A study of the dynamic changes of stability taking place during virgin olive oil storage period examined by mesh cell-FTIR spectroscopy

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ABSTRACT

Virgin olive oil (VOO) stability needs to be controlled to guarantee that the oil keeps the quality declared on the label during distribution. However, the stability is changing as storage progresses and its estimation under moderate conditions are difficult to accomplish, due to the challenge of implementing rapid stability evaluation under the environmental variables given in long storage periods. The main objective of this work was to study the dynamic changes of VOO stability taking place during storage. For this purpose, a realistic storage approach of 27 months was designed. The stability of the oils was evaluated at different months by incubating the oil on mesh cell and scanning FTIR spectra during this incubation (mesh cell-FTIR). Although the storage of the oils did not show remarkable spectral changes, the spectra from mesh cell incubation showed variations at different months, which evidenced a change in the stability as the oil aged. The results revealed that the stability changes were abrupt during VOO ageing. In general terms, the results showed that these changes mainly occurred during the first 6 months. The incubation in mesh cell-FTIR spectroscopy is able to detect the minor chemical changes occurring in VOO during storage under moderate conditions.

1. Introduction

Virgin olive oil (VOO) stability is one of the properties most widely evaluated in the olive oil sector to estimate VOO shelf life and to assure the quality of the product. During storage, VOO chemical composition is slowly changing as a consequence of the oxidation process (Choe, 2017; Krichene, Salvador, & Fregapane, 2015; ;Tena, Lobo-Prieto, Aparicio, & García-González, 2018), and thereby these changes modify the stability measured by any procedure compared with the measurements carried out in the initial fresh oils. The ongoing changes of oil stability taking place over time can be explained by the modification of the chemical composition of antioxidants/prooxidants and their ratio. Thus, minor changes can lead to an unexpected resistance/sensitivity to oxidation. This time-domain change of the measured stability makes the estimation of the "best before date" difficult to accomplish even when transport and distribution are done under controlled and stable conditions.

The current regulation does not provide for a method to determine VOO stability and the "best before date" yet (IOC, 2018a). Numerous studies (Castillo-Luna, Criado-Navarro, Ledesma-Escobar, López-Bascón, & Priego-Capote, 2021; Díaz, Pega, Primrose, Sancho, & Nanni, 2019; Esposto, Taticchi, Servili, Urbani, Sordini, Veneziani, Daidone, & Selvaggini, 2021; Mousavi et al., 2021) have been focused on the effect of storage conditions on the oxidative degradation of VOOs. The majority of the stability studies have focused their effort in analysing the stability in a static point of view, only at the moment of the measurement and without considering the possible stability changes that take place in the oil during distribution and storage due to the inevitable minor chemical changes. Thus, for a correct handling of the oil during distribution, and a proper interpretation of the stability measurements, there is still a need to develop new studies to determine the VOO stability with a dynamic perspective over time based on moderate storage conditions. The multiple reactions and compounds involved in the oxidation process turn spectroscopy into a promising alternative to study the stability of VOO from a global perspective. In fact, several studies have used spectroscopic techniques to assess VOO stability under different storage conditions (Aparicio-Ruiz, Romero,

Aparicio, García González, & Morales, 2017; Hernández-Sánchez, Lleó, Ammari, Cuadrado, & Roger, 2017; Tena, Aparicio, & García-González, 2017); Baltazar, Hernndezánchez, Diezma, & Lleó, 2020), due to the potential of these techniques to register absorptions assigned to different chemical species related with different oxidation stages in a single measurement and with minimum sample handling.

In this work, a methodology based on a sampling accessory called mesh cell (García-González & Van de Voort, 2009) combined with FTIR spectroscopy has been applied for assessing the dynamic changes of VOO stability during storage. Mesh cell-FTIR spectroscopy has been recently proved and successfully optimized for studying the stability of VOO and other edible oils at moderate conditions (Tena et al., 2017; Tena, Aparicio, & García-González, 2018; Trypidis et al., 2019). The previous study (Tena et al., 2017) optimized the method parameters and also studied the best moderate conditions to carry out the incubations. This was a first approach carried out on fresh samples. The fact that mesh cell is able to accelerate the oxidation under moderate light and temperature conditions makes this accessory suitable for studying the changes of stability that takes place during the storage. In fact, the previous studies (Tena et al., 2017; Tena, Aparicio, et al., 2018; Trypidis et al., 2019) proved that it is possible to identify the effect that minor differences in the oil composition, even from the same cultivar, exerts on the production of hydroperoxides and secondary oxidation products when the oil is incubated under moderate conditions. Thus, in this study, it was hypothesized that the minor composition changes caused during the storage may also cause variations in the measurements of stability by mesh cell-FTIR even from the first months. Then, the aim of this work was to develop a strategy to acquire a dynamic perspective of the chemical changes occurring in VOO during the storage and to evaluate their effect on VOOs stability when they are subjected to incubation under defined light and temperature conditions. The work has been planned to know the dynamic changes in the stability associated to different scenarios, with mild heating, light or both simultaneously.

2. Materials and methods

2.1. Sample collection

Four monovarietal VOOs, from three different cultivars (Picual, Hojiblanca and Arbequina), were used with the purpose of studying how the oxidative process affects different chemical compositions, related to the olive cultivar. The olives were collected in the harvest year of 2015/2016 (in October–December) and they were directly processed in the olive oil mill on the same day of production. The virgin olive oil samples were provided by Spanish producers and extracted in olive oil mills located in Andalusia (Southern Spain). The samples were collected on site (at the olive oil mill on the same day of production) to guarantee the origin and freshness of the oils. After collection, they were taken directly to the laboratory. The transport was carried out avoiding temperature higher than 20 °C and protecting the samples from light. The codes used to identify the VOOs and their respective cultivars were: VOO1, Hojiblanca; VOO2, Arbequina-1; VOO3, Picual; and VOO4, Arbequina-2.

2.2. Sample storage: experimental design

Once in the laboratory, the moisture analysis (ISO 662) confirmed that the moisture contents were lower than 100 mg/100 g in the filtered samples, which is within the normal range for extra virgin and virgin olive oil according the current International Olive Council standard (IOC, 2022). Then, each fresh oil was transferred to 28 clear plastic bottles, one per each month of storage (27 months) and one for the fresh oil (0 months). The bottle material was made of transparent polyethylene terephthalate (PET), which is one of the most commonly used materials for packaging olive oil. They were supplied by Aceites del Sur-Coosur, S.A. (Dos Hermanas, Seville, Spain). The bottles had a volume of 500 mL and their dimensions were 24 × 5 × 5 cm.

A long-term storage (27 months), simulating real conditions, was designed. The VOOs were stored during 27 months in a compartment with controlled conditions. Inside the compartment, the samples were stored under diffuse lighting (light intensity of ≈1000 lx) with 12 h of light/dark cycles under air conditioning (set at 25 °C), simulating the conditions of a supermarket shelf. The temperature and humidity were controlled daily during the 27 months of storage study by means of a temperature-humidity data logger (TFA Dostmann Hygrometer, Wertheim, Germany), recording a maximum and minimum value per each one, 29.7–16.3 °C and 70-21%, respectively. The experimental design was performed to take a bottle every three months of storage, excepting the three initial months (0–3 months) and the last three months of storage (25–27 months) in which the measurements were carried out every month. This sampling was in line with the expected changes according to previous experiences of the group (Aparicio-Ruiz et al., 2017; Lobo-Prieto, Tena, Aparicio-Ruiz, Morales, & García-González, 2020). The oil analysed every month was extracted from a bottle just opened.

2.3. Incubation of stored samples on mesh cells (autooxidation/photooxidation) and FTIR spectroscopy monitoring

The samples collected from the storage experiment at different times were subjected to incubation on mesh cell and their FTIR spectra were acquired daily during the incubation time. The method used was the one optimized and described by Tena et al. (2017). Unlike this previous work, in which the stability of the fresh virgin olive oil was assessed, the objective of the present work was to implement the developed method to monitor the changes of stability during virgin olive oil storage under moderate conditions. Thus, the method was applied in the samples collected at different months of storage to subject these samples to different incubation conditions and to identify the moment when the main changes of stability occur.

