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Variability in volatile compounds from lipoxygenase pathway in extra virgin olive oils from Tuscan olive germoplasm by quantitative SPME/GC-MS

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Abstract

A quantitative method, based on SPME GC-MS, for the quantification of volatile compounds derived from lipoxygenase pathway, considered the most important for the aroma of high-quality olive oil, was developed. The method was used to study the variation within the extra virgin olive oils from 67 cultivars of the Tuscan olive germplasm conserved at "Santa Paolina" experimental farm (Follonica, Italy). A great variability was observed among the 67 cultivars both for the total amount of volatile compounds and for the different ratios between the groups of volatile compounds from common precursors. The aim was to obtain basic information on the characteristics and the quality of the oils obtainable from nonwidely cultivated olive varieties. These data can support the reintroduction in the production chain of old autochthonous varieties and for exploitation in breeding programs as a source of positive characters to transmit to the progeny.

KEYWORDS

aroma, cultivar, extravirgin olive oil, germplasm, lipoxygenase pathway, solid phase microextraction

1 | INTRODUCTION

Italy possesses one of the widest olive genetic assets in the world; 538 cultivars have been registered so far,[1] but a very limited number of cultivars are used intensively. Each region has its own typical local varieties that in many cases have been genetically characterised. Despite the tree characterisation, information about the quality of olive oils that can be obtained from these varieties is still scarce or absent at all. The majority of the olive germplasm is at risk of genetic erosion because it was abandoned and only marginally cultivated. The reduction of plant biodiversity is a worldwide issue. This process is further accelerated in cultivated species when few varieties are intensively cultivated bringing to the disappearing of older varieties or cultivars. In Italy, from the 1950s until the 1980s, erosion rates between 0.48% and 4% p.a. were estimated.[2] There is an increasing interest in the production of high-quality olive oils as well as monocultivar olive oils linked to limited production areas. The "Santa Paolina" experimental farm in Follonica is a centre for plant biodiversity conservation maintaining about 1000 accessions of olive tree 82 of them collected within the Tuscany region. While trees are well characterised for morphological aspects[3] and in some cases for genetic diversity,[4] the produced oils or that can be obtained by these varieties have been only partially characterised.

To collect information on the characteristics of the oils related to Tuscan accessions, 130 monocultivar EVOOs from 67 genotypes were produced over 2 harvesting seasons.

Aroma is one of the most important and the first to be perceived attribute of high-quality olive oil. The most important and impacting volatile compounds of olive oil aroma are the C5 and C6 compounds[5,6] derived from the enzymatic oxidation of linolenic and linoleic acids through the so-called lipoxygenase (LOX) pathway.[7,8] In the first step of this pathway, LOX using linoleic (LA) and linolenic (LnA) acids as substrate catalyses the productions of 9-hydroperoxides and 13-hydroperoxides with a preference for the latter.[9,10] Subsequently, specific hydroperoxide lyases generate C6 aldehydes from 13-

hydroperoxides. C6 aldehydes are then reduced, by alcohol dehydrogenase, to the corresponding alcohols which are the substrates of the alcohol acetyl transferases producing esters.[10,11] Additional branch of the LOX pathway involving LnA brings to the formation of stabilised 1,3-pentene radicals that can dimerise leading to C10 hydrocarbons (known as pentene dimers) or couple with a hydroxy radical present in the medium producing C5 alcohols, which can be enzymatically oxidised to corresponding C5 carbonyl compounds.[12]

In this contribution, we present a developed method based on SPME GC-MS to quantitatively measure volatile compounds in the analysed olive oils derived from lipoxygenase pathway. The selected compounds comprise straight chain C6 volatile compounds originated from LA (LA-C6) and from LnA (LnA-C6) and the straight chain C5 originated from LnA (LnA-C5) being quantitatively and qualitatively the major volatile compounds in high-quality virgin olive oils[13] and responsible for the green-type sensory descriptors of virgin olive oils.[14] The method was then applied to screen the variation, of these groups of volatile compounds important for olive oil quality, within the 130 monocultivar olive oils from the 67 different genotypes from Tuscan olive germoplasm produced over 2 seasons to take into account seasonal variation.

2 | MATERIALS AND METHODS

2.1 | Samples

Plants and fruits. Olive trees belong to a collection of autochthonous cultivars collected within the Tuscany region maintained by the National Research Council of Italy at Santa Paolina experimental station in Follonica (42° 56′ 30″ N, 10° 46′ 19″ E). Each cultivar, represented by 4 cloned plants, has been morphologically and genetically characterised in previous works.[3,4] The olive orchard, located under typical Mediterranean environmental condition, is cultivated in dry farming with traditional management practices. During the autumn 2010 and 2013 along a 2-month period (from October to December), all the cultivars that had enough fructification for the production of the oil were harvested following the stage of ripening. All the fruits were hand harvested in the morning and processed the same day. Forty kilos of healthy fruits was collected for each 4-plant group. The date of harvesting, expressed as day of year (DOY), is reported in Table 1 per each cultivar.

