

Fatty acid composition and $\delta^{13}\text{C}$ of bulk and individual fatty acids as marker for authenticating Italian PDO/PGI extra virgin olive oils by means of isotopic ratio mass spectrometry[†]

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European Regulation (EEC) 2568/91 has been setting the minimum requirements in order to allow labeling of oil as extra virgin. These general requirements, are based on physical–chemical and organoleptic parameters directly linked to the freshness and quality of the product.

Isotope ratio mass spectrometry (IRMS) was demonstrated to be a useful tool for the discrimination of the origin of unknown samples, because the obtained data are practically independent of the cultivar employed and the production technique.

In this work, the evaluation of the composition of fatty acid methyl esters (FAME) alongside with the determination of stable isotope ratio of C in bulk oils and in main FAME constituents have been investigated as a tool to improve geographical discrimination of Italian Protected Designation of Origin/Protected Geographical Indication (PDO/PGI) samples.

For this purpose, authentic PDO/PGI extra virgin olive oils were sampled at oil mills and grouped into different sets according to their areas of provenience.

The use of principal component analysis and partial least squares discriminant analysis multivariate analysis techniques demonstrated that discrimination of olive oil samples can be done using geographical and pedoclimatic parameters predominantly by using $\delta^{13}\text{C}$ results of bulk and individual fatty acids. Results showed that $\delta^{13}\text{C}$ values are a more reliable marker of origin with respect to fatty acid composition. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: extra virgin olive oil; PDO/PGI; IRMS; FAME composition; chemometric analysis

Introduction

Extra virgin olive oil (EVOO) is today recognized as a fundamental landmark of the Mediterranean diet because of its noticeable nutritional and organoleptic characteristics well appreciated by consumers.

Most of its beneficial properties originate from its special (traditional) production process: EVOO is obtained from the olive fruit using only physical treatments that do not cause alteration of the glyceric structure of the oil and preserve its original characteristics.

European Regulation EEC 2568/91 sets the minimum requirements for labeling oil as extra virgin.^[1] These general requirements, which are indicated in annex I of the regulation, are based on physical–chemical and organoleptic parameters, directly linked to freshness and quality of the product (free acidity, peroxide index and ultraviolet absorption, panel test and, more recently, alkyl esters); further requirements, called purity parameters are intended to prevent fraudulent mixing with cheaper oils (refined, pomace or seed oils).^[2]

EVOOs exhibit great differences either from the organoleptic or nutritional/functional point of view (e.g. polyphenols and tocopherols content, aroma, etc.) that originate from the different

and/or the production techniques and/or the geographical origin. These peculiarities have granted to some EVOOs the labeling under a Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) oils (Council Regulation EU 1151/2012).^[3]

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The latter represent distinct regimes of geographical indications within the legal framework of the Protected Geographical Status defined by European Union to protect the 'quality brand' regional foods. For PDO/PGI oils, the production area, including human know-how, is definitively important.

The European Commission already listed in the 'Register of protected designations of origin and protected geographical indications' 42 PDO and 1 PGI olive oils, produced in Italy.

As expected, because of the higher cost associated with these products, fraud is always possible. Selling oils that do not fulfil the requirements of a PDO/PGI as well as selling mixtures of lower quality oils should be treated as counterfeit.

Unfortunately, the lack of official methods of analysis for assessing the origin of the product implies that official control must rely only on checking the accompanying paper documentation.

To address the geographical characterization of EVOOs, various approaches have been proposed, taking into account the aspects (parameters) that are traceable back to origin.

In 1982, Forina and Tiscornia have performed a multivariate approach to classify Italian oils on the basis of compositional data.^[4] A similar approach was applied to the characterization of triacylglycerol and fatty acid profiles of olive oil^[5] in combination with multivariate techniques used to distinguish VOOs according to their geographical origin. Fingerprinting techniques such as NMR,^[6–8] NIR,^[9–11] FT-IR, FT-MIR and FT-Raman spectroscopies,^[12] DNA fingerprinting,^[13] GC/GC-TOF-MS^[14] and HS-SPME coupled with GC-MS have also been used for the determination of oil authenticity.^[15–17]

Sensory analysis was also investigated as a tool to assess differences among monovarietal oils.^[18]

Isotopic ratio mass spectrometry (IRMS) methods were used for the geographical characterization of olive oil in previous works.^[19–21] This technique presents the advantage of giving output data that are practically independent of the employed cultivar and the production technique and, in some cases, has already been introduced as an official method.^[22–24] This kind of methods relies on commodity databases updated each year.^[25]

The isotopic fractionation of C and H and O is linked to pedoclimatic factors (soil, climate, latitude and rain); therefore, they are strictly linked to the region where olive trees come from.^[26] The study of $\delta^{13}\text{C}$ variability of olive oils in several harvests demonstrated that it is not dependent on either the degree of ripeness or maturity state of the olives or the variety.^[27] Angerosa *et al.*^[26] determined the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ of bulk, sterols and aliphatic alcohols of olives from different European regions, developing a model for their geographical classification. However, they observed a certain degree of overlapping for olives coming from neighboring countries with similar climates.

