




Article

Potential of the Oxidized Form of the Oleuropein Aglycon to Monitor the Oil Quality Evolution of Commercial Extra-Virgin Olive Oils

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Abstract: The quality of commercially available extra-virgin olive oils (VOOs) of different chemical compositions was evaluated as a function of storage (12 months), simulating market storage conditions, to find reliable and early markers of the virgin olive oil (VOOs) quality status in the market. By applying a D-optimal design using the Most Descriptive Compound (MDC) algorithm, 20 virgin olive oils were selected. The initial concentrations of oleic acid, hydrophilic phenols, and α -tocopherol in the 20 VOOs ranged from 58.2 to 80.5%, 186.7 to 1003.2 mg/kg, and 170.7–300.6 mg/kg, respectively. K_{270} , ΔK , (*E*, *E*)-2,4-decadienal and (*E*)-2-decenal, and the oxidized form of the oleuropein aglycon (3,4-DHPEA-EA-OX) reflected the VOO quality status well, with 3,4-DHPEA-EA-OX being the most relevant and quick index for simple monitoring of the “extra-virgin” commercial shelf-life category. Its HPLC-DAD evaluation is easy because of the different wavelength absorbances of the oxidized and non-oxidized form (3,4-DHPEA-EA), respectively, at 347 and 278 nm.

Keywords: extra-virgin olive oil quality evolution; light exposure; extra-virgin olive oil quality markers; K_{270} ; ΔK ; (*E*, *E*)-2,4-decadienal; (*E*)-2-decenal; the oxidized form of the oleuropein aglycon (3,4-DHPEA-EA-OX)



Citation: Esposto, S.; Urbani, S.; Selvaggini, R.; Taticchi, A.; Gallina Toschi, T.; Daidone, L.; Bendini, A.; Veneziani, G.; Sordini, B.; Servili, M. Potential of the Oxidized Form of the Oleuropein Aglycon to Monitor the Oil Quality Evolution of Commercial Extra-Virgin Olive Oils. *Foods* **2023**, *12*, 2959. <https://doi.org/10.3390/foods12152959>

Academic Editor: Antonella De Leonardis

Received: 19 June 2023

Revised: 1 August 2023

Accepted: 3 August 2023

Published: 4 August 2023



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1. Introduction

The consumption and production of VOO have seen a huge international increase mainly because of its recognized health benefits and peculiar sensorial characteristics [1–5]. However, this significant rise in interest makes producers and packagers eager to improve olive oil quality protection during its market shelf life. The shelf life is defined as the period during which the food product will remain safe for consumption; keep the desired sensory, chemical, physical, and microbiological characteristics; will stay acceptable for consumers; and will uphold any declaration reported on the label [1].

The storage of VOOs might promote quality loss, according to the oxidative phenomena correlated to the oxygen concentration and light exposition. Several studies have demonstrated that light exposure over storage is the main factor responsible for quality loss [2–8].

Photo-oxidation compromises VOO’s legal, health-promoted, and sensory quality once it is marketed because of the accumulation of hydroperoxides and their rapid homolytic cleavage in C7-C11 volatile compounds responsible for the “rancid” defect [4–8]. Consequently, discrepancies could cause contradiction between the quality declared on the label, the relative consumers’ expectations, and the actual VOO quality in the market.

Indeed, suitable instruments for monitoring VOO's shelf life must be provided considering the wide variability in terms of fatty acid composition and antioxidant content affecting the huge number of VOOs in the real market [9].

Peroxide value, extinction coefficients, volatile compounds related to the "rancid" defect and the overall and sensory assessment [4–8,10–15], chlorophylls, and their derivatives pyro pheophytins (PPPs) and diacylglycerols (DAGs) [16–23] have been recognized as potential markers of the quality status of VOOs.

Furthermore, since the recognized involvement of secoiridoid derivatives in the oxidative processes, new studies have focused on their oxidative products as discriminants of the actual VOO quality under market conditions.

Despina et al., monitoring the evolution of the phenolic composition in VOOs subjected to heat treatment, highlighted the accumulation of oxidized phenolic compounds in products [24]. Di Maio et al. [25,26] concluded that hydroxytyrosol (3,4-DHPEA) is involved in reactions of VOO oxidation processes and that the oxidation product of the dialdehydic form of the elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA or oleacein) that accumulates early in the VOO can be used as a molecular marker of the oil the VOO's oxidation status [25,26]. Esposto et al. confirmed the constant and rapid accumulation during deep oil frying, or storage in normal conditions of the oxidized form of 3,4-DHPEA-EDA, according to the initial concentration of 3,4-DHPEA-EDA [7,27]. More recently, Di Serio et al. [28] included the oxidized phenols in the development of an analytical method to predict the remaining shelf-life of individual marketed VOOs [28]. Tsolakou et al. [29] investigated the presence of oleocanthalic acid, in VOOs stored in different conditions, observing its increase with storage time while the oleocanthal concentration decreased. The conversion of oleocanthal in oleocanthalic acid and its relative accumulation in VOOs kept at different storage conditions was also observed by Esposito Salsano et al. [15]. Abbattista et al. [30] proposed a reverse-phase liquid chromatography coupled with high-resolution/accuracy Fourier transform mass spectrometry with electrospray ionization to monitor the accumulation of oxidative forms of oleuropein and ligstroside aglycones, oleacin, and oleocanthal during storage up to 6 months under controlled conditions. Antoniadi et al. [31], after the characterization of the main oxidized olive oil secoiridoids, in several commercial Greek VOOs, hypothesized that they could contribute as chemical markers for the quality categorization of VOOs as extra-virgin, virgin, or lamp [31].

For the first time, the potentiality of the oxidized form of the oleuropein aglycon (3,4-DHPEA-EA-OX), as a new marker of the quality status of the VOO stored under market conditions, was evaluated because of the more rapid and earlier involvement of oleuropein derivatives to contrast VOO photo-oxidation [5,7,8,27,32]. A simple and clear identification method of 3,4-DHPEA-EA was also demonstrated.

2. Materials and Methods

2.1. Solvents and Reagents

For the chromatographic analysis, methyl alcohol, n-hexane, isopropyl alcohol, and glacial acetic acid, all of HPLC grade, and water, methyl alcohol, and glacial acetic acid, all of LC-MS grade, were obtained from VWR (Milan, Italy). Analytical- and HPLC-grade water was obtained using purification systems. Phenolic compounds such as (p-hydroxyphenyl) ethanol (p-HPEA) and (3,4-dihydroxyphenyl) ethanol (3,4-DHPEA), vanillic acid, caffeic acid, and α -tocopherol were supplied by Merck (Milan, Italy). The isomer of oleuropein aglycon (3,4-DHPEA-EA), the dialdehydic forms of elenolic acid linked to tyrosol and hydroxytyrosol (p-HPEA-EDA and 3,4-DHPEA-EDA, nowadays named olecanthal and oleacein, respectively), and lignans (+)-1-acetoxypinoresinol and (+)-pinoresinol were extracted from a VOO as reported by Selvaggini et al. [33]. Pure analytical standards of volatile compounds were purchased from Merck (Milan, Italy).

2.2. VOO Samples

Twenty-three VOOs were selected from among several VOOs purchased from Italian, EU and extra-EU providers according to their legal quality parameters (acidity, peroxide number, K_{232} , K_{270} , ΔK), acidic composition and antioxidant composition (α -tocopherols and total phenols). Among those 23 samples, we selected the 6 most representative VOOs (named A, C, D, E, F and G) covering the realistic quality range in the market [9]. Through a statistical set-up, 51 virtual VOOs, characterized by different contents of oleic acid, hydrophilic phenols, and α -tocopherol, were virtually created by mixing of the 6 “mother” samples at different percentage ratios (50%/50% or 25%/75%). Among the 51 virtual VOOs, according to the D-optimal design (Figure S1) using the Most Descriptive Compound (MDC) algorithm, 20 virgin olive oils, were selected because of their low, medium, and high concentrations of oleic acid, phenolic compounds, and α -tocopherol. The different percentages of the 6 “mother samples” used to realistically produce the 20 VOOs selected, are reported in Table S1. An alphanumeric code from S1 to S20 was attributed to each VOO (Table S1).

2.3. Simulation of the VOO Real-Time Shelf-Life

The twenty VOOs selected and realistically produced as described above were packaged in 500 mL UVA-grade green glass bottles, sealed with screw caps, and placed on shelves for a 12-month real-time shelf-life light exposure (500 lux for 12 h/day) at a temperature of 25 °C. The bottles were moved two times per week, from the first to the last position, into the same row, to ensure equal light exposure over the experimental period.

One bottle of each VOO was taken monthly to carry out the full analytical evaluations. Before analysis, the samples were stored at 12 °C in the darkness. All the analyses were carried out within 1 week of the VOO being withdrawn.

2.4. VOO Analytical Evaluations

2.4.1. Regulatory Quality and Freshness Indices

The free acidity, peroxide values (PV), and extinction indices, of VOOs from T0 to T12 were determined according to the official methods of the European Commission Regulation [34]. Fatty acid (FA) composition (only for T0 samples) was estimated in accordance with the regulations of the European Union [34]. The FA and 1,2-diacylglycerol (DAG) content of the oils (only for VOOs at T0) were determined using a Dani Master GC (DANI Instruments, Milan, Italy) equipped with a flame ionization detector (FID) in accordance with the procedures described in EU Reg. 2022/2104 and ISO 29822:2009, respectively [34,35].

