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Effect of Storage on Secoiridoid and Tocopherol Contents and Antioxidant Activity of Monovarietal Extra Virgin Olive Oils

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The degradation of secoiridoid, tocopherol, and antioxidant activity in extra virgin olive oils (EVOOs) was studied during 8 months of storage in closed bottles in the dark, at 40 and 25 °C. Picual, Arbequina, Taggiasca, and Colombaia monovarietal EVOOs possessing quite different fatty acid and antioxidant contents were used. The secoiridoid aglycones, namely, the oleuropein and ligstroside derivatives, and α-tocopherol decreased following pseudo-first-order kinetics. In all EVOOs oleuropein derivatives were less stable than the corresponding ligstroside derivatives and α-tocopherol. Accordingly, overall antioxidant activity decreased following pseudo-first-order kinetics, with rate constants ranging from 0.85×10^{-3} to 4.1×10^{-3} days^{-1} at 40 °C and from 0.8×10^{-3} to 1.5×10^{-3} days^{-1} at 25 °C. According to both the antioxidant activity and the hydrolysis and oxidation indices established by EU regulation to assess EVOO quality, Colombaia oil was the least stable, followed by Taggiasca, Arbequina, and Picual oils. Despite antioxidant degradation, EVOOs with high antioxidant contents were still "excellent" after 240 days of storage at 40 °C. These data led to the conclusion that the beneficial properties of EVOOs due to antioxidant activity can be maintained throughout their commercial lives.

KEYWORDS: Extra virgin olive oil; storage; oleuropein derivatives; ligstroside derivatives; α-tocopherol; antioxidant activity; 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl

INTRODUCTION

Extra virgin olive oil (EVOO) consumption is associated with a reduced risk of coronary heart disease and some types of cancers (*1*−*5*). The nutritional properties of EVOO have been ascribed to its antioxidant components, especially polar phenolics (compounds I−**VI** in **Figure 1**) deriving from olive secoiridoids, which are found only in plants belonging to the Oleaceae family (*6*). Olive antioxidants include tocopherols, squalene, and, in lesser quantities, carotenoids.

The amounts of antioxidants contained in EVOO depend on various factors, such as olive cultivar, climatic and environmental conditions, ripeness, processing, and duration of storage (*7*). Polar phenolics and tocopherols, but not squalene, are partially degraded during oil refining (*8*), so the former can be considered specific quality indices for EVOO.

According to their content in polar phenolic compounds, EVOOs have been grouped into low content (50−200 mg/kg), medium content (200−500 mg/kg), and high content (>500 mg/kg) varieties (*9*). In EVOOs the content in tocopherols [which are mainly represented by the α-isomer (compound **VII** in **Figure 1**)] ranges from 5 to 300 mg/kg (*10*); in good-quality EVOOs the concentration of compound **VII** is in the range of 100−300 mg/kg (*11*). As a consequence of these differences in antioxidant concentration, EVOOs differ greatly in terms of antioxidant activity measured in vitro (*12*).

In a previous study we reached the conclusion that EVOO quality can be differentiated further in terms of oxidation and hydrolysis indices, which are already listed in Eurpean Union (EU) regulations (*13*), namely, acidity, peroxide value, and spectroscopic indices in the UV region. Especially the measurements of antioxidant contents and antioxidant activity can be used as indices to identify "excellent" EVOOs (*12*).

However, the autoxidation and hydrolytic processes that occur during storage of EVOO could reduce its antioxidant contents. In fact, under accelerated storage conditions (60 °C, dark, open bottles) EVOO antioxidants are completely degraded within 100 days, according to an established pattern: polar phenolics diminish at the highest rate, followed by compound **VII**, carotenoids, and chlorophylls, whereas squalene is stable (*11*).

