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Comparison of lipid profile of Italian Extra Virgin Olive Oils by using rapid chromatographic approaches

Marianna Oteri [a], Francesca Rigano [b,*], Giuseppe Micalizzi [b], Monica Casale [c], Cristina Malegori [c], Paola Dugo [b,d], Luigi Mondello [b,d,e,f]

[a] Department of Veterinary Sciences, Section of Animal Production, University of Messina, Messina I-98168, Italy
[b] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina I-98168, Italy
[c] Department of Pharmacy, University of Genoa, Genoa I-16148, Italy
[d] Chromaleont s.r.l., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina I-98168, Italy
[e] Unit of Food Science and Nutrition, Department of Medicine, University Campus Bio-Medico of Rome, Rome I-00128, Italy
[f] BeSep s.r.l., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina I-98168, Italy

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ABSTRACT

The following research focuses on the characterization of the major components of Extra Virgin Olive Oils (EVOOs), *viz.* triacylglycerols (TAGs), analyzed both in their intact form and after conversion into fatty acid methyl esters (FAMEs). Samples coming from different Italian regions were evaluated in order to obtain quali/quantitative information, which can be correlated to the cultivar, label and/or place of origin. Two fast chromatographic methods were developed for the determination of TAGs and FAMEs in 200 EVOOs by liquid and gas chromatography, respectively. In both cases, a mass spectrometry library with embedded Linear Retention Indices was explored for the identification of single molecular species. Principal components analysis was performed as a multivariate display method; the score plots showed a clear demarcation between southern and central-northern regions, as well as a discrimination based on the variety of olives. In particular, southern EVOOs were satisfactorily differentiated on the basis of the cultivar, while the central-northern EVOOs showed a partial overlapping, unless a significant reduction of the total variability was carried out, considering only EVOOs produced according to a specific regulation, *e.g.* oils labelled with Protected Designation of Origin.

1. Introduction

The Extra Virgin Olive Oil (EVOO) is a typical and indispensable ingredient of the Mediterranean diet; it is considered the "*liquid gold of the Mediterranean area*", mainly due to its health benefits (Piroddi et al., 2017). Its unique properties are the result of its specific composition, including high proportions of oleic acid, a balanced presence of polyunsaturated fatty acids (Esposto et al., 2017) and other minor components, such as α-tocopherol and phenolic compounds (Dugo et al., 2020; Bulotta et al., 2014; Mateos et al., 2003), which guarantee its high antioxidant potential (Xiang et al., 2017; Kalogeropoulos & Tsimidou, 2014). Italy is the first country in Europe for the number of EVOO labelled with Protected Designation of Origin (PDO) (EC/info/geographical indications register). Considering the Italian geographical variability, like atmospheric conditions, between the North and the South of Italy, and different production techniques, each PDO oil shows specific chemical compositions and organoleptic features (Panico et al., 2014).

The construction of databases and statistical models containing a huge number of samples represents a starting point to evaluate which factor mostly contributes to the differentiation between all these high-quality olive products: cultivar, geographical area, production technique, label (Cerretani et al., 2006; D'Imperio et al., 2007a, b; Mannina et al., 2003; Mangraviti et al., 2021).

Actually, there is a lack of sufficiently powerful and fast analytical methods in the classification of olive oils, which allow the rapid identification of discriminant factors. Fingerprinting methods based on spectroscopic techniques such as nuclear magnetic resonance (NMR) (Mannina et al., 2003; Lukić et al., 2019; Consonni & Cagliani, 2018; D'Imperio et al., 2007b), Near Infrared spectroscopy (NIR) (Mustorgi

* Corresponding author.
E-mail addresses: moteri@unime.it (M. Oteri), frigano@unime.it (F. Rigano), giumicalizzi@unime.it (G. Micalizzi), monica@difar.unige.it (M. Casale), malegori@difar.unige.it (C. Malegori), pdugo@unime.it (P. Dugo), lmondello@unime.it (L. Mondello).

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et al., 2020; Sinelli et al., 2008) and direct-MS were successfully investigated in this field, exploiting the great advantage of minimal requirements for sample preparation and the benefit arising from a very short total analysis time (Mangraviti et al., 2021). However, only few details about the chemical composition are normally obtained, such as only major components can be identified.

The aim of the present research work was the analysis of Italian EVOOs, labeled as PDO, PGI (Protected Geographical Indication) and organic farmed oil (BIO), using fast and innovative LC and GC methods, characterized by a high analytical throughput that allowed the characterization of a large number of samples. The macro constituents, namely triacylglycerols (TAGs), were considered in the present study. The TAG profile is normally obtained by Liquid Chromatography (LC) methods coupled to evaporative light scattering (ELS) or MS detection, while the total fatty acid (FA) composition is usually elucidated through gas chromatography (GC) coupled with flame ionization detection (FID) or mass spectrometry (MS) (Eder, 1995; Delmonte et al., 2020) after transesterification reaction in order to convert intact lipids (*e.g.* sterol esters, TAGs and phospholipids) into more volatile and less polar fatty acid methyl esters (FAMEs). The FAME analysis provides a deeper insight into the TAG molecular structure, since FA isomers are satisfactorily separated during the GC run. Moreover, the FAME quali-quantitative profile represents a support for TAG identification since FAs are combined in the TAGs according to their relative percentages (Beccaria et al., 2014).

In order to speed up the entire analytical work-flow, MS libraries with embedded Linear Retention Indices (LRIs) were used in both LC and GC methods, thus enabling a fast, automatic and reliable identification of single molecular species. In GC-MS, the high reproducibility of Electron Ionization MS (EI-MS) spectra, achieved under high vacuum and high energy conditions, allowed for the building of universal EI-MS libraries, that can be used by all operators in any part of the world, since they are independent from the experimental conditions (Zellner et al., 2008; Mondello et al., 2005). However, retention data are necessary to distinguish analytes characterized by almost identical fragmentation pattern, as in the case of double-bond positional FAME isomers. Being the LRI system a robust tool in GC as introduced by Kováts (1958) and van Den Dool and Kratz (1963) more than fifty years ago, it is possible to assess that the use of dual-filter MS database is a widespread approach in GC-MS.

Conversely, in LC, MS spectra obtained by means of atmospheric pressure ionization (API) techniques, much more compatible with the amount of liquid phase coming from the LC column, strongly depend on the experimental and environmental parameters, therefore spectral database can be applied only at intra-laboratory level, by using the same analytical conditions, the same instruments and in a restricted time period.

Moreover, API-MS, being soft ionization techniques, are insufficient for the univocal peak assignment. Some ion sources generate some diagnostic fragments, as in the case of atmospheric pressure chemical ionization (APCI) (Byrdwell, 2001; Byrdwell & Emken, 1995), but the lack of universally applied spectral libraries results in the need of manual interpretation of each spectrum, thus making data processing really slow and prone to errors.

In 2018, Rigano et al. (2018) developed a new LC method based on the Linear Retention Index (LRI) approach to achieve a fast and automatic identification of intact lipids (TAGs) only on the basis of their retention behavior, thus avoiding the need of expensive instrumentation (*i.e.* tandem MS systems). Such an approach was already applied to vegetable samples (Zarai et al., 2018) and fish oils (Zarai et al., 2020; Rigano et al., 2020). Also, thanks to the higher batch-to-batch reproducibility in LC column packaging technologies and instrumentations, the LRI identification system resulted stable by changing different experimental condition, such as column length and inner diameter, flow rate and gradient steepness. Hence, with respect to previous works, the LC analysis was significantly shortened by using a fast gradient that enabled the baseline separation of intact TAGs in less than 20 min. The additional goal was the development of a dedicated software for the handling of a dual-filter library for the first time in LC.

As for FAME analysis, the novelty consisted in the development of a fast GC-FID method that provided the profile of the total FA composition in less than 3 min. Finally, multivariate statistical analyses were mandatory to compare the lipid content of 200 oils from different Italian regions and to classify them mainly according to both olive variety and geographical origin.

2. Materials and methods

2.1. Chemical and reagent

n-heptane and methanol (reagent grade), potassium hydroxide (KOH), acetonitrile and 2-propanol (LC-MS grade) were purchased from Merck Life Science (Darmstadt, Germany). C_4-C_{24} even carbon saturated FAMEs standard mixture (1000 μg/mL each in *n*-hexane), standard of trinonanoin (C9C9C9), triundecanoin (C11C11C11), tritridecanoin (C13C13C13), tripentadecanoin (C15C15C15), triheptadecanoin (C17C17C17), trinonadecanoin (C19C19C19) were all purchased from Merck Life Science. Standard of TAGs for the building of a spectral library were reported in Table S1, along with their abbreviation and suppliers.

2.2. Samples

A total of 200 EVOOs were collected. They were supplied by different Italian farms that produce PDO, monocultivar, PGI and BIO EVOOs and refer to the harvesting year 2017. Table 1 shows the list of the analyzed EVOOs. They came from twelve Italian region and were labeled as: PDO, PGI, BIO, monocultivar (MV), bicultivar (BV) and blend (mixture of more than 2 cultivar at different percentages).

2.3. Sample preparation

2.3.1. Fatty Acid transesterification procedure for GC analysis

Fatty acid composition of EVOOs was determined according to the International Oleic Council (IOC) guidelines (IOC, 2017). The preparation of FAMEs was performed by transesterification with a methanolic solution of potassium hydroxide (KOH) at room temperature. Specifically, 25 mg of EVOO was weighted into a 5 mL screw-top test tube. FA transesterification was carried out by adding 0.1 mL of KOH in methanol (2 N) to the tube. The FAMEs were extracted using 1 mL of *n*-heptane. The reaction mixture was stirred using a vortex mixer. After gravitational separation of phases, the upper layer was collected and transferred into 2 mL autosampler vial for GC analyses.