2.4.2. Oxidative Stability Index (OSI) Time

The test to determine the OSI time (hour), was carried out only on the 20 VOOs at time 0, as follows: with a stream of purified air (120 mL min⁻¹ air flow rate), which passed through a 5 g of sample, and the effluent air from the oil was then bubbled through a vessel containing deionized water. The effluent air contained volatile organic acids such as formic acid and other volatile compounds formed during thermal oxidation of the oil, increasing the conductivity of the water. The temperature at which this test was carried out was 110 °C. The OSI (or OSI time) was expressed in hours [36].

2.4.3. α -Tocopherol

A total of 1 g of oil was dissolved in 10 mL of n-hexane, filtered with a 5- μ m polyvinylidene difluoride (PVDF) syringe filter (Whatman, Clifton, NJ, USA) and injected into the HPLC system. The HPLC analysis was conducted using an Agilent Technologies Model 1100 consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatic column compartment, a diode array detector (DAD) and a fluorescence detector (FLD). α -Tocopherol was evaluated according to the procedure of Psomiadou and Tsimidou [37] with the following modifications: n-hexane/2-propanol (99.5:0.5 v/v) (A) and n-hexane/2-propanol (70:30 v/v)

(B) were used as eluents. The gradient was as follows: 100% A for 2 min, to 89% A in 8 min and maintained for 6 min, to 45% A in 2 min, then to 20% A in 2 min, maintained for 6 min, 100% A in 4 min, and maintained for 5 min. The total run time was 35 min, and the injection volume was 50 μ L. α -Tocopherol was detected at an excitation wavelength of 294 nm and at an emission wavelength of 330 nm. Quantification was performed by using the curve constructed with the standard solutions of α -tocopherol. Results were expressed as mg/kg of oil.

2.4.4. Polyphenols

The extraction of VOO phenolic compounds was performed in accordance with Taticchi et al. [38]. The HPLC analyses of the phenolic extracts were conducted according to Selvaggini et al. [33] with a reversed-phase column using an Agilent Technologies system Model 1100 (Agilent Technologies, Santa Clara, CA, USA), which was composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostatic column compartment, a DAD, and a fluorescence detector (FLD). The C18 column used in this study was a Spherisorb ODS-1 250 mm \times 4.6 mm with a particle size of 5 μ m (Waters, Milford, MA, USA); the injected sample volume was 20 μ L. The mobile phase was composed of 0.2% acetic acid (pH 3.1) in water (solvent A)/methanol (solvent B) at a flow rate of 1 mL/min and the gradient changed as follows: 95% A/5% B for 2 min, 75% A/25% B in 8 min, 60% A/40% B in 10 min, 50% A/50% B in 16 min, and 0% A/100% B in 14 min; this composition was maintained for 10 min and then returned to the initial conditions and equilibration in 13 min; the total running time was 73 min. The oxidized form of the oleuropein aglycon (3,4-DHPEA-OX) was detected by using the DAD set at 339 nm; all the other phenolic compounds were detected by DAD at 278 nm, the analyses and the elaboration of chromatographic data were conducted using the ChemStation software version A.10.02 (Agilent Technologies, Palo Alto, CA, USA). The quantitative evaluation of the phenols was carried out using single calibration curves for each compound, and the results are expressed as mg/kg of oil. The 3,4-DHPEA-OX was quantified using the response factor of caffeic acid, because the standard compound was not commercially available.

2.4.5. Oxidized (Acidic) Form of the Oleuropein Aglycon (3,4-DHPEA-OX)

The confirmation of 3,4-DHPEA-OX was performed by UHPLC-DAD-Q-TOF/MS using an Ultra High Performance Liquid Chromatography (UHPLC) system Agilent Technologies mod. 1260 Infinity composed of a degasser, a binary pump, an autosampler, a thermostatic column compartment, and a diode array detector (DAD), coupled with a quadrupole-time of flight (Q-TOF) mass spectrometer with an electrospray ionization source (Dual ESI) Agilent mod. 6530 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA). The column used was a Poroshell 120, EC-C18, 3.0 \times 50 mm, 2.7 μ m (Agilent Technologies, Santa Clara, CA, USA). The injected sample volume was 1 μ L, and the elution was performed with a flow of 0.7 mL/min using water with 0.2% acetic acid as solvent A and methanol with 0.2% acetic acid as solvent B. The elution gradient was changed as follows: at 0 min, the solvent ratio was 85% of A and 15% of B; at 10 min, the ratio was 40% of A and 60%; at 15 min, the ratio was 100% of B, and this composition was maintained for 5 min. The system was then restored to its initial condition and allowed to equilibrate for 5 min. The total time of the analysis was 20 min. DAD spectra were acquired in the range of 190–640 nm. The MS analysis was acquired in negative mode in the m/z range of 50–1600 with a scan speed of 1 spectrum/s, and for accurate mass measurements, two reference masses (by using a calibrating solution infused through the second nebulizer) of 112.985587 and 980.016375 m/z were used. The parameters of the Dual ESI source were as follows: drying gas flow, 9 L/min; gas temperature, 325 $^{\circ}$ C; nebulizer pressure, 35 psig; capillary voltage, VCap 3500 V; fragmentor, 150 V; skimmer, 65 V, octapole 1 RF, 750 V. To confirm the structure of 3,4-DHPEA-OX a MS/MS analysis, using as precursor ion 393.1194 m/z , was always performed always in negative mode with the following parameters: VCap, 3500 V; collision energy, 15 V and scan speed of 1.5 spectrum/s, with

all the other parameters unchanged. The analyses and data processing were performed using Agilent “MassHunter” software version 10.1, and for the elucidation of the structure of 3,4-DHPEA-OX, Molecular Structure Correlator (MSC) version 8.2 was used (Agilent Technologies, Santa Clara, CA, USA) (Figure S2).

2.4.6. Volatile Compounds

Evaluation of volatile compounds in VOOs was performed via headspace solid-phase microextraction, followed by gas chromatography-mass spectrometry (HS-SPME/GC-MS), according to the method of Taticchi et al. [38]. A total of 3 g of oil was mixed with 2-methylpropyl acetate as internal standard at the concentration of 9.8 mg/kg. To sample the headspace volatile compounds, solid-phase microextraction (SPME) was applied as follows: all of the vials were held at 35 °C for 10 min, and then the SPME fiber (a 50/30 µm 2 cm long DVB/Carboxen/PDMS, Stableflex; Supelco, Inc., Bellefonte, PA, USA) was exposed to the vapor phase for 30 min to sample the volatile compounds. The gas chromatography mass spectrometry analyses (GC-MS) were performed using an Agilent Technologies GC 7890B with “Multimode Injector” (MMI) 7693A coupled to a single quadrupole MSD mod. 5977B using an EI Extractor (XTR) source (Agilent Technologies, Santa Clara, CA, USA); a thermostatic PAL3 RSI 120 autosampler equipped with a fiber conditioning module and an agitator (CTC Analytics AG, Zwingen, Switzerland) was also employed. The chromatographic conditions used to analyze volatile compounds were the same reported by Taticchi et al. [38]. Volatile compounds were identified by comparing their mass spectra and retention times with those of authentic reference compounds. and with spectra in the NIST 2014 mass spectra library. The quantitation of the volatile compounds was performed using the calibration curves for each compound with the internal standard method and the results are expressed as mg/kg of oil.

2.5. Statistical Analysis

SigmaPlot software v12.3 (Systat Software Inc., San Jose, CA, USA) for performing a priori one-way analysis of variance (ANOVA) with the Tukey test ($p < 0.05$).

The SIMCA 13.0 chemometric package was used (Umetrics AB, Umeå, Sweden) to perform multivariate statistical analyses. For this statistical elaboration, the analytical data were previously put in a matrix with the samples (n objects) in rows and the analytical parameters (k variables) in columns. The raw data were normalized, with the subtraction of the mean, and auto-scaled, dividing these results by the standard deviation. The number of significant components was found by applying cross-validation. The results of modelling are presented in graphical form.

3. Results and Discussion

3.1. Evaluation of EVOO Quality at Time T0

The initial quality indices of the 20 VOOs at the time of packaging (T0), such as free acidity; PV, K_{232} and K_{270} extinction coefficients; and FAEs' fatty acids composition; phenols and the oxidative form of the oleuropein aglycon; oxidative stability; and volatiles' compositions, was evaluated and reported in the following Tables S2–S4. The legal parameters were within the limits defined for the “extra virgin marketable” category designated by the current EU regulation [34].

Furthermore, the 20 samples had remarkably high variability (in terms of oleic acid, α -tocopherol, total phenols and volatiles), which is strongly correlated to genetic, agro-nomic, and technological factors, and reflected the current variability of the commercial VOOs [9].

