GRASAS Y ACEITES 68 (1)
January–March 2017, e178
ISSN-L: 0017-3495
doi: http://dx.doi.org/10.3989/gya.0940162

Improving oxidative stability of olive oil: Incorporation of Spirulina and evaluation of its synergism with citric acid

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Submitted: 14 September 2016; Accepted: 13 December 2016

SUMMARY: The effects of different Spirulina concentrations used alone and in combination with citric acid on the oxidative stability of olive oil were assessed. The amounts of primary and secondary oxidation products produced in Spirulina samples were lower than that of the control. The improved oxidative stability indices of Spirulina samples with and without citric acid were in the range of 85.20–94.47% and 258.10–260.21%, respectively. In comparison with the control, Spirulina samples manifested significantly higher carotenoid and chlorophyll contents at the beginning and end of the storage period. The presence of these bioactive compounds results from the presence of Spirulina in the medium and can thus retard the oxidation of olive oil. A higher oxidative stability was reached using BHT in comparison with Spirulina samples. Furthermore, no synergistic action was observed in possible connections between citric acid and Spirulina. In conclusion, Spirulina can enhance oxidative stability and improve the shelf life of olive oil.

***KEYWORDS*:** *Arthrospira platensis; Citric acid; Natural antioxidant; Olive oil; Spirulina; Synergistic effect*

RESUMEN: ***Mejora de la estabilidad oxidativa del aceite de oliva: Incorporación de Espirulina y evaluación de su sinergismo con ácido cítrico*.** Se evaluaron los efectos de diferentes concentraciones de Espirulina usadas solas y en combinación con ácido cítrico sobre la estabilidad oxidativa del aceite de oliva. Las cantidades de productos de oxidación primarios y secundarios producidos en muestras de Espirulina fueron menores que las del control. Además, la estabilidad oxidativa de muestras de Espirulina con y sin ácido cítrico estaban en el intervalo de 85,20–94,47% y 258,10–260,21%, respectivamente. En comparación con el control, las muestras de Espirulina mostraron un contenido significativamente mayor de carotenoides y clorofila al inicio y al final del período de almacenamiento. La presencia de estos compuestos bioactivos y la presencia de Espirulina en el medio pueden retardar la oxidación del aceite de oliva. Se obtuvo una mayor estabilidad oxidativa usando BHT en comparación con muestras de Espirulina. Además, no se observó ninguna acción sinérgica en las posibles combinaciones entre el ácido cítrico y la Espirulina. En conclusión, la Espirulina puede mejorar la estabilidad oxidativa y la vida útil del aceite de oliva.

***PALABRAS CLAVE*:** *Aceite de oliva; Ácido cítrico; Antioxidante natural;* Arthrospira platensis*; Efecto sinérgico; Espirulina*

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Citation/Cómo citar este artículo: Alavi N, Golmakani MT. 2017. Improving oxidative stability of olive oil: Incorporation of Spirulina and evaluation of its synergism with citric acid. *Grasas Aceites* **68**, e178. http://dx.doi.org/10.3989/gya.0940162

1. INTRODUCTION

Olive oil is a major vegetable oil obtained from the mesocarp of the fruits of the olive tree (*Olea europaea*). Olive oil contains high contents of monounsaturated fatty acids (MUFAs) and the presence of several minor natural antioxidants, which commonly possess fragrant features and nutritional value. The event of oxidation occurring in olive oil can lead to changes in color and flavor, produce toxic compounds, and reduce nutritional value (Barjol, 2013).

The use of antioxidants from different natural sources has recently attracted considerable attention (Taghvaei and Jafari, 2013). The microalga Spirulina (*Arthrospira platensis*) belongs to the group of cyanobacteria and the family Oscillatoriaceae. Spirulina's annual production is about 3000 tons (Raheem *et al.,* 2015). Spirulina can synthesize large amounts of protein with a high quality profile of amino acids, lipids with fatty acids of the ω6 family such as gamma-linolenic acid, and carbohydrates. Furthermore, Spirulina contains vitamins and minerals, and is rich in pigments such as phycobiliproteins, chlorophylls, and carotenoids, and antioxidant enzymes such as superoxide dismutase and peroxidase (Spolaore *et al.,* 2006; Golmakani *et al.,* 2012a; Ismaiel *et al.,* 2014). Spirulina has been used in numerous experimental investigations for its chemical and biological properties. Spirulina or its constituents manifested antioxidant capacity by several mechanisms based on free radical scavenging and metal-chelating attributes (Santoyo *et al.,* 2006; Bermejo *et al.,* 2008). Accordingly, Spirulina can be used as a promising source of safe and natural antioxidants. Some efforts have been made to highlight the antioxidant activity of Spirulina in foodstuffs. Cervejeira Bolanho *et al.,* (2014) prepared cookies with Spirulina and discovered an increase in antioxidant capacity of cookies which included Spirulina.

Citric acid (CA) is widely used as a synergist with a mechanism attributed to a chelating metal (Pokorny, 2007). In this regard, the antioxidant activity was evaluated by focusing on the synergy between CA and tanshen (*Salvia miltiorrhiza* Bunge) extract in lard which ultimately proved the synergy hypothesis correct (Gordon and Weng, 1992). However, Luzia *et al.,* (1998) observed no synergistic effect between 5-caffeoylquinic acid and CA in soybean oil.

The present study aims to assess the effects of different concentrations of Spirulina on improving the oxidative stability of olive oil, individually or in combination with CA. Likewise, the antioxidant activity of Spirulina was compared with that of butylated hydroxy-toluene (BHT).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, citric acid, BHT, and *p*-anisidine reagent (4-Methoxyaniline) were purchased from the Sigma-Aldrich Company (St. Louis, MO). All other chemicals and solvents were of analytical or chromatography grade and were purchased from Merck Company (Darmstadt, Germany).

2.2. Spirulina

Spray-dried Spirulina was purchased from Swisse Wellness Pty Ltd. (Melbourne, Australia) and was stored in a vacuum-packed condition at 4 °C until the time of analysis. The protein, fat, carbohydrates, ash, and moisture contents of Spirulina were analyzed according to the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC, 1997).

