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^{1}H–NMR fingerprinting and supervised pattern recognition to evaluate the stability of virgin olive oil during storage

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ABSTRACT

Metabolomic fingerprinting of virgin olive oil (VOO) by ^{1}H NMR spectroscopy was used to study its stability during storage simulating normal shelf life conditions during its commercialization. A representative set of VOOs covering the full range of possible chemical compositions were exposed to light (500 lux for 12 h/day) at 25 °C for 12 months or stored in the dark at 25 °C, 30 °C and 35 °C for 24 months. Multivariate data analysis of the ^{1}H NMR spectra of the oil samples provided classification models to evaluate VOO freshness and to verify the light exposure of the VOO during storage, as well as regression models to determine VOO storage time and tentatively the best before date of a fresh VOO. These predictive models disclosed the chemical compounds responsible for the compositional changes in VOO due to hydrolytic and oxidative degradation taking place during its storage, and confirmed that light and increasing temperature enhance these processes. The presence of characteristic resonances of hydroperoxides (primary oxidation products) and the decrease of ^{1}H signals assigned to phenolic compounds, mainly secoiridoid derivatives, and other minor compounds such as fatty acids, squalene and native (*E*)-2-hexenal present in fresh VOO revealed its oxidative degradation. Further, the emergence of low intensity ^{1}H signals of saturated aldehydes meant that the secondary oxidation process has started at a low rate and yield. Moreover, the decrease of the ^{1}H signals of triacylglycerides and *sn*-1,2-diacylglycerides, and the increase of *sn*-1,3-diacylglycerides indicated that hydrolytic degradation of VOO and diacylglyceride isomerisation was occurring. ^{1}H NMR fingerprint of VOO together with pattern recognition techniques afford relevant information to assess the quality of VOOs taking into consideration legal, sensory and health-promoting aspects.

1. Introduction

Virgin olive oil (VOO) is a high added value agricultural product of the Mediterranean diet from nutritional, health, sensory and commercial points of view. The exclusive nutritional, health-promoting and sensory properties of VOO are due to its particular chemical composition. VOO is a complex mixture of triacylglycerides (more than 98%) and a wide range of minor components (Aparicio & Harwood, 2013). Triacylglycerides in VOO are rich in monounsaturated fatty acids (such as oleic acid), which confer nutritional value and a high stability to this edible oil. Besides, its natural antioxidants, mainly phenolics and tocopherols, contribute to protect VOO from its oxidative degradation (Esposto et al., 2017), as well as human health from several diseases (López-Miranda et al., 2010). Mostly secoiridoid derivatives, but also

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simple phenols (hydroxytyrosol and tyrosol), flavonols and lignans are the families of phenolic compounds found in VOO. Phenolic compounds attribute the positive sensory characteristics of pungency and bitterness to VOOs (Dais & Hatzakis, 2013; Servili et al., 2009). However, VOO organoleptic properties are principally influenced by their volatile compounds, e.g. aldehydes, aliphatic alcohols and esters (García--González, Vivancos, & Aparicio, 2011; Morales, Aparicio-Ruiz, & Aparicio, 2013). VOO is a plant source of the hydrocarbon squalene, which is considered a valuable functional ingredient (Tsimidou, 2010). Other important minor constituents of VOO are β-sitosterol and α-tocopherol, being the most abundant phytosterol and tocopherol respectively; carotenoids, chlorophylls, pheophytins, terpenic alcohols, free fatty acids, diacylglycerides and monoacylglycerides (Aparicio & Harwood, 2013; Dais & Hatzakis, 2013). VOO chemical composition depends on the olive cultivar, agronomical, environmental and pedo-climatic factors, time of olive harvest, olive storage after harvest, extraction technology and storage conditions (Aparicio & Harwood, 2013; Servili et al., 2014). As a result, VOO can present a wide assortment of compositions (Servili et al., 2015). Therefore the assessment of quality and traceability of VOO is still a challenging task for the olive oil industry and the food control laboratories due to the complexity and variability of this multicomponent food matrix.

The quality of VOO is related to its oxidative stability, storage history, sensory characteristics and nutritional properties (Dais & Hatzakis, 2013). VOO oxidation leads to the formation of off-flavour substances, the degradation of its bioactive antioxidants and the accumulation of degradation compounds, which causes the loss of its sensory and health-promoting qualities as well as its commercial value and consumer acceptance. The resistance of a VOO to oxidation depends on its chemical composition and its exposure to pro-oxidant factors such as oxygen, light, temperature and activators (chlorophylls and transition metals) during storage (Esposto et al., 2015).

The oxidative stability of VOO has been evaluated by several methods reviewed elsewhere (Alonso-Salces, Holland, & Guillou, 2011; Frankel, 2010): Oil stability index (OSI), also known as Rancimat test, peroxide value, conjugated dienes, conjugated trienes, K_{232} and K_{270} UV indexes, acidity, total phenol content, TBARS test, DPPH assay, ORAC assay, analysis of individual components (phenolic compounds, tocopherols, squalene) by liquid or gas chromatography, electron paramagnetic resonance analyses, among others. Most of the studies on the oxidative stability of VOO previous to 2011 employed questionably high-temperatures, which contributed to the understanding of the autoxidation process under thermal stressing, but unfortunately were not conclusive to predict stability of olive oil under usual conditions during its transport and storage, as well as the role of its natural antioxidants during oxidation process taking place along its shelf life. The evolution of VOO oxidation is a slow process that usually requires from 9 to 24 months (Dais & Hatzakis, 2013; Esposto et al., 2017). ^{1}H NMR has proved to provide data closely related with the evolution of the classical parameters, such as iodine value, peroxide value, conjugated dienes, etc., used to evaluate the oxidation stability of VOO. The major drawbacks of previous approaches to evaluate VOO stability is that they focus on a single metabolite or a single family of metabolites, whereas the stability of VOO is a complex phenomenon and depends on the different constituents in the oil, as said above. Moreover, synergisms among oil components in relation to oxidation have been observed. This means that to have a global picture of VOO stability, the quantification of different metabolites may require different analytical methods and techniques making even more complicate the evaluation of the oil stability. Fingerprinting methods such as NMR are particularly attractive since they are non-selective, require little or no sample pre-treatment, use small amounts of organic solvents or reagents, and are typically less time-consuming, allowing high and fast throughput analysis (Alonso-Salces et al., 2011; Dais & Hatzakis, 2013).

Currently the quality of VOO is determined by chemical-physical measurements together with sensory assessment carried out by panel test. All the limits and reference values of these measurements are described by the International Olive Council (IOC) as well as by the Codex Alimentarius. However, among these legal parameters and analytical methods, no method is proposed to evaluate the quality decay of VOO during storage considering the complexity of alteration processes and their effect in both health-promoting and sensory properties. Fatty acids, squalene, tocopherols and phenolic compounds are the main bioactive phytochemicals of VOOs; and volatile compounds and phenolics contribute to define the sensory quality of VOOs regarding olfactory and taste-tactile aspects respectively (Servili et al., 2009). Thus, the evaluation of the quality of a VOO should include not only the legal aspects, but also those related to human health and sensory ones as part of a whole (Servili et al., 2015). In this sense, ^{1}H NMR can provide a useful analytical tool to contribute to this aim, since this technique can provide information related to legal, health-promoting and sensory features of VOO in the same analysis. Indeed, major and several minor components in VOO, as well as its degradation products can be determined by ^{1}H NMR. The ^{1}H NMR spectrum of a VOO is considered its fingerprint, which can be further analysed by pattern recognition techniques to extract the information required for each subject in question.

According to the European Regulation 1335/2013, the storage conditions of olive oil should be clearly indicated on the label to ensure that the consumer is properly informed about the best conditions for its preservation. However, the labelling of the harvest year, which is an indicator related to the freshness of the oil, is not compulsory. Moreover, this regulation establishes neither a storage time nor the control of VOO deterioration during storage. In this regard, the Directorate General of the European Commission for Agriculture and Rural Development (DG-AGRI) expressed the imperative of studying the storage period, i.e. the best-before date, for olive oil (DG-AGRI, 2012). In the framework of this need, the present study was developed. ^{1}H NMR fingerprinting was used to evaluate the stability of VOO under usual commercial storage conditions. Multivariate data analysis of the ^{1}H NMR spectral data allowed to study the evolution of VOO with time, disclosing the chemical compounds responsible for the compositional changes taking place in VOO due to its hydrolytic and/or oxidative degradation. Thus, classification models were built to discriminate between fresh and non-fresh VOO for the control of VOO freshness, and to verify the light exposure of the oil during storage. Besides, regression models were developed to determine the age of a VOO, i.e. the VOO storage time, and tentatively, the best before date of a fresh VOO.

