JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. XXXX, XXX, XXX−XXX

pubs.acs.org/JAFC

New Volatile Molecular Markers of Rancidity in Virgin Olive Oils under Nonaccelerated Oxidative Storage Conditions

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Supporting Information

ABSTRACT: Evolution of the volatile profile of two extra-virgin olive oils with very different fatty acid composition (monounsaturated fatty acid/polyunsaturated fatty acid ratio) stored in several nonaccelerated oxidative conditions was studied by a validated headspace solid-phase microextraction-gas chromatography−mass spectrometry (HS-SPME-GC−MS) method. The role of C8 volatile compounds in oxidative processes was highlighted, and controversial aspects regarding the origin of some volatiles were clarified. Specific volatile markers for rancidity were proposed: sum of pentanal, hexanal, nonanal, *E*-2-heptenal, propanoic acid, and hexanoic acid for oils stored in the dark; sum of pentanal, heptanal, nonanal, decanal, *E*-2-heptenal, *E*-2-decenal, *E,E*-hepta-2,4-dienal, and *E,E*-deca-2,4-dienal, octane for oils stored under light exposure; sum of pentanal, nonanal, decanal, *E*-2-heptenal, *E*-2-decenal, *E,E*-hepta-2,4-dienal, nonan-1-ol, propanoic acid, octane, 6-methylhept-5-en-2-one, and oct-1-en-3-ol for oils stored under light exposure with oxygen in headspace. A simplified marker (sum of pentanal, nonanal and *E*-2-heptenal) suitable for all conditions was also proposed.

KEYWORDS: *volatile organic compounds, lipid autoxidation, photo-oxidation, markers of rancidity, panel test, extra-virgin olive oil, HS-SPME-GC−MS*

INTRODUCTION

Virgin olive oil (VOO) is recognized as the most valuable product among the edible oils, mainly thanks to the high percentage of monounsaturated fatty acids (MUFA) as oleic acid, to antioxidants as phenolic compounds and tocopherols present in greater amount than in other edible oils,[1] and its pleasant taste and smell.[2] Phenolic compounds are responsible for the bitter taste and the pungency of VOO,[3−5] and many attempts have been made for improving the quality of the VOOs, trying to increase the amount of phenolic compounds avoiding decreases of extraction yield.[6−9] The other pleasant sensory attributes (green and fruity notes) are mainly due to the volatile organic compounds (VOCs) originated by the lipoxygenase (LOX) cascade, a series of enzymatic transformations mainly occurring on free polyunsaturated fatty acids (PUFA) with a 1-cis,4-cis-pentadiene system, as linoleic and linolenic acids, which lead to C5 and C6 VOCs as aldehydes, alcohols, and esters.[10] In olives, the LOX pathway leads to C6 rather than C9 volatile compounds, with a high reaction rate in the range of 0−20 °C.[11] Regarding the negative sensory attributes, the main defects can originate from both microbiological and oxidative activities.[10]

The oxidative activities are recognized as one of the major causes of the lipid food spoilage and lead to the rancid defect.[12] Autoxidation of food lipids affects molecules with one or more allyl groups, proceeds via free-radical mechanism, and, after degradation reactions of the formed hydroperoxides, leads to a great number of volatile oxygenated compounds. Several factors affect lipid autoxidation, including the presence of pro- and antioxidants, the partial pressure of oxygen, the surface exposed to the air, and storage conditions, and particularly exposure to light, which triggers the transition of oxygen from a triplet state ($^3O_2$) to a singlet state ($^1O_2$), thus promoting the start of autoxidation in the lipid media (photo-oxidation). Some pigments (i.e., chlorophylls *a* and *b*, pheophytins *a* and *b*) act as sensitizers, while other ones, as carotenoids, act as effective $^1O_2$ quenchers, so protecting fat/oil from photo-oxidation.[12−15] In general, PUFAs are more susceptible to oxidation than MUFAs as oleic acid, with the following relative rates of oxidation: arachidonic/linolenic/linoleic/oleic ≈ 40:20:10:1.[16] Consequently, olive oils with different fatty acid composition have different susceptibility to oxidation,[17−19] particularly when the samples are not correctly stored in the dark.

Reactions occurring on food lipids during oxidation are exceedingly complex. Several model systems with oleate, linoleate, and linolenate methyl esters have been used to hypothesize and ascertain the mechanistic pathways, but a generalization is not always justified and the number and type of VOCs originated from oxidation in a real olive oil are difficult to predict. Furthermore, the extreme conditions applied in many of the accelerated studies reported in the literature[11,17,19] are not completely suitable to simulate the

Received: September 15, 2019
Revised: November 4, 2019
Accepted: November 5, 2019
Published: November 5, 2019

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

actual real-time storage conditions, thus leading to a difficulty in selecting markers of oxidation.[18,20,21] As an example, at the high temperatures used in such experiments, the rate of lipid oxidation is dependent on O_2 pressures and some polymerization and cyclization of PUFAs became important.[17] Finally, very few studies focused their attention on the evolution of the VOCs profile under typical market storage (filled close bottle), only applying a semiquantitative approach to not more than 40 different volatiles.[21,22]

As a consequence of this state of the art, definition of the molecules related to specific defects in general and rancidity in particular[23] is still necessary. Regarding the rancid defect, some researchers tried to correlate the level of rancidity to specific VOCs (e.g., *E*-2-heptenal, *E*-2-decenal, nonanal, hexanal/nonanal ratio),[13,17,24,25] but a definitive group of VOCs suitable as markers of rancidity has not been yet defined.

An headspace solid-phase microextraction-gas chromatography−mass spectrometry (HS-SPME-GC−MS) method for a reliable quantitation of approximately 70 VOO-VOCs based on the use of 11 internal standards for area normalization has been recently optimized and validated.[26]

In this research, such method was applied to study, for the first time, the evolution of the volatile profile of extra-virgin olive oils (EVOOs) characterized by a very different MUFAs/PUFAs ratio and stored in different nonaccelerated oxidative conditions designed to stress light and/or oxygen exposure. By this way, we simulated ideal (no oxygen in headspace in the dark) and nonideal storage conditions, similar to those of oils stored in the marketplace and/or at home. The content of typical parameters of oxidation other than VOCs was also measured over time. The main goal was a more in-depth comprehension on the origin and behavior over time of some VOCs and proposing markers for measuring the level of rancidity in EVOOs stored in ideal and nonideal conditions.

MATERIAL AND METHODS

Chemicals and Standards Preparation. All chemicals and standards of analytical reagent grade were from Sigma-Aldrich (Steinheim, Germany). The authentic standards of 73 volatile organic compounds (VOCs) used for external calibration and their purity are reported in the Supporting Information (Table S1). A stock standard solution containing specific amounts of the 73 authentic standards of VOCs (ExtSTD solution) and a stock standard solution of internal standards (ISTD solution) were prepared in a refined olive oil (ROO) free from VOCs. The ISTD solutions were prepared by weighing 4-methyl-2-pentanol (≥98.0%), 6-chloro-2-hexanone (≥97.0%), 3-octanone (≥98.0%), ethyl acetate-d_8 (≥99.0%), toluene-d_8 (≥99.6%), butanol-d_{10} (≥99.0%), 3,4-dimethylphenol (≥98.0%), trimethylacetaldehyde (≥96.0%), and acetic acid-2,2,2-d_3 (≥99.0%) (all from Sigma-Aldrich, Steinheim, Germany) in the ROO. Six diluted solutions were then prepared and used for building the six-point calibration lines to be used for VOCs quantitation: in each diluted solution, we added the same amount of ISTD solution and different amounts of ExtSTD solution, with ranges of concentrations chosen according to previous works[26] to simulate their contents in olive oils. Standard solutions were stored in the dark at −20 °C until chromatographic analyses.

Samples and Experimental Design. Two EVOOs were chosen according to their fatty acid composition (Table 1). They were (i) an Italian sample rich in MUFAs (cultivar Coratina, code It-EVOO) and a Tunisian sample rich in PUFAs (cultivar Chemlali, code Tun-EVOO). The content of oleic acid was close to the upper (It-EVOO) and lower (Tun-EVOO) legal limit (55−83%) for the EVOO category.[27] After initial chemical and sensorial analysis, aimed to confirm the classification of the EVOOs and to characterize them, the samples were divided into several aliquots of 100 mL and stored in the following conditions: (i) in the dark in the absence of oxygen (i.e., no headspace in the bottles) as an ideal storage condition; (ii) under exposure to light in open bottles (i.e., unlimited oxygen availability), as extreme oxidative storage conditions; (iii) under exposure to light in the absence of oxygen, a condition similar to that often applied by large retailers; (iv) in the dark in the presence of oxygen in the headspace (i.e., approximately a third of the bottle was left unfilled); and (v) under exposure to light in the presence of the oxygen in the headspace. The last two types of storage can be considered similar to household conditions. Table S2 summarizes the given codes associated with the different storage conditions. Samples were randomly disposed on a table, at temperature daily ranging from 18 to 24 °C and stored in the period October 7 to April 7 (a total of 26 weeks = 6 months) and were exposed to a mix of natural and artificial light. For each of the five storage conditions and for each of the two types of samples, three bottles randomly selected were taken and analyzed after the following storage time: 3 weeks, 8 weeks, and 26 weeks. The samples were analyzed in triplicate.