2.2.1. Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared from Spirulina according to the method described by Golmakani *et al.,* (2012a). FAMEs were analyzed on a gas chromatography (GC) system (SP-3420A, Beijing, China), equipped with a flame ionization detector (FID), and fitted to a BPX70 fused silica capillary column (30 m long, 0.25 mm internal diameter, and 0.25 μm film thickness). The injector and detector temperatures were adjusted to 250 and 300 °C, respectively. The carrier gas was nitrogen. The injection volume was 1.0 μL while the injector was set in the split mode (at a ratio of 1:10). The initial oven temperature was held at 140 °C for 5 minutes. Then, it was increased up to 180 °C by the gradual increase of 20 °C per minute. The temperature was kept at 180 °C for 9 minutes. Finally, the temperature was gradually raised to 200 °C by increasing at a rate of 20 °C per minute, and was held at that temperature for 3 minutes. The identification of fatty acids was carried out by comparing their retention times with those of injected standards. The result was expressed as percentages of relative peak areas and was also reported according to the unsaturation degree of fatty acids as in the saturated fatty acid (SFA), MUFA, and polyunsaturated fatty acid (PUFA).

2.2.2. Antioxidant properties

Before commencing the antioxidant property experiments, a methanolic extract of Spirulina was prepared. The extraction method involved consecutive steps that were performed as follows: five grams

of Spirulina powder were mixed with 25 mL methanol and shaken vigorously for 2 minutes. Then, the mixture was centrifuged (SW14R, Froilabo, Lyon, France) at 5000 rpm for 5 minutes. The supernatant was filtered through Whatman No.1 filter paper. This extraction procedure was executed on the residue twice. In the final stage, all supernatants were combined, centrifuged, and filtered. The extract volume was ultimately increased to 100 mL by adding methanol. The prepared extract was kept at 4 °C until it was considered to be tested in due course.

The method by Şükran *et al.,* (1998) was employed to estimate the carotenoid and chlorophyll contents of Spirulina, and the results were expressed as mg per gram of Spirulina. The ability of the Spirulina extract to donate a hydrogen atom or electron was probed to be assessed, along with the capacity of its hydrophilic and lipophilic antioxidants. These criteria were measured by using the DPPH and cupric reducing antioxidant capacity (CUPRAC) assays, respectively. The DPPH radical scavenging activity of the Spirulina extract (0.01–1.00 mg/mL) was analyzed (Shalaby and Shanab, 2013). A positive control BHT at concentrations of 0.01–0.10 mg/mL was used for comparison of the activities. The results were given as sample concentrations providing 50% DPPH scavenging activity against the radicals present in the test medium (IC_{50} value). The CUPRAC of Spirulina extract (1 mg/mL) was determined according to the method described by Apak *et al.,* (2004). For preparation of a standard curve, 0.01–0.10 mg/mL ascorbic acid solutions were used. CUPRAC was expressed as mg ascorbic acid equivalent per gram of Spirulina.

2.3. Olive oil

Olive oil was provided by The Edible Oil Industries Group of the Etka Organization. It was stored in a dark bottle with no head space volume and was kept at 4 °C until analysis.

2.3.1. Analytical indices

Free acidity (Ca 5a-40), peroxide value (PV; Cd 8–53), *p*-anisidine value (AV; Cd 18–90), and specific extinction coefficients (ultraviolet spectrophotometric indices) at 232 nm (K_{232} value) and 268 nm (K_{268} value) (Ch 5–91) were evaluated according to the Official Methods of the American Oil Chemists' Society (AOCS, 1998). Totox value (TV) was calculated as 2PV+AV.

2.3.2. Fatty acid composition

Olive oil fatty acids were esterified into FAMEs according to the method described by Golmakani *et al.,* (2012b). All technical features and GC system conditions were similar to those described earlier for the identification of the fatty acid composition of Spirulina. Results are expressed as the percentage pertaining to relative peak areas for each identified fatty acid, and are also reported as SFA, MUFA, and PUFA.

2.4. Oxidative stability of olive oil incorporated with Spirulina

Spirulina was ground to a fine powder by using a mill grinder (MJW176P, Matsushita Electric Industrial Company, Osaka, Japan). Spirulina powder was added at concentrations of 0.5, 1.0, and 1.5% (w/w) to the olive oil. Then, Spirulina samples were sonicated with an ultrasound probe (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). The ultrasound probe conditions were set at 50 W for 10 minutes total working time (20 seconds ultrasound time and 10 seconds interval time) at 25 °C. For the purpose of comparison, BHT was added to olive oil at a concentration of 0.01% (w/w). Along with different antioxidants used alone in this study, the binary mixtures of the antioxidants and the CA (0.01%, w/w) were also prepared.

Samples were heated in an incubator (Memmert GmbH + Co. KG, Schwabach, Germany) set at 60±1 °C for 16 days. The samples were placed in the dark. Spirulina samples were initially filtered through Whatman No.1 filter paper and were then analyzed. The oxidative stability of samples was monitored every 4 days by determining the PV, AV, TV, K_{232} value, and K_{268} value. The carotenoid and chlorophyll contents of the samples were determined (Minguez-Mosquera *et al.,* 1991) at the beginning and at the end of the storage period, and were respectively expressed as mg lutein and pheophytin-a per kg of olive oil. Also, antioxidant indices of the samples were estimated and were reported as the induction period (IP), protection factor (PF), antioxidant activity (AA), improved oxidative stability (IOS), and synergism degree resulting from the combination of antioxidants. The IP is defined as the number of days necessary to reach the PV of 20 meq O_2/kg (Keramat and Golmakani, 2016). The IP was calculated by the extrapolation of PV curve. AA index is a function of antioxidant concentration (Antolovich *et al.,* 2002). Accordingly, AA values of those samples containing synergistic modes of action were estimated based on the concentrations of antioxidants involved and the concentration of the CA. PF, AA, IOS, and synergism were estimated based on the IPs according to the following equations:

$$PF = \left(\frac{IP_{sample}}{IP_{control}} \right) \qquad \text{eq. (1)}$$

$$AA = \left(\frac{\left(IP_{sample} - IP_{control} \right)}{[\text{sample concentration}](IP_{control})} \right) \quad \text{eq. (2)}$$

$$IOS\ (\%) = \left(\frac{\left(IP_{sample} - IP_{control} \right)}{IP_{control}} \times 100 \right) \quad \text{eq. (3)}$$

$$\text{Synergism}\ (\%) = \left[\frac{\left(IP_{\text{sample combinations}} - IP_{control} \right) - \left(IP_{sample} - IP_{control} \right) - \left(IP_{CA} - IP_{control} \right)}{\left(IP_{\text{sample combinations}} - IP_{control} \right)} \times 100 \right] \quad \text{eq. (4)}$$

Color parameters of $L^*a^*b^*$ were determined for the samples at the beginning and at the end of the storage period following the method described by Habibi *et al.,* (2015). In the $L^*a^*b^*$ coordinate system, the L^* value represents brightness varying from 0 (black) to 100 (white), while a^* value varies from −100 (greenness) to +100 (redness), and the b^* value varies from −100 (blueness) to +100 (yellowness). Moreover, the color difference $\Delta E = \left(\left[\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2} \right]^{\frac{1}{2}} \right)$ was also calculated in comparison with the control (with or without CA).

2.5. Statistical analysis

All experiments and analyses were performed in triplicate. The results were reported as mean values along with ± standard deviations. Statistical analyses were carried out using Statistical Analysis Software (SAS) version 9.1 (SAS Institute Inc., Cary, NC). All data were treated with the general linear model (GLM) procedure. Significant differences ($P < 0.05$) were determined among the mean values using the Duncan's multiple range tests.

3. RESULTS AND DISCUSSION

3.1. Spirulina

Spirulina consisted of protein (63.60%), carbohydrates (17.51%), fat (6.38%), ash (7.03%), and moisture (5.48%). Spirulina was characterized by high protein content and low moisture content.

3.1.1. Fatty acid composition

As shown in Table 1, palmitic acid (54.03%), γ-linolenic acid (21.03%), and linoleic acid (18.59%) were the major fatty acids identified in Spirulina. Golmakani *et al.,* (2012a) also evaluated the fatty acid compositions of Spirulina cultivated under different conditions. Similar to the findings herein, they reported that the major fatty acids present in Spirulina were palmitic acid (47.5–50.4%), γ-linolenic acid (23.6–25.4%), and linoleic acid (12.5–14.6%).

Table 1. Fatty acid composition of Spirulina and olive oil

	Relative peak area (%)	
Fatty acid	**Spirulina**	**Olive oil**
Myristic acid (C14:0)	1.68 ± 0.17*	ND**
Palmitic acid (C16:0)	54.03 ± 0.92	17.14 ± 0.05
Palmitoleic acid (C16:1 ω-7)	0.87 ± 0.74	ND
Stearic acid (C18:0)	ND	0.42 ± 0.16
Oleic acid (C18:1 ω-9)	3.81 ± 0.26	75.44 ± 3.77
Linoleic acid (C18:2 ω-6)	18.59 ± 0.06	5.71 ± 3.03
α-Linolenic acid (C18:3 ω-3)	ND	1.29 ± 0.53
γ-Linolenic acid (C18:3 ω-6)	21.03 ± 0.18	ND
Σ Saturated fatty acid (SFA)	55.71 ± 0.75	17.59 ± 0.16
Σ Monounsaturated acid (MUFA)	4.68 ± 0.99	74.90 ± 2.75
Σ Polyunsaturated fatty acid (PUFA)	39.61 ± 0.24	7.51 ± 2.60

* Mean ± SD (n=3).
** Not detected.

Only small amounts of PUFAs (0.02, 0.03, and 0.04%) can be liberated into the olive oil by adding 0.5, 1.0, and 1.5% Spirulina, respectively. Therefore, these minor amounts cannot be deemed the cause for significant alterations in the PUFA contents of the olive oil.

3.1.2. Antioxidant properties

The initial carotenoid and chlorophyll contents of Spirulina were found to be 0.51 and 11.98 mg/g, respectively. Also, Mendiola *et al.,* (2009) determined the composition of *Spirulina pacifica* and detected that it contained 1.00 mg/g carotenoids and 2.16 mg/g chlorophylls.

The IC_{50} value of the Spirulina extract was found to be 0.364 ± 0.003 mg/mL. Therefore, Spirulina was proven to have an apt role as a radical scavenger. BHT manifested an IC_{50} value of 0.0363 mg/mL. Therefore, BHT exhibited higher DPPH radical scavenging activity than Spirulina.

3.2. Olive oil

3.2.1. Analytical indices

The free acidity, PV, AV, TV, K_{232} value, and K_{268} value of olive oil were 1.76 ± 0.15 g oleic acid/100 g, 4.18 ± 0.21 meq O_2/kg, 3.77 ± 0.05 mg/kg, 12.13 ± 0.05, 1.25 ± 0.13, and 0.10 ± 0.00, respectively.

3.2.2. Fatty acid composition

According to Table 1, the major fatty acids were found to be oleic acid (75.44%), palmitic acid (17.14%), and linoleic acid (5.71%). The oil was rich in MUFA (74.90%).