2. Material and methods

2.1. Samples

Twenty three extra virgin olive oils (EVOOs) from the harvest 2016/2017 were supplied by EU and extra-EU providers. The legal control parameters, i.e free acidity content (g of oleic acid/100 g of oil), peroxide value (PV, amount of hydroperoxides expressed as milliequivalents of O_2/kg of oil), K_{232} and K_{270} extinction coefficients, ΔK and the fatty acid profile of the EVOOs were determined according to the official methods of the Commission Regulation (EEC) No 2568/91 and the Commission Delegated Regulation (EU) 2015/1830. Their α-tocopherol contents were determined according to Esposto et al. (2015); and their phenolic compounds were extracted as reported by Montedoro, Servili, Baldioli, and Miniati (1992) and analysed according to Selvaggini et al. (2006). The legal quality parameters and the antioxidant compositions (α-tocopherol and phenolic compounds) of the 23 EVOOs were used to select 6 of them according to the following criteria: (*i*) free acidity $< 0.5\%$ and PV < 12 meq O_2/kg oil; and (*ii*) cover the concentration range of oleic acid, total phenols and α-tocopherols found in the market. These 6 EVOOs ("mother" samples) were mixed virtually according to different percentage ratios issued by an experimental design, resulting in 51 virtual EVOO samples characterised by different contents of oleic acid, hydrophilic phenols, and α-tocopherol. According

to the D-optimal design using the Most Descriptive Compound (MDC) algorithm, the most descriptive 20 EVOOs among the 51 virtual blends (0%, 50%, 75%, 100%) were selected from the Principal Component Analysis (PCA) space, in order to cover the experimental domain with low, medium and high concentration combinations of the three selected chemical parameters (oleic acid, total phenols and α-tocopherols). The selection was made to define the mixing proportions of the 6 "mother" samples suitable for the experimental aim (different shelf life conditions of temperature, exposure to light, dark, and time of storage). In order to evaluate the influence of the temperature in dark conditions on the shelf life of EVOOs, 5 EVOOs out of the 51 virtual blends were chosen according to the above D-optimal design. Finally, the selected 20 EVOO samples were obtained by blending the 6 "mother" EVOOs in the established proportions (Table S1 in the supplementary material), and were analysed for their full characterisation. The legal quality parameters (free acidity content, PV, K_{232}, K_{270} and ΔK) and the compositions of fatty acids, diacylglycerides, phenolic compounds and α-tocopherol of the 20 EVOOs were determined as mentioned above; and their volatile profiles according to Servili, Selvaggini, Taticchi, and Montedoro (2001). The 20 EVOOs were bottled in 500 mL-glass UVA grade bottles, sealed with screw cap and placed on shelves at different shelf life conditions regarding temperature, light exposure and time of storage. Light exposure was 500 lux for 12 h/day. The 20 EVOOs selected were studied at 25 °C both in light and dark conditions during 12 months. The 5 EVOOs chosen to evaluate the influence of temperature in the dark were studied at 25 °C, 30 °C and 35 °C during 24 months. Thus, up to 50 EVOO samples of experimental blends of the 6 "mother" EVOOs were stored (20 samples exposed to light; and 30 samples in darkness at three different temperatures). One bottle of each EVOO was taken monthly for samples exposed to light and every two months for those stored in the dark to be analysed.

Table S2–S4 in the supplementary material gather the time (month) at which the EVOOs exceeded the threshold values of the selected parameters, considering the limits established by Commission Regulation (EEC) No 2568/91: 0.220 for the K_{270} for samples exposed to light or 0.190 for the K_{270} for samples stored in the dark (assumed since none of the samples has reached the legal limit of 0.220 stored in the dark); 0.01 for ΔK; 250 mg of total phenols/kg of oil (as required by the Health Claim according to the EU Reg 432/2012); and 410 μg of (*E*)-2-decenal/kg of oil or 354 μg of (*E,E*)-2,4-decadienal/kg of oil (representing the thresholds for the rancid defect). For the external validation of the predictive model for the EVOO stored at 25 °C exposed to light, the identical protocol for the real-time shelf life study (see above) in terms of bottling, storage, management of samples and analytical evaluation was carried out using 18 industrial blends of EVOOs from different olive cultivars and geographical origin (EU and extra-EU countries), bottled at the beginning of their commercial life (Table S5 in the supplementary material). At time zero, all the EVOOs studied were characterized by good quality indexes, including free fatty acid percentage and indicators of primary (PV and K_{232}) and secondary (K_{270}) oxidation products, all being below the upper legal limit values established in current EU Reg. 1830/2015. The set of EVOOs studied also presented a large variability in the composition of fatty acids, phenolic compounds, and α-tocopherol; as well as in the profiles of volatile compounds in terms of high contents of molecules responsible for the positive sensory attributes, and low ones of those considered to be related to off-flavours (Esposto et al., 2017). The total absence of the (*E,E*)-2,4-decadienal was also confirmed.

2.2. Chemicals

Deuterated chloroform for NMR analysis (99.8 atom % D) was provided by Sigma-Aldrich Chemie (Steinheim, Germany).

2.3. NMR analysis

Aliquots of 150 μL of each oil sample were dissolved in 750 μL of deuterated chloroform, shaken in a vortex, and placed in a 5 mm NMR capillary. The ^{1}H NMR experiments were performed at 300 K on a Bruker (Rheinstetten, Germany) Avance 500 (nominal frequency 500.13 MHz) equipped with a 5 mm broadband inverse probe with Z-gradients. The spectra were recorded using a 6.1 μs pulse (90°), an acquisition time of 3.5 s (50 k data points) and a total recycling time of 7.0 s, a spectral width of 7100 Hz (14 ppm), 32 scans (+4 dummy scans), with no sample rotation. Prior to Fourier transformation, the free induction decays (FIDs) were zero-filled to 64 k and a 0.3 Hz line-broadening factor was applied. The chemical shifts were expressed in δ scale (ppm), referenced to the residual signal of chloroform (7.26 ppm). The spectra were phase- and baseline-corrected manually, binned with 0.02 ppm-wide buckets, and normalized to total intensity over the region 4.10–4.26 ppm (glycerol signal). The region of the NMR spectra studied comprised from 0 ppm to 11 ppm. TopSpin 2.1 (2013) and AmixViewer 3.7.7 (2006) from Bruker BioSpin GMBH (Rheinstetten, Germany) were used to perform the processing of the spectra. The data table generated with the spectra of all samples, excluding the eight buckets in the reference region 4.10–4.26 ppm, was then used for pattern recognition.

2.4. Multivariate data analysis

Datasets were made up of the 542 buckets of the ^{1}H NMR spectra (variables in columns) measured on the VOO samples (samples in rows). A total number of 638 VOO samples were analysed (20 VOOs stored at 25 °C exposed to light during one year, and stored in darkness during two years; 5 VOOs stored in the dark at 30 °C and 35 °C during two years; and 18 VOOs of the external set). Datasets were analysed by univariate procedures (ANOVA, Fisher index, Box & Whisker plots and simple least squares regressions); and by multivariate techniques, unsupervised such as PCA, and supervised as partial least squares discriminant analysis (PLS-DA) and partial least squares regression (PLS-R) (Berrueta, Alonso-Salces, & Héberger, 2007). Data analysis was performed by means of the statistical software package Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA, 1984–2004) and The Unscrambler V9.7 (Camo Software AS, 1986–2007).

PCA, PLS-DA and PLS-R were applied to the standardized (or autoscaled) data matrix of ^{1}H NMR spectra (542 variables) of the oil samples. The presence of outliers in the dataset was analysed by PCA. In PLS-DA and PLS-R, the optimal number of PLS components are estimated by cross-validation by plotting the PRESS (predicted residual error sum of squares) or RMSEP (root mean square error in the prediction) against the number of PLS components. Sometimes there are several almost equivalent local minima on the curve; the first one should be preferred to avoid overfitting (according to the principle of parsimony). The model with the smallest number of features should be accepted from among equivalent models on the training set. In PLS-DA, once the number of PLS components is optimised, the predictions in the training-test set are represented in a box and whisker plot in order to define the half of the distance between the quartiles as the boundary. The weighted regression coefficients (Bw) of the PLS-components indicate the importance of the NMR variables on the model: the larger the regression coefficient, the higher the influence of the variable on the PLS-DA model.

Classification and regression models achieved by PLS-DA and PLS-R respectively were validated by 3-fold cross-validation or leave-one-out cross-validation for parameter optimization, and by external validation when an external set of samples was available. Binary classification models can lead to artefacts if they are not used and validated properly (Kjeldahl & Bro, 2010). The reliability of the classification models developed was studied in terms of recognition ability (percentage of the samples in the training set correctly classified during the modelling step), prediction ability in the cross-validation (percentage of the samples in the test set correctly classified by using the model developed in the training step), and prediction ability in the external validation (percentage of the samples in the external set correctly classified by

using the optimised model) (Berrueta et al., 2007). The goodness of the regression model fit was evaluated by means of the prediction error, which is an expression of the error expected when using the calibration model to predict; the correlation coefficient between predicted and measured values in calibration and validation (R-cal and R-val); and the evaluation of the residuals, which show how well each individual object is modelled and predicted. The RMSEP expresses the average error to be expected associated with future predictions, i.e. the estimated precision. Thus, the RMSEP is the practical average prediction error as estimated by the validation set, and therefore an empirical error estimate, which is expressed in the original measurement units. The result is expressed as the predicted Y-value ± 2 RMSEP (Esbensen, Guyot, Westad, & Houmøller, 2002). The mean of the differences between Y-reference and Y-predicted by the model and its confidence interval at 95% were determined.