Under conditions promoting photo-oxidation (12100 lx, 25 °C, closed bottles) a considerable loss of compound **VI** occurred within 20 h, whereas the losses of polar phenolic compounds and squalene were limited (*14*). During storage of EVOO in the dark and under ambient temperature, the concentrations of

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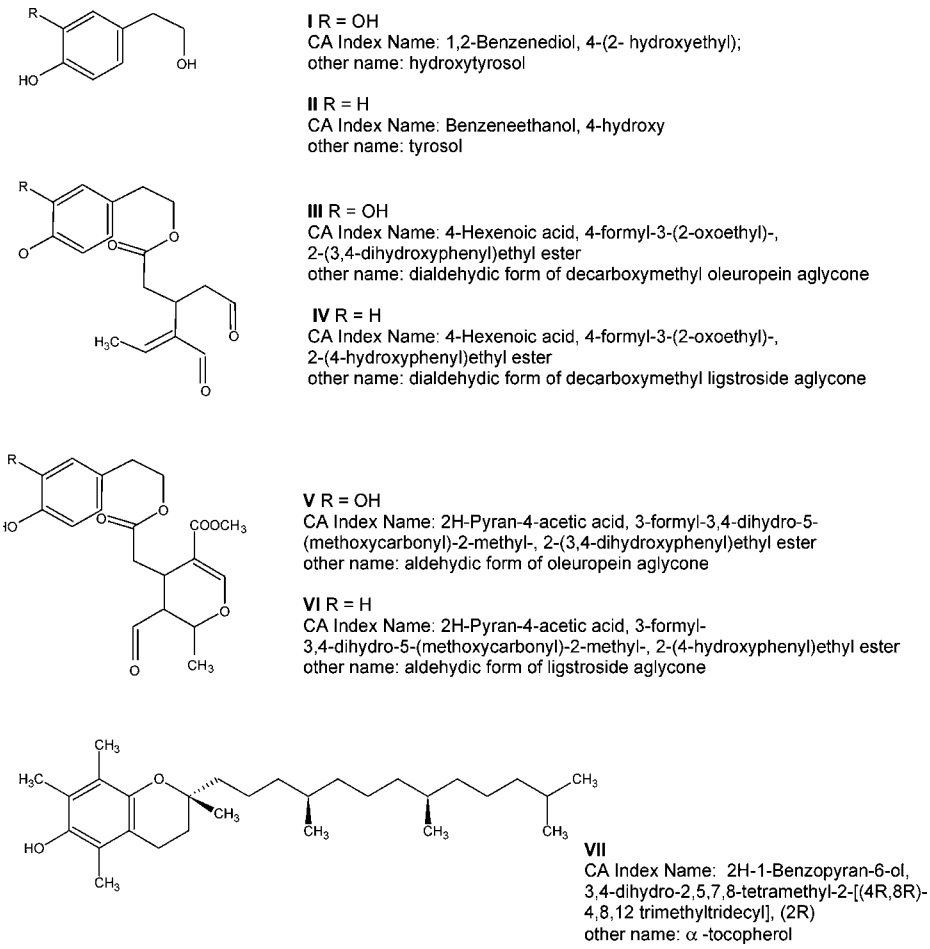

Figure 1. Main antioxidants found in EVOO and discussed in this paper. Possible formation pathways of compounds **III** and **V** from the secoiridoid glucoside oleuropein and of compound **IV** from the secoiridoid glucoside ligstroside have been described elsewhere (*22*, *24*).

polar phenols and compound diminished, whereas the concentration of squalene remained stable (*15*−*17*). The low levels of carotenoids in EVOO make their detection difficult; results are unreliable and do not permit any conclusion related to their participation in the autoxidation process.

Most of the previous studies on EVOO stability have been carried out under accelerated conditions. EVOO degradation under conditions that mimic its routine storage during the period from production to sale has not been fully elucidated. Furthermore, no information exists on the degradation of polar phenolics (compounds **I**−**VI**) and of overall antioxidant activity during storage. This is an interesting issue, as the degradation of complex phenolics (compounds **III**−**VI**) can result in the increase of simple phenolics (compounds **I** and **II**) or not, depending on the process involved.

The aim of this work was to study the extent of degradation of compounds **I**−**VII** and of overall antioxidant activity in EVOOs during storage at a medium and a relatively high ambient temperature (25 and 40 °C), in closed bottles in the dark. This study will show whether the potential properties of EVOO antioxidants are maintained or lost during the commercial life of the product.

