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Beyond current quality indices: Quantitative volatilomics unrevealed cultivar traits, harvesting practices impact, and aroma blueprint of extra-virgin olive oils

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A B S T R A C T

Virgin olive oil, derived from *Olea europaea* L., is a staple in the Mediterranean diet and is classified into quality categories by EU regulations and the International Olive Council. Extra Virgin Olive Oil (EVOO), the highest quality, is valued for its nutritional and sensory attributes, driving consumer willingness to pay a premium. However, this makes EVOO susceptible to fraud, necessitating robust quality control methods. This study combines advanced untargeted fingerprinting and quantitative volatilomics using headspace solid-phase microextraction (HS-SPME) coupled to comprehensive two-dimensional gas chromatography with parallel mass spectrometry and flame ionization detection (GC×GC-MS/FID) on EVOOs from *Hojiblanca* and *Picual* cultivars to define robust markers of quality, named cultivar markers and cultivation practice indicators. The comprehensive analysis identified over 190 peak features and 84 compounds, revealing distinct volatile profiles influenced by cultivar, cultivation methods, and ripening stages. Of the 125 diagnostic volatile features (Fisher value > 4) for cultivar discrimination, alkenes, carbonyls, and alcohols predominate. Key compounds, such as (*E*)-2-octenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol, reflect lipoxygenase pathway activity and maturation stages across cultivars. Quantification confirmed distinct aroma blueprints between cultivars, driven by differences in OAVs of key-odorants. However, minimal differences between odor active markers for organic and conventional cultivation practices, suggest consumers are unlikely to perceive variations in aroma between the two.

1. Introduction

Virgin olive oil, derived from *Olea europaea* L., is central to the Mediterranean diet and primarily produced in the Mediterranean region (Lioupi et al., 2022; MAPAMA, n.d.). Classified by organizations like Codex Alimentarius, International Olive Council, an subjected to EU Regulations ("European Commission Regulation (EC) Reg. 2568/91," 1991), its quality categories depend on sensory and physicochemical parameters. Extra Virgin Olive Oil (EVOO), the highest quality category, is prized for its nutritional traits and sensory attributes (Caporaso et al., 2015; Cecchi et al., 2021), driving consumer demand and willingness to pay premiums for specific qualities, such as health benefits, organic certification, or geographic origin (Arroyo-Manzanares et al., 2019). However, this high demand also increases the risk of fraud, necessitating stringent quality control measures to maintain consumer confidence (Conte et al., 2020; M. P. Segura-Borrego et al., 2022a; M. Pilar Segura-Borrego et al., 2022b).

Characterizing EVOO involves analyzing a wide range of sensory and physicochemical parameters, with a growing need for advanced analytical methods due to the limited effectiveness of current techniques

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and evolving adulteration methods (Conte et al., 2020). Technological advancements have been embraced by the olive sector to meet quality demands, with producers and researchers focusing on optimizing production under various agronomic and environmental conditions. Efforts aim to ensure the highest quality of olive oils despite these variables (Cecchi et al., 2022; Hong et al., 2017; Quintanilla-Casas et al., 2020b).

Aroma is a crucial quality criterion for EVOO, significantly influenced by factors such as olive variety/cultivar, geographic origin, fruit maturation, irrigation, crop season, agronomic practices, and oil extraction conditions (Beltrán et al., 2005; Benito et al., 2012; Jiménez et al., 2017; Mansouri et al., 2015; Morelló et al., 2004; Romero et al., 2003; Vidal et al., 2019). The volatile profile and overall aroma are especially affected by olive variety/cultivar and ripening stage, driving interest in monovarietal oils. Despite promising analytical methods, no official approach exists to authenticate monovarietal EVOOs, highlighting the need for robust authentication methods (Herrera et al., 2012; Jimenez et al., 2015, 2014; Jiménez et al., 2017).

The type of farming, particularly organic versus conventional methods, is another significant factor due to consumer demand for organic products and strict agricultural regulations (Jiménez-Herrera et al., 2019). While organic olive farming has grown, studies comparing organic and conventional EVOOs are limited and often inconclusive due to uncontrolled variables. Future research needs controlled samples to advance knowledge on organic EVOOs, prevent fraud, and provide valuable information to the industry, producers, and consumers.

Gas chromatography-mass spectrometry (GC-MS) is widely used to profile olive oil volatile compounds. Combined with headspace (HS) approaches like solid-phase microextraction (SPME) or dynamic headspace (D-HS), it enables high-throughput profiling and monitoring of many aroma-active compounds (Cecchi et al., 2022; Cordero et al., 2015; Quintanilla-Casas et al., 2020b). The introduction of comprehensive two-dimensional GC (GC×GC) has significantly improved the understanding of EVOO's chemical complexity, providing higher peak capacity, resolution, lower detection thresholds, and more confident analyte identification (Lukić et al., 2019; Marriott and Nolvachai, 2021; Purcaro et al., 2014; Ryan and Marriott, 2003). Moreover, adopting non-targeted data processing [e.g., combined untargeted and targeted fingerprinting *UT fingerprinting* (Magagna et al., 2016)], the investigation of complex fraction of volatile organic compounds (VOCs) by GC×GC has opened to volatilomics, the discipline that connects the chemical information encrypted in the volatilome (or volatome) with biological properties (Broza et al., 2015; Phillips et al., 2013; Stilo et al., 2021a). The volatilome "*contains all of the volatile metabolites as well as other volatile organic and inorganic compounds that originate from an organism*" (Amann et al., 2014) super-organism, or ecosystem; as part of the metabolome it brings information worthy to be investigated for product valorization and treaciability.

Analytical platforms that implement parallel detection, combining mass spectrometry (MS) and flame ionization detector (FID), offer great opportunities allowing for reliable identification and accurate quantification of analytes without external calibration. This enables true quantitative results for robust marker selection and correlation with quality traits (Stilo et al., 2023; Stilo et al., 2021). The use of GC×GC-MS/FID in olive oil research in particular, allows for a comprehensive mapping of detectable VOCs, provides accurate amounts on selected analytes, and enables causal correlation with biological variables such as olive cultivar, ripening stages, geographical origin, agricultural practices, and technological processing (Magagna et al., 2016; Reichenbach et al., 2019; Stilo et al., 2023; Stilo et al., 2021). The quantitative mapping of key-aroma compounds delineates the aroma blueprint of EVOOs, i.e., the unique pattern of odor active compounds responsible of the aroma identity of the product (Granvogl and Schieberle, 2022)

This study, for the first time, combines advanced untargeted fingerprinting and quantitative volatilomics using HS-SPME-GC×GC-MS/FID on a selection of monocultivar EVOOs to define both robust quality tracers and distinctive chemical blueprints predicting aroma features. Samples were obtained from *Hojiblanca* and *Picual* olive cultivars harvested in the Cordoba region (Spain) under different farming procedures (organic and conventional) and ripening stages from November 2021 to January 2022.

2. Materials and methods

2.1. Extra virgin olive oil samples

Extra virgin olive oils (EVOOs) were obtained from *Picual* and *Hojiblanca* olive cultivars harvested in 2021. Olive trees were subjected to two different cultivation methods, *organic* and *conventional*. Trees were located at the Agricultural Research Training Centre in "Cabra" in the province of Cordoba (Spain) [plot coordinates 37°29'36.4"N 4°25'45.8"W], which has a continental Mediterranean climate with dry summers and mild winters. Rainfall occurs from autumn to spring, with a mean annual rainfall of 400 mm. The average annual temperature is 17 °C, reaching −1.3 °C in winter and 43 °C in summer. Soils have low depths, being mainly over limestone and siliceous stones. They have a loamy texture and an alkaline pH (8–8.5 range). The electrical conductivity is low, as is the organic matter content and the carbon/nitrogen ratio. Twenty olive trees were randomly selected (always among the most loaded to guarantee sampling) of the *Picual* and *Hojiblanca* cultivars from conventional cultivation and twenty trees of the two cultivars from organic cultivation. Organic cultivation was conducted according to EU Regulations and certified by Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA) (Junta de Andalucía) ("Regulation (EU) 2018/848 of the European Parliament and of the Council of 30 May 2018 on organic production and labelling of organic products and repealing Council Regulation (EC) No 834/2007," 2018).

Olive harvesting was done by randomly hand-picking healthy fruits (without any type of infection or physical damage), beginning in November 2021 and ending in January 2022, and comprising different harvest periods. It conforms to a total of 3 ripening stages: 15 of November (stage I), 13 of December (stage II), and 1 of January (stage III). The ripening index (RI) of each harvest was determined according to the methodology proposed by Uceda and Frias (Uceda and Frias, 1975).

Table 1
EVOO samples characteristics and coding.

Sample coding	Cultivar	Cultivation method	Olives harvesting	Maturity stage	Ripening Index
EVOO_1	*Picual*	Conventional	November 15	I	3.5
EVOO_2	*Picual*	Conventional	December 13	II	4.8
EVOO_3	*Picual*	Conventional	January 1	III	5.7
EVOO_4*	*Picual*	Conventional	December 13	II	4.8
EVOO_5	*Picual*	Organic	November 15	I	4.1
EVOO_6	*Picual*	Organic	December 13	II	4.8
EVOO_7	*Picual*	Organic	January 1	III	5.9
EVOO_9	*Hojiblanca*	Conventional	November 15	I	1.4
EVOO_10	*Hojiblanca*	Conventional	December 13	II	3.8
EVOO_11	*Hojiblanca*	Conventional	January 1	III	4.0
EVOO_12*	*Hojiblanca*	Conventional	December 13	II	3.8
EVOO_13	*Hojiblanca*	Organic	November 15	I	1.2
EVOO_14	*Hojiblanca*	Organic	December 13	II	2.6
EVOO_15	*Hojiblanca*	Organic	January 1	III	4.7

* Technological replicates from a different batch

The fruit RI values for each modality are shown in Table 1.