Oils. Immediately after harvesting, the olives were washed and then processed by a 2-phase Oliomio® continuous mill (Toscana Enologica Mori, Tavarnelle V.P., Italy). The system reproduces, at a small scale, the industrial method of oil extraction so that the resulting EVOOs were as much as possible similar to those produced in an industrial plant. All the operational conditions (temperature, time of malaxation, speed of the centrifuge, flux of water in the separator) were kept steady to lower the variability among oil samples produced along the season, and the extractions were carried out by a single operator. The oils at the exit of the horizontal centrifuge were immediately filtered with a cotton laboratory filter, divided in 100 mL sample bottles, and kept at 12°C protected from the light. The analyses to define the quality grade of the oils (fatty acid composition, number of peroxides, acidity, and sensory analysis) were completed within 15 days from the extraction. The produced oils presented chemical and sensory characteristics typical of extravirgin olive oils with values of acidity and number of peroxides below 0.8% and 20 milliequivalents of active oxygen/kg oil respectively and absence of sensory defects (data not shown) determined according to Commission Regulation EC number 2568/91 and amendments. The analyses of volatile compounds were executed after 3 months of storage (12°C in the dark) to be closer to an oil ready for commercialisation.

2.2 | SPME/GC-MS analysis

Calibration curves. All the standards, with purity equal or higher than 95%, were from Sigma-Aldrich. Pure standards were dissolved in deodorised sunflower oil at 6 concentrations (blank included) covering the range of concentrations expected for extravirgin olive oils (from literature data). The solutions prepared were used to create calibration curves for the following 11 volatile compounds, the concentrations of which are shown in parentheses: hexanal (33, 186, 1734, 3486, 17 252 μg kg^{-1}), n-hexan-1-ol (22, 123, 1140, 2851, 11 342 μg kg^{-1}), and hexyl acetate (37, 205, 1908, 3618, 18 983 μg kg^{-1}) belonging to the LA-C6 group; (E)-2-hexenal (24, 185, 1140, 2261, 11 199 μg kg^{-1}), (E)-2-hexen-1-ol (17, 130, 803, 1603, 7886 μg kg^{-1}), (Z)-3-hexen-1-ol (34, 187, 1740, 3670, 17 305 μg kg^{-1}), and (Z)-3-hexenyl acetate (30, 230, 1421, 2997, 13 959 μg kg^{-1}) belonging to the LnA-C6 group; and (2Z)-2-penten-1ol (15, 119, 737, 1533, 7240 μg kg^{-1}), 1-penten-3-ol (16, 124, 768, 1505, 7546 μg kg^{-1}), (E)-2-pentenal (21, 160, 987, 2115, 9693 μg kg^{-1}), and 1-penten-3-one (24, 182, 1123, 2133, 11 035 μg kg^{-1}) belonging to the LnA-C5 group. Each concentration level was measured in triplicate. Table 2 reports the range of concentrations where the response was linear. In preliminary tests, headspace profiles of different oils were analysed under the same SPME and chromatographic conditions to identify the most appropriate to create calibration curves. Deodorised sunflower oil was chosen because presenting only few compounds (acetaldehyde, butanal, 2-propanone, hexane, acetic acid, 2-ethyl hexan-1-ol, phenol, and few other aromatic compounds) detected at trace level not interfering with the quantification of the selected volatile compounds. As further check, samples of pure sunflower oils and of sunflower oils spiked with standards were compared as well.

Volatile compounds analysis. A 3 g of oil was placed in a 20-mL glass vial, spiked with 50 μL of a solution of 4-methyl-2-pentanol (Aldrich, Milan, Italy), prepared in deodorised sunflower oil, at the concentration of 9.92 mg kg^{-1} as internal standard, capped, after the introduction of a magnetic stir bar, and housed in the autosampler (CTC Analysis AG, Zwingen, Switzerland). The same amount of 4-methyl-2-pentanol was added to the blank and calibration curve solutions as well. Olive oil samples were equilibrated at 40°C while stirring (750 rpm) for 10 minutes; after that, volatile compounds were extracted on a fused silica fibre (2 cm), coated with DBV/CAR/PDMS 50/30 μm (SUPELCO Bellefonte, USA), exposed to the sample headspace for 30 minutes, and then desorbed at 250°C in the injector port of a GC coupled to a mass detector which operates in electron ionisation mode (EI; 70 eV) with a scan range *m/z* 40 to 300 (GC Clarus 500, PerkinElmer, Norwalk, CT, USA). Separation was achieved on a HP-Innowax fused-silica capillary

TABLE 1 Name of the cultivars belonging to the Tuscan olive germplasm used for the production of the 67 monocultivar VOOs and 2-year mean day of harvesting