Isotopic ratios ($\delta^{13}\text{C}$, $\delta^2\text{H}$ and $\delta^{18}\text{O}$) of both bulk and extracted glycerol were also found to be a relevant tool for discriminating the origin of EVOO from different regions of Italy^[28] and of Europe.^[19,29]

In detail, $\delta^{13}\text{C}$ content was lower at higher latitudes and/or altitudes, as well as $\delta^2\text{H}$, whereas $\delta^{18}\text{O}$ was strictly related to the mean $\delta^{18}\text{O}$ of annual precipitation. Thus, the isotopic ratios must be regarded as relevant tools for the geographical discrimination of olive oils.

In the present paper, the possibility to use $\delta^{13}\text{C}$ of both bulk oil and individual fatty acids together with fatty acid methyl esters (FAME) composition data as traceability markers in Italian PDO/PGI extra virgin oils was investigated.

Principal component analysis (PCA) was used as a preliminary tool for inspecting data structure using a combination of the

parameters. This unsupervised pattern recognition method is a very useful tool for variable reduction and separation into classes.^[30] Data improvement classification was then demonstrated with a partial least squares discriminant analysis (PLS-DA) approach.

This chemometric approach has been used in this study because it allows straightforward control of results. The PLS-DA is a well-known classification model applied in many scientific fields.^[31,32]

Experimental

Samples

Sampling of authentic EVOO was made by the 'Department of Central Inspectorate for the protection of the quality and fraud repression of food products' of the Italian Ministry of Agricultural and food Policies (ICQRF) inspective personnel at oils mills during the 2010/2011 period. Sampled oils were aimed to be certified under a Protected Designation of Origin Status according to Reg. EU 1151/2012. Three samples at different maturation stages, collected in 60 days, were used.

Oil samples were collected in 250-ml dark glass bottles covered with a black plastic envelope and then shipped to the laboratory. Upon receive, oils were filtered by means of a 0.45- μm barrel type nylon filter, in order to remove any sediment formed which may deteriorate the product. Samples were kept at 8 °C until the analysis.

For every sampling, the following information was recorded:

PDO/IGP zone (and sub zone was applicable), orchard position, olive variety, olive maturity index according to I.O.C guidelines^[33], date of olive pick up, date of transformation, fertilization and irrigation methods, method utilized for olive picking and description of milling process.

Reagents and materials

All reagents were of analytical grade and were supplied by Sigma-Aldrich (St. Louis, MO, USA). High purity gases were supplied by SIAD Spa (Bergamo, Italy).

Fatty acid analysis by GC/FID

Samples were trans-esterified with methanolic potassium hydroxide according to the official method reported in annex XA of EC Regulation 702/2007 (analysis by gas chromatography of methyl esters of fatty acids). Briefly, 0.100 \pm 0.02 g of oil was weighted in a 5-ml vial, and 2-ml heptane and 0.2 ml of 2N methanolic potassium hydroxide solution were added and shaken for 30 s.

The solution was let stratify until the upper solution became clear. One microliter of the heptane solution was then injected into the gas chromatograph.

Fatty acid composition was determined on a Thermo Trace GC/FID chromatograph equipped with a fused silica capillary column RTX-2330 (10% cyano-propyl-phenyl, 90% bis cyanopropyl poly siloxane, L=60 m, i.d.=0.25 mm, f.t.=0.25 μm) Restek (Bellefonte, Pennsylvania, USA). Helium was used as a carrier gas with a constant flow rate of 0.7 ml min⁻¹. The injected sample amount was 1 μl with a split ratio of 1:99; injector temperature was 250 °C. The oven temperature program was 10 min at 165 °C, raised at 1 °C/min to 175 °C, then raised at 5 °C/min to 220 °C and finally held for 10 min at 220 °C; the total time was 39 min. The identification of individual FAME was performed by comparison

Table 1. The three zones used to divide the data set of the work. The classification was based on both geographical and pedoclimatic typology. The geological and altitude parameters were from Ispra (<http://www.isprambiente.gov.it/en>). MLS = mean sea level