Table 1. Fatty Acid Composition of the Two Extra-Virgin Olive Oils Selected for the Experiment[a]

	Tun-EVOO	It-EVOO
Miristic acid	<0.1	<0.1
Palmitic acid (% w/w)	17.6	10.3
Palmitoleic acid (% w/w)	**2.5**	**0.4**
Margaric acid	<0.1	<0.1
Margaroleic acid	**<0.1**	**<0.1**
Stearic acid (% w/w)	2.3	2.2
Oleic acid (% w/w)	**57.3**	**78.7**
Linoleic acid (% w/w)	**18.5**	**6.4**
Linolenic acid (% w/w)	**0.8**	**0.7**
Arachic acid (% w/w)	0.4	0.4
Eicosenoic acid (% w/w)	**0.2**	**0.4**
Behenic acid (% w/w)	0.1	0.1
Lignoceric acid (% w/w)	0.1	0.0
trans C18:1(% w/w)	<0.1	<0.1
trans C18:2 + C18:3 (% w/w)	<0.1	<0.1
∑saturated fatty acids (% w/w)	20.5	13.0
∑monounsaturated fatty acids (% w/w)	**60.0**	**79.5**
∑polyunsaturated fatty acids (% w/w)	**19.3**	**7.1**

[a]Mono- and Polyunsaturated fatty acids are reported in bold.

Legal Quality Indices. Free fatty acids, peroxide number, UV spectrophotometric indices (K_{232}, K_{268}, ΔK), and fatty acid composition were determined following the analytical method described in the European Regulations EEC 2568/1991.[27]

Evolution of Pigments and Phenolic Compounds. Analysis of pigments was performed with the aim to follow their degradation under the experimental conditions. To this aim, the absorbance at specific wavelengths was measured working on the oils without solvent dilution, using a glass cell (thickness, 10 mm). The content of chlorophylls was measured according to the IUPAC method:[28] absorbance values at λ = 630, 670, and 710 nm were recorded and the content of chlorophylls, expressed as mg of pheophytin per kg of oil (mg_{ph}/kg), was calculated by the formula

$$C = [345.3 \times (A_{670} - 0.5 \times A_{630} - 0.5 \times A_{710})]/L$$

where C is the content of chlorophylls in mg_{ph}/kg, L is the thickness of the spectrophotometer cell (mm), and A_{XXX} is the absorbance at the respective wavelength (nm).

To follow the evolution of carotenoids, two wavelengths were selected: 460 and 490 nm: they are close to the λ_{MAX} value in the UV−vis spectra of carotenoids, and chlorophylls show very low absorptions at these λ values.[29] Results were expressed as the sum of absorbance at λ = 460 nm and at λ = 490 nm.

The total content of phenolic compounds was evaluated according to the IOC official method.[30] Briefly, extraction was carried out by a

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 2. Evolution of the Content of the Volatile Organic Compounds Quantified over 6 Months of Storage in Different Conditions[a]

			D			DO_{HS}			L			LO_{HS}			LO_{OB}		
		T0	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks
heptane	Tun	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.097	0.236	0.190	0.203
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	0.128	<0.097	nd	<0.097	<0.097	0.239	0.122	0.171
octane	Tun	<0.150	<0.150	<0.150	<0.150	<0.150	<0.150	<0.150	0.556	0.491	0.692	0.186	0.361	0.636	nd	nd	nd
	Ita	<0.150	<0.150	<0.150	<0.150	<0.150	<0.150	<0.150	0.338	0.690	1.309	<0.150	0.420	1.461	nd	nd	<0.150
methyl acetate	Tun	0.055	0.089	0.084	0.100	0.079	0.067	0.089	0.088	0.067	0.112	0.073	0.061	0.105	nd	nd	nd
	Ita	0.830	0.793	0.906	1.070	0.644	0.738	0.999	0.830	0.824	1.036	0.782	0.635	1.018	nd	nd	nd
ethyl acetate	Tun	0.130	0.122	0.111	0.116	0.117	0.119	0.106	0.118	0.121	0.140	0.136	0.133	0.163	<0.095	<0.095	nd
	Ita	0.686	0.643	0.687	0.788	0.611	0.578	0.777	0.573	0.506	0.846	0.628	0.656	0.964	<0.095	<0.095	nd
methanol	Tun	3.443	3.383	3.164	3.588	2.905	2.186	2.431	3.899	2.915	3.875	3.063	2.258	2.949	<0.184	<0.184	<0.184
	Ita	6.957	4.175	6.825	7.333	5.423	5.133	5.694	6.140	4.612	7.754	5.826	4.741	5.722	<0.184	<0.184	<0.184
butan-2-one	Tun	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	0.024	0.037	0.095	0.040	0.129
	Ita	nd	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	0.040	0.095	0.037	0.110
methyl propanoate	Tun	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	0.009	0.023	0.031	0.131	0.010	<0.008	0.023
	Ita	<0.008	<0.008	<0.008	0.009	<0.008	<0.008	<0.008	<0.008	<0.008	0.026	0.026	0.013	0.566	0.014	0.012	0.244
2-methylbutanal	Tun	0.059	0.054	0.045	0.045	0.043	0.045	0.040	0.045	0.052	0.062	0.044	0.043	0.047	<0.007	<0.007	0.012
	Ita	0.048	0.075	0.056	0.043	0.043	0.044	0.061	0.048	0.047	0.050	0.045	0.044	0.059	<0.007	<0.007	0.009
isovaleraldehyde	Tun	0.040	0.043	0.037	0.032	0.032	0.033	0.024	0.033	0.035	0.043	0.028	0.028	0.033	nd	nd	nd
	Ita	0.023	0.029	0.029	0.025	0.022	0.020	0.021	0.027	0.023	0.023	0.019	<0.019	0.022	nd	nd	nd
ethanol	Tun	3.641	4.254	3.639	4.156	3.934	3.362	4.044	4.283	3.475	4.724	3.756	3.002	4.173	<0.185	<0.185	<0.185
	Ita	2.982	2.899	2.481	3.241	2.442	2.511	2.828	2.508	2.117	3.031	2.436	2.329	2.983	<0.185	nd	<0.185
ethyl propanoate	Tun	nd	nd	<0.010	<0.010	nd	nd	nd	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	nd	nd	nd
	Ita	<0.010	nd	<0.010	nd	nd	<0.010	<0.010	<0.010	nd	<0.010	<0.010	<0.010	<0.010	nd	nd	nd
pentan-3-one	Tun	0.160	0.173	0.187	0.190	0.171	0.162	0.178	0.175	0.153	0.210	0.166	0.162	0.288	0.024	0.009	0.027
	Ita	0.980	0.928	0.945	1.043	0.842	0.906	1.085	0.805	0.724	1.159	0.902	0.911	1.238	0.055	0.013	0.017
pentanal	Tun	0.154	0.188	0.181	0.216	0.170	0.196	0.203	0.183	0.185	0.276	0.214	0.251	0.480	0.118	0.051	0.143
	Ita	0.082	0.085	0.093	0.150	0.097	0.129	0.175	0.114	0.115	0.253	0.367	0.423	0.628	0.228	0.130	0.095
butan-2-ol	Tun	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
pent-1-en-3-one	Tun	0.069	0.072	0.051	0.056	0.058	0.058	0.053	0.064	0.065	0.078	0.062	0.068	0.072	0.012	0.008	0.016
	Ita	0.121	0.228	0.166	0.127	0.130	0.120	0.159	0.162	0.136	0.141	0.152	0.137	0.220	0.019	0.010	0.019
propanol	Tun	nd	<0.063	nd	<0.063	nd	nd	nd	<0.063	nd	<0.063	nd	nd	nd	nd	nd	nd
	Ita	nd	<0.063	nd	nd	nd	nd	<0.063	nd	nd	nd	nd	nd	nd	nd	nd	nd
ethyl butanoate	Tun	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
butyl acetate	Tun	nd	nd	nd	nd	nd	nd	0.052	nd	nd	0.088	nd	nd	0.065	<0.017	<0.017	<0.017
	Ita	nd	nd	nd	nd	nd	nd	0.039	nd	nd	nd	nd	nd	0.030	<0.017	<0.017	<0.017
hexanal	Tun	1.095	1.492	1.427	1.820	1.524	1.404	1.750	2.487	2.421	2.837	1.218	1.343	2.282	0.630	0.429	1.462
	Ita	1.469	1.395	1.545	1.924	1.466	1.833	2.248	1.784	1.715	2.134	0.916	1.462	1.234	0.364	0.386	0.417
isobutanol	Tun	<0.012	0.028	0.025	0.033	0.031	0.024	0.027	0.033	0.031	0.037	0.026	0.024	0.034	0.015	<0.012	0.019
	Ita	0.014	0.038	0.041	0.054	0.041	0.039	0.033	0.048	0.046	0.048	0.025	0.057	0.033	0.016	<0.012	<0.012
pentan-2-ol	Tun	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.005	nd	<0.005	<0.005	nd	nd	nd