MATERIALS AND METHODS

Oil Samples. Olive fruits of the Picual and Arbequina cultivars were processed at Aceites Malagon, Malagon (Ciudad Real, Spain), and at Aceites Eladio Rodriguez, Consuegra (Toledo, Spain). Fruits of the Taggiasca and Colombaia cultivars were processed at Azienda Agricola Domenico Ruffino, Finale Ligure (Savona, Italy). EVOOs were analyzed within a few days from production and then stored in the dark, in closed bottles (10% headspace), both at 25 and at 40 °C for a period of 8 months. For each cultivar six bottles were taken from the incubator for analysis at scheduled times (monthly).

Acidity, peroxide number, and spectroscopic indices K_{232} and K_{270} in the UV region were determined according to the EU official method (*18*).

Fatty acid composition was determined according to the EU official method (*18*). In brief, the fatty acid methyl esters were prepared by vigorous shaking of a solution of EVOO in hexane (0.2 g in 3 mL) with 0.4 mL of 2 N methanol potash and analyzed by GC with an Agilent Technologies (HP 6890) chromatograph equipped with a FID. A fused silica column (50 m length, 0.25 mm i.d.), coated with SGL-1000 phase (0.25 mm thickness; Sugerlabor) was used. Helium was employed as carrier gas with a flow through the column of 1 mL/min. The temperatures of the injector and detector were set at 250 °C, and the oven temperature was set at 210 °C. The injection volume was 1 mL.

α-Tocopherol (compound **VII** in **Figure 1**) was determined according to AOCS official method Ce 8-89 (*19*). A solution of EVOO in hexane was analyzed by an Agilent Technologies HPLC series 1100 system on a silica gel Lichrosorb Si-60 column (particle size, 5 μm; 250 mm × 4.6 mm i.d.; Sugerlabor, Madrid, Spain),which was eluted with hexane/2-propanol (98.5:1.5) at a flow rate of 1 mL/min. A fluorescence detector (Thermo-Finnigan FL3000) with excitation and emission wavelengths set at 290 and 330 nm. respectively, was used.

Quantification. The content of compound **VII** was calculated by a calibration curve obtained with a commercial standard (Merck, Darmstadt, Germany).

Polar phenolics (compounds **I**−**VI** in **Figure 1**) were determined as reported previously (*20*, *21*). To EVOO (2.5 g) was added 250 μL of a solution of internal standard (15 mg/kg of syringic acid in

methanol). The solvent was evaporated in a rotary evaporator at 35 °C under vacuum, and then the EVOO was dissolved in 6 mL of hexane. A diol-bonded phase cartridge (Supelco Co., Bellefonte, PA) was used to extract the phenolic fraction as described by Mateos et al. (*21*), and the residue was dissolved in 250 μL of methanol/water (1:1 v/v) and analyzed by an Agilent Technologies HPLC series 1100 system equipped with a column oven and a diode array UV detector. A Spherisorb S3 ODS2 column (250 × 4.6 i.d. mm, 5 μm particle size) (Waters Co., Milford, MA) was used, maintained at 30 °C, with an injection volume of 20 μL and a flow rate of 1.0 mL/min. Mobile phase was a mixture of water/acetic acid (95:5 v/v) (solvent A), methanol (B), and acetonitrile (C). The elution gradient was from 95% A−2.5% B−2.5% C to 34% A−33% B−33% C in 50 min, followed by 100% B for 15 min to clean the column.

Reference Compounds. 4-Hydroxybenzene ethanol (compound **II** in **Figure 1**) was obtained from Merck; , 4-(2-hydroxyethyl)-1,2-benzenediol (compound **I** in **Figure 1**) was synthesized according to the method of Montedoro et al. (*22*); 3-formyl-3,4-dihydro-5-(methoxycarbonyl)-2-methyl-2*H*-pyran-4-acetic acid, 2-(3,4-dihydroxyphenyl)-ethyl ester (compound **V** in **Figure** 1), was obtained according to the method of Limirioli et al. (*23*) from oleuropein glycoside (Extrasynthese, Genay, France), by enzymatic reaction using β-glycosidase from almonds (Sigma, St. Louis, MO); 4-formyl-3-(oxoethyl)-4-hexenoic acid, 2-(3,4-dihydroxyphenyl)ethyl ester (compound **III** in **Figure 1**), was isolated from olive leaves according to the procedure of Paiva-Martins and Gordon (*24*). 4-Formyl-3-(2-oxoethyl)-4-hexenoic acid, 2-(4-hydroxyphenyl)ethyl ester, and 3-formyl-3,4-dihydro-5-(methoxycarbonyl)-2-methyl-2*H*-pyran-4-acetic acid, , 2-(4-hydroxyphenyl)ethyl ester (compounds **IV** and **VI** in **Figure 1**), were identified by a comparison with a reference sample obtained by Dr. Arturo Cert, Instituto de la Grasa (Sevila, Spain).

