

3.3. Fatty acids profile

Fatty acids profile of EVOO's without and with different concentrations of *S. almeriensis* extracts are reported in Table 2. The fatty acids profile of the five EVOO's is mainly composed by oleic acid ($C_{18:1}$), followed by palmitic acid ($C_{16:0}$) and linoleic acid ($C_{18:2}$). In the EVOO's studied the addition of *S. almeriensis* extracts didńt brought significant changes in the fatty acids profile, except in some specific situations. In two olive oils (B and E) the content of palmitic acid increase significantly, while in olive oil C the same was observed regarding linoleic acid. The contents of linolenic acid ($C_{18:3}$) in olive oils B and D increase considerably with statistical meaning (Table 2). Concerning the different fatty acids fractions that compose the studied olive oils, as expected due to the high contents in oleic acid, MUFA (monounsaturated fatty acids) were the most abundant (>77%), followed by SFA (saturated fatty acids) between 14.40% and 17.15%, and by PUFA (polyunsaturated fatty acids) with values ranging from 3.59% and 4.70% (Table 2). *Trans* fatty acids were always equal or inferior to 0.05% (Table 2).

3.4. Tocopherols composition

Tocopherols detailed composition of the EVOO's with different concentrations of *S. almeriensis* extracts is reported in Table 3. In the five olive oils analyzed, three tocopherols were detected and quantified: α-, β-, and γ-tocopherol. α-Tocopherol was present in higher amounts, as expected for olive oils (Malheiro, Casal, Lamas, Bento, & Pereira, 2012), with contents ranging from 119 to 429 mg/kg in control olive oils. β-Tocopherol values were between 4 and 6 mg/kg while γ-tocopherol ranged from 40 to 55 mg/kg in all samples studied (Table 3). Total tocopherols amount varied from 159 mg/kg (olive oil C in control samples) to 511 mg/kg (olive oil B with 0.1 mg/mL). The addition of *S. almeriensis* extracts does not affect considerably total tocopherols amount as well as individual tocopherols, except in some particular cases (Table 3). These observations are in accordance with the observed absence of tocopherols in the *S. almeriensis* extract. Since tocopherols, mainly α-tocopherol, possess important antioxidant and vitaminic action, the fact that the addition of *S. almeriensis* do not compromise their content is a good aspect, since both important biological properties are preserved in the olive oils.

3.5. Pigments composition

Analysis carried out by HPLC in the *S. almeriensis* extracts, prior to the inclusion in the EVOO's, supported its richness in

Table 2
Detailed fatty acids composition (g/100 g of fatty acids) of EVOO's without (I) and with *S. almeriensis* extract (II = 0.1 mg/mL; III = 0.21 mg/mL).

