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Ground-breaking comparison of target stable isotope ratios vs. emerging sesquiterpene fingerprinting for authenticating virgin olive oil origin

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ABSTRACT

This study presents a pioneering comparison of target stable isotope ratios analysis and sesquiterpene (SH) fingerprinting for authenticating virgin olive oil (VOO) geographical origin. Both methods were selected for being among the most promising targeted and untargeted approaches, respectively. These methods were applied to the same sample set of nearly 400 VOO samples, covering diverse harvest years, cultivars and producers. PLS-DA classification models were developed to differentiate between Italian and non-Italian VOOs, as well as VOOs from three closely located Italian regions. Isotopic models based on bulk $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ achieved over 75 % classification accuracy in distinguishing Italian from non-Italian VOOs, while SH fingerprinting outperformed with over 90 % accuracy and greater sensitivity to regional differences, as assessed in external validation. This systematic comparison provides insights into the strengths and weaknesses of each method, and the results will guide future research to enhance their reliability in VOO geographical authentication.

1. Introduction

Food fraud has gained increasing concern over the years and currently remains a critical issue undermining food chain integrity (Bannor et al., 2023; Everstine et al., 2024). The olive oil supply chain, particularly extra virgin olive oil (EVOO), is highly vulnerable to fraud, as evidenced by its persistent ranking among foods with the highest fraud incidence (The EU Agri-Food Fraud Network, 2021; Joint Research Center of the European Commission JRC, 2024). A substantial percentage of fraud cases in official reports were related to mislabelling, including the falsification of mandatory origin declarations required on VOO label under European regulations (Commission Delegated Regulation (EU) 2022/2104). Since the country of origin significantly influences consumer preferences, it impacts the market price, particularly in Italy, where the "Made in Italy" label boosts global demand for high-quality products, such as VOO (Cappelli et al., 2017; Carbone & Henke, 2023). As a result, Italian-declared EVOO commands the highest prices in both international and domestic markets (International Olive Council IOC, 2023; Bimbo et al., 2020), with prices 35 % and 45 % higher than those from other European and non-European countries, reflecting its reputation and higher production costs (Bimbo et al., 2020).

This situation fosters fraud of Italian EVOOs with cheaper alternatives. In this sense, the lack of an official analytical method to verify the

Abbreviations: COW, Correlation Optimized Warping; DVB/CAR/PDMS, divinylbenzene/carboxen/polydimethylsiloxane; EA-IRMS, Elemental Analysis-Isotope Ratio Mass Spectrometry; EIC, Extracted Ion Chromatograms; ESP, Spain; EU, European Union; EVOO, extra virgin olive oil; GRC, Greece; HS-SPME-GC–MS, Headspace-solid phase microextraction-gas chromatography–mass spectrometry; ITA, Italy; LV, latent variables; MIPAAF, Italian Ministry of Agricultural, Food and Forestry Policies; PCA, Principal Component Analysis; PDO, Protected Designation of Origin; PGI, Protected Geographical Indication; PLS-DA, partial least squares discriminant analysis; POR, Portugal; PV, predicted value; RMSEcv, Mean Squared Error of Cross Validation; ROC, receiver operating characteristic; SH, sesquiterpene hydrocarbons; TUN, Tunisia; TUR, Turkey; SIM, selected ion monitoring; VOO, virgin olive oil..

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geographical origin of VOOs, despite legal requirements on labels, is a critical gap in preventing fraud that significantly concerns stakeholders and needs urgent attention (Casadei et al., 2021; Conte et al., 2020). In response, researchers have intensified their efforts on developing reliable methods for VOO geographical authentication (Maléchaux et al., 2020), leading to significant advancements in the state of the art (Bajoub et al., 2017; Conte et al., 2020; Zaroual et al., 2021). The research has concentrated on developing efficient, cost-effective, and rapid screening methods to detect fraud, while also addressing challenges such as reproducibility and transferability, which are crucial for adoption by regulatory authorities.

One of the most recognized methods for establishing the geographical origin of food is stable isotope analysis, a targeted technique that identifies and quantifies a selected set of predefined isotopes in samples. This method relies on the strong influence of production zone factors, such as geology and hydrogeology on the isotopic composition of agricultural products (Laursen et al., 2016). Isotopic analysis of light bioelements (C, H, O, N, S) has been widely applied to verify the geographical origin of various foodstuffs and, in some cases, has even been proposed for legal verification (Camin et al., 2017). Regarding its application for tracing the origin of VOO, bulk $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ enabled characterising VOO Italian production (Camin et al., 2010; Chiocchini et al., 2016; Portarena et al., 2014), differentiating part of Italian VOOs from Tunisian oils (Camin et al., 2016) and other EU oils (Camin et al., 2010), and establishing significant differences among Italian macro-regions (Bontempo et al., 2009; Camin et al., 2010; Portarena et al., 2014).