The oil samples (16 μL) collected along the storage were deposited on the mesh in one of the two apertures of the cell using a micropipette. The sample was uniformly distributed on the surface with the micropipette tip, avoiding any surface uncovered and any excess of oil. After that, the mesh cell was placed in a vertical position for 5 min to drain off any excess of sample. Finally, the loaded mesh cells were incubated in an especial compartment designed for that purpose (Utility model ES1217719) (Tena, García-González, & Aparicio, 2018). Inside the compartment, the cells were horizontally exposed to controlled conditions of light and temperature for 576 h. The incubation conditions used were: i) in the dark and at 35 °C, ii) 400 lx and 23 °C, and iii) 400 lx and 35 °C, which were selected based on previous works (Tena et al., 2017, Tena, Aparicio, et al., 2018; Trypidis et al., 2019). During the incubation experiments, the spectral changes in oil samples collected during the storage were monitored by FTIR spectroscopy every 24 h along the experiment. The mesh cell-FTIR spectra before incubation (0 h) were also acquired for all the samples. The experiments were run in triplicate, including the loading of each sample in three different mesh cells and the subsequent FTIR spectrum acquisition during the incubation period.

A Bruker Vertex 70 FTIR spectrometer (Bruker, Optics, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector was used in this study. During the analysis, the mesh cell was mounted in the transmission cell holder provided with the instrument. All spectra were collected in the range 5000–400 cm^{-1} by co-addition of 32 scans and a resolution of 4 cm^{-1} using weak Norton-Beer apodization. The spectra were collected and manipulated with OPUS version 7.2 (Bruker Optics, Ettlingen, Germany). To facilitate the comparison between the samples, all of the spectra obtained were normalized to an effective path length of 110 μm.

2.4. Spectrum processing

FTIR spectra were normalized according to the procedure described by Tena et al. (2017). The peak heights were measured relative to a

selected single-point baseline by implementing macros programmed with the Macro/Basic tool provided by Omnic 7.3 (Thermo Electron Inc., Madison, WI, USA). The baseline points were optimized for each band: the alcohol band (3535 cm^{-1}) was measured using 4600 cm^{-1}, the hydroperoxide band (3430 cm^{-1}) was measured relative to 3324 cm^{-1}, and the unsaturated aldehydes band (1685 cm^{-1}) using 1576 cm^{-1} as baseline point. This value allows comparing the evolution of the different functional groups monitored during the storage and incubation time.

The influence of the storage and incubation conditions upon the FTIR spectra was studied by the total variance spectra, which was computed with all the spectra obtained from the whole experiment. The change of the spectral properties during the storage period was studied by means of the cumulative variance spectra (García-González & Van de Voort, 2009; Tena et al., 2017) computed with Omnic 7.3.

2.5. Chemical and physical-chemical parameters determination

The quality parameters were determined in the VOOs following the methods for chemical analysis of olive oil, which are specified in "Trade standard applying to olive oils and olive pomace oils (COI/T.15/NC No 3/Rev. 18)" (IOC, 2022). The quality parameters determined were: peroxide value (PV), following COI/T.20/Doc. No 35/Rev.1 (IOC, 2017b), ultraviolet absorptions at wavelengths 232 nm (K$_{232}$) and 270 nm (K$_{270}$), using the standard COI/T.20/Doc. No 19/Rev. 5 (IOC, 2019), free fatty acids (FFA), determined using the standard COI/T.20/Doc. No 34/Rev. 1 (IOC, 2017a), and the organoleptic assessment, using the standard COI/T.20/Doc. No 15/Rev.10 (IOC, 2018b). An accredited panel (UNE-EN-ISO/IEC 17025) carried out the organoleptic assessment and the results were expressed as the median of defect and the median of the fruity attribute.

The determination of α-tocopherol was carried out following the method specified by ISO 9936:2016 (ISO, 2016a) regulation. This analysis was carried out by HPLC (Agilent Technologies 1200, Santa Clara, CA, USA), equipped with a fluorescence detector and a Superspher RP-18 (250 mm length X 4 mm I.D.), particle size 5 μm packed into a LiChroCART® 250-4 HPLC cartridge (Merck, Darmstadt, Germany).

The method for the determination of phenolic compounds was based on the previously described by Aparicio-Ruiz et al. (2016). The sample (2.5 g) was solved in 6 mL hexane together with p-hydroxyphenylacetic (0.12 mg/mL) and o-coumaric (0.01 mg/mL) as internal standards. The phenolic fraction was extracted with methanol by solid phase extraction using diol-bonded phase cartridges. The extracted phenolic fraction was injected in the HPLC system (Agilent Technologies 1200, Santa Clara, CA, USA), equipped with a diode array detector and a LiChrospher 100RP-18 column (4.0 mm i.d. × 250 mm; 5 μm, particle size) (Merck KGaA, Darmstadt, Germany) maintained at 30 °C. The gradient elution, at a flow rate of 1.0 mL/min, water/ortho-phosphoric acid (99.5:0.5 v/v) (solvent A) and methanol/acetonitrile (50:50 v/v) (solvent B) was as follows: from 95% (A)-5% (B) to 70% (A)-30% (B) in 25 min; 65% (A)-35% (B) in 10 min; 60% (A)-40% (B) in 5 min; 30% (A)-70%(B) in 10 min and 100% (B) in 5 min, followed by 5 min of maintenance. The chromatographic signals were obtained at 235, 280 and 335 nm (Mateos et al., 2001).

The determination of the degradation products of chlorophyll a, pheophytin a and pyropheophytin a, was based on the standard method ISO 29841:2016 (ISO, 2016b). The analyses were carried out using a LaChrom Elite HPLC system (Hitachi, Tokyo, Japan), equipped with a diode array detector and a Lichrospher RP18 HPLC column, 250 mm length, 4.0 mm internal diameter, filled with reversed-phase, particles size 5 μm (Merck, Darmstadt, Germany). The quantification was carried out by the calibration curve. Thus, seven different volumes of the standard solution, in duplicate, were injected into the HPLC. The volumes varied from 1 μL to 75 μL, covering the normal range of these compounds in virgin olive oil. The calibration factors of pheophytin a and pyropheophytin a were calculated from the slope values of their calibration curves. The expression of the results was in concentration values (mg/kg).

The oxidative stability of the fresh samples was determined by Rancimat (Oil Stability Index, OSI). This determination was carried out according to the AOCS standard method Cd 12b-92 (AOCS, 2017).

2.6. Statistical analysis

The statistical analysis was carried out with the mean spectra obtained from the triplicate analyses. The STATISTICA 8 package (Statsoft, Tulsa, OK, USA) was used to perform a t-test analysis comparing the intensity (peak height) of each band in the spectra acquired at 0 h and 576 h for the months 0–3 vs. 24–27 (fresh vs. aged oils). Significance was accepted when $p < 0.05$.

The Unscrambler 11 Client (Camo Analytics, Oslo, Norway) was used to carry out the spectral data processing. Principal component analyses (PCA) was used to distinguish the stored samples in terms of stability. A principal component analysis (PCA) was performed with the spectra from the four monovarietal VOOs collected during 27 months of storage at moderate conditions and after 576 h of incubation in mesh cell. The number of principal components (PCs) was selected according to their explained variance.