In each harvesting stage, 200 kg of olives were collected without any discriminant selection before immediate processing in the experimental mill of IFAPA Venta del Llano (Mengíbar, Jaén, Spain) to obtain the oils. Thus, the extraction of the oils was carried out in a two-phase semi-industrial continuous system (Il Molinetto, Gruppo Pieralisi, Italy) equipped with a hammer mill, a thermo-blender, and a horizontal centrifuge. The 200 kg of olives were ground and then, the resulting paste was beaten for 45 min at 28 °C, after which the oil was separated by centrifuging the beaten paste at ≈ 3250 G. The oils obtained were decanted and filtered. Samples for volatilome screening were immediately sent to the University of Turin laboratory in dark-glass bottles. Before analysis they were kept at −18°C away from UV exposure. Sample descriptions and details are reported in Table 1.

Olive oils were also subjected to the evaluation of the sensory panel of the Priego de Córdoba Protected Denomination of Origin (PDO), following the standards of the International Olive Council (IOC) (Ríos-Reina et al., 2021). According to sensory results, all samples considered in this work resulted “Extra-Virgin” Olive Oils.

2.2. Reference standards and solvents

Pure reference compounds for identity confirmation of marker volatiles and potent odorants listed in Table 2, *n*-alkanes (*n*-C7 to *n*-C25) for Linear Retention Indices (I^T) calibration, and α/β-thujone used as internal standards for response stability check were obtained from Merck (Milan, Italy).

The external standard for predicted relative response factors (RRF) based on combustion enthalpies calibration was hexanal (99 % purity, Merck). The reference solution was prepared in cyclohexane (99 % purity, Merck) at a final concentration of 10.00 g/L and then diluted in dibutyl phthalate (99 % purity, Merck) to suitable concentrations for linearity evaluation.

2.3. Multiple headspace solid phase microextraction: devices and conditions

Volatiles from EVO oils were extracted by HS-SPME with a divinylbenzene/carboxen/polydimethyl siloxane (DVB/CAR/PDMS) fiber (d_f 50/30 μm; 2 cm length) from Supelco (Bellefonte, PA, USA) according to a previously optimized procedure (Stilo et al., 2019). The SPME fiber was conditioned before use as recommended by the manufacturer. Sampling was carried out on 0.100 ±0.003 g of oil, precisely weighed in a 20 mL headspace vial, and kept at 40 °C for 60 min under constant agitation. The very low amount of sample was chosen to match for HS linearity conditions for most of the characteristic analytes of the EVO oil volatilome (Stilo et al., 2021). After extraction, the SPME device was automatically transferred to the split/splitless injection port of the GC×GC system, kept at 250 °C, and thermal desorption was for 5 min. Samples were analyzed in four replicates randomly distributed over two weeks.

Multiple headspace extraction by SPME (MHS-SPME) of samples and calibration solutions were conducted by applying the above-indicated conditions, and the number of consecutive extraction steps was set to four, achieving an almost exhaustive extraction of the analytes under study (Stilo et al., 2021).

2.4. Comprehensive two-dimensional gas chromatography: instrument set-up and conditions

Automated MHS-SPME was performed by a multipurpose sampler, model MPS-2 (Gerstel, Mülheim a/d Ruhr, Germany), installed on a GC×GC system equipped with a reverse-inject differential-flow modulator based on capillary flow technology™ (Agilent Technologies, Little Falls, DE, USA). The Agilent 7890B GC unit was coupled to an Agilent 5977B equipped with a high-efficiency source (HES) and fast quadrupole MS analyzer (Agilent Technologies). The MS was operating in electron ionization mode at 70 eV. Ion source and transfer-line temperatures were set at 280 °C, and the quadrupole temperature was set at 240 °C. The scan range was set between 40 and 250 *m/z*, achieving a data acquisition frequency of 30 Hz. Parallel detection was by a fast FID with base temperature held at 280 °C; H_2 flow 40 mL/min, air flow 350 mL/min, and sampling frequency 200 Hz.

The column set was configured as follows: first dimension (^{1}D) HeavyWax™ column (100 % polyethylene glycol - PEG; 20 m ×0.18 mm dc ×0.18 μm d_f) coupled with second dimension (^{2}D) DB17 column (50 % phenyl-methylpolysiloxane; 1.8 m ×0.18 mm d_c ×0.18 μm d_f), both from Agilent Technologies. The connection between the ^{2}D column and deactivated silica capillaries (Agilent Technologies) toward MS (0.5 m ×0.1 mm d_c) and FID (1.1 m x 0.18 mm d_c) for parallel detection was by a three-way unpurged capillary microfluidic splitter (G3181B, Agilent Technologies). The resulting split ratio was 70:30 FID/MS.

The GC split/splitless injector port was set at 250 °C and operated in pulsed-split mode (250 kPa overpressure applied to the injection port until 2 min) with a split ratio 1:20. The carrier gas was helium at a nominal flow of 0.4 mL/min along the ^{1}D column and 10 mL/min along the ^{2}D column. The oven temperature program was set as: from 40 °C (2.29 min) to 240 °C (11’) at 3.06 °C/min. The modulation period (P_M) was set at 3 s and pulse time at 250 ms. The *n*-alkanes liquid sample solution for I^T determination was analyzed under the following conditions: split/splitless injector in split mode, split ratio 1:50, injector temperature 250 °C, and injection volume 1 μL.

2.5. Analytes identification criteria

Analyte identification was by combining retention data (carried out using experimental I^T with ± 10 units tolerance *vs.* tabulated ones) and comparing electron ionization (EI)-MS spectral signature with reference spectra in commercial and in-house databases by using the NIST identity search algorithm with direct match factor (DMF) and reverse match factor (RMF) scores threshold ≥ 900.

2.6. Analytical data acquisition, processing and mining

2.6.1. Combined untargeted and targeted (UT) fingerprinting by smart templates

A template is a pattern of 2D peaks and/or graphic objects (features) created over a reference chromatogram or image (single or cumulative). This template is used to identify similar patterns of 2D peaks in a set of analyzed chromatograms or images (Bressanello et al., 2018; Reichenbach et al., 2019). The process involves specific matching functions that establish correspondences between features across multiple chromatograms. Specificity is achieved by defining confidence thresholds for retention times and MS spectral similarity to account for variable peak detection, and by using suitable transform functions to correct for retention time inconsistencies between runs (Squara et al., 2023b; Stilo et al., 2019). Once the correspondences between features are established, the template’s metadata (chemical name, retention times, mass spectra, informative ions, and their relative ratios) are transferred to candidate peaks and/or graphic objects (peak regions) in the analyzed chromatogram. This feature template, which includes untargeted reliable peaks and peak regions, is obtained through a fully automated workflow in GC Image Investigator™ (GC Image™, GC Image LLC).

The workflow consists of the following steps:

(a) Match peak patterns between all chromatogram pairs from a set of representative samples, using optimized parameters: S/N threshold of 50, MS constraint of 700 for both the direct match factor (DMF) and reverse match factor (RMF) based on the NIST similarity algorithm (Squara et al., 2023b; Technology, 2005).

(b) Select reliable peaks across the analyzed chromatograms, using a relaxed reliability criterion that includes peaks matching at least 50 % +1 of the chromatograms (Reichenbach et al., 2013). This template of

Table 2
Identified compounds in the volatile fraction of EVOOs together with analytical information. See text for identification criteria.

