Effects of Olive Fruit Quality and Oil Storage Practices on the Diacylglycerol Content of Virgin Olive Oils

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The evolution of 1,3- and 1,2-isomers of diacylglycerols (DGs) in olive oils obtained from healthy olives and the influence of the olive quality was studied. Healthy olive fruits yielded oils containing almost exclusively 1,2-isomers whereas altered olives produced oils with significant amounts of 1,3-isomers. Virgin olive oils obtained from various olive cultivars and stored at different temperatures showed triacylglycerol hydrolysis and diacylglycerol isomerization depending on the acidity and temperature. The results indicated that the relationship between acidity and total diacylglycerol content has scarce utility for detecting mild refined oil in virgin olive oil. On the other hand, the 1,3-/1,2-DG isomers ratio is useful for assessing the genuineness of virgin olive oils with low acidities during the early stages of storage.

Keywords: *Diacylglycerol; 1,3-isomer; 1,2-isomer; olive fruit quality; oil storage; virgin olive oils*

INTRODUCTION

Extra virgin olive oils are obtained from healthy olive fruits using extraction processes that produce minimal changes in oil composition. The determination of this oil category is mainly on the basis of the following parameters: free acidity, UV-absorption, peroxide index, and panel test score. It is technically possible to reduce the acidity level and undesirable odor of poor quality oils to obtain neutral oils that can be blended with extra virgin oils. This mild refining process essentially consists of neutralization, washing, and deodorization of the oil at low temperature under vacuum. Admixtures of virgin olive oils with mild refined oils are undetectable by the quality parameters included in the European Economic Community (EEC) olive oil regulations.

For the past 10 years the diacylglycerols (DGs) have been investigated for their usefulness as a marker of this practice. In virgin olive oils, DGs are present in a range of 1 to 3% and they are found as 1,2- and 1,3-isomers. The 1,2-isomers are attributed to the incomplete biosynthesis of triacylglycerols (TGs), whereas the 1,3-isomers are attributed to enzymatic or chemical hydrolysis of TGs occurring before or during the oil extraction process (*1*). Consequently, freshly made "just-obtained" olive oils from healthy olive fruits contain almost solely 1,2-DGs (*2*); on the other hand, those coming from poor-quality fruits show a significant increase of 1,3-isomers and free fatty acids (*3*). Alkaline neutralization of the oil produces a decrease of total DGs up to 10% and an increase of the 1,3-/1,2-DG ratio (*4*), but the effect of the deodorization process has not been investigated. During the storage of virgin olive oils, the amount of 1,2-DGs decreases, whereas the 1,3-DGs, the 1,3-/1,2-DG ratio, and the total DGs amounts were observed to increase. From these facts, several authors (*3*−*6*) suggested that the 1,2-DGs isomerize toward 1,3-DGs, the 1,3-/1,2-DG ratio is indicative of oil freshness, and the correlation between free fatty acids and total DGs is able to detect blends of virgin oil with mild refined oils.

The above results are mainly based on the analysis of numerous samples but not on the study of the hydrolysis and isomerization processes. In this work the influence of the fruit quality, the changes during refining process, and the kinetics during the oil storage are investigated, in order to determine the significance of the mentioned parameters. Factors such as temperature, acidity, and olive cultivar that can affect the isomerization and hydrolysis processes are taken in account. For DG determination, a simple analytical method using solid-phase extraction (SPE) and GC analysis on polar columns was used (*2*). Using this method the isomerization of DGs during isolation and GC analysis is negligible (*2*, *7*).

Following are the abbreviations that are used for referring to the fatty acids: L, linoleic acid; O, oleic acid; P, palmitic acid.

EXPERIMENTAL PROCEDURES

Samples. Several sets of olive oils samples were prepared. *Set 1.* Extra virgin olive oils were obtained from fruits of *Picual* cultivar. The olive fruits were picked from trees growing in the Instituto de la Grasa (Seville, Spain) from September to December. Oils were obtained in triplicate, using Abencor equipment (*8*), within 24 h after harvest. The oils were kept at −20 °C until analysis.

Set 2. Extra virgin olive oils from three olive cultivars (*Picual*, *Lechin* and *Hojiblanca*) were obtained in an olive oil mill using the "two phases" centrifugation mode. The olive fruits were of good quality and usual ripeness stage. Aliquots of these three oils were spiked with oleic acid up to 0.5 and 1.0% of acidity.