3. Results and discussion

Prior to the spectroscopic characterization of the oils, the quality of the fresh samples prior to bottling was determined by the physical-chemical analysis. The results of the quality parameters (PV, ultraviolet absorbance, FFA and organoleptic characteristics), total phenolic compounds, α-tocopherol concentration and the degradation products of chlorophyll a (pheophytin a and pyropheophytin a) obtained for the fresh samples and for the samples collected during the storage are gathered in a dataset (Lobo-Prieto, Tena, Aparicio-Ruiz, Morales-Millán, & García-González, 2022). According to the results obtained for FFA, PV, K$_{270}$, K$_{232}$, and the sensory assessment for the fresh samples, all the values were below the limits stated in EC regulation (European Union, 1991) for the classification as "extra virgin olive oil" category, except for VOO4. This sample was initially categorised as "virgin olive oil" due to the presence of a winey-vinegary defect detected by the panellists (Lobo-Prieto et al., 2022). In regards to the concentration of phenols and α-tocopherol, VOO3 showed the highest initial concentration of phenols (534.82 mg/kg), while VOO2 showed the highest concentration of α-tocopherol (272.28 mg/kg). On the other hand, VOO1 showed the lowest total phenol content (226.71 mg/kg), and the lowest concentration of α-tocopherol was found in VOO4 (192.94 mg/kg) (Lobo-Prieto et al., 2022). The analysis of the degradation products of chlorophyll a revealed that, in fresh samples, the highest concentration of pheophytin a was found in VOO3, with a value of 23.43 mg/kg, whereas the rest of the oils showed values ranged of 3.02–7.06 mg/kg. The highest concentration of pyropheophytin a was found in VOO4, with a value of 0.11 mg/kg. In order to assess the chemical changes that take place during the VOO shelf life, all these parameters were determined monthly during the whole storage in the four samples. The tracking results of these parameters during storage were described by Lobo-Prieto et al. (2022).

The identification of the FTIR spectral regions more susceptible to changes when the samples were subjected to incubation under controlled conditions was necessary, as well as the selection of the incubation conditions that produce more variability in the VOO spectra. A previous work (Tena et al., 2017), which optimized the method by studying frehs VOO samples, was focused on this purpose. The three moderate conditions to carry out the incubations were mild heating (35 °C) in the dark, room temperature (23 °C) under light conditions (400 lx), and mild heating (35 °C) combined with light conditions (400 lx). Likewise, three FTIR spectral bands were characterized as the most informative about the degradation of the oils on mesh cells. These FTIR bands (henceforth monitoring bands) were the following: the band

assigned to hydroperoxides (3430 cm^{-1}), which informs on the formation of the primary oxidation products and about the induction period of the samples, and the bands assigned to alcohols (3535 cm^{-1}), and unsaturated aldehydes (1685 cm^{-1}), informing on secondary oxidation products. Although other bands may appear in the spectra as consequence of oil degradation, these bands allowed establishing differences in the oil stability and they can be easily measured under moderate conditions.

3.1. Influence of incubation conditions upon VOO stability by mesh cell-FTIR

Due to the different initial quality of the samples, the resistance of the fresh four monovarietal VOOs to the chemical degradation was determined by FTIR spectroscopy in order to study the influence of the different conditions upon the oils stability. Thus, the four fresh oils were incubated on mesh cell under the three moderate conditions. Fig. 1 shows the intensity of the bands assigned to primary (hydroperoxides) and secondary (alcohols and aldehydes) oxidation products at 0 h (before incubation) and 576 h of mesh cell incubation under the three moderate conditions. The final intensity of the monitoring bands at 576 h of incubation provided information about the final state of the oils, but it is also conditioned by their initial values and hence by the initial oxidation state of the oils. For that reason, with the purpose of assessing the relative response of the monitoring bands in relation to the initial state of the oil, the ratio of the intensity of each monitoring band at 576 h to the intensity at 0 h was calculated in the four oils per each incubation condition. Table 1 shows that the highest ratios were observed when the oils were incubated under light conditions (either 23 °C or 35 °C) compared with the oils kept at mild heating in the dark, and the main increment of intensity was observed in the hydroperoxide bands. Thus, in these incubations, the ratios for this band were higher than 9 for VOO1, VOO2, and VOO3 and 4.984 for VOO4. In general, lower ratios were observed for VOO4 in relation to all the FTIR bands, denoting a better stability according to these spectroscopic results. The ratios for the band assigned to alcohols pointed out that the intensity at 0 h was approximately multiplied by 2 when the oils were exposed to 400 lx and 23 °C and by 2–4.5 when the oils were exposed to 400 lx and 35 °C, except in VOO4 (Table 1). Finally, the band assigned to aldehydes only showed ratios near to 2 for VOO1, VOO2 and VOO3 incubated at 400 lx and 35 °C.

In contrast to what was observed in the incubation including light, the ratios of the intensity of the three bands assigned to hydroperoxides, alcohols, and aldehydes, showed to be lower than 2 when the oils were kept in the dark and at 35 °C (Table 1). Thus, the response of the three monitoring bands showed that the combination effect of light and temperature (400 lx and 35 °C) produced the most drastic alteration of the oils during the incubation (Fig. 1 and Table 1).
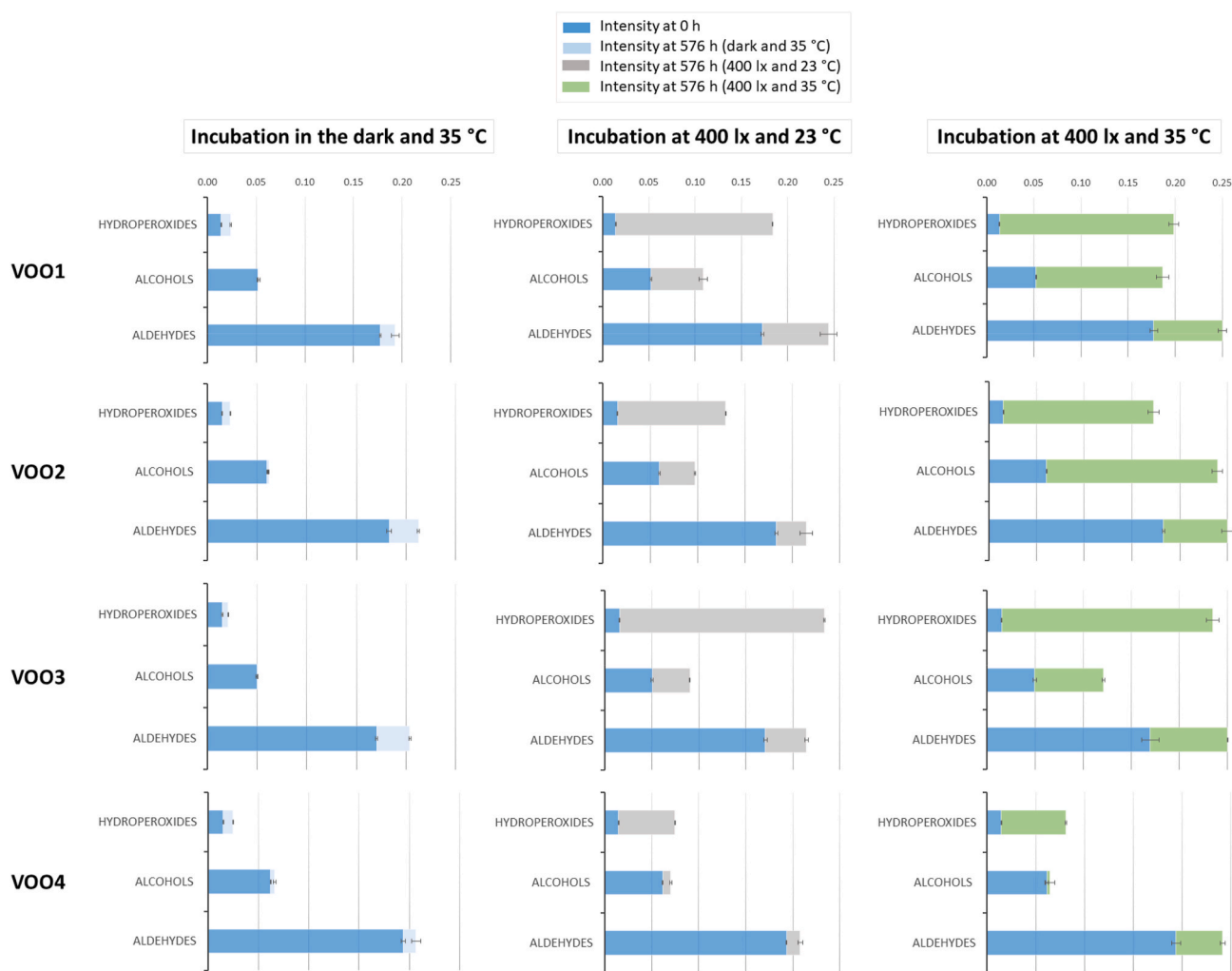

Fig. 1. Intensities, measured as peak height values, of the three monitoring bands: hydroperoxides (3430 cm^{-1}), alcohols (3535 cm^{-1}) and aldehydes (1685 cm^{-1}), registered at 0 h and 576 h of incubation under three moderate conditions: in the dark and 35 °C; 400 lx and 23 °C; and 400 lx and 35 °C. The error bars indicated the standard deviations computed from the three replicates.