3. Results and discussion

3.1. ^{1}H NMR spectra of virgin olive oils

The ^{1}H NMR spectra of the 638 VOO samples were recorded. The chemical shifts of ^{1}H NMR signals of the major and some minor compounds in VOOs, as well as their assignments to protons of the different functional groups are gathered in Table 1. These ^{1}H NMR signals are the ones found in all VOOs analysed at time zero. The ^{1}H signals of triacylglycerides, the major components of olive oil, are well-known (Mannina & Segre, 2002; Sacco et al., 2000). Triacylglycerides present different substitution patterns depending on the length, degree and kind of unsaturation of the acyl groups (Aparicio & Harwood, 2013). Minor components are only observed by ^{1}H NMR when their signals are not overlapped with those of the main components and their concentrations are high enough to be detected; this is the case for the signals of cycloartenol at 0.318 ppm and 0.543 ppm; β-sitosterol at 0.669 ppm; stigmasterol at 0.687 ppm; squalene at 1.662 ppm; *sn*-1,2 diacylglyceryl at 3.71 ppm and 5.10 ppm; three unknown terpenes at 4.571 ppm; 4.648 ppm and 4.699 ppm; phenolics at 5.73 ppm, 5.99 ppm, 6.55 ppm, 6.61 ppm, 6.75 ppm and 7.03 ppm; aldehydic protons of secoiridoids at 9.215 ppm and 9.626 ppm, and of (*E*)-2-hexenal at 9.51 ppm; and hydroxyl protons of volatile compounds in the region 8.06–8.14 ppm (Alonso-Salces, R. M. et al., 2010; Alonso-Salces et al., 2011; Alonso--Salces, Rosa M. et al., 2010; Alonso-Salces et al., 2015; Christophoridou & Dais, 2009; D'Imperio et al., 2007; Guillén & Ruiz, 2001; Mannina & Segre, 2002; Mannina, Sobolev, & Segre, 2003; Owen et al., 2000; Papadia et al., 2011; Pérez-Trujillo, Gómez-Caravaca, Segura-Carretero, Fernández-Gutiérrez, & Parella, 2010; Rotondo, Salvo, Giuffrida, Dugo, & Rotondo, 2011; Sacchi et al., 1996; Shi et al., 2019; Ün & Ok, 2018). Besides, throughout the elapsed storage time, ^{1}H NMR signals of other minor compounds resulting from the oxidative and hydrolytic degradation of VOO were observed (Table 2), e.g. β-methylene protons of alkanals at 1.64–1.66 ppm, α- and β-protons of 2,3-branched saturated aldehydes in the region 2.51–2.57 ppm and 2.32–2.36 ppm respectively; epoxy ring protons at 2.63 ppm; *sn*-1,3 diacylglyceryl group protons at 4.07 ppm; protons of unsaturated alcohols at 4.57 ppm; and hydroperoxide protons at 5.57 ppm, 5.99 ppm, 6.55 ppm and 8.11 ppm (Dais & Hatzakis, 2013; Dugo et al., 2015; Guillén & Ruiz, 2001; Guillén & Uriarte, 2012; Sacchi et al., 1996; Salvo, Rotondo, La Torre, Cicero, & Dugo, 2017; Ün & Ok, 2018).

3.2. Stability of virgin olive oil

The high stability of VOO is basically due to the rather low unsaturation degree of its fatty acids, its relatively high contents of oleic acid, which is a monounsaturated fatty acid, and to the antioxidant activity of some of its unsaponifiable components, mainly phenolic compounds (Esposto et al., 2017; Frankel, 2010; Fregapane, Gómez-Rico, Inarejos, & Salvador, 2013; Krichene, Salvador, & Fregapane, 2015). α-Tocopherol

Table 1
Chemical shift assignments of the ^{1}H NMR signals of the main components of VOOs.

#	Chemical shift (ppm)	Multiplicity[a]	Functional group	Attribution
01	0.318	d	–C***H***$_2$– (cyclopropanic ring)	cycloartenol
02	0.527	s	–C***H***$_2$–	alcohol, sterol
03	0.543	d	–C***H***$_2$– (cyclopropanic ring)	cycloartenol
04	0.669	s	–C***H***$_3$ (C18-steroid group)	β-sitosterol
05	0.687	s	–C***H***$_3$ (C18-steroid group)	stigmasterol
06	0.740	t	–C***H***$_3$ (^{13}C satellite of signal at 0.87 ppm, acyl group)	
07	0.866	t	–C***H***$_3$ (acyl group)	saturated, oleic (or ω-9) and linoleic (or ω-6)
08	0.960	t	–C***H***$_3$ (acyl group)	linolenic (or ω-3)
09	0.987	t	–C***H***$_3$ (^{13}C satellite of signal at 0.87 ppm, acyl group)	
010	1.19–1.37		–(C***H***$_2$)$_n$– (acyl group)	
011	1.243		–(C***H***$_2$)$_n$– (acyl group)	saturated (palmitic, stearic)
012	1.256		–(C***H***$_2$)$_n$– (acyl group)	oleic
013	1.288		–(C***H***$_2$)$_n$– (acyl group)	linoleic and linolenic
014	1.51–1.65		–OCO–CH$_2$–C***H***$_2$– (acyl group)	
015	1.662	s	–C***H***$_3$	squalene
016	1.96–2.07		–C***H***$_2$–CH=CH– (acyl group)	
017	2.26–2.32	m	–OCO–C***H***$_2$– (acyl group)	
018	2.40–2.45	m	–OCO–C***H***$_2$– (^{13}C satellite of signal at 2.26–2.32 ppm, acyl group)	
019	2.72–2.82		= CH–C***H***$_2$–CH= (acyl group)	
020	2.754	t	= CH–C***H***$_2$–CH= (acyl group)	linoleic
021	2.789	t	= CH–C***H***$_2$–CH= (acyl group)	linolenic
022	3.69–3.73	d	–C***H***$_2$OH (glyceryl group)	*sn*-1,2-diacylglycerides
023	4.09–4.32		–C***H***$_2$OCOR (glyceryl group)	triacylglycerides
024	4.571	d		terpene
025	4.648	s		terpene
026	4.699	s		terpene
027	5.05–5.15	m	>C***H***OCOR (glyceryl group)	*sn*-1,2-diacylglycerides
028	5.22–5.28	m	>C***H***OCOR (glyceryl group)	triacylglycerides
029	5.28–5.38	m	–C***H***=C***H***– (acyl group)	
030	5.52–5.43	m	–C***H***=C***H***– (^{13}C satellite of signal at 5.28–5.38 ppm, acyl group)	
031	5.72–5.76	dt	= C***H***– (phenolic ring)	phenolic compounds
032	5.986		= C***H***– (phenolic ring)	phenolic compounds
033	6.551	dt	= C***H***– (phenolic ring)	phenolic compounds
034	6.607	dd	= C***H***– (C8'; phenolic ring)	dialdehyde of oleuropein lacking a carboxymethyl

(*continued on next page*)

Table 1 (*continued*)

#	Chemical shift (ppm)	Multiplicity[a]	Functional group	Attribution
				group, aldehydic form of oleuropein
035	6.79–6.73	d	= C***H***– (C5′, C7’; phenolic ring)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group, aldehydic form of secoiridoid (oleuropein, ligstroside)
036	7.05–7.00	dt	= C***H***– (C4′, C8’; phenolic ring)	dialdehyde of ligstroside lacking a carboxymethyl group, aldehydic form of ligstroside
037	7.562	s	= C***H***–O– (C3)	aldehydic form of secoiridoid (oleuropein, ligstroside)
038	8.14–8.06		>C(O***H***)OR	volatile compounds
039	9.215	d	–C***H***O (C1)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group
040	9.51	d	–C***H***O	*E*-2-alkenals (*E*-2-hexenal)
041	9.626	dd	–C***H***O (C3)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group
		dd	–C***H***O (C1)	aldehydic form of secoiridoids (oleuropein, ligstroside)

[a] Signal multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet; R, alkyl chain.

also exhibited notorious antioxidant activity (Esposto et al., 2015, 2017), and even showed a synergistic effect in association with some phenolic compounds (Deiana et al., 2002). Phenolics are hydrophilic antioxidants that inhibit oxidation by singlet oxygen quenching, free radical scavenging and metal chelating mechanisms; whereas α-tocopherol is a lipophilic antioxidant that acts scavenging lipid peroxy radicals and also quenching singlet oxygen (Esposto et al., 2017).

During the VOO oxidation process, oxygen is incorporated into the triacylglyceride structure by a free radical mechanism. As a result, very reactive species are generated, causing the breakdown of the acyl group chains, and producing primary oxidation compounds called hydroperoxides. These primary oxidation products may degrade into secondary oxidation products such as aldehydes, ketones, lactones, alcohols, acids, etc. Moreover, reactions between different acyl group chains may occur resulting in oligomeric and polymeric systems. The oxidation of edible oils is a matter of major concern also from a safety point of view due to some oxidation products with toxicological implications (Guillén & Ruiz, 2001, 2006). Regarding the sensory relevance of oxidation products, several saturated and unsaturated aldehydes were found to be responsible for rancid sensory defect in VOO (Morales, Luna, & Aparicio, 2005), as well as for off-odours (Kalua et al., 2007), altering its organoleptic characteristics. Furthermore, VOO can also undergo hydrolytic degradation. During this process, triacylglycerides hydrolyse, which increases the contents of free fatty acids and therefore the acidity of the oil, which implies a deterioration of its quality.

