

Kinetics of Diglyceride Formation and Isomerization in Virgin Olive Oils by Employing ^{31}P NMR Spectroscopy. Formulation of a Quantitative Measure to Assess Olive Oil Storage History

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Diacylglycerol isomers and free acidity were determined for five extra virgin olive oils of different initial acidities by employing a facile ^{31}P NMR methodology as a function of storage time and storage conditions. The kinetic treatment of the hydrolysis of triacylglycerols (TGs) and the isomerization of 1,2-diacylglycerols (1,2-DGs) to 1,3-diacylglycerols (1,3-DGs) during storage of 18 months at ambient temperature in the dark and light and at 5 °C in the dark showed that the isomerization is strongly dependent on the rate of the TGs hydrolysis, the initial free acidity (H_0) of the virgin olive oil samples, and storage conditions. Although the time-evolution of the diacylglycerols (DGs) depends on the TGs hydrolysis, the ratio D of the concentration of 1,2-DGs to the total amount of DGs was found to be independent of this factor. From the kinetic expression of the ratio D , a quantitative measure was formulated that allows the estimation of the storage time or age of virgin olive oils. Application of this quantitative measure to several olive oil samples of known and unknown storage history resulted in a very good agreement with respect to the actual storage time for up to 10–12 months of storage. For a longer storage period, where the isomerization of DGs is close to its equilibrium state, the calculated age index is only indicative.

KEYWORDS: olive oil; TGs; diacylglycerols; hydrolysis; isomerization; kinetics; storage time

INTRODUCTION

Diacylglycerols, usually termed as diglycerides (DGs), are found in virgin olive oils at low concentrations (1–3% w/w). They are formed as intermediate products of incomplete biosynthesis of triacylglycerols or triglycerides (TGs) (1) as well as upon hydrolysis of TGs during olive oil extraction and storage (2). Extraction and storage of olive oil produce further changes in DGs composition, due to the isomerization of 1,2-diacylglycerols (1,2-DGs) to 1,3-diacylglycerols (1,3-DGs) (3–6). Knowledge of the amounts and composition of DGs is of great importance for the evaluation of the quality of the olive oil. In particular, the ratio of 1,3-DGs/1,2-DGs or the ratio of 1,2-DGs to the total amount of DGs [$D = 1,2\text{-DGs}/(1,2\text{-DGs} + 1,3\text{-DGs})$] was found to be a useful index to assess the storage history of virgin olive oil (3, 4, 7–9). Nevertheless, these investigations consider the problem of olive oil age from a qualitative point of view, because they are unable to specify the duration of storage reflected on the DGs concentration. A few attempts have been made in the past to correlate the diacylglycerol content with storage history and/or to detect possible adulteration of virgin olive oil with deodorized and refined oils (3, 10). Perez-Camino et al. (3) reported the evolution of DGs and the ratio of 1,3-DGs/1,2-DGs for Spanish virgin olive oils as a function of storage time, temperature, and

free acidity. These investigators noted that the 1,2-DGs to 1,3-DGs isomerization depends on these parameters, and in fact it becomes faster with increasing temperature and free acidity. However, no quantitative (kinetic) information was extracted from these data. Serani et al. (10) studied the kinetics of the DGs isomerization in Italian olive oils of different free acidity with storage time in light. They also concluded that the rate of the 1,2-DGs to 1,3-DGs isomerization increases with free acidity. However, they did not include in their kinetic analysis the hydrolysis of TGs and did not study the specific effect of the exposure of olive oil in light during storage. Both factors are expected to influence the fate of DGs in olive oils. The TGs hydrolysis is expected to affect the kinetics of DGs isomerization upon increasing the concentrations of DGs and free fatty acids, whereas light exposure of olive oil is an important factor for commercial products stored for prolonged periods of time on the supermarket shelves.

In the present paper, we report a detailed kinetic study of DGs formation and isomerization in fresh extra virgin olive oil samples of Greek origin with varying initial acidity and DGs content as a function of storage time (18 months) at three different storage conditions: ambient temperature in the dark and in the light, and at 5 °C in the dark. The kinetic information will be used to establish quantitative measures that relate the olive oil age with DGs concentration and free acidity.

In previous publications (9, 11), we have introduced a facile ^{31}P NMR method to determine the DGs composition and free

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Table 1. Initial^a DGs Composition, Ratio D_0 , and Free Acidity (H_0) of Five Extra Virgin Olive Oils in $\mu\text{Mol/g}^b$ of Oil

region	variety	1,2-DGs ^b	1,3-DGs ^b	Total DGs ^b	D_0	H_0^c
Sitia	Koroneiki	25.63	1.57	27.20	0.94	8.7
Lesvos	Adramitiani	23.33	2.39	25.72	0.91	9.0
Iraklion	Koroneiki	27.69	5.72	33.41	0.83	14.8
Pilion	Local	41.43	5.19	46.62	0.89	29.8
Peza ^d	Koroneiki	26.78	3.27	30.05	0.91	8.5

^a At $t = 0$. ^b $\mu\text{mol/g}$ are converted to mg/g multiplying by a factor of 0.621. ^c Acidity values are converted to % oleic acid dividing by the factor 35.5. ^d This virgin olive oil was used as a test sample.

acidity in olive oils. This method is based on the phosphitylation of the labile hydrogens of the hydroxyl and carboxyl groups of DGs and free fatty acids, respectively, with the reagent 2-chloro-4, 4, 5, 5-tetramethyl dioxaphospholane and the use of the ³¹P chemical shifts to identify the phosphitylated compounds. The same analytical approach will be used in the present study to detect and quantify the DGs and free acids in olive oils.