2.3.2. Sample preparation for LC analysis

A standard mixture of C9C9C9, C11C11C11, C13C13C13, C15C15C15, C17C17C17, C19C19C19 was prepared at a concentration of 1000 mg/L each in 2-propanol.

EVOO samples were prepared by dissolving 10 mg of oil in 1 mL of 2-propanol.

2.4. GC-MS analysis of FAMEs

The separation and identification of FAMEs were carried out by using a GCMS-QP2020 (Shimadzu, Duisburg, Germany) equipped with a split-splitless injector (280 °C) and an AOC-20i autosampler. A medium-polarity ionic liquid (IL) capillary column, namely SLB-IL60i 30 m × 0.25 mm i.d., 0.20 μm d_f (Merck Life Science), was used for the chromatographic separation. The temperature program was as follows: 70–280 °C at 5.0 °C/min. Injection volume was 0.2 μL with a split ratio of 1:50. Helium was used as carrier gas, at an initial inlet pressure of 31.7 kPa and at an average linear velocity of 30 cm s^{-1}. MS parameters

Table 1
List of Analyzed EVOOs, classified according to region of provenance, denomination, label and cultivar.

Region	Denomination	Label	Cultivar (n. samples)
Sicily (n = 24)	PDO (n = 10)	Monti Iblei	*Tonda Iblea (1)*
		Monti Iblei Gulfi	*Tonda Iblea (1)*
		Monti Iblei Monte Lauro	*Tonda Iblea (1)*
		Valle del Belice	*Nocellara del Belice (2)*
		Val di Mazara	*Biancolilla (1)*
			Nocellara del Belice (2)
			Nocellara del Belice-Biancolilla (1)
			Blend (1)
	IGP (n = 2)		*Nocellara del Belice (1)*
			Blend (1)
	BIO (n = 4)		*Nocellara del Belice (1)*
			Nocellara Messinese (1)
			Nocellara Etnea-Nocellara Messinese (1)
			Tonda Iblea (1)
	MV (n = 8)		*Tonda Iblea (2)*
			Nocellara del Belice (3)
			Biancolilla (1)
			Cerasuola (1)
			Nocellara Etnea (2)
Apulia (n = 37)	PDO (n = 15)	Terra di Bari	*Coratina (3)*
			Coratina-Ogliarola (1)
		Terra di Bari-Castel del Monte	*Coratina (7)*
		Terre di Bari - Bitonto	*Ogliarola Barese-Coratina (1)*
			Coratina (2)
		Canosa di Puglia	*Coratina (1)*
	BIO (n = 3)		*Ogliarola Garganica (1)*
			Peranzana (1)
			Coratina (1)
	MV (n = 19)		*Ogliarola Barese (4)*
			Peranzana (2)
			Coratina (11)
			Frantoio (2)
Tuscany (n = 62)	PDO (n = 16)	Chianti Classico	*Blend (7)*
			Frantoio (2)
			Moraiolo-Leccino (1)
		Seggiano	*Olivastra Seggianese (5)*
		Terre di Siena	*Blend (1)*
	PGI (n = 17)		*Frantoio (1)*
			Moraiolo (2)
			Frantoio-Leccino (1)
			Blend (13)
	BIO (n = 6)		*Frantoio (2)*
			Moraiolo (1)
			Blend (3)
	MV (n = 19)		*Nocellara (1)*
			Frantoio (5)
			Moraiolo (3)
			Leccio del Corno (4)
			Pendolino (3)
			Maurino (1)
			Coratina (1)
			Raggiolo (1)
	BV (n = 1)		*Moraiolo-Frantoio (1)*
	Blend (n = 3)		*Blend (3)*
Lazio (n = 29)	PDO (n = 14)	Sabina	*Blend (9)*
		Colline Pontine	*Itrana (1)*
		Tuscia	*Blend (1)*
			Caninese (3)
	BIO (n = 8)		*Frantoio (1)*
			Leccino (1)
			Leccino-Bolzone (1)
			Itrana-Leccino (2)
			Canino (1)
			Itrana (2)
			Blend (1)
	MV (n = 4)		*Leccino (2)*
			Itrana (1)
			Caninese (1)
	Blend (n = 3)		*Blend (2)*
Umbria (n = 11)	PDO (n = 3)	Colli Orvietani	*Blend (2)*
		Colli Assisi Spoleto	*Blend (1)*
	BIO (n = 2)		*Frantoio (1)*
			Coratina (1)
	MV (n = 1)		*Moraiolo (1)*
	Blend (n = 5)		*Blend (5)*
Calabria (n = 10)	PDO (n = 2)	Lametia	*Carolea (2)*
	BIO (n = 3)		*Nocellara del Belice (1)*
			Blend (2)
	MV (n = 4)		*Carolea (1)*
			Ottobratica (3)
	BV (n = 1)		*Blend (1)*
Campania (n = 1)	MV (n = 1)		*Ortice (1)*
Liguria (n = 4)	PDO (n = 2)	Riviera Ligure di Levante	*Lavagnina (1)*
		Riviera Ligure, Riviera dei Fiori	*Blend (1)*
	MV (n = 2)		*Taggiasca (2)*
Veneto (n = 7)	PDO (n = 5)	Garda Orientale	*Casaliva-Leccino (2)*
			Blend (1)
		Veneto Valpolicella	*Blend (1)*
			Grignano (1)
	BIO (n = 1)		*Blend (1)*
	MV (n = 1)		*Grignano (1)*
Sardinia (n = 8)	PDO (n = 2)	Sardegna PDO	*Bosana-Semidana (1)*
			Blend (1)
	BIO (n = 1)		*Nera di Oliena (1)*
	MV (n = 4)		*Semidana (1)*
			Bosana (3)
	BV (n = 1)		*Bosana-Semidana (1)*
Trentino-South Tyrol (n = 4)	PDO (n = 1)	Garda Trentino	*Casaliva (2)*
	MV (n = 2)		*Casaliva (1)*
			Coratina (1)
	BIO (n = 1)		*Blend (1)*
Lombardy (n = 3)	PDO (n = 2)	Garda Bresciano	*Blend (1)*
			Blend (1)
	MV (n = 1)		*Casaliva (1)*

were as follows: mass range 40–550 amu, the ion source temperature 220 °C, and the interface temperature 250 °C. The GCMSsolution software (version 4.50 Shimadzu) was used for data collection and handling. Peak identification was carried out by using both spectral similarity (over 85%) and a ± 5 LRI tolerance window. In such respect, a C_4-C_{24} FAMEs standard solution (Merck Life Science) was used for LRIs determination of FAMEs. LIPIDS Mass Spectral Library Version 1.0 (Shimadzu Europe, Duisburg, Germany) and a lab-made LRI database were used for peak assignment.

2.5. GC-FID analysis of FAMEs

FAMEs quantification was carried out using a GC-2010 (Shimadzu, Duisburg, Germany) equipped with a split-splitless injector (280 °C), an AOC-20i+s autosampler, and a flame ionization detector (FID) detector. Conventional GC-FID analyses were carried out using a SLB-IL60i capillary column 30 m × 0.25 mm i.d., 0.20 µm *df* (Merck Life Science), under the same experimental parameters used for GC-MS analyses. The inlet pressure was 103.5 kPa and the injector temperature was set at 280 °C. The injection volume was 0.2 µL with a split ratio of 1:50. Gas flows of FID detector were 40 mL/min for hydrogen, 30 mL/min for

make up (nitrogen) gas and 400 mL/min for air. Fast GC analyses were performed on the same Shimadzu instrument, by using a SLB-IL60i 15 m × 0.10 mm i.d., 0.08 µm *df* (Merck Life Science) capillary column. The program temperature was as follows: 180–230 °C at 15 °C/min. Injection volume was 0.2 µL with a split ratio of 1:250. Hydrogen was used as carrier gas at an inlet pressure of 606.4 kPa and at an average linear velocity of 90 cm s^{-1}. Temperature and gas flows of FID were the same as described for the conventional GC-FID analysis.

Data were collected and processed using the LabSolution software (version 5.92, Shimadzu). Each EVOO was analyzed in triplicate.

2.6. LC/APCI-MS instrumentation and analytical conditions

The TAG analysis was carried out on a Nexera X2 HPLC system (Shimadzu, Kyoto, Japan), consisting of a CBM-20A controller, two LC-30 CE dual-plunger parallel-flow pumps (120.0 MPa maximum pressure), a DGU-20A5R degasser, a CTO-20AC column oven and a SIL-30AC autosampler. The HPLC system was coupled to a single quadrupole mass spectrometer (LCMS-2020) detector (Shimadzu) through an APCI interface (Shimadzu).

Chromatographic separation was achieved on a C18 Ascentis Express, 50 × 2.1 mm I.D., 2.7 µm *dp* column (Merck Life Science) using (A) ACN and (B) IPA as mobile phase in gradient mode elution: 0–17.50 min, 20%− 50% B; 17.50–20.00, 50%, then returning to the initial condition in 0.1 min with an equilibration time of 3 min. The flow rate was set at 500 µL/min with oven temperature of 35 °C; injection volume was 2 µL.

MS parameters were as follow: APCI source operating in positive ionization mode; mass spectral full scan range: 250–1200 *m/z*; event time: 0.6 s; detector voltage: 1.50 kV; nebulizing gas (N_2) flow rate: 3.0 L/min; interface temperature: 450 °C; DL (Desolvation Line) temperature, 250 °C; heating block temperature, 300 °C; drying gas flow (N_2), 5.0 L/min.

Data acquisition was recorded using the LabSolution software (Version 5.91, Shimadzu).

2.7. Data processing for TAG analysis

A mixture of six standard TAGs with odd carbons number from C9C9C9 to C19C19C19 was used as reference homologue series, then injected at the beginning and at the end of each batch of EVOO samples to calculate the LRI of all analytes and evaluate the inter-sample repeatability of the method in terms of both retention time and LRI.

