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Novel Approach to Study Oxidative Stability of Extra Virgin Olive Oils: Importance of α-Tocopherol Concentration

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The stability of extra virgin olive oils is mainly due to their relatively low fatty acids unsaturation and to the antioxidant activity of some of the unsaponifiable components. We studied the activity of α-tocopherol in extra virgin olive oil in its natural state, using new and simple oxidizing conditions in "thin layer" and "bulk phase". Oxidation time course was monitored at 37 °C or 75 °C. A storage test was also performed. Two parameters were evaluated: depletion of α-tocopherol and formation of PUFA hydroperoxides, measured as conjugated dienes (CD) and peroxide value. The value of complex polyphenols was also measured. α-Tocopherol concentration decreased in function of time and temperature and showed a strong inverse correlation with the increase of CD. When α-tocopherol reached a "threshold value" of 60−70 mg/kg, a significant increase of CD formation was observed, together with a good correlation between CD and peroxide value. The initial amount of α-tocopherol is one of the components that influences oil oxidative susceptibility.

KEYWORDS: α-Tocopherol; conjugated dienes; olive oil; oxidation; polyphenols

INTRODUCTION

Olive oil is the major edible vegetable oil of the Mediterranean countries. In recent years, in all of the industrialized countries the olive-oil-rich Mediterranean-style diet has been highly promoted because of the growing body of evidence of its beneficial health effects (*1*). In this context, olive oil is becoming a widespread food product of great value.

The quality of olive oil is determined by flavor criteria, as stated by the International Olive Oil Council (IOOC), and by different physical and chemical parameters, as indicated by the European Community Codex (EC-91 and following updates) (*2*). The top grades are the extra virgin olive oils that come out from the first pressing and contain most of the lipophilic constituents of the olive in their natural, unaltered state, and so have a high nutritional quality (*1*).

Although virgin olive oils are known to be more resistant to oxidation than other edible oils (*3*), the autoxidation process that may occur during extraction and following storage can compromise their nutritional qualities. It has been reported that the consumption of products from oxidized fats seems to be involved in several pathological conditions (*4*). Thus, there is considerable interest in the evaluation of the oxidation state of the oils. Although established criteria, such as peroxide value, are important in assessing olive oil oxidative stability (*5*), to predict its shelf life and so its quality, it is necessary to take into consideration the action of the several antioxidant and prooxidant factors that determine this state.

The stability of virgin oils is mainly due to their relatively low fatty acids unsaturation and to the antioxidant activity of some of the unsaponifiable components (*3*). Among the latter, as widely reported in the literature (*6*), the main antioxidant activity is exerted by the large fraction of the so-called hydrophilic phenolic compounds (*7*, *8*). However, such an activity seems to be exerted mainly at the initial stage of oil autoxidation (*9*). The amount of this fraction, that is 200−500 mg/kg on average, shows a great variability (from 50 to 1000 mg/kg) (*10*), depending on various factors such as cultivar, climate and environmental factors, ripeness, processing, and following storage of the oil (*11*). α-Tocopherol, which comprises about 90% of the total tocopherol fraction, with an average concentration of 100−300 mg/kg, still quite variable (*10*), seems to become effective when the activity of the polar phenolic fraction is reduced and primary products of autoxidation reach a critical concentration (*9*). A slight prooxidant activity of α-tocopherol at the early stages of olive oil autoxidation, depending on its concentration, has also been reported (*3*, *9*); moreover, α-tocopherol seems to have a synergistic effect in association with some phenolic compounds with significant antioxidant activity, acting, in a model system, as a scavenger

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of peroxyl radicals (*7*, *12*). Nevertheless, the literature related to its role on olive oil stability is limited.

The objective of this work was to investigate the contribution of α-tocopherol to olive oil stability in new and simple oxidizing conditions.