Definitively, despite of the recognised VOO stability, hydrolytic,

Table 2
Chemical shift assignments of the ^{1}H NMR signals related to the most important variables (buckets of ^{1}H NMR spectra) on the PLS-DA model for freshness (fresh VOO *vs* non-fresh VOO).

#	Bucket (ppm)	Multiplicity[a]	Functional group	Attribution
1	9.63	dd	–C***H***O (C3)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group
		dd	–C***H***O (C1)	aldehydic form of secoiridoids (oleuropein, ligstroside)
2	9.51–9.49	d	–C***H***O	*E*-2-alkenals (*E*-2-hexenal)
3	9.23–9.21	d	–C***H***O (C1)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group
4	8.11	bs	–OO***H*** (hydroperoxide group)	hydroperoxide derivatives
5	7.57–7.55	s	= C***H***–O– (C3)	aldehydic form of secoiridoid (oleuropein, ligstroside)
6	7.05–7.03	dt	= C***H***– (C4′, C8’; phenolic ring)	dialdehyde of ligstroside lacking a carboxymethyl group, aldehydic form of ligstroside
7	6.79–6.73	d	= C***H***– (C5′, C7’; phenolic ring)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group, aldehydic form of secoiridoid (oleuropein, ligstroside)
8	6.61–6.59	dd	= C***H***– (C8’; phenolic ring)	dialdehyde of oleuropein lacking a carboxymethyl group, aldehydic form of oleuropein
9	6.55–6.53	t	–C***H***=C***H***–C***H***=C***H***– (*cis, trans* conjugated diene system)	hydroperoxide derivatives
10	5.99–5.95	t	–C***H***=C***H***–C***H***=C***H***– (*cis, trans* conjugated diene system)	hydroperoxide derivatives
11	5.57	m	–C***H***=C***H***–C***H***=C***H***– (*cis, trans* conjugated diene system)	hydroperoxide derivatives
12	5.49	m	–C***H***=C***H***– (^{13}C satellite of signal at 5.28–5.38 ppm, acyl group)	
13	5.25	m	>C***H***OCOR (glyceryl group)	triacylglycerides
14	5.15–5.05	m	>C***H***OCOR (glyceryl group)	*sn*-1,2-diacylglycerides
15	4.59–4.55			unsaturated alcohols
16	4.45–4.41	m	–C***H***$_2$OCOR (^{13}C satellite of signal at 4.24–4.32 ppm, glyceryl group)	triacylglycerides
17	4.35		>C***H***–OOH (methine proton of hydroperoxide group)	hydroperoxide derivatives
18	4.33–4.27	m	–C***H***$_2$OCOR (glyceryl group)	triacylglycerides

(*continued on next page*)

Table 2 (*continued*)

#	Bucket (ppm)	Multiplicity[a]	Functional group	Attribution
19	4.09–4.05	q	>C***H***–OH (glyceryl group)	*sn*-1,3-diacylglycerides
20	3.73–3.69	d	–C***H***$_2$OH (glyceryl group)	*sn*-1,2-diacylglycerides
21	3.61	m	–C***H***(OHC–C ═)–CH$_2$–CHO (C5)	dialdehyde of secoiridoids (oleuropein, ligstroside) lacking a carboxymethyl group
22	2.81–2.79	m	–CO–C***H***$_2$–C***H***$_2$–CHO	4-oxo-alkanals
23	2.65–2.61	m	–CH$_2$–C***H***OHC–C***H***$_2$–	9,10-epoxystearate
24	2.55–2.49		–R'CHX–C***H***R–CHO	2,3-branched saturated aldehydes
25	2.43–2.41	m	–OCO–C***H***$_2$– (^{13}C satellite of signal at 2.26–2.32 ppm, acyl group)	
26	2.35–2.33		–R'C***H***X–CHR–CHO	2,3-branched saturated aldehydes
27	2.31–2.27	m	–OCO–C***H***$_2$– (acyl group)	
28	2.15	m	–OCO–C***H***$_2$– (^{13}C satellite of signal at 2.26–2.32 ppm, acyl group)	
29	1.97–1.95		–C***H***$_2$–CH═CH– (acyl group)	
30	1.67	s	–C***H***$_3$	squalene
31	1.65		–C***H***$_2$–CH$_2$–CHO	alkanals
32	1.61		–OCO–CH$_2$–C***H***$_2$– (acyl group)	
33	1.33		–(C***H***$_2$)$_n$– (acyl group)	
34	1.25		–(C***H***$_2$)$_n$– (acyl group)	oleic
35	0.97	t	–C***H***$_3$ (acyl group)	linolenic (or ω-3)

[a] Signal multiplicity: bs, broad signal; s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; dd, doublet-doublet; dt, doublet-triplet; R, R′, H or alkyl chain; X, electron withdrawing group.

autoxidation and photo-oxidation processes can occur during its production and storage, which can compromise its quality and nutritional properties. Consequently, the importance of having analytical tools to assess the stability of VOO in order to predict its shelf life, and therefore its quality, is evident. In this regard, ^{1}H NMR fingerprinting of VOO has been already proved to be a valuable tool for the quality traceability of VOOs (Alonso-Salces et al., 2011). The ^{1}H NMR analysis of VOOs were able to detect changes in the ^{1}H signals of the original constituents of VOO and the appearance of some low intensity signals due to the oxidative and hydrolytic degradation of the VOO stored at room temperature in the dark during 43 months. In the present work, several analytical approaches based on ^{1}H NMR fingerprinting together with pattern recognition techniques are proposed to determine VOO freshness, the exposition of VOO to light during its storage or the time a VOO has been stored under certain shelf life conditions, as well as to estimate the best before date of a fresh VOO. With these purposes, the ^{1}H NMR spectra of 638 VOO samples in the spectral region of 0–11 ppm (542 NMR buckets) were submitted to multivariate data analysis in order to achieve the corresponding predictive models, and disclose the main compounds involved in the deterioration process of VOO during its storage under different shelf life conditions.

3.2.1. Freshness of virgin olive oil

In previous studies, the evaluation of VOO quality was performed by the analysis of the legal parameters (free acidity content, PV, K_{232}, K_{270} and ΔK) and its fatty acid profile. VOO health-promoting characteristics were assessed by its phenolic profile and α-tocopherol content; and its sensory properties were defined by its volatile compounds. These measurements allowed to study the influence of light exposure, temperature, headspace oxygen and olive cultivar on both the quality and the stability of VOOs with a wide range of chemical compositions stored under simulated market shelf life conditions (Benito, Abenoza, Oria, & Sánchez-Gimeno, 2011; Conte et al., 2020; Esposto et al., 2017; Iqdiam et al., 2020; Kotsiou & Tasioula-Margari, 2015; Stefanoudaki, Williams, & Harwood, 2010). The parameter K_{270}, phenolic compounds, α-tocopherol and volatile compounds were proved to be the most important factors for VOO quality control, since they were related to the resistance of VOO to oxidation and the rancid defect (Benito et al., 2011; Lobo-Prieto, Tena, Aparicio-Ruiz, Morales, & García-González, 2020; Morales et al., 2005). Besides, phenolics and volatiles were confirmed to be responsible for the bitter and pungent tastes of VOO (Servili et al., 2015). In the present work, these legal parameters and chemical measurements traditionally analysed in olive oil were used as a reference to establish whether the VOO samples were fresh or not according to the legal limits established by EU Reg. 1830/2015 (Table S2−S4 in the supplementary material). Then, the ^{1}H NMR spectra of the VOOs exposed to light at 25 °C during 12 months or stored in darkness at different temperatures during 24 months were analysed by PLS-DA in order to build predictive models to determine VOO freshness. Several PLD-DA models were achieved using the complete NMR dataset or subsets (light or dark stored samples), and the freshness information provided by the different traditional parameters determined in the samples (Table 3). Satisfactory PLS-DA models for the determination of VOO freshness were achieved. These classification models were stable and robust since recognition and prediction abilities in cross-validation were close to each other and the former was greater than the latter, for both categories. All models achieved similar results: 81–91% of fresh VOOs and 82–96% of non-fresh VOOs were correctly recognised; and 80–89% of fresh VOOs and 80–91% of non-fresh VOOs were correctly predicted in cross-validation. All samples available for the external validation set were time zero, and thus fresh VOOs. The predictions of all models in the external set were acceptable. All models presented better prediction abilities in the external validation than in cross-validation, except for total phenol model for samples stored in the dark. Usually the opposite occurs, i.e. the predictions in cross-validation are higher or equal than in the external validation. The reason for the overestimated results achieved in the external validation was the limited samples available in the external set (regarding both number and variability covered).

The chemical composition of fresh VOO (time 0) evolves during storage. As a result, there may come a time when the oil can no longer be considered fresh according to legal, sensory and/or health-promoting aspects. Table 2 collects the most important variables on the PLS-DA models developed for the determination of VOO freshness (Table 3). All these variables were influential in the models built with all (light and dark) data or dark data. However, the NMR signals #1, #2, #3, #4, #5, #6, #9, #10, #11, #12, #17, #21, #22, #23, #25, #31, #33, #34 and #35 were not present or did not change significantly during the 12 months of storage in light conditions in order to differentiate fresh VOO from non-fresh VOO.