Compound Name	^{1}D t_R min	SD	^{2}D t_R sec	SD	Exp I^T	Lit. I^T	MW	Formulae	PRRF	β (±SD)	Odor quality	OT (ng/g)*
Analytes subjected to accurate quantification												
Propanal	4.83	0.073	0.42	0.019	792	786	58.1	C_3H_6O	1.38	0.90 (±0.07)	Fresh, fruity, malty	9.4 [1]
Ethyl acetate	6.44	0.028	0.37	0.070	897	898	88.1	$C_4H_8O_2$	1.60	0.92 (±0.08)	Fruity, sweet, winey	940 [3]
1-Penten−3-one	10.15	0.016	0.68	0.049	1017	1021	84.1	C_5H_8O	1.12	0.65 (±0.05)	Pungent, spicy	1.6 [1]
α-Pinene	10.24	0.087	1.68	0.055	1017	1017	136.2	$C_{10}H_{16}$	0.79	0.93 (±0.07)	Herbal, woody,terpenic	274 [4]
Hexanal (Internal Standard for FID)	12.57	0.027	0.91	0.048	1079	1080	100.2	$C_6H_{12}O$	-	0.73 (±0.07)	Green, grass	300 [1]
β-Pinene	13.34	0.062	1.85	0.067	1097	1100	136.2	$C_{10}H_{16}$	0.79	0.92 (±0.06)	Herbal, pine	-
(*E*)−2-Pentenal	14.45	0.011	0.73	0.049	1123	1131	84.1	C_5H_8O	1.12	0.75 (±0.02)	Pungent, apple-like	300 [3]
2-Pentanol	14.99	0.016	0.44	0.052	1136	1138	88.1	$C_5H_{12}O$	1.02	0.88 (±0.11)	Musty, fermented	380 [2]
(Z)−3-Hexenal	15.14	0.020	0.77	0.050	1139	1133	98.1	$C_6H_{10}O$	1.04	0.56 (±0.03)	Green, grassy	1.7 [1]
δ−3-Carene	15.16	0.040	1.77	0.085	1139	1144	136.2	$C_{10}H_{16}$	0.79	0.9 (±0.04)	Citrus, pine, herbal	770 [4]
1-Penten−3-ol	15.75	0.006	0.42	0.052	1153	1157	86.1	$C_5H_{10}O$	1.07	0.57 (±0.02)	Pungent, butter	400 [3]
Heptanal	16.99	0.024	1.02	0.062	1181	1181	114.2	$C_7H_{14}O$	0.96	0.93 (±0.05)	Citrus-like, fatty	500 [1]
Limonene	17.48	0.027	1.67	0.050	1192	1195	136.2	$C_{10}H_{16}$	0.79	0.92 (±0.06)	Citrus, terpenic	250 [2]
3-Methyl−1-butanol	17.80	0.006	0.45	0.050	1199	1211	88.1	$C_5H_{12}O$	1.02	0.63 (±0.03)	Malty, ethereal	100 [3]
Eucalyptol	17.88	0.036	1.86	0.089	1201	1205	154.2	$C_{10}H_{18}O$	0.90	0.94 (±0.03)	Herbal, minty	15 [2]
(*E*)−2-Hexenal	18.40	0.006	0.79	0.051	1212	1216	98.1	$C_6H_{10}O$	1.04	0.6 (±0.02)	Bitter almond, green, fruity	320 [1]
Hexyl acetate	21.05	0.014	1.15	0.050	1271	1275	144.2	$C_8H_{16}O_2$	1.09	0.7 (±0.08)	Fruity, pear-like	1040 [3]
Octanal	21.70	0.006	1.10	0.053	1285	1289	128.2	$C_8H_{16}O$	0.92	0.94 (±0.04)	Citrus-like, fatty	140 [1]
(Z)−2-Penten−1-ol	22.50	0.006	0.42	0.052	1303	1306	86.1	$C_5H_{10}O$	1.07	0.57 (±0.02)	Green, almond	250 [2]
(*E*)−2-Penten−1-ol	22.84	0.022	0.40	0.047	1310	1313	86.1	$C_5H_{10}O$	1.07	0.68 (±0.03)	Mushroom, earthy	250 [2]
(Z)−3-Hexenyl acetate	23.01	0.025	1.01	0.047	1315	1317	142.2	$C_8H_{14}O_2$	1.12	0.55 (±0.04)	Green	200 [1]
(*E*)−2-Heptenal	23.17	0.025	0.86	0.031	1318	1320	112.2	$C_7H_{12}O$	0.99	0.81 (±0.05)	Green, fatty	1200 [1]
6-Methyl−5-hepten−2-one	23.80	0.006	0.95	0.054	1332	1339	126.2	$C_8H_{14}O$	0.95	0.69 (±0.03)	Pungent, green, fruity-like	1000 [3]
1-Hexanol	24.30	0.044	0.48	0.054	1343	1348	102.2	$C_6H_{14}O$	0.97	0.66 (±0.02)	Fruity, banana	400 [3]
(Z)−3-Hexen−1-ol	25.69	0.022	0.46	0.048	1374	1380	100.2	$C_6H_{12}O$	1.00	0.6 (±0.01)	Banana, fresh, grass	1100 [5]
(E,E)−2,4-Hexadienal	26.30	0.006	0.63	0.051	1388	1397	96.1	C_6H_8O	1.08	0.68 (±0.12)	Green, soapy	270 [2]
Nonanal	26.35	0.006	1.13	0.054	1389	1390	142.2	$C_9H_{18}O$	0.90	0.95 (±0.02)	Fatty, waxy, pungent	610 [1]
(*E*)−2-Hexen−1-ol	26.55	0.011	0.62	0.050	1394	1398	100.2	$C_6H_{12}O$	1.00	0.7 (±0.07)	Green, grass	500 [1]
(*E*)−2-Octenal	27.70	0.009	0.40	0.054	1420	1427	126.2	$C_8H_{14}O$	0.95	0.92 (±0.04)	Fatty, nutty	120 [1]
Acetic acid	28.51	0.023	0.24	0.051	1440	1440	60.1	$C_2H_4O_2$	3.62	0.73 (±0.09)	Sour, vinegary	350 [1]
(E,E)−2,4-Heptadienal	29.20	0.006	0.70	0.055	1456	1461	110.2	$C_7H_{10}O$	1.02	0.86 (±0.05)	Fatty, green, oily	710 [1]
α-Copaene	30.55	0.016	2.37	0.052	1489	1491	204.4	$C_{15}H_{24}$	0.78	0.94 (±0.06)	Woody	-
Benzaldeyde	31.59	0.022	0.66	0.051	1513	1524	106.1	C_7H_6O	0.92	0.91 (±0.04)	Almond, burnt sugar, erthy	60 [4]
Popanoic acid	32.15	0.013	0.26	0.055	1526	1534	74.1	$C_3H_6O_2$	2.06	0.81 (±0.05)	Acidic, pungent	720 [4]
(*E*)−2-Nonenal	32.26	0.026	0.91	0.058	1530	1534	140.2	$C_9H_{16}O$	0.92	0.95 (±0.02)	Fatty, green, soapy	140 [1]
1-Octanol	33.01	0.022	0.55	0.055	1548	1553	130.2	$C_8H_{18}O$	0.90	0.86 (±0.03)	Nut, mushroom	27 [2]
Butanoic acid	35.75	0.006	0.25	0.053	1616	1613	88.1	$C_4H_8O_2$	1.60	0.85 (±0.05)	Cheesy, sour	34 [1]
(E,E)-α-Farnesene	40.60	0.015	1.56	0.051	1742	1744	204.4	$C_{15}H_{24}$	0.78	0.78 (±0.03)	Sweet, floral	-
Analytes not quantified												
Hexane	3.58	0.135	0.45	0.049	600	600	86.2	C_6H_{14}			Alkane	
Octane	4.87	0.066	0.99	0.063	800	800	114.2	C_8H_{18}			Solvent	940 [5]
Acetone	5.05	0.017	0.45	0.049	850	845	58.1	C_3H_6O			Pungent	-
2-Methyl butanal	7.08	0.140	0.64	0.073	924	926	86.1	$C_5H_{10}O$			Malty	10 [5]
Ethanol	7.35	0.015	0.37	0.051	928	933	46.1	C_2H_6O			Ethanol-like	-
3,4-Diethyl−1,5-hexadiene (RS/SR)	7.90	0.018	1.21	0.049	946	952	138.2	$C_{10}H_{18}$			-	-
3-Methylbutanal	8.00	0.133	0.72	0.066	939	930	86.1	$C_5H_{10}O$			Malty	13 [5]
3,4-Diethyl−1,5-hexadiene (meso)	8.45	0.145	1.40	0.083	956	956	138.2	$C_{10}H_{18}$			-	-
Pentanal	8.76	0.027	0.75	0.050	975	974	86.1	$C_5H_{10}O$			Almond-like, pungent, malt	150 [4]
(Z)−1-Methoxy−3-hexene	9.74	0.025	1.17	0.050	1007	997	114.2	$C_7H_{14}O$			-	-
(5Z)−3-Ethyl−1,5-octadiene	9.93	0.026	1.69	0.050	1012	1006	138.2	$C_{10}H_{18}$			-	-
(5E)−3-Ethyl−1,5-octadiene	10.47	0.027	1.69	0.049	1026	1032	138.2	$C_{10}H_{18}$			-	-
Toluene	10.73	0.050	0.91	0.057	1029	1036	92.1	C_7H_8			Chemical-like	-
(E,Z)−3,7-Decadiene	12.52	0.028	1.92	0.051	1078	1068	138.2	$C_{10}H_{18}$			-	-
(E,E)−3,7-Decadiene	12.85	0.016	1.89	0.049	1085	1082	138.2	$C_{10}H_{18}$			-	-

(*continued on next page*)

Table 2 (*continued*)

