PAPER

EXTRA VIRGIN OLIVE OIL STORED IN DIFFERENT CONDITIONS: FOCUS ON DIGLYCERIDES

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ABSTRACT

The effects of storage conditions of extra virgin olive oil (EVOO) on the isomerization of diglycerides (DGs) have been investigated. Aliquots of EVOO were stored for 14 months under four different conditions: at 20°C in darkness and in light, at 4-6°C in light and at 20°C in light with argon in the headspace. Samples were analysed bimonthly: 12 DGs with C34 and C36 (1,2 and 1,3 isomers) were tentatively identified and quantified by GC-FID. After 14 months, a clear tendency towards a decrease of 1,2-DGs and a significant increase of 1,3-DGs during storage was observed for all samples. 1,2-DGs were always predominant compared to 1,3-DGs and, for both types, C36 DGs were prevalent compared to C34 DGs. Overall, EVOO stored at 4-6°C in light showed the highest preservation of 1,2-DGs.

- Keywords: Extra Virgin Olive Oil, Diglycerides,1,2/1,3-DGs ratio, GC, Storage conditions -

INTRODUCTION

Extra virgin olive oil (EVOO) is fresh olive (*Olea europaea L.*) juice obtained by mechanical and physical processes (Lozano-Sanchez *et al.*, 2012), and it is well known as one of the major components of the diet of Mediterranean countries. EVOOs consist of triglycerides as the main components (about 98%) and other minor components including diglycerides, free fatty acids, squalenes, sterols, phospholipids, phenolics and different volatile compounds (BOSKOU, 1996). Some of these minor components, in addition to a high content of mono-unsaturated fatty acids, play a major role in keeping EVOO more stable against oxidation during storage compared to other vegetable oils (BENDINI *et al.*, 2009a). Elimination of air in the head space, either by fully filling the EVOO bottles or by its replacement with inert conditioning gas, has been found to add marked improvement in terms of oxidation quality, stability, shelf life and slow down the oxidation process of EVOO (URDA- ROMACHO, 2009; GIOVACCHINO *et al.*, 2002).

Newly produced EVOO contains a low concentration of diglycerides (DGs) (1-3%), which are formed as intermediate products of the incomplete biosynthesis of triglycerides (SPYROS *et al.*, 2004) and partial hydrolysis of triglycerides. During storage many changes may occur in DG composition due to isomerisation of 1,2-DGs, the predominant form in fresh EVOO, to 1,3-DGs (SACCHI *et al.*, 1991). The effects of storage temperature and exposure to light during different periods of time on the quality of EVOO have been investigated by different authors (VELASCO and DOBARGANES, 2002; MENDEZ and FALQUE, 2007), while other studies have assessed the amount of DGs as an indicative parameter of the freshness of EVOO. CATALANO *et al.* (1994) investigated DGs isomerisation occurring in EVOO stored in darkness, at room temperature and at 4°C. In particular, the results revealed that the 1,2-DGs remained less than 1.5 % after one year of storage for all samples analysed, while about 10% and 45% of the samples stored at room temperature and at 4°C, respectively, contained less than 0.4% 1,3-DGs. Furthermore, PÉREZ-CAMINO *et al.* (2001) studied the evolution of the two DG isomer classes in oils obtained from olives of different qualities stored at different temperatures, concluding that triacylglycerol hydrolysis and DG isomerisation depended not only on the value of free acidity, but also on the storage temperature. In addition, the 1,3/1,2-DG ratio was a useful parameter for assessing the genuineness of EVOOs with low free acidity during early storage stages.

Another interesting study was carried out by SPYROS *et al.*, (2004), assessing olive oil through investigation of 1,2 and 1,3-DG isomerisation during 18 months of storage at room temperature, at 5°C with light and in darkness. The result of the isomerisation process was mainly dependent on the initial quality parameters of the oil, and in particular the free acidity. Another study based on the evaluation of olive oil quality in relation to storage conditions through the analysis of DG isomerisation was carried out by COSSIGNANI *et al.* (2007) on samples produced from different olive cultivars stored at 15°C and at 30°C in darkness for 12 months. The results showed important differences in the percentage of each individual DG and in the ratio among classes; in particular, samples analysed at time zero exhibited the highest percentage of 1,2-DGs and the lowest of 1,3-DGs, whereas samples stored at 30°C showed the highest content of 1,3-DGs suggesting that temperature plays an important role in the isomerisation process. More recently, a study carried out by CAPONIO *et al.* (2013) investigated the effects of storage of EVOO in green glass bottles in light and darkness for 24 months, providing evidence that the degree of isomerisation was affected by the initial hydrolysis level of the oil and by the storage time, although other storage conditions did not show any effect. Overall, these results suggest that the content of DGs and the ratio between isomers might be considered as possible markers to establish the freshness state of an EVOO alongside with other quality parameters defined by official regulations (EU Reg. 61/2011).