The appearance in the ^{1}H NMR spectra of VOOs of some signals due to hydroperoxide protons (#4), protons of the conjugated diene systems of hydroperoxide derivatives (#9, #10 and #11), and the methine proton of the hydroperoxide group (#17) indicated that primary oxidative degradation of the oil took place in the dark at all temperatures studied. Besides, a decrease of the ^{1}H signals #9, #10, #11 and #17 with increasing temperature in dark conditions disclosed the degradation of hydroperoxide derivatives into secondary oxidation products, such as aldehydes (#22, #24, #26, #31), alcohols (#15) and epoxides (#23). Aldehydes signals #24 and #26 increased during storage with increasing temperature in darkness, as well as in light exposure. This evolution of primary and secondary oxidation products during storage of VOOs under the moderate temperature and light/dark conditions studied was in agreement with previous results reported in literature (Farhoosh & Hoseini-Yazdi, 2013; Korifi et al., 2016; Tena,

Table 3
PLS-DA models to discriminate between olive oils according to their freshness.[a]

PLS-DA model	Data	PLS-comp	Boundary	Class[b]	Class code	n	p	%R	%P-CV	%P-EV
K_{270}	All	4	0.6663	non-fresh	0	137	0.22	82	80	
				fresh	1	483	0.78	81	80	100
Total phenol	All	4	0.5261	non-fresh	0	226	0.36	89	85	
				fresh	1	394	0.64	85	82	89
(*E,E*)-2,4-decadienal	All	5	0.6248	non-fresh	0	175	0.28	90	84	
				fresh	1	445	0.72	83	82	100
K_{270}	Light	5	0.5186	non-fresh	0	104	0.40	96	91	
				fresh	1	156	0.60	90	87	100
Total phenol	Light	4	0.5205	non-fresh	0	103	0.40	87	83	
				fresh	1	157	0.60	88	84	94
(*E*)-2-decenal	Light	3	0.5720	non-fresh	0	119	0.46	89	85	
				fresh	1	141	0.54	84	84	100
K_{270}	Dark	3	0.6205	non-fresh	0	91	0.24	90	86	
				fresh	1	289	0.76	88	84	100
Total phenol	Dark	2	0.5993	non-fresh	0	126	0.33	89	85	
				fresh	1	254	0.67	83	81	78
(*E,E*)-2,4-decadienal	Dark	5	0.5718	non-fresh	0	126	0.33	94	86	
				fresh	1	254	0.67	91	89	100

[a] Abbreviations: n, number of samples; PLS-comp, number of PLS components selected; p, prior probability; %R, % of recognition ability; %P-CV, % of prediction ability in 3-fold crossvalidation; %P-EV, % of prediction ability in the external validation.
[b] Freshness criteria: $K_{270} < 0.220$ in fresh VOO stored in the light or $K_{270} < 0.190$ in fresh VOO in the dark; total phenol content >250 mg/kg in fresh VOO; (*E*)-2-decenal content <410 μg/kg in fresh VOO (rancid defect threshold); (*E,E*)-2,4-decadienal content <354 μg/kg in fresh VOO (rancid defect threshold).

Aparicio, & García-González, 2017). It was proved that the oxidation of triacylglycerides or the free fatty acids resulted from the hydrolytic degradation of triacylglycerides lead to the formation of relative stable hydroperoxides at temperatures below 65 °C. This fact allows the detection of these primary oxidation products during shelf life storage conditions. Moreover, increasing temperature provides additional energy for activating the hydroperoxide decomposition into the secondary oxidation product, and extra free radicals that will accelerate the propagation reactions (Farhoosh & Hoseini-Yazdi, 2013). Furthermore, photo-oxidation of VOOs was confirmed to take place even at milder conditions (23 °C and 400 lux) than those of present study (Tena et al., 2017). Hydroperoxides are intermediate reaction products, thus during their oxidation, they could be formed and decomposed at the same time. Hence, the evolution of their contents that is observed depends on the formation and decomposition rates (Conte et al., 2020). In the present study, these rates were closed to each other during the one year storage of VOOs exposed to light at 25 °C, therefore the changes in the ^{1}H signals due to hydroperoxides were not significant in the classification model for light conditions. In contrast, the rise in the intensity of saturated aldehydes signals (#24 and #26) was relevant under these storage conditions. From the data at 25 °C (light *vs* dark), it was evidenced that the formation of hydroperoxides was enhanced by light; and that the formation and decomposition rates of hydroperoxides were similar in VOOs exposed to light, whereas the decomposition rate was higher than the formation one in darkness. Therefore the intensity of the hydroperoxides signals decreased during storage, meanwhile those of aldehydes increased, as previously reported (Esposto et al., 2017).

Changes in the intensities of the ^{1}H signals of unsaturated aldehydes responsible for the negative rancid off-flavours of VOOs (Esposto et al., 2017) were not detected in the VOOs stored during one year in the light or two years in the dark at different temperatures. This is explained by the fact some protons in the alkenal structures overlap with those of major components of VOOs (Sacchi et al., 1996). Moreover, some other aldehydic protons (9.3–9.9 ppm) from unsaturated aldehydes generated during the secondary oxidation reactions overlap with those signals of secoiridoids, present in higher concentrations, and the increase of those aldehydes during storage might have been not significant to be detected in contrast with the decrease of secoiridoids. These results related to the second oxidation products (aldehydes) measured in VOOs agreed with those achieved using other spectroscopic approach based on mesh cell-FTIR used to study the oxidation of VOO under accelerated mild storage conditions emulating transport and storage stages (Tena et al., 2017). Indeed, FTIR aldehyde band intensities of VOOs stored in similar conditions (dark at 23 °C and 35 °C, and light at 400 lux and 23 °C) to those of the present study were not significantly different among the studied conditions.

The ^{1}H signal intensities of VOO phenolic compounds (#1, #3, #5, #6, #7, #8 and #21) decreased during storage. This behaviour, which contributed to distinguish fresh and non-fresh VOOs, was in accordance with the role that these substances play as antioxidants during the oxidative degradation process of VOO (Esposto et al., 2017; Koidis & Boskou, 2014; Krichene et al., 2015; Stefanoudaki et al., 2010). Phenolic compounds act as primary antioxidants in VOOs, playing a critical role in VOO stability. Under dark conditions, the deterioration of VOO is mainly related to autoxidation of its antioxidants, preferentially its secoiridoid derivatives (Krichene et al., 2015; Stefanoudaki et al., 2010). In contrast, photo-oxidation is the main responsible of VOO deterioration in the presence of light (Esposto et al., 2017; Koidis & Boskou, 2014). The involved ^{1}H signals were due to secoiridoids, which are dialdehydic forms of oleuropein and ligstroside lacking a carboxymethyl group (#1, #3, #6, #7, #8 and #21) and aldehydic forms of oleuropein and ligstroside (#1, #5, #6, #7 and #8). No significant differences were observed in the dialdehydic signals #1, #3, #7 and #21 between light and dark conditions during the first year of storage; whereas signals #3 and #21 decreased in darkness with increasing temperature (Krichene et al., 2015). The intensities of the aldehydic signal #5, #6 and #8 were slightly higher in VOOs in the dark than in those exposed to light, which means that less degradation of the VOOs happened in darkness (Esposto et al., 2017; Krichene et al., 2010, 2015); no influence of temperature was noticed. The ^{1}H signals of both the aldehydic (#5) and dialdehydic forms of oleuropein (#8) and ligstroside (#6) derivatives, denoted that the degradation behaviour of both families of secoiridoids (hydroxytyrosol and tyrosol derivatives, respectively) was quite similar (Esposto et al., 2017; Krichene et al., 2015). Secoiridoids degradation rate was shown to be dependent of the initial concentrations in the VOO: higher contents of secoiridoids lead to higher resistance of the VOO to oxidation (Esposto et al., 2017; Krichene et al., 2015). The effects of light, temperature and initial phenolic contents on VOO stability are important issues to be considered to ensure the quality of VOO and its health-promoting properties. Hence, a VOO stored at reduced temperatures and protected from light increases its shelf life, and consequently will comply with the European Regulation Health Claim during a longer period of time.