Olive oil	$C_{16:0}$	$C_{16:1}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{18:3}$	$C_{20:1}$	$C_{22:0}$	SFA	MUFA	PUFA	*Trans*
A														
I	11.76 ± 0.45a	0.79 ± 0.06a	0.10 ± 0.02a	2.87 ± 0.26a	78.09 ± 0.84a	3.46 ± 0.44a	0.37 ± 0.02a	0.57 ± 0.03a	0.24 ± 0.01a	0.09 ± 0.02a	15.24 ± 0.56a	79.22 ± 0.85a	4.02 ± 0.41a	0.04 ± 0.01a
II	11.35 ± 0.44a	0.77 ± 0.01a	0.10 ± 0.00a	2.60 ± 0.23a	79.32 ± 0.61a	3.18 ± 0.04a	0.35 ± 0.01a	0.58 ± 0.00a	0.22 ± 0.00a	0.11 ± 0.00a	14.52 ± 0.66a	80.42 ± 0.61a	3.76 ± 0.05a	0.02 ± 0.00a
III	11.07 ± 0.37a	0.80 ± 0.01a	0.10 ± 0.00a	2.74 ± 0.06a	79.21 ± 0.36a	3.30 ± 0.01a	0.35 ± 0.01a	0.60 ± 0.00a	0.22 ± 0.00a	0.11 ± 0.01a	14.40 ± 0.45a	80.34 ± 0.36a	3.9 ± 0.01a	0.02 ± 0.01a
B														
I	11.43 ± 0.64a	1.33 ± 0.03a	0.11 ± 0.00a	2.41 ± 0.32a	77.67 ± 0.66a	3.58 ± 0.12a	0.36 ± 0.01a	0.77 ± 0.02a	0.25 ± 0.01a	0.12 ± 0.02a	14.45 ± 0.95a	79.35 ± 0.69b	4.35 ± 0.13a	0.04 ± 0.01b
II	12.01 ± 0.27a,b	1.36 ± 0.06a	0.10 ± 0.00a	2.29 ± 0.17a	77.33 ± 0.14a	3.73 ± 0.04a	0.37 ± 0.01a	0.81 ± 0.01b	0.25 ± 0.00a	0.11 ± 0.02a	14.92 ± 0.13a,b	79.04 ± 0.08a,b	4.54 ± 0.04a	0.01 ± 0.00a,b
III	13.00 ± 0.78b	1.34 ± 0.03a	0.11 ± 0.01a	2.71 ± 0.16a	76.22 ± 0.71a	3.73 ± 0.08a	0.36 ± 0.01a	0.80 ± 0.01a,b	0.25 ± 0.00a	0.10 ± 0.00a	16.30 ± 0.65b	77.91 ± 0.69a	4.53 ± 0.08a	0.03 ± 0.01a
C														
I	10.85 ± 0.56a	0.88 ± 0.02a	0.10 ± 0.01a	2.99 ± 0.17a	78.77 ± 0.58a	3.56 ± 0.08a	0.34 ± 0.01a	0.63 ± 0.05a	0.22 ± 0.02a	0.11 ± 0.02a	14.42 ± 0.72a	79.96 ± 0.58a	4.19 ± 0.10a	0.05 ± 0.02a
II	11.22 ± 0.34a	0.93 ± 0.09a	0.13 ± 0.04a	2.86 ± 0.18a	78.69 ± 0.25a	3.66 ± 0.05a,b	0.38 ± 0.04a	0.60 ± 0.03a	0.27 ± 0.06a	0.11 ± 0.01a	14.73 ± 0.53a	80.02 ± 0.14a	4.26 ± 0.03a,b	0.04 ± 0.01a
III	11.35 ± 0.11a	0.90 ± 0.04a	0.10 ± 0.00a	2.94 ± 0.05a	78.73 ± 0.09a	3.73 ± 0.03b	0.35 ± 0.01a	0.63 ± 0.01a	0.23 ± 0.00a	0.10 ± 0.00a	14.86 ± 0.05a	79.96 ± 0.12a	4.36 ± 0.04b	0.04 ± 0.01a
D														
I	13.36 ± 0.74a	1.17 ± 0.02a	0.11 ± 0.03a	2.28 ± 0.10a	77.09 ± 1.01a	2.94 ± 0.05a	0.33 ± 0.02a	0.65 ± 0.02a	0.24 ± 0.02a	0.10 ± 0.01a	16.19 ± 0.78a	78.61 ± 1.03a	3.59 ± 0.07a	0.05 ± 0.03a
II	13.14 ± 0.52a	1.16 ± 0.01a	0.10 ± 0.00a	2.32 ± 0.10a	77.33 ± 0.24a	3.07 ± 0.11a	0.35 ± 0.03a	0.69 ± 0.01a,b	0.24 ± 0.00a	0.11 ± 0.01a	16.05 ± 0.44a	78.84 ± 0.24a	3.77 ± 0.11a	0.02 ± 0.01a
III	12.18 ± 0.71a	1.17 ± 0.04a	0.10 ± 0.00a	2.27 ± 0.13a	78.29 ± 0.74a	3.31 ± 0.05a	0.34 ± 0.01a	0.74 ± 0.03b	0.24 ± 0.01a	0.10 ± 0.01a	15.02 ± 0.68a	79.82 ± 0.77a	4.05 ± 0.08b	0.04 ± 0.02a
E														
I	12.17 ± 0.64a	1.18 ± 0.40a	0.08 ± 0.03a	3.16 ± 0.26a	76.94 ± 0.76a	3.85 ± 0.20a	0.36 ± 0.01a	0.63 ± 0.03a	0.22 ± 0.03a	0.11 ± 0.01a	15.94 ± 0.88a	78.41 ± 0.42a	4.48 ± 0.23a	0.04 ± 0.03a
II	11.92 ± 0.87a,b	0.94 ± 0.01a	0.09 ± 0.00a	2.98 ± 0.03a	77.19 ± 0.74a	4.03 ± 0.06a	0.37 ± 0.01a	0.67 ± 0.01a	0.24 ± 0.01a	0.11 ± 0.00a	15.52 ± 0.86a	78.47 ± 0.72a	4.70 ± 0.07a	0.04 ± 0.02a
III	13.55 ± 0.26b	0.96 ± 0.05a	0.09 ± 0.01a	3.02 ± 0.14a	76.04 ± 0.39a	3.98 ± 0.06a	0.35 ± 0.02a	0.67 ± 0.02a	0.23 ± 0.01a	0.10 ± 0.01a	17.15 ± 0.42a	77.32 ± 0.41a	4.65 ± 0.08a	0.03 ± 0.01a

carotenoids, with β-carotene representing 13.3% of total extract, followed by 0.25% of lutein and 1% of other unidentified carotenoids. Spectrophotometric analyses corroborated the previous results, with an average of 14.4% of total carotenoid-like compounds in the *S. almeriensis* extracts.