Therefore, the main aim of this study was to investigate the isomerisation processes related to diacylglycerols, and in particular the amounts of 1,2- and 1,3-DGs and relative C34 and C36 sub-classes as well as the 1,2/1,3-DG ratio in EVOO during storage under different conditions for 14 months. The purpose was to investigate how these compounds were influenced by different variables such as temperature, light and headspace gases.

MATERIALS AND METHODS

Samples

EVOO samples used in this study were produced from olives of the Arbequina cultivar (Coop. Sant Bartomeu, Soller, Spain) using an industrial plant working with a three-phase decanter. Once in the laboratory, the EVOO was poured into 250 mL transparent glass bottles. The headspace in each bottle was about 2 mL. The bottles were hermetically sealed and divided into four batches. The first batch was stored in darkness inside a thermostatic chamber at 20°C (Cond. 1); the second batch was stored at 20°C under diffuse light (600 Lux for 12 h/day 11 W; 595 lm; 6400°K) simulating the conditions of a supermarket shelf (Cond. 2); the

third batch was stored in a refrigerated chamber at 4-6°C with diffuse light (Cond. 3); finally, the fourth batch was stored with argon in the headspace of bottles at 20°C with diffuse light (Cond. 4). Samples were analysed in triplicate after 2, 4, 6, 8, 10, 12 and 14 months of storage after production.

Basic chemical analysis

Free acidity, peroxide value and UV absorption (K_{232}, K_{270}) were determined according to the official methods described in EEC Reg. 2568/91 for all samples at the initial period of storage (2 months) and after the end of storage simulation (14 months).

Gas chromatographic (GC) determination of diglycerides

The silylated samples were prepared according to a previous work (SWEELEY *et al.*, 1963) and DGs were determined according to a modified version of the method suggested by SERANI *et al.*, (2001) using a GC Carlo Erba MFC500 with a Rtx-65TG (Restek, Bellefonte, PA) fused silica capillary column (30 m length x 0.25 mm i.d. x 0.10 μm f.t.) coated with 35 % dimethyl-65 % diphenylpolysiloxane. The oven temperature was programmed from 250 to 320°C at a rate of 2°C min^{-1} and then increased to 365°C at a rate of 5°C min^{-1}. The final temperature was maintained for 21 min. The injector and FID temperatures were both set at 360°C. Helium was used as carrier gas at a pressure of 130 kPa. The split ratio was 1:70. Identification of DGs was carried out by comparing peak retention times and GC traces with those of DG standards and chromatograms reported in the literature (SERANI *et al.*, 2001; BENDINI *et al.*, 2009b). The results, expressed as mg of each DG per 100 mg of oil, were quantified with respect to dilaurin, added as internal standard (0.5 mL of a solution 2 mg mL^{-1} of dilaurin dissolved in chloroform, added to 100 mg of oil).

Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate the data by analysis of variance (ANOVA, Fisher LSD, $p < 0.05$).

RESULTS AND DISCUSSION

The free acidity, peroxide values and extinction coefficients (K_{232} and K_{270}), shown in Table 1, indicated that at the end of the storage period all samples were within the accepted limits established by EU regulations for the EVOO category (EU Reg. 61/2011).

Fig. 1 shows a comparison between the gas chromatography traces of DG fractions of EVOO stored for 2 and 14 months in dark at 20°C. Twelve different DGs were tentatively identified and quantified as 1,2 and 1,3 isomers with 34 or 36 carbon atoms (C34, C36). Only a co-elution was present (peak 11) between 1,3 isomers of the oleic-linoleic and linoleic-linoleic couples. The peaks numbered from 1 to 6 (Fig. 1) were relative to C34 DGs whereas from 7 to 11 belonged to C36, and palmitic-oleic (PO) and oleic-oleic (OO) were the most abundant DGs for the two classes, respectively. Observing the GC traces (Fig. 1), it is also possible to note that the 1,2 isomers eluted before the 1,3 ones for both groups with 34 and 36 carbon atoms.