MATERIALS AND METHODS

Samples. Five virgin olive oil samples were obtained from three regions of Crete (Sitia, Peza, and Iraklion) and the regions of Lesvos and Pilion. The virgin olive oils samples were provided by the local cooperatives and produced by the same method of extraction (centrifugation). Virgin olive oils were extracted within 48 h after harvesting and stored immediately at $-20\text{ }^\circ\text{C}$. The samples from Crete were extracted from the same olive variety (Koroneiki), whereas those from Lesvos and Pilion originated from different varieties (adramitiani from Lesvos and a local variety from Pilion). All samples were collected during the periods 2000–2001 and were virgin according to the official analytical methods and limits (12, 13). **Table 1** summarizes the initial composition values of DGs, the initial ratio D_0 , and the initial free acidity (H_0) of the five olive oil samples as determined by ³¹P NMR spectroscopy. The virgin olive oil from Peza was used as a test sample for storage time prediction, and hence, it was excluded from the kinetic treatment.

Aliquots of each olive oil sample were stored for eighteen months in the dark at $5\text{ }^\circ\text{C}$, at ambient temperature (in dark glass bottles), and at ambient temperature in light (in transparent bottles). Two batches of samples were periodically analyzed.

NMR Experiments. Details of the experimental protocol for the synthesis of the phosphitylated agent, sample preparation, and the ³¹P NMR experiments can be found elsewhere (9, 11, 14). The applicability of this method to quantitative analysis, as well as its reproducibility and repeatability, has been tested thoroughly in previous publications (9, 11, 14, 15).

Analytical solutions and numerical calculations of the differential equations obtained from kinetic schemes were performed by using the software package Mathematica 4.1 for Windows (Wolfram Research Inc.). Nonlinear fittings of the experimental data to the appropriate equations (see below) were carried out with Statistica 5.1B for Windows (Statsoft Inc.).

RESULTS AND DISCUSSION

Data Analysis. **Figure 1** depicts the concentration changes of 1,2-DGs, 1,3-DGs, total DGs, and free acidity (H) in the four virgin olive oil samples during eighteen months of storage under different environmental conditions; at ambient temperatures and at $5\text{ }^\circ\text{C}$ in the dark (AD and 5D); and in light at ambient temperature (AL). Inspection of **Figure 1** reveals several interesting trends: (a) The concentration of 1,2-DGs decreases with storage time, while that of 1,3-DGs increases due to isomerization. Both changes are greatly accelerated with increasing temperature (compare the evolution 5D with AD). Temperature appears to have a similar effect on 1,2-DGs

diminution and 1,3-DGs growth under light exposure, although both processes occur with a faster rate (compare the evolution of AD with that of AL in **Figure 1**). (b) The parameters total DGs and free acidity (H) remain effectively constant at $5\text{ }^\circ\text{C}$ even after 18 months of storage, whereas the same parameters of the aliquots maintained at ambient temperature in the dark increase linearly with time. This observation supports an effective TGs hydrolysis at higher temperature, increasing thus the amounts of DGs and free fatty acids. Also, total DGs and free fatty acids increase with time in light (AL), although the growth is faster by a factor of 2–3 compared to that in the dark (AD). (c) Olive oil samples characterized by higher initial acidities (e.g., sample from Pilion) show faster evolution of both total DGs and free acidity at all storage conditions, indicating that both TGs hydrolysis and DGs isomerization are accelerated in olive oils of higher free acidities. This implies that free fatty acids act as catalysts for both procedures. Similar observations were made by Perez-Camino et al. (3), studying the evolution of DGs of virgin olive oils from *Picual* cultivar as a function of temperature and acidity, except perhaps that the growth of total DGs was not linear, especially at high free acidity (1.0% oleic acid). The nonlinear behavior at high free acidity could be attributed to the fact that their samples were spiked with oleic acid, thus perturbing the natural hydrolysis of TGs.

It is worth noting that no glycerol was detected in any of the virgin olive oil samples in this study. 1-Monoacylglycerols were detected in trace amounts ($<3\text{ }\mu\text{mol/g}$ of oil) only in the sample characterized by the highest acidity (Pilion) and at long storage times. It appears that further hydrolysis of DGs to monoacylglycerols in the present olive oil samples occurs very slowly.

The effect of free acidity on the concentration changes of DGs during storage is illustrated in **Figure 2a**, where the ratio D for the four olive oil samples at ambient temperature in the dark (AD) is plotted against time. It is evident that olive oils with high free acidity values (e.g., Pilion, Iraklion) approach the equilibrium value of $D \sim 0.3$ at shorter storage time. A similar plot is obtained for samples stored at ambient temperature in light (not shown). The dependence of DGs content on free acidity can be shown in an alternative way by plotting the ratio D/D_0 (D_0 is the value of D at $t = 0$) versus storage time. This plot is shown in **Figure 2b** for the olive oil samples stored at $5\text{ }^\circ\text{C}$ in the dark (5D). Again, the ratio D/D_0 for the olive oil samples characterized by higher free acidity goes faster toward its equilibrium value.

Hydrolysis of TGs in Olive Oils. As mentioned before, total DGs and H increase linearly with time (**Figure 1**) at both storage conditions AD and AL, owing to hydrolysis of TGs. Assuming that hydrolysis follows a first order reaction, and taking into consideration that the TGs concentration is practically constant (it is much higher than that of DGs), we can fit the total DGs and free acidity data in **Figure 1** using the following equations:

$$[\text{DGs}] = [\text{DGs}]_0 + k_h t \quad (1)$$

$$H = H_0 + k_h t \quad (2)$$

where $[\text{DGs}]_0$ and H_0 are the initial total DGs concentration and free acidity (at time $t = 0$), respectively (**Table 1**), and $k_h = k_h'$ $[\text{TGs}]_0$ is the apparent rate constant of TGs hydrolysis. The calculated k_h values for the four olive oil samples at different storage conditions and the statistics of the fitting are summarized in **Table 2**. The data in this table indicate that total DGs and free acidity grow faster as a function of storage time for olive oils with high initial free acidities H_0 (Pilion, Iraklion) at both storage conditions. Also, k_h appears to be 2–3 times larger for AL compared to AD.