3.2. Evolution of VOOs' Overall Quality during 12 Months of Light Exposure

The whole data set of the results obtained analyzing the 20 experimental blends each month was firstly elaborated by building a PCA (Principal Component Analysis, Figure S3),

showed a clear discrimination of the samples according to the time to the light exposure, along the first component (from the left to the right of the score, Figure S3A).

Successively, an orthogonal-partial least square (OPLS, Figure 1) of latent variables analysis was carried out to observe a potential correlation between the evolution of the VOOs' quality and the duration of storage to light exposure. The score plot (Figure 1A) confirmed the distribution of the samples according to the time of storage, with those VOOs characterized by the longest period of conservation (from 7 to 12 months), positioned to the right and to the top side of the score plot, and the VOOs with the earliest period of conservation (from 0 to 6 months), to the left and to the lower side of the score (Figure 1A). The relative loading plot (Figure 1B) highlighted that the latent dependent variable Y (time to the light exposure), was positively correlated, particularly with the extinction coefficients K_{270} and ΔK , C₇-C₁₀ aldehydes (*E*)-decenal and (*E*, *E*)-2,4-decadienal, and DHPEA-EA-OX. On the contrary, negative correlations between Y and all the antioxidant compounds, especially with the oleuropein derivatives oleacein (3,4-DHPEA-EDA) and 3,4-DHPEA-EA, were demonstrated. Furthermore, we observed that K_{232} and the ligestroside derivatives oleocanthal (*p*-HPEA-EDA) and *p*-HPEA-EA, as well as lignans, together with volatile compounds such as hexanal, nonanal, decanal and propionic acid, occupying the center of the loading plot, did not show correlation with time (Figure 1B).

This general exploration allowed us to conclude that, among all the chemical substances evaluated on the 20 VOOs over the storage to the light exposure evolution of K_{270} and ΔK , aldehydes (*E*, *E*)-2,4-decadienal and (*E*)-2-decenal, the phenolic composition and in particular the oleuropein derivatives, like 3,4-DHPEA and 3,4-DHPEA-EA-OX, were strongly correlated to the quality /freshness evolution of the VOOs.

Those data were very similar to those published by Esposto et al. (2017) [7], where PCA and PLS models showed the same positive correlations with the time to exposure to the light of different VOOs and parameters like K_{270} , C₇-C₁₁ aldehydes, and the oxidative products of polyphenols [7]. However, in this research, besides K_{270} , ΔK and the aldehydes (*E*, *E*)-2,4-decadienal and (*E*)-2-decenal also appeared more positively correlated to the time of conservation of the VOOs.

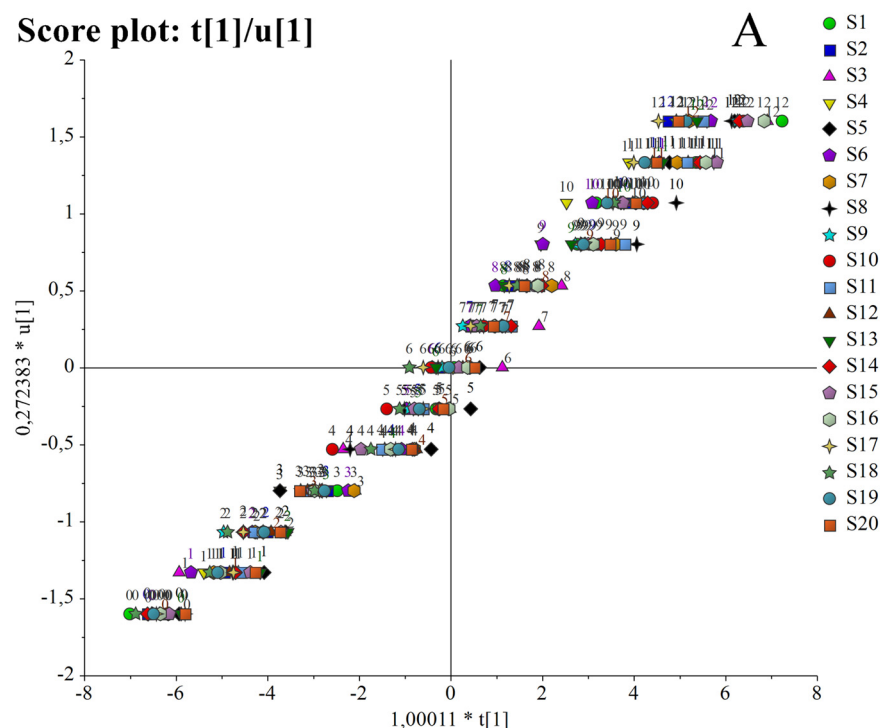


Figure 1. Cont.

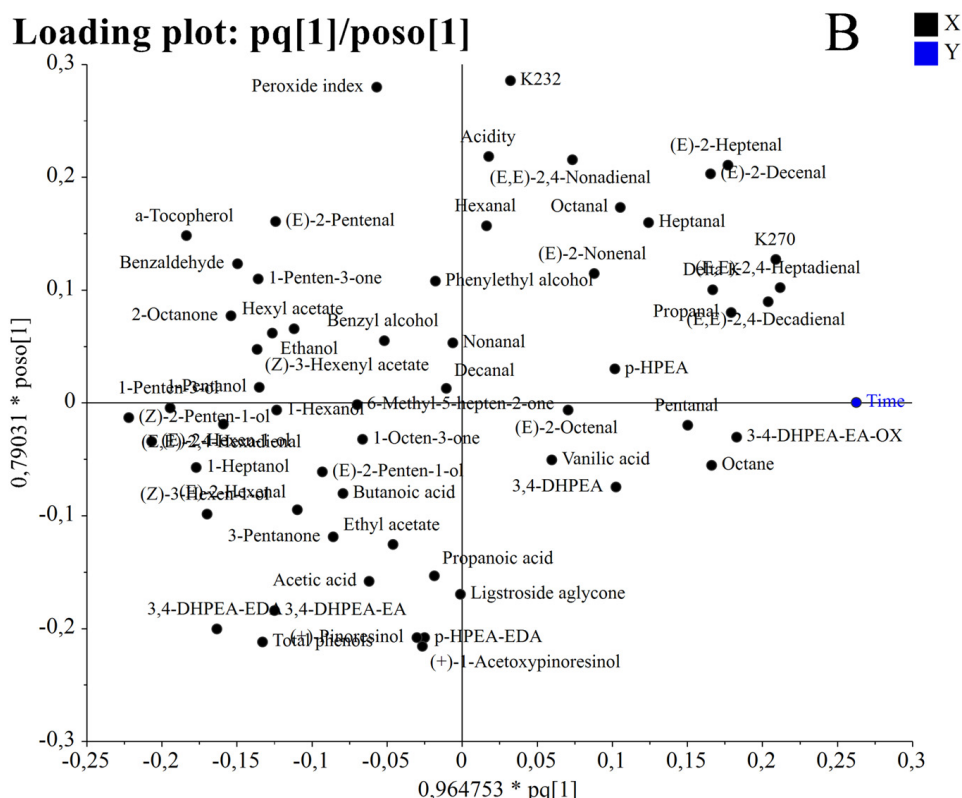


Figure 1. Score plot (t1/u1) (A) and loading plot (B) (pq1/poso1) of the first latent variable of the OPLS model built with all the analytical evaluations (independent variable X) for the 20 VOOs exposed to light for 12 months (latent variable Y representing the TIME). The model, explained 61% of the total variance of X (R^2X) with five components (1 predictive and 4 orthogonal, each explaining 24%, 14%, 12%, 7%, and 4%, respectively) and 98% of the overall explained variance (R^2).

Using the previous separation of the 20 samples into two groups, according to the initial low-medium and high concentration of polyphenols (VOO_{lmp} and VOO_{mhp} groups, respectively), we followed the evolution of those parameters that more significantly influenced the quality evolution of the VOO, according to the PLS model.

3.3. Evolution of Extinction Indexes K_{270} and ΔK during 12 Months of Light Exposure

In this study, the evolution of K_{270} showed a general tendency to increase during the storage period (Figure 2; Table S5) for all the samples analyzed with strong differences in terms of the number of months in which the samples remained in the extra-virgin category (limit of 0.20) [34], according to the initial concentration of polyphenols.

In fact, Figure 2A,B highlighted that in those VOO_{lmp}, the legal limit [34] was reached by S12 and S2 within the first four months; by S11, S19, and S20 within 6 months; and by the rest of the VOO_{lmp} samples (S13 and S17) within the 10 months of storage to the light exposure. On the contrary, S7 and S18 remained under 0.20 until the end of the experimentation.

On the contrary, in VOO_{mhp} (Figure 2B), only S1 exceeded the limit between the fourth and sixth month of shelf life, and only at the end of the experimentation did all the other VOO_{mhp} (except S4) no longer belong to the extra-virgin category [34]. These results confirmed that a lack of polyphenols promotes a quick increase in those volatile substances produced at the end of the oxidation phase, which are absorbed at 270 nm [34].