Zone A							
Region	N of samples	City	N of samples	Department	Geology	Altitude (MSL) m	Label
Abruzzo	10	Chieti	3	Casoli	Paleocene-Eocene	300–400	A1–A3
		Pescara	6	Loreto aprutino-Pianella	Paleocene-Eocene	200–300	A4–A9
		Teramo	1	Morro D'oro	Paleocene-Eocene	250	A10
Calabria	8	Catanzaro	2	Lamezia Terme	Mesozoic	150	A11–A12
		Cosenza	2	Cerchiaro Di Calabria- Corgliano calabro	Metamorphic	500	A13–A14
		Cosenza	2	Vaccarizzo	Metamorphic	600	A15–A16
		Crotone	2	Belvedere Spinello	Mesozoic	300	A17–A18
Lazio	5	Rieti	1	Fara Sabina	Paleocene-Eocene	300	A19
		Viterbo	4	Canino-Stocchi Luciano	Volcanic	250–300	A20–A23
Puglia	22	Bari-Brindisi-Foggia-Lecce	22	Several	Mezoic	100–200	A24–A45
Sicilia	9	Agrigento	3	Sciaccia	Mezoic	200	A46–A48
		Trapani	6	Castel vetrano-Trapani	Paleocene-Eocene	150	A49–A54
Umbria	2	Terni	2	Orvieto	Volcanic	300	A55–A56
Zone B							
Region	N of samples	City	N of samples	Department	Geology	Altitude (MSL) m	Label
Campania	8	Napoli	2	Sorrento-Ebali-S. mezzarria-Serre-V. Lucania	Oligocene-Neogene	100	B1–B8
		Salerno	6	Several	Oligocene-Neogene	300–400	B9–B10
Emilia	5	Ravenna-Forlì-Cesena	2	Several	Oligocene-Neogene	150	B11–B13
Friuli	3	Trieste	3	Montegridolfo-Saludicio	Oligocene-Neogene	100	B14–B16
		Savona	3	S. dorligo della valle	Oligocene-Neogene	70	B17,B19
Liguria	3	Pesaro-Urbino	3	Albenga-Andora-Garlienda	Oligocene-Neogene	200–250	B20–B22
Marche	3	Campobasso-Isernia	3	Several	Oligocene-Neogene	150–300	B23–B25
Molise	3	Catania	3	Adrano-Bronte	Oligocene-Neogene	500	B26–B28
	6	Ragusa	3	Chiaromonte	Oligocene-Neogene	300–500	B29–B31
Toscana	2	Grosseto	1	Grosseto	Oligocene-Neogene	70	B32
		Siena	1	Trequanda	Oligocene-Neogene	350	B33
Umbria	12	Perugia	9	Several	Oligocene-Neogene	200–400	B34–B42
		Terni	3	Amelia-Sangemini	Oligocene-Neogene	200–400	B43–B45
Zone C							
Region	N of samples	City	N of samples	Department	Geology	Altitude (MSL) m	Label
Abruzzo	4	Chieti	2	Vasto	Quaternary	40	C1–C2
		Teramo	2	Atri-Canzano	Quaternary	40	C3–C4
Calabria	9	Catanzaro	1	Lamezia Terme	Quaternary	70	C5
		Cosenza	8	Bisignano-C. di Calabria-Mongrassano-S. Albenese-T. da Sibari	Quaternary	90–150	C6–C13
Liguria	6	Genova	2	Chavari-Lavagna	Quaternary	50–60	C14–C15
		Imperia	3	Diano s. Pietro-Imperia-Pietrabruna	Quaternary	150	C16–C18
		La Spezia	1	Levanto	Quaternary	150	C19

Zone C		N of samples		City		N of samples		Department		Geology		Altitude (MSL) m		Label	
Region	Lombardia	9		Brescia-Lecco		9		Several		Quaternary		100		C20-C28	
	Sicilia	3		Messina		3		Torrenova		Quaternary		400		C29-C31	
	Toscana	9		Lucca-Pisa-Siena		9		Several		Quaternary		80-90		C32-C40	
	Trentino	3		Trento		3		Rovereto		Quaternary		150		C41-C43	
	Umbria	1		Terni		1		Orvieto		Quaternary		150		C44	
	Veneto	10		Padova-Treviso-Verona		10		Several		Quaternary		80-100		C45-C54	

of retention times with those of a standard FAME mixture (Supelco, Bellefonte, PA).

The quantification was made with the internal normalization method; results were expressed as percentage over the total of fatty acids, assuming complete elution of the components.

C isotope ratio analysis of bulk oil by EA/IRMS

The carbon isotope ratio ($\delta^{13}\text{C}$) was determined according to the elemental analysis (EA)/IRMS technique. The experiments were carried out by a flash combustion on an elemental analyzer (EA) Flash 2000 HT (Thermo Fisher, Bremen, Germany), connected to a Delta V Plus (Thermo Fisher, Bremen, Germany) isotope ratio mass spectrometer operating in continuous helium flow mode, via a ConFlo IV interface (Thermo Fisher, Bremen, Germany). Following the flash combustion, the products CO_2 and N_2 were previously split (1:9) and then injected, via the interface, into the IRMS.

An aliquot of about 0.80 ± 0.01 mg of oil was weighted on a semi-microanalytical balance and then wrapped in a cylindrical tin capsule, (8 i.d. \times 5 mm). The oil was combusted in the EA under a continuous stream of helium and on a 3-s flash of oxygen in a quartz reactor packed with Cr_2O_3 , $(\text{Co}_3\text{O}_4)_2\text{Ag}$, Cu/CuO at 920°C , according to the producer's instructions.

The combustion produces a mixture of CO_2 , NO_x and H_2O . NO_x was reduced to N_2 by metallic Cu, while water was removed by using a trap of anhydrous $\text{Mg}(\text{ClO}_4)_2$, placed in series with the combustion reactor.

CO_2 was separated from N_2 in a 5-m-long Pora-PLOTQ packed column with a 1/4-in i.d. (Varian, PaloAlto, CA) at 45°C . The flow was split before entering the interface and then injected in the IRMS, where the isotopic ratios of the masses 44, 45 and 46 were determined. The values of $\delta^{13}\text{C}$ of the samples were calculated against pulses of a CO_2 reference gas from tank. Delta values were calculated by the Isodat 3.0 software (from Thermo Bremen, Germany).

The value of delta (δ) notation is reported as delta per thousand (‰), according to the following expression:

$$\delta^{13}\text{C}^0/00 = (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \cdot 10^3$$

where R is the isotopic ratio $^{13}\text{C}/^{12}\text{C}$. The usual reference standard is the Vienna-Pee Dee Belemnite (V-PDB), a calcareous mineral, and $\delta^{13}\text{C}$ values are expressed as 'vs. V-PDB'.

The R ratio was calculated according to the signal of masses 45 (for ^{13}C) and 44 (for ^{12}C). The 'Craig correction'^[34] was also applied, based on the signal of mass 46, too.