The main strengths of stable isotope analysis are its high precision under repeatable measurement conditions (<0.05 % RSD), the use of certified reference materials for bias correction, and, in the case of bulk analysis, the minimal sample manipulation and short analysis time. It can also be implemented by accredited food testing laboratories according to ISO and AOAC standards (Bayen et al., 2024). However, isotopes are highly dependent on the harvest season (Camin et al., 2010), and significant differences in the isotopic profiles of bulk VOO from various geographical areas often overlap considerably, making direct differentiation difficult. Therefore, it is often necessary to combine bulk isotopic analysis with compound-specific isotopic analysis (Bontempo et al., 2019; Faberi et al., 2014) or other techniques and markers, such as elemental profile (Camin et al., 2010; Camin et al., 2010) or metabolite analysis (Faberi et al., 2014; Lukić et al., 2020; Portarena et al., 2017).

Untargeted metabolomics represents a cutting-edge approach for food authentication, providing more comprehensive data for fraud detection compared to traditional targeted methods (Quintanilla-Casas et al., 2025). This is because the untargeted approach is not limited to predefined compounds but considers comprehensive data, enhancing authentication efficiency (Amaral, 2020; Ballin & Laursen, 2019). Among untargeted methods, the fingerprinting approach combines raw analytical data with chemometric techniques, proving highly effective for authenticating VOO (Quintanilla-Casas et al., 2025; Quintanilla-Casas, Bertin, et al., 2020; Quintanilla-Casas, Marin, et al., 2020; Torres-Cobos et al., 2021). For instance, sesquiterpene hydrocarbon (SH) chromatographic fingerprints analysed by headspace-solid phase microextraction-gas chromatography–mass spectrometry (HS-SPME-GC–MS) in combination with Partial Least Square-Discriminant Analysis (PLS-DA) successfully distinguish VOOs based on their origin across different levels, including EU-wide and single-country labels, as well as adjacent PDO (Quintanilla-Casas, Torres-Cobos, Guardiola, Romero, et al., 2022; Quintanilla-Casas, Torres-Cobos, Guardiola, Servili, et al., 2022). SHs are robust geographical markers for VOOs, strongly associated with olive cultivars and growing regions, and stable during processing and storage (Quintanilla-Casas, Bertin, et al., 2020; Vichi et al., 2018). Moreover, their analysis requires affordable and automatable instrumentation and need minimal sample manipulation, offering extremely high classification accuracy when used under a fingerprinting approach (Quintanilla-Casas, Bertin, et al., 2020). However, unlike stable isotope analysis, the transferability of chromatographic fingerprints between laboratories remains challenging due to absence of clear guidelines to assess the analytical performance of these methods (Bayen et al., 2024; Quintanilla-Casas et al., 2025; Riedl et al., 2015).

Therefore, both stable isotope analysis and SH fingerprinting offer distinct advantages and capabilities, making them appear as suitable strategies for authenticating the geographical origin of Italian VOO. However, it is essential to objectively evaluate and contrast their performance to identify the most reliable and effective method for VOO geographical authentication and to identify areas for further advancement in these approaches. The results in literature obtained by both methods are not easily comparable, underscoring the need for a systematic comparison under standardized conditions. Such a comparative study should test both methods on the same sample set, applying consistent statistical treatments, and assessing their performance across diverse challenging scenarios in terms of sample variability and level of VOO geographical differentiation to help identify the most reliable and effective method for detecting origin fraud in VOO.

With the aim, this work compares models developed on stable isotope data and SH fingerprinting data obtained from the exact same sample set of VOO samples, to differentiate between Italian and non-Italian VOOs, as well as among VOO from closely situated Italian production regions.

2. Material and methods

2.1. Samples

The sample set consisted of 393 traceable VOOs from various countries and geographical regions (Table 1), produced from 2016–17 to 2019–20. Part of these samples were from Italy (ITA, $n = 242$), while the remaining samples were oils from other 5 Mediterranean countries [non-ITA, $n = 151$: Spain (ESP), $n = 51$; Greece (GRC), $n = 39$; Portugal (POR), $n = 23$; Turkey (TUR), $n = 21$; Tunisia (TUN), $n = 17$]. Italian samples were produced in different regions: Apulia ($n = 73$), Calabria ($n = 58$), Sicily ($n = 40$), and other regions (Lombardia/Emilia Romagna, $n = 16$; Tuscany, n = 7; Liguria, $n = 6$; Sardinia, n = 4; Basilicata, n = 4; Umbria, n = 3; Abruzzo, n = 2; Campania, n = 2; Marche, n = 1) or were from not specified Italian regions ($n = 26$) (Fig. 1). Therefore, oil samples were produced over multiple harvest years and showed significant variability in cultivars, producers, and processing techniques, providing a challenging scenario to test the methods under investigation. Additional information about the samples is available in **Table S1** of **Supplementary information**. The samples were stored under a nitrogen (N_2) atmosphere at −20 °C until analysis.

Table 1
Geographical origin and harvest year of the 393 VOOs analysed.