Table 1

Ratio of the intensities at 576 h/0 h for the three monitoring bands (hydroperoxides, alcohols and aldehydes) in the four VOOs incubated under the three moderate conditions. The standard deviations of the values are shown between parenthesis.

	Dark and 35 °C				400 lx and 23 °C				400 lx and 35 °C			
	VOO1	VOO2	VOO3	VOO4	VOO1	VOO2	VOO3	VOO4	VOO1	VOO2	VOO3	VOO4
Hydroperoxides band (3430 cm^{-1})	1.731 ($\pm$0.010)	1.661 ($\pm$0.011)	1.380 ($\pm$0.010)	1.626 ($\pm$0.011)	12.969 ($\pm$0.011)	9.174 ($\pm$0.004)	15.147 ($\pm$0.025)	4.984 ($\pm$0.009)	14.626 ($\pm$0.002)	12.722 ($\pm$0.010)	15.288 ($\pm$0.003)	5.508 ($\pm$0.005)
Alcohols band (3535 cm^{-1})	1.021 ($\pm$0.005)	1.194 ($\pm$0.011)	1.007 ($\pm$0.002)	1.067 ($\pm$0.008)	2.072 ($\pm$0.011)	1.865 ($\pm$0.005)	1.790 ($\pm$0.011)	1.142 ($\pm$0.008)	3.565 ($\pm$0.012)	4.571 ($\pm$0.005)	2.431 ($\pm$0.007)	1.043 ($\pm$0.009)
Aldehydes band (1685 cm^{-1})	1.086 ($\pm$0.010)	1.196 ($\pm$0.003)	1.196 ($\pm$0.005)	1.067 ($\pm$0.011)	1.415 ($\pm$0.003)	1.244 ($\pm$0.004)	1.255 ($\pm$0.008)	1.075 ($\pm$0.004)	1.837 ($\pm$0.009)	1.971 ($\pm$0.022)	1.644 ($\pm$0.006)	1.247 ($\pm$0.007)

3.2. Study of the chemical degradation of VOO by mesh cell-FTIR during storage

The computation of the total variance spectra allowed identifying the main spectral changes in the three monitoring bands (Tena et al., 2017). Thus, the total variance spectra were computed from the FTIR spectra collected during the 27 months of storage (VOO-0m - VOO-27m) without incubation in mesh cell (0 h) and after incubation in mesh cell (576 h). Since three incubation conditions were tested, each VOO was characterized with four total variance spectra.

Fig. 2 shows the total variance spectra of the spectral region 3700–3100 cm^{-1}, in which the bands assigned to hydroperoxides and alcohols are located, for the four oils (VOO1 – VOO4). This figure shows no relevant changes in the spectra of the stored samples before incubation (0 h) (red line in Fig. 2). In fact, the absorption variance in the spectra at 0 h was <0.001 absorbance units (AU) in all of the cases, which revealed that the spectra acquired during the whole storage

before mesh cell incubation showed no apparent differences in their spectral properties as the oil is ageing. Nevertheless, the monitoring of the quality and chemical parameters during the storage revealed that the composition of the oil was changing (Lobo-Prieto et al., 2022) slowly as the storage progressed. These chemical changes were not enough to bring about variations in the absorption of the bands as the oil ages. On the other hand, Fig. 3 shows the total variance spectra of the spectral region ranged between 1750 and 1550 cm^{-1}, in which the band assigned to unsaturated aldehydes is found. In this case, no change in the absorption was either observed for the band assigned to unsaturated aldehydes when no incubation was performed (spectra at 0 h). Nevertheless, the total variance spectra of the stored samples incubated in mesh cell during 576 h evidenced that there were changes in the composition of the oil. These changes were enough to produce variations in the intensity of the bands assigned to hydroperoxides, alcohols and aldehydes when the oils were subjected to the incubation conditions in mesh cell (Figs. 2 and 3). Thus, while the intensity of the total variance
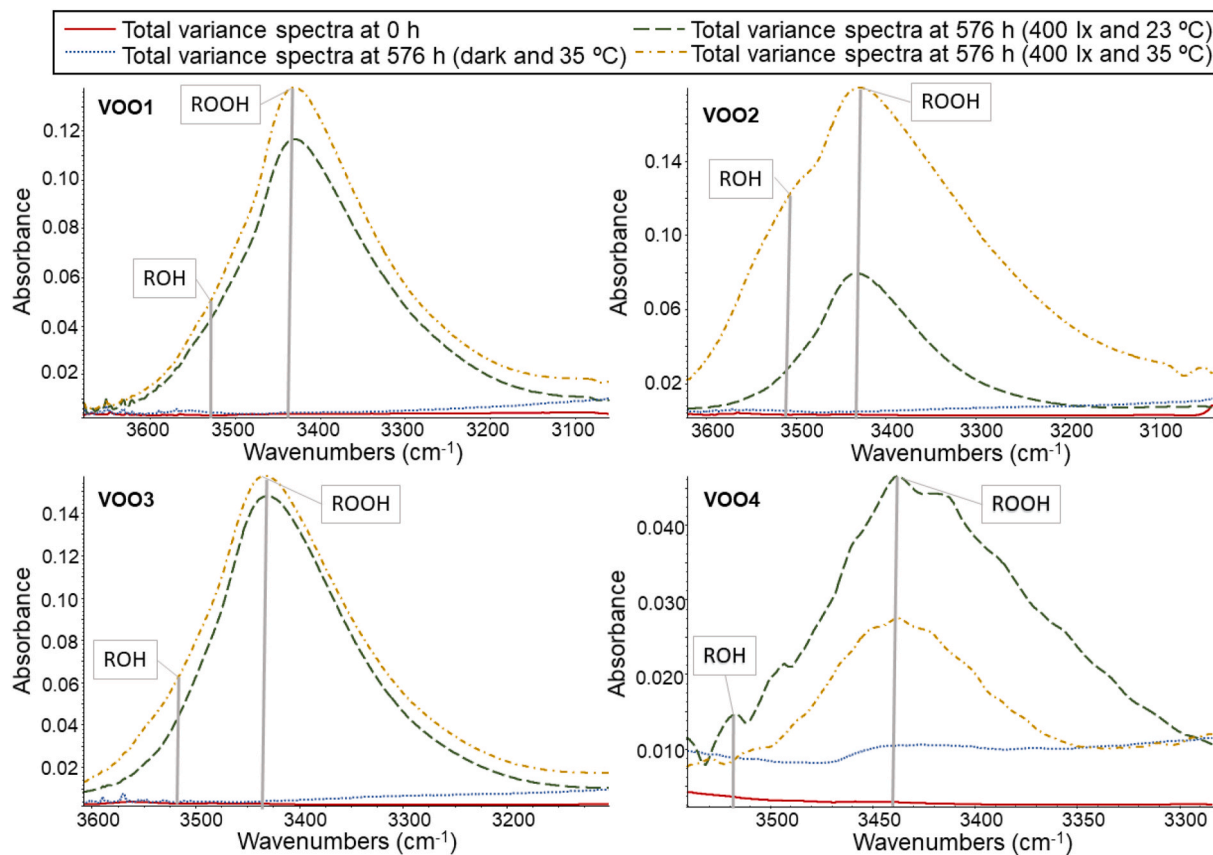