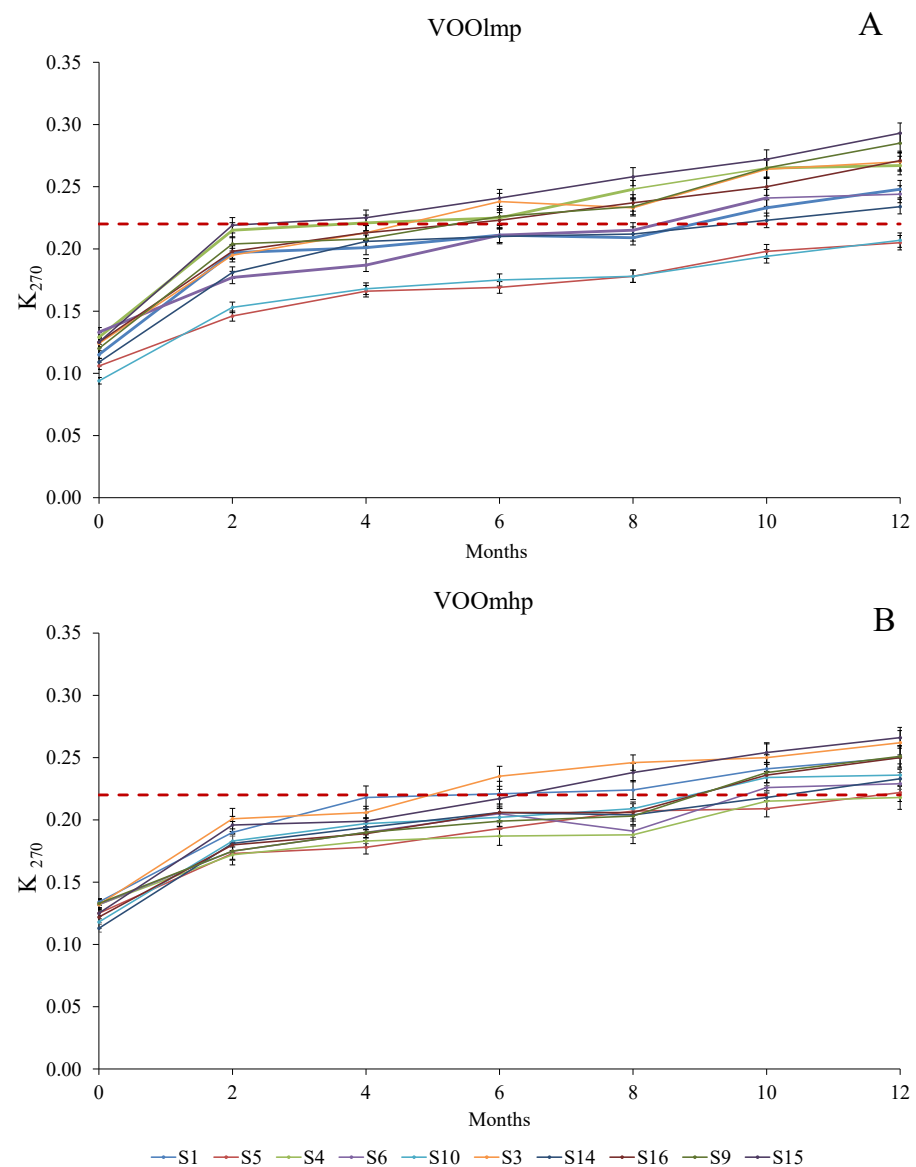


Figure 2. Evolution (reported every two months) of K_{270} over 12-month storage with light exposure in VOOlmp (A) and VOOmhp (B) samples. The dashed red line indicates the K_{270} legal limit (0.20) for the extra-virgin olive oil category [34]. Legend: VOOlmp: Virgin olive oils with low-medium polyphenol content; VOOmhp: Virgin olive oils with medium-high polyphenol content.

Moreover, the usefulness of K_{270} as a simple and cheap parameter for evaluating the oxidative status of a VOO [6–8,39,40], was confirmed.

In regard to ΔK evolution (Figure 3A,B; Table S6) a similar tendency to increase over storage was revealed: the legal limit (0.01) for the extra-virgin category [34] was first reached by VOOlmp samples starting from the period ranging between the second and the third month (samples S8 and S11) and between the fifth and the sixth month of conservation for almost all the 10 VOOlmp, S7 and S18 samples excluded.

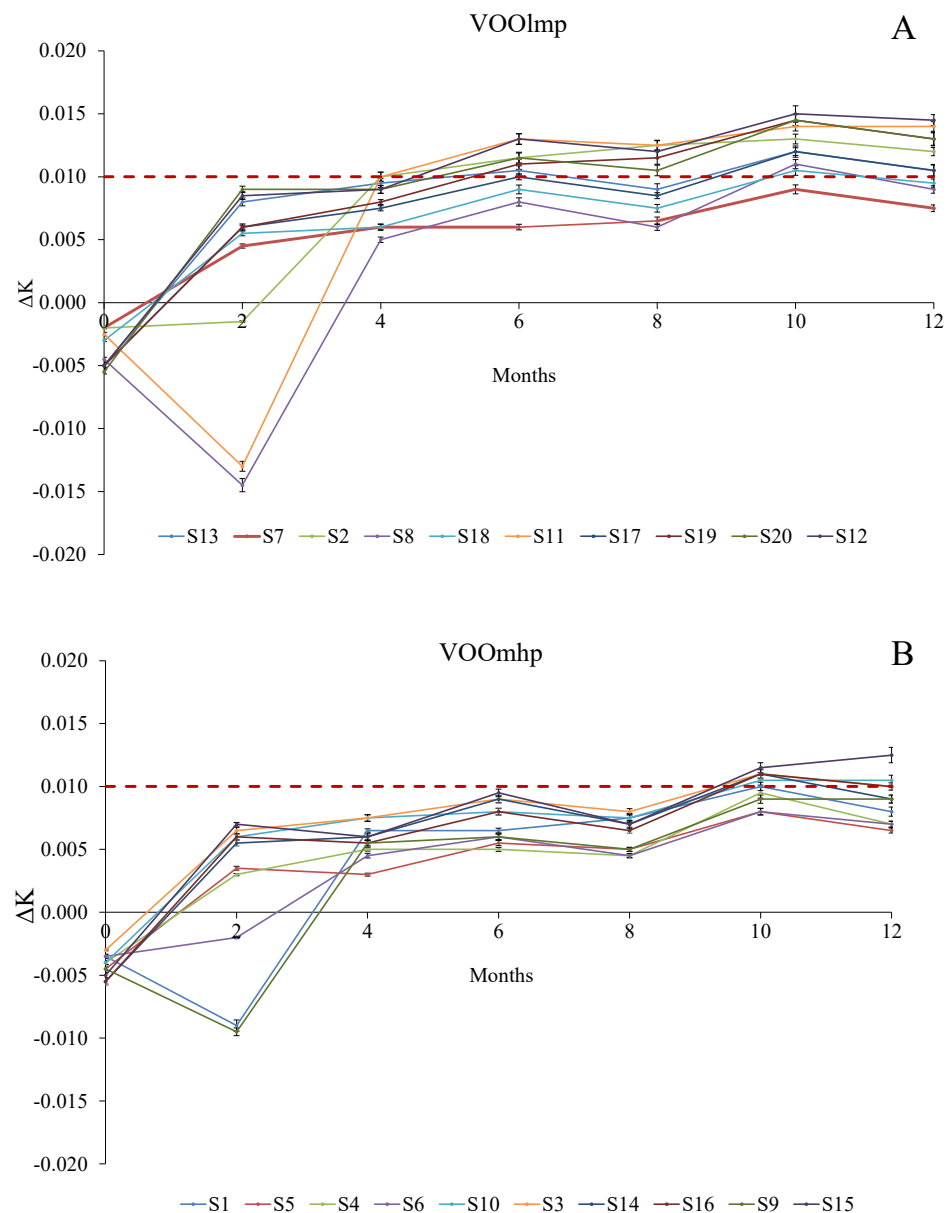


Figure 3. Evolution (reported every two months) of ΔK over 12-month storage with light exposure in VOOLmp (A) and VOOMhp (B) samples. The dashed red line indicates the ΔK legal limit (0.01) for the extra-virgin olive oil category [34]. Legend: VOOLmp: Virgin olive oils with low-medium polyphenol content; VOOMhp: Virgin olive oils with medium-high polyphenol content.

On the contrary, higher stability was observed in VOOMhp samples since four of ten samples (respectively S5, S4, S9, and S6) never exceeded the legal limit, whereas the other six VOOs (respectively, S1, S3, S6, S10, S14, and S15) showed value higher than 0.01 by the eighth month of storage.

However, between ΔK and K_{270} , K_{270} showed higher sensibility in revealing the oxidative status of the samples: S5, S6, and S9, which remained within the extra-virgin marketable definition according to the ΔK limit, were not according to the K_{270} parameter by the ninth month of storage.