The pigments composition of the five EVOO's with and without extracts of *S. almeriensis* is displayed in Fig. 2. From the results obtained is observed that *S. almeriensis* extracts are mainly rich in carotenoids, β-carotene and lutein, the first one being the major component. In the five EVOO's analyzed, lutein content in control olive oils varied from 4.5 to 6.3 mg/kg (Fig. 2B). The addition of *S. almeriensis* extracts increases considerably lutein content in olive oils (except olive oil B). The increase of lutein with 0.1 mg/mL was only statistically significant in oils A and C, while the addition of 0.21 mg/mL was significant in olive oils A, C and D in comparison with the control. Therefore the addition of 0.1 mg/mL would be the best option in order to improve lutein content in olive oils. Meanwhile, we observed that the extracts of *S. almeriensis* are highly rich in other carotenoid, β-carotene (Fig. 2A). β-carotene content in control olive oils varied between 1.2 (olive oil C) and 4.1 mg/kg (olive oil D). When we added 0.1 mg/mL, β- carotene increase 6.5, 6.0, 11.1, 4.4, and 8.3 times in olive oils from A to E, respectively. With an addition of 0.21 mg/mL β-carotene content increased 9 times in olive oil D and 25.8 times in olive oil C. β-carotene content in olive oils with 0.21 mg/mL varied between 30.1 and 37.1 mg/kg (olive oil B and D respectively; Fig. 2A). Comparatively to EVOO's enriched with olive leaves during extraction process (Malheiro, Casal, Teixeira, Bento, & Pereira, 2013b), EVOO's with *S. almeriensis* report lower lutein content but much higher β-carotene, resulting from extract addition, without the significant increment in chlorophylls and pheophytins as observed with olive leaves addition during extraction process, regarded as potentially reducing olive oil photostability.

Such increment in carotenoids caused significant changes in olive oils color and quality as witnessed in Sections 3.1 and 3.2 of the present manuscript and in Fig. 1 and Table 1.

3.6. Oxidative stability

The oxidative stability of olive oils was assessed in order to verify if the addition of *S. almeriensis* extracts could display antioxidant, pro-oxidant or no activity in the EVOO's. The results obtained are presented in Fig. 2C and they clearly show that *S. almeriensis* extracts act as antioxidants, protecting olive oils from oxidation. A significant increase in resistance to oxidation was verified in all olive oils. Meanwhile, by adding 0.21 mg of extract per mL of olive oil no significant increases in oxidative stability were observed comparatively to the addition of 0.1 mg/mL (except olive oil D). From this observation we could infer the addition of 0.21 mg/mL of *S. almeriensis* extracts to olive oil could be excessive since no further improvements were observed, and could also possibly act as prooxidants. The addition of 0.1 mg/mL increased oxidative stability of olive oils from 11.85% (olive oil C) to 37.80% (olive oil A), while 0.21 mg/mL raised oxidative stability between 15.76% (olive oil C) and 42.06% (olive oil B). The increase in the oxidative stability is related to the composition of the extracts of *S. almeriensis*, especially rich in β-carotene. β-carotene is a recognized antioxidant compound (Sies & Stahl, 1995), and according to Aparicio, Roda, Albi, and Gutiérrez (1999), in normal conditions, carotenoids are responsible for about 6% of olive oils stability. In this kind of olive oils, with the great amounts of β-carotene in the olive oils, carotenoids contribution to oxidative stability is greatly increased. Such observations are important since shelf-life of olive oils could be considerably increased, being interesting to study the impact and stabilization of olive oils with *S. almeriensis* extracts during storage period and thermal processing.

Table 3
Tocopherols composition (mg/kg of olive oil) of EVOO's without (I) and with *S. almeriensis* extract (II = 0.1 mg/mL; III = 0.21 mg/mL).