Compound Name	$^{1}D\ t_R$ min	SD	$^{2}D\ t_R$ sec	SD	Exp I^T	Lit. I^T	MW	Formulae	PRRF	β (±SD)	Odor quality	OT (ng/g)*
(Z)−2-Pentenal	13.49	0.017	0.74	0.049	1101	1105	84.1	C_5H_8O			Pungent, apple-like	300 [3]
3-Methylbutyl acetate	14.42	0.053	0.97	0.060	1123	1126	130.2	$C_7H_{14}O_2$			Banana-like, fruity	
2-Heptanone	16.85	0.027	0.98	0.086	1178	1171	130.2	$C_7H_{14}O$			Sweet, fruity	470 [4]
(Z)−2-Hexenal	17.66	0.022	0.82	0.048	1197	1193	98.1	$C_6H_{10}O$			Fruity	-
γ-Terpinene	19.59	0.024	1.71	0.058	1239	1247	98.1	$C_6H_{10}O$			Petrol-like	-
trans-β-Ocimene	19.95	0.006	1.44	0.050	1246	1249	136.2	$C_{10}H_{16}$			Citrus-like, soapy, geranium-like	-
o-Cymene	20.72	0.027	1.42	0.050	1264	1268	134.2	$C_{10}H_{14}$			-	-
3-Hydroxy−2-butanone	21.45	0.021	0.46	0.059	1279	1283	88.1	$C_4H_8O_2$			Buttery	-
2-Octanone	21.50	0.014	1.07	0.052	1280	1284	128.2	$C_8H_{16}O$			Mould, green	510 [4]
(*E*)−3-Hexen−1-ol acetate	22.49	0.020	1.57	0.050	1303	1300	142.2	$C_8H_{14}O_2$			Fruity	-
Dioxa−1,6-spiro[4.5]decane	23.54	0.032	1.44	0.077	1326	-	142.2	$C_8H_{14}O_2$			-	-
3-Methyl-cyclopentanol	23.68	0.034	0.65	0.064	1329	1342	100.1	$C_6H_{12}O$			-	-
(*E*)−3-Hexen−1-ol	24.78	0.031	0.47	0.044	1354	1352	100.2	$C_6H_{12}O$			green, grassy	-
Methoxymethyl-benzene	26.05	0.006	0.93	0.053	1382	1382	122.2	$C_8H_{10}O$			-	-
(Z)−2-Hexen−1-ol	26.62	0.035	0.43	0.029	1396	1405	101.2	$C_6H_{12}O$			Green, grass	-
Dimethyl Sulfoxide	32.60	0.015	0.49	0.051	1537	1549	78.1	C_2H_6OS			-	-
3-Methyl−2-hexen−4-one	34.60	0.000	0.47	0.054	1587	1586	112.2	$C_7H_{12}O$			-	-
Benzoic acid, methyl ester	35.66	0.028	0.75	0.060	1613	1614	136.1	C_8H_8O			Starfruit-like, sweet	-
1,4-Cyclohex−2-enedion	39.93	0.025	0.53	0.047	1725	-	110.1	$C_6H_6O_2$			-	-
Pentanoic acid	39.93	0.024	0.25	0.050	1725	1730	102.1	$C_5H_{10}O_2$			Sweaty, fruity	400 [1]
2,3-Dimethylbenzaldehyde	39.99	0.052	0.66	0.049	1725	1736	134.2	$C_9H_{10}O$			-	-
5-ethyl−2(5 H)-Furanone	40.73	0.024	0.51	0.045	1746	1757	112.1	$C_6H_8O_2$			Sweet, spicy	-
Methyl salicylate	41.53	0.029	0.72	0.046	1768	1765	152.1	$C_8H_8O_3$			Fatty, tallowy, terpene-like	-
2-(2-butoxyethoxy)Ethanol	42.09	0.024	0.55	0.054	1783	1786	162.2	$C_8H_{18}O_3$			-	-
Hexanoic acid	43.84	0.022	0.26	0.049	1831	1839	116.2	$C_6H_{12}O_2$			Goat-like, sweaty	460 [1]
Benzyl alcohol	44.78	0.025	0.38	0.046	1858	1857	108.1	C_7H_8O			Sweet, fruity	-
Phenylethyl alcohol	46.02	0.029	0.44	0.053	1893	1904	122.2	$C_8H_{10}O$			Floral, honey-like	-
(*E*)−2-Hexenoic acid	47.89	0.022	0.30	0.047	1947	1941	114.1	$C_6H_{10}O_2$			-	-
Phenol	49.10	0.006	0.26	0.054	1983	1987	94.1	C_6H_6O			Ink-like, phenolic	-
Benzoic acid, 2-methoxy-, methyl ester	51.31	0.045	0.66	0.060	2049	2032	166.2	$C_9H_{10}O_3$			-	-

*Odor threshold references: [1] (Neugebauer et al., 2020); [2] (Van Gemert, 2003); [3] (Luna et al., 2006); [4] (Squara et al., 2023a); [5] (Purcaro et al., 2014)

Retention times ($^{1}t_R$ min, $^{2}t_R$ sec), standard deviation (SD), experimental and tabulated linear retention indexes (I^T), molecular weight (MW), predicted FID relative retention factor (RRF), decay function (β), odor threshold (OT) in ng/g reliable peaks is used for re-aligning chromatograms in the temporal domain.

(c) Align chromatograms of representative samples to the average retention times of the reliable peaks, then sum/fuse them into a composite chromatogram.

(d) Generate a comprehensive untargeted feature template from the composite chromatogram, including reliable peaks and peak regions defined by the footprint of all detected peaks (Reichenbach et al., 2013).

This process was used to generate a composite image from a selection of sample chromatograms representative of the different expressions of functional variables. From the composite image, a feature template consisting of 190 UT features (*i.e.*, peak regions corresponding to untargeted and targeted components) was defined and applied to single chromatogram images (*i.e*, samples and replicates) for reliable tracking and alignment of features. The list of targeted features is reported in Table 2 together with chemical names, ^{1}D and ^{2}D retention times ($^{1}t_R$; $^{2}t_R$), experimental I^T, tabulated I^T, odor quality, and odor thresholds (OTs) in oil as from reference literature.

2.6.2. *Data acquisition and statistical analysis*

Raw chromatographic data were acquired by MassHunter Workstation (Agilent Technologies). Raw data were processed by GC Image™ V2020 r1.2 suite (GC Image, LLC Lincoln, NE, USA).

Statistical analysis and chemometrics were performed using GC Investigator™ (GC Image), XLSTAT statistical and data analysis solution (Addinsoft 2020, New York, USA), and Microsoft Office Excel 2016 (Microsoft, Redmond, WA USA).

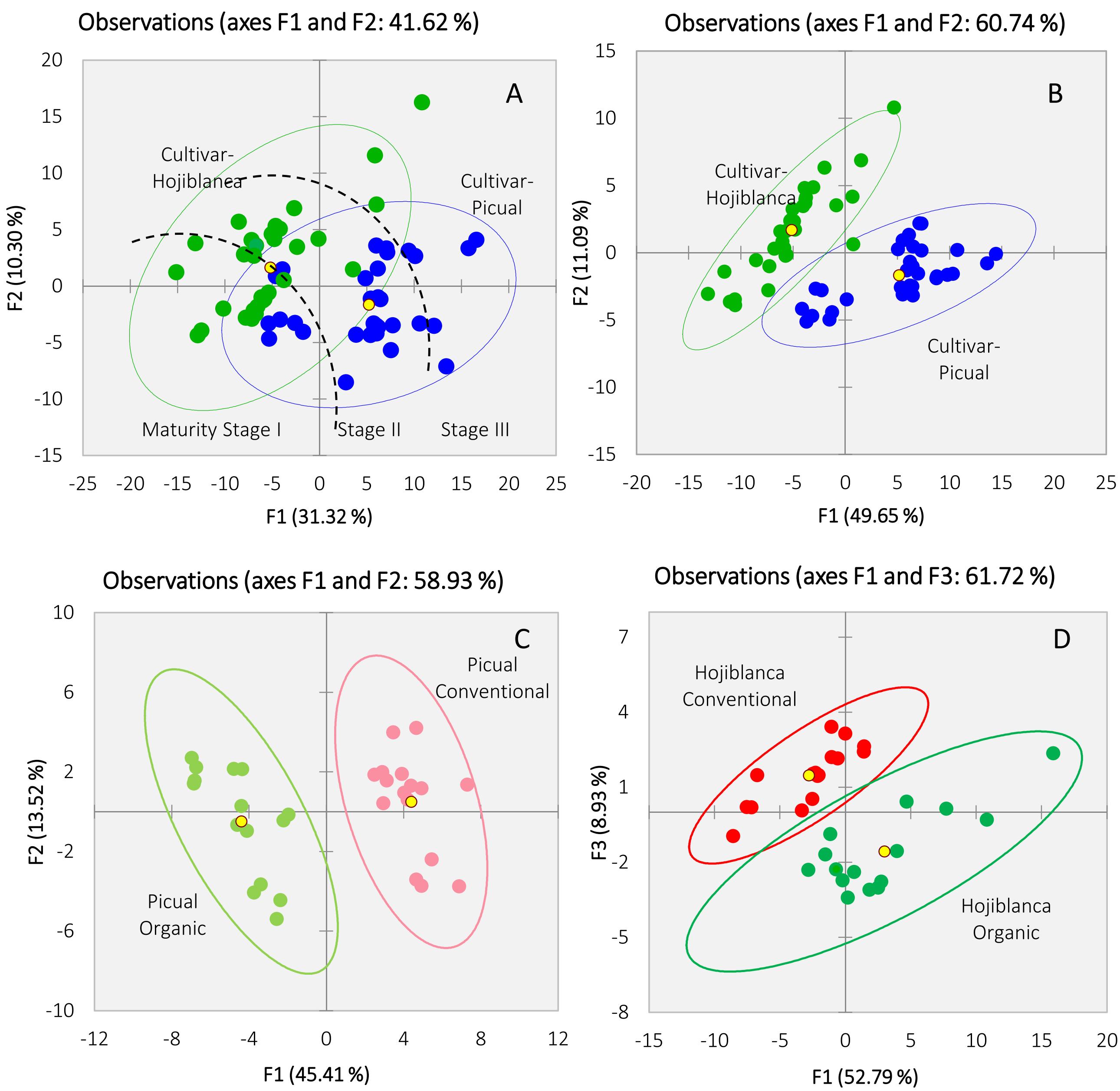

Fig. 1. PCA on untargeted and targeted (UT) peak features (analytes) % responses; ellipses set at 95 % of confidence level, show cultivar natural clusters. In Fig. 1**A** all 190 UT features were computed for all samples; dotted lines indicate Maturity Stage evolution according to sample loadings. Fig. 1**B** results from Fisher ratio features reduction considering cultivar discrimination (*Picual vs. Hojiblanca*) the PCA refers to 92 UT peaks with a F > 10. Fig. 1**C** Fisher ratio features reduction considering cultivation type on *Picual* oils (organic *vs.* conventional) the PCA refers to 49 UT peaks with a F > 10. Fig. 1**D** Fisher ratio features reduction considering cultivation type on *Hojiblanca* oils (organic *vs.* conventional) the PCA refers to 49 UT peaks with a F > 10.