3.3. Oxidative stability of olive oil incorporated with Spirulina

3.3.1. Primary, secondary, and total oxidation products

The samples began to oxidize during accelerated storage and the oxidation progress was analyzed by measuring primary, secondary, and total oxidation products. The PV of the control was increased until day 8 of storage (Figure 1a). The control had a maximum PV of 21.81 meq O_2/kg after 8 days of storage, which led to its increased oxidation by 45.21–57.63% more than other samples. Thereafter, the control underwent a decrease in the PV as the oxidation progressed and the value reached 18.97 meq O_2/kg. Simultaneously, the AV result of the control showed a rapid increase in the formation of secondary oxidation products from day 8 onwards of storage. Rapid oxidation can be a result of the instability of hydro-peroxides in the control, thereby generating further secondary oxidation products. The control yielded a significantly higher PV than other samples throughout the storage period. The PVs of samples other than the control exhibited an increasing trend during the storage period. The PVs of samples containing 0.5, 1.0, and 1.5% Spirulina at the end of the storage period were measured to be14.62, 14.57, and 14.00 meq O_2/kg, respectively, which is significantly lower than the value of the control after 8 days of storage (21.81 meq O_2/kg). The release of bioactive compounds from Spirulina can delay the oxidation of olive oil. Similarly, Farvin and Jacobsen (2015) evaluated the antioxidant activity of the extracts of seaweeds *Fucus serratus* and *Polysiphonia fucoides* in a fish oil-in-water emulsion. Their results showed that the PV of the emulsion containing the ethanolic extract of *Polysiphonia fucoides* was lower than that of the control throughout the storage period.

After 12 days of storage, the PV of the BHT sample (9.91 meq O_2/kg) was significantly lower than those of the Spirulina samples (13.52–13.83 meq O_2/kg). Thereafter, the BHT sample, however, showed a PV of 14.03 meq O_2/kg, similar to those of the Spirulina samples (14.00–14.62 meq O_2/kg). Siriwardhana *et al.,* (2004) compared the PVs of brown alga *Hizikia fusiformis* extracts and BHT in fish oil. They reported that both *Hizikia fusiformis* extracts and BHT had significantly lower PVs than the control; however, the highest concentration of *Hizikia fusiformis* manifested a slightly higher efficiency than BHT.

The PVs of samples containing CA were observed to increase along with a longer storage period (Figure 1d). The control presented the highest PV during storage. At the end of the storage period, the PVs of the Spirulina samples at concentrations of 0.5, 1.0, and 1.5% were significantly lower by 33.01, 32.69, and 35.11%, respectively, compared to the control. Different Spirulina concentrations showed fairly similar PV patterns of change in value during the storage period, suggesting that a further antioxidant potential failed to be achieved at higher concentrations of Spirulina. At the end of the storage period, there were no significant differences among the PVs of samples containing BHT and Spirulina. At this stage, the PVs of all samples containing CA were significantly lower than those of their corresponding samples without CA.

The AVs of samples increased during the storage period (Figure 1b). The AV of the control was significantly higher than those of other samples throughout the storage period and finally reached 7.61 mg/kg. At the end of storage period, the Spirulina samples (0.5, 1.0, and 1.5%) exhibited significantly lower AVs, reaching amounts of 7.21, 6.23, and 5.65 mg/kg, respectively. Consequently, the formation of secondary oxidation products can be retarded by adding Spirulina. In the case of the Spirulina samples, the AVs decreased when higher concentrations of Spirulina were applied. Also, at the end of the storage period, the lowest AVs were observed in samples containing 1.5% Spirulina (5.65 mg/kg) and BHT (5.58 mg/kg).

The AVs of the samples containing CA increased during the storage period (Figure 1e). At the end of the storage period, the control and the sample containing 0.5% Spirulina showed the highest AVs (6.88 and 7.04 mg/kg, respectively). At this stage, the AVs of the samples containing 1.0% and 1.5% Spirulina were 5.59 mg/kg, and were similar to the AV of the BHT sample (5.46 mg/kg). Although the AVs of the control and also the sample containing 1.0% Spirulina with CA were significantly lower than those of their corresponding samples without CA, there were no significant differences among the AVs of 0.5% Spirulina, 1.5% Spirulina, and the BHT samples with and without CA throughout the storage period.

The trends observed in the TV results were similar to those in the PV results. The TV of the control reached a maximum value of 50.80 after 8 days of the storage, which was 1.69–2.17 times higher than the TVs observed in the Spirulina samples (Figure 1c). Subsequently, the TV of the control decreased, measuring 45.55 at the end of the storage period. In comparison with the control, the TVs of the Spirulina samples (0.5, 1.0, and 1.5%) were significantly lower, measuring 36.44, 35.37, and 33.66, respectively, at the end of the storage period. Kindleysides *et al.,* (2012) evaluated the antioxidant activities of the extracts of two brown seaweeds (*Ecklonia radiata* and *Macrocystis pyrifera*) and two