Olive Oil	α-Tocopherol	β-Tocopherol	γ-Tocopherol	Total tocopherols
A				
I	257 ± 6.1 a	4.9 ± 0.1 a	50 ± 0.9 b	312 ± 6.8 a
II	273 ± 1.2 b	4.7 ± 0.2 a	50 ± 0.1 b	328 ± 1.8 b
III	261 ± 4.4 a	4.5 ± 0.1 a	46 ± 0.4 a	311 ± 4.6 a
B				
I	429 ± 9.8 a	5.7 ± 0.2 a	55 ± 2.4 a	489 ± 11.4 a
II	451 ± 20.3 a	5.5 ± 0.3 a	54 ± 1.9 a	511 ± 20.2 a
III	435 ± 5.2 a	5.4 ± 0.1 a	52 ± 2.5 a	492 ± 6.7 a
C				
I	119 ± 21.4 a	4.1 ± 0.1 a	44 ± 3.5 a	159 ± 24.7 a
II	121 ± 19.9 a	4.1 ± 0.0 a	42 ± 2.8 a	168 ± 22.6 a,b
III	137 ± 20.6 a	4.2 ± 0.1 a	42 ± 3.3 a	183 ± 24.0 b
D				
I	336 ± 25.8 a	5.2 ± 0.1 b	42 ± 5.1 a	383 ± 30.8 a
II	330 ± 26.5 a	5.0 ± 0.2 a,b	40 ± 5.0 a	376 ± 31.5 a
III	334 ± 31.7 a	4.7 ± 0.2 a	39 ± 4.3 a	378 ± 35.6 a
E				
I	297 ± 2.6 a	5.0 ± 0.1 a	51 ± 0.6 b	353 ± 3.2 a
II	297 ± 2.0 a	4.9 ± 0.1 a	49 ± 0.6 a,b	351 ± 2.6 a
III	307 ± 11.7 a	4.8 ± 0.1 a	49 ± 1.4 a	360 ± 13.2 a

3.7. Olive oils discrimination by Scenedesmus almeriensis extract addition

Scenedesmus almeriensis extracts addition at different concentrations clearly affected color, quality, composition and oxidative stability of EVOO's in a way that it was possible to discriminate them according to the concentration of extract added in each olive oil. In Fig. 3A is shown the two-dimensional representation of the two principal component factor scores that explain 58.61% of the total variance of the data obtained. It is perceived that the first principal component (PC1) separates control EVOO's (without extracts) from olive oils with *S. almeriensis* extracts. Control EVOO's, represented in the entire negative region of PC1 (Fig. 3A), were characterized by worst quality indexes (PV, FA, and TOTOX value), while EVOO's with 0.1 and 0.21 mg/mL, mainly represented in the positive region of PC1, are associated with higher values in color parameters (a^*, b^*, L^*, ΔE, and YI – Table 1 and Fig. 1), higher lutein and β-carotene content (Fig. 2A and B), and higher oxidative stability (Fig. 2C).

Together with the unsupervised PCA method, a stepwise linear discriminant analysis (LDA) was applied to the obtained data in order to create a discriminant model. The model obtained resulted in seven significant discriminant functions that explained 100% of the variance, although only the first two were used, since they explain 89.3% of the variance of the experimental data (Function 1 52.8% and Function 2 36.5%; Fig. 3B). From the initial 32 variables, the model was created and based in 7 variables: b^*, L^*, YI, β-carotene, lutein, α-tocopherol and γ-tocopherol. Once more, chemometrics proved that extracts addition have an important effect in the EVOO's color descendent from pigments content in a way that is possible to correctly classify samples. The model showed an extremely satisfactory performance since it allowed classifying correctly all samples according to the olive oil they belong and to the correct concentration of *S. almeriensis* added. The same results were observed for the cross-validation procedure (sensitivities and specificities of 100%), showing that the classification of the original groups was not overestimated. In Fig. 3B is possible to observe that the discriminant functions separate EVOO's accordingly to the concentration of *S. almeriensis* extracts added, and among the same concentration is even possible to separate