3. Results and discussion

EVO oil *volatilome* encrypts information on many key-quality variables (*e.g.*, olives cultivar and geographical origin, cultivation methodologies, olives harvesting stage, oil auto-oxidation level, and sensory profile (Luna et al., 2006; Melucci et al., 2016; Morales et al., 2005), by GC×GC it was possible to consistently monitor more than 190 *reliable* peak features (including targeted and untargeted compounds (Magagna et al., 2016)), of which 84 were identified according to [1]D retention index ($I^T \pm 10$ units of tolerance) combined to EI-MS spectral similarity (Direct Match Factor threshold >900) with commercial and in-house databases. Table 2 lists putatively identified compounds (*i.e.*, targeted features) and potent odorants together with retention data, odor qualities, odor thresholds (OTs) in oil, and FID predicted RRFs calculated according to hexanal as reference internal standard. Table S1, provided as supplementary material, lists targeted and untargeted features comprehensively mapping the detectable volatilome of analyzed samples.

As a first approach to understanding the existence of natural samples' clusters according to volatile patterns distribution, unsupervised exploration by Principal Component Analysis (PCA) was conducted. Fig. 1**A** illustrates PCA results obtained by examining the data matrix including untargeted and targeted (UT) peak features (analytes) % responses for all *Picual* oils (blue indicators) and *Hojiblanca* oils (green indicators) (190 × 32 – features × samples replicates). It represents a total explained variance of 41.62 % (PC1 and PC2) and, along the PC1 with 31.32 % of the total explained variance, the two *cultivars* form natural clusters as shown by ellipses, set at 95 % confidence. Besides some outliers, the cultivars' chemical signature is almost clear confirming that the volatile fraction *in toto* encrypts information on olives' chemotype useful for their discrimination. This result agrees with other authors who observed that botanical traits have the most influence on the volatile profile of EVOOs (Ríos-Reina et al., 2021).

Nevertheless, a certain degree of overlap between the two clusters suggests that for some samples the volatilome has similar composition, possibly due to the influence of the harvest region with its common pedoclimatic and soil characteristics. However, the simultaneous presence of many variables influencing the volatiles' qualitative and quantitative profiles, such as the ripening stage of the fruits, might have a confounding effect. According to previous investigations, the ripening stage of the fruit was, after the variety/cultivar, the most influential factor on the volatile fraction (Magagna et al., 2016; Ríos-Reina et al., 2021; Stilo et al., 2021b). A deeper observation of the samples' distribution on the Cartesian plane (Fig. 1A), indicates a clear trend along PC1 with oils obtained by olives harvested at Stage I characterized by lower loadings and those obtained by olives at Stage III with higher values. This is in accordance to the increasing ripening index as reported in Table 1. Analytes responsible for this distribution are, among the others: (*E,E*)-α-farnesene, (5*Z*)-3-ethyl-1,5-octadiene, octane, (*E,Z*)-3,7-decadiene, 3,4-diethyl-1,5-hexadiene (meso), 3,4-diethyl-1,5-hexadiene (RS+SR), (*Z*)-3-hexenal, (*Z*)-2-hexenal, phenylethyl alcohol, 6-methyl-5-hepten-2-one, (*Z*)-3-hexen-1-ol, (*E,E*)-3,7-decadiene, 1-penten-3-one, 1-penten-3-ol, and nonanal. Results are in keeping with previous studies on ripening indices (Angerosa et al., 1998; Magagna et al., 2016; Stilo et al., 2021b).

To identify cultivar discriminating variables, the UT features % response data were filtered by the Fisher ratio (F) value criterion (Schöneich et al., 2022). Since F_{crit} (1,15) with α=0.05 is 4.54, UT variables with a $F_{calc} > 10$ (arbitrarily set above the critical value) were retained (n= 92 UT features), and by applying this features selection criterion, the resulting PCA shown in Fig. 1**B** clearly clusters samples according to the cultivar type. The total explained variance raises 60.74 % (PC1 and PC2) and samples are now independently clustered and discriminated along the PC1 (49.65 % of the total explained variance). F values referred to UT features discriminating cultivars are reported in Table S1.

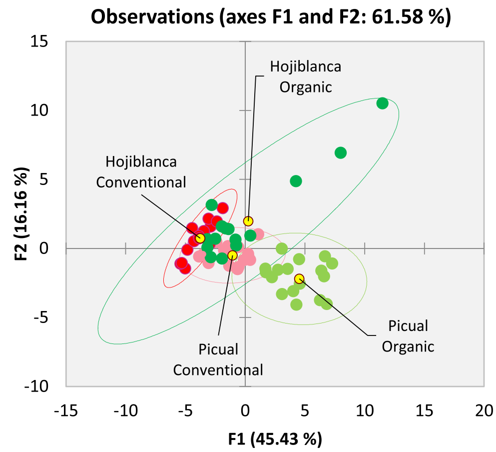

Fig. 2. PCA on untargeted and targeted (UT) peak features (analytes) % responses after Fisher ratio reduction according to classes organic *vs.* conventional and F>10 (35 UT features). Red indicators are for *Hojiblanca* conventional, pink is for *Hojiblanca* organic, dark green is for *Picual* conventional, and light green is for *Picual* organic.

The impact of cultivation methodology (*i.e.*, conventional *vs.* organic) was then explored. The PCA was conducted on the data matrix including UT peak features (analytes) % responses with an F value > 10 for *organic vs. conventional* classes. F values discriminating cultivation methods are listed in Table S1. PCA loadings plot based on the 35 features with F>10 is shown in Fig. 2. Results confirm the major role played by cultivar traits on the volatilome expression, with a total explained variance of 61.58 % the first two PCs do not show any independent clustering for organic and conventional cultivated olive tree samples. However, for *Picual* oils, a distinction of the two sub-groups along the PC1 appears (pink and light green indicators).

Due to the concurrent effect of external variables, as cultivar and cultivation practices, the identification of marker volatiles for organic cultivation was conducted on samples belonging to the same cultivar with the aid of supervised strategies.

3.1. *Picual olive oils: organic vs. conventional cultivation diagnostic volatiles signature*

Considering the *Picual* cultivar, the unsupervised statistics on the two cultivation methodologies ($F_{calc} > 5$ for organic *vs.* conventional classes) show two independent groups along PC1, with a total explained variance of 58.93 % (*e.g.*, Fig. 1**C**). At the same time, heatmap visualization (*e.g.*, Fig. S1A) captures diagnostic patterns of UT volatiles and by hierarchical clustering (HC) based on Pearson correlation of variables form two independent clusters corresponding to cultivation methodologies. Sub-clusters are coherent with biological and technical replicates.

To select statistically relevant yet informative compounds related to cultivation methodologies on *Picual* oils, supervised analysis by partial least squares-discriminant analysis (PLS-DA) was adopted (Lee et al., 2018). The model was developed on an estimation set consisting of 70 % randomly selected measures/samples (20 samples over 28) and validated on the residual 30 % (8 samples over 28). Model performances, after 10 reiterations, were good referring of a 98 % of correctness. The variable importance in the projection scores (VIPs) were used to identify meaningful variables, resulting from the classification model on *Picual* EVO oils conventional *vs.* organic, they are visualized as histogram in Fig. 3 (orange bars). **Table sS2** lists VIPs values together with relative standard deviation. Discriminant analytes between the cultivation methodologies belongs to the class of potent odorants with 2-pentanol, 1-hexanol, (*Z*)-3-hexen-1-ol likely discriminating the *Hojiblanca* cultivar from the *Picual* which has a characteristic pattern of odorants with (*E*)-2-penten-1-ol, heptanal, and (*E*)-2-pentenal. Variables showing

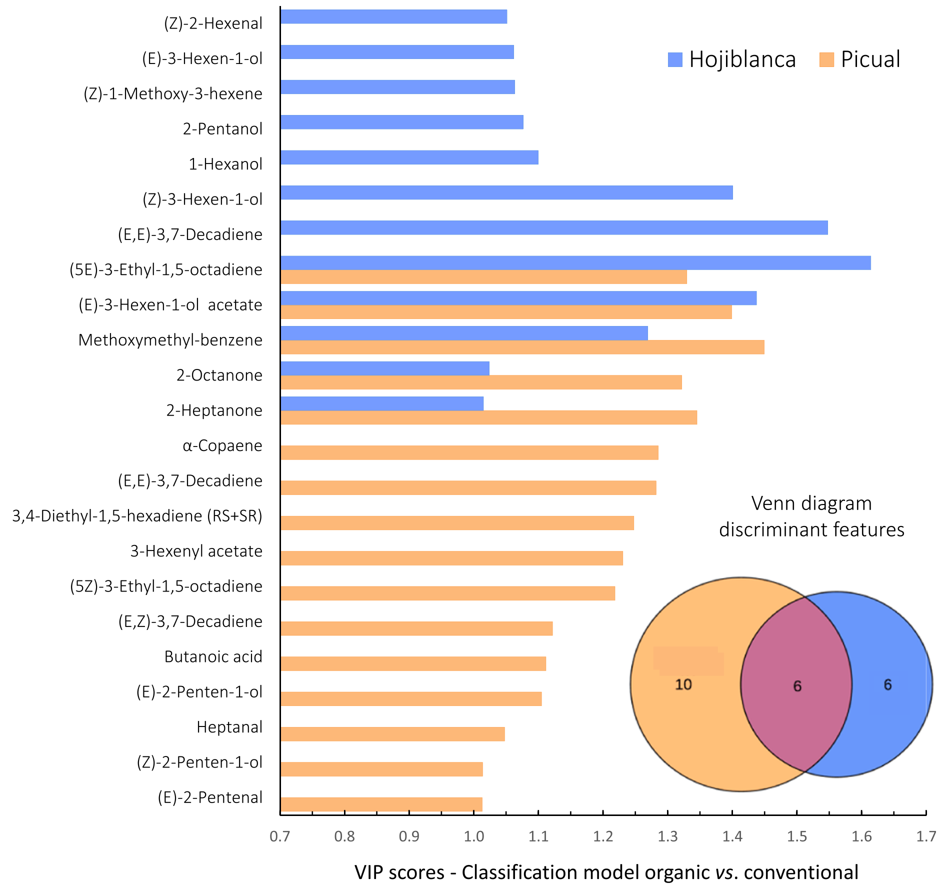

Fig. 3. **:** Histogram showing the VIPs values derived by the PLS-DA classification models conventional *vs*. organic classes. Blue bars are for *Hojiblanca* and orange bars for *Picual*. The Venn diagram visualizes the number of targeted analytes (features) in common (n=6) or unique (n=10 for *Picual* and n=6 for *Hojiblanca*) for the two cultivars.