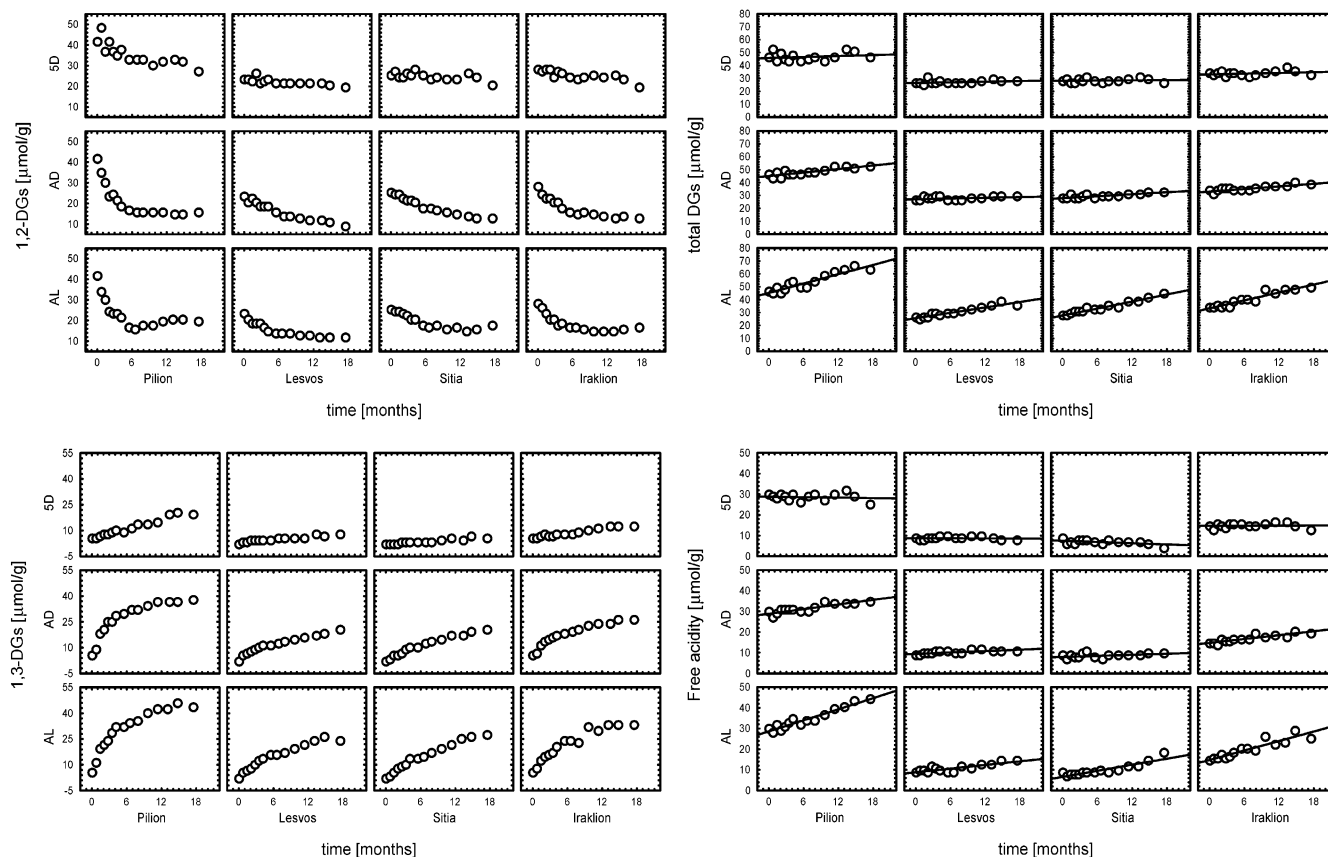
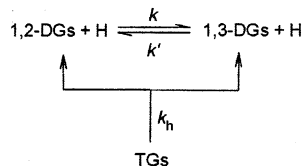


Figure 1. Time-evolution of 1,2-DGs, 1,3-DGs, total DGs, and free acidity for four different extra virgin olive oils of Greek origin stored at ambient temperatures in the dark (AD) and in light (AL), and at 5 °C in the dark (5D) for a period of 18 months.

It has been suggested (16) that during storage the DGs in olive oils are formed by acid-catalyzed and/or enzymatic hydrolysis of TGs. The observed scaling of k_h with H_0 suggests that the acid-catalyzed pathway is more important during prolonged storage of olive oil samples. Moreover, the 3-fold increase in the rate of TGs hydrolysis for olive oils stored under light could be explained on the basis that the oxidation products of the unsaturated fatty acids contained in olive oils may have a strong accelerating effect on the TGs hydrolysis.