Quantification. Polar phenolics were quantified at 280 nm using syringic acid as internal standard and the response factors determined by Mateos et al. (*21*).

Oxidative stability was expressed as the oxidation induction time (hours) measured with the Rancimat 679 apparatus (Metrohm AG, Basel, Switzerland), using an EVOO sample of 3.5 g warmed to 100 °C and an air flow of 10 L/h (*25*).

Antioxidant activity was evaluated by measuring the radical scavenging effect of EVOO methanolic extract toward the synthetic radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH), as reported previously (*12*, *26*). Briefly, after the addition of methanol (5 mL), EVOO (2 g) was vigorously stirred for 1 h at room temperature and then centrifuged (4500*g* at 15 °C for 10 min) to separate the polar and the lipid fractions. Different dilutions of the methanolic extracts of EVOO were added to a 25 mg/L methanolic solution of DPPH. The decrease in absorbance was determined at 515 nm after 15 min (when a constant value was reached). The percent decrease in DPPH concentration was calculated, and a dose−response curve was constructed. The amount of EVOO required to lower the initial DPPH concentration by 50% (I_{50}) was interpolated by the dose−response curve. A calibration curve was determined using Trolox as a standard, and the antioxidant activity was expressed as micromoles of Trolox equivalents per kilogram (TE), that is, the ratio of the I_{50} of Trolox (μmol) to the I_{50} of the sample (kg).

Statistical Analysis. Data regression and analysis of variance were conducted with Statgraphics 5.1 (STCC Inc., Rockville, MD); Fisher's least significant difference (LSD) procedure ($P < 0.05$) was used to discriminate among the means.

RESULTS

The fatty acid composition of the four monovarietal EVOOs studied is shown in **Table 1**. The main factors that affect major EVOO components, namely, triacylglycerols, are genetic; the effects of environmental factors are less important. The high monounsaturated-to-polyunsaturated fatty acid ratio, which is typical of olive oil, is one of the main reasons for the higher stability of olive oil with respect to other edible oils (*7*). However, this ratio varies widely according to olive variety.

Table 1. Fatty Acid Composition (Percent) of the Monovarietal EVOOs Studied

fatty acid	Colombaia	Taggiasca	Arbequina	Picual
C16:0	13.5	13.1	12.37	9.43
C16:1	1.44	1.11	1.33	0.68
C17:0	0.44	0.51	0.11	0.06
C17:1	0.08	0.09	0.25	0.09
C18:0	2.54	0.01	1.76	3.41
C18:1	62.5	71.5	74.0	81.2
C18:2	18.4	9.74	8.88	3.64
C18:3	0.68	0.79	0.53	0.57
C20:0	0.44	0.43	0.38	0.46
C20:1	0.25	0.31	0.34	0.31
C22:0	0.12	0.13	0.12	0.13

Among the olive varieties chosen for the present study, Picual has the highest monounsaturated-to-polyunsaturated fatty acid ratio (19.5), whereas Colombaia has the lowest (4.4). These values represent the upper and lower limits reported for olive oil triacylglycerol composition (*27*). Taggiasca and Arbequina EVOOs have intermediate values.

The second major reason for EVOO stability is its antioxidant content (*7*), which depends on genetic, environmental and technological factors. For the present study we selected Picual EVOO with 709 mg/kg of polar phenolics and 194 mg/kg of compound **VII**, Taggiasca EVOO with 527 mg/kg of polar phenolics and 190 mg/kg of compound **VII**, Colombaia EVOO with 389 mg/kg of polar phenolics and 81 mg/kg of compound **VII**, and Arbequina EVOO with 84 mg/kg of polar phenolics and 238 mg/kg of compound **VII**. On the basis of fatty acid and antioxidant contents, Picual EVOO and Colombaia EVOO would be expected to be the most and least stable ones, respectively.