LRIs were calculated by using the following equation:

$$LRI = 100\left[z + 6\frac{t_{Ri} - t_{Rz}}{t_{R(z+6)} - t_{Rz}}\right]$$

which takes into account the retention time of the unknown TAG (t_{Ri}) and those of the reference compounds eluted immediately before and after the unknown TAG (t_{Rz} and t_{Rz+6}, respectively).

z is the partition number (PN) of the reference TAG eluted immediately before the unknown TAG. Considering that PN is related to the carbon chain length CN and double bond number DB (PN=CN-2DB), *z* ranges from 27 for C9C9C9 to 57 for C19C19C19; 6 is the difference in *z* units between adjacent reference standard TAGs.

Taking into account that, up to now, differently from GC, there are not commercially available software able to perform an automatic identification based on the application of both LRI and MS similarity filters in LC-MS an in-house software was applied to the present work in order to realize a dual-filter identification strategy (quite similar to GC-MS). The following steps are required:

1) Peak integration of both the homologue series and the unknown sample.

2) Loading of the LRI database and integrated reference series analysis in the LRI window for the automatic calculation of LRI for the peaks in the sample.

3) Automatic search in the LRI database and in the MS library.

4) Data reporting containing peak identification, the MS spectral similarity and the comparison between tabulated and experimental LRI.

As a preliminary work of the present research, an MS library of 80 diacylglycerols (DAGs) and TAGs, which can be detected in vegetable oils and available in the laboratory, was built by using the LabSolution software (Version 5.91, Shimadzu). The list of compounds injected under the same experimental conditions and included in the library is reported in Table S1, Supplementary Material. The main fragments and their relative intensity are also reported.

2.8. Statistical analysis

Principal Component Analysis (PCA) (Joliffe, 2002) was performed as a multivariate display method on the whole data matrix, after column autoscaling, in order to extract the useful information embodied within the data and to visualize the data structure. Explorative data analysis is the first fundamental step for obtaining information about the samples, the variables and their relations. The explorative analysis and representation techniques are useful for identifying general data features, finding similarities between the samples or the variables by which they are characterized, detecting anomalies, in order to devise the appropriate classification or correlation strategies. In this study, PCA was performed using the multivariate data analysis software CAT (Chemometrics Agile Tool), freely accessible by http://www.gruppochemiometria.it/index.php/software.

3. Results and discussion

The present work is focused on the determination of FAs and TAGs of 200 samples of EVOOs from different Italian regions. The simplicity of the matrix, combined to the availability of novel analytical methods and/or stationary phases, allowed for the separation of FAs and TAGs in less than 3 and 20 min, respectively, compared to conventional separations obtained in more than 60 min (Beccaria et al., 2016; Holčapek et al., 2005; Holčapek et al., 2003).

3.1. GC analysis of FAMEs

GC-MS analysis was carried out to elucidate the total FA composition. Two independent identification filters were applied for the univocal identification of FAMEs. In detail, the first filter regarded the mass spectral similarity, set at a minimum of 85% to be included in the list of candidates, while the second one considered an LRI tolerance of ± 5 units, so that compound falling out this window will be automatically excluded. As in previous research papers (Micalizzi et al., 2020; Rigano et al., 2021), the use of retention data is needed to distinguish some positional isomers, *e.g.* oleic (C18:1n9) and vaccenic acid (C18:1n7). The MS similarities and experimental LRIs compared with the indices reported in the lab-made database are summarized in Table S2. Spectral similarities higher than 90% and ΔLRI (difference between experimental and tabulated values) minor than 7 units were achieved for all the compounds.

Considering the large number of EVOO samples to be investigated, a fast method was developed. The conventional GC separation on the 30 m × 0.25 mm i.d. capillary column was transferred to a 10 m × 0.1 mm i.d. micro-bore column for the fast GC-FID separation by using H_2 as gas carrier rather than He. The temperature program was restricted to the range 180–230 °C, roughly corresponding to the elution temperature of FAMEs identified in EVOOs. The linear velocity and temperature gradient were increased from 30 to 90 cm s^{-1} and from 5° to 15 °C/min, respectively. Then, the fast GC separation was achieved in about 2.5 min, against the 30 min separation time of the conventional analysis.

Fig. S1 (Supplementary Material) shows the comparison between the

chromatographic profiles obtained under conventional and fast GC conditions. Table S2 reports the relative quantification performed by both methods, pointing out that not significant differences were obtained in terms of relative quantitative results ($p > 0.05$ running a t-test). Hence, the developed method resulted suitable for the quantification of 12 FAMEs in EVOO samples, leading to a 10-time gain in term of analysis time, with respect to conventional analyses.

According to previous research works (Di Lecce et al., 2020; D' Imperio et al., 2007a; Mannina et al., 2003; Bucci et al., 2002, Bianchi et al., 2001), the results here presented highlighted significant differences in the FA composition of EVOOs coming from different Italian regions and obtained from different cultivar ($p < 0.01$, by running a one-way ANOVA for all FAs, Table S3 and Tables 2–3). Particularly, most of the previous works focused on the differentiation of EVOOs according only to one of the investigated variables, *viz.* the region of cultivation or the olive variety, while no large studies have been performed on the effect of the PDO label. For instance, D' Imperio et al., 2007a and Mannina et al. (2003) centered their research on the analysis of numerous monocultivar EVOOs, all coming from Sicily, thus limiting the total variability. Similarly, Bucci et al. (2002) analyzed only monocultivar EVOOs from Lazio, while Bianchi et al. (2001) considered few varieties coming from 4 Italian regions. Even when the final purpose was the characterization of different PDO trademarks, the statistical analysis based on the FA composition revealed that EVOOs were mainly differentiated according to the main cultivar (Consonni & Cagliani, 2018).

For this reason, in the present study, which include a large set of EVOOs obtained for many variety of olives, coming from 12 Italian regions and labelled with different trademarks, different comparisons have been proposed, aiming to investigate about the most discriminant variables.

First, relative quantitative results are reported in Table S3 for all the analyzed samples, grouped according to the region of origin, with the exception of the Campania EVOO, for which only one sample was available. For an easier visualization of quantitative data, a histogram

Table 2
Percentage content of the main FAs, along with their standard deviation (SD) and coefficient of variations (CV%), in analyzed EVOOs, grouped according to trademark.

	C16:0	C18:0	C18:1n9	C18:1n7	C18:2n6
PDO Monti IBlei (n = 3)	15.12 A a	2.30 BCE b	67.02 C c	2.72 A ab	9.85 A a
SD	1.01	0.12	1.60	0.32	0.41
CV%	6.70	5.03	2.38	11.83	4.19
PDO Val di Mazara (n = 5)	11.77 BCE cd	3.02 A a	72.23 B b	1.86 BCE c	8.43 AB ab
SD	0.79	0.37	2.68	0.44	1.04
CV%	6.69	12.25	3.71	23.50	12.35
PDO Terra di Bari (n = 14)	10.29 C d	2.33 B b	77.57 A a	1.37 C d	6.35 C c
SD	1.03	0.25	1.53	0.26	0.52
CV%	10.06	10.64	1.98	18.67	8.11
PDO Sabina (n = 9)	13.37 AB bc	1.94 BCE bc	72.26 B b	2.54 A b	7.20 BCE bc
SD	0.90	0.15	2.19	0.19	1.32
CV%	6.71	7.64	3.04	7.29	18.33
PDO Tuscia (n = 4)	13.59 AB ab	1.74 C c	71.60 BCE b	3.09 A a	7.40 BCE bc
SD	0.67	0.21	1.92	0.45	0.87
CV%	4.90	12.27	2.68	14.68	11.69
PDO Chianti Classico (n = 10)	13.07 AB bc	2.09 BCE bc	73.02 B b	2.64 A ab	6.73 C c
SD	0.51	0.13	1.22	0.14	0.56
CV%	3.91	6.09	1.67	5.24	8.27
PDO Seggiano (n = 5)	13.16 AB bc	2.28 BCE b	72.65 B b	2.43 AB b	7.05 BCE bc
SD	1.06	0.21	2.29	0.25	0.77
CV%	8.03	9.06	3.16	10.30	10.97
PDO Garda (n = 6)	12.41 B bc	1.95 BCE bc	74.02 B b	2.80 A ab	6.28 C c
SD	0.84	0.43	1.45	0.19	0.50
CV%	6.81	21.81	1.96	6.89	8.01

Mean values with different letters within the same column are significantly different, A–C at $p < 0.01$ and a–c at $p < 0.05$.

Table 3
Percentage content of the main FAs in analyzed EVOOs, along with their standard deviation (SD) and coefficient of variations (CV%), grouped according to cultivar.