MATERIALS AND METHODS

Chemicals. All solvents used were HPLC grade (Merck, Darmstadt, Germany). Fatty acids and tyrosol (THY) standards were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol was purchased from Fluka AG (Buchs, Switzerland), and desferal (deferroxamine methanesulfonate) was obtained from CIBA-Geigy (Basel, Switzerland). Carbaryl, 99.8% purity, was purchased from Ehrenstorfer (Augsburg, Germany). All the other reagents and chemicals purchased (Carlo Erba, Milan, Italy) were of the highest available purity.

Pure samples (>90%) of phenols, ester of hydroxyphenilethanol with the dialdheydic form of elenolic acid (HPEA-EDA), and ester of 3,4-dihydroxyphenilethanol with the dialdheydic form of elenolic acid (3,4-DHPEA-EDA), were isolated from an oil extract (500 mg) by preparative HPLC on Kromasil columns (250 × 10 mm i.d., 10 μm, Chrompack, Bohus, Sweden) and under the elution conditions reported by Montedoro et al. (*13*). Analytical standards of oleuropeine aglycon and hydroxytyrosol (HTHY) were obtained as described elsewhere (*14*). Demethyloleuropeine was identified as described by Esti et al. (*15*).

Storage Test. For the storage test, samples of extra virgin olive oil were stored in the dark in fully loaded screw-capped glass bottles (50 mL) at room conditions (20−25 °C) for seven months; the ongoing oxidation process was monitored by measuring monthly the concentrations of α-tocopherol and complex polyphenols, the peroxide value, and the level of conjugated dienes (CD). The peroxide value of all samples was measured according to the CEE official method.

Oxidation Trials. Oxidation trials were conducted on five extra virgin olive oils from several cultivars of Sardinian olives, kindly donated by the Consorzio Interprovinciale per la Frutticoltura di Cagliari, Oristano e Nuoro. Freshly obtained samples, from organic cultures and pesticide-free, were analyzed to measure the initial composition of unsaturated fatty acids, α-tocopherol, and complex polyphenols. Samples of extra virgin olive oils in their natural state were oxidized in "bulk phase" and in "thin layer", an experimental model already used in our laboratory to test antioxidant activity of lipophilic compounds during pure fatty acid autoxidation (*16*). The oxidation time course was monitored at 37 °C or 75 °C, and the formation of unsaturated fatty acids hydroperoxides was evaluated by the standard method of the peroxide value and by the quali-quantitative analysis of conjugated dienes (CD), never before used in vegetable oils.

Aliquots of neat extra virgin olive oil were oxidized at 37 °C or at 75 °C in a water bath for different lengths of time, depending on the initial amount of the oil to be oxidized. Oxidation experiments in thin layer were carried out as follows: 3 mL of oil solution (1 mg/mL $CHCl_3$) was dried down in a glass test tube under vacuum, and the samples were incubated at 37 °C for 24, 48, 72, and 120 h and at 75 °C for 4, 8, 16, and 24 h. To perform the oxidation experiments in bulk phase, 1.5 g of neat oil was incubated in a glass test tube at 75 °C for 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, and 144 h. Control samples were kept at 0 °C. Samples were then cooled on ice. Aliquots of neat oil were used to extract the phenolic fraction or redissolved in ethanol to proceed to the preparation of free fatty acids, α-tocopherol, and the second derivative spectrophotometric analyses.

Preparation of Fatty Acids. Preparation of free fatty acids from olive oil samples was obtained by a mild saponification as previously described (*17*). Briefly, aliquots (3 mg) of oil were dissolved in 5 mL of ethanol, and then 100 μL of desferal (25 mg/mL H_2O), 1 mL of a 25% solution of ascorbic acid, and 0.5 mL of 10 N KOH were added. The solutions were left in the dark at room temperature for 14 h. After addition of 10 mL of *n*-hexane and 7 mL of H_2O, samples were acidified with 0.35 mL of 37% HCl, to pH 3−4, and were centrifuged for 1 h at 900*g*. The hexane phase containing free fatty acid was collected, the solvent was evaporated, and the residue was dissolved in 0.5 mL of CH_3CN/CH_3CO_2H (100/0.14, v/v). Aliquots of the latter were injected into the HPLC system.