During storage at 40 °C antioxidant degradation occurred with a similar pattern in all the monovarietal EVOOs. An example of the behavior of Picual and Colombaia EVOOs is provided in **Figure 2**. Secoiridoid aglycones, namely, compounds **III**−**VII** decreased following pseudo-first-order kinetics (**Table 2**). The goodness-of-fit of the model was evaluated by the correlation coefficients (R), the P values, and the comparison between the observed and calculated values. The same kinetic behavior was observed for total polar phenolics and compound **VII**, when EVOO oxidation occurred under illumination, at 30 °C (*28*). In the present study the degradation rate constants of individual phenolics were determined within cultivars. Oleuropein derivatives, namely, compounds **III** and **V**, were less stable than the corresponding ligstroside derivatives, namely, compounds **IV** and **VI**. This result is interesting, as a previous investigation showed that the main determinants of EVOO antioxidant activity are phenolic compounds that share ortho-diphenolic structures, such as compounds **I**, **III**, and **V**, rather than compound **II** and its derivatives (*12*).

The degradation rates of compound **VII** were similar to those of compound **VI** except in Colombaia EVOO, in which the degradation of compound **VII** was faster than that of compound **VI**. Losses by oxidation would be expected to dominate in this EVOO.

Conversely, a previous study reported that compound **VII** was the first to be oxidized during EVOO autoxidation (*16*). This difference may be attributed to the noticeably higher phenolic content in the EVOOs considered in the present study with respect to the work cited above.

The degradation constant rate of compound **III** was higher in Colombaia and Taggiasca EVOOs than in the other EVOOs, whereas the degradation constant rate of compound **VII** was

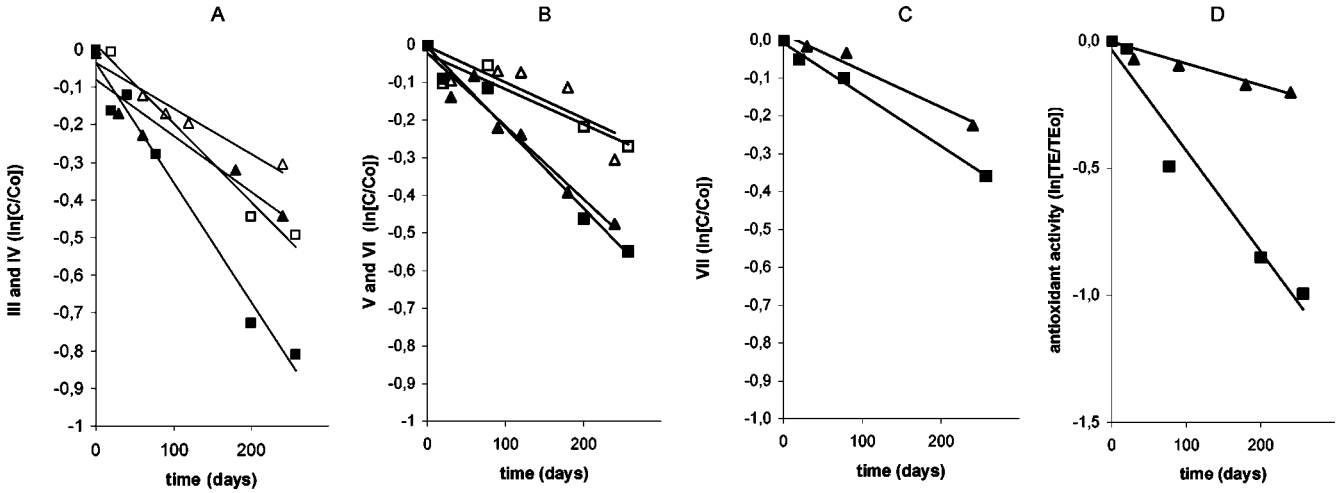

Figure 2. Degradation of compounds **III** (■ and ▲ in **A**), **IV** (□ and △ in **A**), **V** (■ and ▲ in **B**), **VI** (□ and △ in **B**), and **VII** (■ and ▲ in **C**) and antioxidant activity (■ and ▲ in **D**) of Picual (triangles) and Colombaia (squares) EVOOs stored at 40 °C. Fitting of data with pseudo-first-order kinetics is reported in **Tables 2** and **4**.