Name	Code	DOY	Name	Code	DOY
Allora	1	346	Maurino	35	289
Americano	2	308	Melaiolo	36	287
Arancino	3	320	Mignolo	37	302
Aretino	4	311	Mignolo Cerretano	38	330
Ciliegino	5	334	Moraiolo	39	315
Colombana	6	294	Morchiaio	40	320
Colombino	7	312	Mortellino	41	329
Correggiolo	8	319	Olivastra di Populonia	42	299
Cucca	9	331	Olivastra di Suvereto	43	294
Cuoricino	10	310	Olivastra Seggianese	44	313
Da Cuccare	11	323	Olivo del Mulino	45	335
Emilia	12	314	Olivo della Strega	46	324
Filare	13	294	Olivo di Casavecchia	47	330
Frantoio	14	330	Olivo di Cerreto	48	324
Ginestrino	15	292	Olivo di San Lorenzo	49	289
Giogolino	16	313	Ornellaia	50	332
Grappolo	17	299	Pegaso	51	332
Gremigna Tonda	18	329	Pendagliolo	52	300
Gremigno di Fauglia	19	294	Pendolino	53	291
Gremignolo di Bolgheri	20	289	Pesciatino	54	314
Lastrino	21	320	Piangente	55	316
Lazzera Reale	22	297	Punteruolo	56	328
Lazzero delle Guadalupe	23	314	Puntino	57	332
Lazzero di Prata	24	297	Quercetano	58	326
Lazzero Pratigiano	25	328	Rosino	59	314
Lazzero di Vallescaja	26	327	Rossellino Cerretano	60	316
Leccino	27	291	Rossello	61	315
Leccio del Corno	28	330	Rossino	62	296
Leccio Maremmano	29	329	San Francesco	63	308
Leccione	30	302	San Donato	64	299
Madonna dell'Impruneta	31	294	Scarlinese	65	292
Madremignola	32	315	Tisignana	66	301
Mansino	33	314	Tondello	67	300
Maremmano	34	331			

The cultivars are listed in alphabetical order, and the correspondent code is used as shorthand designation in subsequent tables and graphics. DOY represents the day of the year.

column (30 m, 0.32-mm ID, 0.5-μm film thickness; Agilent Technologies, Palo Alto, CA, USA). The GC oven temperature program consisted in 50°C for 5 minutes, 50°C to 250°C at 5°C minute^{-1}, 250°C for 1 minute. He was used as carrier gas (2 mL minute^{-1}). Selected volatile compounds were quantified using the developed calibration curves. The response of each analyte was normalised to the response of the reference standard, and the response factors calculated as the slopes of calibration curves in the linear range. The limit of detection (LOD) and the limit of quantification (LOQ) per each analyte were estimated considering a signal to noise ratio of 3 and 10 respectively taking into account the injection of the lowest concentration in the dynamic range (Table 2). Mass spectra were inspected for each quantified compound per each sample to verify the absence of overlapping signals, or to subtract such possible signals, interfering with the correct quantification.

2.3 | Statistical analysis

Descriptive statistics, normality test (Shapiro-Wilk), and correlation analysis were performed using Statistica 9.1 software (StatSoft, Inc., Tulsa, OK). For multivariate analyses, variables were log transformed than scaled to unit variance prior to principal component analysis (PCA) performed by the software package Simca P+ v.12 (Umetrics, Sweden).

3 | RESULTS AND DISCUSSION

3.1 | Calibration curves and method validation

In Table 2, the linear dynamic range, the squared regression coefficient (R^2), the sensitivity (slope of the calibration straight line), and the LOD

TABLE 2 Characteristics of calibration curves

	Range (μg kg^{-1})		Linear Regression		Linearity	(μg kg^{-1})		Repeatability (n = 9)		Recovery (n = 3)
Compound	Min	Max	Slope	Intercept	R^2	LOD	LOQ	Low (%RSD)	High (%RSD)	%
(E)-2-Hexen-1-ol	130	3936	2.02E^{-04}	−3.60E^{-02}	0.992	6.8	22.8	4.6	5.0	101.4
(E)-2-Hexenal	24	5590	2.66E^{-04}	−3.53E^{-02}	0.990	3.1	10.4	20.5	8.0	88.8
(E)-2-Pentenal	160	987	1.85E^{-04}	3.10E^{-03}	0.994	26.7	89	5.7	8.6	98.8
(Z)-2-Penten-1-ol	119	3614	2.90E^{-04}	−2.58E^{-02}	0.992	119.4	398	10.9	6.2	87.8
(Z)-3-Hexen-1-ol	34	8634	3.31E^{-04}	−2.17E^{-02}	0.998	9.1	30.5	10.6	5	95.5
(Z)-3-Hexenyl acetate	30	6967	3.58E^{-04}	−7.89E^{-02}	0.989	3.4	11.5	12.1	7.1	86.3
1-Penten-3-ol	124	3766	3.17E^{-04}	−9.90E^{-03}	0.991	22	73.2	11.3	10.3	98.5
1-Penten-3-one	182	11035	4.87E^{-04}	−1.55E^{-02}	0.998	28.7	95.8	9.1	8.1	98.9
Hexanal	186	17252	3.38E^{-05}	−2.59E^{-02}	0.990	111.8	372.8	10.0	6.5	98.5
Hexyl acetate	37	9471	1.92E^{-04}	−2.30E^{-03}	0.999	7.4	24.5	10.4	13.3	99.8
n-Hexan-1-ol	22	5659	3.22E^{-04}	−9.20E^{-03}	0.999	5.1	16.9	20.1	6.5	97.3

Range: range of linearity of the calibration curves. LOD: limit of detection estimated considering a signal to noise ratio of 3. LOQ: limit of quantification estimated considering a signal to noise ratio of 10.

and LOQ per each calibration curve are reported. The linear dynamic range was adequate for the quantification of all compounds, but (E)-2-hexenal and hexanal whose concentrations, in different samples, were outside the range of linearity of the calibration curves. The samples showing estimated concentration of (E)-2-hexenal and hexanal outside the dynamic range of the calibration curves were opportunely diluted using sunflower oil (the same used to build the calibration curves) and reinjected for a correct quantification. (E)-2-pentenal has the lowest dynamic range between 187 and 987 μg kg^{-1}, while hexanal showed the widest between 186 and 17252 μg kg^{-1}. The LOD and LOQ ranged from 3.1 and 10.4 μg kg^{-1} for (E)-2-hexenal to 119.4 and 398.0 for (Z)-2-penten-1-ol.

