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Relation between the Endogenous Antioxidant System and the Quality of Extra Virgin Olive Oil under Accelerated Storage Conditions

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Three monovarietal extra virgin olive oils (EVOOs) were subjected to accelerated storage conditions (60 °C, dark) representative of the autoxidation process during shelf life. Oxidation markers, i.e., the peroxide value, conjugated dienes, the oil stability index, and minor components, were monitored. The changes in minor components, related to the stage of ongoing oxidation and expressed as a percentage of the induction period (IP), followed a similar pattern in all oils: *o*-diphenols diminished by the highest rate (halved within 15% of the IP), followed by α-tocopherol (halved within 35% of the IP). Carotenoids and chlorophylls were also affected by autoxidation, whereas squalene showed high stability (<20% loss within 100% of the IP). Polar phenols (especially *o*-diphenols) and α-tocopherol were deduced to be the most potent antioxidants of EVOO. They efficiently inhibited oxidative lipid deterioration and subsequent development of sensory defects (rancidity, discoloration), which occurred only after substantial depletion of these antioxidants. Therefore, they could also be used as markers for the oxidative status of EVOO particularly in the early stage of oxidation.

KEYWORDS: Olive oil; oxidation; stability; quality; rancidity; polar phenols; tocopherol; carotenoids; chlorophylls; squalene

INTRODUCTION

The diet in the countries surrounding the Mediterranean Sea is characterized by the use of olive oil as the principal source of fat. A recent increase in popularity of olive oil among consumers in other parts of the world is related to the growing interest in minimally processed, "natural" foods as well as in ethnic cooking styles including the traditional Mediterranean diet, which is believed to be associated with the effective prevention of certain diseases, particularly coronary heart disease and cancer (*1*).

Virgin olive oil (VOO) is obtained from the olive fruit purely by mechanical means, without further treatment other than washing, filtration, decantantion, or centrifugation, and is consumed directly without any further treatment. This process retains most of the lipophilic compounds originally present in the olive fruit, which are responsible for the characteristic taste, flavor, and color of the oil and which probably also contribute to the beneficial health effects of VOO (*2*−*4*). VOO of high sensorial and physicochemical quality is entitled to carry the denomination "extra virgin" if specific criteria laid down in the EU legislation (*5*, *6*) and trade standards of the International Olive Oil Council (IOOC) (*7*) are met.

Apart from the health benefits, VOO exhibits in comparison with other vegetable oils a high resistance against oxidation (*8*). Lipid oxidation is the main process leading to the deterioration of edible oils, e.g., during production, transportation, and mainly storage. A characteristic feature of the autoxidation process is that it proceeds very slowly at the initial stage (induction period, IP), and after a certain point is reached, an exponential acceleration of oxidation suddenly occurs (*8*, *9*).

The physicochemical changes associated with autoxidation result in the loss of sensory and nutrition values. If certain limits of lipid oxidation products (hydroperoxides, conjugated dienes and trienes) are exceeded and/or rancid off-flavors occur, the olive oil may lose the permission to carry the label "extra virgin" or even "virgin". In the first instance labeling regulations would be infringed, whereas in the latter case the oil may not even be sold for consumption anymore.

The remarkable oxidative stability of VOO is attributed to both its characteristic fatty acid composition (a low proportion of polyunsaturated fatty acids) and a significant amount of minor components with antioxidant properties. Their concentrations reported in the literature are rather variable and for good-quality oils are usually in the range 100−300 mg/kg for α-tocopherol (*3*) and 200−1500 mg/kg for polar phenolic compounds (*10*). Other minor components that may also exhibit effects on the oxidative stability of VOO are squalene, 1000−8000 mg/kg (*11*), carotenoids, up to 10 mg/kg, and chlorophylls, up to 20 mg/kg (*3*).

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Table 1. Initial Compositional Characteristics of Extra Virgin Olive Oil Samples

parameter (±SD)	Koroneiki	Coratina	Picual
acidity, % oleic acid	0.58 ± 0.03	0.55 ± 0.03	0.38 ± 0.02
POV, mequiv of O_2/kg	9.6 ± 0.2	8.7 ± 0.1	7.9 ± 0.1
K_{232}	2.05 ± 0.07	1.61 ± 0.08	1.84 ± 0.12
K_{270}	0.14 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
fatty acid concn, % peak area			
C16:0	11.1 ± 0.2	9.6 ± 0.2	10.6 ± 0.3
C16:1	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.1
C18:0	2.6 ± 0.1	2.3 ± 0.1	2.7 ± 0.1
C18:1	76.2 ± 0.8	78.0 ± 0.4	80.3 ± 1.1
C18:2	7.1 ± 0.1	7.6 ± 0.1	3.6 ± 0.1
C18:3	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
C20:0	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
C20:1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
total polar phenolics concn, mg/kg (mmol/kg)	340 ± 22 (1.12 ± 0.08)	734 ± 28 (2.50 ± 0.11)	603 ± 41 (1.18 ± 0.08)
o-diphenols concn, mg/kg (mmol/kg)	144 ± 10 (0.47 ± 0.03)	272 ± 8 (0.88 ± 0.03)	319 ± 18 (0.93 ± 0.06)
α-tocopherol concn, mg/kg (mmol/kg)	158 ± 4 (0.37 ± 0.01)	208 ± 4 (0.48 ± 0.02)	145 ± 5 (0.34 ± 0.01)
β-tocopherol concn, mg/kg	2 ± 1	3 ± 1	12 ± 1
γ-tocopherol concn, mg/kg	6 ± 1	12 ± 1	13 ± 1
β-carotene concn, mg/kg	1.1 ± 0.1	1.7 ± 0.1	1.3 ± 0.2
lutein concn, mg/kg	1.6 ± 0.2	2.5 ± 0.2	3.3 ± 0.2
chlorophylls concn, mg/kg	12 ± 1	14 ± 1	14 ± 1
squalene concn, mg/kg	3690 ± 200	3450 ± 200	5700 ± 300
OSI, h (100 °C, 20 L/h)	25.6 ± 0.4	37.3 ± 0.6	58.5 ± 1.9