Set 3. Olive oils were obtained from altered olive fruits using the Abencor system. In order to obtain fusty oils, 500 kg of olives were piled and fruits from inside of the pile were periodically sampled.

Sample Treatments. *Storage.* Aliquots of Set 2 oils were stored at three different temperatures: 5, 25, and 40 °C for 1 year. Two batches of samples were periodically analyzed.

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Table 1. Diacylglycerol Composition of Oils Obtained from Healthy and Altered Olive Fruits

olive oil cultivar	alteration	acidity (%)	total DG (mg/g)	theor. DG max.[a] (mg/g)	Σ1,2[b] (mg/g)	Σ1,3[b] (mg/g)	Σ1,3/Σ1,2 ratio
Picual	none	0.10	12.1	10.0	10.9	0.6	0.06
	piled for 14 days	8.90	65.2	204	48.8	14.8	0.30
	piled for 28 days	8.80	27.1	201	14.1	11.7	0.83
	piled for 42 days	10.80	32.4	245	15.6	15.8	1.01
Picual	fungus	0.90	17.2	27.6	7.0	9.9	1.41
Hojiblanca	fungus	1.39	17.4	38.4	5.4	11.3	2.09
Lechín	fly	0.50	10.9	18.8	6.0	4.6	0.78
Hojiblanca	frozen	0.45	12.7	17.7	9.1	3.2	0.35

[a] Calculated by eq 1. [b] Σ1,2 and Σ1,3 represent the sum of the main DG isomers (PO, OO, and LO).

Neutralization. Olive oils were neutralized with a 10% aqueous solution of NaOH at 40 °C and then washed out with water until neutral pH was obtained.

Deodorization. Aliquots of Set 2 oils were subjected, in duplicate, to a deodorization process at 100, 150, and 250 °C for 1.5 h under vacuum.

Material and Reagents. All reagents were of analytical-reagent grade, unless otherwise specified. The internal standard 1,3-dipalmitoyl rac-glycerol was purchased from Sigma (St. Louis, MO). The solid-phase extraction cartridge (3 mL), packed with diol-bonded phase was purchased from Supelco (Bellefonte, PA). Silylating reagent was prepared by adding 3 mL of hexamethyldisilazane and 1 mL of trimethylchlorosilane to 9 mL of anhydrous pyridine.

Determination of Diacylglycerols. The diacylglycerols were determined using the method previously described (*2*). An aliquot of 500 μL of oil solution in hexane (0.2 mg/mL) and 200 μL of standard solution [1,3-dipalmitoyl rac-glycerol (1 mg/mL)] were charged onto the column. The first fraction was obtained by passing 6 mL of a solution of hexane/methylene chloride/ethyl ether (89:10:1) and then discarded. A second fraction was collected by passing 4 mL of a solution of chloroform/methanol (2:1). The latter fraction was evaporated until dryness in a rotary evaporator under reduced pressure. The residue was treated with 200 μL of silylating reagent and left at room temperature for a few minutes. An aliquot of 1 μL of solution was injected onto the gas chromatographic system.

The chromatographic analysis was performed using a Chrompack CP900 gas chromatograph (Middelburg, Netherlands) fitted with a flame ionization detector and a split injection system (split ratio 1:30). Separations were carried out on a high-temperature fused-silica capillary column (25 m × 0.25 mm i.d.) coated with 65% methyl-phenylsilicone. Following are the operating conditions that were used: oven temperature, 270 °C for 4 min, then increased at 1 °C/min to 295 °C and held at 295 °C for 1min; injector temperature, 300 °C; detector temperature, 325 °C; and carrier gas, hydrogen at 100 kPa. Data acquisition and processing were carried out by a Chrom-Card Data System (Fisons, Altrincham, UK).

Determination of Acidity. Acidity was determined by titration following European Economic Community (EEC) regulation 2568/91 (*9*). Results were expressed as percentage of oleic acid.

RESULTS AND DISCUSSION

Extra Virgin Olive Oils. The just-obtained oils from healthy olive fruits of the *Picual* cultivar in different ripeness stages showed almost exclusively the 1,2-isomers, in agreement with the hypothesis that the DGs are intermediates in the synthesis of TGs (*10*). The oils obtained from mature olive fruits maintained the acidity value and total DGs content at approximately 0.10% and 10 mg/g, respectively.