The repeatability of the method, for each volatile compound, was assessed by analysing 9 blank (deodorised sunflower oil) samples spiked at 2 different levels corresponding to the lowest and highest concentrations in the range of linearity of the calibration curves (Table 2). Results show a good repeatability with a RSD below 21% for (E)-2-hexenal and n-hexan-1-ol and below or equal to 13.3% for all the other compounds.

The accuracy of the method was assessed calculating the percentage of recovery of each analyte from a blank sample (deodorised sunflower oil) spiked with the highest concentrations in the range of linearity of the calibration curves (Table 2) per each compound. For example in the case of (E)-2-hexen-1-ol, the blank was spiked with 3936 μg kg^{-1} of (E)-2-hexen-1-ol.

3.2 | Quantification of volatile compounds in oils

In Table 3, the intervals of variation (min, max) and the means and medians of the quantified volatile compounds in the 130 oils from the 67 cultivar studied over the 2 production seasons are reported. In general, there is great variability among the different monovarietal oils in amount of volatile compounds that originate from LOX pathway. The total amount of these compounds ranges from 5.06 to 159.05 mg kg^{-1}. In Figure 1, two examples of chromatograms are reported for oils containing low (upper panel) and high (lower panel) amount of LOX pathway-originated compounds. Significant differences, in total amount of volatile compounds, were found also for the 2 seasons for many cultivars. Because all the trees share the same harvesting conditions and agronomic practices, the fruits were picked with minimal differences in ripening degree (no correlation was found between ripening index and any of the volatile compounds quantified but a weak correlation for (E)-2-hexenal (r = −0.273, P = .003)) and the oils produced under the same controlled conditions, most of the differences observed could be attributed to different response each cultivar has for the climatic conditions. The second year of the experiment was warmer than the first. Over the 90-day period between August and October, when 90% of the oil accumulates in the olive fruits, the mean difference in temperature was of +1.8°C.

(E)-2-hexenal, with few exceptions, was the most abundant volatile compound from LOX pathway in both the production seasons, ranging from 1.99 to 105.09 mg kg^{-1} and from 2.47 to 134.52 mg kg^{-1} in 2011 and 2013 respectively. The cultivars that showed the highest amount of (E)-2-hexenal, reported in parenthesis as percentage of LOX compounds, in both the seasons were *Lazzero Pratigiano* (86 ± 0.4%), *Aretino* (85%, only 1 season available), *Piangente* (82 ± 1%), *Gremignolo di Bolgheri* (80 ± 1%), *Olivo di Casavecchia* (78 ± 6%), and *Da Cuccare* (77 ± 4%). On the opposite, those showing lower amount of (E)-2-hexenal in both the seasons were *Olivo di Cerreto* (31%, only 1 season), *Tisignana* (33 ± 10%), *Allora* (37 ± 5%), *Ornellaia* (39%, only 1 season), *Lazzera Reale* (39%, only 1 season), *Lazzero Vallescaja* (42%, only 1 season), *Melaiolo* (44 ± 17%), *Lastrino* (47 ± 8%), and *Scarlinese* (49 ± 1%). Differences have been recorded between the 2 seasons for (E)-2-hexenal but were not statistically significant (r = 0.38, P = .685). (E)-2-hexenal was reported as to be involved in the leaf odour of olive oils. Angerosa and coworkers reported that (E)-2-hexenal was the most important with a positive contribution to lawn perception while contributed negatively to banana and almond odour perceptions.[15] Other authors reported, on the contrary, a positive contribution of (E)-2-hexenal to the almond note.[16] The odour thresholds of (E)-2-hexenal in oil determined nasally and retronasally, by means of aroma extract dilution analyses and gas

TABLE 3 Range of variability and distribution of the quantified volatile compounds in the 130 oils from 67 cultivars