Fig. 2. Total variance spectra of the spectral region ranged between 3700 and 3100 cm^{-1} of the VOO samples collected every month during the storage experiment. This region was assigned to hydroperoxides (ROOH, 3430 cm^{-1}) and alcohols (ROH, 3535 cm^{-1}). A total variance spectrum was calculated before the incubation experiment (0 h) and the other three total variance spectra were computed from the spectra acquired after 576 h of incubation in the mesh cell under the three moderate conditions (dark and 35 °C, 400 lx and 23 °C, and 400 lx and 35 °C). Each spectrum corresponds to the mean spectrum computed from the three replicates.
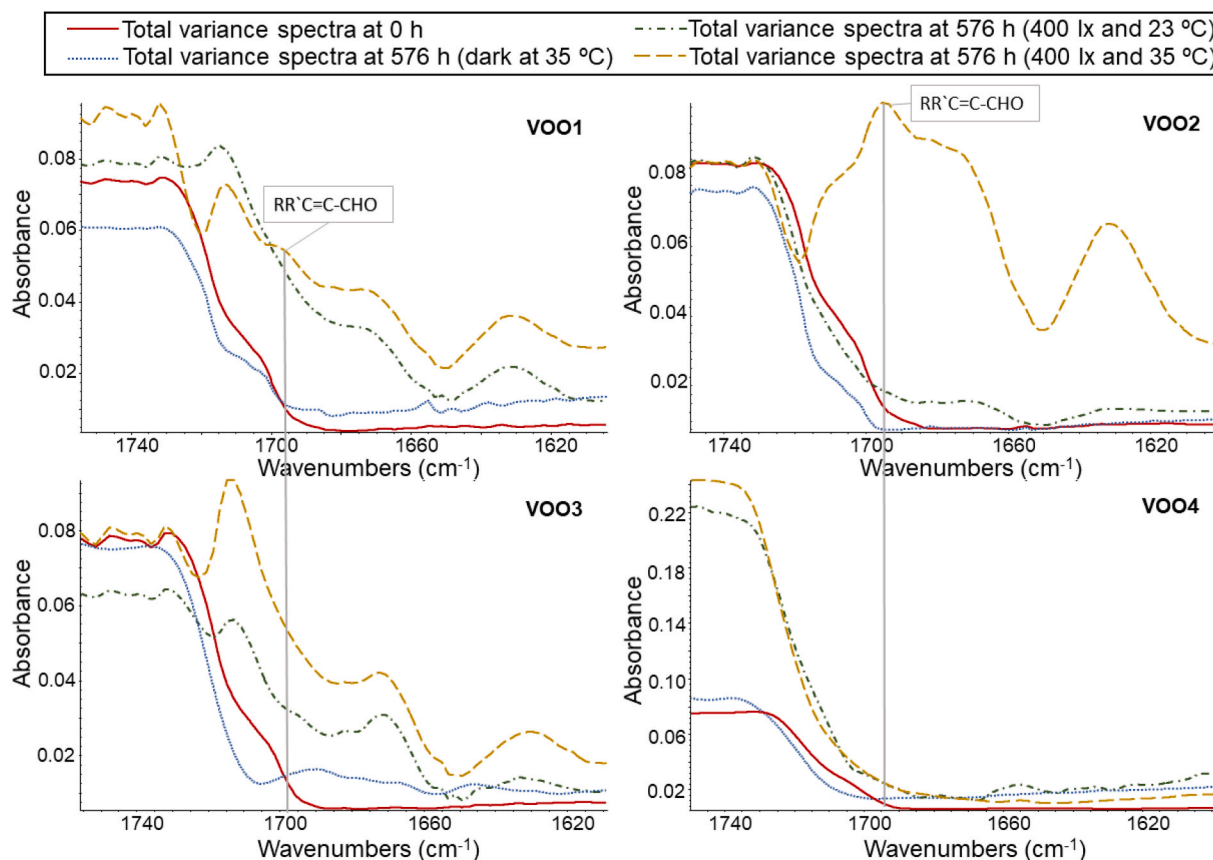
Fig. 3. Total variance spectra of the spectral region ranged between 1750 and 1550 cm^{-1} of the VOO samples collected every month during the storage experiment. One total variance spectrum was calculated before the incubation experiment (0 h) and the other three were analysed after 576 h of incubation under the three moderate conditions previously selected. The band assigned to aldehydes are marked. Each spectrum corresponds to the mean spectrum computed from the three replicates.

spectra computed with the spectra collected during the storage without incubation was always <0.001 AU in the three monitoring bands, the total variance spectra obtained from the collected samples after 576 h of incubation showed intensity values much higher, especially in the two conditions applying light (Figs. 2 and 3). The hydroperoxide band registered the highest variance in all the oils, with values ranging between 0.050 and 0.087 AU in the two experiments carried out with light, while the experiment in the dark and at 35 °C reached values not far from the acquired without mesh cell incubation (0.002–0.005 AU). The band assigned to alcohols and aldehydes also showed the highest variance when the VOOs were incubated under light exposure (Figs. 2 and 3), showing values ranging between 0.003 and 0.045 AU, except for VOO2 when it is incubated at 400 lx and 35 °C (0.066–0.094 AU). These observation points out a relevant effect of temperature (23 °C vs. 35 °C) upon this sample (Figs. 2 and 3), while in the other oils the temperature effect was not so marked. Thus, according to the results obtained through the total variance, the incubation in mesh cell is able to detect and to evidence the effect of minor chemical changes occurring in VOO during storage under moderate conditions.

To identify the most relevant changes and the incubation conditions giving rise to the greatest changes in the spectra, the significant changes in the absorption during the storage were evaluated for the three monitoring bands. A *t*-test ($p < 0.05$) was performed comparing the intensity of each band (measured as peak height value) in the acquired spectra for the months 0–3 vs. 24–27 (fresh vs. aged oils). This *t*-test was carried out for each stored VOO with the spectra collected during the storage without incubation (0 h) and after 576 h of incubation in mesh cell. Table 2 shows the monitoring bands that showed significant changes ($p < 0.05$) for each oil. When no incubation was applied, the *t*-

test showed that there were significant changes only in the intensity of the band assigned to hydroperoxides, while no significant differences were found in the band assigned to alcohols and unsaturated aldehydes. However, the peak height values of the hydroperoxide band in the aged oils (0.016–0.018 AU) were only slightly higher than the values registered in the fresh oils (0.014–0.016 AU). This explains that the total variance spectra computed with all the spectra did not show variations during the storage (Figs. 2 and 3).

The *t*-test results, computed with the spectra obtained after 576 h of incubation between fresh and aged samples (0–3 months vs. 24–27 months), revealed that the incubation experiments producing the highest variations were those carried out with light and the two studied temperatures (400 lx and 23 °C, 400 lx and 35 °C) (Figs. 2 and 3). In fact, the *t*-test showed that 9 from the 12 peak heights measured (3 spectral bands studied in the four VOOs) showed significant differences when comparing fresh and aged oils incubated under 400 lx and 35 °C and 7 peak heights showed significant differences in the experiments performed at 400 lx and 23 °C (Table 2). In contrast, in the experiment carried out in the dark at 35 °C, only 1 peak height (hydroperoxides) showed significant changes. This result pointed out the weak capability of temperature, compared with light, to evidence the spectral variations associated to the stability changes occurring during VOO storage at mild condition. For this reason, the experiment carried out in the dark at 35 °C was not considered in the subsequent statistical analyses for studying the VOO stability changes. Furthermore, the band assigned to hydroperoxides was selected to perform the further analyses because it showed significant changes in the four VOOs in both incubations applying light, except in VOO4 when it was subjected to 400 lx and 23 °C (Table 2).

Table 2

Results obtained from *t*-test performed comparing the intensities of each band in the months 0–3 vs. 24–27 (fresh vs. aged oils) at 0 h and 576 h of incubation. The spectral bands showing significant changes ($p < 0.05$) are indicated for each stored VOO and each experiment carried out.