3.4. Evolution of (E, E)-2,4-Decadienal and (E)-2-Decenal during 12 Months of Light Exposure

In both groups of VOOs studied, we observed a constant increase in these two substances, with differences depending on the concentration of polyphenols at time 0. The

evolution of (*E, E*)-2,4-decadienal (Figure 4; Table S7) showed that, by the fourth month of conservation of the 20 samples, a general accumulation was revealed. However, in both VOOmhp and in VOOLmp samples, the concentration stayed below the perception threshold [41] until the sixth and the tenth month of the shelf life, respectively, in VOOLmp and VOOmhp samples head spaces. Furthermore, while all VOOLmp exceeded the perception threshold [41] in VOOSmhp S1, S4, S6, S14, and S15 (50% of the group) never reached this sensory limit.

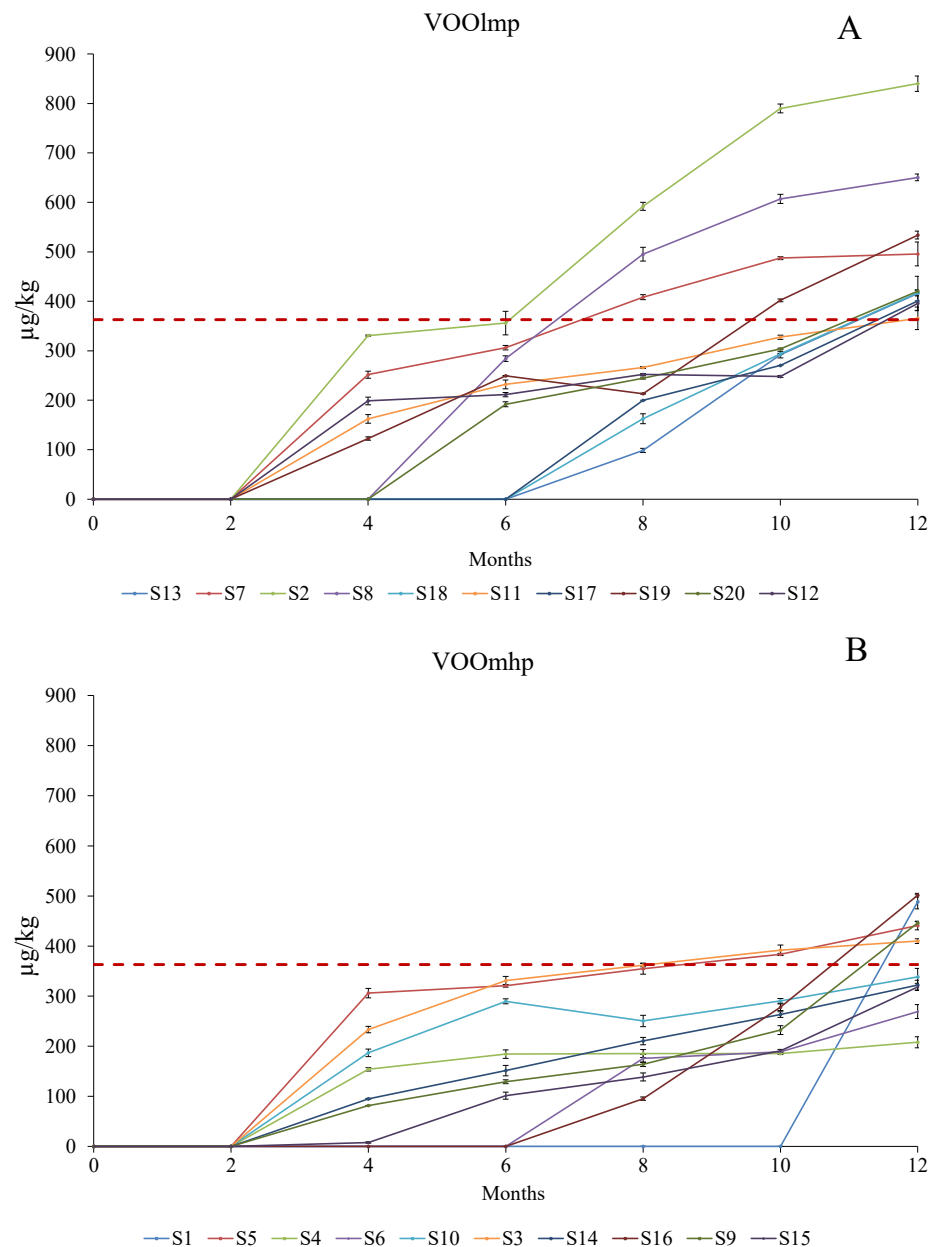


Figure 4. Evolution (reported every two months) of (*E, E*)-2,4-decadienal ($\mu\text{g}/\text{kg}$) over 12-month storage with light exposure in VOOLmp (**A**) and VOOmhp (**B**) samples. The dashed red line indicates the concentration representing the perception threshold ($363 \mu\text{g}/\text{kg}$) [41]. Legend VOOLmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content.

The volatile compound (*E*)-2-decenal, already revealed at time 0 in all the 20 samples, had a similar increase in (*E, E*)-2,4-decadienal and was dependent on the initial phenol concentration (Figure 5, Table S8).

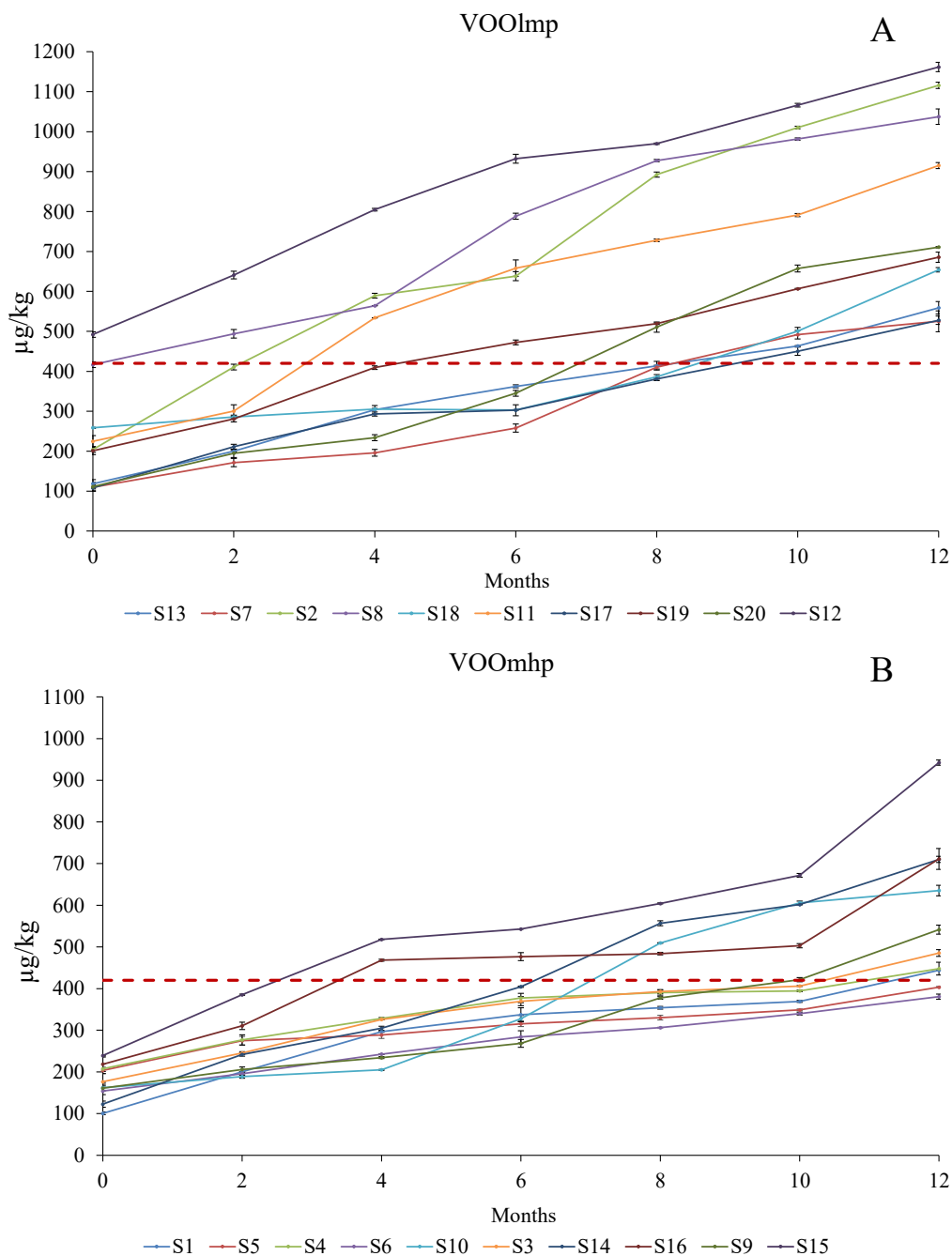


Figure 5. Evolution (reported every two months) of (*E*)-2-decenal ($\mu\text{g}/\text{kg}$) over 12-month storage with light exposure in VOOImp (A) and VOOmhp (B) samples. The dashed red line indicates the concentration representing the perception threshold ($420 \mu\text{g}/\text{kg}$) [41]. Legend VOOImp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content.

In fact, four samples belonging to the VOOImp group (S2, S8, S11, and S12) (Figure 5A) exceeded the perception threshold ($420 \mu\text{g}/\text{kg}$) [41] by T0 within the fourth month of storage and reached a concentration of $800\text{--}1100 \mu\text{g}/\text{kg}$ at the end of the storage. However, except for S19 and S20, all the VOOImp reached the perception threshold within 12 months (Figure 5A).