Isomerization of DGs During Storage. Figure 3 illustrates the variation of the parameters D (in dark and light) and D/D_0 (at 5 °C in dark) for the four olive oil samples with the product of H and storage time t (i.e., $H \times t$). It is notable that 120 D values (Figure 3a) and 60 D/D_0 values (Figure 3b) fall on the same master curves, indicating that the four virgin olive oils share common DGs isomerization kinetics at each temperature, when the effect of different free acidity values is taken into account. A detailed kinetic scheme for the DGs isomerization in olive oils should take into consideration the following conditions: First, the isomerization process is an acid-catalyzed reaction by the free fatty acids liberated from TGs hydrolysis. Second, it cannot proceed in isolation from TGs hydrolysis. Third, it should account for the increase of both total DGs and free acidity with storage time. Such a kinetic scheme is shown below:



This kinetic scheme is different from that proposed by Serani et al. (10), because TGs hydrolysis and the catalytic effect of free fatty acids are implicitly incorporated. k and k' are the rate constants for the isomerization process. The evolution of 1,2-DGs, 1,3-DGs, total DGs, and the ratio D ($[1,2\text{-DGs}]_t / [\text{total DGs}]_t$) as a function of time for a range of k_h values between 0 and $1 \mu\text{mol g}^{-1} \text{month}^{-1}$ is followed by numerically solving the three differential equations (Appendix) resulted from the kinetic scheme. Initial values ($t = 0$) for the DGs and free acidity (H_0) of the virgin olive oil sample from Pilion (Table 1) were used, as well as those of $k_s = 17.4 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{month}^{-1}$ and $D_{\text{eq}} = 0.32$ estimated from the experimental data (see below). It should be noted that the kinetic equations in the appendix assume that both 1,2-DGs and 1,3-DGs are produced by TGs hydrolysis (17, 18) and that the probability of 1,2-DGs molecules formation is twice that of 1,3-DGs molecules, because there exist two sn-1,3 and one sn-2 positions on TGs molecules.

The results of the calculations shown graphically in Figure 4 indicate that the change in the concentration of 1,2-DGs, 1,3-DGs, and total DGs depends strongly on the rate of TGs hydrolysis (Figure 4a). The same is true for H depicted in Figure 4a. Interestingly, the ratio D is independent of the rate of TGs hydrolysis (Figure 4b), even in cases where TGs hydrolysis produces solely 1,2-DGs or 1,3-DGs (not shown). In this respect, we decided to use a simpler kinetic scheme that retains the growth of free acidity during storage but omits the production of 1,2-DGs and/or 1,3-DGs from TGs. The new kinetic scheme describes the isomerization of 1,2-DGs to 1,3-DGs, but contrary to that proposed by Serani et al. (10), incorporates implicitly the effect of free acidity (as a catalyst). Now, the differential equations describing the concentration changes of 1,2-DGs and 1,3-DGs with time can be solved

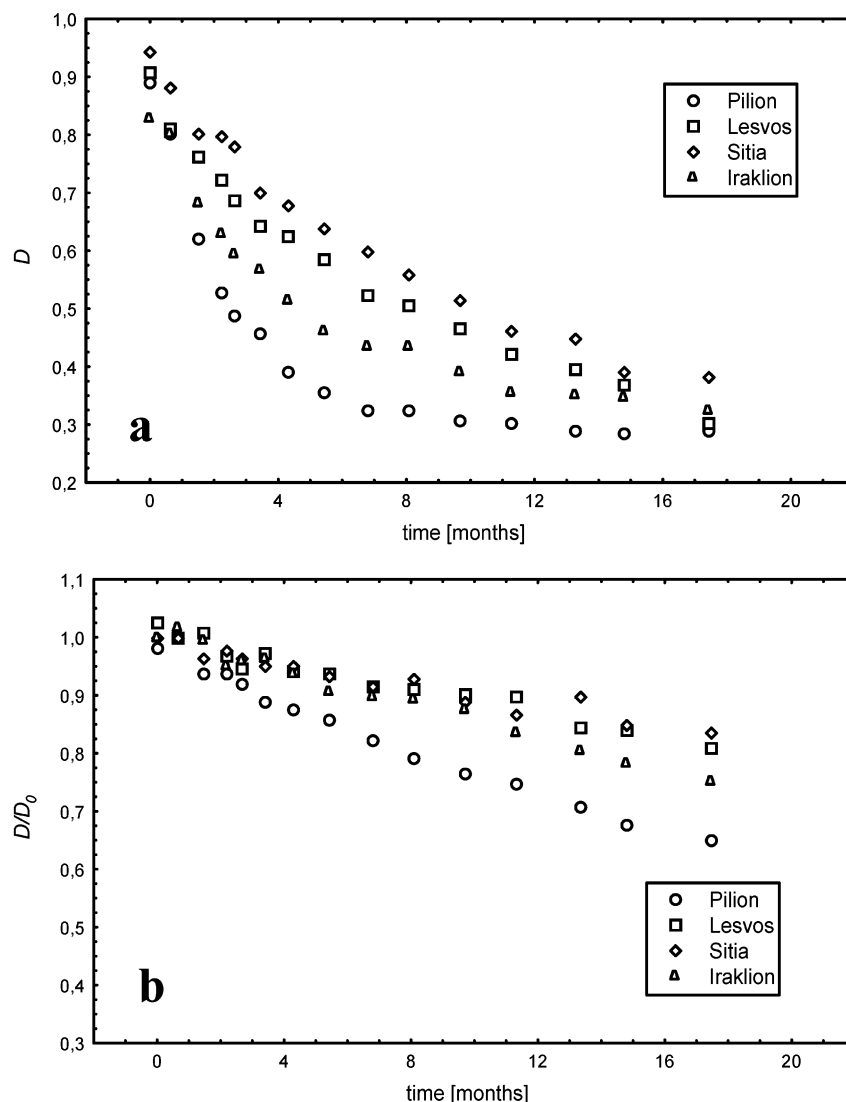


Figure 2. (a) Time-dependence of the ratio D ($D = 1,2\text{-DGs}/\text{total DGs}$) for four different extra virgin olive oils of Greek origin stored at ambient temperatures in the dark (AD) and in light (AL); (b) time-dependence of the ratio D/D_0 for the same samples stored at 5 °C in the dark (5D) for a period of 18 months. D_0 is the value of D at time zero.