Incubation time in mesh cell	Experiment	Stored VOO	Spectral bands with significant changes
0 h	**Before incubation**	VOO1	Hydroperoxides (3430 cm^{-1})
		VOO2	Hydroperoxides (3430 cm^{-1})
		VOO3	Hydroperoxides (3430 cm^{-1})
		VOO4	Hydroperoxides (3430 cm^{-1})
576 h	**Dark and 35 °C**	VOO1	–
		VOO2	–
		VOO3	Hydroperoxides (3430 cm^{-1})
		VOO4	–
	400 lx and 23 °C	VOO1	Alcohols (3535 cm^{-1})
			Hydroperoxides (3430 cm^{-1})
			Unsaturated aldehydes (1685 cm^{-1})
		VOO2	Hydroperoxides (3430 cm^{-1})
		VOO3	Alcohols (3535 cm^{-1})
			Hydroperoxides (3430 cm^{-1})
			Unsaturated aldehydes (1685 cm^{-1})
		VOO4	–
	400 lx and 35 °C	VOO1	Alcohols (3535 cm^{-1})
			Hydroperoxides (3430 cm^{-1})
			Unsaturated aldehydes (1685 cm^{-1})
		VOO2	Hydroperoxides (3430 cm^{-1})
			Unsaturated aldehydes (1685 cm^{-1})
		VOO3	Alcohols (3535 cm^{-1})
			Hydroperoxides (3430 cm^{-1})
			Unsaturated aldehydes (1685 cm^{-1})
		VOO4	Hydroperoxides (3430 cm^{-1})

3.3. Study of changes in stability measurements by mesh cell-FTIR during VOO storage

As the oil degradation proceeds, the composition of antioxidants and prooxidants changes and, therefore, stability measurements over time are expected to change as well. Each oil collected at a given time (month) during the storage was characterized with mesh cell-FTIR spectra acquired during mesh cell incubation experiments under the three moderate conditions mentioned above. The spectral region at 3700–3100 cm^{-1}, which contains the band assigned to hydroperoxides, was studied by calculating their cumulative variance spectra to identify the stability changes of the oils during the storage period. Unlike the total variance spectra, the cumulative variance spectra are able to point out the sequential gathered changes taking place in the spectrum over the storage time, detecting the moment when the oils underwent the highest changes in their chemical composition. This procedure allowed to identify the spectral differences in a given month and to compare the results from different months to evaluate the dynamic change of stability during storage.

Fig. S1 (Supplementary material) shows the cumulative variance spectra computed for the stored samples taking into account the spectra collected during the incubation in mesh cells at 0 h (Fig. S1–A, D, G, J) and 576 h at 400 lx and 23 °C (Fig. S1–B, E, H, K) and at 400 lx and 35 °C (Fig. S1–C, F, I, L). The spectra marked in green colour are those in which the intensity of the band increased during those months, while red colour in the spectra indicates that the intensity of the band remained constant or even decreased. The results obtained before mesh cell incubation (time 0 h) did not show any variations between the spectra obtained during the storage (Fig. S1–A, D, G, J). The cumulative variance spectra at 576 h (Fig. S1 – B-C, E-F, H-I, K-L) pointed out that the samples underwent less changes of stability in the last months of storage. Thus, the intensity of this spectral region, where hydroperoxide and alcohol bands are located, showed less increment as the storage experiment progressed, reaching a moment (month of storage) when the cumulative variance started to keep constant or even decrease. This moment took place in different months depending on the VOO, and depending on the incubation conditions applied (Fig. S1). In general terms, Fig. S1 showed that the stability variations were no linear during the storage and they took places mainly at some specific months during the storage. In the stored samples, the main variation in the spectra take places during the six first months of storage, which revealed that the VOO are more susceptible to stability changes during the first stage of the commercialization process. This suggests that during the first months of the VOO's shelf life, the product handling must be carefully controlled, especially during transport and distribution.

To extract conclusions on the stability changes during the storage, the intensity measured at 576 h in the cumulative variance spectra was selected as indicative of stability of the oils in each month of storage. Two parameters were selected for this purpose: 1) the maximum intensity of the hydroperoxide band observed in the cumulative variance spectra, and 2) the time (month of storage) when the band starts increasing its intensity in these spectra. Thus, lower maximum intensity and longer time means that the stability changes in lesser degree throughout the storage during the early stages of commercialization. Fig. 4 shows the absorbance intensity (peak height value) of the band assigned to hydroperoxides in the cumulative variance spectra (Fig. S1) for the four stored VOOs under the two incubation experiments at 576 h. On the one hand, the maximum intensity value of the band assigned to hydroperoxides ranged between 0.059 and 0.088 AU for the four VOOs in both incubation conditions, except for VOO4, which showed a lower value (0.030 AU) when it was exposed to 400 lx and 23 °C. Under this incubation condition, VOO4 maintained its stability constant throughout 25 months of storage. The main changes of stability in these samples took place during the last two months of storage (months 26 and 27) and they were always less remarkable than the stability changes observed in the other oils (Fig. 4). Thus, the band assigned to hydroperoxides only reached an intensity of 0.030 AU in this oil at the end of the incubation whereas the other VOOs reached intensity values higher than 0.059 AU. By contrast, the highest intensity of the hydroperoxide band was registered in VOO3 for the two incubation conditions (0.089 AU at 400 lx and 23 °C, and 0.087 AU at 400 lx and 35 °C), which indicated that this oil showed the highest changes of stability during the storage compared with the other stored oils.

On the other hand, when the time (month of storage) in which the bands start increasing their intensity in the cumulative variance spectra was analysed, the oils that showed the fastest changes in the stability were VOO1 and VOO2 under the two incubation conditions, and VOO4 when it was incubated at 400 lx and 35 °C. These three oils showed variations in the intensity of the band assigned to hydroperoxides from the beginning of the storage, pointing out an abrupt change of stability in the first six months (Fig. 4). Nevertheless, VOO3 (for both incubation conditions) and VOO4 (400 lx and 23 °C) showed later variations of this band, which indicated no changes of stability in first months of storage. In fact, the variations of this band were observed after 6 months of storage in VOO3 and after 25 months of storage in VOO4. The small variation in the first 6 months can be explained by the protective effect of the phenolic compounds. Thus, VOO3 and VOO4 were the oils with the highest concentration of these compounds in the fresh oils
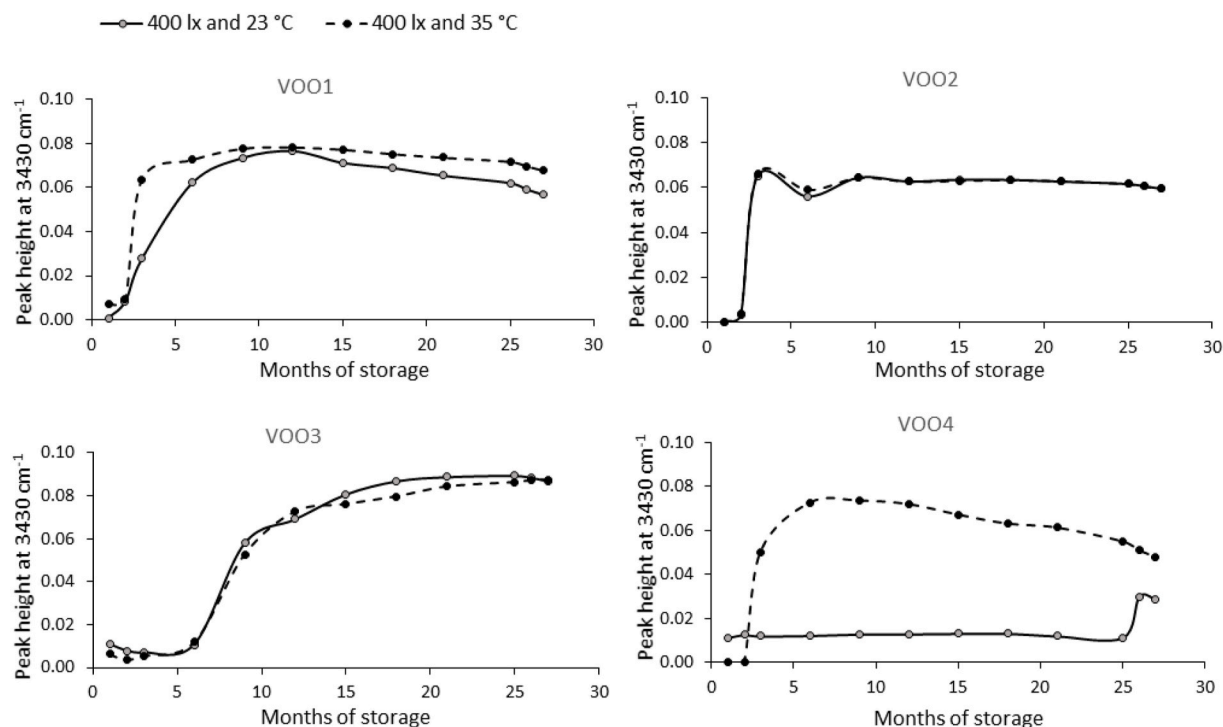
Fig. 4. Absorbance intensities of the band assigned to hydroperoxides (3430 cm^{-1}) reached in the cumulative variance spectra of the monthly collected samples when they were subjected to 576 h of incubation at 400 lx and 23 °C and 400 lx and 35 °C. The results correspond to the mean of three replicates, the standard deviation being always lower than 0.025.