	2011 µg·kg^{-1}				2013 µg·kg^{-1}			
	Min	Max	Mean	Median	Min	Max	Mean	Median
Hexanal	1286	32 636	6392	4305	2146	27 981	8555	7327
n-Hexan-1-ol	67	1731	285	170	39	1388	307	205
Hexyl acetate	4	3347	205	37	0	3098	172	53
(E)-2-Hexenal	1993	105 092	21 686	17 281	2466	134 525	23 125	17 513
(E)-2-Hexen-1-ol	46	1603	337	246	<LOD	3721	597	336
(Z)-3-Hexen-1-ol	89	5450	991	741	<LOD	<LOQ	<LOD	<LOD
(Z)-3-Hexenyl acetate	59	4327	953	541	<LOD	6027	611	298
(2Z)-2-Penten-1-ol	<LOD	1533	427	<LOQ	<LOD	1073	<LOQ	<LOQ
1-Penten-3-ol	115	2106	664	509	74	1066	307	292
(E)-2-Pentenal	<LOD	<LOD	<LOD	<LOD	<LOD	143	<LOQ	<LOQ
1-Penten-3-one	110	37 58	987	762	132	2258	682	634
Σ LA C6	1478	36 403	6881	4698	2331	31 817	9035	7773
Σ LnA C6	2923	112 067	23 967	19 126	2902	136 710	24 336	18 296
Σ LnA C5	296	7348	2079	1684	215	4540	1255	1203
Σ LOX	5064	138 726	32 928	28 769	6652	159 052	34 625	25 104

Results are reported separately for the 2 seasons.

Abbreviations: LOD, limit of detection estimated considering a signal to noise ratio of 3; LOQ, limit of quantification estimated considering a signal to noise ratio of 10.

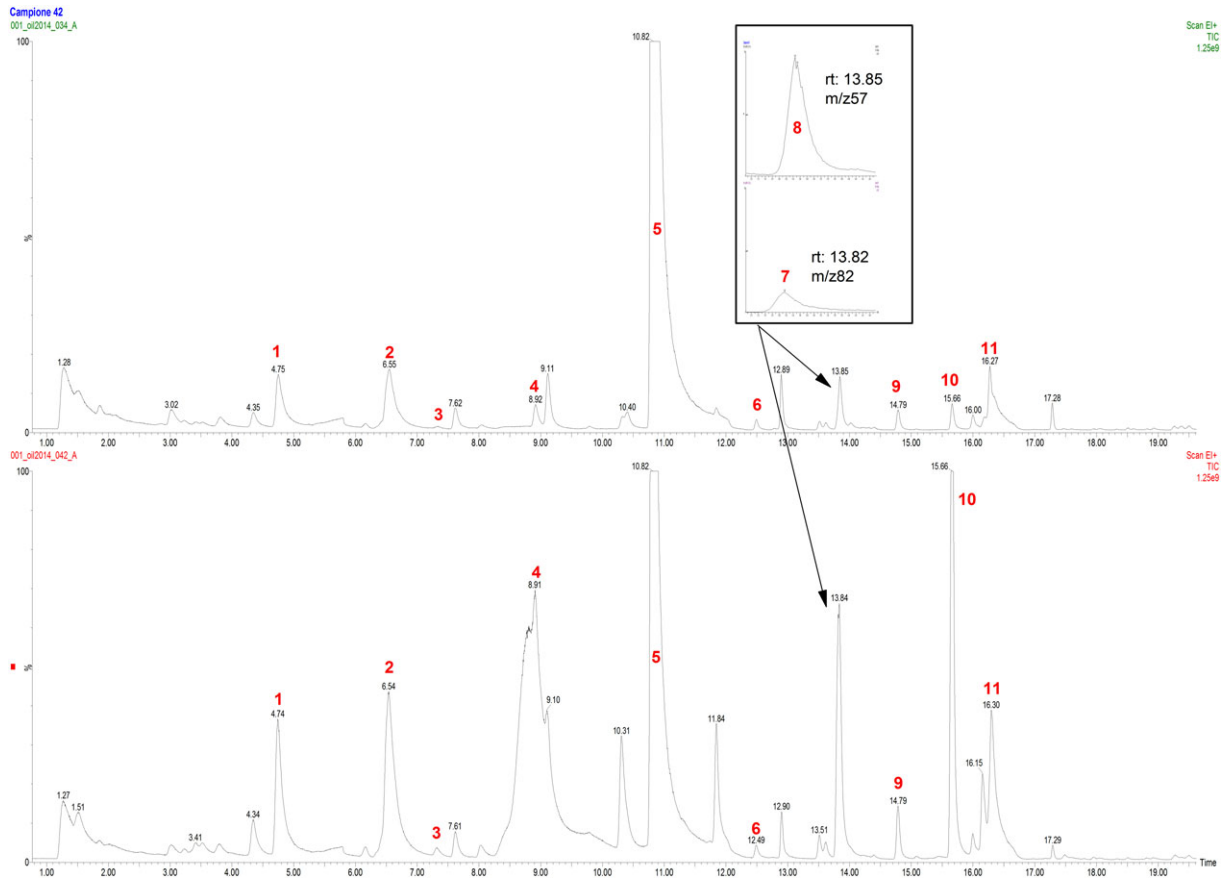

FIGURE 1 Two examples of chromatograms for oils emitting lower (upper panel) and higher (lower panel) amount of compounds originates from lipoxygenase pathway. 1: 1-Penten-3-one; 2: hexanal; 3: (E)-2-pentenal; 4: 1-penten-3-ol; 5: (E)-2-hexenal; 6: hexyl acetate; 7: (Z)-3-hexenyl acetate; 8: (2Z)-2-penten-1-ol; 9: n-hexan-1-ol; 10: (Z)-3-hexen-1-ol; 11: (E)-2-hexen-1-ol. The box reports the traces recorded at *m/z* 82 and *m/z* 57 used to resolve the peaks of (Z)-3-hexenyl acetate and (2Z)-2-penten-1-ol respectively [Colour figure can be viewed at wileyonlinelibrary.com]

chromatography olfactometry by Reiners and Grosch, were 424 and 257 μg kg^{-1} respectively.[17] In all, the oils exanimated concentrations of (E)-2-hexenal if far above these thresholds.