analytically. Substituting the concentrations of 1,2-DGs and 1,3-DGs in the ratio of D [$= 1,2\text{-DGs}/(1,2\text{-DGs} + 1,3\text{-DGs})$], we obtain the following exact relationship:

$$D = \left(\frac{k'}{k + k'} \right) + \left(\frac{[1,2\text{-DGs}]_0 k - k' [1,3\text{-DGs}]_0}{(k + k') [\text{DGs}]_0} \right) \cdot e^{-(1/2)(k+k')[H_0+H]t} \quad (3)$$

where $[1,2\text{-DGs}]_0$, $[1,3\text{-DGs}]_0$, and $[\text{DGs}]_0$ are the initial concentrations of DGs and total DGs and H_0 is the initial free acidity (at $t = 0$). At long storage times ($t \rightarrow \infty$), the isomerization reaction of the DGs has reached the equilibrium state, and the ratio D equals its equilibrium value D_{eq} . In this case, eq 3 becomes

$$D_{\text{eq}} = \frac{k'}{k + k'} \quad (4)$$

The value of D_{eq} has been reported by several authors (3, 4, 8, 9, 16, 19) in the past to be in the range between 0.3 and 0.33. This means that the forward isomerization rate constant k

Table 2. Kinetic Rate Constants k_h of TGs Hydrolysis in Extra Virgin Olive Oils Stored at Ambient Temperature in the Dark and in Light

region	k_h [$\mu\text{mol g}^{-1} \text{month}^{-1}$]	
	dark ^a	light ^b
Sitia	0.17 ± 0.12	0.70 ± 0.29
Lesvos	0.10 ± 0.02	0.50 ± 0.28
Iraklion	0.30 ± 0.06	0.89 ± 0.18
Pilion	0.41 ± 0.06	1.05 ± 0.22

^a $r = 0.841\text{--}0.812$ and $0.874\text{--}0.828$ upon fitting the experimental data of DGs and H , respectively, for the four olive oil samples. ^b $r = 0.972\text{--}0.932$ and $0.962\text{--}0.907$ upon fitting the experimental data of DGs and H , respectively, for the four olive oil samples.

is about twice as large as the backward rate constant k' . Substituting in eq 3, D_{eq} from eq 4, the initial ratio $D_0 = [1,2\text{-DGs}]_0/[\text{DGs}]_0$, and by defining $k_s = k' + k$, we obtain the simple equation

$$D = D_{\text{eq}} + (D_0 - D_{\text{eq}}) \cdot e^{-(1/2)k_s[H_0+H]t} \quad (5)$$

At low temperatures, such as 5 °C, the isomerization reaction is very far from equilibrium, and practically no TGs hydrolysis

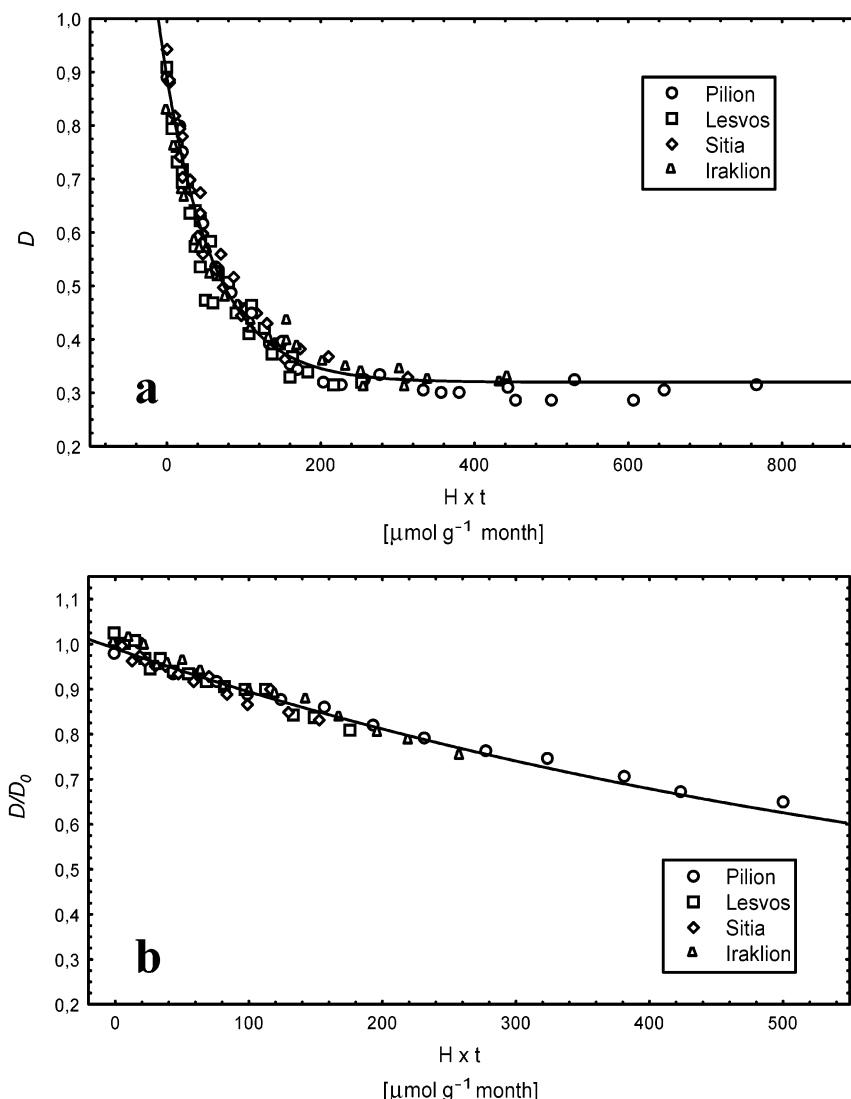


Figure 3. (a) Plot of the ratio D ($D = 1,2\text{-DG}/\text{total DGs}$) for four extra virgin olive oils of Greek origin and different initial free acidities (H_0) as a function of the product $H \times t$, where H is the free acidity of each sample at storage time t at ambient temperature in the dark or light for a period of 18 months; (b) plot of D/D_0 for the same samples stored at 5°C in the dark. D_0 is the value of D at time zero. The solid lines in both plots represent the fit of eq 5 to the experimental data.