	C16:0	C18:0	C18:1n9	C18:1n7	C18:2n6
Tonda Iblea (n = 5)	14.91 A a	2.35 ABCD bc	67.10 E f	2.77 A ab	9.90 AB ab
SD	0.99	0.27	1.38	0.24	0.48
CV%	6.61	11.68	2.05	8.83	4.90
Nocellara Del Belice (n = 10)	12.82 A abc	3.03 A a	71.56 CDE cde	2.34 AB b	7.66 CDE cd
SD	0.88	0.30	1.54	0.44	0.82
CV%	6.89	9.82	2.15	18.71	10.67
Coratina (n = 28)	10.39 B cd	2.31 BCD bcd	77.51 AB ab	1.44 B c	6.28 EF de
SD	0.87	0.29	1.45	0.27	0.63
CV%	8.41	12.78	1.87	18.96	10.03
Peranzana (n = 3)	13.02 A ab	2.47 ABC ab	70.32 CDE cdef	2.41 AB ab	9.28 ABC ab
SD	0.96	0.09	2.20	0.26	0.87
CV%	7.37	3.74	3.13	10.70	9.40
Moraiolo (n = 7)	13.43 A ab	1.84 CD cd	72.27 CD cd	2.86 A ab	7.22 DEF cde
SD	0.68	0.22	1.42	0.28	0.72
CV%	5.05	12.15	1.96	9.68	9.98
Leccino (n = 4)	13.65 A ab	2.05 BCD bcd	72.83 BCE cd	2.54 A ab	6.60 EF de
SD	2.61	0.10	3.96	1.04	0.46
CV%	19.09	4.96	5.44	40.98	7.00
Leccio del Corno (n = 3)	10.36 B d	1.77 CD cd	78.17 A a	2.30 AB b	5.67 F e
SD	1.01	0.04	1.92	0.33	0.58
CV%	9.75	2.43	2.46	14.17	10.22
Frantoio (n = 14)	13.08 A ab	2.04 BCD bcd	72.58 C cd	2.76 A ab	7.16 DEF cde
SD	1.07	0.27	1.43	0.32	0.32
CV%	8.18	13.46	1.97	11.70	4.49
Caninese (n = 5)	14.21 A ab	1.83 CD cd	70.32 CDE cdef	3.21 A a	7.68 CDE cd
SD	0.78	0.24	1.83	0.23	0.90
CV%	5.48	13.20	2.61	7.18	11.67
Olivastra seggianese (n = 5)	13.16 A ab	2.28 BCD bcd	72.65 C cd	2.43 A ab	7.05 DEF de
SD	1.06	0.21	2.29	0.25	0.77
CV%	8.03	9.06	3.16	10.30	10.97
Pendolino (n = 3)	12.65 AB abcd	1.69 D d	73.42 ABC bcd	2.92 A ab	6.95 DEF de
SD	0.24	0.30	0.64	0.28	0.37
CV%	1.86	18.01	0.88	9.56	5.29
Casaliva (n = 3)	12.06 AB bcd	2.05 BCD bcd	74.10 ABC abc	2.61 A ab	6.83 EF de
SD	0.58	0.04	1.28	0.28	0.40
CV%	4.85	1.94	1.73	10.62	5.81
Carolea (n = 3)	14.16 A ab	2.63 AB ab	70.37 CDE cdef	3.02 A ab	6.39 EF de
SD	0.64	0.13	1.16	0.39	0.60
CV%	4.55	4.96	1.65	12.80	9.46
Ottobratica (n = 3)	14.47 A ab	2.28 BCD bcd	69.44 CDE def	2.49 A ab	8.63 BCD bc
SD	1.57	0.14	3.34	0.18	1.27
CV%	10.84	6.25	4.80	7.14	14.74
Bosana (n = 3)	13.81 A ab	2.35 ABCD bc	67.68 DE ef	2.80 A ab	10.79 A a
SD	0.87	0.32	1.57	0.29	0.24
CV%	6.33	13.62	2.32	10.46	2.20

Mean values with different letters within the same column are significantly different, A–F at $p < 0.01$ and a–f at $p < 0.05$.

comparing the main FAs for EVOOs coming from the 11 Italian regions is reported in Fig. S2 A. The greatest differences regarded the most abundant FAs, such as oleic acid (C18:1n9), linoleic acid (C18:2n6) and palmitic acid (C16:0). For example, Calabria EVOOs showed the highest amount of C16:0 (13.84 ± 1.23%), while Apulian EVOOs showed the lowest content (11.09 ± 1.46%). EVOOs from Apulia were the richest of C18:1n9 (76.02 ± 3.04%), while Sardinia and Sicily EVOOs showed the lowest levels with an average of 69.14% and 70.13%, respectively. On the other hand, the FA profiles of Sicily and Sardinia samples showed the highest C18:2n6 content with values of 9.12 ± 2.33% and 10.03 ± 1.66%, respectively. C18:2n6 content in EVOOs from other Italian regions ranged from a minimum value of 5.77 ± 0.68% registered in Veneto to 7.56 ± 1.57% in Calabria.

A significant variability (high standard deviations are reported in Table S3) exists within the same region, due to the multitude of labels and olive varieties included in the present study, so that the FA composition is affected not only by pedoclimatic conditions, but only by production techniques and genetic features. This is particularly true for the Southern regions of Sicily, Apulia and Calabria.

Therefore, other classification systems were evaluated, based on the trademark or the cultivar and only EVOOs for which a minimum of 3 (n = 3) samples was available were taken into account. Tables 2 and 3 reports the percentage content of the main FAs along with their standard deviation (SD) and coefficient of variation (CV) for the most common PDO and monocultivar EVOOs, while Fig. S2 B-C show the corresponding histograms. It was observed that, by grouping EVOO according to the PDO trademark (Table 2), the CV was over 15% only in 4 cases. Two of them correspond to the C18:1n7 content for PDO Val di Mazara (23.50%) and PDO Terra di Bari (18.67%), the latter is however much lower than the CV% obtained for the region of Apulia (31.93%, Table S3). Even from the histogram (Fig. S2 B), it is quite clear that the high variability within Sicilian samples can be mostly referred to the PDO Val di Mazara, rather than to PDO Monti Iblei, being the latter mainly produced from a single olive variety (Tonda Iblea), while the PDO Val di Mazara are normally obtained from different cultivars at different percentages. As for the Apulian EVOOs, the error bar for each FA were significantly reduced by considering only the PDO Terra di Bari EVOOs.

High CV% were also obtained for the linoleic acid (C18:2n6) and stearic acid (C18:0) content of PDO Sabina (18.33%) and PDO Garda (21.81%), respectively.

Giving a look to Table 1, such a behavior can be related to the mixing of many cultivars at different percentages in PDO Sabina EVOOs (all PDO Sabina included in the present study are blend oils) and the provenance from different regions of the PDO Garda (Veneto, Trentino and Lombardy). Similarly, the classification of monocultivar oils according to the olive variety (Table 3) also points out a high variability only when the same cultivar is cultivated in more regions, as in the case of Nocellara del Belice cultivated in Sicily and Calabria (CV 18.71% for C18:1n7), Coratina cultivated in Apulia, Umbria, Tuscany and Trentino (CV 18.96% for C18:1n7) and Leccino cultivated in Lazio and Tuscany (CV 19.09% for C16:0% and 40.98% for C18:1n7). Particularly, the Leccino monocultivar EVOOs are characterized by the biggest error bars for all FAs. On the other hand, the Frantoio, Moraiolo and Casaliva varieties, cultivated in different regions, showed a CV% minor than 14% for all the compounds.

3.2. Quali-quantitative analysis of TAGs

In the present work, a fast non aqueous-reversed phase (NA-RP)LC method coupled to MS *via* APCI ion source was developed for the analysis of TAGs in the EVOOs under investigation. The new method enabled the TAG chromatographic separation in less than 15 min by using a 5 cm length partially porous (fused core) C18 column, against analysis times between 60 and 120 min using a stationary phase length of 10, 15 or 20 cm. The calculated LRI values were in perfect agreement with those reported in the database built by using a slower method (Rigano et al., 2018). Specifically, they fall within the error range (± 15 units) established in a previous work (Rigano et al., 2018).

Then, the well-known TAG profile of EVOO was used to definitely assess the stability of the LRI approach, even by changing the operating conditions, for the first time in LC. Beside the column length and gradient steepness, the detection system was also changed, moving from an Evaporative Light Scattering Detector (ELSD) to an APCI-MS detector.

Fig. 1 shows the LC-APCI-MS chromatograms of both reference homologue series (Fig. 1A) and a Sicilian EVOO sample (Fig. 1B), along with peak identification and LRI.

Furthermore, as a proof of concept, an APCI-MS library was tested for the identification of TAGs in the EVOO samples. As reported in the insert of Fig. 2, spectral similarities higher than 80% were achieved for the majority of peaks included in the database (GOO and AOO are not present in the spectral library; the latter it is not even included in the LRI database), with the exception of trilinolein (LLL) and dilinolein-palmitin (LLP), for which a spectral similarity of 64% and 76% was registered, due to the low signal and the partial coelution with adjacent abundant peaks, respectively. The compounds olein-linolein-palmitolein (OLPo) and olein-linolein-palmitin (OLP) are also negatively affected by the coelution with the abundant peaks olein-dilinolein (OLL) and diolein-linolein (OLO), so that the MS search totally fails in both cases, even performing an accurate baseline subtraction. Both have been identified as the partially coeluting peaks by using MS libraries, while the right compound was selected by the list of candidates resulted from the search into the LRI database, in which neither OLL (Fig. 2A) or OLO appear. Actually, Fig. 2A shows as the peak at 5.591 min could be wrongly identified as olein-palmitolein-myristin on the basis of the LRI search, but it can be reliably discarded considering the GC profile with does not report the presence of myristic acid. On the other hand, the MS search can exclude some compounds from a list of candidates having similar LRI values, thus demonstrating the complementarity between MS and LRI filters. As an example, Fig. 2B show the identification only on the basis of LRI for the peak at 8.769 min. A clear mismatching with TAGs at the same PN occurs, while the further application of the MS library allows to achieve the univocal identification of the TAG diolein-palmitin (OOP or OPO), as in Fig. 2C.

In other words, the combination of both filters corroborates the correct peak assignment by selecting the common candidates against both databases. It is noteworthy that for the first time, a new software developed *ad hoc* for LC data processing, was used for the simultaneous application of LRI and MS filters (as in Figs. 2B and 2C), differently from a previous paper (Rigano et al., 2020), in which the software enabled the identification only on the bases of the LRI criterion, while the MS spectrum was manually interpreted.