The second most abundant compound in the olive oils analysed was hexanal whose concentration ranged from 1.29 to 32.64 mg kg^{-1} and from 2.15 to 27.98 mg kg^{-1} in 2011 and 2013 respectively. Only 14 samples (13 for 1 of the 2 production seasons and 1 for both production seasons) out of the 116 studied showed amount of hexanal to be similar or higher than (E)-2-hexenal. Different studies indicates (E)-2-hexenal as the most abundant volatile compounds in most European extravirgin olive oils[8,18,19] although a great variability has been reported for monovarietal extravirgin olive oils from Marche region[16]. (E)-2-hexenal content in olive oils decreases with storage; Cavalli and coworkers reported a quick decrease over a few months during conservation in ambient temperature in darkness.[18] During storage, hexanal, initially present in fresh oil, can be depleted as well, but further, hexanal can be produced from secondary oxidation from hydroperoxydes.[20] Most of the studies, when indicated, measured volatile compounds few days after the production directly or after storage between 4 and −20°C until analysis.[16,19,21-25] Our samples were measured after 3 months of storage, protected from the light, at 12°C that is the typical time occurring between production and commercialisation. Hexanal has been reported to be negatively correlated with leaf and lawn attributes other than to flower and tomato attributes while was positively correlated to almond attribute and, in combination with (Z)-3-hexenyl acetate, to banana odour.[15] Its odour threshold in oil was reported to be 300 μg kg^{-1} when perceived nasally and 73 μg kg^{-1} when perceived retronasally,[17] far below concentrations reported for all the examined oils (Table 3).

The concentrations of LnA-C5 compounds in the 130 olive oils ranged from trace amount to 7.35 mg kg^{-1}. Among them, the amount of 1-penten-3-one ranges from 110 to 3785 μg kg^{-1} over the 2 seasons far above its odour thresholds in oils of 0.73 and 3.2 μg kg^{-1} perceived nasally and retronasally respectively[17] This compound was