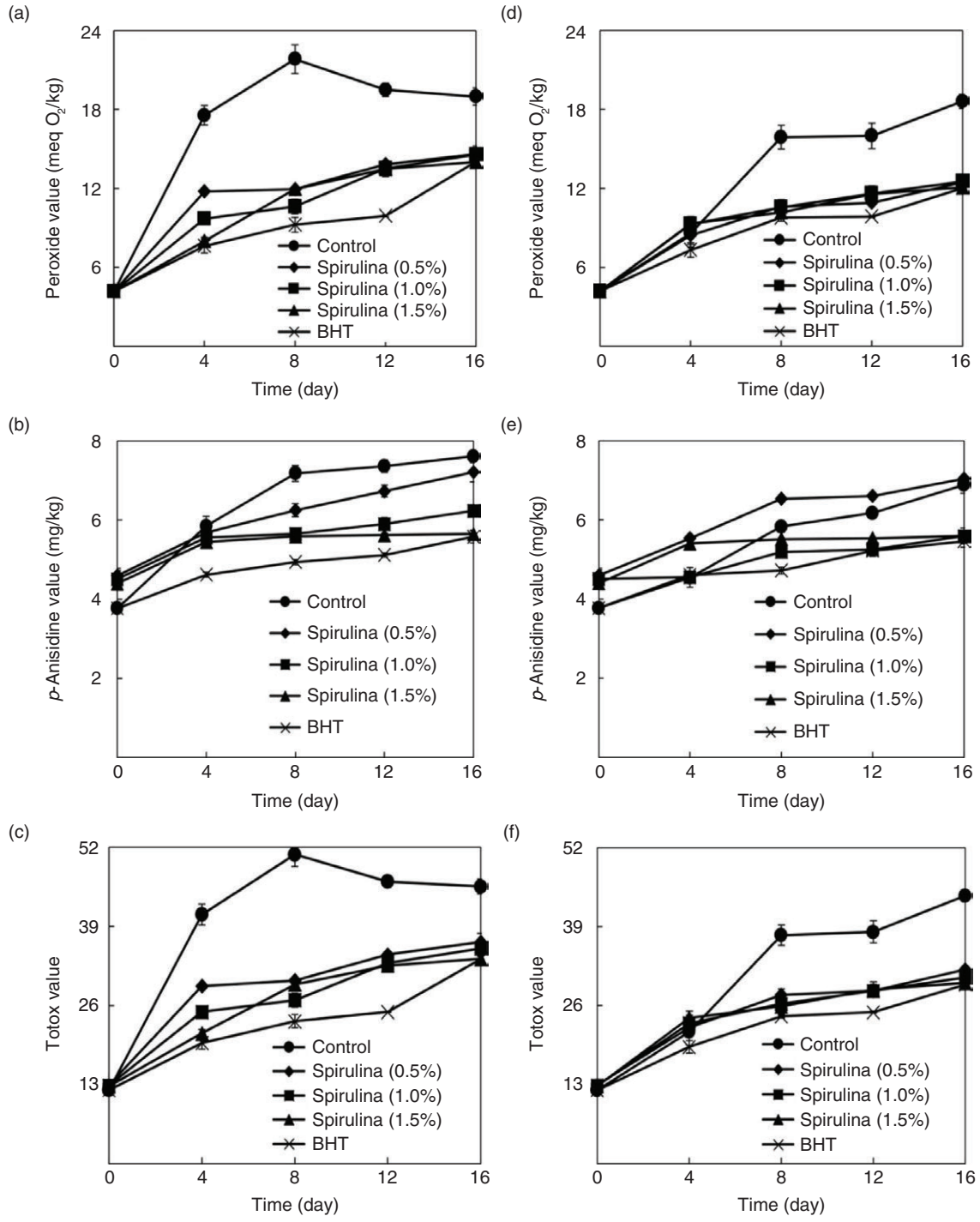

FIGURE 1. Effects of different concentrations of Spirulina on peroxide, para-anisidine, and Totox values of olive oil with (d–f) and without (a–c) citric acid.

red seaweeds (*Champia* sp. and *Porphyra* sp.) in hoki oil. Their results showed that all seaweed extracts exhibited lower TVs than the control.

At the end of the storage period, the TV of BHT sample measured 33.64, exhibiting a similar value compared with the condition where the concentrations of 1.0 and 1.5% Spirulina were applied.

The TVs of the samples containing CA increased as a result of longer storage periods (Figure 1f). The control exhibited the highest TV during storage. At the end of the storage period, the TVs of

the Spirulina samples (0.5, 1.0, and 1.5%) were significantly lower by 27.51, 30.55, and 32.57%, respectively, than the control. The TVs of the samples containing 1.0 and 1.5% Spirulina finally reached 30.62 and 29.73, respectively, and were similar to that of the BHT sample (29.49). The TVs of all the samples containing CA were significantly lower than those of their corresponding samples without CA at the end of the storage period.

3.3.2. Antioxidant indices

To compare the efficiencies of the various antioxidants used in this research, some indices were evaluated (Table 2). In comparison with the control, which exhibited an IP of 6.50 days, significantly higher IPs were observed when adding different concentrations of Spirulina to the olive oil. The inclusion of Spirulina in the olive oil increased the IP to a range of 23.28–23.46 days. This finding can be attributed to the activity of bioactive compounds which are most probably known to emanate from Spirulina. Chakraborty *et al.,* (2016) applied the rancimat method to evaluate the effects of combining the extracts from the seaweeds *Kappaphycus alvarezii*, *Hypnea musciformis*, and *Jania rubens* on improving the oxidative stability of concentrated FAMEs obtained from sardine oil. They found that the IP of the sample containing the seaweed combination (6.80 h) was significantly higher than that of the control (0.28 h).

According to Table 2, there were no significant differences among the IPs of the Spirulina samples. Therefore, adding Spirulina at the concentration of 0.5% can be considered the most economically sufficient concentration for extending the shelf life of olive oil. Applying lower but effective concentrations of alga can be valuable from an economic standpoint (Kindleysides *et al.,*2012). The BHT sample manifested the highest IP, which was 28.03 days.

The IPs of the Spirulina samples containing CA ranged from 29.91 to 31.41 days and were significantly higher than the IP of the control, which was 16.15 days. No significant differences, however, were observed among the Spirulina samples. Also, the BHT sample containing CA had a significantly higher IP than those of the Spirulina samples. The IPs of the control, 0.5%, 1.0%, and 1.5% Spirulina, and the BHT sample containing CA were significantly higher than those of their corresponding samples without CA.

Spirulina samples (with or without CA) showed significantly higher PFs in comparison with their corresponding control (Table 2). However, by increasing the Spirulina concentration from 0.5% to 1.5%, the PF values remained almost constant. The PF values of the BHT samples (with or without CA) were significantly higher than those of their corresponding Spirulina samples.

Unlike IP and PF, the AA index is dependent on the concentration of antioxidants (Antolovich *et al.,* 2002). Although there were no significant differences among the AAs of various concentrations of Spirulina samples (with or without CA), The AA of the BHT sample (with or without CA) was significantly higher than those of their corresponding Spirulina samples (Table 2).