As for quantitative results, the percentage areas of all TAGs identified in all the samples, grouped according to the region, are shown in Table S4 as an intra-region average along with their standard deviation. Once again, it is noteworthy that most previous works already demonstrated that TAG composition can significantly discriminate Italian EVOOs according to pedoclimatic conditions (Damiani et al., 1997; D'Imperio et al., 2007b) or cultivar (Cerretani et al., 2006). However, also in this case, only well-delimited study areas were considered, thus reducing the number of discriminant variables. On the other hand, different classification types have been explored in the present work, thus highlighting differences based not only on cultivar and pedoclimatic conditions of the entire Italian national territory, but also on the PDO denomination.

Focusing on the first classification according to the Italian region, the percentage areas show the highest quantity of triolein (OOO) for Trentino EVOOs (43.66 ± 1.73%), immediately followed by Apulia (42.89 ± 4.38%) and not significant differences ($p > 0.05$ by running a one-way ANOVA) were observed with respect to other north-central regions (Lombardy, Veneto, Umbria, Tuscany and Liguria). Conversely,

TAG species	MS sim (%)	LRI_{exp}	LRI_{ref} (±15)
LLL	64	4171	4160
OLPo	-	4299	4286
OLL	81	4327	4342
LLP	76	4349	4358
OLO	87	4521	4516
OLP	-	4544	4539
SOLn; PLP	-	4575	4563; 4571
OOO	88	4714	4729
OOP	90	4740	4745
POP	86	4770	4776
GOO	-	4906	4905
SOO	86	4941	4948
SOP	89	4969	4961
AOO	-	5138	-

Fig. 1. LC-APCI-MS analysis of A) the reference homologue series (odd carbons number TAGs from C9C9C9 to C19C19C19) and B) of a Sicilian EVOO. Abbreviations: Po: Palmitoleic acid (C16:1); P: Palmitic acid (C16:0); S: Stearic acid (C18:0); O: Oleic acid (C18:1); L: Linoleic acid (C18:2); Ln: Linolenic acid (C18:3); A: Arachidic acid (C20:0); G: Gadoleic acid (C20:1); PN: Partition Number.

significant differences ($p < 0.01$ by running a one-way ANOVA) were obtained with respect to the south regions of Calabria,Sardinia and Sicily which showed the lowest levels of triolein (31.51% ± 3.36, 32.97% ± 3.25% and 34.18% ± 5.07 respectively),. On the other hand, Calabria EVOOs are characterized by the highest content of TAGs containing palmitic acid, such as dioleoin-palmitin (OOP), less represented in Apulian oils (29.33 ± 1.54% *vs* 24.29 ± 2.45%). According to GC data, TAGs containing linoleic acid were more abundant in Sardinia and Sicily EVOOs and significantly lower in the other regions: trilinolein (LLL) in Trentino EVOOs was one-third that of Sardinia EVOOs ($p < 0.05$ running a one-way ANOVA); olein-linolein-palmitolein (OLPo) content in Sardinia and Sicily samples were significantly higher (up to three times) than in Trentino and Veneto ($p < 0.01$ running a one-way ANOVA); maximum levels of diolein-linolein (OLO) were found in Sardinia and Sicily EVOOs (14.88 ± 1.47% and 14.48 ± 1.93%), while minimum amounts were detected in Veneto (11.83 ± 1.02%), Trentino (11.98 ± 0.63%) and Umbria (11.99 ± 1.20%). Finally, the TAG olein-linolein-palmitin-glycerol, containing both linoleic and palmitic acid, was present at the highest concentration in Sardinia EVOOs (9.09 ± 1.66%) and lowest concentration in Apulia EVOOs (5.73 ± 1.41%), followed by the northern EVOOs from Veneto (6.09 ± 0.60%) and Lombardy (6.22 ± 0.57%). All these considerations highlight that the intact lipid profile, that is the combination of FAs in the more complex structure of TAGs, is more sensitive to the cultivation area compared to the FA composition, as previously reported by Cerretani et al. (2006). Even in this case, a histogram comparing the percentages of the main TAGs for 11 Italian regions is reported in Fig. S2 D for visualization purposes. The comparison with Fig. S2 A immediately points out that TAGs are more subject to variations compared to the total FA, thus providing helpful information for the correlation between the geographical origin and the lipid profile.

However, even in this case, high standard deviations were obtained, especially for minor TAGs, thus asking for different classification methods, based on the trademark or the cultivar. The results are reported in Tables 4 and 5, while the corresponding histograms are reported in Fig. S2 E-F. Only EVOOs for which a minimum of 3 (n = 3) samples was available were included. Starting from the PDO classification method, it can be immediately pinpointed that, taking into account only the main above discussed TAGs, the CV decreases from 23% calculated for OLL in Sicilian EVOOs to about 5% and 11% calculated for the same TAG in PDO Monti Iblei and Val di Mazara, respectively, as a consequence of the reduced variability within EVOOs labelled with the same trademark, *viz.* produced according to a specific regulation with olives cultivated in a more delimited geographical area (EC, 2006; EU, 2012).

The same consideration can be applied to the TAG OLP determined in Sicilian and Apulian EVOOs with a CV of 24–25%, which decreases up to 6% in PDO Monti Iblei EVOOs, 15% in PDO Terra di Bari and 19% in PDO Val di Mazara. Interestingly, CV above 20% were obtained only for the TAG OLL in PDO Sabina and SOO in PDO Garda, as a consequence of the high standard deviation registered for C18:2n6 in PDO Sabina and C18:0 in PDO Garda, by GC analysis.

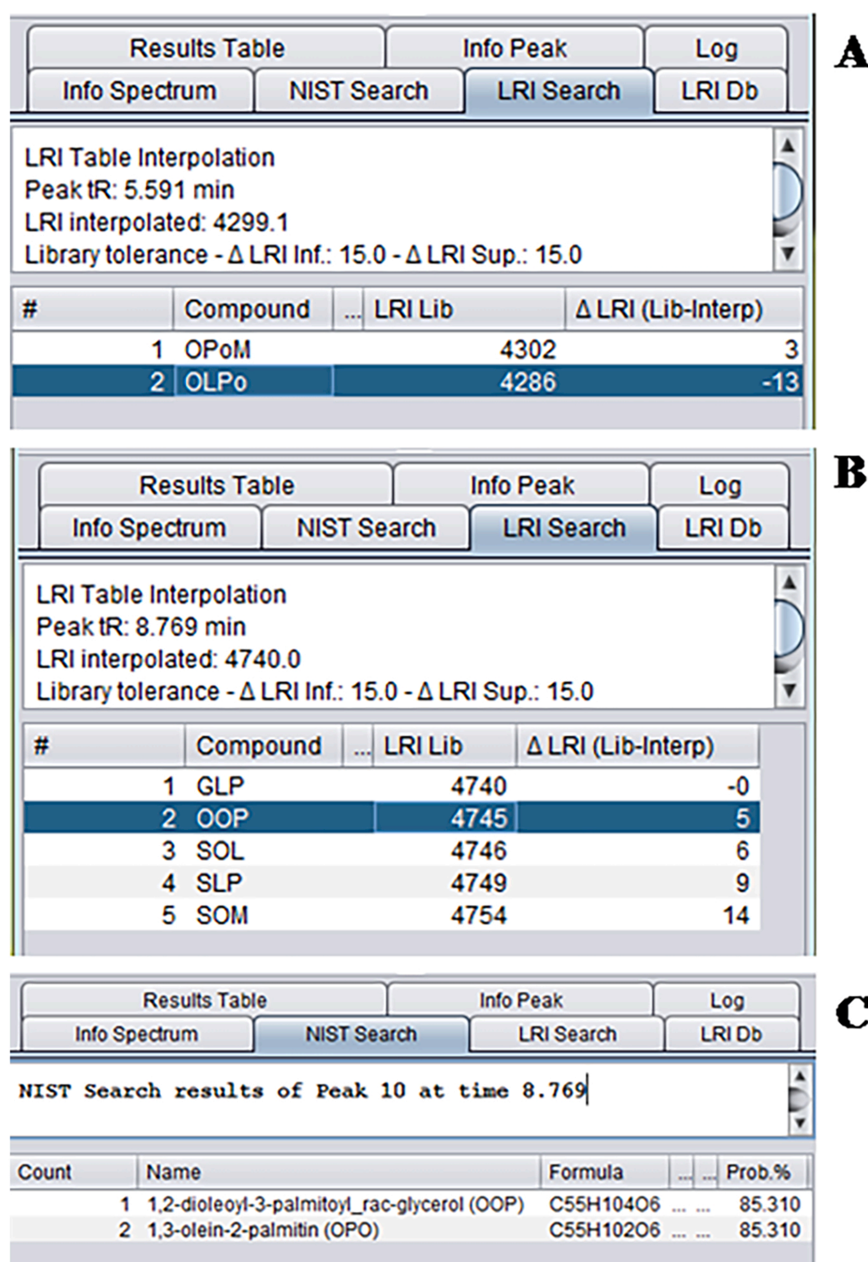

Fig. 2. Screenshot of the ChromLinear software: A) LRI search for peak at 5.591 min; B) LRI search for peak at 8.769 min; C) NIST-MS search for peak at 8.769 min.

As for the monocultivar EVOOs, CV% near to or higher than 20% were registered only for the OLP content in Coratina (CV 19.20%) and Leccino (CV 23.47%) samples, generally highlighting also in this case a minor variability compared to the more general classification according to the region of cultivation. The histograms in Fig. S2 D-F make evident the reduction of the error bars from the first classification based on the region to the other classification methods.

3.3. Multivariate statistical analysis

Given the multitude of samples and the extensive sampling, the relative quantitative analyses of both FAMEs and TAGs for all the 200 EVOOs were evaluated through multivariate analysis, specifically PCA, and more considerations were made on the basis of both origin, trademark and cultivar of EVOOs.

The first 6 principal components, explaining the 73.9% of the total variance, were computed and found to be significant. Among all the combinations of principal components (PCs), the first ones, presenting the greater percentages of total variance, are reported.