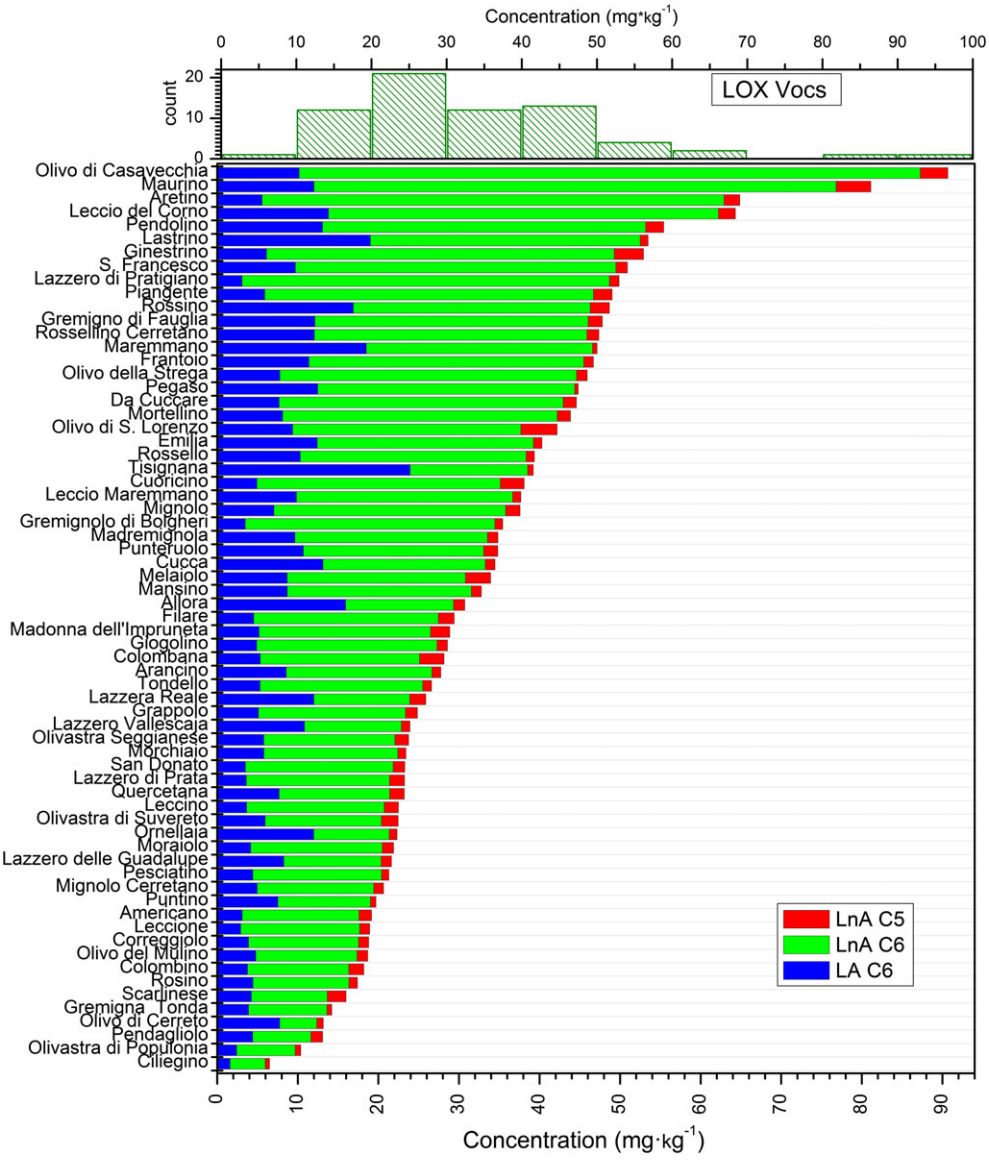

FIGURE 2 Distribution of the 3 groups of volatile compounds derived from lipoxygenase pathway. LnA-C5: Sum of C5 volatile compounds from linolenic acid (red color). LnA-C6: Sum of C6 volatile compounds from linolenic acid (green color). LA-C6: Sum of C6 volatile compounds from linoleic acid (blue colour) [Colour figure can be viewed at wileyonlinelibrary.com]

found to be involved in the elicitation of several sensory attributes and in particular was found to be negatively correlated to green fruity attribute, in combination with (E)-2-pentenal, and positively correlated to leaf and tomato odours other than associated to the bitter taste.[15] The concentration of (E)-2-pentenal was above the limit of detection only for 32 out 130 samples, corresponding approximatively to 25% of the samples. The amount of (Z)-3-hexen-1-ol in the produced oils was found to be dependent to the season; in 2011, its concentration ranged from 89 to 5450 μg kg^{-1}, while in 2013, its concentration was below the limit of quantification and even below the limit of detection for most of the samples. In general, in 2013, it was observed a slight decrease of alcohols. Statistically significant differences between the 2 seasons were found for the alcohols derived from LnA namely (Z)-3-hexen-1-ol (r = −0.59, P < .001), (2Z)-2-penten-1ol (r = −0.39, P < .001), and 1-penten-3-ol (r = −0.47, P < .001). (Z)-3-hexen-1-ol was found to be weakly correlated with sensory attributes in olive oil; anyway, it correlated negatively with positive sensory odours.[15] (2Z)-2-penten-1ol was found in a quantifiable amount only in a reduced number of samples as well; its concentration ranged from trace amount to 1533 μg kg^{-1}. (2Z)-2-penten-1ol in oil was found to contribute to almond sensory attribute.

In Figure 2, the amount of the 3 groups of volatile compounds (LA-C6, LnA-C6, LnA-C5) originated from LOX pathway, averaged over the 2 seasons, are reported per each cultivar. About 69% of the cultivars exhibited a concentration of volatile compounds from LOX pathway between 20.65 and 49.88 mg kg^{-1}, 19% between 6.50 and 19.72 mg kg^{-1}, and the remaining 12% between 50.92 and 90.69 mg kg^{-1}. The cultivars that exhibited the highest concentration of volatile compounds originated from LOX pathway far above the other cultivars are *Olivo di Casavecchia* (90.69 mg kg^{-1}) and *Maurino* (81.13 mg kg^{-1}). These 2 cultivars are extremely different from the others, in fact looking at the distribution of the sum of volatile compounds from LOX pathway (upper panel of Figure 2), all cultivars but *Olivo di Casavecchia* and *Maurino* follow a normal distribution (Shapiro-Wilk test = 0.963, DF = 65, P = .047). The lowest concentration of volatile compounds originated from LOX pathway was recorded for *Ciliegino* (6.50 mg kg^{-1}), the only cultivar below 10.00 mg kg^{-1}. The group of LnA-C6 volatile compounds is confirmed