		2016/2017	2017/2018	2018/2019	2019/2020
Italy (ITA)	n				
Apulia	73	4	1	35	33
Calabria	58	1	1	21	35
Sicily	40	3	0	15	22
Other regions	45	6	6	27	6
No specified	26	1	2	1	22
Total ITA	242	15	10	99	118
Other countries (non-ITA)	n				
Spain	51	0	0	25	26
Greece	39	0	0	9	30
Portugal	23	0	12	6	5
Turkey	21	10	11	0	0
Tunisia	17	1	5	0	11
Total non-ITA	151	11	28	40	72

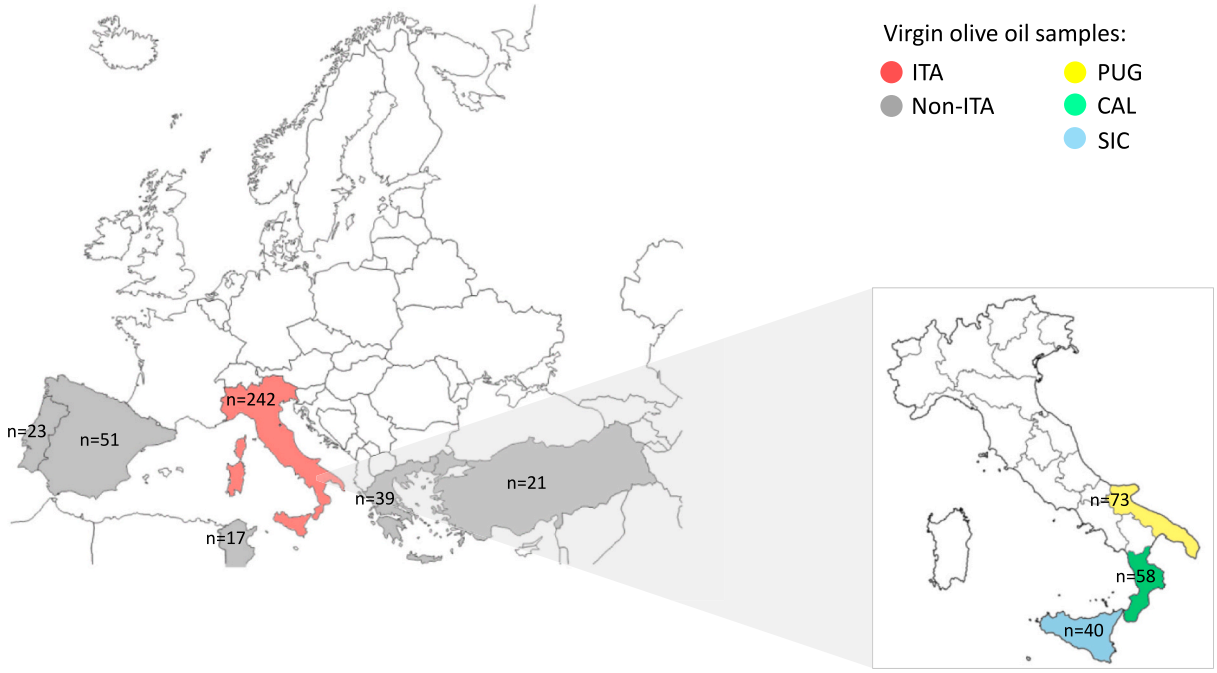

Fig. 1. Virgin olive oil sampling. ITA: Italy (red) non-ITA: the other countries of origin (grey), PUG: Apulia (yellow), CAL: Calabria (green), SIC: Sicilia (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Isotopic analysis of bulk VOO by elemental analysis-isotope ratio mass spectrometry (EA-IRMS)

VOO samples were weighed (ca 0.3 mg) and placed in tin capsules to measure the $\delta^{13}C$ using an isotope ratio mass spectrometer (Elementar Analysensysteme GmbH, Langen-selbold, Germany) after total combustion in an elemental analyser (Vario Isotope Cube; Elementar Analysensysteme GmbH).

The $\delta^{18}O$ and $\delta^{2}H$ ratios were obtained by weighing approximately 0.25 mg of the sample in silver capsule and introducing it into a TC/EA (Finnigan DELTATC/EA, high temperature conversion elemental analyser, Thermo Scientific). The samples were measured in duplicate for carbon as well as for oxygen and hydrogen.

In accordance with IUPAC protocol, isotopic values are expressed as delta relative to the international standards: V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$, V-SMOW (Vienna-Standard Mean Ocean Water) for $\delta^{2}H$ and $\delta^{18}O$, and Air (atmospheric N_2) for $\delta^{15}N$, as described in Eq. (1):

$$\delta^{i}\left(E_{sample/standard}\right) = \frac{R\left({}^{i}E/{}^{j}E\right)_{sample}}{R\left({}^{i}E/{}^{j}E\right)_{standard}} - 1 \quad (1)$$

where 'standard' refers to the international measurement standard, 'sample' is the analysed specimen, and ${}^{i}E/{}^{j}E$ represents the isotope ratio between heavier and lighter isotopes. Delta values are multiplied by 1000 and are commonly expressed in per mil (‰) or, in accordance with the International System of Units (SI), as 'milliurey' (mUr)."

Carbon isotopic values $\delta^{13}C$ were calculated relative to the USGS 88 standard ($\delta^{13}C$ -16.06 ‰). The isotopic value of the sample was obtained by algebraically summing the difference between the true value of the international standard and its instrumental value. Additionally, millet flour (USGS 90, $\delta^{13}C$ -13.75 ‰) and an in-house working standard – a wheat flour ($\delta^{13}C$ -25.95 ‰) calibrated against fuel oil (NBS-22 $\delta^{13}C$ -30.03 ‰), L-glutamic acid (USGS 40, $\delta^{13}C$ -26.39 ‰), and sucrose (IAEA-CH-6, $\delta^{13}C$ -10.45 ‰) were used to assess linearity and as a further check of measurement quality.