During the hydrolytic degradation of VOO, triacylglycerides

hydrolyse resulting in a decrease of the intensity of the triacylglyceride signals. The glyceryl proton signals of triacylglycerides (#13 and #18), and the α-methylene (#27) and β-methylene (#32) protons of the acyl group of fatty acids disclosed the occurrence of hydrolytic degradation during storage both in the light and in the dark. The degradation of VOOs stored in darkness increased with increasing temperature. The decrease in the triacylglyceride contents of VOOs during storage was also reported by other authors (Aparicio & Harwood, 2013; Farhoosh & Hoseini-Yazdi, 2013; Köseoğlu, Sevim, & Kadiroğlu, 2019; Méndez & Falqué, 2007). The intensities of the glyceryl proton signals of triacylglycerides (#16), the vinylic (#12), the α-methylene (#25 and #28), and the methylene (#33) proton of the acyl group of fatty acids were higher in VOOs kept in the dark than those in the light, suggesting less degradation in dark conditions during the first year of storage; little or no influence of temperature was observed on these signals during dark storage over two years. As a result of the hydrolysis reaction of triacylglycerides, fatty acids are released, increasing VOO acidity (Lukić et al., 2020). The maximum acidity level of VOO is stablished by the EU Regulation 2015/1830. Several research studies showed that the free fatty acid content of VOOs increased throughout storage in both light and dark conditions (Baiano, Terracone, Viggiani, & Del Nobile, 2014; Ghanbari Shendi, Sivri Ozay, Ozkaya, & Ustunel, 2018; Köseoğlu et al., 2019; Méndez & Falqué, 2007; Stefanoudaki et al., 2010). However, the hydrolytic process of triacylglycerides was confirmed to be sensitive to the action of light, which led to a greater increase in the acidity of VOOs exposed to light (Méndez & Falqué, 2007). Besides, triacylglyceride hydrolysis occurred to a greater extent as the temperature and acidity of VOO increased; the former being the most significant factor (Pérez-Camino, Moreda, & Cert, 2001; Spyros, Philippidis, & Dais, 2004).

The signal intensities of the allylic (#29) protons of the acyl group, the methylene proton of oleic acyl group (#34) and the methyl proton of the linolenic acyl group (#35) were lower in non-fresh samples, revealing that fatty acids were degrading during storage in the light and in the dark, but no influence of temperature was discerned. The degradation of fatty acids is a consequence of their oxidation (Méndez & Falqué, 2007).

During storage in both light and dark conditions and with increasing temperature, the intensity of the glyceryl proton signal of *sn*-1,3-diacylglycerides (#19) increased, corresponding to a decrease of *sn*-1,2-diacylglyceride signals (#14 and #20). This evidenced the loss of quality and freshness of the VOO, because fresh good quality olive oils contain mostly native *sn*-1,2-diacylglyceride and only small amounts of *sn*-1,3-diacylglyceride. Diacylglycerides result from either incomplete biosynthesis of triacylglycerides in olive fruits or their later acid-catalysed and/or enzymatic hydrolysis during processing and storage (Pérez-Camino et al., 2001; Spyros et al., 2004). 1,2-Diacylglycerides are considered to come mainly from the incomplete biosynthesis of triacylglycerides, whilst 1,3-diacylglycerides from their enzymatic or chemical hydrolysis. During VOO storage, 1,2-isomers are transformed to the thermodynamically more stable.1,3-isomers by an intermolecular transposition (Circi et al., 2018; Pérez-Camino et al., 2001; Sacchi et al., 1996; Spyros et al., 2004). Diacylglyceride isomerisation rate rises as temperature and free acidity increases (Pérez-Camino et al., 2001; Spyros et al., 2004). Besides, light exposure favours that this isomerisation reaction occurs at a faster rate (Spyros et al., 2004). All these trends and findings of former studies are confirmed by the results of present work. Several approaches regarding diacylglyceride ratios have been previously proposed to estimate VOO storage time and freshness (Spyros et al., 2004); (Caponio et al., 2013) (Castellani, Serrilli, Bonadies, & Bianco, 2008).

The signals due to the methyl proton of squalene (#30) and the aldehydic proton of (*E*)-2-hexenal (#2) decreased with the storage time in both light and dark, and little or no influence of temperature was noted, as previously observed (Frankel, 2010; Fregapane et al., 2013; Salvo et al., 2017; Stefanoudaki et al., 2010). The intensity of the squalene signal (#30) was higher in light exposed VOOs than in those stored in the dark. On the one hand, this observation contrasted with the results of a previous study, in which the squalene content decreased significantly during six month storage but was not affected by storage conditions regarding light/dark exposure (Silva, Anjos, Cavalcanti, & Celeghini, 2015). On the other hand, current observation agreed with the findings of another work, which reported that the presence of squalene seemed to protect purified olive oil triacylglyceride fraction from oxidation in the dark, justifying its lower contents in VOOs stored in dark conditions (Tsimidou, 2010). The main volatile compound in fresh VOO is the native (*E*)-2-hexenal, which is responsible of the positive green odour notes in VOOs (Fregapane et al., 2013; Stefanoudaki et al., 2010). (*E*)-2-hexenal, as well as other native volatiles compounds in VOOs, was also reported to decrease during storage (Frankel, 2010; Fregapane et al., 2013).

The validity and fundamentals of the classification models achieved for the determination of VOO freshness are supported by the justified behaviour of the most influential compounds in the models, which agrees with the findings and observations of previous studies. The conformity among the results provided by the predictive models obtained with the data of the different parameters related to the legal, health-promoting and sensory aspects considered for the evaluation of VOO quality (Servili et al., 2015), implies that all these aspects are included in the models. Therefore, the present approach based on ^{1}H NMR fingerprinting and chemometrics can assess by itself the overall quality of VOOs.

3.2.2. *Light exposure of virgin olive oil*

Photo-oxidation is considered the most damaging factor for the oxidative sensibility of VOOs. Several studies have demonstrated that light and temperature play a main role in the changes that VOOs experiment during their storage in their chemical composition, which is directly related with their health-promoting and sensory properties (Aparicio & Harwood, 2013; Esposto et al., 2017; Tena et al., 2017). Other processes that cause these effects are the autoxidation of the lipid substrate of VOOs and the hydrolytic processes (Krichene et al., 2010; Tena et al., 2017). The hydrolytic degradation of acylglycerides in VOOs is sensitive to light, being more intense than in darkness (Méndez & Falqué, 2007). However, the response of a VOO to these alteration processes depends on its chemical composition, particularly natural antioxidants and fatty acids, which can directly or indirectly influence those phenomena respectively (Choe & Min, 2006, 2009), as well as the presence of other pro-oxidant factors such as oxygen and activators such as chlorophylls and transition metals (Choe & Min, 2006; Esposto et al., 2017; Psomiadou & Tsimidou, 2002b). Regarding all these premises, VOOs stored in different light exposure and temperature conditions are supposed to present characteristic chemical compositions, which may be reflected in their ^{1}H NMR fingerprints. In this regard, ^{1}H NMR spectral data of the VOOs stored in the light during 12 months and in the dark at different temperatures during 24 months were studied by pattern recognition. PLS-DA provided a robust classification model to distinguish the samples exposed to light from the samples stored in the dark at different temperatures (Table 4). The recognition and prediction abilities in cross-validation of the PLS-DA model were 95% and 94% respectively for the VOO samples exposed to light, and 92% and 91% for those stored in darkness, respectively. Table 5 shows the most influential variables in the classification model. The signal intensities of the glyceryl protons of triacylglycerides (#16), and the vinylic (#12), α-methylene (#25), allylic (#36), β-methylene (#37) and methylene (#38) protons of the acyl groups of fatty acids were lower in oils exposed to light than in those stored in darkness, indicating that degradation arose to a greater extent under light exposure. No influence of temperature was observed on these signals during dark storage, except for signal #12 which slightly decreased at 35 °C. Spyros et al. (2004) suggested that the oxidation products of fatty acids may have a strong accelerating effect on triacylglyceride hydrolysis, in order to explain the increase of the triacylglyceride hydrolysis rate in VOOs exposed to light during their

Table 4
PLS-DA models to discriminate between olive oils according to their exposure to light or dark conditions.[a]

PLS-DA model	Data	PLS-comp	Boundary	Class	Class code	n	p	%R	%P
Exposure	All	3	0.5106	dark	0	360	0.58	92	91
				light	1	260	0.42	95	94

[a] Abbreviations: n, number of samples; PLS-comp, number of PLS components selected; p, prior probability; %R, % of recognition ability; %P, % of prediction ability; 3-fold crossvalidation.

storage. In this sense, the decline in the contents of both triacylglycerides and fatty acids were larger in VOOs stored in the light than in the dark.

In contrast, the signals of α- and β-protons of 2,3-branched saturated aldehydes (#24 and #26), and β-methylene protons of alkanals (#31), which are secondary oxidation products, followed similar trends at 25 °C in both light and dark conditions, but their intensities increased with increasing temperature (25–35 °C) in darkness. Indeed, temperature provides additional energy to form secondary oxidation products (Conte et al., 2020; Farhoosh & Hoseini-Yazdi, 2013). Secondary oxidation processes in VOOs were found not to be affected by light exposure conditions at ambient temperature, however the evolution of these processes were influenced by moderate temperatures (35–65 °C) (Tena et al., 2017).

The glyceryl proton signals of triacylglycerides (#13 and #18) and *sn*-1,2-diacylglycerides (#14 and #20), as well as the α-methylene (#27) and methyl (#07) protons of the acyl group of fatty acids, disclosed the occurrence of degradation of triacylglycerides and native diacylglycerides, which took place at similar rates at 25 °C in both light and dark conditions during the first year of storage; however degradation increased with increasing temperature in the dark. The changes in the contents of *sn*-1,2-diacylglycerides, fatty acids and triacylglycerides during storage are interrelated because the isomerisation of 1,2-diacylglycerides is acid-catalysed by the free fatty acids produced during the hydrolysis of triacylglycerides. Furthermore, diacylglyceride isomerisation was quantitatively proved to be strongly affected by temperature during dark storage (Spyros et al., 2004).