The ideal test for measuring the changes in VOO during storage would be a test in which the oil is stored under realistic storage conditions and the changes are regularly evaluated over time. An obvious drawback of such an approach is that it takes a very long time. The shelf life of VOO guaranteed by manufacturers ranges from 18 to 24 months, which implies that completing the oxidation process would take several years. Several authors have followed VOO samples under realistic storage conditions (e.g., refs *12*−*15*). However, in these trials samples were only monitored during the initial stage of the oxidation process.

On the other hand, the widely applied oxidative stability tests (e.g., Rancimat) performed at high temperature (100−130 °C) while air is bubbled through the hot oil sample (*11*, *16*, *17*) can give quick information on the oxidative stability of VOO. These high-stress oxidation conditions may correlate to hot kitchen applications, but can hardly mimic realistic storage conditions.

To obtain meaningful results both for the IP and the exponential phase of the oxidation process within a reasonable period, accelerated storage conditions have to be applied that do not alter the oxidation mechanism. Several catalytic systems that stimulate the oxidation process are therefore not acceptable, e.g., the addition of prooxidants (e.g., metal ions) and exposure to irradiation (normal or UV light) or very high temperature. Only the experiments at slightly elevated temperatures give good correlation with experiments carried out under normal storage conditions provided that the temperature is below 85 °C, preferably a maximum of 60 °C (*18*). Accelerated storage trials respecting these considerations have been performed with VOO by several authors (*13*, *14*, *19*, *20*).

In the present study, we investigated the compositional changes, the quality indices, and the oxidative stability of extra virgin olive oil (EVOO) stored at elevated temperature in darkness. Both ongoing oxidation, measured by the rate of formation of hydroperoxides and conjugated dienes, and degradation of minor components were monitored with the primary effort to report on the relationship between these two phenomena. In contrast to previous studies the different stages of the oxidation process were expressed as a percentage of the IP, which enabled us for the first time to directly compare different oils of different stability. With this approach also results of different accelerated storage studies can be compared because it eliminates the differences in the chosen parameters such as temperature and surface-to-oil volume ratio as long as the mechanism of autoxidation is not changed.

MATERIALS AND METHODS

Samples and Material. Twenty samples of EVOOs were screened for their composition, quality indices, and oxidative stability (*21*). Out of these, three monovarietal EVOOs—Koroneiki, Coratina, and Picual collected from Greece, Italy, and Spain, respectively, in the 2001−2002 season—were selected for this study. Samples were stored at −20 °C until the start of the oxidation trial carried out in 2003.

Solvents and Standards. All solvents and reagents were of appropriate grade for spectrophotometric or chromatographic analyses. Lutein and β-carotene were purchased from Fluka AG (Buchs, Switzerland), *dl*-α-tocopherol was purchased from Merck (Darmstadt, Germany), and squalene and *p*-hydroxyphenylacetic acid were purchased from Sigma Chemicals Co. (St. Louis, MO).

Accelerated Storage Test. Aliquots (40.0 ± 0.1 g) of each EVOO sample were poured into glass vessels (100 mL, 45 mm i.d.) kept open in the oven at 60 ± 1 °C. Samples were taken out of the oven at regular intervals. The ongoing oxidation process was monitored by immediate measurement of the peroxide value (POV), extinction coefficient K_{232}, oil stability index (OSI), and titratable acidity. The remainder of the sample was stored in the freezer (−20 °C) under a nitrogen blanket. Tocopherols, polar phenolics, carotenoids, chlorophylls, and squalene were determined in representative samples after the completion of the oxidation trial.

Analytical Methods. The determination of legal quality characteristics of EVOO, i.e., titratable acidity, POV, and extinction coefficients (K_{232}, K_{270}), was carried out following the analytical methods described in the EC Regulations (*5*; Annex II, III, and IX, respectively). Titratable acidity was expressed as the amount of oleic acid %, POV was expressed as milliequivalents (mequiv) of active oxygen per kilogram of oil, and extinction coefficients K_{232} and K_{270} were expressed as specific extinctions of a 1% (w/v) solution of oil in 2,2,4-trimethylpentane in a cell of 1 cm path length.