Oils Obtained from Low-Quality Olive Fruits. A common alteration of oils is named "fusty", and it arises from the piling of olive fruits during long periods of time before the oil extraction. Comparing the DG composition of oil from healthy *Picual* olives with that of oils just obtained from olives piled for 14 days (Table 1), results in a growth in the content of total, 1,2-, and 1,3-DGs. Oils obtained from olives piled for longer periods showed lesser concentration of total and 1,2-DGs, slight changes in the 1,3-DG contents, and a gain in the 1,3/1,2-DG ratios. In all these oils, the acidity was rather high and the monoacylglycerols (MGs) were less than 0.3 mg/g.

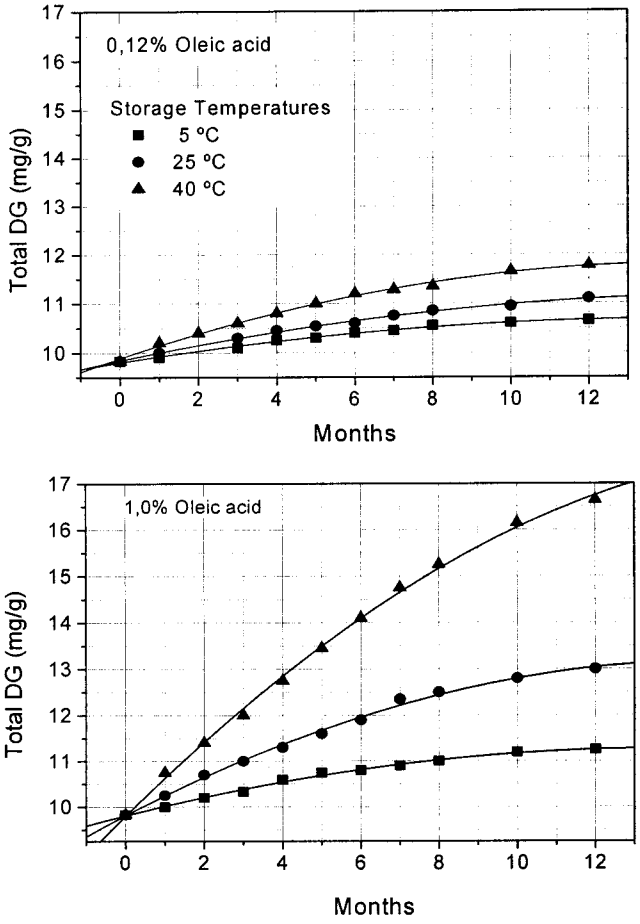

Figure 1. Evolution of total DG in *Picual* extra virgin olive oil during oil storage at various acidities and temperatures (each point is the mean value of two samples).

These results indicated that TGs were hydrolyzed to 1,3- and 1,2-DGs, which suffer a subsequent hydrolysis. The absence of MGs can be due to either the insolubility of such compounds in the oil or to hydrolysis to glycerin. Other olive alterations such as olives infected by fungus *Colletotricum gloesporoides*, olives attacked by the fly *Bractrocera oleae (Dacus oleae)*, or frozen olives yield oils with lesser increase of acidity and total DGs.

Assuming that the increase of the acidity value is only due to the free fatty acids arising from the hydrolysis of the TG to DG, the minimum acidity is 0.10%, and

Table 2. DG Content in Genuine Virgin Olive Oils Obtained by Industrial Processes

sample	cultivar	acidity (%)	total DG (mg/g)	theor. DG[a] max. (mg/g)	ΔDG[b]	Σ1,3/Σ1,2 ratio[c]
1	Picual	0.12	10.2	10.5	0.3	0.04
2	Hojiblanca	0.16	11.6	11.3	−0.3	0.01
3	Picual	0.25	13.3	13.3	0.0	0.35
4	Lechín	0.26	13.0	13.5	0.5	0.10
5	Picual	0.33	14.1	15.1	1.0	0.45
6	Hojiblanca	0.46	16.0	17.9	1.9	0.62
7	Koroneki	0.47	15.0	17.1	2.1	1.04
8	Picual	0.50	11.2	18.8	7.6	0.78
9	Koroneki	0.64	19.3	21.9	12.6	1.31
10	Picual	0.81	16.9	25.6	8.7	1.83
11	Hojiblanca	1.01	12.0	30.0	18.0	0.95
12	Picual	2.20	13.5	56.2	42.7	2.31
13	Picual	2.25	25.4	57.3	31.9	1.39

[a] Calculated by eq 1. [b] $\Delta DG = DG_{\text{theor.}} - DG_{\text{total}}$. [c] Σ1,2 and Σ1,3 represent the sum of the main DG isomers (PO, OO, and LO).