The oxygen $\delta^{18}O$ and deuterium $\delta^{2}H$ values were first corrected for instrumental drift and then calculated relative to two international standards (USGS 84 $\delta^{18}O$ 26.36 ‰, $\delta^{2}H$ -140.4 ‰ and USGS 86 $\delta^{18}O$ 18.76 ‰, $\delta^{2}H$ -207.4 ‰) through the creation of a two-point linear equation; the standards were selected to cover the typical range of variation for this type of sample.

The calibration line was obtained by averaging the standards measured in duplicate at the beginning, middle, and end of the run; the sample value was then corrected according to this line. The accepted maximum standard deviations for repeatability were 0.3 ‰ for $\delta^{13}C$, 0.5 ‰ for $\delta^{18}O$, and 4 ‰ for $\delta^{2}H$.

2.3. Sesquiterpene fingerprinting by HS-SPME-GC–MS

The SH fingerprint of VOO samples was analysed by GC–MS after extraction by HS-SPME according to Torres-Cobos et al. (2021), based on the original protocol from Vichi et al. (2006). For this, a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) was used, in combination with an Agilent 6890 N Network GC system coupled to a quadrupolar mass selective analyser Agilent 5975C Inert MSD (Agilent Technologies, Santa Clara, California, USA). Briefly, 2 g of oil was weighed into a 10 mL vial fitted with a PTFE/silicone septum and maintained at 70 °C under constant agitation (250 rpm). After 10 min of sample conditioning, a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (2 cm length, 50/30 μm film thickness) provided by Supelco (Bellefonte, PA) was exposed to the sample headspace for 60 min. The fiber was then desorbed in the gas chromatograph injection port at 260 °C for 10 min, with the injector operating in split-less mode for the first 5 min of desorption. Separation was performed on a Supelcowax-10 capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness) (Supelco, Bellefonte, PA), using helium as the carrier gas, at 1.5 mL/min.

Mass spectra acquisition was carried out in selected ion monitoring (SIM) mode, targeting m/z 93, 119, 157, 159, 161, 189, and 204, which are recognized as the main specific ions of SHs (Vichi et al., 2010). Extracted Ion Chromatograms (EICs) obtained for the target ions were considered from 21 min to 42 min (3197 scans for each EIC). A fingerprinting approach was then applied using the scans intensities of the EICs. A data matrix was constructed for each ion, with all samples (rows, $n = 393$) and the scan intensities of each EIC as variables (columns) (7 different data matrices with 3197 scans × 7 ions = 22,379 variables).

To correct differences between injections, each EIC was normalized to the maximum intensity (row wise). Subsequently, the EICs of each ion matrix were aligned among them using the Correlation Optimized Warping (COW) algorithm in Matlab® (Nielsen et al., 1998) to correct the retention time shifts among samples. Finally, the 7 aligned EIC matrices were concatenated conforming a two-way unfolded matrix (393 samples × 22,379 variables).

2.4. Statistical analysis

2.4.1. Univariate analysis of isotopic data

To examine differences in isotopic ratios across various origins (ITA/non-ITA, individual countries, Italian regions), we used statistical tests to evaluate population distribution and compare population medians in IBM SPSS Statistics v29.0© (IBM Corp., Armonk, New York, USA). As isotopic ratios did not follow a normal distribution, determined by the Shapiro-Wilk test, or had fewer than 30 samples in the compared populations, the independent samples median test (non-parametric) was used to compare the medians of ITA vs non-ITA, the countries of origin and the Italian regions (Calabria/Sicily/Apulia). In all cases, $p < 0.05$ was considered significant.

2.4.2. Development of partial least square-discriminant analysis (PLS-DA) classification models

First, for both approaches, Principal Component Analysis (PCA) was carried out to explore the data ($n = 393$) and to identify potential outliers based on Hotelling's T^2 range and Q-residuals model parameters. No outliers were detected according to these parameters.

The data matrices obtained by stable isotope analysis and SH fingerprinting were separately used to construct and validate individual PLS-DA classification models with SIMCA v13.0© (Sartorius, Göttingen, Germany). For each method (stable isotope analysis and SH fingerprinting), two types of classification models were developed: i) a binary ITA/non-ITA model (n = 393) to differentiate ITA VOOs ($n = 242$) from those produced in five other major Mediterranean countries (non-ITA, $n = 151$); and ii) a multi-class regional model ($n = 171$) to distinguish among three major ITA producing regions (Apulia, $n = 73$; Calabria, $n = 58$; and Sicily, $n = 40$).

For each type of authentication model (ITA/non-ITA or regional) and method (stable isotope analysis or SH fingerprinting), the sample set was split following a stratified random sampling strategy into a training set [80 % of samples from each category: ITA vs non-ITA model, $n = 313$ (ITA, $n = 193$; non-ITA, $n = 120$); regional model, $n = 136$ (Apulia, n = 58; Calabria, $n = 46$; and Sicily, $n = 32$)] and a validation set (20 % of samples from each category: ITA/non-ITA model, $n = 80$; regional model, $n = 35$). This splitting process was repeated three times (3 iterations) to assess the impact of sample set composition and enhance the robustness of the external validation. Details of the sample set splitting, including the training and validation sets, are provided in **Table S1** of the **Supplementary Information**. To enable a rigorous comparison between methods, the exact same splitting was applied to models based on stable isotope data and SH fingerprinting data.