OSI was measured by a Rancimat apparatus, model 679 (Metrohm, Herisau, Switzerland). A flow of air (20 mL/h) was bubbled through 5.0 g of oil heated to 100 °C. The volatile oxidation products were stripped from the oil and dissolved in cold water, increasing its conductivity. The time taken to reach an inflection point at the induction curve was measured.

The fatty acid composition was determined by capillary GC analysis according to a standard methodology (*22*) after the conversion of triglycerides to methyl esters using sodium methoxide in methanol/diethyl ether (1:1, v/v).

Tocopherols were determined after HPLC separation on a silica gel column by fluorescence detection (*23*).

Polar phenolics were extracted from samples by aqueous methanol and separated by HPLC (*10*); individual phenolics were quantified according to Mateos et al. (*24*).

Chlorophyll and related pigments were determined by spectrophotometric measurements at 630, 670, and 710 nm (*25*) and carotenoids by the HPLC method coupled with a UV−vis detection (*26*).

Squalene was determined after the saponification of the sample by capillary GC analysis according to ref *5* (Annex V).

Statistical Analysis. Two vessels per sample, taken out of the oven at regular intervals, were independently analyzed. The determinations were carried out in duplicate. Statistical analysis (mean, standard deviation, Student's *t* test) was performed by using the Excel standard software package (Microsoft Corp., Redmond, WA). Differences were considered as statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The three monovarietal EVOOs obtained from the olive varieties Koroneiki (K), Coratina (C), and Picual (P) represent some of the most common olive varieties grown in Greece, Italy, and Spain, respectively. The three samples were comparable in quality indices related to oxidative status, but they considerably varied in fatty acid composition, initial amount of minor components, and OSI, as summarized in **Table 1**.

The fatty acid composition of the oils, one of the characteristics predominantly determined genetically, with a lesser impact of the environmental factors, was in accordance with published data on Koroneiki (*27*), Coratina (*28*), and Picual (*29*) VOO.

The levels of minor components in the samples ranged within the intervals typical for a good-quality VOO. The combination of fatty acid profile and natural antioxidant mixtures leads to a great variation in oxidative stability. The OSI of the three differently stable oils fell, however, within the range of values measured under comparable experimental conditions (*29*).

The progress of the oxidation process was monitored by measuring POV and K_{232}; the former expresses the hydroperoxide content, and the latter indicates the level of conjugated dienes resulting from a shift in a double-bond configuration. The POV measurement, performed at regular intervals, allowed construction of the typical induction curves (**Figure 1a**). IP, a variable characteristic for any lipid material and strongly depending on the conditions of measurement, was calculated from these curves. IPs determined for the oils K, C, and P at 60 °C were 40 ± 3, 46 ± 3, and 88 ± 4 days, respectively, which reasonably reflects the trend found by OSI measurement at 100 °C (K < C ≪ P, **Table 1**). The development of K_{232} followed a similar profile. The acceleration in the oxidation rate at the end of the IP was, however, less pronounced (**Figure 1b**).

The titratable acidity closely followed the POV for all samples; it remained relatively stable during the IP with a dramatic increase throughout the exponential phase of oxidation (data not shown), which was in agreement with published results (*30*).

The changes in the OSI of the oils, regularly evaluated by the Rancimat test, showed a gradual decrease during the time of the accelerated trial (**Figure 2**). Once the oxidation process approaches the end of the IP, OSI becomes barely measurable by this method.

The different oxidative stabilities of the three samples are the combined result of the fatty acid composition and the effect of various pro/antioxidants present in the oils. The explanation of the considerably higher stability of oil P lies probably in a relatively low content of polyunsaturated fatty acids, traditionally expressed by the oleic/linoleic ratio (22.0, against 10.7 and 10.3 for samples K and C, respectively) and higher content of *o*-diphenols. As the fatty acid composition is similar in oils K