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 2. continued

			D			DO_{HS}			L			LO_{HS}			LO_{OB}		
		T0	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.005	nd	<0.005	<0.005	nd	nd	nd
E-2-pentenal	Tun	0.026	0.028	0.025	0.027	0.026	0.024	0.024	0.029	0.030	0.031	0.029	0.026	0.032	0.006	nd	0.009
	Ita	0.053	0.062	0.064	0.069	0.060	0.062	0.070	0.071	0.073	0.097	0.057	0.068	0.086	0.011	<0.004	<0.004
pent-1-en-3-ol	Tun	0.227	0.217	0.197	0.225	0.211	0.206	0.224	0.213	0.210	0.254	0.215	0.207	0.246	nd	nd	nd
	Ita	0.594	0.592	0.585	0.661	0.586	0.594	0.680	0.615	0.587	0.701	0.584	0.595	0.700	<0.168	nd	nd
heptan-2-one	Tun	nd	<0.044	nd	<0.044	nd	nd	<0.044	nd	nd	<0.044	<0.044	<0.044	<0.044	nd	nd	nd
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
heptanal	Tun	0.044	0.052	0.045	0.061	0.049	0.048	0.052	0.146	0.156	0.192	0.046	0.062	0.094	0.039	0.036	0.098
	Ita	0.013	0.009	0.012	0.013	0.008	0.012	0.015	0.030	0.055	0.102	0.011	0.020	0.037	0.016	0.023	0.050
limonene	Tun	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	<0.121	0.229
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.121	<0.121	0.255
Z-3-hexenal	Tun	0.074	0.068	0.061	0.067	0.067	0.065	0.067	0.067	0.064	0.074	0.068	0.063	0.069	0.018	nd	nd
	Ita	0.076	0.075	0.072	0.080	0.073	0.074	0.083	0.078	0.073	0.084	0.074	0.074	0.081	0.018	nd	nd
2-methylbutanol + 3-methylbutanol	Tun	0.145	0.135	0.113	0.117	0.132	0.123	0.119	0.144	0.136	0.157	0.135	0.131	0.154	<0.093	nd	nd
	Ita	0.383	0.386	0.383	0.390	0.385	0.399	0.420	0.420	0.411	0.501	0.402	0.381	0.466	0.131	nd	nd
E-2 hexenal	Tun	8.934	8.707	7.620	8.808	8.406	8.053	8.505	8.628	8.175	9.477	8.594	8.080	9.030	3.825	<1.054	nd
	Ita	20.823	20.255	20.093	22.064	20.691	20.947	23.365	21.870	20.740	24.043	20.973	20.587	23.861	8.935	2.264	nd
pentanol	Tun	0.024	0.025	0.022	0.027	0.025	0.023	0.025	0.025	0.025	0.031	0.026	0.025	0.037	0.013	0.006	0.021
	Ita	0.051	0.049	0.051	0.056	0.051	0.053	0.057	0.056	0.050	0.059	0.054	0.053	0.057	0.023	0.007	0.011
hexyl acetate	Tun	0.158	0.169	0.146	0.183	0.166	0.154	0.169	0.175	0.159	0.190	0.175	0.159	0.188	0.125	0.083	0.034
	Ita	0.053	0.050	0.053	0.059	0.050	0.052	0.060	0.058	0.054	0.063	0.053	0.053	0.063	0.036	0.025	0.010
octan-2-one	Tun	0.017	0.019	0.016	0.019	0.018	0.017	0.018	0.017	0.015	0.020	0.017	0.017	0.019	0.016	0.012	0.007
	Ita	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006
octanal	Tun	0.033	0.045	0.036	0.058	0.041	0.036	0.048	0.043	0.043	0.070	0.030	0.033	0.083	0.036	0.036	0.151
	Ita	<0.029	<0.029	<0.029	0.031	<0.029	<0.029	0.029	<0.029	<0.029	0.047	<0.029	<0.029	0.049	<0.029	0.043	0.133
oct-1-en-3-one	Tun	<0.004	<0.004	<0.004	0.004	<0.004	<0.004	0.004	0.004	0.004	0.006	0.006	0.011	0.025	0.007	0.019	0.237
	Ita	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	0.005	0.012	<0.004	0.007	0.072
E-2-pentenol	Tun	0.028	0.029	0.025	0.028	0.028	0.026	0.027	0.029	0.027	0.031	0.029	0.027	0.030	0.011	<0.004	nd
	Ita	0.065	0.066	0.067	0.073	0.067	0.067	0.073	0.070	0.065	0.079	0.068	0.066	0.075	0.022	0.005	nd
Z-2-pentenol	Tun	0.011	0.010	0.008	0.011	0.010	0.010	0.010	0.010	0.011	0.011	0.010	0.009	0.011	<0.008	<0.008	nd
	Ita	0.017	0.018	0.018	0.019	0.017	0.018	0.019	0.018	0.017	0.019	0.018	0.017	0.017	0.008	nd	nd
heptan-2-ol	Tun	0.115	0.116	0.102	0.116	0.112	0.108	0.110	0.115	0.109	0.123	0.113	0.106	0.115	0.043	0.012	nd
	Ita	0.347	0.357	0.355	0.384	0.356	0.357	0.390	0.369	0.342	0.395	0.359	0.345	0.383	0.121	0.027	nd
Z-3-hexenyl acetate	Tun	0.587	0.528	0.472	0.593	0.552	0.515	0.562	0.577	0.539	0.633	0.580	0.524	0.629	0.417	0.278	0.093
	Ita	0.158	0.153	0.164	0.179	0.157	0.163	0.187	0.176	0.168	0.193	0.165	0.157	0.197	0.112	0.073	0.023
E-2-heptenal	Tun	<0.107	0.118	0.112	0.161	0.116	0.121	0.165	0.224	0.254	0.391	0.297	0.598	1.200	0.212	0.422	4.684
	Ita	<0.107	<0.107	<0.107	<0.107	<0.107	<0.107	<0.107	<0.107	<0.107	0.241	<0.107	0.231	0.801	<0.107	0.179	1.697
E-2-hexenyl acetate	Tun	0.006	0.005	0.005	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.005	0.006	0.005	0.004	nd
	Ita	0.025	0.025	0.025	0.029	0.023	0.026	0.028	0.027	0.026	0.029	0.026	0.026	0.030	0.017	0.012	0.005
6-methylhept-5-en-2-one	Tun	0.019	0.018	0.014	0.018	0.015	0.018	0.023	0.014	0.016	0.020	0.025	0.056	0.051	0.083	0.213	0.151
	Ita	0.029	0.027	0.031	0.018	0.027	0.034	0.024	0.021	0.025	0.024	0.148	0.315	0.075	0.404	1.400	0.255

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DOI: 10.1021/acs.jafc.9b05809

Table 2. continued

			D			DO_{HS}			L			LO_{HS}			LO_{OB}		
		T0	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks
hexanol	Tun	0.570	0.557	0.482	0.544	0.541	0.513	0.522	0.564	0.528	0.580	0.558	0.510	0.554	0.365	0.201	0.063
	Ita	3.023	2.984	2.959	3.190	3.009	3.028	3.323	3.195	2.983	3.358	3.041	2.977	3.299	1.881	0.995	0.121
E-3-hexenol	Tun	0.146	0.015	0.013	0.013	0.014	0.118	0.121	0.016	0.014	0.138	0.010	0.121	0.122	0.008	<0.005	<0.005
	Ita	0.238	0.041	0.042	0.044	0.042	0.042	0.045	0.045	0.043	0.049	0.044	0.042	0.046	0.020	0.010	nd
Z-3-hexenol	Tun	0.334	0.325	0.287	0.329	0.319	0.306	0.317	0.324	0.307	0.347	0.327	0.303	0.332	0.192	0.088	<0.016
	Ita	0.544	0.538	0.540	0.587	0.542	0.550	0.606	0.574	0.539	0.619	0.549	0.539	0.606	0.295	0.126	<0.016
nonan-2-one	Tun	nd	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.007
nonanal	Tun	0.366	0.485	0.403	0.486	0.291	0.405	0.482	1.341	1.360	1.867	0.306	0.502	0.837	0.247	0.341	1.229
	Ita	0.274	0.338	0.404	0.447	0.266	0.424	0.482	1.015	1.795	3.339	0.196	0.577	1.255	0.348	0.415	1.042
E-2-hexenol	Tun	0.773	0.768	0.676	0.770	0.747	0.713	0.744	0.782	0.739	0.837	0.765	0.710	0.762	0.547	0.346	0.347
	Ita	3.397	3.305	3.275	3.573	3.402	3.428	3.813	3.613	3.392	3.904	3.432	3.385	3.844	2.123	1.120	0.334
Z-2-hexenol	Tun	nd	nd	nd	nd	nd	nd	nd	0.221	nd	nd	nd	nd	nd	0.149	nd	nd
	Ita	<0.078	<0.078	<0.078	<0.078	1.010	<0.078	<0.078	1.109	<0.078	<0.078	1.037	<0.078	<0.078	0.635	nd	nd
E,*E*-hexa-2,4-dienal	Tun	0.150	0.147	0.119	0.123	0.134	0.127	0.108	0.122	0.122	0.121	0.117	0.119	0.109	<0.091	nd	nd
	Ita	0.285	0.318	0.270	0.274	0.264	0.246	0.254	0.251	0.243	0.294	0.257	0.244	0.278	0.128	<0.091	nd
octan-2-ol	Tun	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
E-2-octenal	Tun	0.018	0.022	0.019	0.024	0.019	0.017	0.023	0.027	0.028	0.048	0.027	0.041	0.152	0.039	0.059	0.345
	Ita	<0.007	<0.007	<0.007	0.008	<0.007	<0.007	<0.007	0.008	0.012	0.027	0.008	0.019	0.071	0.015	0.048	0.163
oct-1-en-3-ol	Tun	0.023	0.022	0.020	0.026	0.022	0.021	0.026	0.024	0.024	0.032	0.032	0.039	0.093	0.031	0.042	1.567
	Ita	0.014	0.013	0.014	0.017	0.013	0.014	0.016	0.015	0.016	0.020	0.017	0.026	0.064	0.017	0.028	0.402
heptanol	Tun	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024
	Ita	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024	<0.024
acetic acid	Tun	1.360	1.340	1.274	2.021	1.244	1.094	1.348	1.298	1.319	1.626	1.619	2.042	1.467	0.665	0.353	0.452
	Ita	7.024	10.304	3.950	5.927	6.248	7.119	6.379	5.521	7.888	8.813	4.882	6.788	4.887	2.567	0.242	0.526
decanal	Tun	<0.028	0.029	<0.028	0.048	<0.028	<0.028	0.036	0.047	0.055	0.126	0.030	0.057	0.128	0.036	<0.028	0.154
	Ita	0.050	0.073	0.056	0.100	0.059	0.056	0.094	0.096	0.116	0.297	0.044	0.100	0.180	0.052	0.042	0.238
E,*E*-hepta-2,4-dienal	Tun	0.026	0.024	0.026	0.031	0.028	0.025	0.032	0.055	0.061	0.103	0.033	0.050	0.096	0.032	0.029	0.118
	Ita	0.059	0.060	0.061	0.076	0.060	0.056	0.083	0.101	0.127	0.315	0.060	0.088	0.214	0.063	0.064	0.124
benzaldehyde	Tun	0.012	0.012	0.007	0.015	0.010	<0.007	0.012	0.012	0.007	0.015	0.010	0.010	0.014	0.024	0.029	0.044
	Ita	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007	0.008	0.008	<0.007	0.011	<0.007	<0.007	0.012	0.022	0.032	0.042
E-2-nonenal	Tun	0.025	0.026	0.027	0.032	0.031	0.025	0.035	0.047	0.039	0.048	0.023	0.034	0.049	0.027	0.031	0.085
	Ita	<0.019	nd	<0.019	nd	<0.019	nd	nd	<0.019	<0.019	0.028	<0.019	<0.019	0.022	<0.019	<0.019	0.049
octanol	Tun	0.044	0.039	0.034	0.041	0.042	0.035	0.038	0.041	0.037	0.047	0.041	0.036	0.049	0.037	0.029	0.106
	Ita	0.034	0.034	0.036	0.034	0.034	0.033	0.036	0.040	0.038	0.041	0.034	0.036	0.046	0.030	0.028	0.059
propanoic acid	Tun	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	<0.080	0.093	nd	nd	<0.080
	Ita	<0.080	<0.080	<0.080	0.096	<0.080	<0.080	0.103	<0.080	<0.080	0.112	<0.080	<0.080	0.141	<0.080	nd	<0.080
butanoic acid	Tun	nd	nd	nd	<0.161	nd	nd	<0.161	nd	nd	<0.161	nd	nd	<0.161	nd	nd	nd
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
E-2-decenal	Tun	0.234	0.252	0.204	0.234	0.254	0.203	0.218	0.300	0.275	0.402	0.257	0.372	0.606	0.156	0.244	1.338