2.4.3. Validation of partial least square-discriminant analysis (PLS-DA) classification models

First, with each training set of each of the three iterations, a PLS-DA model was calibrated and internally validated through leave-10 %-out cross-validation (Riedl et al., 2015). In each iteration, the number of latent variables (LV) and optimal pre-processing (which was mean centering and scaling to unit variance) were chosen based on the lowest Root Mean Squared Error of Cross Validation (RMSEcv) criteria. Subsequently, potential overfitting of the models was assessed using permutation tests ($n = 20$ permutations) and ANOVA on the cross-validated predictive residuals (p-value). Following internal validation, each training model was externally validated by predicting the class of samples in the corresponding validation set, which had not been used in model development. Therefore, for each type of model, three training PLS-DA models were generated from the three iterations of the sample set splitting, and three external validations were conducted by predicting the corresponding validation sets. This procedure ensures that results were not driven by specific influential samples and increased the robustness of the external validation.

In PLS-DA binary models, classes were represented using PLS dummy variables (1 for non-ITA class and 0 for ITA class). In multi-class PLS-DA models, each class is modelled individually against the rest of samples. In this case, the dummy Y matrix contained vectors corresponding to each class, where each vector assigned a value of 1 to its specific class (Apulia, Calabria, Sicily) and 0 to all other classes (non-Apulia, non-Calabria, non-Sicily). Subsequently, each sample was assigned to the class corresponding to the vector with the highest PLS predicted value (PV), provided it exceeded the classification threshold set by receiver operating characteristic (ROC) analysis (section 2.4.4). Samples that did not meet the threshold for any vector were left unassigned (no class).

The performance of each PLS-DA model was assessed by the Q^2 values and the percentage of correct classification in external validation, expressed as mean value of correct classification rate ± standard deviation obtained from the 3 iterations. For the binary models ITA/non-ITA, the sensitivity (true positives/ [true positives + false negatives]) and specificity (true negatives/ [true negatives + false positives]) were also assessed, according to Magnusson and Örnemark (2014).

2.4.4. Optimisation of classification thresholds by receiver operating characteristic (ROC) analysis

To maximize the performance of the developed models, classification thresholds were optimized generating the receiver operating characteristic (ROC) with PVs obtained from internal leave-10 %-out cross-validation. The ROC curve plots the sensitivity against 1-specificity resulting from varying the PV threshold to assign samples to a diagnostic category (ITA or non-ITA; and Apulia, Calabria or Sicily) (Fawcett, 2006). In this case, the positive classes were non-ITA, for the binary model, and Apulia, Calabria and Sicily for the corresponding regional models. ROC analysis was applied on PV values from each individual PLS-DA model. Thus, a total of 24 ROC curves (3 random training sets for 4 diagnostic categories: 1 for the ITA/non-ITA model and 3 for the regional model) were generated for each method (stable isotope analysis and SH fingerprinting). The optimal thresholds for classifying the validation samples, detailed in **Table S2** of the **Supplementary Information**, were those that maximized the sum of sensitivity and specificity (Quintanilla-Casas, Marin, et al., 2020).

3. Results and discussion

3.1. Stable isotope analysis

Median $\delta^{18}O$ values determined in bulk VOOs produced across four harvest seasons, were significantly different between ITA ($\delta^{18}O = 24.0$) and non-ITA ($\delta^{18}O = 25.8$) classes ($p < 0.001$), unlike $\delta^{13}C$ and $\delta^{2}H$ values (ITA: $\delta^{13}C = -29.7$; $\delta^{2}H = -144.0$; non-ITA: $\delta^{13}C = -29.6$; $\delta^{2}H = -143.2$) (**Table S3** of **Supplementary information**). As previous studies have indicated that the differentiation between geographical macro-areas is explained by the distinct characteristics of the sub-areas that compose them (Bontempo et al., 2019; Quintanilla-Casas, Marin, et al., 2020), the behaviour of the different isotopic markers was evaluated across the six countries and the three Italian regions studied (**Table S3** of **Supplementary information**). The median isotopic values obtained were consistent with previous reports (Bontempo et al., 2009; Bontempo et al., 2019; Camin et al., 2016, Chiocchini et al., 2016), and in most cases, showed significant differences between countries and regions, explaining the observed significant differences between ITA and non-ITA classes. In particular, $\delta^{18}O$ presented the lowest value in ITA compared with all the non-ITA countries analysed, while $\delta^{13}C$ and $\delta^{2}H$ in ITA oils significantly differed from GRC, TUN, and TUR; and from GRC,

POR, TUN, and TUR oils, respectively (**Table S3** of **Supplementary information**). However, despite the differences in median values between ITA and non-ITA oils, as well as between individual countries or regions, the corresponding quartile and minimum-maximum ranges indicated that no single marker can clearly distinguish VOOs of any provenance.