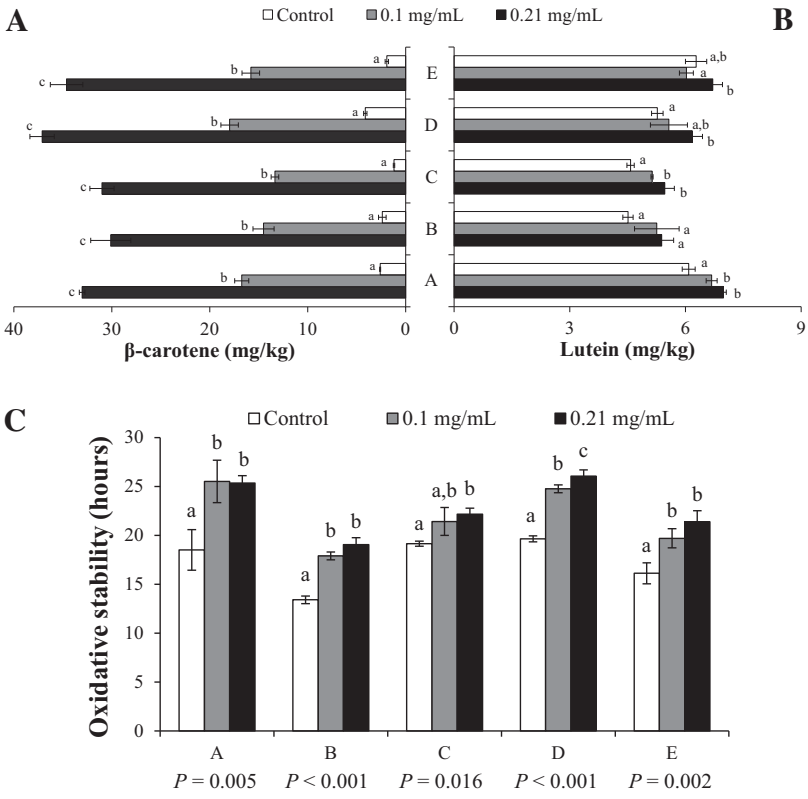

Fig. 2. β-Carotene (mg/kg; A), lutein (mg/kg; B) and oxidative stability (hours; C) of EVOO's without (I) and with *S. almeriensis* extract (II = 0.1 mg/mL; III = 0.21 mg/mL). In each olive oil mean values with different letters differ significantly ($P < 0.05$).

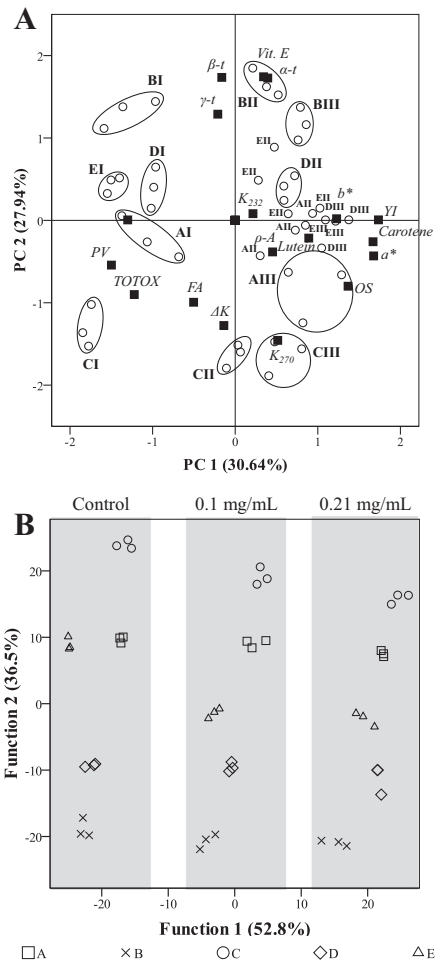

Fig. 3. Principal component analysis (PCA – A) and linear discriminant analysis (LDA – B) of EVOO's with (I) and without enrichment of *S. almeriensis* extract (II = 0.1 mg/mL; III = 0.21 mg/mL) (*p*-A – *p*-anisidine value; OS – oxidative stability). PCA factors and discriminant functions explain 58.58% and 89.3% of the total variance, respectively.

the different olive oils studied. Such data treatment helped recognizing the effects of the extracts addition in the EVOO's, and to observe in an integrated and resumed way the entire data.