Regarding VOOmhp, even if they exceeded the limit for storage (Figure 5B), except for two samples, S1, S3, S4, S5, and S6 stayed under the limit until the tenth month (Figure 5B). The general lower accumulation of (*E*)-2-decenal in VOOmhp is in line with (*E*, *E*)-2,4 decadienal

data, confirming that higher VOO polyphenols attenuate the accumulation of secondary volatiles products linked to the “rancid” defect [5,8,38,42,43].

3.5. Evolution of Polyphenols during 12 Months of Light Exposure

The evolution of all the phenolic compounds expressed as the sum of 3,4-DHPEA, *p*-HPEA, oleacein (3,4-DHPEA-EDA), 3,4-DHPEA-EA, oleocanthal (*p*-HPEA-EDA), and lignans ((+)-1-pinoresinol and (+)-acetoxypinoresinol) was also evaluated.

The data (Figure 6; Table S9) showed a general decrease in the initial concentration, with a loss ranging between 57.1 and 86.9% of the total amount. However, while VOOLmp (Figure 6A) exhibited a depletion from 76.7 to 86.9%, in VOOmhp, (Figure 6B), the loss was attenuated by approximately 57.1 up to 85.9%; in particular, lower decreases (57.1–65.8%) were registered in those VOOmhp with an initial concentration of polyphenols equal to or higher than 900 mg/kg (samples S1, S4, S5, and S6) (Figure 6B).

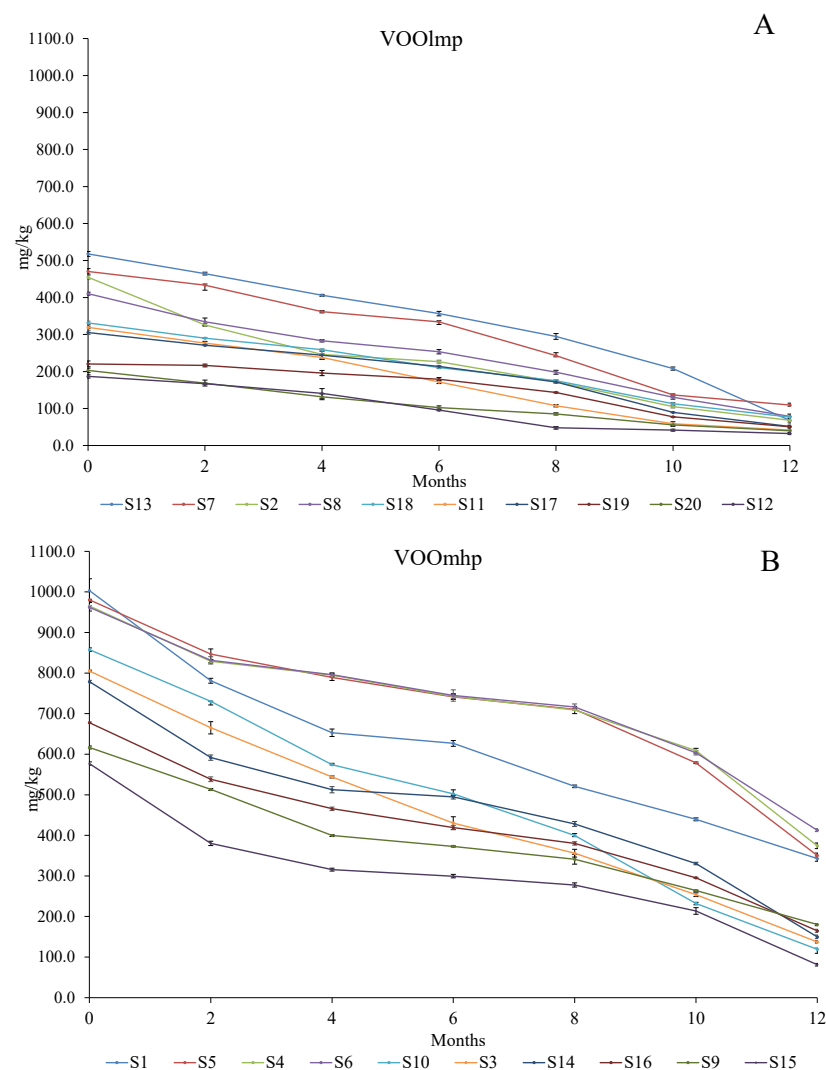


Figure 6. Evolution (reported every two months) of the polyphenol concentrations (mg/kg) over 12-month storage with light exposure in VOOLmp (A) and VOOmhp (B). Legend: VOOLmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content. The values are expressed as the sum of hydroxytyrosol (3,4-DHPEA), tyrosol (*p*-HPEA), oleacein (3,4-DHPEA-EDA), oleuropein aglycon (3,4-DHPEA-EA), oleocanthal (*p*-HPEA-EDA), ligstroside aglycon (*p*-HPEA-EA) and lignans ((+)-1-acetoxypinoresinol and (+)-pinoresinol).

Accordingly, Diamantakos et al. recently observed a significant decrease in phenolic content loss (46%) in Greek VOOs during usual storage with light exposure over 12 months [44].

Additionally, we showed that VOOsmhp with higher polyphenol contents were the same demonstrating higher resistance to oxidation due to their lower values of K_{270} , ΔK , (*E, E*)-2,4-decadienal and (*E*)-2-decenal. Thus, the antioxidant effect of VOO polyphenols, in contrast the photo-oxidation was highlighted.

These findings confirmed the results of Esposto et al. [7,8], who observed, in previous VOOs' real-time shelf-life studies, higher resistance to oxidation in accordance with the initial polyphenol concentration [7,8].

Even Salsasno Esposito et al. [15] observed a huge decrease in oleocanthal and oleacein during the storage of 3 VOOs stored at 25 °C under daylight exposure, but at different rates: in fact, in VOOs with low polyphenol contents, oleocanthal and oleacein decreased quickly, and after only 8 months of storage, they were no longer detectable. Conversely, in the other two VOOs, containing a concentration of polyphenols, the two secoiridoids gradually decreased and were undetectable after 11 and 12 months, respectively [15].

3.6. Evolution of the Oxidized and Non-Oxidized Form of the Oleuropein Aglycon during 12 Months of Light Exposure

This is the first study where the 3,4-DHPEA-EA degradation product (oxidized form of oleuropein aglycon) was monitored over time to evaluate its potential capability in monitoring the oxidative status of the VOO as an earlier and reliable indicator.

In this regard, Di Maio et al. [25,26] and Esposto et al. [7] showed the accumulation of the oxidized form of 3,4-DHPEA-EDA in VOO, already demonstrating its potentiality as a marker of VOO freshness.

Similarly, Di Serio et al. [28], following the evolution of the VOOs' quality real-time shelf life, concluded that it is also possible to predict the residual shelf-life of a VOO by measuring the relative quantity of oxidized phenols [28].

In our study, we chose to monitor only the oxidized form of the oleuropein aglycon instead of the sum of the oxidized forms or the single forms of *p*-HPEA-EDA and/or 3,4-DHPEA-EDA because the chemical structures of the last two ones usually absorb at a wavelength of 278 nm, which is the same wavelength absorbance of the relative non-acidic forms [15]. This means that spectral discrimination between the non-acidic and acidic forms, could be a long and difficult procedure, limiting the chemical evaluation as a reliable and easy method for monitoring VOO freshness over storage.

On the contrary, the discrimination between the oxidized and non-oxidized form of the oleuropein aglycon, is widely easier because of their different wavelength absorbances, respectively at 347 and 278 nm (Figures S4 and 7). With this regard, the lack of this substance in all 20 samples at time 0 showed the freshness of the oils at the beginning of the trial (Figure 8; Table S10). However, its appearance in each sample started after the first month of light exposure, and after that, its accumulation differentiated according to the initial concentration of the oleuropein aglycon.

Specifically, we observed that the higher the initial level of 3,4-DHPEA-EA (Figure 9; Table S11), the higher and faster the accumulation of the relative oxidative product (Figure 8; Table S10). With this regard, samples of the VOOlmp group showed a slow and low accumulation of the oxidized 3,4-DHPEA-EA, which, except for sample S2, did not exceed the concentration of 30 mg/kg. On the contrary, in the VOOsmhp group, all samples reached this value, and except for S4, S9, S10, and S15, the final amounts ranged between 40 and 60 mg/kg.