The accuracy of each analysis batch was checked according to the following procedure.

In every analytical sequence, two different reference materials were employed, in order to correct instrumental bias. The first reference material was used to evaluate the daily bias of the instrument; the bias determined in such a way was added to each experimental value. The second reference material, with a different $\delta^{13}\text{C}$, was used to check the accuracy of this correction.

All the experimental values were accepted if the corrected value of the second reference material fell within its reference interval.

The reference material isotopic values were determined using certified reference materials (IAEA CH-6, IAEA NBS22, IAEA USGS40 from International Atomic Energy Agency, Vienna, Austria).

The repeatability and intermediate precision of the EA/IRMS method, defined as the observed variability from separately replicate analyses of laboratory standard materials and vegetable oil samples, were better than 0.1‰ (SD) for $\delta^{13}\text{C}$.

C isotope analysis of individual fatty acids oil by GC/IRMS

Carbon isotope ratio ($^{13}\text{C}/^{12}\text{C}$) of the individual methyl esters was carried out on an ISOLINK Thermo Fisher Scientific GC apparatus consisting of a Thermo Trace GC gas chromatograph coupled to an IRMS mass spectrometer via a combustion interface operating under a continuous flow of helium (GC/C/IRMS).

The interface consisted of two ceramic furnaces: a combustion reactor operating at a temperature of 940 °C made of CuO/NiO/Pt and a reduction reactor consisting of a filament of Cu maintained at 600 °C which reduces NO_x to N₂ products. Finally, water was removed by passing it through a Nafion tube.

The fatty acid methyl ester preparation procedure and GC conditions were the same carried out for GC/FID.

The performance of the GC/C/IRMS system, including GC and combustion furnace, was evaluated in terms of accuracy and precision. Precision was assessed by performing replicate determinations on the same sample.

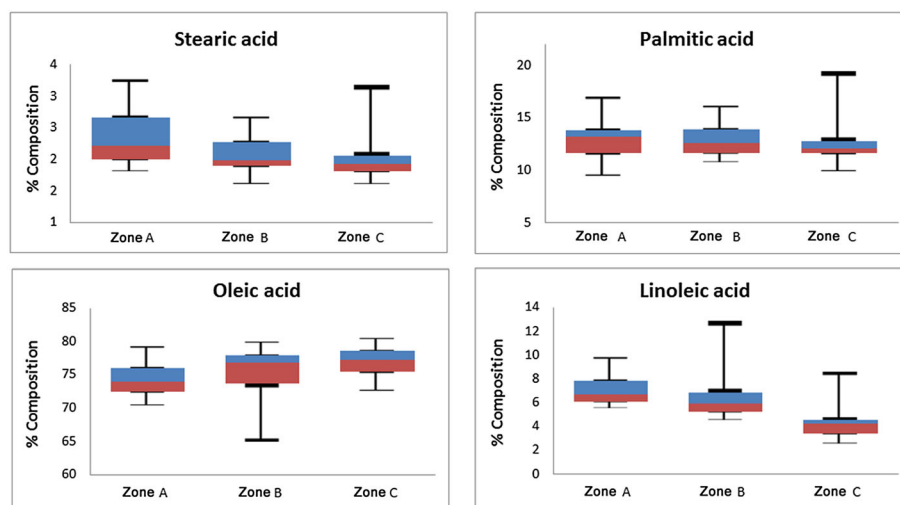


Figure 1. Box and whiskers plots of the distribution of FAME in the selected zones.

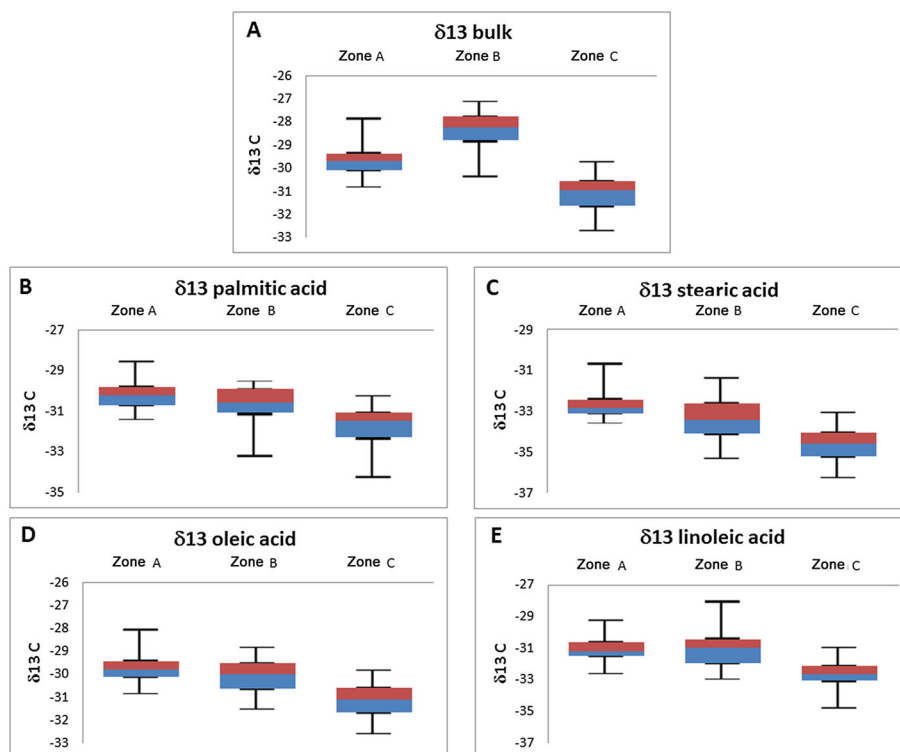


Figure 2. Distribution of $\delta^{13}\text{C}$ of bulk samples (A) and of four FAME: (B) palmitic, (C) stearic, (D) oleic, and (E) linoleic acid.