occurs. This means that H at time t is practically constant and equal to H_0 at time zero, as observed in **Figure 1**. In this case, eq 5 becomes

$$D = D_0 e^{-k_s[H_0]t} + D_{\text{eq}}(1 - e^{-k_s[H_0]t}) \quad (5a)$$

Because k_s is expected to be much smaller at 5°C than at ambient temperature (see above), the second term in eq 5a is very close to zero for short times, since the exponential in parentheses of eq 5a is ~ 1 . This term starts making a measurable contribution ($\sim 5\%$) only after $t > 14$ months. Therefore, this term can be removed safely from eq 5a, fitting the data at low temperature to eq 6.

$$D = D_0 \cdot e^{-K_s[H_0]t} \quad (6)$$

The rate constant k_s and the equilibrium value D_{eq} can be obtained by fitting the experimental data of 1,2-DGs, 1,3-DGs, and H from **Figure 1** and those of D_0 from **Table 1** to eq 5 (storage conditions AD and AL) and eq 6 (storage condition 5D). The fit of 120 data points gave $k_s = 17.4 \times 10^{-3} \text{ g mol}^{-1}$

month^{-1} and $D_{\text{eq}} = 0.32$ at ambient temperature in dark and light, whereas the fit of 60 data points gave $k_s = 1.39 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$ at 5°C in the dark. k_s is more than an order of magnitude smaller at 5°C compared to that calculated at ambient temperature. This finding proves in a quantitative manner that temperature has a strong effect on DGs isomerization during storage. The small extent of isomerization at 5°C (**Figure 1**) does not permit the calculation of D_{eq} for the three olive oil samples of low acidity by employing eq 6. However, application of this equation to the data of the olive oil with the highest free acidity (Pilion) (**Table 1**) allows an estimation of the equilibrium value D_{eq} , which was found to be 0.32 at 5°C . The calculated values of D_{eq} at all temperatures and storage conditions are within the range of values (0.30–0.33) reported in the literature (3, 4, 8, 9, 16, 19). The same equilibrium value has been reported earlier (3) for olive oils subjected to neutralization and deodorization. The extreme conditions applied in such procedures (strong basic medium, intense heating) accelerate the isomerization of DGs.

To further support the present kinetic analysis of DGs isomerization, we plotted the evolution of D and D/D_0 according