Fig. 2 illustrates the evolution of 1,2/1,3-DG ratios, and Tables 2-5 highlight the trends of 1,2-DGs (C34, C36) and 1,3-DGs (C34, C36) for EVOOs stored under the four different experimental conditions. For the samples kept at 20°C in darkness (Cond. 1), a rapid and significant decrease was observed in the 1,2/1,3-DG ratio for the first 8 months; this ratio continued to decrease slowly until the end of storage period (Fig. 2). A similar trend was also seen for the 1,2-DGs C34 and C36 under the same condition (Table 2), and the rapid decrease continued for up to 8 months. At the end of storage period, total 1,2-DG remained about 60 % (data not shown) of

Table 1 - Results for free acidity (FA, g of oleic acid per 100 g of oil), peroxide values (PV, Meq O_2 Kg^{-1}) and extinction coefficient at 232 and 270 nm (K_{232}, K_{270}) at time zero and after 14 months of storage under the four different conditions (Cond. 1 - 4)*.
* Cond. 1, stored at 20°C in dark, Cond. 2, stored at 20 °C in light, Cond. 3, stored at 4-6°C in light, Cond. 4 stored at 20°C in light with argon in the headspace.
Different letters (a-e) represent significant differences among mean values for a same parameter during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 2 and 14 months of storage.

	2 months of storage				14 months of storage			
	FA	PV	K232	K270	FA	PV	K232	K270
Cond. 1	0.15 ± 0.01 b,x	11.63 ± 1.29 a,xy	2.11 ± 0.03 b,x	0.10 ± 0.00 b,z	0.20 ± 0.01 a,x	12.74 ± 0.55 a,y	2.34 ± 0.02 a,x	0.15 ± 0.01 a,y
Cond. 2	0.15 ± 0.01 b,x	14.00 ± 0.04 a,x	2.00 ± 0.09 b,xy	0.17 ± 0.01 b,x	0.20 ± 0.01 a,x	14.74 ± 1.02 a,xy	2.19 ± 0.07 a,y	0.18± 0.01 a,x
Cond. 3	0.15 ± 0.01 b,x	10.59 ± 0.01 b,y	1.94 ± 0.12 b, y	0.13 ± 0.00 b,y	0.17 ± 0.01 a,y	15.47 ± 0.80 a,x	2.19 ± 0.06 a,y	0.14 ± 0.00 a,y
Cond. 4	0.16 ± 0.01 b,x	14.00 ± 0.15 a,z	2.15 ± 0.04 b,x	0.17 ± 0.00 b,x	0.20 ± 0.01 a,x	14.70 ± 0.40 a,xy	2.24 ± 0.03 a,xy	0.18 ± 0.01 a,x