Accuracy was assessed by injecting simultaneously and separated in the syringe by an air bubble 0.5 μl of a 0.01% w/v solution of heptadecanoic acid methyl ester in heptane whose isotopic ratio was previously standardized by EA/IRMS analysis (average of δ obtained by 10 EA/IRMS analysis).

Finally, the isotopic shift due to the carbon introduced in the fatty acid methylation was corrected by a mass balance equation:

$$\delta^{13}\text{C}_{\text{FAME}} = [(C_n + 1)\delta^{13}\text{C}_{\text{FA}} - \delta^{13}\text{C}_{\text{MeOH}}]/C$$

where $\delta^{13}\text{C}_{\text{FAME}}$, $\delta^{13}\text{C}_{\text{FA}}$ and $\delta^{13}\text{C}_{\text{MeOH}}$ are the carbon isotopic values of the FAME, fatty acid and methanol, respectively, and C_n is the number of C atoms in the fatty acid. The $\delta^{13}\text{C}$ of methanol was calculated via EA/IRMS as a mean of 10 determinations.

Statistical evaluation

Statistical analysis was performed using two different multivariate approaches, PCA and PLS-DA by means of MatLab R2009b (Mathworks, Natick, MA, USA) integrated with a classification toolbox for MATLAB from Milano Chemometrics and QSAR Research Group version 3.0. Data have been autoscaled (zero mean and unitary variance). Data vectors belonging to the same geographical origin were first analyzed by unsupervised PCA. Then, a numerical evaluation of the models achieved was validated by cross-validation 'venetian blinds' technique with the number of cv groups equal to 5. Using confusion matrices, the reliability of the classification models achieved was studied in terms of recognition ability (percentage of the members of the training set correctly classified) and prediction ability (percentage of the members of the test set correctly classified by using the rules developed in the training step).