Adding 0.5, 1.0, and 1.5% Spirulina increased the IOS, ranging from 258.10 to 260.21% (Table 2). There were no significant differences among the IOS values of Spirulina samples, suggesting that adding 0.5% Spirulina to the olive oil can be sufficient to

TABLE 2. Effects of different concentrations of Spirulina on antioxidant indices of olive oil

Sample	Induction period (IP; day)	Protection factor (PF)	Antioxidant activity (AA)	Improved oxidative stability (IOS; %)	Synergism (%)
Without citric acid					
Control	$6.50 \pm 0.22^{c*}$	1.00 ± 0.00^{c}	-	-	-
Spirulina (0.5%)	23.28 ± 1.03^{b}	3.58 ± 0.16^{b}	5.16 ± 0.32^{b}	258.10 ± 15.81^{b}	-
Spirulina (1.0%)	23.46 ± 1.28^{b}	3.61 ± 0.20^{b}	2.61 ± 0.20^{b}	260.21 ± 19.76^{b}	-
Spirulina (1.5%)	23.41 ± 0.31^{b}	3.60 ± 0.05^{b}	1.74 ± 0.03^{b}	260.21 ± 4.75^{b}	-
BHT	28.03 ± 0.82^{a}	4.31 ± 0.13^{a}	331.28 ± 12.67^{a}	331.28 ± 12.67^{a}	-
With citric acid					
Control	$16.15 \pm 0.97^{c*}$	1.00 ± 0.00^{c}	-	-	-
Spirulina (0.5%)	30.43 ± 0.19^{b}	1.88 ± 0.01^{b}	1.77 ± 0.03^{b}	88.44 ± 1.18^{b}	-10.41 ± 4.20^{a}
Spirulina (1.0%)	29.91 ± 1.34^{b}	1.85 ± 0.08^{b}	0.85 ± 0.08^{b}	85.20 ± 8.32^{b}	-13.83 ± 6.84^{a}
Spirulina (1.5%)	31.41 ± 0.93^{ab}	1.94 ± 0.06^{b}	0.63 ± 0.03^{b}	94.47 ± 5.71^{ab}	-6.78 ± 5.09^{a}
BHT	33.00 ± 0.86^{a}	2.04 ± 0.05^{a}	52.17 ± 2.66^{a}	102.85 ± 5.66^{a}	-17.80 ± 6.40^{a}

* Mean ± SD (n=3); In each column and for each part (i.e. with or without citric acid), means with different letters are significantly different ($P < 0.05$).

exert its optimum effect. The IOS value of the BHT sample measured 331.28%, and was significantly higher than those of the Spirulina samples.

The Spirulina samples containing CA recorded IOS values in the range of 85.20–94.47% (Table 2). The BHT sample containing CA (102.85%), however, showed a significantly higher IOS value than the Spirulina samples containing CA.

Synergistic effects were not detected between CA and Spirulina since synergism values were negative, ranging from −13.83 to −6.78% (Table 2). The Cooperative actions of synergism were not observed between BHT and CA either.

3.3.3. K_{232} and K_{268} values

Complementary oxidative stability indices such as K_{232} and K_{268} values represent the presence of conjugated diene and triene compounds, respectively (AOCS, 1998; Katsoyannos *et al.,* 2015). The K_{232} values of the samples were increased by prolonged storage periods (Figure 2a). The control staged the highest K_{232} value throughout the storage period. The K_{232} values of the Spirulina samples (0.5, 1.0, and 1.5%) were significantly lower (by 31.94, 36.46, and 29.86%, respectively) compared to the control at the end of the storage period. The observed efficiency acted upon the olive oil can be due to the progressive release of bioactive compounds from Spirulina into the olive oil.

The K_{232} values of the samples containing CA were increased by longer storage periods (Figure 2c). The control was recorded to have a significantly higher K_{232} value than those of other samples throughout the storage period. In comparison with the control, the K_{232} values of the Spirulina samples (at concentrations of 0.5, 1.0, and 1.5%) were significantly lower (by 18.43, 23.53, and 21.57%, respectively) at the end of the storage period.

The K_{268} values of all the samples increased as a result of longer storage periods (Figure 2b). After 8 days of storage, the Spirulina samples had significantly lower K_{268} values than the control. Thereafter, K_{268} values of the samples containing 1.0 and 1.5% Spirulina were similar to that of the control. The BHT sample recorded a K_{268} value similar to those of samples containing 1.0 and 1.5% Spirulina at the end of the storage period.