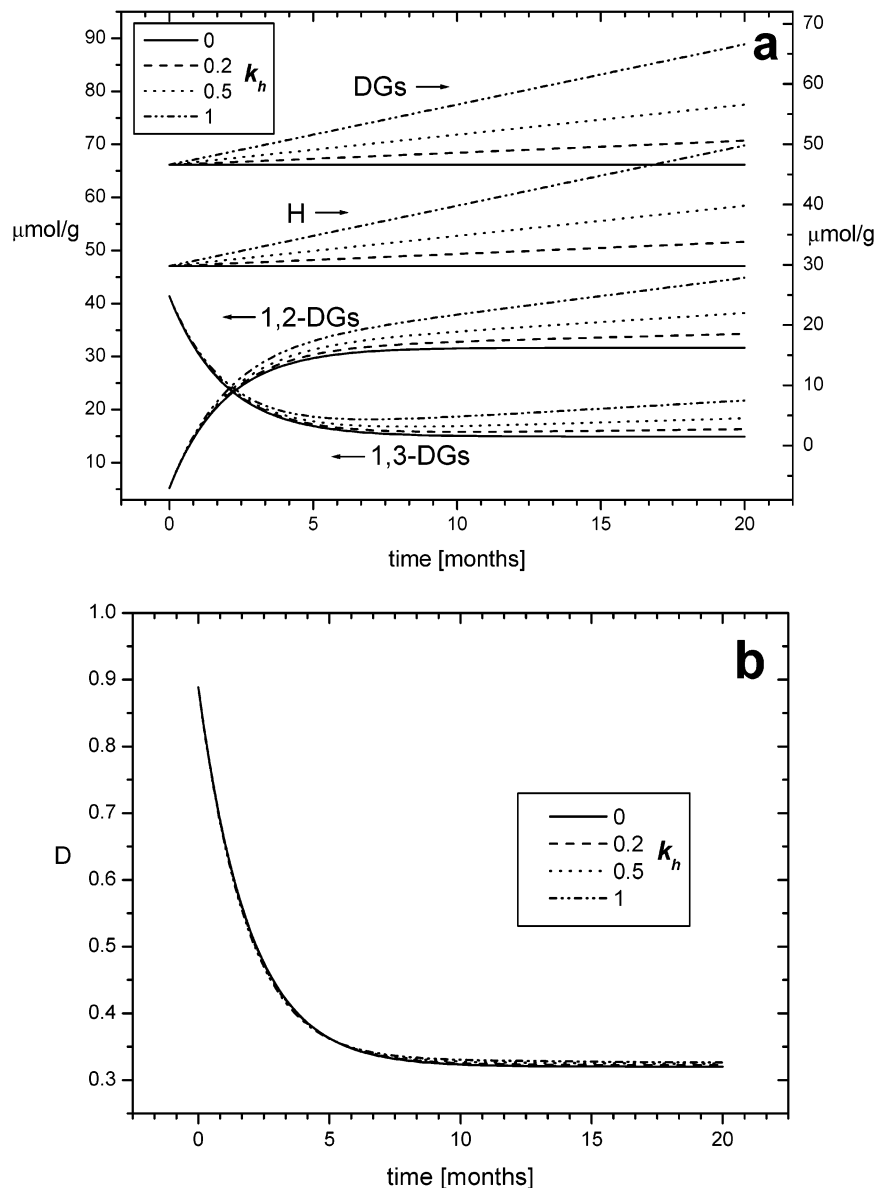


Figure 4. Time-evolution of (a) 1,2-DGs, 1,3-DGs, total DGs, free acidity (H), and (b) the ratio D as a function of the rate constants k_h of TGs hydrolysis. Results were obtained through numerical solution of the differential equations (Appendix) of the kinetic scheme (see text) by using initial values of $[1,2\text{-DG}]_0$, $[1,3\text{-DG}]_0$, H_0 from Table 1 (sample from Pillion), $k_s = 17.4 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$, and $D_{\text{eq}} = 0.32$.

to eqs 5 and 6 using the values of k_s and D_{eq} obtained above at each temperature. This procedure resulted in D and D/D_0 values that lied in the corresponding master curves shown by the solid lines in Figure 3. This confirms an earlier conclusion that the DGs isomerization in the four olive oil samples studied share a common kinetic scheme regardless of storage conditions. Finally, we applied eqs 5 and 6 to the 1,2-DGs and 1,3-DGs data published by Perez-Camino et al. (3) for the DGs evolution during storage of two virgin olive oils from the *Picual* cultivar with different acidities (0.5 and 1% oleic acid) at 5 and 25 °C in the dark and for a period of one year (Figure 3 in ref 3). Values of $k_s = 1.4 \pm 0.2 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$ at 5 °C, and $k_s = 16 \pm 2 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$ and $D_{\text{eq}} = 0.31 \pm 0.01$ at 25 °C were obtained upon fitting these data, in very good agreement with our present results.

Formulation of a Quantitative Measure of Storage Time. The ratio D appears to be a promising measure for determining the age of virgin olive oil, because it is independent of the rate

of TGs hydrolysis (eq 5). Moreover, it is well established (3, 4, 6, 8, 9, 14) that the initial D_0 values for freshly extracted virgin olive oils is close to 1, usually in the range of 0.90–0.95. The problem of unknown H_0 can be surpassed if we consider the form of the exponential term in eq 5. Because of TGs hydrolysis, $H_0 < (H_0 + H)/2 < H$ for any virgin olive oil at any storage time $t > 0$. The time-dependent D values can be fitted equally well if one uses solely the parameters H_0 or H in eq 5, the only difference being the magnitude of the best-fitted k_s value. This is expected to be slightly larger ($k_s = 18.7 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$) using H_0 , or slightly smaller ($k_s = 16.5 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$) using H , compared with that obtained upon fitting the exact form of eq 5. The former k_s value overestimates the true value ($k_s = 17.4 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$) by 7%, whereas the latter rate constant underestimates the true value by 5%. Thus, by using $k_s = 16.5 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$, the value of 0.32 for D_{eq} , replacing the exponential $[(H_0 + H)/2] \times t$ by $H \times t$ in eq 5, and taking the logarithm of both sides

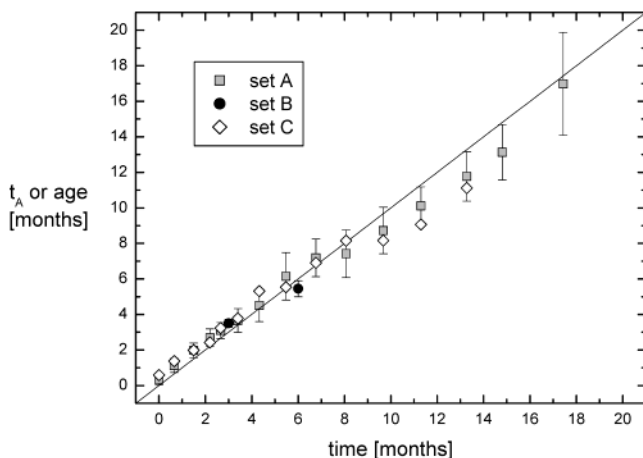


Figure 5. Plot of the calculated mean storage time (t_A , or age) vs actual storage time t for three data sets of extra virgin olive oil samples (see text). Data were obtained by using eq 7 with $k_s=16.5 \times 10^{-3} \text{ g } \mu\text{mol}^{-1} \text{ month}^{-1}$, $D_0 = 0.94$ and $D_{\text{eq}} = 0.32$. Error bars show the standard deviations of t_A for the individual samples within each set from the corresponding mean value.

of eq 5, we obtain an expression, which provides the age (t_A) of a virgin olive oil stored under ambient conditions.

$$\text{age} = t_A = \frac{-\ln\left(\frac{D - 0.32}{D_0 - 0.32}\right)}{0.0165 \times H} \quad (7)$$

For storage at 5°C , the relevant expression is given by

$$\text{age} = t_5 = \frac{-\ln\left(\frac{D}{D_0}\right)}{1.39 \times 10^{-3} \times H} \quad (8)$$

The denominator in eq 7 becomes $0.586 \times H$, if free acidity is expressed in % oleic acid. Equations 7 and 8 allow the determination of the storage time or the age of an olive oil sample, provided that simple measurements for 1,2-DGs, 1,3-DGs, and free acidity have been performed at time t .