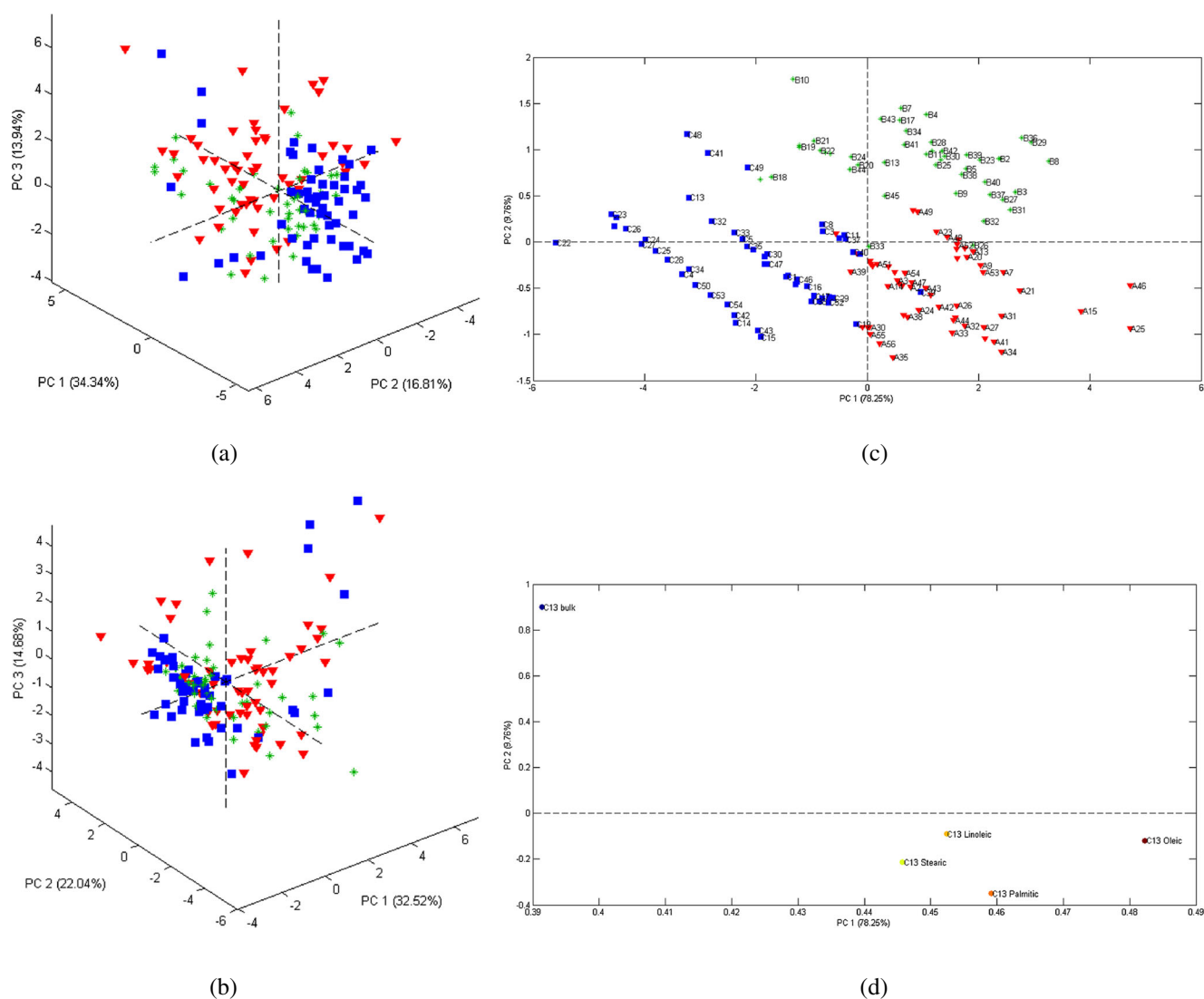


Figure 3. (A). PCA score plot of the first, second and third principal components of the 155 olive oil samples using all the 18 variables. PCA model calculated on the whole data set. Data were autoscaled before analysis. Red = zone A, green = zone B and blue = zone C. (B). PCA Scores plot of the first, second and third principal components of the 155 olive oil samples using only the 13 fatty acid variables. PCA model calculated on the whole data set. Data were autoscaled before analysis. Red = zone A, green = zone B, and blue = zone C. (C). PCA score plot of the first and second principal components of the 155 olive oil samples using only the isotopic variables. PCA model calculated on the whole data set. Data were autoscaled before analysis. Red = zone A, green = zone B and blue = zone C. (D). PCA loading plot of the first and second principal components of the 155 olive oil samples using only the isotopic variables. PCA model calculated on the whole data set. Data were autoscaled before analysis. Color scale represents the weight on PC1. (This figure is available in colour online at wileyonlinelibrary.com/journal/jms.)

Results and discussion

The data set was split in three zones, named A, B and C as reported in Table 1. These were selected on the basis of the geological variety of the Italian areas. Zone A, which includes areas having Mesozoic and lower Cenozoic rocks mostly present in southern Italy; zone B having Cenozoic rocks mostly present in central Italy; zone C refers to areas having Neozoic rocks mostly present in northern Italy.

The reason of this distribution lies in the need to obtain sampling areas which are homogeneous in terms of pedoclimatic typology. In general the Italian geological environment has strong variability, as can be seen in Table 1, but there is a significant correlation between geology and altitude with some exceptions due to rapid changes of Italian land shape.

Some exceptions to the pattern of increasing $\delta^{13}\text{C}$ with latitude and altitude have been reported.^[35] These apparent abnormalities are linked to local pedoclimatic factors, but also soil characteristics have been proposed to be partially responsible of this trend.^[36,37] For this reason, some samples from Sicilia and Umbria were included in different zones, samples from Abruzzo and Calabria were included in zone A and C and samples from Toscana and Liguria were included in zone B and C.

Thirteen FAME (i.e. myristic, palmitic, palmitoleic, heptadecanoic, heptadecenoic, stearic, oleic, linoleic, arachidonic, linolenic, eicosenoic, behenic and lignoceric acid) were quantitatively analyzed in the investigated samples. It was found that the median concentration of stearic and linoleic acid was partially related with the different areas, increasing from north to south (Fig. 1); oleic acid had an opposite trend with greater concentrations in the northern samples, while no specific trends were found for the other FAME. However, these parameters were not sufficient to provide a clear differentiation between samples, because of the great variability on the composition for the samples belonging to each area.

The isotope $\delta^{13}\text{C}$ bulk was subsequently determined for the 155 olive oil samples; it was found that the carbon isotope ratio was lower in the samples belonging to zone C; however, also in this case, no clear classification was possible (Fig. 2A). When samples were subdivided in narrower areas belonging to the same territory, the classification was improved, since $\delta^{13}\text{C}$ is a parameter influenced by geographical variables such as latitude, rainfall, distance from the sea and altitude,^[27] and the three zones are not really homogenous from these points of view.

Median values obtained were generally lower in the northern samples, while A and B zones resulted mostly superposed (Fig. 2B–E).

Since single parameters were not sufficient to provide a clear discrimination, such variables were submitted to multivariate statistical procedures. The 13 fatty acids, the isotope $\delta^{13}\text{C}$ bulk and the four fatty acids isotopes $\delta^{13}\text{C}$, determined for the 155 Italian olive oil samples, represented the entire data set used for multivariate analysis.

As reported in Fig. 3, PCA was applied to the 155 olive oil samples using different variable combinations: all the 18 variables, only the amount of the 13 fatty acids variables and only the 5 isotopic variables.

PCA offers the possibility to project data from a higher to a lower dimensional space having a data overview without any preliminary assumptions.^[38] The PCA evidenced that for all variables tested the first three principal components accounted for 65% of the total variance. The score plot on the first three components did not show optimal discrimination. No additional contribution to zone separation was done by the 13 fatty acids, whereas using only the isotope variables, zones were differentiated by the first two components having a total variance of 88%.