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DOI: 10.1021/acs.jafc.9b05809

Table 2. continued

			D			DO_{HS}			L			LO_{HS}			LO_{OB}		
		T0	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks	3 weeks	8 weeks	26 weeks
	Ita	0.173	0.190	0.179	0.185	0.182	0.188	0.212	0.253	0.264	0.520	0.181	0.380	1.240	0.206	0.282	1.711
nonanol	Tun	0.052	0.052	0.048	0.050	0.052	0.046	0.050	0.060	0.052	0.068	0.055	0.060	0.083	0.035	0.040	0.164
	Ita	0.047	0.043	0.048	0.048	0.046	0.048	0.050	0.058	0.058	0.086	0.045	0.063	0.144	0.041	0.044	0.130
E,E-nona-2,4-dienal	Tun	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	0.029	0.020	0.034	<0.020	<0.020	<0.020	<0.020	<0.020	0.033
	Ita	nd	nd	<0.020	nd	<0.020	nd	nd	<0.020	<0.020	0.025	nd	<0.020	<0.020	nd	nd	<0.020
pentanoic acid	Tun	<0.022	nd	<0.022	0.055	<0.022	<0.022	0.025	<0.022	<0.022	0.022	<0.022	<0.022	0.056	0.034	0.038	0.056
	Ita	0.025	0.060	0.026	0.025	0.036	0.025	0.024	0.034	0.023	0.029	0.051	0.049	0.067	0.075	0.071	0.085
E,E-deca-2,4-dienal	Tun	0.173	0.120	0.133	0.135	0.131	0.115	0.129	0.326	0.374	0.605	0.181	0.228	0.390	0.165	0.147	0.319
	Ita	0.038	0.020	0.035	0.051	0.033	0.032	0.040	0.177	0.303	0.860	0.039	0.091	0.453	0.041	0.051	0.214
hexanoic acid	Tun	0.107	0.223	0.079	0.447	0.200	0.141	0.200	0.133	0.165	0.270	0.156	0.172	0.339	0.154	0.194	0.712
	Ita	0.155	0.361	0.222	0.255	0.253	0.209	0.235	0.216	0.283	0.258	0.251	0.237	0.236	0.239	0.235	0.378
guaiacol	Tun	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034	<0.034
	Ita	0.040	0.039	0.039	0.043	0.040	0.041	0.041	0.037	0.041	0.041	0.042	0.042	0.041	0.038	0.039	0.037
phenylethanol	Tun	0.235	0.269	0.139	0.253	0.247	0.223	0.234	0.172	0.223	0.215	0.245	0.234	0.218	0.173	0.189	0.122
	Ita	0.329	0.327	0.304	0.431	0.327	0.352	0.365	0.246	0.329	0.357	0.380	0.355	0.335	0.317	0.310	0.236
phenol	Tun	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ita	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.097
4-ethylguaiacol	Tun	0.023	0.026	0.018	0.024	0.021	0.020	0.025	0.019	0.020	0.023	0.022	0.021	0.023	0.016	0.021	0.019
	Ita	0.020	0.019	0.018	0.020	0.019	0.020	0.022	0.018	0.018	0.021	0.019	0.019	0.021	0.017	0.019	0.019
4-ethylphenol	Tun	0.158	0.184	0.105	0.197	0.168	0.141	0.168	0.125	0.152	0.184	0.169	0.130	0.170	0.113	0.149	0.123
	Ita	0.058	0.045	0.052	0.070	0.050	0.055	0.058	0.045	0.047	0.052	0.050	0.051	0.056	0.059	0.053	0.050
total VOCs	Tun	24.651	25.499	22.429	27.228	23.969	21.892	24.476	28.803	26.200	33.011	24.710	24.048	30.561	9.872	5.985	15.795
	Ita	52.982	52.848	48.611	55.690	50.529	51.186	56.242	54.211	53.259	68.134	50.116	51.245	60.929	20.710	9.460	10.158
total LOX	Tun	12.885	12.970	11.477	13.461	12.655	12.047	12.975	13.950	13.273	15.380	12.631	12.028	14.237	6.188	2.502	2.228
	Ita	30.392	29.721	29.601	32.605	30.251	30.933	34.687	32.285	30.542	35.434	30.081	30.117	34.282	13.910	5.027	1.084

[a]nd, not detected (i.e., the VOC was below the LOD). Identification of each quantitated VOC was based on injection of authentic standards and by comparison with spectra from mass spectral database (NIST08/Wiley98). Ita, Italian EVOO; Tun, Tunisian EVOO; D, dark; O, oxygen; L, light; HS, headspace; OB, open bottle.

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 3. Evolution Over Time of the Sensory Attributes of the Two Extra-Virgin Olive Oils Stored in Several Conditions[a]

	It-EVOO	fruity	bitterness	pungency	rancid	score		Tun-EVOO	fruity	bitterness	pungency	rancid	score
ID	t0	4.5	3.8	5.4	0.0	7.0	TD	t0	3.4	1.9	2.8	0.0	7.0
ID	t1 (3 weeks)	3.0	3.0	3.5	0.0	6.8	TD	t1 (3 weeks)	1.6	1.4	2.2	0.8	6.2
ID	t1 (8 weeks)	3.5	3.2	3.4	0.0	6.5	TD	t1 (8 weeks)	2.0	1.2	1.3	0.5	6.0
ID	t1 (26 weeks)	4.0	3.8	3.6	0.0	6.5	TD	t1 (26 weeks)	2.2	1.0	0.5	0.5	5.5
IDO_{HS}	t0	4.5	3.8	5.4	0.0	7.0	TDO_{HS}	t0	3.4	1.9	2.8	0.0	7.0
IDO_{HS}	t1 (3 weeks)	3.4	3.0	3.7	0.4	6.4	TDO_{HS}	t1 (3 weeks)	1.5	1.5	2.0	0.3	6.2
IDO_{HS}	t1 (8 weeks)	3.6	3.3	3.9	0.0	6.6	TDO_{HS}	t1 (8 weeks)	1.8	1.0	1.3	0.5	5.8
IDO_{HS}	t1 (26 weeks)	4.2	3.6	4.0	0.0	6.7	TDO_{HS}	t1 (26 weeks)	2.0	0.8	0.5	1.0	5.5
IL	t0	4.5	3.8	5.4	0.0	7.0	TL	t0	3.4	1.9	2.8	0.0	7.0
IL	t1 (3 weeks)	3.2	2.8	3.8	0.0	6.7	TL	t1 (3 weeks)	1.4	1.0	1.4	1.0	6.0
IL	t1 (8 weeks)	2.0	1.5	1.5	2.3	5.5	TL	t1 (8 weeks)	1.0	0.8	1.0	2.0	5.0
IL	t1 (26 weeks)	0.0	0.0	0.0	5.0	4.5	TL	t1 (26 weeks)	0.0	0.0	0.0	4.0	4.5
ILO_{HS}	t0	4.5	3.8	5.4	0.0	7.0	TLO_{HS}	t0	3.4	1.9	2.8	0.0	7.0
ILO_{HS}	t1 (3 weeks)	2.5	2.2	2.4	1.6	5.8	TLO_{HS}	t1 (3 weeks)	0.8	0.6	1.0	2.6	5.3
ILO_{HS}	t1 (8 weeks)	1.0	0.8	1.1	2.5	5.0	TLO_{HS}	t1 (8 weeks)	0.5	0.5	0.5	3.0	5.0
ILO_{HS}	t1 (26 weeks)	0.0	0.0	0.0	3.8	4.5	TLO_{HS}	t1 (26 weeks)	0.0	0.0	0.0	4.0	4.5
ILO_{OB}	t0	4.5	3.8	5.4	0.0	7.0	TLO_{OB}	t0	3.4	1.9	2.8	0.0	7.0
ILO_{OB}	t1 (3 weeks)	1.8	1.8	1.3	2.6	5.0	TLO_{OB}	t1 (3 weeks)	1.2	1.1	1.4	1.8	5.7
ILO_{OB}	t1 (8 weeks)	0.5	3.5	0.5	3.0	5.0	TLO_{OB}	t1 (8 weeks)	0.5	3.0	0.5	2.0	5.0
ILO_{OB}	t1 (26 weeks)	0.0	7.9	0.0	3.5	4.5	TLO_{OB}	t1 (26 weeks)	0.0	7.9	0.0	3.5	4.5

[a]I, Italian EVOO; T, Tunisian EVOO; D, dark; O, oxygen; L, light; HS, headspace; OB, open bottle.