Table 2. Kinetic Parameters for Antioxidant Degradation in the Monovarietal EVOOs Stored at 40 °C[a]

		C_0 (μmol/kg)						$C_{240days}$ (μmol/kg)			
				confidence limits						confidence limits	
compound	oil[b]	obsd	calcd	lower	upper	$K \times 10^3$ (days^{-1})	R	obsd	calcd	lower	upper
secoiridoid **III**	C	428 ± 21	412	411	413	−3.2 ± 0.3	−0.98	190 ± 40	191	174	204
	T	375 ± 25	365	364	366	−3.8 ± 0.2	−0.95	136 ± 17	144	134	156
	P	985 ± 8	888	887	889	−1.5 ± 0.3	−0.92	620 ± 34	632	596	665
	A	97 ± 2	92	91	93	−2.1 ± 0.3	−0.75	53 ± 3	54	50	60
secoiridoid **IV**	C	450 ± 66	478	477	479	−2.4 ± 0.2	−0.90	275 ± 18	271	249	291
	T	497 ± 22	572	571	573	−2.2 ± 0.2	−0.91	315 ± 22	338	312	365
	P	506 ± 12	502	501	503	−1.9 ± 0.1	−0.90	377 ± 11	319	306	363
	A	64 ± 4	74	73	75	−1.8 ± 0.3	−0.93	39 ± 2	40	36	45
secoiridoid **V**	C	31 ± 2	32	31	33	−1.9 ± 0.2	−0.91	20 ± 2	20	15	25
	T	400 ± 5	459	458	460	−2.5 ± 0.2	−0.94	232 ± 14	255	238	274
	P	505 ± 17	497	496	498	−2.0 ± 0.1	−0.94	311 ± 24	339	290	374
	A	67 ± 1	72	71	73	−2.2 ± 0.3	−0.91	55 ± 13	45	40	50
secoiridoid **VI**	C	97 ± 22	90	89	91	−0.92 ± 0.20	−0.88	77 ± 5	72	66	78
	T	142 ± 44	118	117	119	−0.95 ± 0.08	−0.97	99 ± 7	94	91	97
	P	178 ± 3	184	178	192	−0.83 ± 0.10	−0.71	131 ± 14	151	144	161
	A	20 ± 2									
α-tocopherol **VII**	C	187 ± 5	187	167	206	−1.4 ± 0.1	−0.96	130 ± 10	131	119	148
	T	442 ± 10	441	403	482	−0.61 ± 0.09	−0.88	378 ± 22	380	344	416
	P	450 ± 10	450	419	478	−0.81 ± 0.07	−0.97	367 ± 13	369	344	395
	A	536 ± 6	539	503	572	−0.77 ± 0.01	−0.94	446 ± 4	445	416	478

[a] Data were fitted to pseudo-first-order kinetics: $\ln(C) = \ln(C_0) + kt$. $P < 0.01$. [b] C, Colombaia; T, Taggiasca; P, Picual; A, Arbequina.

higher only in Colombaia EVOO. There were no additional differences in the degradation constant rates of the other compounds among the different EVOOs.

Some authors found that the concentrations of compounds **I** and **II** increased during storage, as a result of hydrolysis of compounds **III**−**VI** (*29*). In the present study, the formation of compounds **I** and **II** fitted pseudo-zero-order kinetics for Picual and Arbequina EVOOs (**Table 3**); however, for Colombaia and Taggiasca EVOOs it was not possible to model the changes of these simple phenolics, as they showed an increase followed by a decrease. In any case, for all EVOOs, the increase in these simple phenolics was markedly lower than the decrease in compounds **III**, **VI**, **V**, and **VI**, indicating that complex phenolic degradation occurred via both hydrolysis and oxidation.