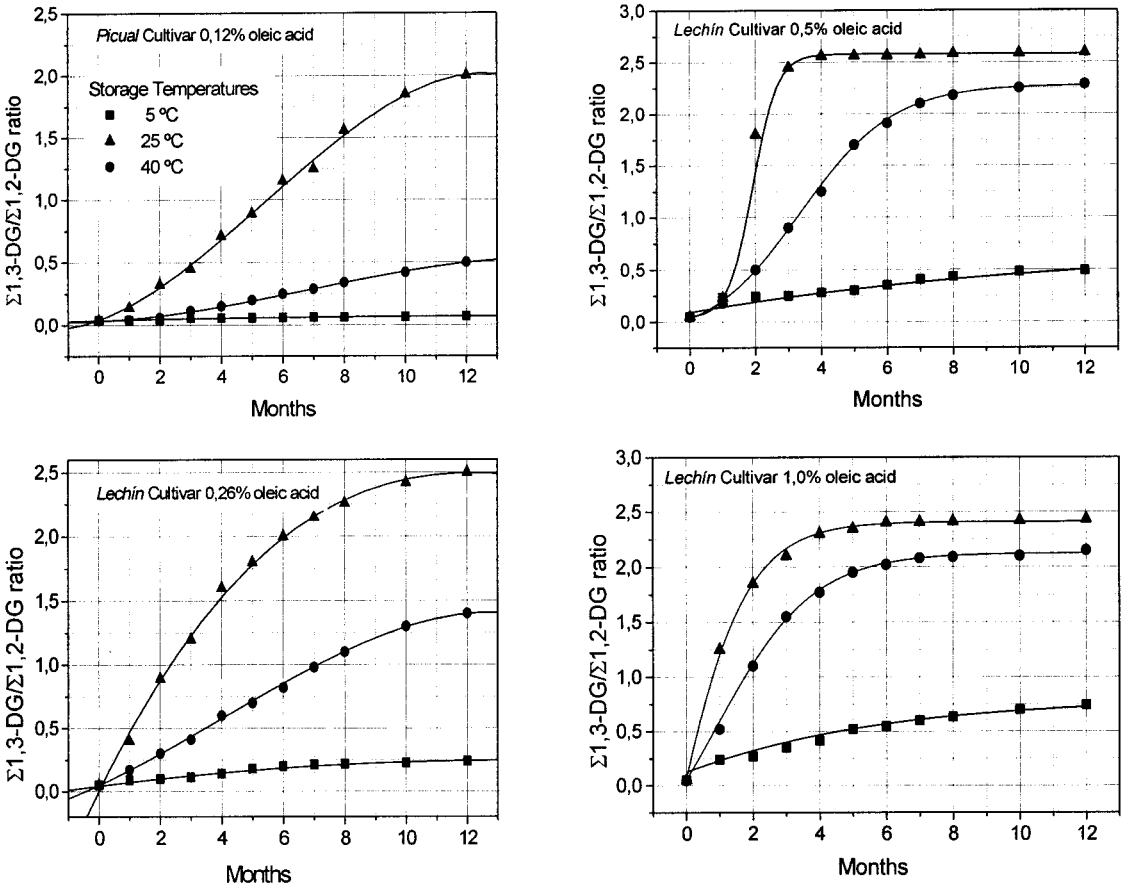

Figure 2. Evolution of 1,3-/1,2-DG ratio in virgin olive oils of several acidities during storage at various temperatures (each point is the mean value of two samples).

the content of biosynthetic 1,2-DG is approximately 10 mg/g, the maximum theoretical DG content can be calculated from the equation:

$$DG_{\text{theor.}}(\text{mg/g}) \approx 22 \times (\%\ \text{acidity} - 0.10) + 10 \quad (1)$$

as a 1% of acidity expressed as oleic acid results in 22 mg/g of DGs.

Applying eq 1 to the oils included in Table 1, it can be observed that the real contents of total DG are lower than the theoretical value.

The observed acidity was not due to the volatile acids originated in the biological activity, because the deodorization of the oils obtained from piled olives under vacuum at 100 °C for 1.5 h did not result in a decrease of the acidity. These results confirm that a percentage of TG is hydrolyzed completely to MG or glycerin, and therefore, there is no relationship between acidity and DG content.