Fig. 3A and B show, respectively, the score and loading plots in the space of the first PCs (PC1 *vs* PC2) for auto-scaled TAG and FAME data relative to all the EVOOs included in the present study, coming from twelve Italian regions (samples are marked with different colors according to the region of cultivation). In this case, a total variance of 45.2% is explained, and a fairly good distinction based on geographical origin is evident. In particular, a clear demarcation between southern and center-northern regions can be immediately pointed out, being the first ones placed mainly at positive values of PC2 and the latter ones at negative values along PC2. Among southern regions, Apulian EVOOs are well-discriminated at negative values of PC1. Conversely, the center-northern EVOOs do not show a satisfactory differentiation on the basis of the region. Interestingly, a clustering according to the cultivar can be also highlighted: samples at higher PC2 value were produced with the olive variety Nocellara Etnea and Nocellara Messinese (both from the

Table 4
Percentage content of main TAGs, along with their standard deviation (SD) and coefficient of variation (CV) in analyzed EVOOs, grouped according to trademark.

	OLL	OLO	OLP	OOO	OOP	SOO
PDO Monti IBlei (n = 3)	3.01 A a	14.53 A a	9.00 A a	30.14 C d	27.27 ab	3.33 BCE b
SD	0.15	0.45	0.52	0.64	1.57	0.35
CV%	4.95	3.07	5.82	2.14	5.77	10.56
PDO Val di Mazara (n = 5)	2.30 AB abc	14.42 AB a	7.00 ABCD abc	36.38 B bc	25.31 ab	4.84 A a
SD	0.26	1.41	1.34	2.56	1.76	0.66
CV%	11.31	9.80	19.21	7.05	6.96	13.61
PDO Terra di Bari (n = 14)	2.01 AB bc	12.07 B b	5.36 D d	44.69 A a	23.54 b	4.30 AB a
SD	0.26	0.98	0.81	2.82	2.58	0.58
CV%	13.11	8.11	15.16	6.31	10.96	13.57
PDO Sabina (n = 9)	2.62 A ab	12.12 AB b	8.10 AB ab	34.90 BCE cd	27.90 a	3.16 C b
SD	0.94	1.51	1.05	3.15	1.30	0.29
CV%	36.00	12.47	12.98	9.04	4.66	9.20
PDO Tuscia (n = 4)	2.20 AB abc	12.85 AB ab	7.95 ABC abc	35.95 BCE bcd	27.27 ab	3.17 C b
SD	0.26	0.78	0.51	1.67	2.14	0.37
CV%	11.99	6.09	6.43	4.64	7.85	11.58
PDO Chianti Classico (n = 10)	2.02 AB bc	12.57 AB ab	6.32 CD cd	39.94 AB b	26.79 ab	3.61 BCE b
SD	0.15	0.93	0.68	2.12	1.63	0.27
CV%	7.48	7.40	10.83	5.31	6.07	7.56
PDO Seggiano (n = 5)	2.10 AB abc	12.62 AB ab	6.75 ABCD bcd	37.79 B bc	26.95 ab	3.89 ABC ab
SD	0.32	0.90	1.13	2.81	1.62	0.26
CV%	15.28	7.11	16.78	7.44	6.00	6.64
PDO Garda (n = 6)	1.70 B c	12.47 AB ab	6.39 BCD cd	40.20 AB b	27.19 ab	3.35 BC b
SD	0.32	0.92	0.48	3.37	2.74	0.68
CV%	18.94	7.39	7.58	8.39	10.07	20.18

Mean values with different letters within the same column are significantly different, A–D at $p < 0.01$ and a–d at $p < 0.05$.

eastern part of Sicily), sometimes mixed together, while the Sicilian samples spread at the center of the score plot are mainly obtained from the olive variety Nocellara del Belice, cultivated in the western part of Sicily, while the monocultivar Tonda Iblea EVOOs are placed at higher values of PC1, near to some Sardinia and Calabria EVOOs. As for the Apulian cluster, it includes some samples coming from other regions; among them, the Umbria EVOO at higher PC2 value (marked with purple rectangle), as well as the Tuscany and the Trentino EVOOs marked with light blue and yellow rectangles, respectively, are all produced from Coratina olives, as the majority of Apulian EVOOs. The Sicilian EVOO falling within Apulian samples was the only one, out of the analyzed samples, produced from Cerasuola olives. This result is in agreement with data reported from D' Imperio et al., 2007a on different Sicilian cultivar, among which Cerasuola was characterized by the highest content of oleic acid, near to Apulian EVOOs, mainly obtained from Coratina olives. The Apulian samples closest to the Sicilian ones are those obtained from Peranzana and Ogliarola olives. This is in accordance with quantitative data obtained for monocultivar EVOOs, also reported in Fig. S2C and F: samples obtained from Peranzana olives significantly differ from Coratina EVOOs (Tables 3 and 5 point out significant differences with $p < 0.01$ for palmitic, oleic and linoleic acids and OLO, OLP and OOO TAGs), whereas they are more similar to Sicilian samples.($p > 0.05$ by running a one-way ANOVA for most FAs and TAGs) Finally, the center-northern samples are completely overlapped probably because they are produced by the same olive varieties. Interestingly the Apulian EVOOs (n = 2) obtained from Frantoio olives still fall within the Apulia cluster, even if very close to Tuscany, Lazio and Veneto EVOOs; they have same score on PC1 and are marked with black rectangle.

The corresponding loading plot is reported in Fig. 3B. It confirmed the findings of the previously discussed quantitative analysis: Apulian and center-northern samples at negative values of PC1 are characterized by the highest content of the FA C18:1n9 and the TAG triolein, which are correlated each other, while Sicily and Sardinia EVOOs, placed at positive values of PC1, could be discriminated because of the highest concentration of C18:2n6 and linoleic acid-contaning TAGs (OLL, OLO, OLPo, OLP, LPP). These results are also supported by literature, which pinpointed a similar content of oleic acid for Coratina and different center-northern monovarietal EVOOs (Di Lecce et al., 2020; Bianchi et al., 2001) and a significantly lower content for Sardinia Bosana monovarietal EVOOs (Bosana cultivar), while linoleic acid has been quantified at minor levels in Apulian samples and highest levels in Sardinia and Sicilian EVOOs (Mangraviti et al., 2021; Bianchi et al., 2001). Interestingly, Mangraviti et al., (2021), despite used a totally different analytical approach, *viz.* the combination between a direct-MS method and a supervised statistical analysis, reported a clusterization quite similar to the one reported in the present study (Fig. 3A), with Southern EVOOs well-distinct each other, while Center-northern ones are partially overlapped and close to the Apulian samples.

In order to better visualize the center-northern EVOO samples, the intra-class variability was reduced, limiting the PCA analysis only to PDO EVOOs, which are produced according to well-defined production specification. The first 6 principal components, explaining the 74.4% of the total variance, were computed and found to be significant. The score plot of PC1 *vs* PC2 (Fig. 4A) explained a total variance of 45.2% and highlighted a clusterization on the basis of the region: Apulian EVOOs are well-separated from the other regions and are placed at negative values of PC1; Sicilian EVOOs were split according to the origin denomination (PDO trademark) with PDO Val di Mazara and PDO Valle del Belice placed at central values of PC1 and positive values of PC2 and PDO Monti Iblei at higher PC1 values, confirming the previously discussed results about the separation of EVOOs produced from Nocellara del Belice olives and those obtained from Tonda Iblea olives; the few PDO samples of Calabria and Sardinia EVOOs are interestingly placed between the two Sicilian PDO EVOOs; the three PDO Umbria EVOOs fall close and are satisfactorily separated from the other center-northern EVOOs at negative values of PC2, despite they are produced by using the same olive varieties (Frantoio-Moraiolo-Leccino) of Tuscany (PDO Chianti Classico) and Lazio (PDO Sabina and PDO Tuscia) EVOOs. In this case, the pedoclimatic conditions of the three center-northern regions could play a major role compared to the genetic features of the cultivar. For the same reason, no separation was pointed out between the main Tuscany PDO trademarks, namely PDO Chianti Classico and PDO Seggiano, despite the latter is produced as a monocultivar EVOO by using the peculiar cultivar Olivastra Seggianese. Also in this case, this finding is supported by quantitative data previously discussed and reported in Tables 3 and 5 and Fig. S2: the FA and TAG profile of Olivastra Seggianese EVOOs are quite similar to the profiles of Frantoio and Moraiolo oils ($p > 0.05$ by running a one-way ANOVA for all FAs and TAGs reported in Table 3 and Table 5). Similar considerations can be made for the PDO Lombardy EVOOs (both PDO Garda Bresciano), well separated at negative PC2 values, although they are produced from Casaliva olives, typical of the Garda region (as other Veneto and Trentino PDO EVOOs), mixed with olive varieties (Frantoio, Moraiolo and Leccino) commonly employed in the other center-northern regions (Umbria, Lazio and Tuscany). As for PDO Veneto EVOOs, the samples are rather dispersed but, it can be noticed that the two PDO Veneto Valpolicella and the three PDO Garda Orientale have same score on PC1, respectively negative the first ones and positive the other three, while the separation along PC2 can be due to the different mixture of olive varieties employed. Specifically, the two PDO Garda Orientale samples at negative values of PC2

Table 5
Percentage content of main TAGs, along with their standard deviation (SD) and coefficient of variation (CV), in analyzed EVOOs, grouped according to cultivar.