In accordance with this latter result, it was found that the antioxidant activity of EVOOs, as evaluated by DPPH radical scavenging activity, decreased during storage (**Table 4**). The decrease in EVOO antioxidant activity followed pseudo-first-order kinetics, with rate constants ranging from 0.85×10^{-3} to 4.1×10^{-3} days^{-1} at 40 °C and from 0.8×10^{-3} to 1.5×10^{-3} days^{-1} at 25 °C. Colombaia EVOO was the least stable, followed by Taggiasca, Arbequina, and Picual EVOOs. As expected in view of its high antioxidant content and low polyunsaturated fatty acid content, Picual EVOO had the highest initial antioxidant activity and was the most stable. The lower stability of Colombaia EVOO can be attributed to its lower antioxidant content, lower oleic acid content, and higher polyunsaturated fatty acid content as compared to other EVOOs. The relatively high stability of Arbequina EVOO, despite its low polar antioxidant content (84 mg/kg), may be partially attributed to its high content in compound **VII** (238 mg/kg). The differences among EVOOs were more evident at 40 °C than at 25 °C.

It is remarkable that, after 240 days of storage at 40 °C, Picual EVOO was still excellent in terms of its antioxidant content

Table 3. Kinetic Parameters for **I** and **II** Increase in the Monovarietal EVOOs Stored at 40 °C[a]

		C_0 (μmol/kg)						$C_{240days}$ (μmol/kg)			
				confidence limits						confidence limits	
compound	oil[b]	obsd	calcd	lower	upper	K (μmol/kg·days^{-1})	R	obsd	calcd	lower	upper
hydroxytyrosol **I**	C	205 ± 21			39	0.47 ± 0.02	0.93	224 ± 23			
	T	133 ± 19			5.5	0.030 ± 0.001	0.85	170 ± 19			
	P	35 ± 1	41	40				147 ± 22	154	148	160
	A	2.8 ± 0.1	5.4	5.3				12 ± 1	13	12	14
tyrosol **II**	C	258 ± 24				0.20 ± 0.01	0.97	255 ± 30			
	T	229 ± 19				0.010 ± 0.003	0.68	256 ± 11			
	P	26 ± 1	28	27	29			77 ± 5	76	73	79
	A	8.0 ± 0.3	9	8	10			12 ± 1	11	10	12

[a] Data were fitted to pseudo-zero-order kinetics: $C = C_0 + kt$. $P < 0.01$. [b] C, Colombaia; T, Taggiasca; P, Picual; A, Arbequina.

Table 4. Kinetic Parameters for Antioxidant Activity Degradation in the Monovarietal EVOOs Stored at 25 and 40 °C[a]

		TE_0[b] (μmol/kg)						$TE_{240days}$ (μmol/kg)			
				confidence limits						confidence limits	
oil[c]	temp (°C)	obsd	calcd	lower	upper	$K \times 10^3$ (days^{-1})	R	obsd	calcd	lower	upper
C	25	794 ± 20	765	706	821	–1.5 ± 0.2	–0.95	532 ± 33	528	488	567
T	25	890 ± 20	880	821	934	–1.3 ± 0.2	–0.90	719 ± 40	645	590	699
P	25	2582 ± 35	2618	2540	2697	–0.8 ± 0.09	–0.95	2136 ± 58	2165	2080	2230
A	25	515 ± 3	503	482	523	–0.98 ± 0.1	–0.94	401 ± 23	399	380	416
C	40	794 ± 20	757	692	845	–4.1 ± 0.3	–0.97	294 ± 7	284	255	321
T	40	890 ± 20	880	820	943	–3.1 ± 0.2	–0.96	405 ± 12	419	387	454
P	40	2582 ± 35	2566	2515	2643	–0.85 ± 0.09	–0.89	2109 ± 99	2101	1918	2298
A	40	515 ± 3	544	523	567	–1.6 ± 0.2	–0.88	391 ± 52	368	350	387

[a] Data were fitted to pseudo-first-order kinetics: $\ln(TE) = \ln(TE_0) + kt$. $P < 0.01$. [b] TE, Trolox equivalents. [c] C, Colombaia; T, Taggiasca; P, Picual; A, Arbequina.