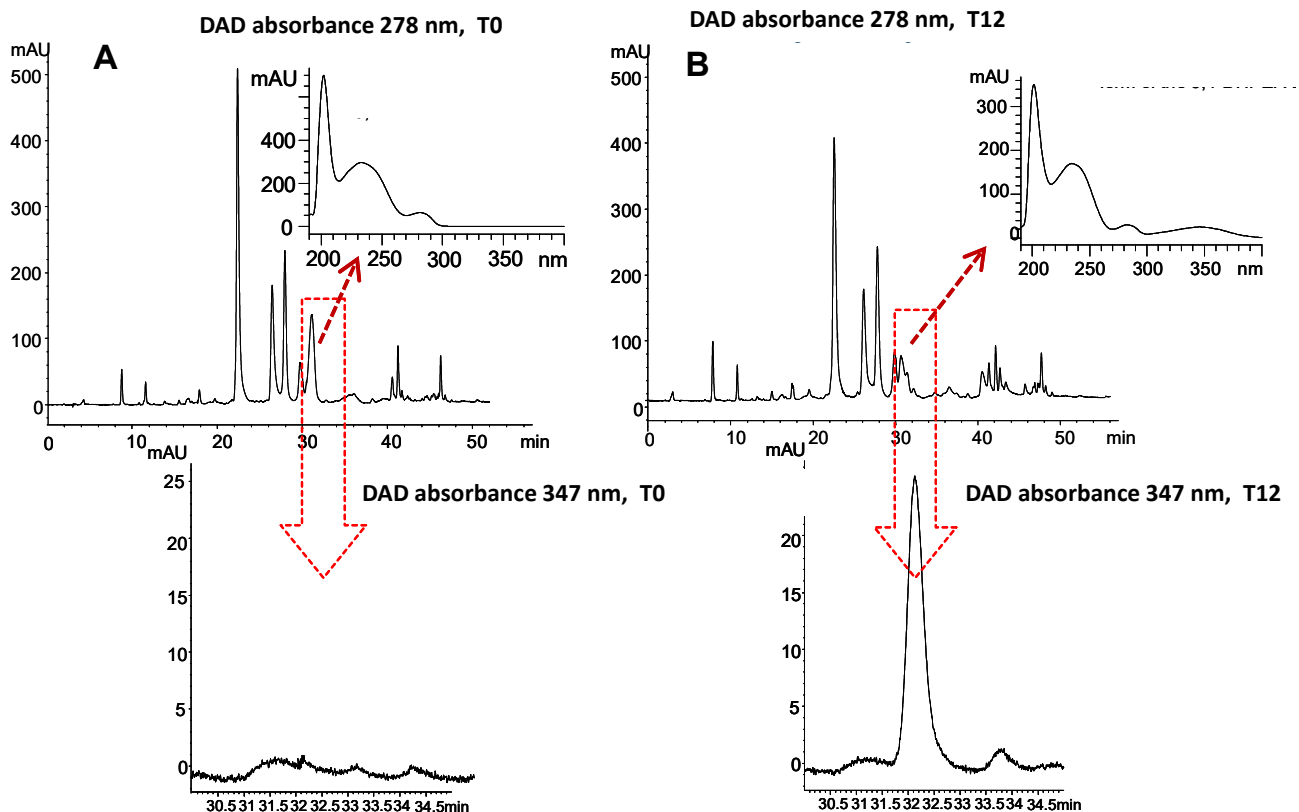


Figure 7. HPLC chromatogram and relative absorbance spectrum at 278 nm and 347 nm of oleuropein aglycon (3,4-DHPEA-EA) (A), and HPLC chromatogram and relative absorbance spectrum at 278 nm and 347 nm of the oxidized form of oleuropein aglycon (3,4-DHPEA-EA-OX) (B), contained in one of the 20 VOOs, at time 0 and at time 12.

The evaluation of the relative decrease in 3,4-DHPEA-EA (Figure 9; Table S11) showed a 100% loss in all VOOs, whereas a residual quantity ranging between 4 and 32% was found in VOOs (except for S15).

Thus, besides the total polyphenol content, it is possible to assume that the higher the initial content of the oleuropein aglycon, the higher its final residual concentration over time.

These data are in accordance with Baldioli et al. [32], who demonstrated that *o*-diphenols 3,4-DHPEA, 3,4-DHPEA-EDA and 3,4-DHPEA-EA had higher antioxidant activity than the other natural antioxidants of the VOO [32]. This finding was also confirmed by other research where oleuropein derivatives 3,4-DHPEA-EA, and 3,4-DHPEA-EDA showed higher and faster capacity in contrasting different oxidative phenomena and in preserving the oxidation of the other VOO antioxidants naturally contained, such as ligstroside derivatives, lignans, and α -tocopherol [7,8].

Hence, the data relative to the 3,4-DHPEA-EA evolution confirm that an estimate of the initial assets in oleuropein derivatives could be used as a predictor of the VOO shelf life in normal conditions.

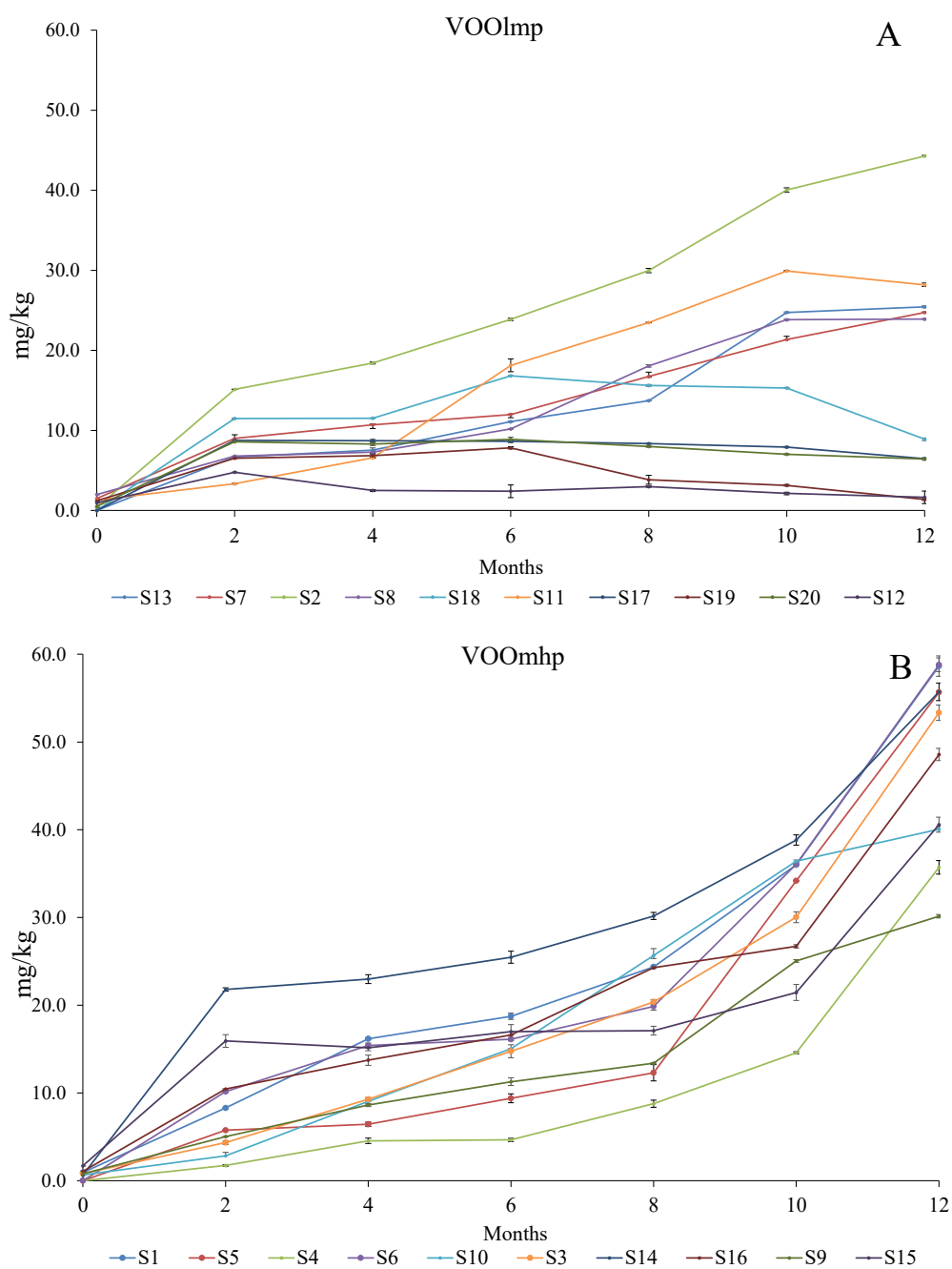


Figure 8. Evolution (reported every two months) of the oxidized form of the oleuropein aglycon concentration over 12-month storage with light exposure in VOOlmp (A) and VOOmhp (B). VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content.