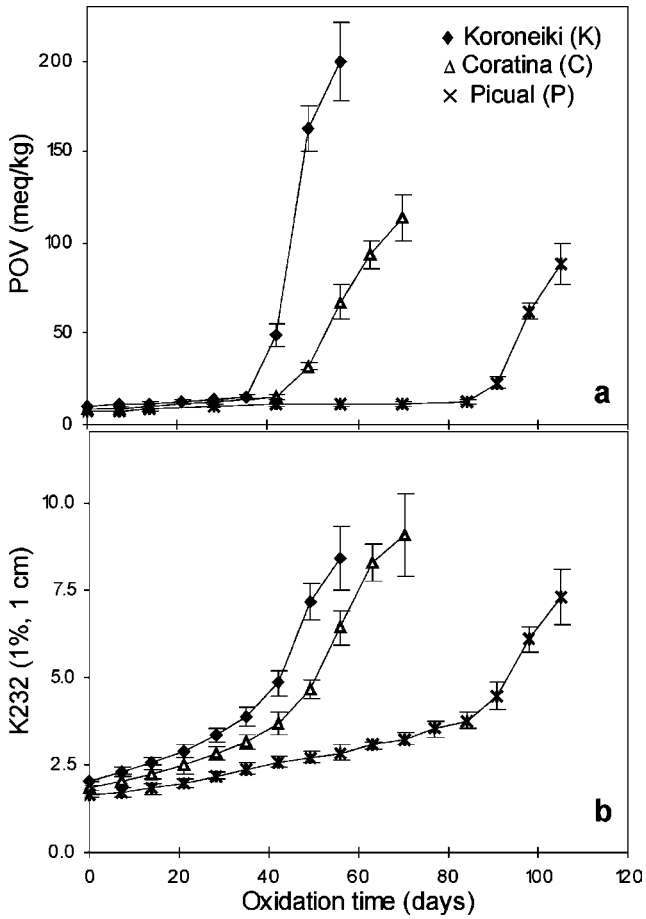

Figure 1. Evolution of hydroperoxides (**a**) and conjugated dienes (**b**) in extra virgin olive oils subjected to accelerated storage conditions at 60 °C. Results are expressed as mean ± SD ($n = 2$). The legal limits for EVOO set by EU (*5*, *6*) are POV ≤ 20 mequiv of O_2/kg (**a**) and K_{232} ≤ 2.5 (**b**).

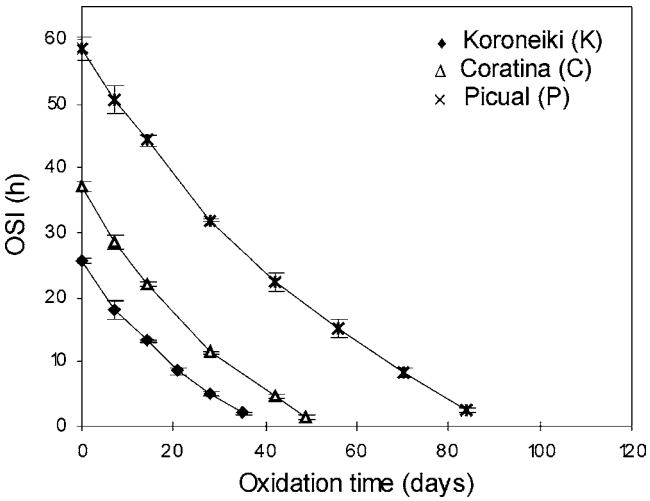

Figure 2. Changes in oxidative stability index (measured at 100 °C) of extra virgin olive oils subjected to accelerated storage conditions at 60 °C. Results are expressed as mean ± SD ($n = 2$).

and C, the considerably higher level of *o*-diphenols may explain the higher stability of oil C compared to oil K. The contribution of other minor components to stability seems to be less pronounced.

As the oils lose their oxidative stability during the oxidation trial (**Figure 2**), it can be anticipated that the antioxidative system is being slowly depleted. To understand better the depletion of antioxidants in oils with different absolute IPs, the compositional changes were expressed on the basis of the relative IP (%). We found that relating these changes to the IP is more informative than relating them to absolute time, as this way of expression is not affected by the conditions of an oxidation trial, which are not standardized.

α-Tocopherol is the predominant representative of tocopherols in olive oils, accompanied by a small amount of the γ-isomer. The degradation of α-tocopherol followed a similar pattern in all samples (**Figure 3a**); a slightly higher retention was found for oil C, probably due to its higher initial concentration and/or the high content of polar phenolics. The α-tocopherol level decreased by half at 30−35% of the IP; its depletion was completed at about 70% of the IP.

As expected, γ-tocopherol exhibited significantly higher resistance to oxidation; a gradual degradation in the first stage of oxidation was observed, after which the slope of the degradation rate changed dramatically (**Figure 3b**). The "breakdown point" lies between 60% and 70% of the IP, i.e., just after the total depletion of α-tocopherol, and the degradation of γ-tocopherol was accomplished by the end of the IP.

The loss of α-tocopherol observed before the exponential phase of oxidation reasonably corresponds to the decrease of α-tocopherol reported for the storage of VOO: 14−32% within 6 months (*31*) and 20−58% within 24 months (*14*). Tocopherols are known as powerful lipid radical scavengers. Their antioxidative effect, however, strongly depends on various parameters such as temperature, concentration, degree of unsaturation of the lipid substrate, and presence of other antioxidants (*9*). The contribution of α-tocopherol to VOO oxidative stability has been recognized (*32*), although its impact, evaluated by the Rancimat test, was found to be smaller compared to those of polar phenolics (*11*, *27*, *29*), fatty acid composition (*29*), and total chlorophylls (*27*).

The polar phenolic fraction of VOO consists of a heterogeneous mixture of compounds, each of which varies in chemical properties and impact on the stability of VOO (*33*). The HPLC method used in our study enabled the quantification of nine major phenolic compounds and thus the distinction among monophenols (tyrosol and aglycons of ligstroside), *o*-diphenols (hydroxytyrosol, hydroxytyrosol acetate, and aglycons of oleuropein), and lignans (pinoresinol and 1-acetoxypinoresinol).

Substantial differences were observed in the degradation of total phenolics between the oils studied (**Figure 3c**). Total phenols were reduced by half at 20−30% of the IP, and 5−15% of the total phenols were still intact at the end of the IP. Different degradation rates and final levels of residual phenolics are based on differences in the phenolic composition of individual samples.