This underscores the importance of investigating the potential of multi-isotopic analysis combined with multivariate techniques to achieve more accurate origin discrimination. Applying PLS-DA to the multi-isotopic data to differentiate VOO between ITA and non-ITA classes, as well as among Italian regions, achieved global classification rates of 74.5 % and 65.2 %, respectively, using internal leave-10 %-out cross-validation (**Tables S4 and S5** of the **Supplementary Information**) (**Fig. S1** of the **Supplementary Information**). To confirm these results, each model, across the three iterations, was externally validated by predicting the class of the corresponding validation samples, which were not used during model development. The external validation of the ITA/non-ITA PLS-DA models developed on the stable isotope data (Table 2) resulted in an overall classification rate > 75 %, with a good sensitivity (0.80) and acceptable specificity (0.73). Examining the identity of the misclassified samples in each validation set (**Table S1** of the **Supplementary Information**) provided valuable insights into the strengths and limitations of the classification model. For the isotopic ITA/non-ITA PLS-DA model, the non-ITA samples with the highest misclassification rates in external validation were from ESP, GRC, and especially from TUR, with 20 %, 25 %, and 50 % of test samples misclassified across the three iterations, respectively. Some misclassification was expected between ESP and ITA samples, given that the preliminary univariate comparison showed only $\delta^{18}O$ to be significantly different between these classes. In contrast, higher classification efficiency was expected for GRC and TUR samples, as their median values for all stable isotopes tested were significantly different from ITA samples. This demonstrates that even when median isotopic values are significantly different between classes, this alone does not guarantee accurate discrimination, as it also depends on the overall dispersion of the samples. Therefore, when single thresholds for these target markers cannot efficiently distinguish sample classes, multivariate classification methods accounting for the complex relationships between isotopic markers, may be helpful for assessing their effective discrimination capacity more accurately. This aligns with previous research indicating the potential of combining multiple isotopic markers with multivariate techniques to improve classification accuracy (Bontempo et al., 2019; Camin et al., 2010; Torres-Cobos et al., 2024). Regarding misclassification of ITA samples in external validation, Sicilian VOOs were the most frequently misclassified (54 %) as non-ITA (**Table S1** of the **Supplementary Information**), which might be due to the particular climate conditions in Sicily, which are more similar to those of other European Mediterranean countries, compared to the other regions in Italy (Camin et al., 2016; Lukić et al., 2020).

Finally, although misclassified samples came from various harvest years and partially reflected their proportions in the sample set, the harvest year did seem to impact the misclassification rate. Specifically, even though the sample set was dominated by VOOs from 2018/19, but particularly from 2019/20, the 2018/19 samples were misclassified more frequently. This higher misclassification rate for the 2018/19 samples might be related to climatic differences registered across the Mediterranean countries during 2018 (Climate Change Knowledge Portal for Development Practitioners & Policy, 2025). In 2018, Italy experienced slightly higher temperatures and lower precipitation compared to other years, which might have made its climatic conditions more similar to those of other Mediterranean countries. Concurrently, some of these countries, such as ESP and TUR, recorded temperatures and especially rainfall that approached Italy's typical annual averages (**Table S6** of the **Supplementary information**). This might have led to the isotopic signatures of Italian VOOs from the 2018/19 harvest resembling those of other Mediterranean VOOs more closely.

Contextualizing classification results of Table 2 with respect to those reported in the literature is challenging because a direct comparison is not always feasible. While many available studies complement bulk isotopic values with compound-specific isotopic values or non-isotopic markers (Bontempo et al., 2019; Faberi et al., 2014), others focusing solely on bulk $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ isotopic markers typically examine VOOs from specific regions (Camin et al., 2010) or single harvest seasons (Jiménez-Morillo, Palma, Garcia, Barrocas Dias, & Cabrita, 2020). These studies cannot be directly compared to the present research, which includes broader geographic areas and multiple harvest years, thereby incorporating significant isotopic variability. Finally, comparisons are further complicated by varying data treatment methods across studies and the infrequent use of multivariate classification techniques for differentiating VOOs based on geographical origin (Bontempo et al., 2009; Camin et al., 2010; Camin et al., 2016; Chiocchini et al., 2016; Portarena et al., 2014)

While direct comparison with previous results is difficult, the findings of this study can be regarded as highly satisfactory given the complexity of the sample set, which includes VOOs from various regions, five Mediterranean countries, and up to four harvest seasons, all based solely on bulk $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ values. Additionally, the robustness of these findings is reinforced by the external validation process, which included three iterations of the sample set to prevent overly optimistic outcomes.

Regarding the differentiation between VOOs from the three adjacent Italian regions, Apulian and Sicilian (**Fig. S2** of the **Supplementary Information**) samples showed satisfactory correct classification rates of 75.6 % and 83.3 %, respectively, in external validation. However, Calabrian samples showed a poor classification rate (36.1 %), and they were often misclassified as Apulian VOOs, resulting in a lower overall classification accuracy (Table 3) compared to the ITA/non-ITA model. This higher misclassification was likely due to the regions' proximity

Table 2
Results of the external validation of the ITA/non-ITA PLS-DA models developed on the stable isotope data. Results are mean values ± standard deviation obtained from three iterations.

	n	Correct classification (%)	non-ITA (n)	ITA (n)	Sensitivity	Specificity
non-ITA	32	**80.2 ± 11.8**	25.7 ± 3.8	6.3 ± 3.8	0.80 ± 0.12	
ITA	48	**72.9 ± 2.1**	13.0 ± 1.0	35.0 ± 1.0		0.73 ± 0.02
Total	80	**75.8 ± 3.8**				

Training model (N = 313, 2 LVs) parameters: mean values obtained with the training sets from 3 iterations: threshold = 0.353 ± 0.012, Q^2 = 0.299, RMSEcv = 0.406. For all models, ANOVA p-value < 0.05.