Extraction of α-Tocopherol. α-Tocopherol was extracted from olive oil samples by a warm saponification method (*18*), modified as follows: aliquots (1 mg) of oil were dissolved in 1.5 mL of ethanol, and 2 mL of a 25% solution of ascorbic acid, and 1 mL of 10 N KOH, were added. The solutions were incubated in a water bath at 55 °C for 30 min in the dark. After cooling on ice, 4 mL of *n*-hexane with BHT (5 mg/L), was added. Samples were then centrifuged for 20 min at 900*g*. The hexane phase containing α-tocopherol was collected, the solvent was evaporated, and the residue was dissolved in 1 mL of methanol. Aliquots of the latter were injected into the HPLC system.

Preparation of the Phenolic Fraction. The extraction procedure of phenolic compounds was performed on oil samples as described elsewhere (*19*).

Second Derivative Spectrophotometric Analyses. A 1-mL aliquot of each oil sample in ethanol solution (1 mg/mL) was dried down and redissolved in cyclohexane, and its simple and second derivative UV absorption spectra between 195 and 350 nm were taken using a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer. The height of the two signals with a minimum at around 233 and 242 nm were measured and added together. The concentration of CD in the samples was determined by using a standard reference curve as previously described (*20*).

All solvent evaporations were performed under vacuum.

HPLC Analyses. *Unsaturated fatty acids.* Separation of unsaturated fatty acids was carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector 1040M. A C-18 Alltech (Deerfield, IL) Adsorbosphere column, 5-μm particle size, 250 × 4.6 mm, was used for unsaturated fatty acids with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70:30:0.12, v/v/v) at a flow rate of 1.5 mL/min. Unsaturated fatty acids were detected at 200 nm. To confirm the identification of the peaks, spectra (195−315 nm) of the eluate were obtained every 1.28 s and were electronically stored, using the Phoenix 3D HP Chemstation software.

α-Tocopherol. Amounts of α-tocopherol were measured by electrochemical detection, which allowed us to detect even tiny amounts of the compound, using a Thermo Separation Products P1000 pump (Milan, Italy) equipped with an electrochemical detector INTRO-Antec Leyden (Leyden, The Netherlands). An automatic injector Triathlon-Spark Holland BV (AJ Emmen, The Netherlands) was used. A C-18 Hewlett-Packard ODS Hypersil column, 5 μm particle size, 100 × 2.1 mm, was used with a mobile phase of methanol/CH_3COONa 0.05 M pH 5.5 (95:5, v/v) at a flow rate of 0.3 mL/min. The electrochemical detector was set at an oxidizing potential of 0.6 V. Data were collected and analyzed using a SC Integrator 1.00.03N Sunicom Oy software (Helsinki, Finland). Quantification of α-tocopherol was performed using a standard reference curve.

Phenolic Fraction. Separation of phenolic compounds was performed using a chromatographic Varian (Palo Alto, CA) 5020 pump equipped with a UV−Vis 100 variable-wavelength detector. An automatic injector (Hewlett-Packard 1050) and a reporting integrator (Hewlett-Packard 3396) were employed. The detector output was also connected to a Perkin-Elmer (Norwalk, CT) LC-235 diode array detector whose signals and UV spectra were processed by an LC-100 computer integrator. An ODS-2 analytical column (Spherisorb, 250 × 4.6 mm i.d., 3 μm, Waddinxveen, The Netherlands) with a 1-cm guard cartridge (C-18, 10 μm) was employed. The column was eluted at a flow rate of 1 mL/min for a total running time of 40 min. The mobile phase was a mixture of A (10^{-3} M H_2SO_4) and B (CH_3CN). The elution profile was: from $t = 0$ to $t = 30$ min, A/B (85:15, v/v); from $t = 30.1$ to $t = 35$ min, A/B (34:66, v/v); from $t = 35.1$ to $t = 40$ min, A/B (8:15, v/v). The detection was performed at 225 nm. The peaks of THY, HTHY, and of the complex polyphenols (CP): HPEA-EDA, 3,4-DHPEA-EDA, oleuropeine aglycon, and demethyloleuropeine, were identified in the chromatograms by comparison of their relative retention times (with respect to Carbaryl as internal standard) with those of pure samples obtained as reported above (*19*).