Unlike the total phenolics, *o*-diphenols were degraded according to a similar pattern (**Figure 3d**), which was characterized by a rapid decrease in the initial stage; the concentration of *o*-diphenols was reduced by half within 15% of the IP. No *o*-diphenols were detected after 80% of the IP.

Individual phenolics within the polar phenolic fraction greatly varied in their stability as shown exemplarily for oil C (**Figure 4**); similar behavior was found in the other two oils. The content of both oleuropein-derived aglycons (3,4-DHPEA-EA, aldehydic

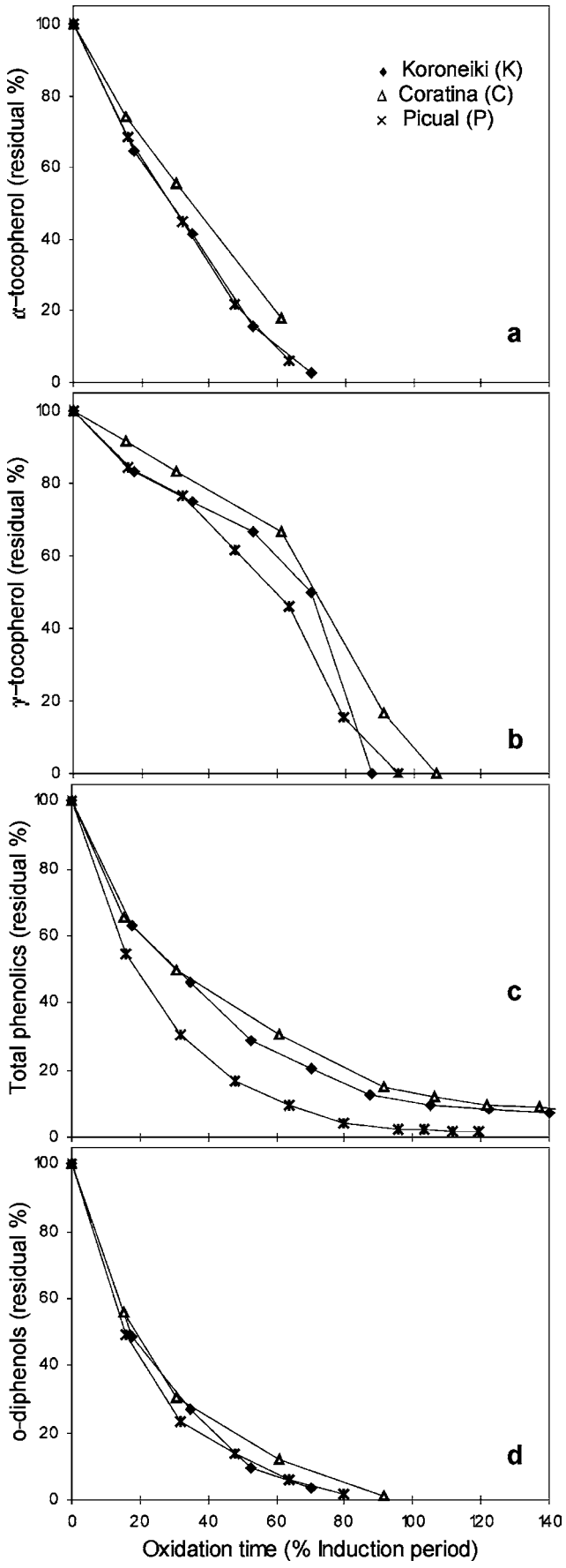

Figure 3. Changes in the residual content of α-tocopherol (**a**), γ-tocopherol (**b**), total polar phenolics (**c**), and *o*-diphenols (**d**) in extra virgin olive oils subjected to accelerated storage conditions at 60 °C. RSD < 5.5% (**a**), < 11.0% (**b**), < 8.0%, (**c**) and < 7.0% (**d**) of the mean values ($n = 2$).

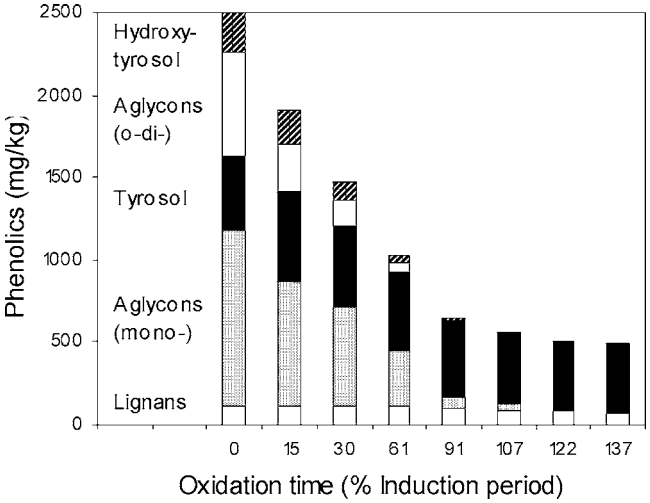

Figure 4. Changes in the total phenol content and phenolic profile in Coratina extra virgin olive oil subjected to accelerated storage conditions at 60 °C. Degradation changes followed a similar pattern in the two other EVOOs (data not shown).