Table 3
Results of the external validation of the regional three-class PLS-DA models developed on the stable isotope data. Results are mean values (± standard deviation) obtained from three iterations.

	n	Correct classification (%)	Apulia (n)	Calabria (n)	Sicily (n)	No class (n)
Apulia	15	**75.6 ± 3.8**	11.3 ± 0.6	2.3 ± 0.6	1.0 ± 1.0	0.3 ± 0.6
Calabria	12	**36.1 ± 21.0**	6.7 ± 1.5	4.3 ± 2.5	1.0 ± 1.0	0.0 ± 0.0
Sicily	8	**83.3 ± 19.1**	0.7 ± 1.2	0.3 ± 0.6	6.7 ± 1.5	0.3 ± 0.6
Total	35	**63.8 ± 11.5**				

Training model (N = 136, 2–3 LVs) parameters: mean values obtained with the training sets from 3 iterations: threshold (Apulia) = 0.341 ± 0.076, threshold (Calabria) = 0.349 ± 0.121, threshold (Sicily) = 0.386 ± 0.087, Q^2 = 0.225, RMSEcv = 0.460. For all models, ANOVA p-value < 0.05.

and their geographical and climatic similarities. This aligns with previous studies that reported similar $\delta^{13}C$ and $\delta^{18}O$ values for Calabrian and Apulian oils, unlike other regions such as Sicily, with these values being correlated to the specific climatic conditions of each region (Chiocchini et al., 2016 ; Portarena et al., 2014). Sicilian VOOs also showed higher $\delta^{2}H$ values, but no previous comparisons of this marker among VOOs from Sicily, Calabria and Apulia are available in the literature. Similarly to the ITA/non-ITA model, the regional model showed that the harvest year influenced classification accuracy, with samples from 2018/19 exhibiting a higher tendency to be misclassified (**Table S1** of the **Supplementary Information**).

3.2. Sesquiterpene fingerprinting

PLS-DA models developed using SH fingerprinting data achieved 99.9 % and 100 % classification accuracy for distinguishing between ITA and non-ITA samples and for identifying the Italian region of origin, respectively, based on internal leave-10 %-out cross-validation (**Tables S4 and S5** of the **Supplementary Information**) (**Fig. S1** of the **Supplementary Information**). As for the isotopic models, SH fingerprinting models were then externally validated by predicting the class of the samples in the corresponding validation sets, and expressing the results as mean values ± standard deviation obtained from the 3 iterations (Tables 4 and 5). The classification accuracy in external validation maintained over 90 % for both classes (ITA and non-ITA) and reached an overall accuracy of 91.7 %, providing sensitivity and specificity values close to 1. These results are in line with those obtained in previous models based on SH fingerprinting, which aimed to distinguish VOO based on their EU, non-EU, single country or PDO origin (Quintanilla-Casas, Torres-Cobos, Guardiola, Servili et al., 2022; Quintanilla-Casas, Torres-Cobos, Guardiola, Romero et al., 2022), confirming the extraordinary efficiency of this method for VOO geographical authentication. Misclassified samples were not clearly related to any specific country of origin or harvest year (**Table S1** of the **Supplementary information**) but rather seemed to be individual samples with specific characteristics.

Considering the differentiation of VOOs from closely located Italian regions, classification models based on SH fingerprinting data achieved an accuracy close to 80 % for all classes (Table 5) (**Fig. S2** of the **Supplementary Information**). Interestingly, Calabrian VOOs, which were not satisfactorily classified by the isotopic model (36.1 %), achieved the highest accuracy with the SH fingerprinting model (83.3 %). Conversely, Sicilian VOOs, which had the highest correct classification rate with the isotopic PLS-DA model (83.3 %), showed slightly lower accuracy with the SH fingerprinting model (79.2 %). This can be attributed to the slightly lower representation of Sicilian samples in the model (Table 1), which likely impacted the performance of SH fingerprinting models more than it affected the isotopic models based on only three markers. This could be justified by the fact that models based on fingerprinting data operate with high-dimensional datasets, which require a larger number of samples to adequately represent the underlying data patterns associated with each class (Brereton, 2006). In contrast, targeted models with fewer variables are less sensitive to sample size because their simpler relationships among variables result in lower noise and a reduced risk of overfitting.

Table 4
Results of the external validation of the ITA/non-ITA PLS-DA models developed on the SH data. Results are mean values (± standard deviation) obtained from three iterations.

	n	Correct classification (%)	non-ITA (n)	ITA (n)	Sensitivity	Specificity
non-ITA	32	**90.6 ± 6.3**	29.0 ± 2.0	3.0 ± 2.0	0.91 ± 0.06	
ITA	48	**92.4 ± 7.3**	3.7 ± 3.5	44.3 ± 3.5		0.92 ± 0.07
Total	80	**91.7 ± 6.2**				

Training model (N = 313, 8–9 LVs) parameters: mean values obtained with the training sets from 3 iterations: threshold = 0.442 ± 0.038, Q^2 = 0.721, RMSEcv = 0.228. For all models, ANOVA p-value < 0.05.