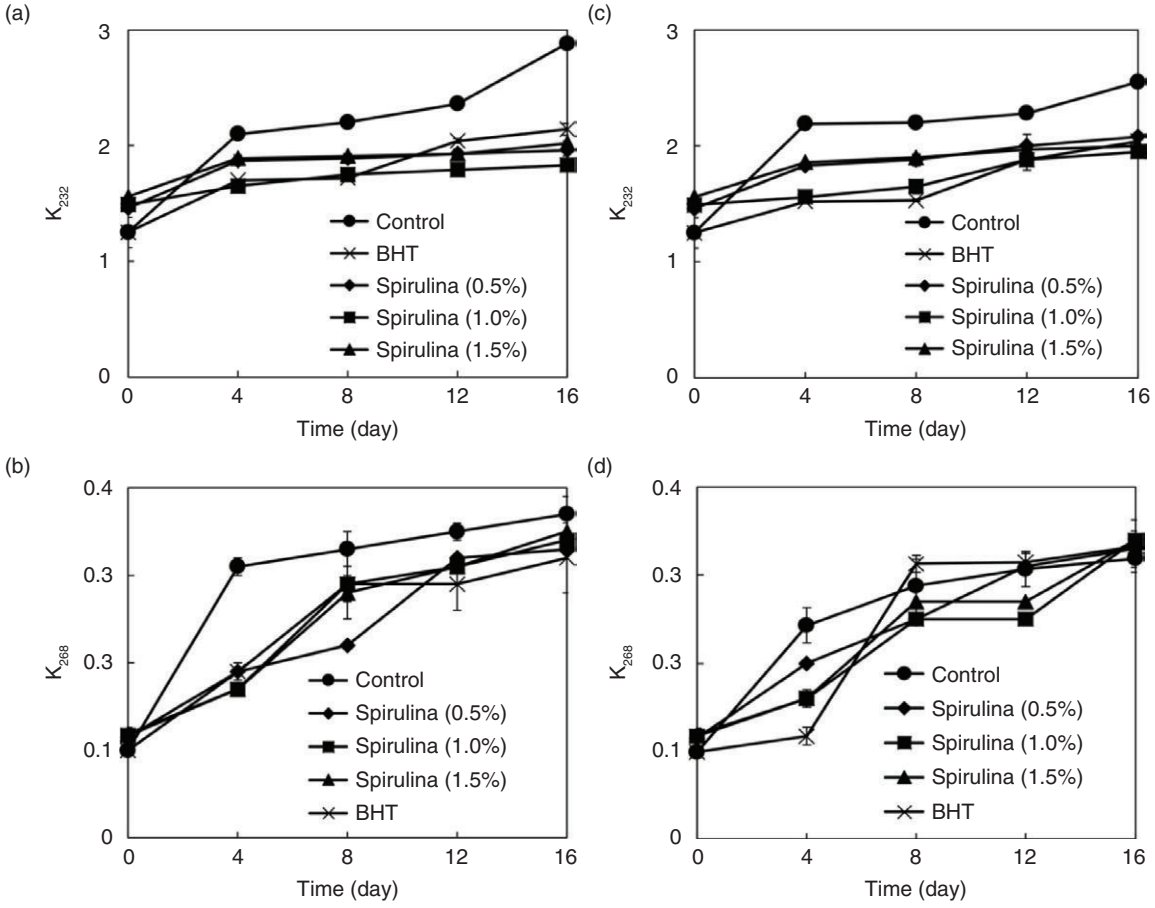

FIGURE 2. Effects of different concentrations of Spirulina on K_{232} and K_{268} values of olive oil with (c,d) and without (a,b) citric acid.

K_{268} values of the samples containing CA increased over time (Figure 2d). At the end of the storage period, the K_{268} values of the Spirulina samples and BHT were similar to that of the control.

3.3.4. Carotenoid and chlorophyll contents

The Antioxidant activity of Spirulina was generally attributed to its carotenoid and chlorophyll contents (Santoyo *et al.,* 2006). Carotenoids can prevent oxidation by scavenging free radicals (reactive oxygen species) or trapping the singlet oxygen. The carotenoid content of the control was 6.16 mg/kg and decreased by 16.17% during the storage period (Table 3). In comparison with the control, however, the Spirulina samples at concentrations of 0.5, 1.0, and 1.5% contained carotenoid contents that were respectively 1.12, 1.36, and 1.50 times higher than the control. This finding can be attributed to the release of Spirulina pigments into the olive oil. Although the carotenoid content of the control sample decreased at the end of the storage period, the carotenoid contents of the Spirulina samples slightly increased at the same time. This progressive trend of increase can be explained by the higher rate of carotenoid liberation compared to its decomposition.

The carotenoid contents of the samples containing 0.5, 1.0, and 1.5% Spirulina (with CA) were respectively 12.5, 31.98, and 51.46% higher than that of the control sample (Table 3). The carotenoid content of the control sample containing CA decreased at the end of the storage period, but the carotenoid contents of the Spirulina samples containing CA were observed to remain almost constant over time.

Chlorophyll is one of the most important molecules acting as an antioxidant agent in a dark environment (Criado *et al.,* 2008). In comparison with the control samples (with or without CA), the chlorophyll contents of the Spirulina samples were significantly higher, both at the beginning and at the end of the storage period (Table 3). The chlorophyll contents of the control samples (with or without CA) decreased at the end of the storage period, but the chlorophyll contents of the Spirulina samples increased.

3.3.5. Color attributes

In comparison with the control samples (with or without CA), the L^* values of the Spirulina samples were significantly lower, both at the beginning and at the end of the storage period (Table 4). The release of pigments from Spirulina caused a reduced brightness of the olive oil. Relevant to this context, Fradique *et al.,* (2010) measured the color attributes of pasta containing *Spirulina maxima* and found a decrease in its L^* value in comparison with the control group of pasta.

Spirulina samples (with or without CA) manifested lower initial a^* values than the control samples, which were accompanied by varied greenness intensities of the olive oil, ranging from a bright green color to an emerald green color (Table 4). At the end of the storage period, the a^* values of the Spirulina samples decreased due to the release of Spirulina's green pigments. The a^* value of the control sample increased at the end of the storage period, which can be due to the degradation of chlorophyll molecules.

TABLE 3. Effects of different concentrations of Spirulina on carotenoid and chlorophyll contents of olive oil

	Carotenoid			Chlorophyll		
Sample	**Initial content (mg/kg)**	**Final content (mg/kg)**	**Relative change (%)**	**Initial content (mg/kg)**	**Final content (mg/kg)**	**Relative change (%)**
Without citric acid						
Control	$6.16 \pm 0.04^{d*}$	5.16 ± 0.06^{e}	-16.17 ± 1.52^{c}	15.49 ± 0.22^{d}	10.04 ± 0.19^{e}	-32.78 ± 0.72^{e}
Spirulina (0.5%)	6.87 ± 0.40^{c}	6.91 ± 0.22^{c}	$+0.69 \pm 2.85^{a}$	19.02 ± 0.35^{c}	25.52 ± 0.09^{c}	$+34.22 \pm 2.50^{c}$
Spirulina (1.0%)	8.36 ± 0.15^{b}	8.54 ± 0.09^{b}	$+2.26 \pm 2.55^{a}$	20.03 ± 0.24^{b}	39.11 ± 0.20^{b}	$+90.96 \pm 1.59^{a}$
Spirulina (1.5%)	9.25 ± 0.14^{a}	9.46 ± 0.07^{a}	$+2.31 \pm 1.52^{a}$	24.46 ± 0.09^{a}	44.14 ± 0.14^{a}	$+81.15 \pm 0.77^{b}$
BHT	6.16 ± 0.04^{d}	5.46 ± 0.04^{d}	-11.36 ± 1.06^{b}	15.49 ± 0.22^{d}	12.26 ± 0.77^{d}	-20.86 ± 4.12^{d}
With citric acid						
Control	$6.16 \pm 0.04^{d*}$	5.30 ± 0.03^{d}	-13.90 ± 1.09^{b}	15.49 ± 0.22^{d}	10.34 ± 0.19^{e}	-33.25 ± 1.72^{e}
Spirulina (0.5%)	6.93 ± 0.03^{c}	7.02 ± 0.11^{c}	$+1.44 \pm 1.32^{a}$	18.44 ± 0.35^{c}	25.04 ± 0.12^{c}	$+35.88 \pm 2.62^{c}$
Spirulina (1.0%)	8.13 ± 0.12^{b}	8.33 ± 0.06^{b}	$+2.46 \pm 0.75^{a}$	19.93 ± 0.12^{b}	37.89 ± 0.06^{b}	$+90.06 \pm 0.99^{a}$
Spirulina (1.5%)	9.33 ± 0.14^{a}	9.59 ± 0.05^{a}	$+2.85 \pm 0.48^{a}$	24.27 ± 0.05^{a}	43.96 ± 0.10^{a}	$+81.13 \pm 0.77^{b}$
BHT	6.16 ± 0.04^{d}	5.48 ± 0.04^{d}	-11.04 ± 0.39^{b}	15.49 ± 0.22^{d}	10.98 ± 0.08^{d}	-29.08 ± 1.47^{d}