form of oleuropein aglycon, and 3,4-DHPEA-EDA, dialdehydic form of decarboxymethyl oleuropein aglycon) was greatly reduced during the first half of the IP. Hydroxytyrosol exhibited slightly higher retention in EVOO during storage; this was obvious especially at the very beginning of the autoxidation trial (<15% of the IP). The disproportion between the concentration decreases of hydroxytyrosol and *o*-diphenolic aglycons can be explained by the fact that hydroxytyrosol is consumed by deactivating lipoperoxyl radicals, but simultaneously also produced by hydrolysis of oleuropein aglycons, as previously shown in VOO during storage (*19*). The concentration of ligstroside-derived aglycons (*p*-HPEA-EA and *p*-HPEA-EDA) was also reduced during accelerated storage, although they showed much higher stability than *o*-diphenolic aglycons. The concentration of tyrosol increased (by 15−40%) in the initial stage of oxidation as a result of monophenolic aglycon hydrolysis and then, in contrast to hydroxytyrosol, its level in EVOO remained relatively steady with a declining tendency. Lignans, which also showed rather high stability against oxidation, were together with tyrosol the only residual phenolics detected in the samples in the exponential phase of oxidation.

Previous experiments performed in the dark showed a slow degradation of VOO phenolics during storage; the loss of total phenolics amounted to 15−20% within 12 months (*19*) and 20−38% within 24 months (*14*). Phenolics are generally considered to be the most effective antioxidants in VOO, which is in conformity with the results of the present study as well. Their contribution to oxidative stability was estimated to be approximately 50% (*16*, *29*). However, the measurement of the total phenolics in these studies was carried out by the Folin−Ciocalteu colorimetric assay, which does not take into account the phenolic profile, which may vary considerably between various VOO samples. Investigation of pure compounds in model systems (*34*) showed that, of the compounds involved in that study (not all VOO phenolics, such as ligstroside aglycons, were included), only *o*-diphenols exhibited high antioxidative capacity. Several other experiments based on different methodologies, e.g., studying the radical scavenging activity (*35*) and Rancimat measurement (*11*), led authors to the same conclusion that *o*-diphenols are the most active antioxidants in VOO also compared to tocopherol. Within this

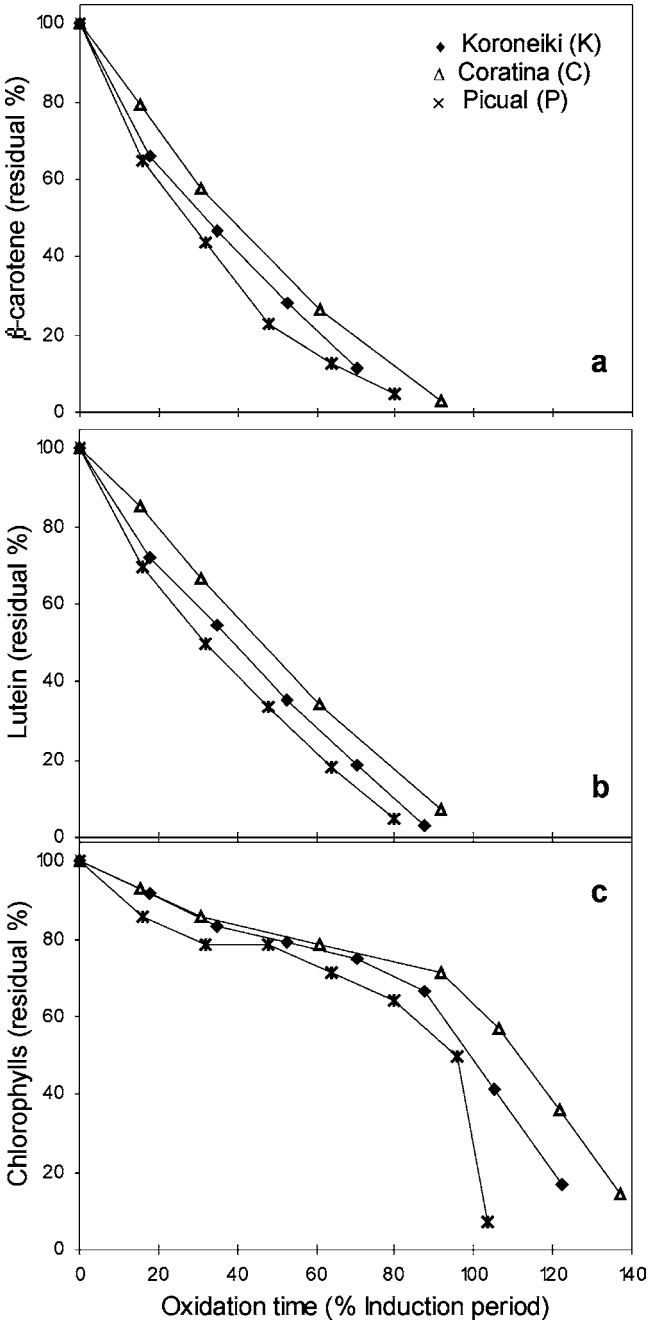

Figure 5. Changes in the residual content of β-carotene (**a**), lutein (**b**), and chlorophylls (**c**) in extra virgin olive oils subjected to accelerated storage conditions at 60 °C. RSD < 5.0% (**a**), < 7.5% (**b**), and < 14.0% (**c**) of the mean values ($n = 2$).

group, no (*11*) or very small (*36*) differences were found between the individual *o*-diphenols occurring in VOO.