Table 5
Results of the external validation of the regional three-class PLS-DA models developed on the SH data. Results are mean values (± standard deviation) obtained from three iterations.

	n	Correct classification (%)	Apulia (n)	Calabria (n)	Sicily (n)	No class (n)
Apulia	15	**82.2 ± 13.9**	12.3 ± 2.1	1.3 ± 1.5	0.7 ± 0.6	0.7 ± 0.6
Calabria	12	**83.3 ± 8.3**	1.0 ± 1.0	10.0 ± 1.0	0.7 ± 1.2	0.3 ± 0.6
Sicily	8	**79.2 ± 7.2**	0.3 ± 0.6	1.3 ± 0.6	6.3 ± 0.6	0.0 ± 0.0
Total	35	**81.9 ± 5.9**				

Training model (N = 136, 13–14 LVs) parameters: mean values obtained with the training sets from 3 iterations: threshold (Apulia) = 0.624 ± 0.024, threshold (Calabria) = 0.472 ± 0.013, threshold (Sicily) = 0.339 ± 0.032, Q^2 = 0.627, RMSEcv =0.292. For all models, ANOVA p-value < 0.05.

3.3. Comparative analysis of strengths and aspects for improvement

This study provides a thorough comparison of the effectiveness of targeted isotopic profiling versus untargeted SH fingerprinting, both selected for their capability in authenticating the geographical origin of VOO. By applying both approaches to the exact same samples and using identical statistical treatments and validation sets, it was possible, for the first time, to compare the efficiency of these methods in terms of measurable indices, such as classification accuracy, sensitivity, and selectivity. This comparison was conducted within the complex scenario of verifying the origin of Italian VOO, a pressing and unresolved issue that demands effective solutions for detecting counterfeiting. This involved, on the one hand, distinguishing ITA VOOs from a highly diverse group of VOOs from various other producing countries, and on the other hand, discriminating between VOOs produced in closely located and relatively similar Italian regions. This context allowed for a thorough evaluation of both methods to assess their effectiveness and limitations. The ITA/non-ITA isotopic models demonstrated satisfactory discrimination power, achieving an overall classification accuracy of over 75 % using just three isotopic markers determined on bulk VOO. This confirms that multi-isotopic methods are among the most effective targeted approaches for geographical authentication. However, authentication models based on untargeted SH fingerprinting achieved classification accuracies over 90 % using the same experimental design. Moreover, they revealed a lower influence from the harvest year in terms of sample misclassification. The ability to discriminate among VOOs from adjacent geographical regions further highlighted the differences between the two analytical approaches. Isotopic markers proved effective only when the origin regions were geographically or climatically distinct; otherwise, they struggled to differentiate VOOs, as seen with Calabrian oils (classification accuracy of 36 %). In contrast, untargeted fingerprinting combined with PLS-DA demonstrated greater sensitivity to regional differences, achieving overall classification accuracy over 80 %. Factors not only related with the presence of distinct local cultivars but also with slight variations in pedoclimatic conditions among neighbouring regions can significantly influence the SH profile, resulting in specific features for each region, as previously reported (Quintanilla-Casas et al., 2022).

On the other hand, it should be borne in mind that the performance of PLS-DA models based on fingerprinting data is more sensitive to sample size than isotopic models based on a limited number of target

variables, due to the higher dimensionality, complexity, and variability of the data. For this reason, more samples are needed to ensure adequate representation, reduce noise, and prevent overfitting, thereby achieving reliable and accurate classification.

4. Conclusions

This pioneering study represents the first systematic comparison between stable isotope analysis and a metabolic fingerprinting approach for VOO authentication, offering valuable insights into the strengths and weaknesses of each method. The results of this research showed that the untargeted SH fingerprinting method outperformed isotopic methods in several aspects, despite facing well-known transferability challenges compared to stable isotope analysis. Targeted isotopic methods offer greater applicability and versatility, as they can be more easily adapted and implemented across various contexts, making them more suitable for widespread use. However, given the strong potential of SH fingerprinting for VOO geographical authentication demonstrated by this study, there is an urgent need to enhance the transferability of this method. Some strategies for in-house validation of chromatographic fingerprinting methods have yielded promising results (Quintanilla-Casas et al., 2020), encouraging further analytical efforts to achieve their full inter-laboratory transferability. This would help overcome complex authentication challenges, such as verifying the origin of Italian VOOs. The results of this study will be useful in guiding future research efforts aimed at enhancing the effectiveness and reliability of the tested methods for VOO geographical authentication.

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CRediT authorship contribution statement

Berta Torres-Cobos: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Luana Bontempo:** Writing – review & editing, Methodology. **Alberto Roncone:** Writing – review & editing, Formal analysis. **Beatriz Quintanilla-Casas:** Writing – review & editing, Investigation, Formal analysis. **Maurizio Servili:** Writing – review & editing, Resources. **Francesc Guardiola:** Writing – review & editing, Supervision. **Stefania Vichi:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Alba Tres:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.143655.

Data availability

The data that has been used is confidential.

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