Statistical Analyses. Data from the oxidation trials are presented as means ± standard deviation of triplicate values obtained in three independent experiments ($n = 9$) from some extra virgin olive oils.

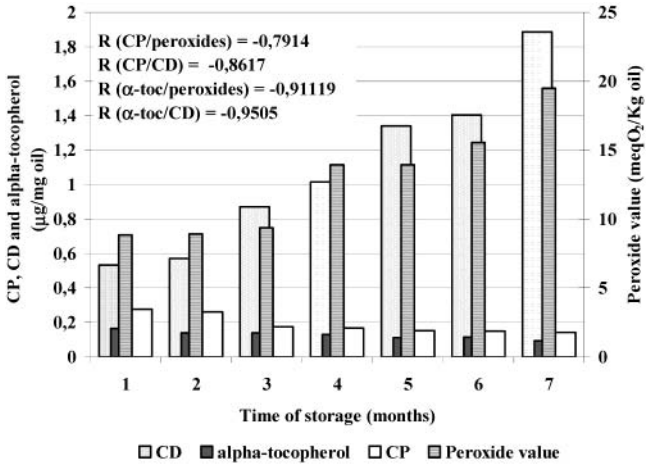

Figure 1. Variation of the concentrations of α-tocopherol, complex polyphenols (CP), peroxide value, and conjugated dienes (CD) in samples from extra virgin olive oil A during seven months of storage at room conditions. The SD values were not more than 10%. The correlation coefficient $R = \text{Cov}(X,Y)/\sigma_x \times \sigma_y$.

Statistical significance within sets of data was determined by one-way analysis of variance with the ANOVA program. It uses the Bonferroni method: the threshold for significance is the traditional value ($p < 0.05$) divided by the number of comparison. Data are reported as changes of concentration of the considered parameters with time. The correlation coefficient R, where R is usually defined as $\text{Cov}(X,Y)/\sigma_x \cdot \sigma_y$, and

$$Cov(X, Y) = \frac{1}{n}\sum_{i-1}^{n}(x_i - \mu_x)(y_i - \mu_y)$$

was calculated between sets of data and is reported in each figure.

RESULTS

Oxidation trials were performed on five extra virgin olive oils (A−E), from several cultivars of Sardinian olives, with different initial amounts of α-tocopherol and polyphenols.

In **Figure 1** are shown the data obtained from extra virgin olive oil A, with an initial concentration of α-tocopherol and CP of 165.04 ng/mg oil and 275.5 ng/mg oil respectively, during seven months of storage. Each month the content of α-tocopherol, CP, and peroxide value were evaluated. As a parameter of the ongoing oxidation process, the level of CD was also measured. As expected, during time there was a decrease of CP and α-tocopherol concentration and an increase of peroxide value and CD.

An inverse correlation was found between CP decrease and peroxide value ($R = -0.7914$) or CD ($R = -0.8617$) increase. A more evident correlation was found between α-tocopherol decrease and peroxide value ($R = -0.9119$) or CD ($R = -0.9505$) increase. A good correlation between peroxide value and CD content in the oil was also observed; in this case the correlation coefficient R is the linear regression coefficient obtained by linear fitting of the two variables, as reported in **Figure 2**. Under these experimental conditions, the value of 20 meq O_2/kg for the peroxide value corresponds to about 1.8 μg/mg oil of CD.