The degradation of lutein and β-carotene, the two major carotenoids of VOO, is depicted in parts **a** and **b** of **Figure 5**, respectively. The results indicate a high susceptibility of carotenoids to oxidation; both compounds show comparable degradation patterns. They steadily (almost linearly) disappeared from the oils, and their degradation was accomplished between 80% and 100% of the IP.

There are no published data relating the degradation of carotenoids in VOO to the IP. However, results of a storage test of four different VOOs showed 20−40% degradation of both carotenoids within 24 months (*14*). Carotenoids (and especially β-carotene as the most studied representative) have

been generally recognized to be very potent inhibitors of photooxidation due to their ability to quench singlet oxygen. However, the knowledge on the contribution of carotenoids to the oxidative stability of VOO in the absence of light, either positive or negative, is still not well established (*37*). Their antioxidative effect in VOO under conditions of autoxidation seems to be very limited (*29*) or even negative due to their oxidation products, which may possibly react with the lipid substrate and thus accelerate oxidation (*38*).

The level of chlorophylls, the green colorants of VOO, decreased slightly until reaching 80−90% of the IP, after which a rapid degradation occurred (**Figure 5c**). That suggests that the changes in the total chlorophyll content (measured by spectrophotometry) are relatively small during storage unless severe oxidation changes take place and/or protecting antioxidants are depleted.

Unlike carotenoids, chlorophylls are known to have a negative impact on the stability of VOO in the presence of light as they act as photosensitizers, i.e., compounds that transfer energy from light into the lipid substrate and thus promote photooxidation. On the other hand, a slight antioxidant activity of chlorophylls in the dark has been reported (*39*, *40*), which was attributed to the possible donation of a hydrogen radical to break free-radical chain reactions. An antioxidant activity of pheophytin *a*, a major chlorophyll derivative in VOO, was recently confirmed in an olive oil model system at elevated temperatures (*14*). Nevertheless, the impact of chlorophylls on the oxidative stability of VOO, i.e., in the presence of other antioxidants, seems to be rather limited (*29*).

As presented above the natural pigments were considerably altered during oxidation, which resulted in visual changes in the color of EVOO samples (photographs not shown). The color was relatively stable during the first half of the oxidation process; however, the color intensity slightly decreased (less vivid color), which can be attributed to the degradation of carotenoids. A reason for the sudden discoloration, which was observed shortly before the end of the IP, is obviously the rapid degradation of chlorophylls. Our results are in line with findings reported by Ceballos et al. (*17*) that the chlorophyll index of VOO is quantitatively less affected by oxidation than the carotenoid index.

Squalene showed high stability toward autoxidation under the conditions of this trial. A steady gradual decline in the concentration of squalene resulted in 15−19% degradation within the complete IP (**Figure 6**).

Although the literature data on squalene degradation are very limited, our results seem to fall within the same order of magnitude as compared with the results of a storage test of four VOOs, which showed 1−13% degradation within 24 months of storage in the dark at ambient temperature (*14*). We did not find any relationship between the degradation of squalene and that of other minor components of VOO. In fact, the protective effect of squalene toward α-tocopherol previously suggested at ambient temperature (*31*) could not be confirmed later at slightly elevated temperature (40 °C) (*41*). On the basis of the low degradation rate and the fact that the rate did not change during the IP, it can be deduced that squalene scarcely participates in the deactivation of lipid radicals. The mechanism of its action seems to be based purely on competitive oxidation (*41*), i.e., a competition between squalene and unsaturated triacylglycerols, possibly resulting in a slight prolongation of the IP. This suggests a very limited contribution of squalene to the oxidative stability of VOO at ambient or slightly elevated temperature, as recently reported by various authors (*11*, *14*). Squalene, however, seems to play a more significant role in protection of olive oil quality during frying temperatures (*42*).

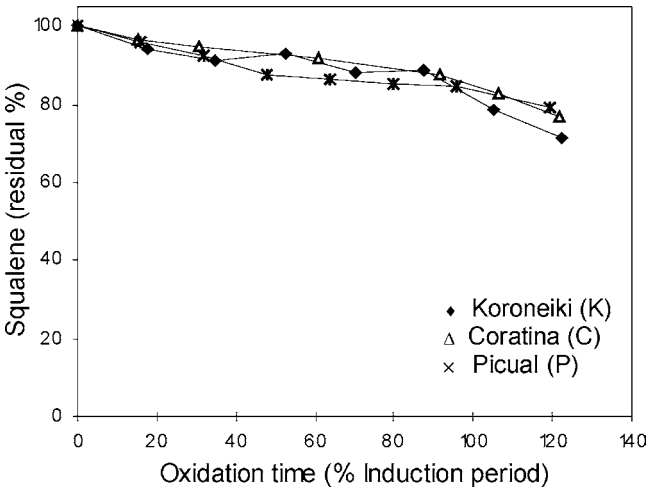

Figure 6. Changes in the residual content of squalene in extra virgin olive oils subjected to accelerated storage conditions at 60 °C. RSD < 7.5% of the mean values ($n = 2$).