MeOH:H_2O 80:20 solution and the chromatographic analysis of the obtained extracts was immediately performed using an HP1200 liquid chromatograph (Agilent Technologies, California). A Sphereclone (Phenomenex, Torrance, CA) RP-18, 5 μm, 250 mm × 4.6 mm id column was used. The gradient reported in the IOC method, using acid H_2O (pH 3.2 HCOOH)/CH_3CN/MeOH, was used for the elution, and chromatograms were registered at 280 nm. Syringic acid and tyrosol were used as internal standard and reference compound, respectively, for quantitation, and the results were expressed as mg_{tyr}/kg_{oil}.

HS-SPME-GC−MS Analysis of Volatile Organic Compounds. The volatile fraction of all samples was analyzed by HS-SPME-GC−MS, using the method proposed by Fortini et al.[26] with slight modifications.[31] Briefly, 4.3 g of sample and 0.1 g of internal standard mix solution were weighed into 20 mL screw cap vials. An SPME fiber 50/30 μm DVB/CAR/PDMS by Agilent (Palo Alto, CA) was exposed for 20 min under orbital shaking at 400 rpm in the vial headspace after sample equilibration for 5 min at 45 °C. The sample was then desorbed for 1.7 min in the injection port of a 6890N GC system equipped with an MS detector, model 5975 (Agilent, Palo Alto, CA), then a fiber backout of 20 min at 260 °C was carried out in a backout unit. An HP-Innowax capillary column of 50 m × 0.2 mm i.d. and 0.4 μm film thickness was employed. The initial oven temperature was kept at 40 °C for 2 min, raised to 156 °C with 4 °C/min gradient, and then to 260 °C with 10 °C/min gradient. The carrier gas was helium at 1.2 mL/min, the temperature of the ion source was 230 °C, and that of the transfer line was 250 °C. Mass detector was set to work in scan mode within the range of 30−350 Th, 1500 Th/s, 70 eV IE energy. Injection of authentic standards (purities are given in Table S1) and comparison of mass spectra (mass spectral database NIST08/Wiley98) and retention times allowed confirming identification of each VOC.

Quantitative analysis of each VOC was carried out using 72 six-point linear least-squares calibration line, with two VOCs, namely, 2-methylbutanol + 3-methylbutanol, which coeluted and were evaluated together. For each VOC, the more suitable ISTD was selected (Table S1 of the Supporting Information) and a calibration line was built plotting the area ratio (analyte peak area over the relative ISTD) versus the amount ratio (analyte amount over ISTD amount). To avoid wrong quantification given by variation of instrumental responses in different days, the 72 calibration lines were rebuilt using the same standard solutions for each analytical sequence carried out on different working days. Furthermore, the repeatability of instrumental response on different days was verified preparing and analyzing, within 2 months, six replicates of a six-level matrix-matched calibration curve. Finally, the same approach described in previous papers[26,31] was applied to validate the method in the Carapelli chemical laboratory, using the following parameters of validation for each quantitated VOC: limit of quantification (LOQ), limit of detection (LOD), linearity (R_{adj}^2 and range of linear calibration), accuracy (trueness and precision), sensitivity, and selectivity.

Sensory Analysis. Samples were assessed according to EEC 2568/91,[27] by a panel that consisted of a panel leader and at least eight trained tasters, and acknowledged by the Italian Ministry of Agricultural Policies (MIPAAF). Each taster smelt and tasted the sample and marked the intensity of negative (rancid, fusty/muddy, musty/humid, winey/vinegary, other) and positive (fruity, bitter, pungent) attributes on a 10 cm unstructured line. The samples resulted extra-virgin olive oil (EVOO) if the median of the defects was 0, and the median for fruity notes was >0; virgin olive oil (VOO) if median of the defects was >0 and <3.5, and the median for fruity was >0; lampante virgin olive oil (LVOO) if the median of the defects was >3.5 and/or the median of the defects was <3.5 and the median for fruity was 0.

Statistical Analysis. All analyses were carried out in triplicate, and the quantitative data have been expressed as mean ± standard deviation. Detailed quantitative data of VOCs have been reported in Table 2 taking into account the parameters gathered during method validation: when the obtained values were below the LOD, the VOC was considered not detected, while when the values were between the LOD and the LOQ, the VOC was given as <LOQ. Data were statistically analyzed by one-way analysis of variance and F-test ($p < 0.05$) using Microsoft Excel statistical software and, when significant differences between the means emerged, Fisher's LSD test was applied using DSAASTAT software v. 1.1 (Onofri, Pisa, 2007).

RESULTS AND DISCUSSION

Characterization of Samples at Time 0. Table 1 shows the fatty acid composition of the selected EVOOs: the Tun-EVOO was characterized by a higher percentage of saturated fatty acids and a lower percentage of MUFAs than the It-EVOO (20.5 vs 13.0% and 79.5 vs 60.0%, respectively). The percentage of PUFAs, the fatty acids more susceptible to

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

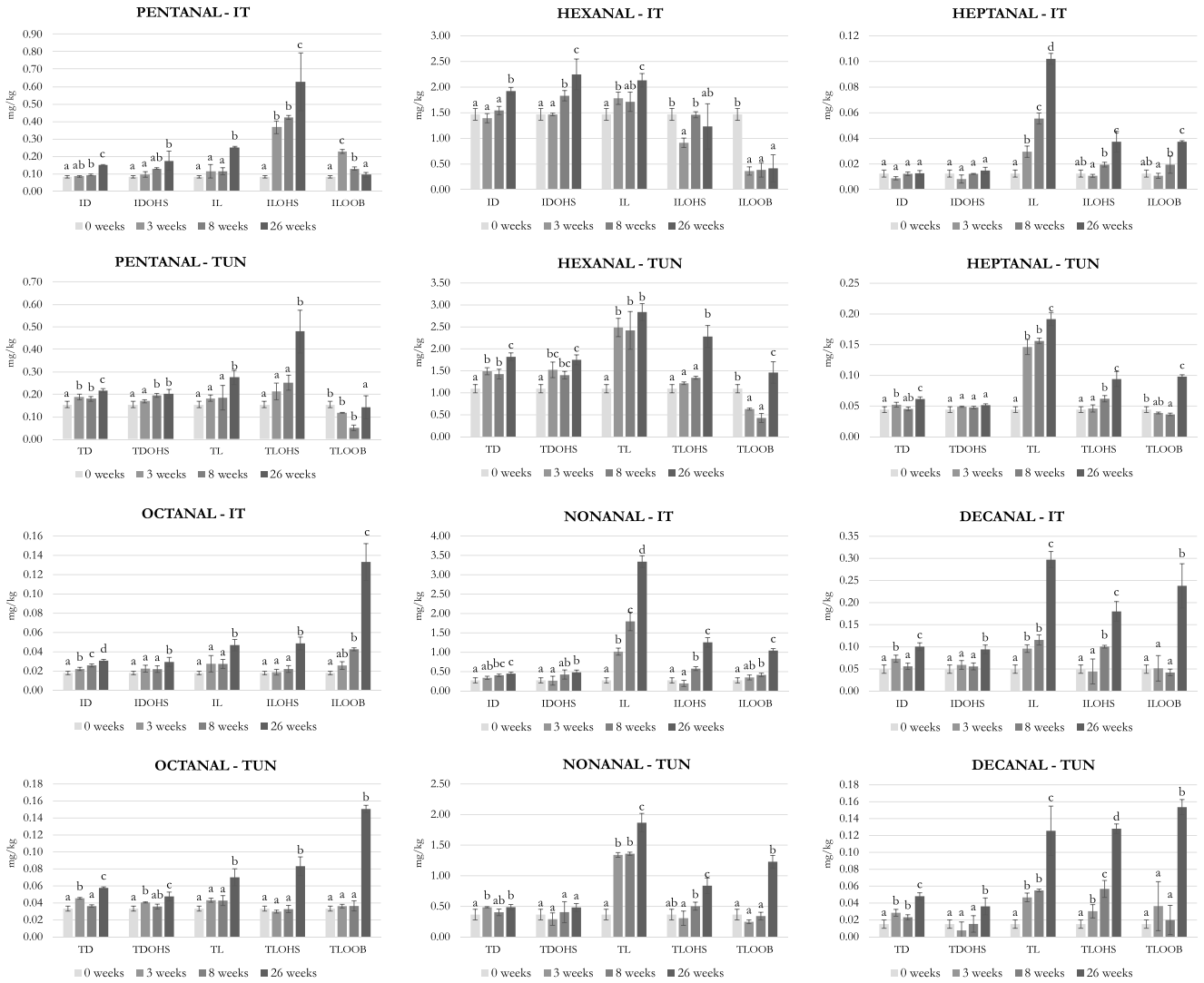

Figure 1. Evolution of the content of volatile saturated aldehydes (C5−C10) over 6 months of storage in different conditions. For each molecule, the upper and lower charts show the evolution in Italian and Tunisian samples, respectively, both over five different storage conditions. All of the measurements are the mean of three determinations, and data are expressed as mg/kg. For each series of data, the different letters over the bars indicate significant differences at $p < 0.05$. I, Italian EVOO; T, Tunisian EVOO; D, dark; O, oxygen; L, light; HS, headspace; OB, open bottle.

autoxidation, was approximately 3 times higher in Tun-EVOO than in It-EVOO (19.3 vs 7.1%), with this difference mainly due to linoleic acid, while linolenic acid was at a very similar content (0.8 vs 0.7%) and within the limit for EVOO category. The choice of these two EVOOs was not aimed to represent the mean fatty acid composition of virgin olive oils but rather to work on samples characterized by extreme composition in terms of fatty acids.