a role in the discrimination are also validated by HC results. Of interest are carbonyls [2-heptanone, 2-octanone, heptanal, (*E*)-2-pentenal] and alkenes [(5*E*)-3-ethyl-1,5-octadiene, (*E,E*)-3,7-decadiene, 3,4-diethyl-1,5-hexadiene (RS+SR), (5*Z*)-3-ethyl-1,5-octadiene, (*E,Z*)-3,7-decadiene]; this last class of chemicals known for their correlation with olives ripening (Angerosa et al., 1998).

The relative distribution of the most informative variables is illustrated by box-plots in Fig. 4. Analytes with higher % response in conventional cultivation are methoxymethyl-benzene [α-methylbenzyl ether already documented(da Silva et al., 2012)], (5*E*)-3-ethyl-1,5-octadiene, (5*Z*)-3-ethyl-1,5-octadiene, 3,4-diethyl-1,5-hexadiene (RS+SR), (*E,E*)-3,7-decadiene, (*E,Z*)-3,7-decadiene, (*Z*)-3-hexenyl acetate, (*E*)-3-hexen-1-ol acetate, and α-copaene. Compounds with an opposite trend are 2-heptanone, 2-octanone, and butanoic acid.

According to the literature, (5*E*)-3-ethyl-1,5-octadiene, (5*Z*)- 3-ethyl-1,5-octadiene, 3,4-diethyl-1,5-hexadiene (RS or SR), (*E*, *E*)-3,7-decadiene and (*E*, *Z*)-3,7-decadiene have been found in *Picual* EVOOs and were considered as markers of early ripening stages, independently of the variety/cultivar and geographical origin (Stilo et al., 2021b). In addition, 3-ethyl-1,5-octadiene, a compound deriving from the lipoxygenase pathway, recorded significant variations according to the cultivar and/or environmental conditions on olive tree growing (Kosma et al., 2020).

(*Z*)-3-Hexenyl acetate is a compound responsible for sensory attributes as *green leaves*, and it was shown to be relevant in the differentiation between non-defective (EVO) and defective (non-EVO) olive oil samples, being present in higher concentration in EVOO samples (Ríos-Reina et al., 2021). Non-defective (EVO) olive oil is of the highest quality and free of sensory defects, while defective (non-EVO) olive oil does not meet these strict standards and is of lower quality. In addition, aligned with the current evidence, (*Z*)-3-hexenyl acetate was differentially distributed in organic and conventional samples considered in a previous study (Jurado-Campos et al., 2021).

α-Copaene is a mono-unsaturated sesquiterpene that has already been detected in Spanish oils obtained from olives of the *Hojiblanca* cultivar and *Picual* (Bortolomeazzi et al., 2001; Guinda et al., 1996), and together with α-muurolene and α-farnesene were the terpenes that aided the discrimination between extra virgin olive oil according to the cultivar and geographical origin (Bubola et al., 2014; Lukić et al., 2018; Zunin et al., 2005) and they have been suggested as markers of olive oil differentiation (Kosma et al., 2020). According to the literature, butanoic acid has been associated with *vinegary*, *musty*, and *rancid* defects (Angerosa, 2002; Cecchi et al., 2019).

3.2. *Hojiblanca olive oils: organic vs. conventional cultivation diagnostic volatiles signature*

A first exploratory approach was conducted on *Hojiblanca* samples considering the cultivation practice. Fig. 1**D** shows the PCA resulting from UT features filtered by Fisher ratio value ($F_{calc} > 10$). Samples, as expected, are clearly clustered according to cultivation practice and the total explained variance for PC1-PC3 achieves 61.72 %. At the same time, heatmap visualization (provided as supplementary material Fig. S1) captures diagnostic patterns of UT volatiles and by hierarchical clustering (HC) based on Pearson correlation of variables form two independent clusters corresponding to cultivation methodologies. Subclusters are coherent with biological and technical replicates. Chemical variables highlighted by PLS-DA as distinctive for the cultivation

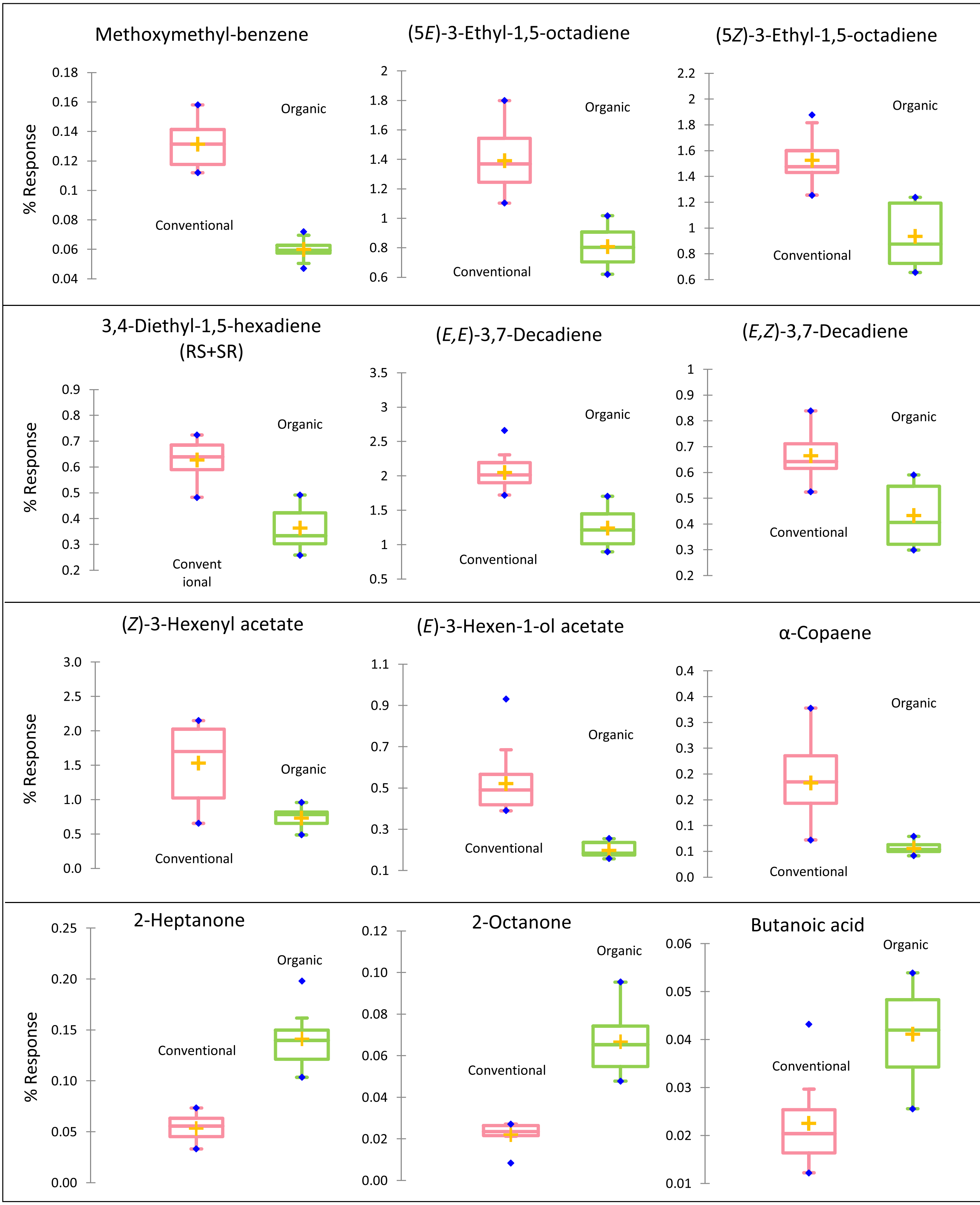

Fig. 4. Box plots of discriminant compounds (from PLS-DA modeling) between organic and conventional cultivation on *Picual* cultivar samples. Green boxes represent data from organic farming (mean/median) while pink boxes are for conventional farming (mean/median).

methods in *Hojiblanca* EVO oil (VIPs > 1) are listed in Table S2 together with VIPs value and relative standard deviation. The model was developed on an estimation set of samples consisting of 70 % randomly selected measures/samples (20 samples over 28) and validated on the residual 30 % of samples (8 samples over 28). Model performances, after 10 reiterations, were good referring of a 90 % of correctness.