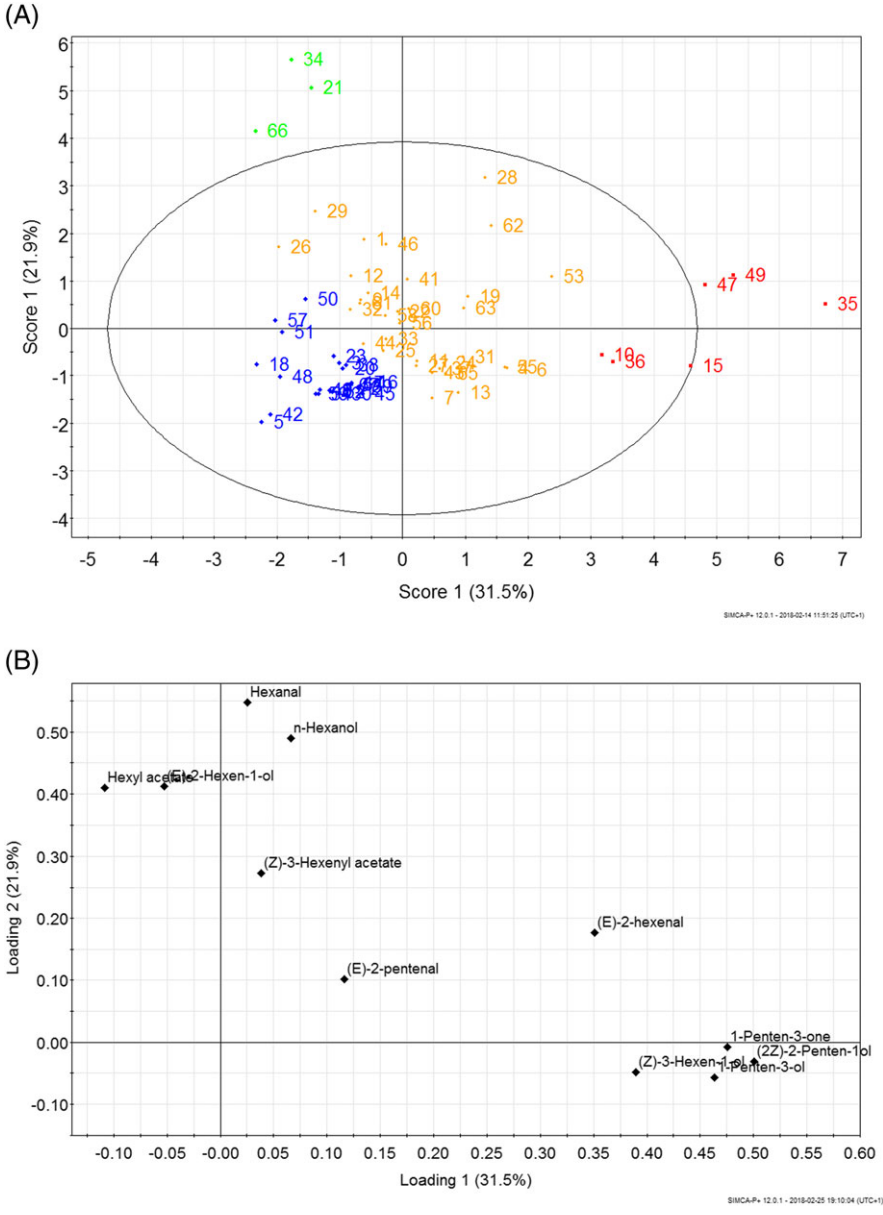

FIGURE 3 PCA score A, and loading B, plots of volatile compounds of the investigated extra virgin olive oils from 67 cultivars

to be the most abundant accounting from 50% to 91% of the compounds derived from LOX pathway in the oils studied with the exception of few cultivars where amount of LA-C6 compounds is similar (*Olivo di Cerreto*) or higher (*Tisignana*, *Ornellaia*, *Allora*, *Lazzera Reale*). The amount of LnA-C5 compounds accounted from 1% to 15% of the compounds derived from LOX pathway with the highest percentages, above or equal to 10%, found for *Scarlinese* (15%), *Pendagliolo* (11%), *Colombana* (11%), *Olivo di S. Lorenzo* (11%), and *Colombino* (10%) cultivars. On the contrary, the cultivar with the lowest fraction of LnA-C5 compounds were *Lazzero di Pratigiano* (2.4%), *Lastrino* (1.8%), *Tisignana* (1.2%), *Maremmano* (1.2%), and *Pegaso* (0.9%).

To highlight common features among the cultivars, a PCA was performed. In Figure 3, the score (A) and loading (B) plots are reported. Using the Ward's method to calculate distances between clusters, it is possible to identify 4 main groups of samples. Cluster 1 (red symbol in Figure 3A) that groups 6 cultivars characterised by higher concentration of LnA-C5 compounds and (Z)-3-hexen-1-ol and a good amount of (E)-2-hexenal (Figure 3B). The cluster 2 (green symbol in Figure 3 A) is composed of 3 cultivars that are differentiated from the other cultivars for higher concentration of hexanal, n-hexan-1-ol, hexyl acetate, and (E)-2-hexen-1-ol (Figure 3B). The 3 cultivars belonging to cluster 2 were described by a sensory panel as to be characterised by olfactory sensations of flowers and fruit.[26] The clusters 3 (blue symbol in Figure 3A) and 4 (orange symbol in Figure 3A), grouping 24 and 34 cultivars respectively, differentiate each other for the total amount of volatile compounds from LOX pathway (Figure 3B) that are more intense in the cultivars belonging to cluster 4. It is thus expected that oils from cultivars belonging to cluster 4 present more intense olfactory attributes.

4 | CONCLUSIONS

Volatile compounds originated from lipoxygenase pathway, recognised to be the most important compounds defining the typical aroma of high-quality EVOOs, have been quantified in oils obtained from 67 cultivars over 2 production seasons. With this work, we demonstrated that within the oils produced from a local restricted olive germplasm collection, it is possible to find a large variation in volatile compounds generated from lipoxygenase pathway, and thus, high variability is expected in aroma associated to the different cultivars. The knowledge acquired on the large aroma variability of these cultivars offers a wide choice for olive breeding programs with the aim of finding new cultivars with improved oil aroma. Further characterisation of these cultivars for fatty acids and for polyphenols composition will provide breeders with a more ample spectrum of combination of characters for their programs.

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