The content of free fatty acids (0.27 ± 0.01% for It-EVOO and 0.25 ± 0.01% for Tun-EVOO), peroxide values (Table S3) and spectrophotometric indices (Table S4) were within the limits for the extra-virgin category. For both the samples, the panel test (Table 3) scored the median of the defect equal to 0, and the median of fruity greater than 0 (4.5 for It-EVOO and 3.4 for Tun-EVOO); thus, the two samples have been confirmed belonging to the extra-virgin olive oil category.

Table S5 shows that the chlorophylls content was 37.5 mg_{ph}/kg for It-EVOO and 12.2 mg_{ph}/kg for Tun-EVOO, in agreement with the different color of samples, i.e., It-EVOO is characterized by a more intense green color than Tun-EVOO. Also the carotenoids level was higher for It-EVOO than for Tun-EVOO (sum of absorbances, 3.7722 vs 1.4559 mAu). The total phenolic content was 354.3 mg/kg for It-EVOO and 210.1 mg/kg for Tun-EVOO.

Characterization of Samples Over 6 Months of Storage. As expected, no significant increases of peroxide number were observed in the absence of oxygen, with values (Table S3) below the EVOO limit (20 meq_{O2}/kg). With the oxygen in headspace and in the dark, a slight increase of the peroxide number was observed only after 8 weeks; the value remained below the EVOO limit over 26 weeks for the It-EVOO, while, at the same time, it exceeded the limit in a slightly significant manner for the Tun-EVOO. Under exposure to light in the presence of oxygen, the peroxide number was over the EVOO limit already after 3 weeks, for both the oils. Then, after this rapid increase, the values remained almost unchanged during the next weeks in the presence of the only oxygen of headspace, while in open bottle (i.e., oxygen availability was limitless), a very fast increasing rate was observed over all of the 26 weeks, reaching values of almost 500 meq_{O2}/kg.

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

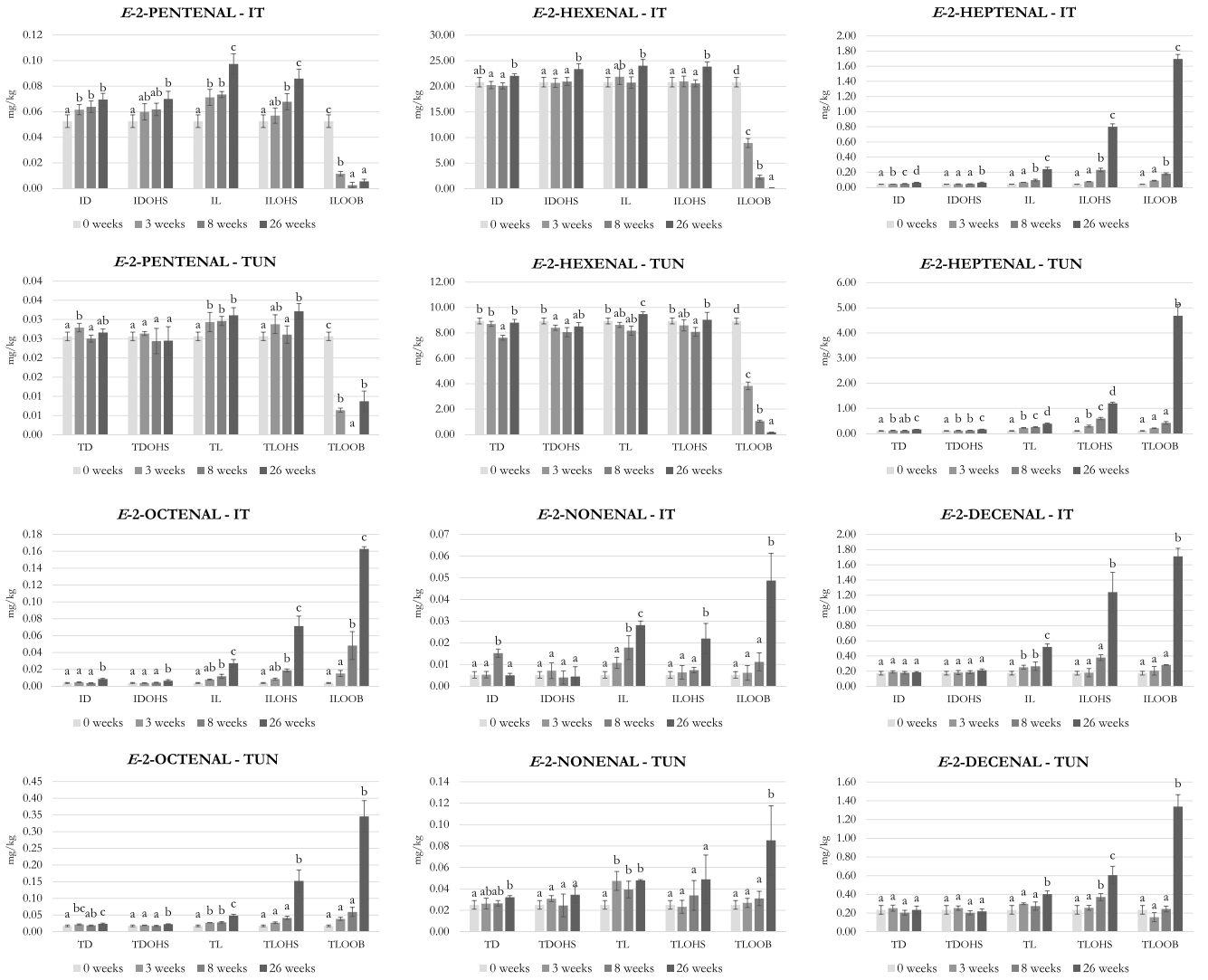

Figure 2. Evolution of the content of volatile monounsaturated aldehydes (C5−C10) over 6 months of storage in different conditions. For each molecule, the upper and lower charts show the evolution in Italian and Tunisian samples, respectively, both over five different storage conditions. All of the measurements are the mean of three determinations, and data are expressed as mg/kg. For each series of data, different letters over the bars indicate significant differences at $p < 0.05$. I, Italian EVOO; T, Tunisian EVOO; D, dark; O, oxygen; L, light; HS, headspace; OB, open bottle.

Table S4 shows the evolution of the spectrophotometric indices: in the absence of oxygen, a slight increase of K_{232} was observed only after 26 weeks; in the presence of oxygen, an increase was observed after 26 weeks for samples stored in the dark, while it was already observed after 3 weeks under exposure to light. It-EVOO significantly exceeded the EVOO limit (i.e., $K_{232} > 2.50$) only when stored in an open bottle, while Tun-EVOO exceeded the limit in all conditions in which oxygen was present.

These observations showed that in noncontrolled and nonaccelerated oxidative conditions, neither the different MUFA/PUFA ratio nor the initial phenolic content (both higher for the It-EVOO) were able to significantly slow down the oxidation processes.

The content of chlorophylls did not change over time in the dark, while, under exposure to light, it quickly decreased (Figure S1). Because this decrease was faster for Tun-EVOO than for It-EVOO and was faster in the absence of oxygen, our data confirmed that chlorophyll degradation is mainly due to light.[14,15] The degradation of carotenoids showed similar trends for both the samples, and it fell down under 50% of the initial value after 26 weeks with samples stored in open bottle under exposure to light. The phenolic compounds content significantly decreased over time only in extreme oxidative conditions (open bottle under exposure to light). This is not surprising, and a similar behavior has already been reported in the literature.[32]

Regarding sensory attributes (Table 3), Italian sample remained extra-virgin (i.e., median of fruity > 0 and median of defect = 0) over the 26 weeks in the dark, while, under exposure to light, it became virgin after 8 weeks in the absence of oxygen, and already after 3 weeks in the presence of oxygen. On the contrary, the Tunisian sample already became virgin after 3 weeks in all conditions, even in the dark. Finally, both the samples became lampante after 26 weeks when stored under exposure to light. Noteworthy, the panelists perceived a very high intensity of the bitterness for the oils stored in open bottle; to the authors' knowledge, this phenomenon has not been reported in the literature to date. We hypothesized that some compounds originating in these extreme but not accelerated oxidative conditions, and different from VOCs

DOI: 10.1021/acs.jafc.9b05809
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