Some analytes capable of discriminating between two different cultivation practices in *Hojiblanca* EVO oils were already found in *Picual* samples, they are visualized with a Venn diagram in Fig. 3 together with a the histogram showing the VIPs values derived by the classification model (blue bars for *Hojiblanca* and orange bars for *Picual*). Fig. S2 illustrates with box plots the relative distribution in all sample classes for (5*E*)-3-ethyl-1,5-octadiene, (*E,E*)-3,7-decadiene, and (*E*)-3-hexen-1-ol, acetate. The trend for *(5E)*-3-ethyl-1,5-octadiene and *(E,E)*-3,7-decadiene is similar in both cultivars; they are more abundant in samples from conventional practices than in organic ones. However, a different behavior was observed for (*E*)-3-hexen-1-ol acetate that was more abundant in the organic oils from *Hojiblanca* and lower in the *Picual*.

Hojiblanca EVO oil samples showed additional markers for organic cultivation, relative distribution is shown in box-plots of Fig. S3; they are (*Z*)-3-hexen-1-ol and 2-pentanol. These analytes were more abundant in organic samples. According to previous studies those cultivars with the highest percentage of C6 alcohols, mainly represented by (*Z*)-3-hexen-1-ol were the same cultivars with the highest content of (*Z*)-3-hexenyl acetate. In our case, (*Z*)-3-hexen-1-ol and (*E*)-3-hexen-1-ol acetate resulted to be more abundant in organic *Hojiblanca* samples (Cecchi et al., 2022). This could be due to a different expression/activity of the isomerase, an enzyme within the Lipoxygenase (LOX) pathway.

3.3. Accurate quantification of aroma active compounds: artificial Intelligence smelling

Many of the informative compounds identified in the first part of the study have minimal or no sensorial impact, therefore they would not be perceived by a consumer during oil consumption. In this perspective, the possibility to highlight possible sensory-relevant compositional differences between oils obtained from olives harvested with organic practices assumes a great interest from a marketing perspective. If the farming does not have an impact on the typical yet univocal sensory trait of the cultivar, consumer's experience is likely aligned to expectations. However, to proceed in this direction, accurate quantification of sensory active analytes is required (Jeleń and Wieczorek, 2023; Sgorbini et al., 2019).

This step of the study performed accurate quantification of an extended list of target analytes to provide a solid foundation for samples' aroma blueprinting (Purcaro et al., 2014; Stilo et al., 2023; Stilo et al., 2021). Moreover, by targeting key-aroma compounds elucidated by sensomics (Neugebauer et al., 2020), the implementation of the Artificial Intelligence smelling concept was possible as the prediction of oils aroma features was reliable and objective (Nicolotti et al., 2019).

The selection of analytes for accurate quantification was driven by their impacting sensory qualities (potent odorants and key aromas) in the definition of high-quality EVO oils aroma and/or information role already described in reference literature (Luna et al., 2006). Alcohols and carbonyls contribute to *fresh-green* and *fruity* notes that are considered within the positive attributes mandatory for EVO oils qualification (International Olive Oil Council, 2009). They are (*E*)-2-pentenal, (*Z*)-2-penten-1-ol, 1-penten-3-ol, (*E,E*)-2,4-hexadienal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, hexanal, and 1-hexanol. Compounds responsible for sensorial defects were also monitored: heptanal, (*E*)-2-octenal, and (*E*)-2-nonenal are linear saturated and unsaturated aldehydes deriving from fatty acids hydroperoxides cleavage. They have *rancid* and *fatty* notes and provide information on the autoxidation process and shelf-life evolution (Cecchi et al., 2019; Neugebauer et al., 2020). The targeted compounds list was completed by analytes correlated to peculiar sensory defects described in the International Olive Council standards (International Olive Oil Council, 2018; Purcaro et al., 2014; Stilo et al., 2021): (*E*)-2-penten-1-ol, with *mushroom-like* and *earthy* sensations; 2-pentanol, related to *musty* and *fermented* perception; and ethyl acetate, responsible for the *winey* note.

Other compounds, deriving from the LOX pathway, are 1-penten-3-one, hexyl acetate, *(Z)*-3-hexenal, (*Z*)-3-hexenyl acetate, and (*E*)-2-hexen-1-ol, all correlated to *green* and *fruity* notes (Reboredo-Rodríguez et al., 2013). Terpenes and terpenoids, as limonene, α-pinene, β-pinene, δ-3-carene, eucalyptol, α-copaene, and *(E,E)*-α-farnesene are mainly known to be cultivar and/or geographical markers (Quintanilla-Casas et al., 2020a) bringing *herbal*, *pine*, and *citrus-like* notes to the overall perception (Quintanilla-Casas et al., 2020c, 2020b; Stilo et al., 2023; Stilo et al., 2021). 6-Methyl-5-hepten-2-one is a ripening indicator with a *fruity-like* odor and propanal is present in high-quality EVOOs and described as *fresh*, *fruity,* and *malty* notes (Luna et al., 2006). Acetic acid, propanoic acid, and butanoic acid are responsible for *vinegary*, *musty*, and *rancid* defects (Neugebauer et al., 2020; Purcaro et al., 2014); 1-octanol is usually found in oxidized samples responsible for a *mushroom-like* odor; and benzaldehyde imparts *moldy* and *earthy* notes. Finally, octanal, nonanal, (*E*)-2-heptenal, and (*E,E*)-2,4-heptadienal contribute to *rancid* and *fatty* sensations.

The analytical strategy involving GC×GC with parallel MS and FID detection offers several advantages for accurate quantification and analyte identity confirmation. MS detection provides suitable specificity through the selection of specific ion traces in case of co-elutions, while FID offers stable and structure-specific response factors over a wide range of concentrations. This allows the use of FID-predicted RRFs, based on combustion enthalpies and molecular structure, for accurate quantification without external calibration. In contrast, 1D-GC, with its lower peak capacity, fails to achieve suitable chromatographic resolution for applying FID response factors. Fig. S4 shows the contour plot of a *Picual* sample (EVOO_3 conventional cultivation, ripening stage III), while enlarged areas highlight elution regions with critical pairs where 1D-GC would fail in providing full/suitable resolution for accurate quantification.

Quantified analytes showed an exponential decay in multiple consecutive extractions from the HS (MHS-SPME approach) indicating that they were not saturating the HS [*i.e.*, HS linearity range (Kolb and Ettre, 2006)]. The use of a multicomponent fiber, *i.e.*, CAR/PDMS/DVB, that mixes partition and adsorption phenomena prevents a correct application of non-exhaustive extraction approaches and consequently the possibility to adopt external calibration. Once the HS linearity for selected analytes was verified, their quantification was by predicted RRFs based on combustion enthalpies. **Equation 1** illustrates how to calculate RRFs (De Saint Laumer et al., 2015).

$$RRF = 10^3 * \left(\frac{MW_i}{MW_{IS}}\right) * (-61.3 + 88.8\eta_C + 18.7\eta_H - 41.3\eta_O + 6.4\eta_N + 64.0\eta_S - 20.2\eta_F - 23.5\eta_{Cl} - 10.2\eta_{Br} - 1.75\eta_I + 127\eta_{benz})^{-1}$$

where η_C, η_H, η_O, η_N, η_S, η_F, η_{Cl}, η_{Br}, η_I, and η_{benz} correspond to the number of carbon, hydrogen, oxygen, nitrogen, sulfur, fluorine, chlorine, bromine, and iodine atoms, and the number of benzene rings; and MW_i and MW_{IS} are the molecular weights of the analyte *i* and the *IS* adopted for the development of the reference model (De Saint Laumer et al., 2015). In this study, quantification was achieved by considering the analytes' response (*i.e.*, 2D peak volume), as resulting by FID signal, and its predicted RRF calculated with **Eq. 1.** To align combustion enthalpies *versus* hexanal, here taken as internal standard and accurately quantified by external calibration, the analyte-specific RRF was corrected to the hexanal/methyl octanoate ratio ($RRF_{i.\ hexanal} = RRF_{i.\ methyl\ octanoate}/0.967$) (Cachet et al., 2016). RRFs are listed in Table 2 together with analytes molecular weights (g/mol), formulae, decay constant β, odor qualities, and odor thresholds (OTs) in oil.