(Lobo-Prieto et al., 2022). However, in VOO4, the probable reduction of phenols concentration in the first months of storage was enough to increase the variance of the hydroperoxide band from the beginning of the storage when it was incubated at 400 lx and 35 °C, showing stability changes in the sample earlier than at 400 lx and 23 °C (Fig. 4). Afterwards, the intensity of the band in the cumulative variance spectra remained constant or even decreased for these oils, indicating that there were not relevant changes of stability measured with mesh cell after those moments.

In both incubations, the hydroperoxides band reached the maximum intensity at the same moment for VOO1 in month 12 (0.077 AU at 400 lx and 23 °C, 0.078 AU at 400 lx and 35 °C) and for VOO2 in months 3 (0.057 AU at 400 lx and 23 °C, 0.065 AU at 400 lx and 35 °C), which indicated that the stability changes occurred at the same time regardless of the incubation condition used. The results of VOO3 did not show large changes between incubation experiments. Thus, the maximum intensity of this band was reached in months 25 and 27 of storage when it was exposed to 400 lx and 23 °C (0.0893 AU) and 400 lx and 35 °C (0.0874 AU), respectively. In contrast, VOO4 showed that the moment when the hydroperoxide band reaches the maximum intensity greatly depended on the incubation condition (Fig. 4). In this sample, the incubation conditions had a marked effect, the maximum intensity was observed in month 26 at 400 lx and 23 °C (0.0295 AU) and in month 6 at 400 lx and 35 °C (0.0727 AU). Thus, in this oil, the application of light only or light combined with mild heating can dramatically accelerate or slow down the moment when the main changes of stability occur (Figs. 3 and 4). The relative low content of pheophytin *a* (4.61 mg/kg) and α-tocopherol (192.94 mg/kg) of this sample could explain this different behaviour, in addition to the fact that this sample was the one that was not EVOO. However, VOO2 also had low content of pheophytin *a* (3.02 mg/kg) and exhibited more sensitivity to photooxidation than VOO4 (Fig. 4). This result illustrates that some oils with small differences in prooxidants/ antioxidant can show different behaviour when moderate conditions are applied.

Afterwards, a principal component analysis (PCA) was performed

with the spectra from the four stored VOOs incubated in mesh cell for 576 h under two moderate conditions: 400 lx and 23 °C, and 400 lx and 35 °C.

Fig. 5 shows the scores (A) and loadings (B) plots in the plane defined by PC1 and PC3 obtained in the incubation at 400 lx and 23 °C. A selected area of the PCA plot is zoomed for a better visualization (C). The principal components (PCs) 1 and 3 explained 95% of the total variance, where most of the variance was explained by PC1 (94%). The scores plot (Fig. 5-A) shows a distribution of the stored samples along PC1 according to their ageing. The samples collected in the first months of storage were characterised with negative scores for PC1 and they had scores closer to positive values as the storage experiment progressed. The loadings plot (Fig. 5-B) revealed that the negative values of the PC1 were associated to the spectral region 3587–3244 cm^{-1} (Fig. 5-B), where the absorption bands of the hydroperoxides (3430 cm^{-1}) and alcohols (3535 cm^{-1}) (Tena, Aparicio, et al., 2018) are located. These loadings explained that the samples collected in the first months of storage were separated from the rest because they were in an early state of degradation in which the formation of hydroperoxides has an important role. On the other hand, the majority of the aged samples were located in the negative side of PC3 (Fig. 5-A), which is associated to the spectral region assigned to C=O stretching (Sikorska, Khmelinskii, & Sikorski, 2014), where the absorption bands attributed to secondary oxidation products, such as unsaturated aldehydes (1685 cm^{-1}) (Tena et al., 2017), are located (Fig. 5-B).

The PCA obtained from the incubation at 400 lx and 35 °C showed similar results to the PCA carried out for the experiment at 400 lx and 23 °C. In this analysis, like in the previous PCA, the samples were distributed along PC1 according to their ageing (Fig. 6-A), and the spectral regions that ranged between 3587 and 3244 cm^{-1} and 1800–1620 cm^{-1} (Fig. 6-B) also showed the greatest influence on the model. Few differences were observed in the PCA results obtained from the two different incubations in mesh cell. The PCA results obtained from the incubation at 400 lx and 23 °C shows that the samples collected in the last months of storage of VOO1 and VOO2 were evolving towards
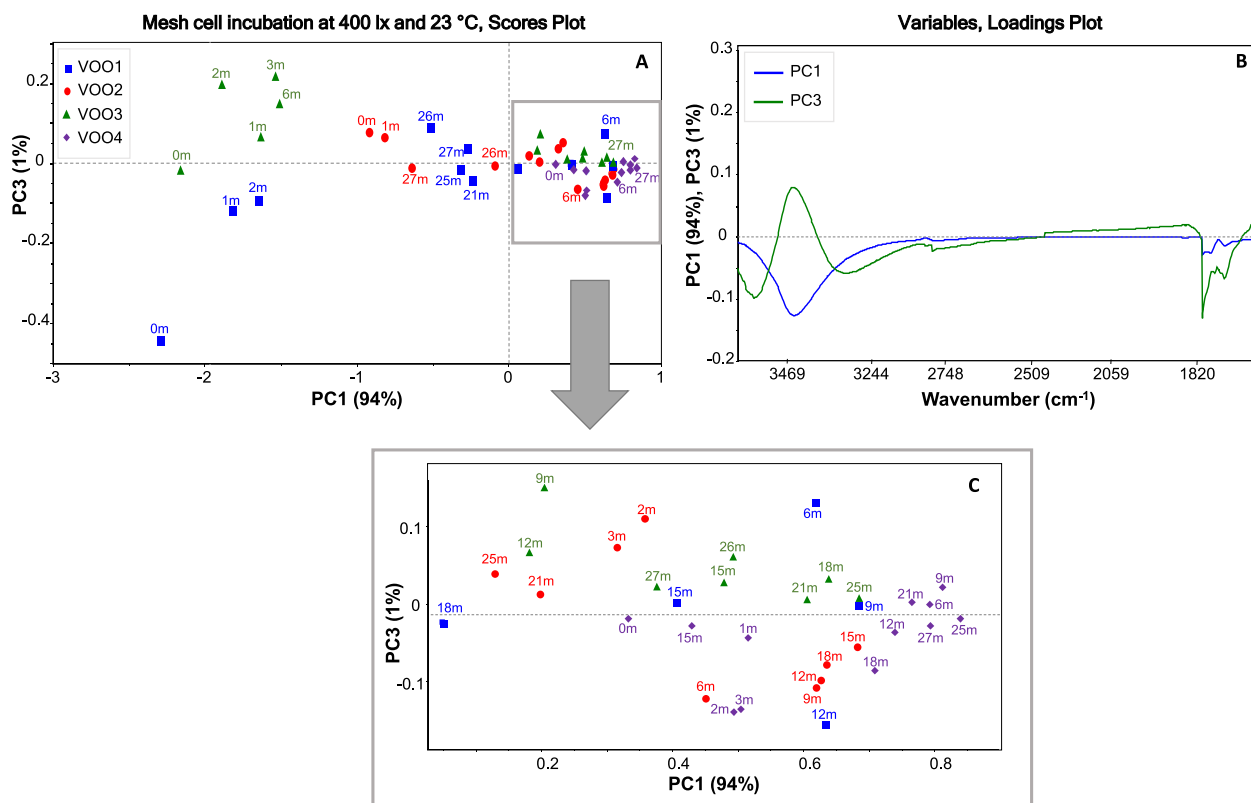
Fig. 5. Results of the principal component analysis (PCA) carried out with the FTIR spectra obtained from the four monovarietal VOOs stored for 27 months and incubated in mesh cells for 576 h at 400 lx and 23 °C. The scores plot (A) and the loadings plot (B) of the principal components 1 (PC1) and 3 (PC3) are shown. A selected area of the PCA plot is zoomed for a better visualization (C).

negative values of PC1 (Fig. 5-A.), which indicated that the spectra of the samples collected in the first and last months of storage under incubation in mesh cell showed similar spectral characteristics in these two oils. This finding could be due to the fact that fresh samples, with more concentration of chlorophylls pigments, were highly sensitive to photooxidation, while the most aged samples, with lower concentration of antioxidants, were also highly sensitive to autoxidation (Lobo-Prieto et al., 2022). However, this was not observed in the incubation at 400 lx and 35 °C, where the fresh samples were better separated from the rest of the stored samples.