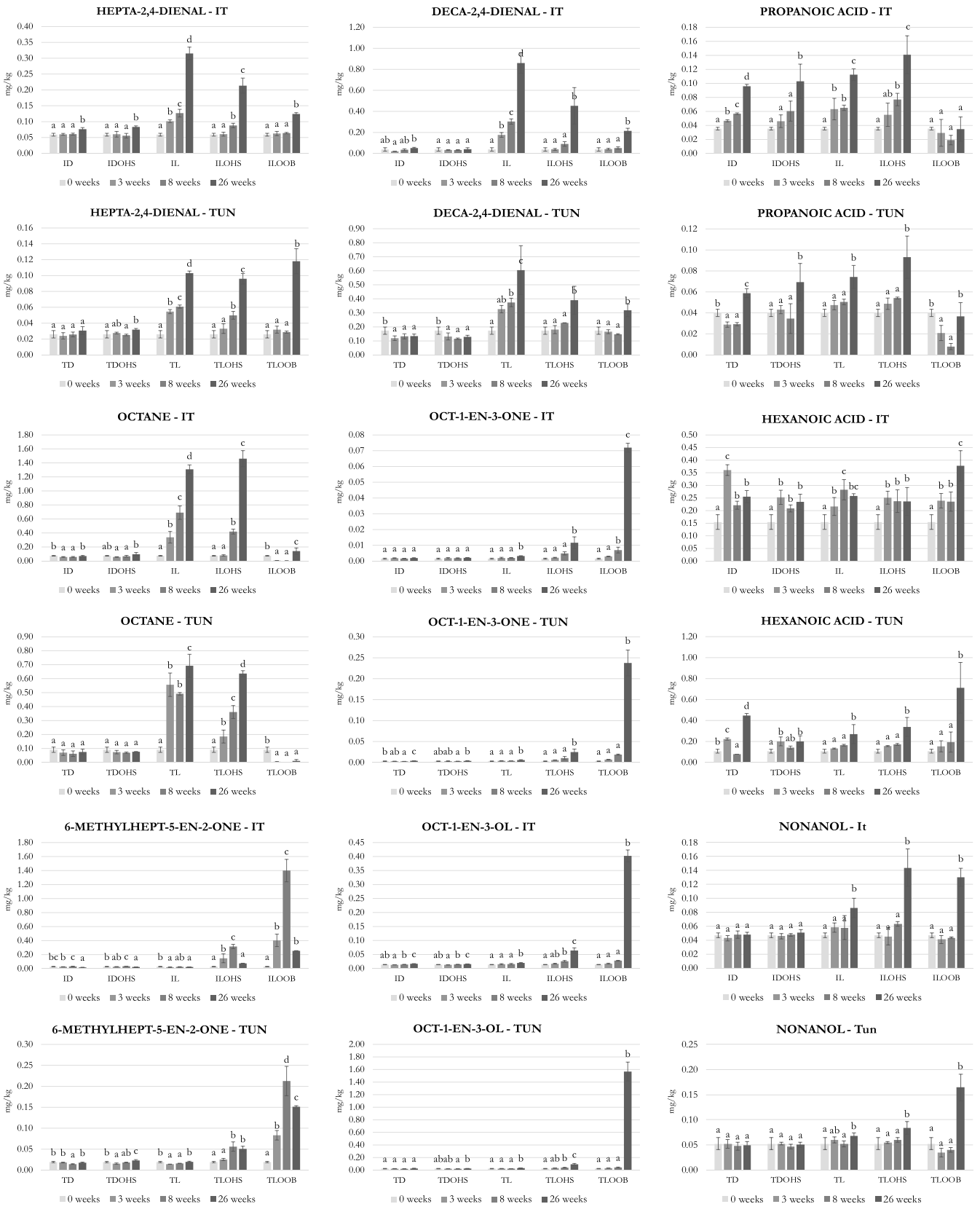

Figure 3. Evolution of the content of other VOCs over 6 months of storage in different conditions. For each molecule, the upper and lower charts show the evolution in Italian and Tunisian samples, respectively, both over five different storage conditions. All of the measurements are the mean of three determinations, and data are expressed as mg/kg. For each series of data, different letters over the bars indicate significant differences at $p < 0.05$. I, Italian EVOO; T, Tunisian EVOO; D, dark; O, oxygen; L, light; HS, headspace; OB, open bottle.

DOI: 10.1021/acs.jafc.9b05809
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detected in headspace, triggered this sharp increase of bitterness in both the oils after 6 months.

Regarding rancidity, It-EVOO never became rancid when stored in the dark, while it became rancid after 8 weeks under light exposure and after 3 weeks under light exposure in the presence of oxygen. Tun-EVOO showed a low level of rancidity already after 3 weeks also when stored in the dark, and this rancidity never exceeded the score of 1.0; under exposure to light, it showed a behavior similar to the It-EVOO, and also for the samples in the absence of oxygen, it become rancid after 3 weeks. These slight differences between the two oils are probably due to the initial higher intensity of the fruity attribute in the It-EVOO, able to partially mask the perception of the rancid defect, and which in turn is due to the higher content of VOCs originated by the LOX pathway (see the next paragraph).

Evolution of Volatile Organic Compounds. The evolution of VOCs mainly affected over storage of samples is shown in Figures 1 and 3, while all quantitative data of the 72 quantitated VOCs are presented in Table 2. The total content of VOCs at time 0 was higher for It-EVOO than for Tun-EVOO (52.982 vs 24.651 mg/kg), and the same was observed for the molecules from the LOX pathway (30.392 vs 12.885 mg/kg, Table 2), data in agreement with the median of the fruity notes of the two samples, higher for the It-EVOO (Table 3). Table 2 also shows that *E*-2-hexenal was the most abundant VOC at time 0 for both the samples, followed by the alcohols methanol, ethanol, and hexanol, by acetic acid, and, mainly for the Italian sample, by *E*-2-hexenol. Butan-2-ol, ethyl butanoate, octan-2-ol, and phenol listed in Table 2 were absent in both the samples stored in all of the different conditions (only phenol was present in amount below the LOQ in the Italian sample stored for 6 months in open bottle).

Aldehydes and Carboxylic Acids. All of the linear saturated aldehydes (C5−C10, Figure 1) were affected by the storage: pentanal (valeraldehyde) increased over time in all of the conditions, except in open bottle, likely due to its volatility. The highest increasing rate was for the samples stored under exposure to light in the presence of oxygen, and it was higher for ILO_{HS} than for TLO_{HS} (increase of approximately 8 times vs 3 times after 6 months, respectively). Hexanal showed a lower increase over time, this increase being maximum for Tun-EVOO stored under exposure to light. Heptanal and nonanal showed very similar trends: very slight changes were observed in the dark, while, under exposure to light, the concentration of these aldehydes increased over time, with the highest increase in the absence of oxygen for IL sample (heptanal increased approximately 8 times, nonanal approximately 12 times). The trends were very similar, but the amounts were very diverse, with nonanal reaching 3.34 mg/kg for IL and heptanal only reaching 0.10 mg/kg in the same sample. For both samples, also decanal showed only a slight increase in the dark and a higher increase under exposure to light, with similar trends in the absence and presence of oxygen and in open bottle. These three aldehydes showed a different behavior from octanal, which showed similar values for It-EVOO and Tun-EVOO over time and an appreciable increase only in extreme conditions in open bottle.

Regarding linear monounsaturated aldehydes (Figure 2), very low amounts (always less than 0.10 mg/kg) of *E*-2-pentenal were observed in all samples, and only slight increases over time for the It-EVOO stored in all conditions, except in open bottle likely because of its high volatility. *E*-2-hexenal, the main VOC originated by the LOX pathway, showed similar amounts in all of the conditions of storage and a fast decrease in open bottle. On the contrary, except *E*-2-nonenal that showed values lower than 0.10 mg/kg over the whole period of storage, all of the C7−C10 linear monounsaturated aldehydes showed significant (and in some cases very strong) increases over time, mainly under exposure to light and in the presence of oxygen. *E*-2-heptenal increased more for It-EVOO (about 6 times for IL and 20 times for ILO_{HS}) than for Tun-EVOO (about 4 times and 10 times for TL and TLO_{HS}), reaching high values for this VOC (approximately 1 mg/kg even in closed bottle), known to have a low odor threshold value.[23] *E*-2-octenal showed a behavior similar to *E*-2-heptenal, but in this case with values in closed bottle never exceeding 0.15 mg/kg. *E*-2-decenal reached values of 1.240 and 0.606 mg/kg in ILO_{HS} and TLO_{HS} samples, respectively, showing a higher increasing rate for the It-EVOO sample. Unlike saturated aldehydes, these three unsaturated aldehydes showed the highest increases under exposure to light in open bottle.

Regarding linear polyunsaturated aldehydes, *E,E*-hexa-2,4-dienal did not increase over time while *E,E*-nona-2,4-dienal never exceeded 0.04 mg/kg (Table 2). On the contrary, *E,E*-hepta-2,4-dienal and, above all, *E,E*-deca-2,4-dienal (Figure 3) strongly increased over time under exposure to light, reaching values up to 0.315 mg/kg (*E,E*-hepta-2,4-dienal) and 0.860 mg/kg (*E,E*-deca-2,4-dienal) in the absence of oxygen after 6 months in It-EVOO. These two polyunsaturated aldehydes showed a behavior quite similar to heptanal and nonanal.

Taking into account the MUFA/PUFA composition of the samples, our findings allowed clarifying the origin of some aldehydes with respect to previous literature, as discussed in the next paragraph.

Propanoic acid (Figure 3) showed only slight increases over time, with values never exceeding 0.14 mg/kg. Hexanoic acid showed a significant increase (up to 0.378 mg/kg) only in open bottle for the It-EVOO, while, for the Tun-EVOO, the increase always became significant after 6 months under exposure to light. Acetic acid and butanoic acid showed no significant increases over time, while pentanoic acid increased in some condition of storage, but its content never exceeded 0.09 mg/kg (Table 2). The slow increasing rate observed for the short-chain fatty acids can be due to the fact that these molecules are products of further degradation, i.e., further oxidation of the corresponding aldehydes or peroxidation of *E,E*-deca-2,4-dienal that originates hexanoic acid.[13]

Role of C8 Compounds in Oxidation. C8 molecules as octane, oct-1-en-3-one, 6-methylhept-5-en-2-one, and oct-1-en-3-ol (Figure 3) increased over time only when samples were stored under exposure to light. Octane strongly increased both in the absence and presence of oxygen but not in open bottle, likely because of its volatility: it increased from 0.074 mg/kg up to 1.461 mg/kg for It-EVOO and from 0.091 mg/kg up to 0.692 mg/kg for Tun-EVOO. Oct-1-en-3-one and oct-1-en-3-ol showed the highest increases in the presence of oxygen and particularly in open bottle. 6-Methylhept-5-en-2-one, a branched unsaturated C8 ketone, showed significant increases only in the presence of oxygen, but in this case, the amounts were greater for It-EVOO than for Tun-EVOO (up to 1.400 vs 0.213 mg/kg); this molecule reached its maximum concentration after 2 months and then decreased. Except for octane, reported to be the main decomposition product from oleic acid hydroperoxides under photo-oxidation conditions together with *E*-2-decenal, the high increase of C8 VOCs was

DOI: 10.1021/acs.jafc.9b05809
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unexpected, in that, to the author knowledge, the most studies in the literature reported they are originated by the activity of microorganisms present in olives.[23,33] On the contrary, regarding the role of C8 compounds in oxidation, only oct-1-en-3-ol has been previously described as a secondary oxidation product of linoleic acid.[12]

The selected oils were filtered, but, since the highest increase was in open bottles, we first hypothesized that, in some way, environmental microorganisms contributed to give rise to these VOCs. In any case, our data demonstrated that different processes originated them since oct-1-en-3-one and oct-1-en-3-ol increased the most in Tun-EVOO while 6-methylhept-5-en-2-one increased the most in It-EVOO even in closed bottles. In this research, working on real EVOO samples stored in nonaccelerated oxidative conditions, the role of C8 molecules in the development of oxidative defect has been pointed out for the first time.