The ^{1}H NMR buckets with negative Bw coefficients were due to signals of fatty acids, which were more degraded in VOO samples exposed to light than in those stored in the dark, and were slightly or not affected by the increase in temperatures. In contrast, the ^{1}H signals of triacylglycerides and *sn*-1,2-diacylglycerides presented positive Bw coefficients, because their degradation rate was similar in VOOs stored in the light to those in the dark, but their degradation increased with increasing temperature. Oppositely, the secondary oxidation products, even though being influenced by the storage temperature as well, presented negative Bw coefficients since their contents increased during VOO degradation. Thus, Bw coefficients of the classification model reflected the different behaviours of the compounds involved in oxidative and hydrolytic degradation of VOOs depending on the VOO storage conditions.

These results prove that VOOs exposed to light or stored in darkness experienced different chemical changes due to the hydrolytic processes of acylglycerides as well as the oxidative degradation of fatty acids, which have significant implications in VOO quality. Therefore, taking into account the importance of avoiding the exposure of VOO to light during its shelf life and storage until its consumption, the existence of analytical tools to check whether a VOO has been correctly stored since it was produced is of great interest in order to preserve the high quality standards of VOO. ^{1}H NMR fingerprinting of VOO together with pattern recognition techniques provide a rapid high throughput approach to control the correct storage of VOO during its commercial life, and have an estimation of its genuine quality depending on its production date.

3.2.3. Storage time of virgin olive oil

The ^{1}H NMR spectral data of VOOs contained information related to the time that passed since the VOO was produced and stored at 25 °C in the light or at different temperatures in the dark (Table 6). The PLS-R models built allowed to determine how long a VOO had been stored, which was related to the degree of deterioration of its quality. The most influential variables in the PLS-R model for VOOs stored at 25 °C in the light are shown in Table S6 in the supplementary material. The signals due to the glyceryl protons of triacylglycerides (#18) and *sn*-1,2-diacylglycerides (#14 and #20) and the α-methylene (#27) and allylic (#36) protons of the acyl groups of fatty acids decreased with the time of storage (Caponio et al., 2013; Méndez & Falqué, 2007; Pérez-Camino et al., 2001; Spyros et al., 2004), resulting in negative Bw coefficients. In contrast, the signals of those compounds that increased with degradation during the storage time presented positive Bw coefficients, such as the glyceryl protons of *sn*-1,3-diacylglycerides (#19) and the α-methylene protons of 2,3-branched saturated aldehydes (#24), as expected (Caponio et al., 2013; Pérez-Camino et al., 2001; Spyros et al., 2004; Tena et al., 2017). The signals of the compounds involved in the degradation of the VOO during storage at 25 °C in the light are correlated to the "time of exceeding the threshold value" of the traditional parameters measured to establish when an oil is not "fresh" anymore, i.e. K_{270}, ΔK, and the contents of total phenol, (*E*)-2-decenal or the (*E*, *E*)-2,4-decadienal, as evidenced in section 3.2.1. Thus, the threshold of the intensity values of the most influential ^{1}H NMR signals (buckets) on PLS-R models were estimated for the months at which the traditional parameters were exceeded. For this purpose, simple least squares regressions of the storage time versus the bucket intensity were built for each influential variable and VOO sample. This way, threshold values were determined for the principal ^{1}H signals to assess whether a VOO is fresh or not (Table S7 in the supplementary material). The intensity threshold values obtained using each traditional parameter were similar and presented satisfactory repeatability values (0.4–20% of relative standard deviation). Table S7 in the supplementary material shows the intensity threshold value for each ^{1}H signal obtained with the data of the traditional parameter which afforded the best precision. The month of exceeding K_{270} threshold value (0.220) provided the best results to establish the intensity thresholds for the ^{1}H signals of triacylglycerides, *sn*-1,2-diacylglycerides, acyl groups of fatty acids and 2,3-branched saturated aldehydes; whereas the month of exceeding the (*E*)-2-decenal content threshold value (410 μg/kg), for the buckets of *sn*-1,3-diacylglycerides. Positive correlations had been already observed between light exposure duration and K_{270} and C_7–C_{11} aldehydes during VOO storage at ambient temperature (Esposto et al., 2017).

Likewise the threshold values for the most influential variables in the PLS-R models for VOOs stored at different temperatures in the dark were estimated (Table S8−S13 in the supplementary material). Most of these variables were coincident with the most important ones in the PLS-R model for VOOs exposed to light at 25 °C, and followed the same trends as well. The signals presenting negative Bw coefficients decreased with the time of storage and were due to the glyceryl protons of triacylglycerides (#13 and #18) and *sn*-1,2-diacylglycerides (#14 and #20) and the α-methylene (#27) and β-methylene (#32) protons of the acyl groups of fatty acids (Farhoosh & Hoseini-Yazdi, 2013) (Caponio et al., 2013; Méndez & Falqué, 2007; Pérez-Camino et al., 2001; Salvo et al., 2017; Spyros et al., 2004). Besides, the signals of the glyceryl protons of *sn*-1,3-diacylglycerides (#19) and the α-methylene protons of 2,3-branched saturated aldehydes (#24), showing positive Bw coefficients, increased with degradation during the storage time (Caponio et al., 2013; Pérez-Camino et al., 2001; Salvo et al., 2017; Spyros et al.,

Table 5
Weighted regression coefficient (Bw) of the most influent variables on the PLS-DA model built to discriminate between olive oils according to their exposure to light or dark conditions.

#	Bucket (ppm)	Bw (3 PLS-comp)	Functional group	Attribution
12	5.49	−6.14E-03	–C***H***=C***H***– (^{13}C satellite of signal at 5.28–5.38 ppm, acyl group)	
	5.47	−6.56E-03		
	5.43	−6.30E-03		
13	5.25	6.83E-03	>C***H***OCOR (glyceryl group)	triacylglycerides
	5.21	6.34E-03		
14	5.05	9.15E-03	>C***H***OCOR (glyceryl group)	*sn*-1,2-diacylglycerides
16	4.39	−6.65E-03	–C***H***$_2$OCOR (^{13}C satellite of signal at 4.09–4.32 ppm, glyceryl group)	triacylglycerides
	4.37	−6.04E-03		
18	4.27	6.25E-03	–C***H***$_2$OCOR (glyceryl group)	triacylglycerides
20	3.75	7.13E-03	–C***H***$_2$OH (glyceryl group)	*sn*-1,2-diacylglycerides
	3.73	8.51E-03		
	3.71	8.08E-03		
	3.69	6.46E-03		
24	2.59	−7.58E-03	–R'CHX–C***H***R–CHO	2,3-branched saturated aldehydes
	2.57	−7.07E-03		
	2.51	−6.56E-03		
	2.49	−8.06E-03		
25	2.47	−7.61E-03	–OCO–C***H***$_2$– (^{13}C satellite of signal at 2.26–2.32 ppm, acyl group)	
	2.45	−7.74E-03		
	2.43	−7.36E-03		
	2.41	−7.24E-03		
	2.39	−8.07E-03		
26	2.37	−8.37E-03	–R'C***H***X–CHR–CHO	2,3-branched saturated aldehydes
	2.35	−7.64E-03		
	2.33	−6.22E-03		
27	2.29	6.22E-03	–OCO–C***H***$_2$– (acyl group)	
36	2.09	−6.39E-03	–C***H***$_2$–CH=CH– (^{13}C satellite of signal at 1.96–2.07 ppm, acyl group)	
37	1.75	−6.60E-03	–OCO–CH$_2$–C***H***$_2$– (^{13}C satellite of signal at 1.51–1.65 ppm, acyl group)	
	1.73	−6.98E-03		
31	1.65	−7.19E-03	–C***H***$_2$–CH$_2$–CHO	alkanals
38	1.51	−6.96E-03	–(C***H***$_2$)$_n$– (^{13}C satellite of signal at 1.19–1.37 ppm, acyl group)	
	1.49	−6.48E-03		
	1.47	−6.65E-03		
	1.45	−8.17E-03		
	1.43	−8.49E-03		
	1.41	−7.99E-03		
	1.39	−7.44E-03		
07	0.89	6.26E-03	–C***H***$_3$ (acyl group)	saturated, oleic (or ω-9) and linoleic (or ω-6)

2004). In VOOs stored at 35 °C in the dark, together with the influential ^{1}H signals at lower temperatures, other signals due to primary oxidation products such as hydroperoxide derivatives (#17), which degraded during storage to secondary oxidation products such as aldehydes (#24 and #26) (Choe & Min, 2006; Conte et al., 2020; Farhoosh & Hoseini-Yazdi, 2013; Tena et al., 2017), were among the most influential variables in the PLS-R model. Moreover, the degradation of secoiridoids (#21) was also important at 35 °C (Krichene et al., 2015).

From the results achieved in the different storage conditions of VOO, it was observed that the best precisions to determine the NMR signal intensity threshold to assess whether VOO freshness is lost were obtained using the data of the extinction coefficient K_{270} and the (*E*)-2-decenal content.