Table 2. PLS-DA important statistical data used for the 155 olive oil sample all variables, only the 5 $\delta^{13}\text{C}$ isotopic variables and only the 13 fatty acid variables. CAL = calibration; CV = cross validation

Class	All variables			$\delta^{13}\text{C}$ isotopic variables			13 fatty acid variables		
	1	2	3	1	2	3	1	2	3
Sensitivity (Cal)	0.95	0.93	0.94	0.98	1.00	0.94	0.82	0.87	0.89
Specificity (Cal)	0.90	0.97	0.89	0.91	0.97	0.92	0.79	0.52	0.60
Sensitivity (CV)	0.91	0.91	0.96	0.98	0.96	0.93	0.79	0.78	0.83
Specificity (CV)	0.89	0.94	0.89	0.89	0.97	0.91	0.78	0.49	0.60
Class. err (Cal)	0.08	0.05	0.08	0.05	0.01	0.07	0.20	0.31	0.25
Class. err (CV)	0.10	0.08	0.07	0.06	0.04	0.08	0.22	0.37	0.28
RMSEC	0.31	0.22	0.30	0.29	0.20	0.29	0.41	0.43	0.40
RMSECV	0.33	0.25	0.31	0.30	0.21	0.30	0.44	0.46	0.42
RMSEP	0.31	0.22	0.30	0.29	0.20	0.29	0.41	0.43	0.40
Bias	-6E-15	8E-15	-3E-15	4E-16	2E-15	-3E-15	8E-16	-2E-16	-6E-16
CV bias	2E-03	1E-03	-3E-03	-2E-04	-2E-04	3E-04	4E-04	-7E-04	3E-04
R ²	0.60	0.77	0.60	0.62	0.80	0.64	0.28	0.09	0.28
CV R ²	0.52	0.69	0.58	0.61	0.78	0.62	0.18	0.01	0.22