3.8. Incorporation of S. almeriensis and olive oil consumption

From the entire data obtained in the studied EVOO's is clear that the main changes inflicted are related to carotenoids concentration of the extracts, namely β-carotene and lutein, as previously expected. Carotenoids are important components of several biological processes in the human organism, having repercussions in human health (Granado et al., 2003). Lutein and β-carotene, present in the extracts of *S. almeriensis* are two good examples. The amounts of carotenoids that are found in human tissues are almost exclusively from dietary origin, mainly from fruits and vegetables, or from supplements but its consumption is deficient in many countries. For instance, and according to the State Indicator Report on Fruits and Vegetables 2013 (CDC, 2013), in the U.S. 37.7% and 22.6% of the adult population consume fruits and vegetables, respectively, less than one time a day. When results report to adolescent population, 36.0% and 37.7% consume fruits and vegetables, respectively, less than one time daily. The fruits median intake in U.S. for adults and adolescents is 1.0 and 1.1 times a day respectively, while for vegetables values increase to a consumption of 1.6 and 1.3 times a day respectively (CDC, 2013). Clearly and according to Kimmons, Gillespie, Seymour, Serdula, and Blanck (2009), few American adolescents and adults consume the recommended amounts of fruits and vegetables. Although the figures for Europe are apparently not so disturbing, in several countries carotenoid intake from natural sources is still reduced (Tennant, Davidson, & Day, 2014). Olive oil production and consumption, in opposition, is steadily increasing worldwide over the last years (IOC, 2013a,b). Lutein and β-carotene are naturally present in olive oil, but in low doses (Malheiro et al., 2013a). With the incorporation of *S. almeriensis* extracts their content increase in olive oil, mainly β-carotene (Fig. 2A). Therefore, the incorporation of *S. almeriensis* extracts in olive oil is a good strategy to encourage the consumption of essential carotenoids to the human organism. From the nutritional point of view the consumption of olive oils enriched with *S. almeriensis* extracts would be a valuable asset to counterbalance the deficient consumption of fruits and vegetables, reducing, the lower ingestion of vital carotenoids. Furthermore, the carotenoids from *S. almeriensis* possess a much higher bioaccesibility for absorption in human organism than many fruits and vegetables or supplements (Granado-Lorencio et al., 2009). Still, more work must be performed before the commercialization of this type of products, especially focusing into the safety for the consumer. Despite being β-carotene and lutein GRAS additives, E160a and E160b, respectively, the identification of other extract components should be fully discriminated. Also, *S. almeriensis* extract preparation, in particular, should be optimized for increased productivity while accomplishing all food industry GMP of, particularly regarding the use of allowed materials and biocompatible solvents.

4. Conclusions

The addition of *S. almeriensis* brought significant changes in olive oils color, quality, composition, and stability. The extracts addition turns olive oils more yellow-orange due to pigments extracts concentration, mainly β-carotene. Oxidative stability is markedly improved by *S. almeriensis* extracts, improving olive oils shelf-life, protecting them from oxidative agents. Olive oils peroxidation is reduced as well as the formation of oxidation products, enhancing olive oils quality. Fatty acids profile is not affected by extracts addition, as well as tocopherols content. Overall, extracts addition is beneficial to olive oils being possible to discriminate the different olive oils according to the amount of extract added. From all the results obtained we concluded that the addition of 0.21 mg/mL does not contribute decisively to olive oils quality and composition comparatively to 0.1 mg/mL, therefore the concentration of 0.1 mg of *S. almeriensis* extracts per mL of olive oil would be an optimal concentration, conserving all properties and characteristics of original olive oils, while providing important doses of bioactive compounds and increased oxidative stability. The possible introduction of this kind of olive oil would increase the daily intake of essential carotenoids in the daily diet, enhancing possible health properties for consumers and conferring important nutritional assets. Nevertheless, future works need to be carried out to assess the stability of this kind of olive oils during storage and during culinary process as well. In this sense, we are going to study olive oil stability as a function of three main degradation variables: time, temperature and light exposure.

With this work we also concluded that *S. almeriensis* microalgae are good sources of bioactive compounds and could be exploited for the extraction of carotenoids for incorporation in food products or to be included in food supplements.

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References

Achir, N., Randrianatoandro, V. A., Bohuon, P., Laffargue, A., & Avallone, S. (2010). Kinetic study of β-carotene and lutein degradation in oils during heat treatment. *European Journal of Lipid Science and Technology, 112*, 349–361.

Acién, F. G., Fernández-Sevilla, J. M., Magán, J. J., & Molina-Grima, E. (2012). Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnology Advances, 30*, 1344–1353.

Aparicio, R., Roda, L., Albi, M. A., & Gutiérrez, F. (1999). Effect of various compounds on virgin olive oil stability measured by Rancimat. *Journal of Agricultural and Food Chemistry, 47*, 4150–4155.

Block, G., Patterson, B., & Subar, A. (1992). Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. *Nutrition and Cancer, 18*, 1–29.

Camacho, F. G., Rodríguez, J. G., Mirón, A. S., García, M. C. C., Belarbi, E. H., Chisti, Y., et al. (2007). Biotechnological significance of toxic marine dinoflagellates. *Biotechnology Advances, 25*, 176–194.