	OLL	OLO	OLP	OOO	OOP	SOO
Tonda Iblea (n = 5)	3.00 A a	14.37 A abc	9.52 AB a	29.77 F f	26.75 AB abc	3.35 BCED cde
SD	0.11	0.51	0.99	1.00	1.40	0.29
CV%	3.57	3.53	10.43	3.34	5.25	8.66
Nocellara Del Belice (n = 10)	2.27 BCD bc	13.66 A abcd	6.81 CD bc	35.69 BCDEF cdef	26.33 AB abc	4.90 A a
SD	0.27	1.51	0.88	2.31	2.04	0.27
CV%	11.72	11.02	12.95	6.48	7.75	5.48
Coratina (n = 28)	2.14 CD cd	12.00 B cd	5.09 D c	44.97 A a	23.72 B bc	4.39 ABC abc
SD	0.28	1.09	0.98	3.22	2.44	0.49
CV%	13.00	9.12	19.20	7.17	10.31	11.22
Peranzana (n = 3)	2.22 BCD cd	15.60 A a	7.71 ABC ab	36.73 BCDEF cde	23.37 B c	4.19 ABC abcd
SD	0.16	1.06	1.07	3.53	2.52	0.28
CV%	7.21	6.81	13.82	9.62	10.78	6.58
Moraiolo (n = 7)	2.09 CD cd	13.24 AB abcd	6.88 CD bc	37.76 ABCDE cd	27.14 AB abc	3.37 CD de
SD	0.15	1.46	0.46	1.79	1.71	0.21
CV%	7.13	10.99	6.75	4.74	6.31	6.11
Leccino (n = 4)	2.36 ABC bc	11.24 B d	6.97 CD bc	36.44 BCDEF cdef	28.83 AB ab	3.59 CD cde
SD	0.33	1.08	1.64	5.50	4.14	0.29
CV%	14.06	9.59	23.47	15.10	14.37	8.05
Leccio del Corno (n = 3)	2.04 CD cd	12.34 AB cd	5.24 D c	45.42 A a	22.95 B c	4.69 AB ab
SD	0.22	1.03	0.63	4.35	2.37	0.15
CV%	10.91	8.38	11.99	9.57	10.34	3.21
Frantoio (n = 14)	1.95 CD cd	13.16 AB abcd	6.73 CD bc	38.49 ABCD bcd	26.70 AB abc	3.82 ABCD bcde
SD	0.15	0.80	0.57	2.27	1.85	0.30
CV%	7.70	6.05	8.40	5.90	6.93	7.98
Caninese (n = 5)	2.22 BCD cd	13.00 AB bcd	7.85 ABC ab	35.86 BCDEF cdef	27.69 AB abc	3.05 D e
SD	0.22	1.05	0.56	1.46	2.26	0.34
CV%	10.03	8.11	7.18	4.07	8.17	11.15
Olivastra seggianese (n = 5)	2.10 CD cd	12.62 AB cd	6.75 CD bc	37.79 ABCDE cd	26.95 AB abc	3.89 ABCD bcde
SD	0.32	0.90	1.13	2.81	1.62	0.26
CV%	15.28	7.11	16.78	7.44	6.00	6.64
Pendolino (n = 3)	1.59 BCD cd	13.44 AB abcd	6.36 CD bc	41.22 ABC abc	26.10 AB abc	3.03 D e
SD	0.20	0.47	0.31	0.63	1.46	0.40
CV%	12.81	3.51	4.94	1.54	5.60	13.11
Casaliva (n = 3)	1.91 CD cd	12.18 AB cd	6.58 CD bc	42.31 AB abc	25.08 AB abc	3.55 CD cde
SD	0.26	0.74	0.08	3.34	2.79	0.19
CV%	13.52	6.04	1.18	7.89	11.14	5.28
Carolea (n = 3)	1.65 D d	11.55 B d	7.12 BCD bc	33.61 CDEF def	30.30 A a	4.70 AB ab
SD	0.15	0.73	0.97	1.42	1.54	0.33
CV%	9.26	6.30	13.64	4.22	5.09	6.99
Ottobratica (n = 3)	2.34 ABC bc	12.46 AB cd	9.56 A a	30.89 DEF ef	29.01 AB ab	4.40 ABC abc
SD	0.31	1.13	1.15	4.12	0.44	0.29
CV%	13.38	9.09	12.07	13.34	1.50	6.49
Bosana (n = 3)	2.82 AB ab	15.45 A ab	9.44 AB a	30.51 EF ef	25.55 AB abc	3.88 ABCD bcde
SD	0.05	0.81	0.75	1.37	2.58	0.06
CV%	1.92	5.27	7.91	4.50	10.08	1.47

Mean values with different letters within the same column are significantly different, A–F at $p < 0.01$ and a–f at $p < 0.05$.

are bivarietal oils (Casaliva-Leccino), while the oil at positive values is a blend EVOO with Casaliva as main cultivar and low percentages of Leccino, Moraiolo, Pendolino, *etc.* Interestingly, the separation between Lombardy PDO Garda Bresciano and Veneto PDO Garda Orientale, along both PC1 and PC2, can be ascribed to different pedoclimatic factors, despite the main cultivar was Casaliva in all the samples. The satisfactory discrimination between different micro-areas around the Lake Garda has been already pinpointed by Schievano et al. (2006) by using an NMR approach. As for the PDO Veneto Valpolicella EVOOs, the one closest to the origin of the axes is a monocultivar oil (Grignano cultivar), while the one at the most negative values of PC2 is a blend EVOO (Grignano-Favarol-Pendolino-Trepp).

The corresponding loading plot (Fig. 4B) confirms the previous findings (Fig. 3B), but adds some information regarding the discrimination of PDO Monti Iblei and PDO Val di Mazara, as well as the not complete overlapping between center-northern regions. PDO Monti Iblei oils at positive values of PC1 are characterized by a higher relative concentration of C16:0 and a lower concentration of C18:1n9 and C18:0 FAs, with respect to PDO Val di Mazara oils ($p < 0.01$, by running a one-way ANOVA, as reported in Table 2 and Table 4). The latter can be also quite distinct due to the highest concentration of the TAGs stearyl-diolein and triolein ($p < 0.01$, by running a one-way ANOVA). Finally, the PDO EVOOs from Umbria, Lombardy and the PDO Veneto Valpolicella (n = 2) at negative values of both PC1 and PC2 are characterized by the lowest content of C18:2n6 and linoleic acid-containing TAGs.

Following all these considerations, the results here presented on the differentiation of Italian PDO EVOOs showed a partial agreement with previous findings (Consonni & Cagliani, 2018), which pointed out as the classification occurred mainly on the basis of the cultivar. In the present study, this is true for the analyzed southern EVOOs, characterized by cultivar which maintain their lipid composition even if cultivated under different pedoclimatic factors, while the effect of the exact geographical origin is more relevant for northern EVOOs. Actually, several papers dealing with the TAG composition as discriminant factor, reported a clear demarcation according to the climatic conditions for EVOOs coming from center-northern Italian regions, such as Umbria (Damiani et al., 1997), and Lazio (D'Imperio et al., 2007b). Moreover, Bianchi et al. (2001) already reported a high similarity in the FA composition of two typical Tuscany cultivars (Frantoio and Moraiolo, also present in Lazio, Umbria and Garda samples), further supporting the partial overlapping experienced in the present study between center-northern EVOOs, independently from the employed variety of olives.

Finally, only monocultivar EVOOs were considered to reduce the total variability. The first 6 principal components, explaining the 77.9% of the total variance, were computed and found to be significant. The