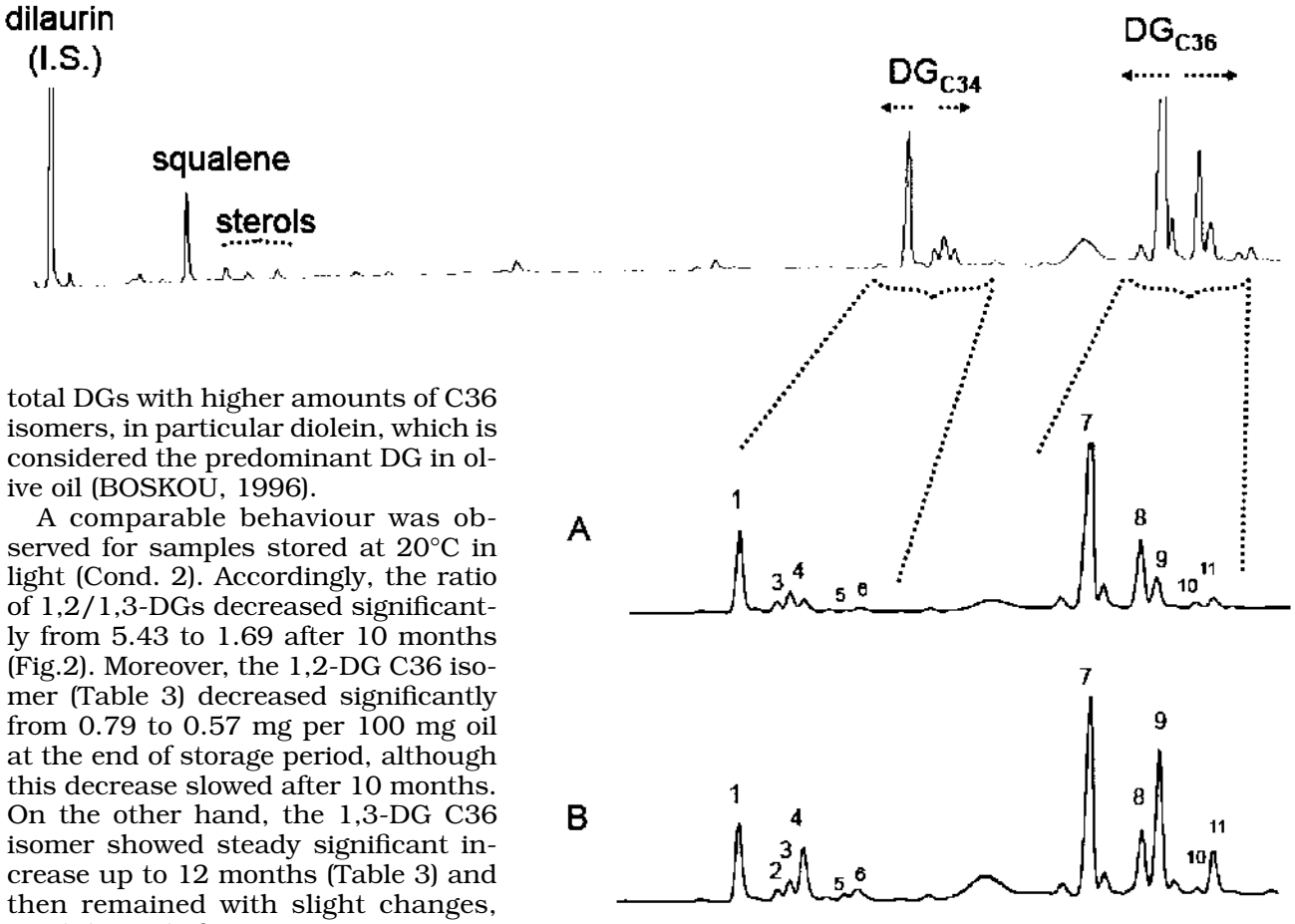

Fig. 1 - Example of full chromatogram of the EVOO sample at 20°C in dark. A) GC tracing of the diglyceride fraction of EVOO stored for 2 months at condition 1; B) GC trace of the diglyceride fraction of EVOO stored for 14 months at condition 1. 1, 1,2-PO; 2, 1,2-PoO; 3, 1,2-PL; 4, 1,3-PO; 5, 1,3-PoO; 6, 1,3-PL; 7, 1,2-OO; 8, 1,2-OL; 9, 1,3-OO; 10, 1,2-LL; 11, 13-OL + 1,3-LL. P = palmitic acid; Po = palmitoleic acid; O = oleic acid; L = linoleic acid.

total DGs with higher amounts of C36 isomers, in particular diolein, which is considered the predominant DG in olive oil (BOSKOU, 1996).

A comparable behaviour was observed for samples stored at 20°C in light (Cond. 2). Accordingly, the ratio of 1,2/1,3-DGs decreased significantly from 5.43 to 1.69 after 10 months (Fig.2). Moreover, the 1,2-DG C36 isomer (Table 3) decreased significantly from 0.79 to 0.57 mg per 100 mg oil at the end of storage period, although this decrease slowed after 10 months. On the other hand, the 1,3-DG C36 isomer showed steady significant increase up to 12 months (Table 3) and then remained with slight changes, until the end of storage. However, 1,3-DG C34 isomers showed a significant slight change toward increases, after 6 months of storage, reaching about 0.14 mg per 100 mg sample after 14 months of storage (Table 2).