The fact that the stored samples were plotted in the different quadrants would be indicative of a difference in the formation kinetics of hydroperoxides and aldehydes along the storage, which is associated to a different stability of the stored samples and different speed in the formation of both kinds of compounds. The moment when the stored oils changed to the positive values of PC1 revealed that the hydroperoxides no longer showed a predominant role, and hence, the oxidation process was starting to be characterized with the presence of secondary oxidation products. The storage months in which the PC1 scores became positive depended on the oil. Thus, VOO3 was always, in the two incubation experiments, the last oil presenting positive PC1 scores during the storage, whereas VOO1 and VOO2 were the first ones. The slower changes in the scores observed in VOO3 compared with VOO1 and VOO2 revealed a high oxidative resistance and slow changes in their stability during the storage period. On the other hand, VOO4 was the only oil where all the samples stored under the two incubation experiments were on the positive side of PC1. (Figs. 5 and 6-C.). This pointed out to be the most degraded oil from the beginning of the storage, which agreed with the initial characterisation of the oil as "virgin olive oil" instead of "extra virgin olive oil".

4. Conclusions

The significant differences found in the FTIR bands of the stored samples without and with incubation in mesh cell pointed out that mesh cell-FTIR spectroscopy is able to highlight the small chemical changes occurred during the VOO storage under moderate conditions, and allows their sequential comparison to study the dynamic changes of stability from a multiparametric perspective during VOO ageing. Thus, in this study, stability was measured as the resistance of the oil to mesh cell incubation, and this resistance showed a non-lineal change in all the cases. In general terms, the main variation of stability measured with mesh cell-FTIR spectroscopy took place during the first 6 months of storage, although the effect of antioxidants and prooxidants delayed or accelerated this variation. The monitoring of the hydroperoxides band along the storage provided relevant information about the VOO chemical changes, allowing the distinction of samples according to their stability changes. In fact, the results obtained through the PCA verified the ability of the FTIR spectra obtained by mesh cell incubation to distinguish, in terms of stability, between VOO samples collected during the storage time (27 months). The study provided a dynamic perspective of the minor changes that takes place since the first months of storage and it may affect the stability of the oils when they are subjected to other conditions of light and temperature. The study also points out the importance of the conditions (light or light plus moderate heating) when they are moderate. Thus, it shows the complexity of predicting stability with simple approaches. The new challenges derived from this work would be focused on studying the stability changes during the storage in VOOs belonging to different categories and on the evaluation of the effect of individual phenols and pigments on the VOO stability, also with a dynamic perspective and including changes of temperature/light conditions during storage.
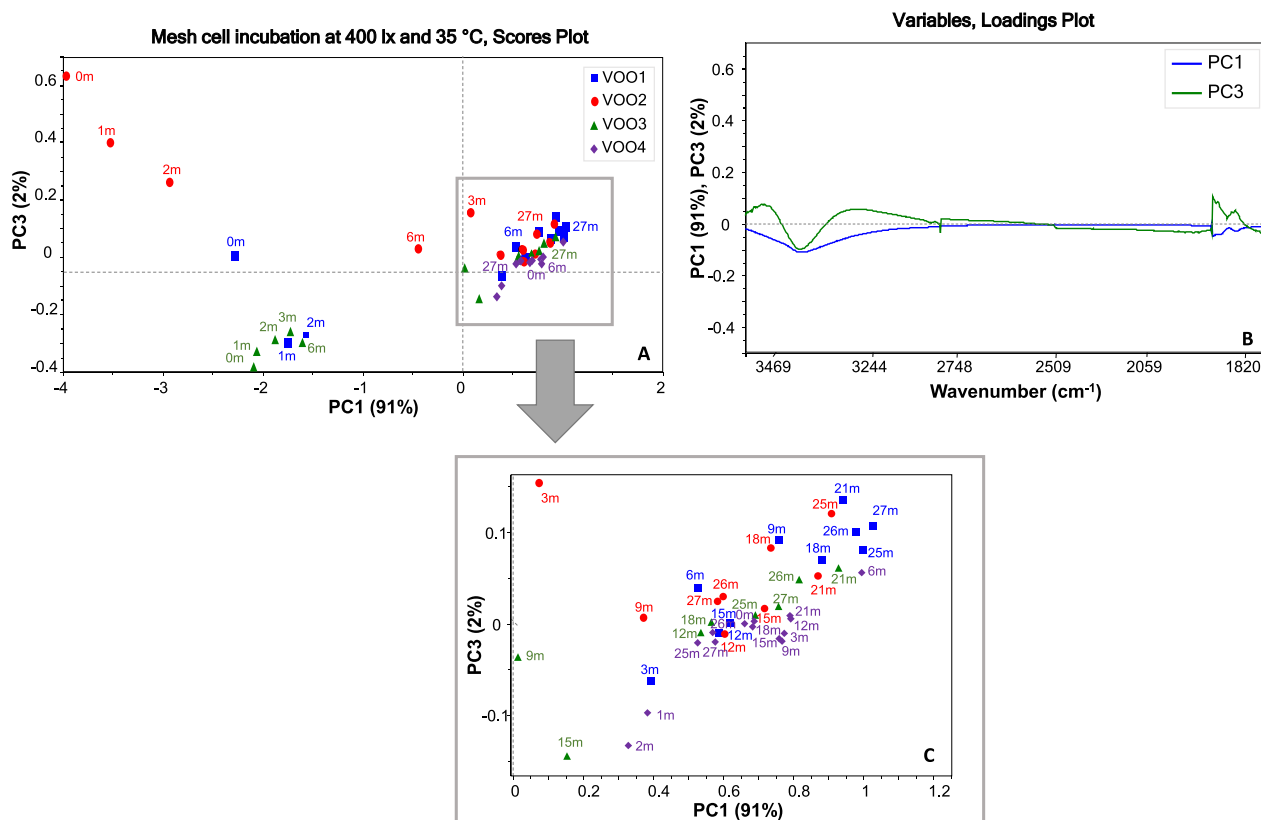
Fig. 6. Results of the principal component analysis (PCA) carried out with the FTIR spectra obtained from the four monovarietal VOOs stored for 27 months and incubated in mesh cells at 400 lx and 35 °C for 576 h. The scores plot (A) and the loadings plot (B) of the principal components 1 (PC1) and 3 (PC3) are shown. A selected area of the PCA plot is zoomed for a better visualization (C).

The information obtained by mesh cell-FTIR spectroscopy could be useful for making informed decision about the commercialization process of VOO, such as the selection of the best condition for storage, transport or distribution. Producers and retailers could gain more knowledge about which factors affect the shelf life of VOO the most and when the product is more susceptible to deterioration. Furthermore, this technique can be applicable to other different lipid matrices with different degree of stability depending on their complex composition, such as other vegetable virgin oils with a particular interest in keeping quality during production and distribution.

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CRediT authorship contribution statement

Ana Lobo-Prieto: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Noelia Tena:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **Ramón Aparicio-Ruiz:** Data curation, Investigation, Methodology, Supervision, Writing – review & editing. **María T. Morales:** Investigation, Methodology, Resources, Supervision, Writing – review & editing. **Diego L. García-González:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114160.

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