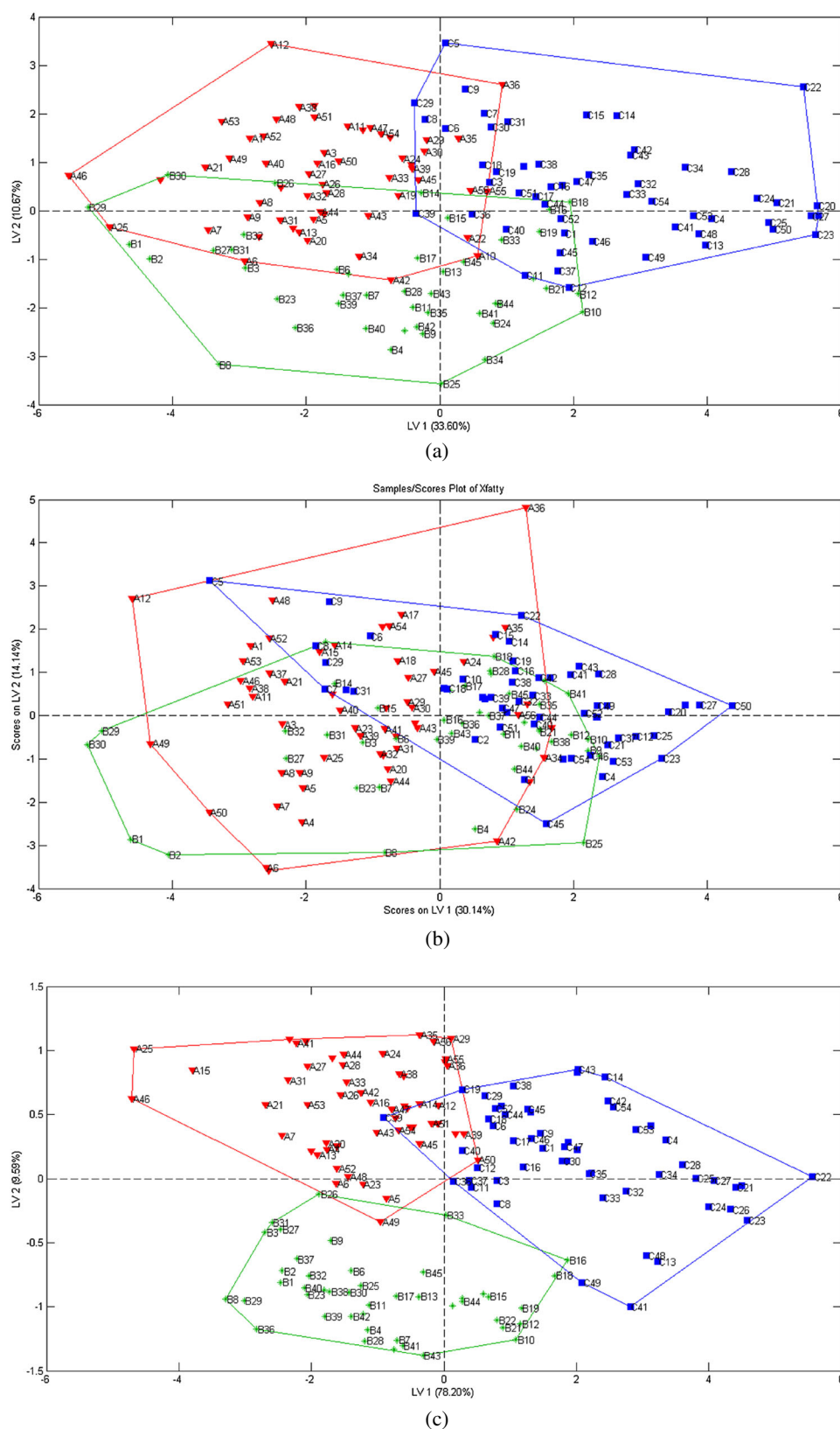


Figure 4. (A). Score plot of PLS-DA model built to classify the data using the 155 olive oil samples using all variables. Data have been linearly normalized and autoscaled (zero mean and unitary variance) in order to remove concentration effects and different signals ranges. Red = zone A, green = zone B and blue = zone C. (B). Score plot of PLS-DA model built to classify the data using the 155 olive oil samples using only the 13 fatty acid variables. Data have been linearly normalized and autoscaled (zero mean and unitary variance) in order to remove concentration effects and different signals ranges. Red = zone A, green = zone B and blue = zone C. (C). Score plot of PLS-DA model built to classify the data using the 155 olive oil samples using only the 5 isotopic variables. Data have been linearly normalized and autoscaled (zero mean and unitary variance) in order to remove concentration effects and different signals ranges. Red = zone A, green = zone B and blue = zone C.

As usual, with multivariate data analysis, each sample corresponds to a vector in a multidimensional space. It is worth to discuss the position of the loadings as they are a measure of the contribution of the individual parameter to the first two principal components and, then, the measurement contribution to the representation of PCA. In particular, it is important to point out that the loadings are orthogonally distributed, and orthogonality in PCA plots means uncorrelated contributions.^[39] Looking at the isotopic variable contribution (Fig. 3 D), the isotopes $\delta^{13}\text{C}$ of fatty acids have a certain degree of correlation with respect to PC 2, but they are inversely correlated to bulk $\delta^{13}\text{C}$. Despite the correlation looking at PC 1, the isotopes $\delta^{13}\text{C}$ of linoleic–stearic–palmitic fatty acids are grouped with respect to $\delta^{13}\text{C}$ of oleic fatty acid. This demonstrates that isotopes $\delta^{13}\text{C}$ bulk and $\delta^{13}\text{C}$ of fatty acids independently contribute to the data set discrimination especially in separating zone A–B from zone C. The relative position of the scores in the PCA plot (Fig. 3 C) provides an indication about the ability of the isotopes $\delta^{13}\text{C}$ of fatty acids to discriminate among the three zones samples herewith considered. The scores data indicated that the isotopes can discriminate between and within different geographical/pedoclimatic typologies. Anyway, a partial overlap is observed for the zones A and B.

To evaluate the real efficiency of the discrimination among the different olive oil samples, based on the multi-isotopic analysis, a multivariate discriminant analysis was applied. As described above, the entire set of samples (total number 155; see Table 1 for details on the sample set) was used for this approach. As in any supervised classification techniques, the classes have to be chosen a priori. The natural choice for the samples in this case was to choose the zones as classes. With this classification scheme, a PLS-DA model has been built.

PLS-DA is an extension of PLS, by projecting intercorrelated X-variables from high dimensional space into low-dimensional space according to a Y-vector that encodes the class membership in a set of categorized variables (1 and 0 values, respectively).^[40] Initially PLS-DA analysis was applied using all the 18 variables

and then using independently the 5 isotopes $\delta^{13}\text{C}$ and the 13 fatty acids.

As reported in Table 2, the use of $\delta^{13}\text{C}$ isotopic variables gave a very good PLS-DA sensitivity and specificity in both calibration and cross validation. Data have been linearly normalized and then autoscaled (zero mean and unitary variance) before analysis. A graphical representation is reported in Fig. 4, respectively, for all the variables (Fig. 4A), the 13 fatty acids (Fig. 4B) and $\delta^{13}\text{C}$ variables only (Fig. 4C).

A clear separation between the data related to the three classes is observed only using the $\delta^{13}\text{C}$ variables.

A numerical evaluation of the classification properties can be obtained by considering the cross validation of the PLS-DA method according to the 'venetian blinds' technique. The results are shown in Table 3, in the form of a confusion matrix. Ninety-six percent of the samples have been correctly classified by $\delta^{13}\text{C}$ variables, 93% using all variables and only 59% using fatty acids variables.

Conclusions

This work has achieved the fatty acid $\delta^{13}\text{C}$ characterization of all the Italian PDO/PGI olive oil related to the crop year 2010/2011, allowing the creation of a databank based of composition and isotopic parameters.

The evaluation of FAME results showed a certain degree of correlation among each individual analyzed fatty acid, but that is not sufficient to discriminate samples on the basis of the typology of area of origin.

On the other hand, as demonstrated by multivariate analysis, discrimination of olive oil samples can be done using geo-climatic parameters predominantly by using $\delta^{13}\text{C}$ results of bulk and individual fatty acids. Results showed that ^{13}C isotopic values are a robust marker of origin with respect to fatty acid composition.

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Table 3. Cross-validation confusion matrix of the PLS-DA method using all variables, only the 13 fatty acids and only the $\delta^{13}\text{C}$ variables. Venetian blind validation method was used for cross validation. True classes are read along the columns and estimated classes along the rows. The total accuracy was also reported

All variables				
Real/predicted	Zone A	Zone B	Zone C	Row (%)
Zone A	52	0	4	93
Zone B	1	44	0	98
Zone C	3	3	48	89
Total (%)		93		
Only 13 fatty acids variables				
Zone A	37	8	11	66
Zone B	12	10	23	22
Zone C	7	2	45	83
Total (%)		59		
Only $\delta^{13}\text{C}$ isotopic variables				
Zone A	54	2	0	96
Zone B	0	44	1	98
Zone C	2	1	51	94
Total (%)		96		

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