The quantification results are reported in Table S3; the analytes' amount in the oil samples is expressed in ng/g (mean value over four

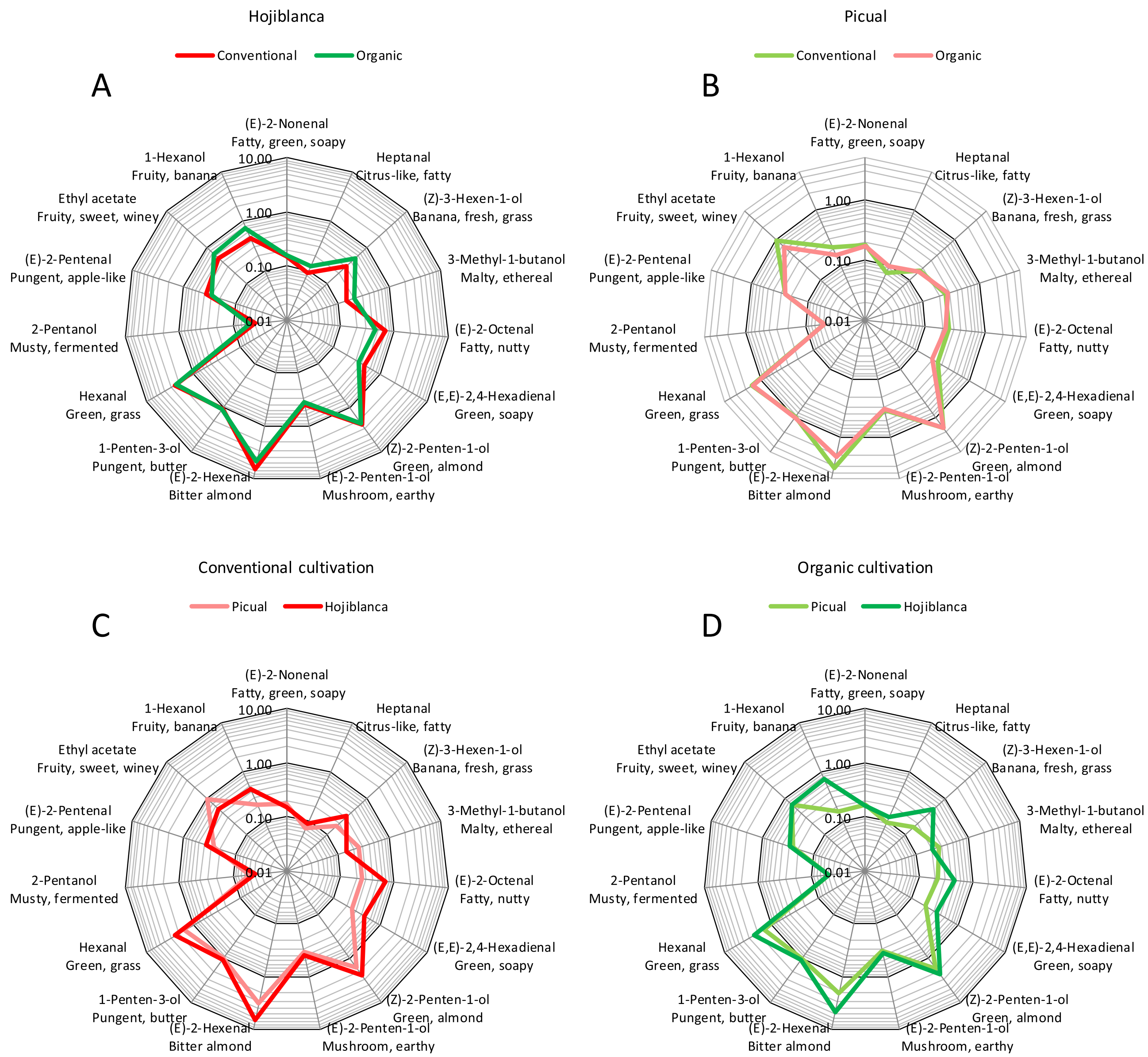

Fig. 5. Spider diagrams illustrating OAVs in log10 scale for the most potent odorants. Cultivation methods are compared for *Hojiblanca* (Fig. 5**A**) and *Picual* (Fig. 5**B**) while cultivar differences on aroma blueprint are highlighted in Fig. 5**C** for the conventional cultivation method and in Fig. 5**D** for the organic one.

replicates) accompanied by absolute uncertainty.

3.4. *Aroma blueprinting of organic extra-virgin olive oils*

Based on quantification results, odor activity values (OAVs), as the ratio between the odorant amount and its odor threshold (OT) in oil, were calculated therefore obtaining a more realistic picture of the actual contribution to the sensory profile of the most potent odorants on the overall EVO oils aroma, *i.e.* the aroma blueprint (Granvogl and Schieberle, 2022; Neugebauer et al., 2020).

In this study, we referred to both known key odorants qualified through the sensomic protocol (Neugebauer et al., 2020) and potent odorants highlighted in the reference literature. They are: (*E*)-2-pentenal, (*E*)-2-penten-1-ol, (*Z*)-2-penten-1-ol, 1-penten-3-ol, 3-methyl-1-butanol, 2-pentanol, ethyl acetate, (*E,E*)-2,4-hexadienal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, hexanal, 1-hexanol, heptanal, (*E*)-2-octenal, and (*E*)-2-nonenal (Aparicio et al., 2013; Kalua et al., 2007; Morales et al., 2005; Quintanilla-Casas et al., 2020c).

Spider-diagrams in Fig. 5**A-C**, evoking descriptive sensory analysis results, show the EVO oils aroma blueprint by plotting OAVs in $\log_{10}$ scale for selected potent odorants. In Fig. 5**A-B** spider diagrams show blueprint differences between cultivation practices on *Hojiblanca* and *Picual* cultivars observed separately while Fig. 5**C-D** show the cultivar aroma blueprint as a function of olives harvest methodology. To note, aroma blueprinting supports effective comparative analysis of samples based on their aroma code but cannot replace a human panel in expressing preferences or likeness.

Considering the impact of cultivation methods on the *Picual* cultivar, the profiles between organic and conventional harvesting are nearly identical. However, discriminating analytes identified through PLS-DA modeling (*Picual* organic vs. conventional, VIPs >1) showed lower absolute amounts of 1-hexanol (-29 %), (*E*)-2-hexenal (-35 %), and (*E,E*)-2,4-hexadienal (-25 %) in oils from organically cultivated olives. For the *Hojiblanca* samples, the most significant differences (PLS-DA modeling, *Hojiblanca* organic vs. conventional, VIPs >1) were a 64 % higher concentration of 1-hexanol in organic samples. Other notable odorants with significant quantitative differences were (*Z*)-3-hexen-1-ol (+57 %) and (*E*)-2-octenal (-34 %). Despite these statistically relevant variations, all were below OAV unit values, suggesting no perceivable differences in the overall aroma profile.

On the other hand, by comparing the impact of olives' harvesting practices on the cultivar aroma identity, it appeared that the organic cultivation methodology had an effect in emphasizing concentration differences of the cultivar's specific blueprint odorants. In conventional cultivation, *Hojiblanca* samples showed higher amounts of (*E*)-2-octenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol (+170.54 %, +107.74 %, +85.58 % respectively) compared to the *Picual* oils (Fig. 5**C**) likely impacting on the *fatty*, *fresh* and *green*, and *bitter almond* notes. In case of organic cultivation, potent odorants differences were even higher (Fig. 5**D**). The (*E*)-2-octenal was +99 %, (*E*)-2-hexenal +122 %, and (*Z*)-3-hexen-1-ol +207 % in *Hojiblanca* vs. *Picual*.

4. Conclusions

The volatilome of extra-virgin olive oil (EVOO) effectively encodes critical quality parameters such as cultivar, geographical origin, cultivation methodologies, harvesting stage, oxidation level, and sensory profile. Through comprehensive analysis using GC×GC, over 190 reliable peak features were monitored, with 84 compounds identified. Principal Component Analysis (PCA) revealed distinct clusters for *Picual* and *Hojiblanca* cultivars, demonstrating the significance of botanical traits in the volatile profiles. However, some overlap indicated shared influences, likely from regional pedoclimatic conditions and fruit ripening stages.

Filtering variables by Fisher ratio and further PCA provided clearer discrimination, especially for cultivar type, explaining up to 60.74 % of the variance. Cultivation methodologies (organic vs. conventional) were less distinguishable, but for *Picual* oils, significant differences were observed using supervised strategies as PLS-DA, which identified key volatiles with high accuracy.

Diagnostic volatiles for cultivar discrimination, 125 UT features above a F>4, included alkenes [(*E,E*)-3,7-decadiene, (5*E*)-3-ethyl-1,5-octadiene, 3,4-diethyl-1,5-hexadiene (RS+SR), 3,4-diethyl-1,5-hexadiene (meso), (5*Z*)-3-ethyl-1,5-octadiene, and (*E,Z*)-3,7-decadiene), carbonyls [1-penten-3-one, (*E*)-2-octenal, (*E*)-2-nonenal, (*Z*)-2-hexenal, 3-hydroxy-2-butanone, (*Z*)-3-hexenal, 3-methylbutanal, (*E*)-2-hexenal, 2-methyl butanal, and 6-methyl-5-hepten-2-one], and alcohols [2-pentanol, (*Z*)-2-penten-1-ol, (*Z*)-3-hexen-1-ol, 1-hexanol, 3-methyl-1-butanol, 1-penten-3-ol, and (*E*)-2-hexen-1-ol] reflecting LOX pathway activity while also intercepting maturation stages likely aligned between cultivars. Accurate quantification of aroma-active compounds confirmed that the two cultivars have distinctive blueprint traits due to the different OAVs for (*E*)-2-octenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol. Differences that were minimal between the cultivation practices suggesting that consumer-would probably not perceived any difference between organic and conventional products.

In conclusion, the study underscores the complex interplay of factors shaping EVOO's volatile profile. While cultivar remains the dominant influence, cultivation practices and ripening stages also play crucial roles. The findings highlight the potential for using volatilome analysis in EVOO quality control and marketing, ensuring alignment of consumer expectations with sensory experiences.

CRediT authorship contribution statement

Erica Liberto: Writing – review & editing, Validation, Supervision. **Fulvia Trapani:** Writing – review & editing, Data curation. **Brígida Jiménez-Herrera:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Angelica Fina:** Writing – review & editing, Formal analysis. **Andrea Caratti:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Raquel L Callejon:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Lourdes Arce:** Writing – review & editing, Writing – original draft, Supervision, Investigation. **Chiara Emilia Irma Cordero:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors Andrea Caratti, Angelica Fina, Fulvia Trapani, Erica Liberto, Brígida Jiménez-Herrera, Lourdes Arce, Raquel M Callejón, Chiara Cordero 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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106975.

Data availability

Data will be made available on request.

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