* Mean ± SD (n=3); In each column and for each part (i.e. with or without citric acid), means with different letters are significantly different ($P < 0.05$).

TABLE 4. Effects of different concentrations of Spirulina on color attributes of olive oil

Sample	L*		a*		b*		ΔE**	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Without citric acid								
Control	57.67 ± 0.82^{a***}	55.00 ± 0.71^{b}	−5.50 ± 0.55^{a}	−4.40 ± 0.55^{a}	56.83 ± 0.98^{a}	51.40±0.55^{b}	-	-
Spirulina (0.5%)	51.78 ± 0.41^{b}	50.50 ± 0.58^{c}	−6.83 ± 0.41^{b}	−7.17 ± 0.41^{b}	50.80 ± 0.45^{b}	50.25 ± 0.50^{c}	8.59 ± 0.09^{c}	5.31 ± 0.69^{d}
Spirulina (1.0%)	50.17 ± 0.41^{c}	49.00 ± 0.00^{d}	−6.83 ± 0.41^{b}	−8.75 ± 0.50^{c}	50.25 ± 0.50^{b}	49.00 ± 0.00^{d}	9.67 ± 0.98^{b}	7.53 ± 0.25^{b}
Spirulina (1.5%)	48.60 ± 0.54^{d}	47.83 ± 0.75^{e}	−7.50 ± 0.58^{b}	−9.50 ± 0.58^{d}	50.20 ± 0.84^{b}	44.25 ± 0.50^{e}	11.15 ± 0.29^{a}	10.84 ± 0.31^{a}
BHT	57.60 ± 0.55^{a}	56.00 ± 0.00^{a}	−5.50 ± 0.57^{a}	−5.00 ± 0.00^{a}	56.50 ± 0.58^{a}	56.75 ± 0.50^{a}	0.35 ± 0.71^{d}	6.31 ± 0.53^{c}
With citric acid								
Control	58.00 ± 0.00^{a1}	57.50 ± 0.58^{a}	−5.50 ± 0.58^{a}	−4.75 ± 0.50^{a}	56.50 ± 0.58^{a}	54.50 ± 0.58^{b}	-	-
Spirulina (0.5%)	50.75 ± 0.50^{b}	49.50 ± 0.58^{b}	−6.75 ± 0.50^{b}	−7.50 ± 0.58^{b}	50.75 ± 0.50^{b}	50.00 ± 0.00^{c}	9.38 ± 0.51^{c}	9.60 ± 0.36^{c}
Spirulina (1.0%)	50.00 ± 0.00^{c}	49.50 ± 0.58^{b}	−6.80 ± 0.45^{b}	−8.25 ± 0.50^{c}	50.00 ± 0.00^{c}	49.25 ± 0.50^{d}	10.27 ± 0.29^{b}	10.28 ± 0.21^{b}
Spirulina (1.5%)	46.75 ± 0.50^{d}	44.75 ± 0.50^{e}	−7.50 ± 0.58^{b}	−9.00 ± 0.00^{d}	49.00 ± 0.00^{d}	44.75 ± 0.50^{e}	13.67 ± 0.65^{a}	16.42 ± 0.75^{a}
BHT	57.80 ± 0.45^{a}	57.00±0.00^{a}	−5.75 ± 0.50^{a}	−5.00 ± 0.00^{a}	56.50 ± 0.58^{a}	56.50 ± 0.58^{a}	0.35 ± 0.71^{d}	2.18 ± 0.12^{d}

** In comparison with its corresponding control as reference.
*** Mean ± SD (n=3); In each column and for each part (i.e. with or without citric acid), means with different letters are significantly different ($P < 0.05$).

According to Table 4, the initial b* values of the Spirulina samples were significantly lower than those of the control samples. The release of pigments during the the storage period led to the reduction of the final b* values in the Spirulina samples.

The ΔE values of the Spirulina samples increased when higher concentrations of Spirulina were applied (Table 4). The ΔE values of the Spirulina samples were significantly higher than those of the BHT samples.

4. CONCLUSIONS

In this study, the effects of different Spirulina concentrations, either used alone or in combination with CA on the oxidative stability of olive oil were assessed. Lower amounts of primary, secondary, and total oxidation products were obtained in samples with different concentrations of Spirulina. No synergistic effects were observed between Spirulina samples and CA. Spirulina can be regarded as an appropriate source of bioactive compounds aimed at preventing oxidation, maintaining nutritional compounds, and enhancing the shelf life of olive oil.

ACKNOWLEDGEMENTS

This research project was financially supported by Shiraz University. We would like to thank the Edible Oil Industries Group of The Etka Organization for providing olive oil. We also thank the Iranian editor Mohsen Hamedpour-Darabi for editing the English language of the paper.

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