To better investigate the correlations between α-tocopherol or CP and CD, we performed some trials by oxidizing samples from oils B and C in bulk phase, using 1.5 g of oil and a high temperature (75 °C) to accelerate the oxidation process, as shown in **Figures 3** and **4**. The initial amounts of CP and α-tocopherol were 57.5 ng/mg and 159.36 ng/mg in oil B, and

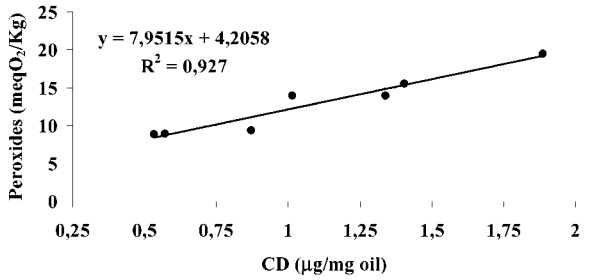

Figure 2. Correlation between the peroxide value and the amount of conjugated dienes (CD) measured in samples from extra virgin olive oil A during seven months of storage. R is the linear regression coefficient obtained by linear fitting of the two variables.

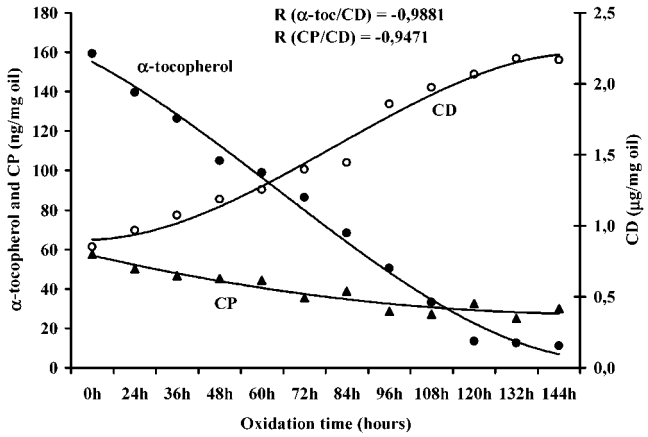

Figure 3. Variation of the concentrations of α-tocopherol, conjugated dienes (CD), and complex polyphenols (CP) in samples of 1.5 g of extra virgin olive oil B oxidized at 75 °C. The SD values were not more than 5%. The correlation coefficient $R = \text{Cov}(X,Y)/\sigma_x \times \sigma_y$.

102.6 ng/mg and 149.63 ng/mg in oil C. The ongoing oxidation was monitored by measuring CD values. The concentration of CP (HPEA-EDA, 3,4-DHPEA-EDA, oleuropeine aglycon, and demethyloleuropeine) decreased with time until it reached a plateau. In this experimental condition the decrease of CP showed a good inverse correlation to the CD increase in the case of the extra virgin olive oil B ($R = -0.9471$) (**Figure 3**). The same correlation was lower ($R = -0.7617$) employing the concentration of the 3,4-DHPEA-EDA instead of that of CP. In a different set of experiments, using samples of extra virgin olive oil C (**Figure 4**), the correlation we observed was still inverse but lower ($R = -0.7091$ and $R = -0.3471$, respectively). An increase of the peaks of THY and HTHY was also observed. However, a linear correlation was not found between the total decrease of CP and the increase of simple polyphenols.

In both oils we observed a good inverse correlation between α-tocopherol decrease and CD increase ($R = -0.9881$ in oil B and $R = -0.9697$ in oil C) thus, we investigated this correlation under more drastic conditions, by oxidizing samples of extra virgin olive oils in thin layer (3 mg) at 37 °C or 75 °C. Thin-layer oxidation time course of oil D was monitored at 37 °C and at 75 °C by the evaluation of two parameters: the depletion of α-tocopherol and the formation of CD. As shown in **Figure 5**, the oxidation pattern at 37 °C was followed for 120 h. Starting from a concentration of 157.86 ng/mg of oil, α-tocopherol value decreased in function of time, while CD value kept almost unchanged until α-tocopherol concentration reached a value of 60−70 ng/mg of oil, after about 36 h. When α-tocopherol concentration reached this threshold value, we could observe a significant increase of CD formation, indicating an enhancement