The results for samples stored at low

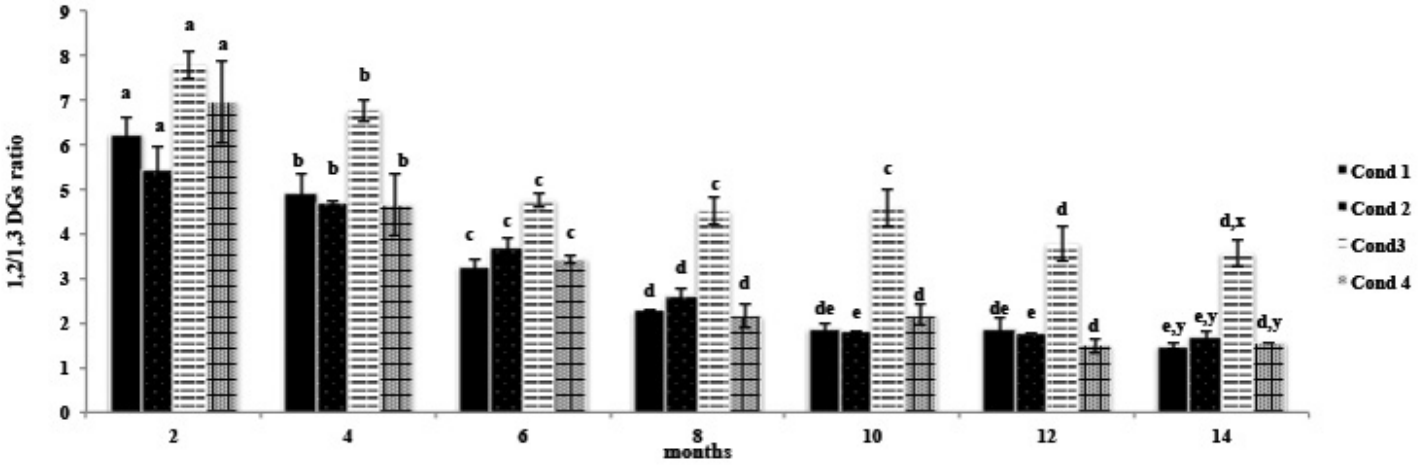

Fig. 2 - Trends of 1,2/1,3 DGs during the EVOO storage of 14 months at the four different conditions (Cond 1-4)*. The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same condition during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months.
* Cond. 1, stored at 20°C in dark, Cond. 2, stored at 20°C in light, Cond. 3, stored at 4-6°C in light, Cond. 4 stored at 20°C in light with argon in the headspace.

Table 2 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 1 (at 20°C in dark). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 1			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.09 ± 0.01 f	0.19 ± 0.03 e	0.48 ± 0.06 a	1.25 ± 0.14 a
4	0.11 ± 0.01 e	0.25 ± 0.02 de	0.47 ± 0.05 a	1.27 ± 0.16 a
6	0.13 ± 0.01 de	0.26 ± 0.01 d	0.38 ± 0.05 b	0.89 ± 0.07 b
8	0.13 ± 0.00 cd	0.33 ± 0.01 c	0.28 ± 0.01 c	0.77 ± 0.02 b
10	0.15 ± 0.00 bc	0.40 ± 0.07 b	0.28 ± 0.01 c	0.75 ± 0.05 b
12	0.16 ± 0.01 b	0.37 ± 0.03 bc	0.27 ± 0.01 c	0.74 ± 0.10 b
14	0.19 ± 0.02 a,x	0.49 ± 0.02 a,x	0.27 ± 0.02 c,yz	0.73 ± 0.05 b,y

temperature (4-6°C) (Cond. 3) showed that, at the end of the storage period, the 1,2/1,3-DG ratio remained about 2 times higher than the values for EVOO samples stored at 20°C (Fig. 2). Furthermore, the 1,2-DGs isomers C36 and C34 showed a significant decrease from 2 to 14 months (Table 4).

Regarding the samples stored with argon in the headspace (Cond. 4), the 1,2/1,3-DGs ratio decreased significantly during the first 8 months of storage, and minor changes were detected up to the end of storage (Fig. 2). Similarly, 1,2-DGs for both C36 and C34 classes decreased after 14 months of storage compared to the initial value, with a fluctuation trend (Table 5), while 1,3-DG C36 isomers showed a significant increase throughout the entire storage period.

By comparing the different conditions, after 2 months of storage the highest 1,2/1,3-DG ratio corresponded to the sample stored at low temperature (4-6°C), followed by the sample stored under light at 20°C with argon in the headspace

Table 3 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 2 (at 20 °C in light). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 2			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.06 ± 0.00 e	0.15 ± 0.01 e	0.35 ± 0.01 ab	0.79 ± 0.15 cd
4	0.09 ± 0.02 d	0.21 ± 0.02 d	0.38 ± 0.05 a	1.06 ± 0.14 a
6	0.12 ± 0.02 cd	0.25 ± 0.02 d	0.37 ± 0.02 a	0.98 ± 0.05 ab
8	0.15 ± 0.01 ab	0.30 ± 0.01 c	0.32 ± 0.01 b	0.85 ± 0.07 bc
10	0.15 ± 0.01 a	0.36 ± 0.01ab	0.22 ± 0.01 c	0.69 ± 0.02 de
12	0.13 ± 0.00 bc	0.39 ± 0.00 a	0.23 ± 0.00 c	0.68 ± 0.00 de
14	0.14 ± 0.01 ab,y	0.32 ± 0.06 bc,y	0.21 ± 0.01 c,z	0.57 ± 0.04 e,z