Table 5. Acidity, Peroxide Value (PV), Spectroscopic Indices in the UV Region, and Oxidative Stability of the Monovarietal EVOOs Stored at 25 and 40 °C[a]

oil[b]	time (days)	temp (°C)	acidity (% oleic acid)	PV (mequiv of O_2/kg)	K_{232}	K_{270}	oxidative stability[c] (h)
C	0		0.40a	7.2a	2.4a	0.20a	29a
	240	25	0.50b	14.6b	3.0b	0.27a	29a
	240	40	0.74c	15.0b	3.1b	0.33b	30a
T	0		0.30a	5.5a	1.9a	0.16a	42a
	240	25	0.37b	13.5b	2.8b	0.19a	39a
	240	40	0.53c	13.0b	2.8b	0.36b	45a
A	0		0.08 ± 0.01a	6.1a	1.5a	0.11a	40a
	240	25	0.10 ± 0.02ab	11.6b	2.3b	0.14b	31b
	240	40	0.12 ± 0.02b	12.7c	2.5b	0.23c	30b
P	0		0.17 ± 0.01a	5.4a	1.5a	0.12a	172a
	240	25	0.18 ± 0.02a	7.4b	1.9b	0.17b	163b
	240	40	0.26 ± 0.03b	9.0c	2.1b	0.16b	150a

[a] Within each variety mean values at time 0 and time 240 days bearing different letters are statistically different ($P \leq 0.05$). [b] C, Colombaia; T, Taggiasca; P, Picual; A, Arbequina. [c] Rancimat conditions: 100 °C and 10 L/h.

and antioxidant activity, which were higher than those of the other EVOOs at the beginning of storage.

As summarized in **Table 5**, the monovarietal EVOOs at the beginning of storage met the quality standards fixed by EU regulation (*13*) for the highest quality EVOO, namely, peroxide value (maximum value = 20 mequiv of O_2/kg), acidity (maximum value = 0.8% oleic acid), and K_{232} and K_{270} (maximum values = 2.5 and 0.20, respectively). However, Colombaia EVOO reached the limit set for the spectroscopic indices in the UV region at the beginning of storage. After 240 days of storage at 40 °C, the K_{232} and K_{270} limits were exceeded in Colombaia, Taggiasca, and Arbequina EVOOs, and the maximum value of free acidity was reached only by Colombaia EVOO. The limit set for the peroxide value was not exceeded. Thus, this index did not have any predictive value in the evaluation of the oxidative status of EVOO, as observed previously (*11*). After EVOOs were stored for 240 days at 25 °C, Colombaia and Taggiasca EVOOs exceeded the limits set for the spectroscopic indices. Therefore, according to the regulatory limits Colombaia EVOO was found to be the least stable, followed by Taggiasca EVOO, Arbequina EVOO, and Picual EVOO, as already shown by the decrease in antioxidant activity. It is remarkable that Picual EVOO met all of the regulatory limits even after 240 days of storage at 40 °C. The free acidity of Arbequina EVOO was very low. Free fatty acids have a pro-oxidant effect (*30*). Therefore, the moderately low C18:2 and C18:3, the high content of compound **VII**, and the low free fatty acid content of Arbequina EVOO can explain its higher stability with respect to Taggiasca and Colombaia EVOOs. The oxidative stability at the beginning of storage was very high in Picual EVOO (172 h), intermediate in Taggiasca and Arbequina EVOOs (40 h), and lower in Colombaia EVOO (29 h). The oxidative stability of EVOOs was not related to their antioxidant activity at the beginning of storage and after storage. Indeed, the oxidative stability test is carried out at elevated temperatures (100 °C), while air is bubbled through the hot oil sample, whereas the former is performed at 25 °C. Therefore, converting the oxidative stability test into the stability of EVOO antioxidants at lower temperatures is misleading, as already observed (*17*).

In the present study, we did not expect any simple relationships between the initial composition of EVOOs and the decrease in antioxidant activity during storage, as the factors that may have a pro-oxidative or antioxidative role in EVOOs

are numerous. Four varieties with different characteristics were chosen to define a range of antioxidant degradation rates during storage.

On the basis of the results reported above, the kinetics of antioxidant degradation was defined, and the behavior of individual compounds was studied under conditions that mimic the storage of EVOO from production to sale, that is, in closed dark bottles, at 25 and 40 °C. Results showed that despite antioxidant depletion, EVOOs with high antioxidant content are still excellent after 240 days of storage at the higher temperature considered. These data led to the conclusion that the beneficial properties of EVOOs due to antioxidant activity can be maintained throughout its commercial life.

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