3.2.4. *Estimation of the best before date*

The composition of VOO, once it has been extracted, influences and determines the evolution of VOO during its storage, which depends on external factors such as light, temperature, oxygen availability and metal contamination from packing materials (Esposto et al., 2017; Velasco & Dobarganes, 2002). The final composition of fresh VOO is the result of a large number of variables involved in the different production stages from the olive cultivation to the olive oil extraction. The well-known high resistance of VOO to oxidative deterioration is due to its composition in triacylglycerides with low contents of polyunsaturated fatty acid, and antioxidants (Psomiadou & Tsimidou, 2002a; Velasco & Dobarganes, 2002). The triacylglycerides and fatty acid profiles of VOO are mostly determined by the olive cultivar genotype, and less influenced by environmental factors and the ripeness stage of olives (Servili et al., 2014; Velasco & Dobarganes, 2002). The ratio between monounsaturated and polyunsaturated fatty acids is considered an appropriate index of olive oil tendency to undergo autoxidation; the higher the ratio, the higher oxidative stability of the VOO (Farhoosh & Hoseini-Yazdi, 2013; Krichene et al., 2010). Indeed, VOO autoxidation rate depends to a great extent on the formation rate of alkyl radicals of fatty acids or acylglycerides, and this rate, in turn, on the types of these constituents in VOO (Choe & Min, 2006). Regarding minor components in VOO, some accelerates VOO oxidation performing as pro-oxidant such as free fatty acids, mono- and diacylglycerides and metals; and others act as antioxidant, such as phenolic compounds, carotenoids, sterols, tocotrienols, phospholipids and squalene. Meanwhile tocopherols present both functions, and chlorophylls is pro-oxidant in the presence of light and antioxidant in the dark (Aparicio & Harwood, 2013; Choe & Min, 2006; Velasco & Dobarganes, 2002). Esposto et al. (2017) reported that the initial contents of antioxidant compounds, particularly oleuropein derivatives and α-tocopherol, highly influence the loss of VOO quality from the health-promoting and sensory points of view. Oleuropein derivatives are involved in the primary inhibition of oxidation processes in VOO, and thus VOOs with high initial contents of these compounds experiment lower losses of secoiridoid derivatives and α-tocopherol, and develop fewer amounts of volatile C_7–C_{11} compounds responsible for the rancid defect during storage (Esposto et al., 2017). High phenolic contents in VOOs seem to limit the involvement of α-tocopherol in the oxidation processes and preserve its antioxidant effect (Esposto et al., 2017). Whereas in VOOs with low phenolic contents, α-tocopherol plays an important role in the inhibition of the oxidation phenomena, and a larger decrease of α-tocopherol contents is observed (Esposto et al., 2015). VOO oxidative stability depends on the correlation between the concentration of the secoiridoid families in fresh VOO and the initial oxidative degradation rate, which is temperature dependent (Krichene et al., 2010, 2015). Squalene presents a moderate antioxidant activity, which is concentration dependent in VOO stored at low temperatures and in the dark (Psomiadou & Tsimidou, 1999), and appears to protect α-tocopherol during the photo-oxidation of VOO (Psomiadou & Tsimidou, 2002b). During light exposure, VOO stability is also influenced by their pigment contents, which is characteristic of the olive cultivar (Aparicio & Harwood, 2013),

Table 6
PLS-R models to determine the storage time of a VOO kept at 25 °C in the light or at different temperatures (25 °C, 30 °C and 35 °C) in the dark.[a]

PLS-R model[b]	Data[c]	n	PLS-comp	R-cal	R-val	RMSEP	Mean of |Yref-Ypred|	Confidence interval (95%)
NMR buckets (X) vs time (Y)	Light (0–12 months)	257	6	0.966	0.953	1.1	0.895	0.084
NMR buckets (X) vs time (Y)	Dark, 25 °C (0–24 months)	260	5	0.946	0.926	2.8	2.27	0.21
NMR buckets (X) vs time (Y)	Dark, 30 °C (0–24 months)	80	4	0.941	0.886	3.8	3.03	0.52
NMR buckets (X) vs time (Y)	Dark, 35 °C (0–24 months)	80	5	0.958	0.925	3.1	2.61	0.39

[a] Abbreviations: n, number of samples; PLS-comp, number of PLS components; R-cal, correlation coefficient in calibration; R-val, correlation coefficient in validation; RMSEP, root mean square error in the prediction; 3-fold crossvalidation.
[b] X matrix = normalized intensities of the NRM buckets; Y matrix = storage time (month).
[c] Samples used to build the model.

since VOO susceptibility to photo-oxidation rather depends on its chlorophyll content (Psomiadou & Tsimidou, 2002b; Tena et al., 2017). The diacylglyceride isomerisation is not affected by storage conditions of temperature and light exposure but by the olive cultivar and storage time (Caponio et al., 2013). The profiles of phenolic compounds, volatile compounds and monounsaturated fatty acids in VOOs are highly related to the olive cultivar and strongly influenced by agronomical and technological factors. VOO composition in some sterols, such as campesterol, is affected by the olive cultivar genetics and pedoclimatic conditions (Servili et al., 2014). Taking into account the knowledge provided by all these previous studies, our hypothesis was that the ^{1}H NMR spectral data of VOOs at time zero, i.e. at the moment of VOO production, would contain information related to the probable evolution of VOOs during their storage, and therefore, would allow to estimate the best before date for its consumption. Despite the reduced number of samples available for this aim in the present study, PLS-R afforded a model to determine the best before date of a VOO stored in the light at 25 °C (Table 7), using the data of the time exceeding the K_{270} parameter threshold for VOOs stored in this conditions (Table S2 in the supplementary material). These preliminary results confirmed that ^{1}H NMR spectral data contains the necessary information to achieve a regression model to estimate the best before date of a VOO by analysing the oil at time zero. However, this preliminary regression model should be put in perspective, and be aware that further research is required with a larger representative sample set of VOOs. These VOOs stored under different storage conditions (light exposure, temperature) have to be analysed at time zero, and then the evolution of their K_{270} parameter followed until the time this parameter is exceeded, in order to achieve robust PLS-R models with appropriate accuracies and precisions.

4. Conclusion

The relevance of present study on the stability of VOO lies in the simulation of real shelf life conditions of VOOs in the market, including a representative sample set of VOOs covering the full range of possible chemical compositions. The most important factors that influence VOO quality and stability during storage, i.e. light exposure, temperature, time of storage and the initial composition of VOO were considered. Metabolic fingerprinting of VOO by ^{1}H NMR spectroscopy combined with chemometrics provide valuable approaches for the quality control of VOOs, as well as to evaluate their stability. The potential of the classification and regression models achieved to determine whether a VOO is fresh, whether it has been stored in light or dark conditions, the storage time of VOO under different storage conditions and the best before date of a fresh VOO was proved. Besides, these predictive models disclosed the chemical compounds responsible for the compositional changes taking place in VOO due to its hydrolytic and oxidative degradation, which helps to understand the quality changes that VOO can experience during its shelf life. None of the signals present in the ^{1}H NMR spectrum of VOOs at time zero disappeared during VOO storage over one year in the light and two years in the dark. Over the time of storage, relatively small changes in the original ^{1}H signal intensities and the emergence of new low intensity signals indicated that some oxidative and hydrolytic degradation of VOO was occurring under normal market conditions. These results confirm the high oxidative stability of VOO at moderate temperatures, even exposed to light, and dismiss any significant changes which could render its consumption hazardous. However, those changes in the ^{1}H NMR fingerprints of VOOs were evident enough to evaluate their quality according to legal, sensory and health-promoting aspects using pattern recognition techniques. Furthermore, this study corroborated that light and increasing temperature accelerate VOO degradation during its shelf life. Therefore, the use of packaging that protects VOO from light (e.g. dark glass bottles), minimal headspace and the control of temperature during transport, distribution and storage of VOO are highly recommended to guarantee VOO quality from production to consumption.

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CRediT authorship contribution statement

Rosa María Alonso-Salces: Conceptualization, Methodology, Formal analysis, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Blanca Gallo:** Resources, Writing - review & editing. **María Isabel Collado:** Investigation. **Andrea Sasía-Arriba:** Investigation. **Gabriela Elena Viacava:** Investigation. **Diego Luis García-González:** Resources, Writing - review & editing. **Tullia Gallina Toschi:** Resources. **Maurizio Servili:** Resources, Data curation. **Luis Ángel Berrueta:** Resources, Writing - review & editing.

Table 7
PLS-R models to determine the best before date of a VOO stored in the light at 25 °C.[a]

PLS-R model[b]	Data[c]	n	PLS-comp	R-cal	R-val	RMSEP	Mean of |Yactual-Ypred|	Confidence interval (95%)
NMR buckets (X) vs time (Y)	Light (time 0)	22	2	0.933	0.841	0.71	0.59	0.18

[a] Abbreviations: n, number of samples used to build the model; PLS-comp, number of PLS components; R-cal, correlation coefficient in calibration; R-val, correlation coefficient in validation; RMSEP, root mean square error in the prediction; leave-one-out crossvalidation.
[b] X matrix = normalized intensities of the NRM buckets; Y matrix = time (month) exceeding the threshold value of K_{270}.
[c] Samples used to build the model.

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.

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Appendix A. Supplementary data

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