Different VOOs greatly vary in oxidative stability, which depends on many intrinsic factors (fatty acid composition, natural level of pro/antioxidants). The natural antioxidant system of VOO is formed by a mixture of compounds, which, despite occurring at much lower concentrations, are more susceptible to oxidation than the unsaturated triacylglycerols. These compounds scavenge lipoperoxyl radicals and prevent them from reaction with a lipid substrate. Thus, the quickly acting antioxidants are likely to play a crucial role in improving the oxidative stability of the oil by interrupting the propagation phase of lipid autoxidation. The observed compositional changes, related to the stage of ongoing oxidation (expressed by a percentage of the IP), indicated that regardless of the length of the IP the changes of EVOO minor components followed a similar pattern. Of the compounds present in EVOO, *o*-diphenols diminished at the highest rate, followed by α-tocopherol. Their concentration was reduced by half within 15% and 35% of the IP, respectively. The difference we found between the degradation patterns of *o*-diphenols and α-tocopherol (compare parts **a** and **d** of **Figure 3**) is in accordance with a recent kinetic study performed under Rancimat test conditions (*11*), demonstrating that *o*-diphenols are degraded substantially prior to α-tocopherol. *o*-Diphenols, namely, hydroxytyrosol and especially highly abundant 3,4-DHPEA-EA were also shown to be more effective (faster) radical scavengers than α-tocopherol (*36*). Because of the difference between phenolics and α-tocopherol in polarity, this can be explained by the theory of the polar paradox (antioxidants possessing a higher polarity are more effective in less polar media) and/or by the hypothesis that *o*-diphenols may be consumed not only as free-radical chain breakers, but also as regenerators of tocopheryl radicals, which are generated during oxidation, and thus prolong the retention of α-tocopherol in VOO.

By actively protecting the integrity of the unsaturated fatty acids, the natural antioxidants are depleted. Although the triacylglycerol quality may still be good and fulfill the legal requirements for (E)VOO (i.e., not exceeding maximum values for conjugated dienes and peroxides), the amount of minor components responsible for the characteristic taste and for some of the potential health benefits of (E)VOO could have already decreased significantly. This decrease starts before the fatty acids are oxidized, leading to the development of rancidity (detected

at 53−64% of the IP) and to levels of lipid oxidation products exceeding legal limits.

Particularly with respect to the detection of sensory defects, it has to be noted that it is very difficult (even for an experienced panelist) to correctly evaluate the oxidative status of (E)VOO within the first half of the IP until the development of rancid off-flavor. Rancid off-flavor can also be masked by the strong characteristic taste top notes of (E)VOO. In fact, this is a complex issue, as some characteristic flavor compounds are derived from (enzymic) lipid oxidation and are in some cases identical to compounds responsible for rancidity. Although this oxidation stage (i.e., the first half of the IP) is not characterized by significant sensory changes, a gradual decrease of (E)VOO quality, as previously mentioned, is not negligible.

Among the maximum limits for lipid oxidation products laid down in regulations and trade standards for (E)VOO (*5*−*7*) are POV and K_{232}. POV increased very slowly during the IP, and the legal limit set by EU for EVOO of ≤20 mequiv of O_2/kg was hardly reached before the exponential phase (see **Figure 1a**). The increase in K_{232} showed a steeper slope (see **Figure 1b**), and the legal EU limit for EVOO of ≤2.5 was exceeded between 30% and 47% of the IP (the limit for VOO of ≤2.6 was exceeded between 35% and 51% of the IP). Thus, in comparison with POV, K_{232} showed a higher predictive value in the evaluation of the oxidative status of EVOO in this experimental condition, which is likely to be valid when applied to the common storage conditions (at ambient temperatures ranging between 15 and 30 °C). The slow evolvement of these two oxidation parameters also illustrates that the integrity of the fatty acids is effectively protected by the antioxidants present. However, after 50% of the IP the oils do not fulfill the requirements any more to carry the label "virgin". Color changes were hardly recognizable until an intensive discoloration, which occurred in the advanced stage of the oxidation process. If this discoloration is observed, it can be assumed that the oil quality is already dramatically deteriorated and will not fulfill the requirements for (E)VOO.

It can be concluded from this study that the determination of those components which are involved in autoxidative protection of the oil could be an alternative or addition to measuring products formed during lipid oxidation. We clearly showed in this study that the oxidation process in EVOO developed simultaneously with the depletion of *o*-diphenols and α-tocopherol, which were primarily consumed in the initial stage of the oxidation process and which can serve as "early indicators" of the oxidative status of (E)VOO.

ABBREVIATIONS USED

$K_{232,270}$, extinction coefficients at 232 and 270 nm, respectively; IP, induction period; POV, peroxide value; VOO, virgin olive oil; EVOO, extra virgin olive oil; OSI, oil stability index.

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