Commercial Oils. The study of genuine oils obtained from industrial oil mills (Table 2) showed that in oils of acidity lower than 0.40% the DG content is similar to the theoretical value, but for those with acidity higher than 0.40%, the DG content is much less than the theoretical value, with the differences being rather diverse. The results were in agreement with those obtained with poor quality olives (Table 1). The dispersion of the 1,3-/1,2-DG ratios in oils with acidity less than 0.40% suggests changes during the storage of the oil.

Evolution of DG Content in Oils During Storage. During 12 months of storage, the evolution of DG content in just-obtained olive oils, as well as those spiked with oleic acid, was very similar in the three

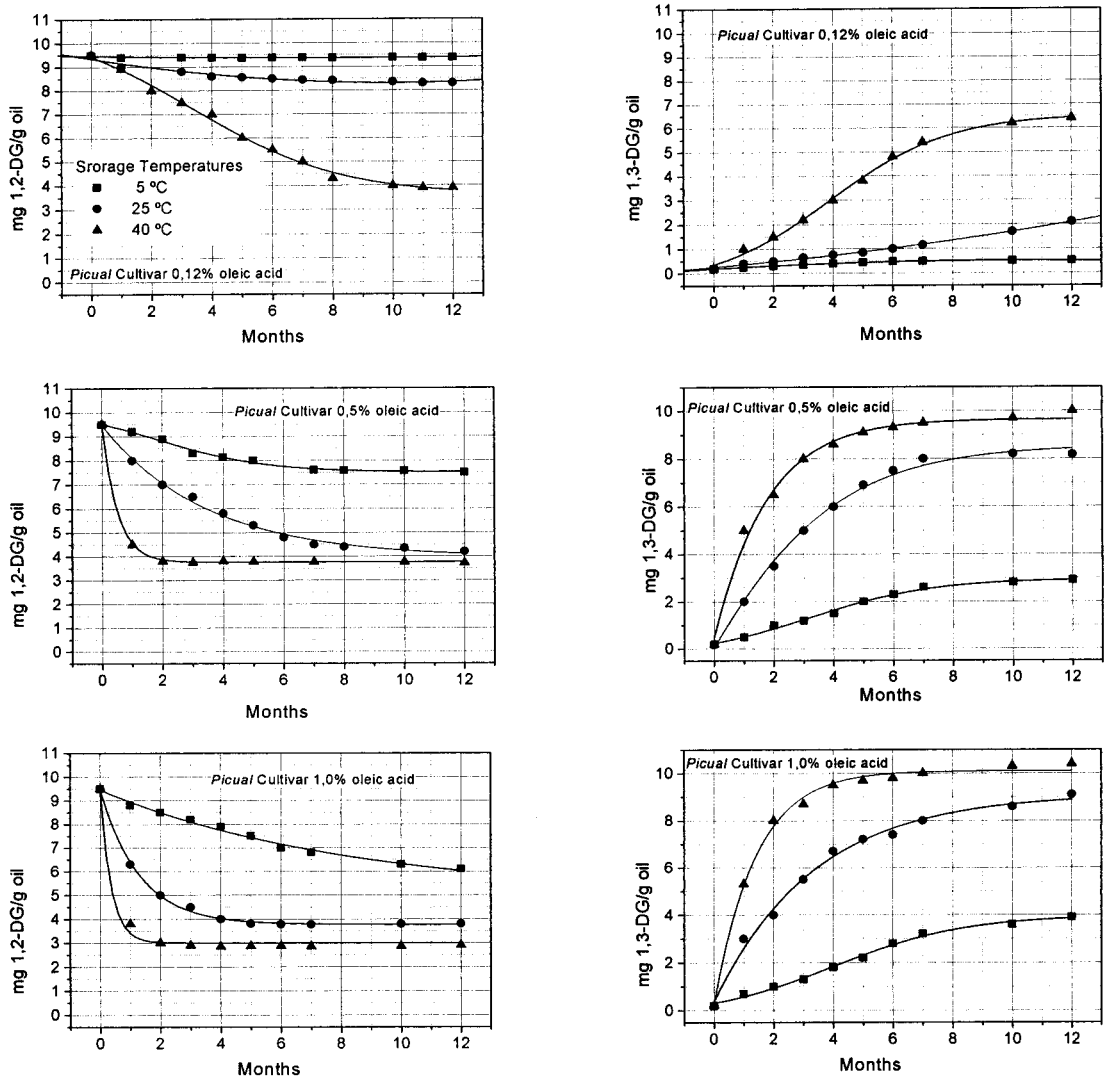

Figure 3. Evolution of 1,2- and 1,3-DG isomers in virgin olive oil of *Picual* cultivar during storage at various temperatures (each point is the mean value of two samples).