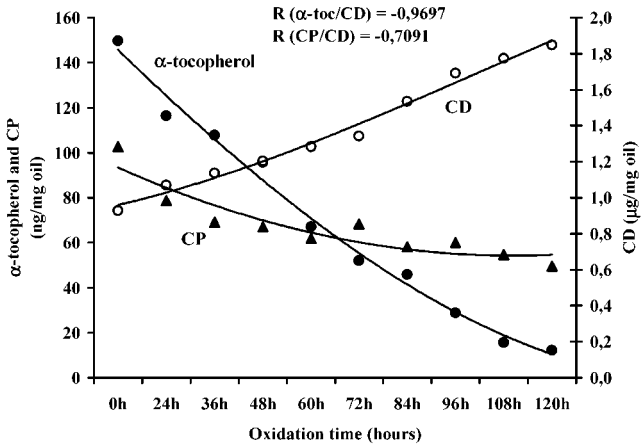

Figure 4. Variation of the concentrations of α-tocopherol, conjugated dienes (CD), and complex polyphenols (CP) in samples of 1.5 g of extra virgin olive oil C oxidized at 75 °C. The SD values were not more than 5%. The correlation coefficient $R = \text{Cov}(X,Y)/\sigma_x \times \sigma_y$.

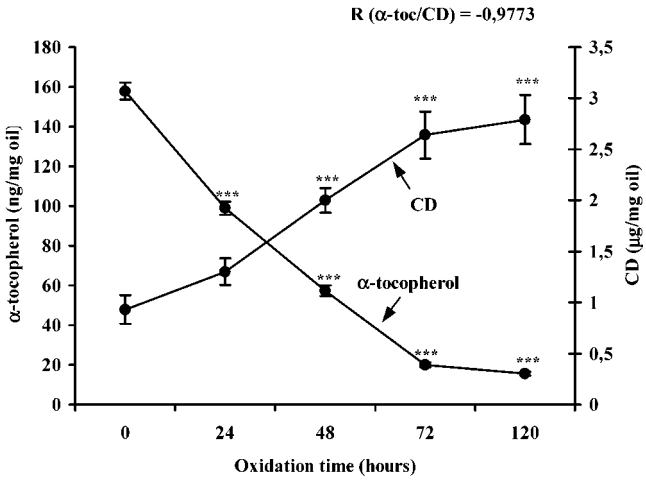

Figure 5. Variation of the concentrations of α-tocopherol and conjugated dienes (CD) in extra virgin olive oil D oxidized in thin layer at 37 °C for 120 h. *** $= p < 0.001$ versus initial values ($T = 0$). The correlation coefficient $R = \text{Cov}(X,Y)/\sigma_x \times \sigma_y$.

of the oxidation process (R α-tocopherol/CD = −0.9773). When samples of the same oil D were oxidized at 75 °C, we obtained a similar pattern (R α-tocopherol/CD = −0.9139) and the oxidation process was followed for only 24 h, as shown in **Figure 6**. In fact, at this higher temperature, we had a faster decrease of the α-tocopherol amount. The threshold value of 60−70 ng/mg of oil was reached after 4 h of incubation, and again we could observe a steep increase of CD formation. **Figure 6** also shows the oxidation pattern at 75 °C of samples of extra virgin olive oil E (R α-tocopherol/CD = −0.7770), with an higher initial concentration of α-tocopherol (198.4 ng/mg). We could observe that, as it was to be expected, as less α-tocopherol was present in the oil (oil D), the faster the threshold value was reached and the oxidation was enhanced.

No significant changes in unsaturated fatty acids concentration were found in any of the oxidizing conditions used.