Prediction of Storage Time (Age) of Virgin Olive Oils.

To test the ability of eq 7 to predict the age of virgin olive oils, three sets of DGs and free acidity measurements were used. Set A comprises the data of the four virgin olive oil samples stored in dark and light and used in the present kinetic study. Set B is composed of four independent virgin olive oil samples originated from the same regions as those of the set A but extracted during the 2001–2002 crop season and stored at ambient temperatures in dark. The DGs and free acidity of these samples were measured after 0, 3, and 6 months of storage. Finally, set C is consisted of the data of one virgin olive oil sample from Peza stored at ambient temperatures under light for 13 consecutive months. Measurements of the diglyceride concentration and free acidity were made at approximately one-month intervals. The data for each individual sample of the test sets B and C are available as supporting material. It should be stressed that the data of sets B and C have not been used to fit eq 5, as described previously. **Figure 5** illustrates the plot of the calculated mean storage times t_A for the three data sets of olive oil samples against the actual storage time. Calculations were performed by using eq 7 and a value of $D_0 = 0.94$, typical for freshly extracted virgin olive oils (3, 4, 6, 8, 9, 14). Additional calculations were conducted for different D_0 values ranging between 0.90 and 0.95 with insignificant changes in

the plot t_A versus actual storage time of **Figure 5**. Very good agreement is observed between the calculated age and the actual storage time for all virgin olive oil samples up to 10–12 months at ambient temperature (**Figure 5**). The slight underestimation of the true storage time for $t > 12$ months, sets an upper limit of the applicability of eq 7. Nevertheless, this discrepancy was expected, because at very long storage ($t > 12$ months), the D values are very close and sometimes smaller than the calculated equilibrium value $D_{\text{eq}} = 0.32$. The difference $D - D_{\text{eq}}$ in the numerator of eq 7 approaches zero or can assume negative values, when $D < D_{\text{eq}}$. Therefore, for DGs isomerization that has reached the equilibrium ($D \sim D_{\text{eq}}$), the calculated storage time of the respective olive oil by employing eq 7 is only indicative, leading to the conclusion that the olive oil is more than one year old. At the lower temperature of 5°C , eq 8 proves to be equally successful in estimating the storage time of the four virgin olive oils of the present study (not shown). However, it appears that this equation is of limited application, because olive oils are seldom stored at such low temperatures.

In a previous publication (9), we reported the DGs content and free acidity measured for several virgin olive oil samples harvested in 2000–2001, as well as for eight commercial extra virgin olive oils. Samples ($n = 29$) of fresh ($D_0 \geq 0.87$) virgin olive oils were selected randomly, and the calculated average age of these samples was found to be 0.5 ± 0.5 months, thus verifying the validity of eq 7. On the contrary, only four commercial extra virgin olive oils were aged lower than 12 months, while the remaining four samples were over aged (between 13 and 15 months). These findings indicate that the commercial olive oil samples were extracted from olive fruits harvested in previous crop seasons.

The concentration changes of DGs upon storage do not have an immediate effect on the organoleptic properties of olive oil. However, they do reflect in a quantitative manner the aging of olive oil as presented in this study. This is quite important, because aging is accompanied by degradation of the natural antioxidants of olive oil, such as squalene, α -tocopherol, and phenolic compounds (simple phenols, secoiridoids, and lignans), thus downgrading the quality of olive oil. After long storage, olive oil becomes rancid with poor nutritional and organoleptic characteristics (20–22). For instance, α -tocopherol was decomposed by 79% in 4 months storage under diffused light, whereas $\sim 45\%$ of the phenols were lost during the same period (23). Storage in dark somewhat impedes the degradation of antioxidants (except perhaps squalene), probably due to the absence of photooxidation (24, 25). Our results on olive oil aging suggest that a suitable way to inhibit rancidity process and obtain good stability of olive oil used in domestic kitchens is by storing olive oil in well-closed dark glass bottles or even in transparent glass bottles in carton boxes stored in dry places at low temperature ($< 15^\circ\text{C}$). Large industrial companies could follow similar storage practices, although the type of packaging (colored glass bottles) may be not appropriate. Usually, metallic and/or plastic containers are used for storage and transportation, with known disadvantages against oxidation (26, 27). At any rate, when plastic containers are used, they should be filled almost completely, reducing the headspace as much as possible, to exclude oxygen. Another possible measure to avoid oxygen and improve stability may be the loading of the headspace with an inert gas, preferably argon.

The present study showed that the quantitative determination of the DGs and free acidity of virgin olive oils are useful parameters to assess their age or storage time. This has been achieved by formulating an equation that relates the storage time

with the DGs concentration and free acidity of olive oils at various storage conditions. This equation was derived on the basis of a kinetic analysis of the isomerization of 1,2-DGs to 1,3-DGs catalyzed by the free fatty acids produced during the hydrolysis of TGs. The amounts of 1,2-DGs, 1,3-DGs, and free acidity are the only parameters needed to calculate with high accuracy the age of virgin olive oils up to 12 months of storage.

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Supporting Information Available: Measurements of the diglyceride concentration and free acidity data for each individual sample of the test sets B and C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

APPENDIX

The set of the differential equations resulting from the kinetic scheme (see text)

$$\frac{d[1,2-DG]}{dt} = -k[1,2-DG]_t[H] + k'[1,3-DG]_t[H] + \frac{2k_h}{3} \quad (9)$$

$$\frac{d[1,3-DG]}{dt} = k[1,2-DG]_t[H] - k'[1,3-DG]_t[H] + \frac{k_h}{3} \quad (10)$$

$$\frac{d[H]}{dt} = k_h \quad (11)$$

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