cultivars (*Picual*, *Hojiblanca,* and *Lechín*). Figure 1 shows the evolution of *Picual* virgin olive oil. The DG content in the aliquots maintained at 5 °C remained almost constant regardless of the acidity value, whereas in those maintained at 25 and 40 °C the growths were greater as the temperature and the acidity increased, with temperature being the factor with the most significance in the oil hydrolysis. The fall of the slope in the curves shown in Figure 1 suggests an equilibrium between the triacylglycerols and the hydrolysis products, similar to that which happens between the wax esters and the fatty alcohols present in the oil (*11*).

Study of the 1,3-/1,2-DG ratios in the oil samples showed that only those of low acidity value stored at 5 °C maintain the ratio nearly constant regardless of the stored time, whereas in the oils spiked with oleic acid a slight increment is observed (Figure 2). The 1,3-/1,2-DG ratios rise rapidly at 25 °C with acidity of 0.26% or greater. At 40 °C the ratio rises quickly in a short time.

This behavior can be explained by the contents of the 1,2- and 1,3-DG isomers. The graphs in Figure 3 show that in the *Picual* cultivar with 0.12% acidity at 25 and 40 °C, the content of 1,2-DG decreases whereas the 1,3-isomer increases because the hydrolysis is negligible. The oils with acidities of 0.5 and 1.0% stored at 5 and 25 °C showed a decrease of the 1,2-DG content dependent on the storage time; on the other hand, the 1,3-isomers contents were increasing during the whole storage time. These facts are explained because initially there are only 1,2-DG isomers and the isomerization is rather quick, reaching the equilibrium in a short time. The latter increment in the ratio suggests a larger formation of 1,3-isomers due to the hydrolysis of the TGs exceeding the equilibrium value, therefore, there is transformation of the 1,3-isomers into the 1,2-isomers.

Neutralized Oils. Alkaline neutralization results in a decrease of the total DG content and the acidity value (Table 3). On the other hand, the 1,3-/1,2-DG ratio tends toward 1.8 because of the catalytic effect of the basic medium over the isomerization, with this value being approximately the equilibrium ratio at room temperature (*2*). In all cases, the variation in the ratio was ±0.2, approximately.

Deodorized Oils. Behavior of the DGs during low temperature deodorization showed that at 100 °C the acidity and the total DGs increased slightly, about 10%, due to TG hydrolysis. Regarding the 1,3-/1,2-DG ratio, there was an increase depending on the acidity (Figure 4). When the deodorization was performed at higher temperatures, the total DG content increased with the acidity and temperature, and the ratio increased rapidly until the equilibrium point was reached. Therefore, the

Table 3. Effects of Alkaline Neutralization on the DG Content of Virgin Olive Oils

oil cultivar	treatment	acidity (%)	total DG (mg/g)	Σ1,2[a] (mg/g)	Σ1,3[a] (mg/g)	Σ1,3/Σ1,2 ratio[a]
Lechín	none	0.26	13.0	11.5	1.2	0.10
	neutralized	0.16	12.9	9.9	2.7	0.27
Hojiblanca	none	0.63	14.4	10.0	4.1	0.41
	neutralized	0.18	14.1	8.5	5.3	0.62
Lechín	none	1.31	19.6	5.7	13.2	2.32
	neutralized	0.23	18.9	5.8	12.4	2.13
Picual	none	8.90	65.2	48.8	14.8	0.30
	neutralized	0.26	62.2	40.4	19.6	0.49

[a] Σ1,2 and Σ1,3 represent the sum of the main DG isomers (PO, OO, and LO).