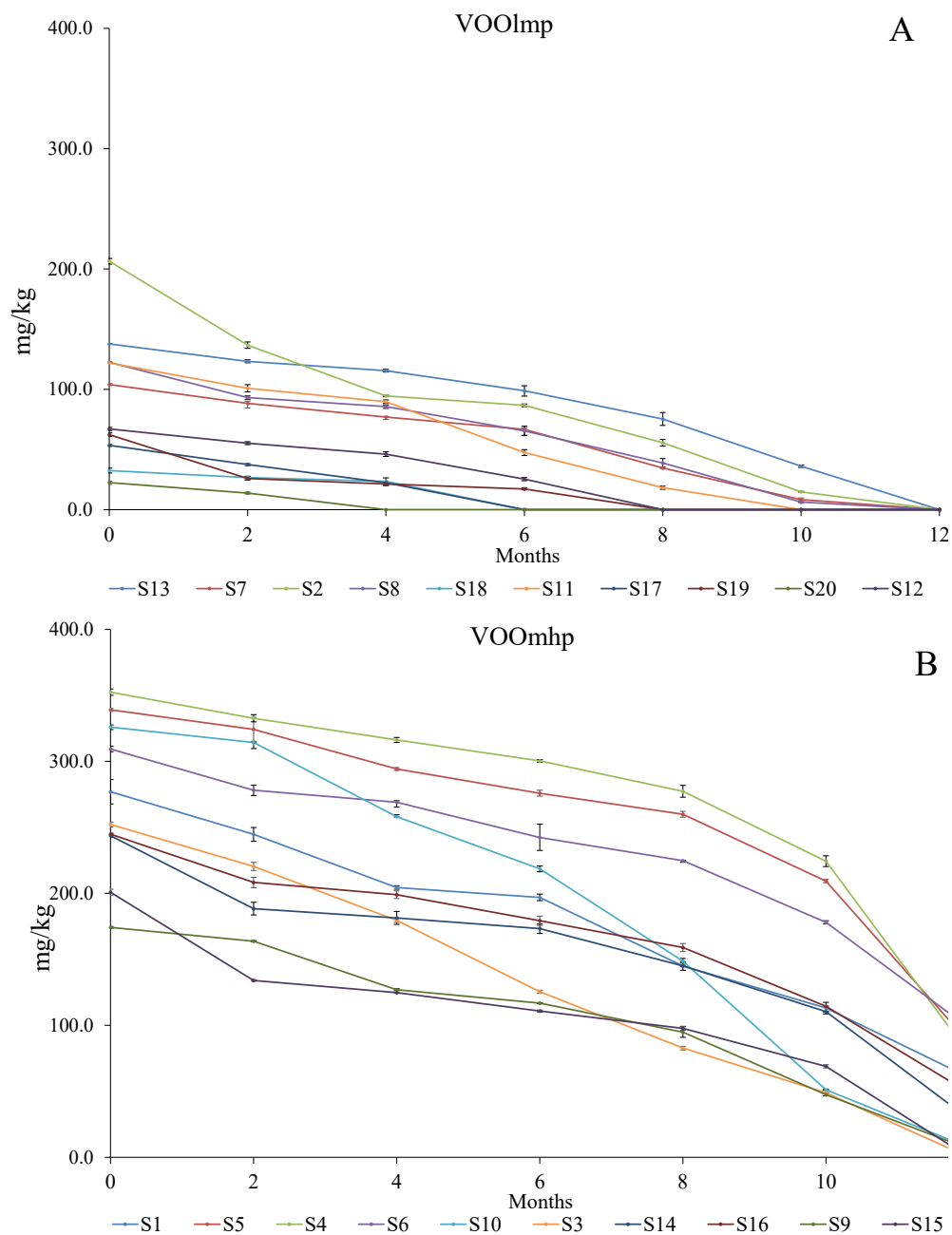


Figure 9. Evolution (reported every two months) of the oleuropein aglycon concentration over 12-month storage with light exposure VOOlmp (A) and VOOmhp (B). VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content.

4. Conclusions

Based on the results obtained in this research, the quality and freshness of commercial VOOs during their storage in market conditions are strongly influenced by photo-oxidation and the initial antioxidant heritage.

However, by studying the evolution of parameters that are directly involved in the modification of VOOs' legal, health-promoted, and sensory qualities, the following was found:

K_{270} and ΔK extinction indexes and (*E*)-2-decenal and (*E, E*)-2,4-decadienal may be useful for monitoring the quality of stored VOOs by determining the time at which they will lose their "extra" status.

The rapid increase in the 3,4-DHPEA-EA-OX contents in the VOOs indicated the immediate and primary involvement of the oleuropein derivatives in inhibiting the oxidation phase.

The different wave lengths of absorbance of 3,4-DHPEA-EA and 3,4-DHPEA-EA-OX simplify the analytical determination of 3,4-DHPEA-EA-OX.

The results of this study clearly demonstrate the effectiveness of 3,4-DHPEA-EA-OX as a faster indicator of VOOs' quality and freshness for the first time.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods12152959/s1>, Figure S1: Score plot of the first two (at left) and of the first three (at right) components of the PCA space of the D-optimal design model; Figure S2: LC-MS-MS chromatogram (A) and LC-MS-MS spectrum (B) of one of the 20 VOOs (specifically S2) after 12 months of light exposure; Figure S3: Score plot (t1/t2) (A) and loading plot (p1/p2) (B) of the first two principal components of the PCA model built using all the analytical evaluations carried out on the 20 VOOs exposed to light for 12 months. The PCA model explains 70% of the total variance with six significant principal components (27%, 19%, 9%, 7%, 5%, and 3%, respectively). Figure S4: Chromatograms of one of the 20 VOOs (specifically S2) after 12 months of light exposure (A, B); LC-MS extracted ion chromatogram in negative mode (393.11911 *m/z*) showing the acidic form of oleuropein aglycone (C); DAD-UV chromatogram absorption at a wavelength of 347 nm and at a retention time of at 8.479 min (D). Table S1: Mixing proportions of the 6 “mother” samples A, C, D, E, F, and G selected for the preparation of the 20 experimental VOOs S1-S20. Table S2: Values of free acidity, peroxide value, extinction coefficients, 1,2 diacylglycerols, oleic acid, MUFA, PUFA and SFA, of the 20 VOOs at time 0 *. The results are the means of two independent determinations \pm standard deviation. Legend S: sample; Table S3: volatiles' composition ($\mu\text{g/kg}$) of the head space of the 20 VOOs at time 0 *. The results are the means of two independent determinations \pm standard deviation. Legend S: sample; Table S4: Composition in antioxidants (mg/kg), value of OSI time (hour), and content of 3,4-DHPEA-EA-OX (mg/kg) of the 20 VOOs at time 0. The results are the means of two independent determinations \pm standard deviation. Legend S: sample; OSI: Oxidative Stability Index; 3,4-DPEA-EA-OX: Oxidized form of 3,4-DHPE-EA; Table S5: Evolution of the extinction coefficient K270 over 12-month storage with light exposure in VOOlmp and VOOmhp samples. The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content; Table S6: Evolution of the extinction coefficient ΔK over 12-month storage with light exposure in VOOlmp and VOOmhp samples. The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content; Table S7: Evolution of (*E*, *E*)-2,4-decadienal ($\mu\text{g/kg}$) over 12-month storage with light exposure in VOOlmp and VOOmhp samples. The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content; Table S8: Evolution of (*E*)-2-decenal ($\mu\text{g/kg}$) over 12 month-storage with light exposure in VOOlmp and VOOmhp samples. The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content; Table S9: Evolution of the sum * of the phenolic fractions over 12-month storage with light exposure in VOOlmp and VOOmhp samples. The values are expressed as the sum of the following phenols: hydroxytyrosol (3,4-DHPEA), tyrosol (p-HPEA), oleacein (3,4-DHPEA-EDA), oleuropein aglycon (3,4-DHPEA-EA), oleochantal (p-HPEA-EDA), ligstroside aglycon (p-HPEA-EA) and lignans ((+)-1-acetoxypinoresinol and (+)-pinoresinol). The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol content; Table S10: Evolution of the oxidized form of the oleuropein aglycon (3,4-DHPEA-EA-OX) over 12-month storage with light exposure in VOOlmp and VOOmhp samples. The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOlmp: Virgin olive oil with low-medium polyphenol content; VOOmhp: Virgin olive oil with medium-high polyphenol

content; Table S11: Evolution of the oleuropein aglycon (3,4-DHPEA-EA) over 12 month-storage with light exposure in VOOLmp and VOOLmhp samples. The results are the means of two independent determinations \pm standard deviation. Different letters in each row indicate statistically different values at $p < 0.05$. Legend: VOOLmp: Virgin olive oil with low-medium polyphenol content; VOOLmhp: Virgin olive oil with medium-high polyphenol content.

Author Contributions: Conceptualization, S.E., S.U., R.S. and M.S.; Methodology, S.E., S.U., R.S., A.T., A.B. and M.S.; Software, S.U. and R.S.; Validation, S.E., S.U. and R.S.; Formal analysis, S.E., S.U., R.S., L.D., G.V. and B.S.; Investigation, S.E., S.U., R.S., A.T. and M.S.; Data curation, S.E., S.U., R.S. and A.T.; Writing—original draft, S.E., S.U., R.S. and A.T.; Writing—review & editing, S.E. and S.U.; Visualization, S.E., S.U. and R.S.; Supervision, S.E., T.G.T. and M.S.; Project administration, T.G.T., A.B. and M.S.; Funding acquisition, T.G.T. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work is supported by the Horizon 2020 European Research project OLEUM “Advanced solutions for assuring the authenticity and quality of olive oil at a global scale”, which received funding from the European Commission within the Horizon 2020 Framework Program (2014–2020), grant agreement No. 635690.

Data Availability Statement: Data is contained within the article or Supplementary Material.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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