CDC (Centers for Disease Control and Prevention) (2013). "*State Indicator Report on Fruits and Vegetables 2013*" from the Division of Nutrition, Physical Activity, and Obesity of the National Center for Chronic Disease Prevention and Health Promotion.

Cerón, M. C., Campos, I., Sánchez, J. F., Acién, F. G., Molina, E., & Fernández-Sevilla, J. M. (2008). Recovery of lutein from microalgae biomass: Development of a process for *Scenedesmus almeriensis* biomass. *Journal of Agricultural and Food Chemistry, 56*, 11761–11766.

Cerretani, L., Bendini, A., Rodriguez-Estrada, M. T., Vittadini, E., & Chiavaro, E. (2009). Microwave heating of different commercial categories of olive oil: Part I. Effect on chemical oxidative stability indices and phenolic compounds. *Food Chemistry, 115*, 1381–1388.

Commission Implementing Regulation (EU), No. 299/2013: Amending Regulation (EEC) No. 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Official Journal of the European Union*.

Commission Regulation (1991). (EEC) No. 2568/91: On the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis. *Official Journal of European Union, L248*, 1–82.

Duyn, M. A. S. V., & Pivonka, E. (2000). Overview of the health benefits of fruit and vegetable consumption for the dietetics professional: Selected literature. *Journal of the American Dietetic Association, 100*, 1511–1521.

European Food Information Council (EUFIC) (2012). EUFIC Review. Fruit and vegetable consumption in Europe – do Europeans get enough? Available at: <http://www.eufic.org/article/en/expid/Fruit-vegetable-consumption-Europe/> Accessed at 23.02.14.

Fernández-Sevilla, J. M., Acién Fernández, F. G., & Molina Grima, E. (2010). Biotechnological production of lutein and its applications. *Applied Microbiology and Biotechnology, 86*, 27–40.

García-González, M., Moreno, J., Manzano, J. C., Florencio, F. J., & Guerrero, M. G. (2005). Production of *Dunaliella salina* biomass rich in 9-cis-β-carotene and lutein in a closed tubular photobioreactor. *Journal of Biotechnology, 115*, 81–90.

Gaziano, J. M., Manson, J. E., Branch, L. G., Colditz, G. A., Willett, W. C., & Buring, J. E. (1995). A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. *Annals of Epidemiology, 5*, 255–260.

Gómez-Caravaca, A. M., Cerretani, L., Bendini, A., Segura-Carretero, A., Fernández-Gutiérrez, A., & Lercker, G. (2007). Effect of filtration systems on the phenolic content in virgin olive oil by HPLC-DAD-MSD. *American Journal of Food Technology, 2*, 671–678.

Granado, F., Olmedilla, B., & Blanco, I. (2003). Nutritional and clinical relevance of lutein in human health. *British Journal of Nutrition, 90*, 487–502.

Granado-Lorencio, F., Herrero-Barbudo, C., Acién-Fernández, G., Molina-Grima, E., Fernández-Sevilla, J. M., Pérez-Sacristán, B., et al. (2009). *In vitro* bioaccesibility of lutein and zeaxanthin from the microalgae *Scenedesmus almeriensis*. *Food Chemistry, 114*, 747–752.

International Olive Council (IOC) (2013). World olive oil figures – production. Available at: <http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures> Accessed 11.02.14.

International Olive Council (IOC) (2013). World olive oil figures – consumption. Available at: <http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures> Accessed 11.02.2014.

ISO 6885 (2006). Animal and vegetable fats and oils – Determination of anisidine value.

ISO 9936 (2006). Animal and vegetable fats and oils – Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography.

Kimmons, J., Gillespie, C., Seymour, J., Serdula, M., & Blanck, H. M. (2009). Fruit and vegetable intake among adolescents and adults in the United States: Percentage meeting individualized recommendations. *The Medscape Journal of Medicine, 11*, 26.

Labrinea, E. P., Thomaidis, N. S., & Georgiou, C. A. (2001). Direct olive oil anisidine value determination by flow injection. *Analytica Chimica Acta, 448*, 201–206.

Laguerre, M., Lecomte, J., & Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. *Progress in Lipid Research, 46*, 244–282.

Malheiro, R., Casal, S., Lamas, H., Bento, A., & Pereira, J. A. (2012). Can tea extracts protect extra virgin olive oil from oxidation during microwave heating? *Food Research International, 48*, 148–154.

Malheiro, R., Casal, S., Ramalhosa, E., & Pereira, J. A. (2011). Microwave heating: A time saving technology or a way to induce vegetable oils oxidation? In S. Grundas (Ed.), *Advances in induction and microwave heating of mineral and organic materials* (pp. 597–614). Rijeka: Croatia.