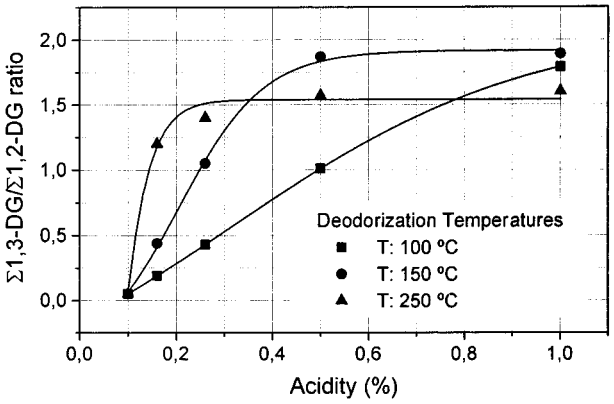

Figure 4. Values of the 1,3-/1,2-DG ratio in virgin olive oils of various acidities deodorized at 100, 150, and 250 °C for 1.5 h under vacuum. The values corresponding to 0.5 and 1.0% acidities are the means of those obtained for the oils from three olive cultivars.

deodorization at 100 °C produces an increase of the 1,3-/1,2-DG ratio in direct relationship with the acidity.

In the neutralized and thereafter deodorized oils, the total DG drop-off occurring in the first step is compensated by the gain in the second step. The 1,3-/1,2-DG ratio increases in both steps, but if the neutralized oils have low acidity (<0.20%) and the deodorization temperature is lower than 100 °C the growth of the ratio is not important.

Detection of Deodorized Oils in Virgin Olive Oils. As has been already stated, virgin olive oils showed DG contents lower than the maximum contents calculated according to eq 1. For acidity values lower than 0.40%, the real DG contents are near the maxima, but for higher acidities, the differences are quite important. In the case of oils with low acidity, it could be thought that the addition of deodorized oils would increase the DG content over the theoretical maxima. However, Table 2 shows that there are numerous virgin olive oils with high acidity and low content of DG. These samples, once neutralized and deodorized, would have acidities and DG content similar to the genuine virgin olive oils. Therefore, values of total DG exceeding the theoretical maxima indicate adulteration of the oil, but lower values do not guarantee that the oil is genuine.

Regarding the 1,3-/1,2-DG ratio, the oils subjected to neutralization and deodorization processes usually show high ratio values because they have high acidities. In addition, both processes increase this ratio around 0.18 and 0.15, respectively. However, data reported previously (*3*) indicated that some virgin olive oils with acidity higher than 0.5% have a ratio of 0.20. Consequently, neutralized oils and mild refined ones will show minimum values of 0.4 and 0.6, respectively. These facts allow the establishment of limits to detect the presence of high percentages of mild refined oils in virgin olive oils stored in bulk at room temperature within certain ranges of acidity and storage time. This limit can be inferred from the behavior of virgin olive oils of highest quality during storage assuming 15 °C as the average temperature during the winter and spring seasons. For oils with acidities less than 0.20% and 4 months of storage, the expected ratio will be given by the equation:

$$\text{1,3-/1,2-}DG\ ratio = (0.005 \times t^2) + (0.005 \times t) + 0.10 \quad (2)$$

where t is the storage time expressed in months.

The addition of significant amounts of neutralized oils (1,3-/1,2-DG ratio ≈ 0.40) to virgin olive oil stored at low temperature (1,3-/1,2-DG ratio < 0.10) will produce values exceeding those given by this equation.

For oils with acidities between 0.2 and 0.30% and for 3 months of storage eq 3 gives the maximum values:

$$\text{1,3-/1,2-}DG\ ratio = (0.050 \times t) + 0.10 \quad (3)$$

In oils with acidities between 0.3 and 0.50% a maximum limit of 0.25 for the first month of storage is proposed.

Finally, in oils with acidity higher than 0.50%, the effect of the temperature is really significant (Figure 2) and it is not possible to establish a limit, because the increase due to the refining is low compared with that due to isomerization. Values of the 1,3-/1,2-DG ratio exceeding the above limits do not mean necessarily adulteration of the oil, because oils obtained from olives of moderate quality or those stored at high temperatures or blends of fresh and aged oils would show high values of this parameter.

From Figure 2 it can be deduced that the 1,3-/1,2-DG ratio is useful for distinguishing between oils from the current crop and those from previous ones, only for oils with low acidity (<0.30%) and during the early months after harvesting. In addition, this ratio is also adequate to evaluate the storage conditions of oils with acidities lesser than 0.50% during a year after the oil obtention.

In summary, for detecting mild refined oils in virgin olive oils, the relationship between acidity and total DG content shows scarce utility because most olive oils contain low concentrations of DGs. The 1,3-/1,2-DG ratio is useful for assessing the genuineness of virgin olive oils of low acidities, determining the oil aging, and evaluating the storage conditions if the DG determination is performed in the early stages after the oil obtention.

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