DISCUSSION

Several factors, with antioxidant or prooxidant activity, operate simultaneously to determine the autoxidation of olive oil; so it is quite difficult to determine the effect of each one on the complex oxidative process. The antioxidant activity of

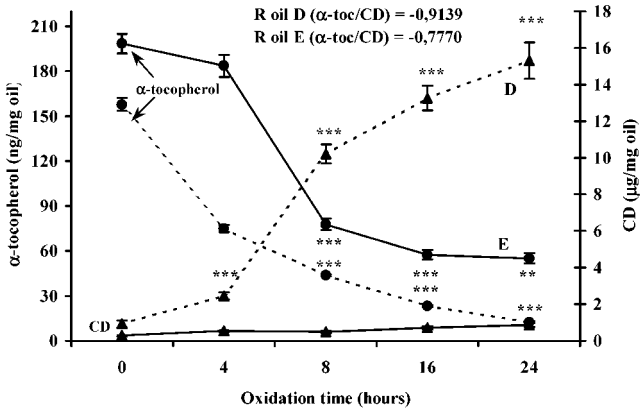

Figure 6. Variation of the concentrations of α-tocopherol and conjugated dienes (CD) in samples of two extra virgin olive oils, D and E, oxidized in thin layer at 75 °C. *** $= p < 0.001$ versus initial values ($T = 0$). The correlation coefficient $R = \text{Cov}(X,Y)/\sigma_x \times \sigma_y$.

the hydrophilic phenolic compounds and α-tocopherol present in extra virgin olive oil has been reported in several papers, but depending on the experimental conditions used, the results do not seem to agree. It is quite clear, however, that among the natural antioxidants present in olive oil, the main activity is exerted by the large fraction of the hydrophilic phenolic compounds (*6*−*8*). Such an activity seems to be exerted mainly at the initial stage of oil autoxidation, and, when it is reduced, α-tocopherol seems to become effective (*9*). Moreover, α-tocopherol seems to have a synergistic effect in association with some phenolic compounds with significant antioxidant activity (*7*, *11*).

Our results show that α-tocopherol itself can be important for olive oil stability. In the experimental conditions used, the decrease of the concentration of antioxidants (CP and α-tocopherol) was inversely correlated with the increase of CD or peroxide value. In our systems, the value of CD, commonly used to indicate the oxidation state of tissues (*21*), could be considered as an adequate measure of the ongoing oxidation of the oil, as there is a good correlation between peroxide value and CD content. This method is simple and allowed us to measure even tiny amounts of oxidation products, using only a few milligrams of oil.

The CP fraction has been reported to have an important influence on the resistance of the oil to oxidation (*22*) and to be correlated to the peroxide value (*7*); in our experimental condition this correlation was evident also toward CD, although it seemed to be greatly dependent on the oil utilized. α-Tocopherol decrease instead was clearly correlated to the increase of CD or peroxide value in all the experimental conditions used, indicating a strict dependence of the oxidation pattern on its amount. During the thin layer autoxidation of the oil, both at 37 °C and at 75 °C, we observed that when α-tocopherol concentration was above a threshold value of 60−70 ng/mg of oil oxidation proceeded slowly, but when it dropped under that value the oxidation process had a significant increase. The initial amount of α-tocopherol greatly determined the pattern of the oxidation process: when less α-tocopherol was present in the oil, the faster the threshold value was reached, and the oxidation was enhanced. The same oxidation pattern was observed during the autoxidation of a larger amount of oil.

The initial amount of α-tocopherol should be considered one of the parameters that influence the olive oil stability to oxidation, its shelf life, and thus its quality. It is known that its initial amount varies depending on the variety of olives and

the extraction process used. It is important to remember, however, that α-tocopherol concentration can also be greatly affected by the storage conditions of the oil, including temperature and light exposure, as reported in the literature (*23*).

Our data show a clear indirect correlation, not always evident in the experimental systems reported in the literature, between α-tocopherol concentration and oxidation products, measured as CD, and even more evident in drastic oxidizing conditions.

ABBREVIATIONS USED

CD, conjugated dienes; PUFA, polyunsaturated fatty acids; HPEA-EDA, ester of hydroxyphenylethanol with the dialdehydic form of elenolic acid; 3,4-DHPEA-EDA, ester of 3,4-dihydroxyphenylethanol with the dialdehydic form of elenolic acid; CP, complex polyphenols; THY, tyrosol; HTHY, hydroxytyrosol.

ACKNOWLEDGMENT

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