Table 4 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 3 (at 4-6°C in light). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 3			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.08 ± 0.01 c	0.14 ± 0.01 d	0.58 ± 0.08 a	1.09 ± 0.18 ab
4	0.08 ± 0.00 c	0.17 ± 0.01 c	0.46 ± 0.02 b	1.25 ± 0.06 a
6	0.12 ± 0.01 ab	0.18 ± 0.01 c	0.41 ± 0.02 bcd	1.01 ± 0.11 b
8	0.12 ± 0.01 ab	0.16 ± 0.01 cd	0.34 ± 0.04 d	0.9 ± 0.12 b
10	0.10 ± 0.03 bc	0.22 ± 0.00 b	0.38 ± 0.01 cd	1.08 ± 0.03 ab
12	0.14 ± 0.02 a	0.25 ± 0.02 b	0.45 ± 0.02 bc	1.03 ± 0.17 b
14	0.13 ± 0.01 a,y	0.28 ± 0.03 a,y	0.39 ± 0.04 cd,x	1.07 ± 0.03 ab,x

Table 5 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 4 (at 20 °C in light with argon in the headspace). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 4			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.07 ± 0.00 c	0.14 ± 0.02 d	0.41 ± 0.08 a	1.07 ± 0.18 ab
4	0.07 ± 0.01 c	0.18 ± 0.01 d	0.32 ± 0.02 bc	0.82 ± 0.13 cd
6	0.14 ± 0.00 b	0.31 ± 0.06 c	0.46 ± 0.05 a	1.09 ± 0.11 a
8	0.15 ± 0.01 b	0.37 ± 0.04 bc	0.29 ± 0.01 c	0.82 ± 0.02 d
10	0.21 ± 0.01 a	0.46 ± 0.04 ab	0.38 ± 0.01 ab	1.06 ± 0.21 abc
12	0.17 ± 0.01 b	0.48 ± 0.06 a	0.25 ± 0.01 c	0.70 ± 0.05 d
14	0.21 ± 0.04 a,x	0.53 ± 0.10a,x	0.31 ± 0.06 bc,y	0.84 ± 0.15 bcd,y

(Fig. 2). Moreover, during the first 4 months, when EVOOs were stored at 20°C under light without headspace modification (Cond. 2), the sample exhibited a lower ratio than the respective sample stored in darkness (Cond. 1). The results also highlighted the positive effect of using inert gas in the head space. The total 1,2-DGs remained after 14 months (data not shown) of storage was about 1.5 times higher, in comparison with their presence in EVOO stored under the same conditions, but with air in the head space. The findings are in accordance with SPYROS *et al.* (2004), suggesting that the length of storage time plays an important role in isomerisation changes of DGs, which is accelerated by temperature.

The formation of oxidation products by photo-oxidation was confirmed by the high values of K_{270} obtained for samples stored under diffuse light, especially for those stored at 20°C after 14 months of storage (Table 1). It should be noted that, at the end of storage period, all the samples remained within EVOO category parameters. As expected, free acidity (Table 1), which is considered to be the main driving factor affecting DG isomerisation (PÉREZ-CAMINO *et al.*, 2001), showed only a minor increase after 14 months of storage.

The results of this study showed that the isomerisation of DGs in EVOOs depends not only on the length of storage, but also on the temperature of storage. This finding is in agreement with the studies of PÉREZ-CAMINO *et al.* (2001) and COSSIGNANI *et al.* (2007). Moreover, the results showed that after 14 months of storage at 20°C (Cond. 1, 2 and 4) there were slight but not significant differences in the 1,2/1,3 ratio among samples stored under diffuse light (Cond. 2 and 4) and for those stored in darkness (Cond. 1), in spite of the fact that light exposure has an adverse effect on the oxidation of EVOO (significantly higher K_{270} values were found for samples stored under diffuse light). This result is in agreement with considerations noted by AFANEH *et al.* (2013).

CONCLUSION

The results of this study confirmed that the isomerisation of DGs in EVOO depends not only on the length of storage, but also on the temperature. By comparing the different conditions, it was found that after 10-14 months of storage the 1,2/1,3-DG ratio remained higher for samples stored at low temperature (4-6°C). Moreover, the presence of argon gas in the headspace of the sample was not sufficient to protect it from DG isomerisation when the EVOO was exposed to light.

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