Malheiro, R., Casal, S., Teixeira, H., Bento, A., & Pereira, J. A. (2013b). Effect of olive leaves addition during the extraction process of overmature fruits on olive oil quality. *Food and Bioprocess Technology, 6*, 509–521.

Malheiro, R., Rodrigues, N., Manzke, G., Bento, A., Pereira, J. A., & Casal, S. (2013a). The use of olive leaves and tea extracts as effective antioxidants against the oxidation of soybean oil under microwave heating. *Industrial Crops and Products, 44*, 37–43.

Morita, M., Watanabe, Y., & Saiki, H. (2002). Photosynthetic productivity of conical helical tubular photobioreactor incorporating *Chlorella sorokiniana* under field conditions. *Biotechnology and Bioengineering, 77*, 155–162.

Nishino, H. (1998). Cancer prevention by carotenoids. *Mutation research/fundamental and molecular mechanisms of mutagenesis, 402*, 159–163.

Omenn, G. S., Goodman, G. E., Thornquist, M. D., Balmes, J., Cullen, M. R., Glass, A., et al. (1996). Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *The New England Journal of Medicine, 334*, 1150–1155.

Organisation for Economic Co-operation and Development (OECD) (2013). Health at a glance 2013. OECD indicators.

Ozawa, Y., Sasaki, M., Takahashi, N., Kamoshita, M., Miyake, S., & Tsubota, K. (2012). Neuroprotective effects of lutein in the retina. *Current Pharmaceutical Design, 18*, 51–56.

Peterson, J. J., Dwyer, J. T., Jacques, P. F., & McCullough, M. L. (2012). Associations between flavonoids and cardiovascular disease incidence or mortality in European and US populations. *Nutrition Reviews, 70*, 491–508.

Pomerleau, J., Lock, K., Knai, C., & McKee, M. (2004). Effectiveness of interventions and programmes promoting fruit and vegetable intake. Background paper for the joint FAO/WHO workshop on fruit and vegetables for health. p. 133.

Sánchez, J. F., Fernández, J. M., Acién, F. G., Rueda, A., Pérez-Parra, J., & Molina, E. (2008a). Influence of culture conditions on the productivity and lutein content of the new strain *Scenedesmus almeriensis*. *Process Biochemistry, 43*, 398–405.

Sánchez, J. F., Fernández-Sevilla, J. M., Acién, F. G., Cerón, M. C., Pérez-Parra, J., & Molina-Grima, E. (2008b). Biomass and lutein productivity of *Scenedesmus almeriensis*: Influence of irradiance, dilution rate and temperature. *Applied Microbiology and Biotechnology, 79*, 719–729.

Seddon, J. M., Ajani, U. A., Sperduto, R. D., Hiller, R., Blair, N., Burton, T. C., et al. (1994). Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. *JAMA, 272*, 1413–1420.

Sies, H., & Stahl, W. (1995). Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *The American Journal of Clinical Nutrition, 62*, 1315S–1321S.

Teikari, J. M., Laatikainen, L., Virtamo, J., Haukka, J., Rautalahti, M., Liesto, K., et al. (1998). Six-year supplementation with alpha-tocopherol and beta-carotene and age-related maculopathy. *Acta Ophthalmologica Scandinavica, 76*, 224–229.

Tennant, D. R., Davidson, J., & Day, A. J. (2014). Phytonutrient intakes in relation to European fruit and vegetable consumption patterns observed in different food surveys. *British Journal of Nutrition, 112*, 1214–1225.

Veer, P., Jansen, M. C. J. F., Klerk, M., & Kok, F. J. (2000). Fruits and vegetables in the prevention of cancer and cardiovascular disease. *Public Health Nutrition, 3*, 103–107.

World Health Organization (2014). Global Startegy on Dier, Physical Activity and Health. Promoting fruit and vegetable consumption around the world. Available at: <http://www.who.int//dietphysicalactivity/fruit/en/> Accessed 23.02.2014.

Yen, H., Sun, C., & Ma, T. (2011). The comparison of lutein production by *Scenedesmus* sp. in the autotrophic and the mixotrophic cultivation. *Applied Biochemistry and Biotechnology, 164*, 353–361.

Zamora, R., Olmo, C., Navarro, J. L., & Hidalgo, F. J. (2004). Contribution of phospholipid pyrrolization of the color reversion produced during deodorization of poorly degummed vegetable oils. *Journal of Agricultural and Food Chemistry, 52*, 4166–4171.