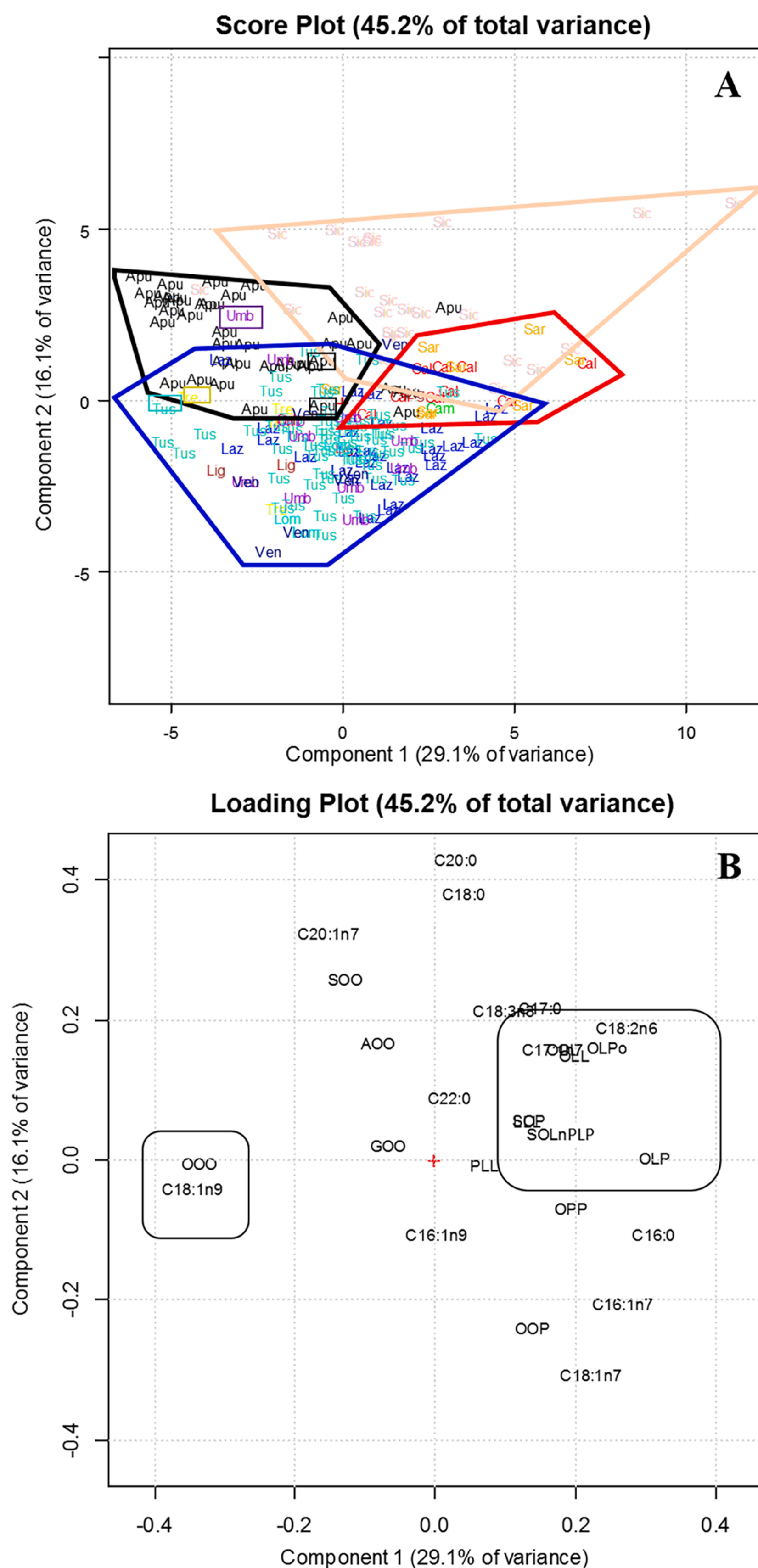

Fig. 3. A) Score plot and B) loading plot of 200 EVOOs in the orthogonal space PC1-PC2 on TAGs and FAMEs auto-scaled data. The names of the twelve Italian regions are abbreviated as follows: Apu = Apulia; Umb = Umbria; Sic = Sicily; Cal = Calabria; Laz = Lazio; Tus = Tuscany; Lig = Liguria; Tre = Trentino-South Tyrol; Sar = Sardinia; Ven = Veneto; Cam = Campania; Lom = Lombardy.

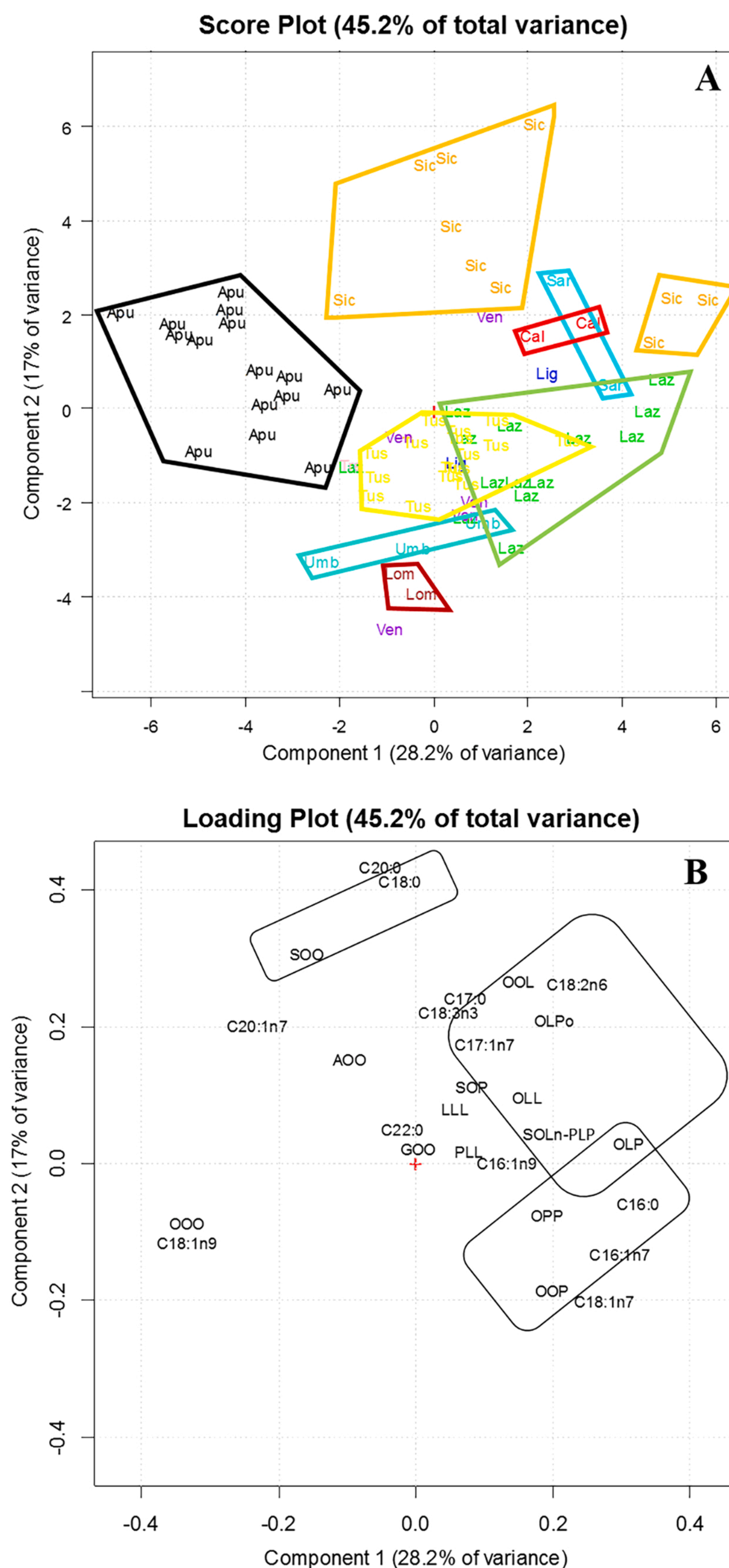

Fig. 4. A) Score plot and B) loading plot of PDO EVOOs in the orthogonal space PC1-PC2 for TAGs and FAMEs auto-scaled data. The names of the Italian regions are abbreviated as follows: Apu = Apulia; Umb = Umbria; Sic = Sicily; Cal = Calabria; Laz = Lazio; Tus = Tuscany; Lig = Liguria; Tre = Trentino-South Tyrol; Sar = Sardinia; Ven = Veneto; Lom = Lombardy.

score plot in the orthogonal space PC1 *vs* PC2 of the auto-scaled TAGs and FAMEs data (data not shown), provided a slightly higher total variance (49.4%) and a clusterization very similar to the one reported in Fig. 3 A, thus confirming the discriminant role of the olive variety in the differentiation of EVOOs. Specifically, the Coratina cultivar seems to maintain its genetic feature even if transplanted from a southern to a central-northern region, while cultivar typical of the central-northern area were less discriminated each-others. This is also in accordance with a previous work reporting the characterization of Apulian EVOOs of different harvesting year (Girelli et al., 2016); in particular, Girelli et al., (2016) pointed out as the chemical profile of EVOOs obtained from Coratina olives did not undergo significant alterations by changing the climatic conditions of different harvest year. By contrast, another paper by Sinelli et al. (2008) showed the classification based on a fingerprinting NIR method for typical center-northern cultivars, namely Casaliva, Leccino and Frantoio, cultivated in Calabria (South of Italy), Tuscany (Center of Italy) and Lombardy (North of Italy) and mixed in blend EVOOs. Calabria EVOOs are totally separated from the other two regions, clearly due to completely different climatic conditions. Tuscany and Lombardia EVOOs are also well clusterized, even if with a lower variance.

4. Conclusion

The present study was aimed to the differentiation of high-quality Italian EVOOs on the basis of the region of cultivation, cultivar and trademark. It was highlighted that lipid components that are the major constituents of the oils can be used for the discrimination of EVOOs not only on the basis of the cultivation region, but also based on the trademark and/or the cultivar. In particular, EVOOs from the southern regions of Sicily, Apulia, Sardinia and Calabria were quite distinct each other and from EVOOs cultivated in the central-northern regions, while the latter were partially overlapped independently from the label (*e.g.* PDO) or the employed variety of olives, leading to conclude that the composition of the typically northern cultivar is mostly affected from pedoclimatic conditions rather than genetic features which should entail the formation of well-defined clusters, as in the case of Sicilian or Apulian samples. Focusing on the central-northern EVOOs, some differentiation was possible by limiting the statistical analysis to only PDO samples, confirming that EVOOs obtained from the same olive varieties in different regions can be separated in the multivariate space (*e.g.* PDO Garda from Lombardy or Veneto, and EVOOs obtained from Frantoio-Moraiolo-Leccino cultivars in Umbria with respect to those obtained from the same cultivars in Lazio and Tuscany), while no separation was achieved for different cultivars in Lazio and Tuscany.

Lipid compounds giving a major contribution to the discrimination were also discussed. They mainly correspond to the most abundant species, such as oleic acid and triolein, as well as linoleic and palmitic acids and the TAGs deriving from their combination.

The other important task regarded the development of fast, reliable and automatic analytical methods, for the determination of both the total FA composition and intact lipids, in order to make the approach fully compatible with the extensive number of samples, required for this kind of studies. To this regard, the development of the LC method included the building of a spectral library and the implementation of a dedicated software for data processing.

Finally, the present work can be used for the realization of a database of Italian EVOOs, grouped according to different characteristics, such as the label, the cultivars or the region of cultivation, with the final aim to valorize the Italian products as important source of bioactive molecules, being in this case the monounsaturated and polyunsaturated FAs, which also can represent discriminant molecules among different oils.

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CRediT authorship contribution statement

Marianna Oteri: Investigation, Visualization, Writing – original draft. **Francesca Rigano:** Validation, Supervision, Software, Writing – review & editing. **Giuseppe Micalizzi:** Investigation, Visualization. **Monica Casale:** Formal analysis, Methodology. **Cristina Malegori:** Formal analysis. **Paola Dugo:** Conceptualization, Supervision, Writing – review & editing. **Luigi Mondello:** Resources, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2022.104531.

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