Other VOCs. Nonan-1-ol, previously associated with the rancid defect by some authors,[23,34] showed a significant increase only when the samples were exposed to light, and the increase was more intense in the presence of oxygen. Other VOCs that showed slight increases over time have been heptane, butan-2-one, methyl propanoate, and limonene (Table 2).

Brief Comparison with the Literature. This experiment, performed working on real EVOOs with different MUFA/PUFA ratios stored in nonaccelerated oxidative conditions, pointed out some data in disagreement with other researches carried out working on model systems and/or in accelerated conditions. Particularly, pentanal increased more for It-EVOO, while it was previously reported as a decomposition product of linoleic acid (Table S6).[11,12,35] Moreover, *E*-2-heptenal and *E*-2-octenal, reported as decomposition products of linoleic acid (Table S6),[11,12,35] showed slightly higher contents in Tun-EVOO, but the highest increasing rate was in It-EVOO. *E,E*-hepta-2,4-dienal and *E,E*-deca-2,4-dienal showed greater values and increases for It-EVOO, in disagreement with previous papers that reported these two VOCs as decomposition products of PUFAs (Table S6).[11,12,17]

Markers for Rancidity for Oils Stored in Non-accelerated Conditions. Several papers in the literature report attempts to define one or more volatile markers for rancidity in extra-virgin olive oil.[13,17,35] It is very difficult that a single VOC could be able to indicate the level of rancidity of an oil sample due to several reasons, among which are the very complex plethora of reactions occurring during autoxidation and photo-oxidation, and the even more complex effects that the different VOCs play on the sensory properties of the oxidized oil. As discussed above, the former effect has been also confirmed by our results, which, in some cases, are in disagreement with what previously reported by other authors about the origin of some VOCs (Table S6),[11,12,17,35] while the latter one is due to different odor thresholds of different VOCs and, increasingly important, to the complex and still not well-defined synergistic and antagonist interactions among VOCs.[36] This also results in difficulty in defining the sensory attributes of specific VOCs, as confirmed by the different sensory attributes assigned to a single VOC.[36] Consequently, even though there are some attempts of using groups of VOCs for the aroma characterization of EVOOs,[36] definitive markers or group of markers useful to follow the evolution of specific defects in general, and rancidity in particular, have not yet been defined. Toward this goal, in our opinion, at least the following two conditions are necessary: (i) the availability of a reliable method for quantitation of the EVOO-VOCs; (ii) specific knowledge on the quantitative evolution of the VOCs content in EVOO samples stored in different nonaccelerated oxidative conditions. The method cited in point (i) has been already proposed and validated[26] and used in this paper for addressing point (ii).

Overall, Figures 1 and 3 and Table 2 show that the highest increases of the VOCs of oxidative origin were under exposure to light, confirming light exposure as the main factor affecting the production of these VOCs from both PUFA and MUFA. In the dark, similar trends were observed in the presence and absence of oxygen with only slight increases for saturated aldehydes (pentanal, hexanal, octanal, nonanal, decanal; Figure 1), for some other VOCs as propanoic and hexanoic acids (Figure 3) and, at very negligible extent, for some monounsaturated aldehydes (*E*-2-heptenal, *E*-2-octenal; Figure 2). We also calculated the correlation coefficients between the evolution of rancidity and each single VOC in the dark (this calculation only for Tun-EVOO since It-EVOO did not show any increase of rancidity perceived by the panel test, likely because of the higher level of fruity). Keeping into account the increases shown in Figures 1 and 3 and the highest correlation coefficient among the VOCs that reached amounts higher than 0.05 mg/kg after 26 weeks, the sum of the content of pentanal, hexanal, nonanal, *E*-2-heptenal, propanoic acid, and hexanoic acid is proposed as a marker for measuring the slow evolution of rancidity in VOOs correctly stored in the dark (Table 4, $\sum R_{dark}$).

Table 4. List of the VOCs To Be Used as Markers of Rancidity in Different Storage Conditions[a]

marker	VOCs
$\sum R_{dark}$	pentanal, hexanal, nonanal, *E*-2-heptenal, propanoic acid, hexanoic acid
$\sum R_{light}$	pentanal, heptanal, nonanal, decanal, *E*-2-heptenal, *E*-2-decenal, *E,E*-hepta-2,4-dienal, *E,E*-deca-2,4-dienal, octane
$\sum R_{lightO2}$	pentanal, nonanal, decanal, *E*-2-heptenal, *E*-2-decenal, *E,E*-hepta-2,4-dienal, nonan-1-ol, propanoic acid, octane, 6-methylhept-5-en-2-one, oct-1-en-3-ol
$\sum R$	pentanal, nonanal, *E*-2-heptenal

[a] $\sum R_{Dark}$ for oils stored in the dark, $\sum R_{light}$ for oils stored under light exposure in the absence of O_2, $\sum R_{lightO2}$ for oils stored under light exposure in the presence of O_2 in headspace. $\sum R$ is a simplified marker suitable for all of the oils.

Under light exposure in the absence of oxygen, the VOCs that showed the highest increase have been heptanal, nonanal, decanal, *E*-2-heptenal, *E,E*-hepta-2,4-dienal, *E,E*-deca-2,4-dienal, octane, and, at a lower extent, pentanal and *E*-2-decenal. Again, taking into account these increases, and the highest correlation coefficient among the evolution of rancidity in this condition and the VOCs that reached amounts of at least 0.20 mg/kg after 26 weeks, the sum of the content of these nine VOCs (Table 4, $\sum R_{light}$) results suitable as a marker to follow the evolution of rancidity in samples stored under exposure to light (typical of the marketplace), maybe after applying suitable correction factors for keeping into account the lower content of some of these VOCs.

Under light exposure in the presence of oxygen in headspace, the VOCs that showed the highest increase and the highest correlation coefficients among the evolution of rancidity were pentanal, nonanal, decanal, *E*-2-heptenal, *E*-2-

DOI: 10.1021/acs.jafc.9b05809
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decenal, *E,E*-hepta-2,4-dienal, propanoic acid, nonan-1-ol, and 3 C8 molecules (octane, oct-1-en-3-ol, and 6-methylhept-5-en-2-one). The sum of these 11 VOCs is proposed as a marker for rancidity of samples stored under light exposure in the presence of oxygen (Table 4, $\sum R_{lightO2}$), conditions that often occur for oils stored at home.

Three molecules, namely, pentanal, nonanal and *E*-2-heptenal, are present in all of the three proposed markers: the sum of these three VOCs could be suggested as a simplified marker for rancidity (Table 4, $\sum R$) that can be calculated measuring the level of only three VOCs in VOOs. Looking at our data (Tables 2 and 3), values of $\sum R$ greater than 0.65 mg/kg appear suitable for indicating the presence of rancidity in virgin olive oils.

The proposed markers were finally correlated with the evolution of rancidity in the different conditions of this research. The best correlation of each marker was with rancidity of the oil stored in that specific condition. $\sum R_{dark}$ correlated very well (R, 0.969); $\sum R_{light}$ showed again very good correlations (0.973 and 0.935 for It-EVOO and Tun-EVOO, respectively); $\sum R_{lightO2}$ showed good but slightly lower correlations: 0.907 and 0.814 for It-EVOO and Tun-EVOO, respectively; and finally, $\sum R$ showed a slightly lower but still good correlation (always higher than 0.800) for the oils stored in all of the different conditions, confirming its suitability as a simplified marker for rancidity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b05809.

List of the used authentic standards with their purity, with also the selected internal standard for each quantified VOC (Table S1); list with the codes and the conditions of the analyzed samples (Table S2); evolution over time of peroxide number and spectrophotometric indices (Tables S3 and S4, respectively); evolution over time of pigments (Table S5); fatty acid precursors of the main VOCs produced during oxidation processes, according to the previous literature (Table S6); evolution of pigments over time (Figure S1); and the appearance of samples after 6 months of storage (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was partially supported by the FOODOLEAPLUS project co-founded by Tuscany region (D.D. no. 6762 (396), 15/01/2018) and by COMPETiTiVE—Claims of Olive oil to iMProvE the market ValuE project (AGER 2 Project, grant no. 2016-0174). The authors have declared no conflict of interest.

■ ABBREVIATIONS

VOO, virgin olive oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LOX, lipoxygenase; VOCs, volatile organic compounds; HS-SPME-GC−MS, headspace solid-phase microextraction gas chromatography−mass spectrometry; EVOO, extra-virgin olive oil; ISTD, internal standard; IOC, International olive council; LVOO, lampante virgin olive oil

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DOI: 10.1021/acs.jafc.9b05809
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