Profiling versus fingerprinting analysis of sesquiterpene hydrocarbons for the geographical authentication of extra virgin olive oils

PROFILING VERSUS FINGERPRINTING ANALYSIS OF SESQUITERPENE HYDROCARBONS FOR THE GEOGRAPHICAL AUTHENTICATION OF EXTRA VIRGIN OLIVE OILS

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Abstract

The verification of the geographical origin of extra virgin (EVOO) and virgin olive oil (VOO) is crucial to protect consumers from misleading information. Despite the large number of studies performed, specific markers are still not available. The present study aims to evaluate sesquiterpene hydrocarbons (SHs) as markers of EVOO geographical origin and to compare the discrimination efficiency of targeted profiling and fingerprinting approaches. A prospective study was carried out on 82 EVOOs from seven countries, analyzed by Headspace Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS). Classification models were developed by Partial Least Square-Discriminant Analysis (PLS-DA) and internally validated (leave 10%-out cross-validation). The % of correct classification was higher for the fingerprinting (100%) than for the profiling approach (45.5-100%). These results confirm the suitability of SHs as EVOO geographical markers and establish the fingerprinting as the most efficient approach for the treatment of SH analytical data with this purpose up to date.

Keywords

Fingerprinting; Geographical origin; Virgin Olive Oil; Sesquiterpene; Food authentication.

1. Introduction

As reported by EU Parliament (Parliament Resolution EU No 2013/2091 (INI)), the cases of food fraud reduce the confidence of consumers in the food chain, compromising its global image and causing a negative influence in the food sector. EU Regulation No 29/2012 states as mandatory the country of origin in labeling extra virgin olive oil (EVOO) and virgin olive oil (VOO) to inform the consumer regarding their geographical origin. The geographical origin reported in the label refers to i) a single EU Member State or third country, ii) oil blends of European Union or non-European Union origin, or iii) certain protected designations of origin or protected geographical indications according to EU Regulation (Regulation (EU) No 1151/2012). The verification of conformity of the label-declared geographical origin of EVOO and VOO plays a key role, not only to protect consumers from misleading information and restore their confidence in the product, but also to detect and prevent fraudulent practices and increase the competitiveness of the sector. A large number of studies have been performed trying to face up the EVOO geographical authentication. They have been based on several chemical compounds such as triacylglycerols, fatty acids, phenolic compounds, pigments, sterols and volatile compounds, by applying different analytical techniques as well as chemometric approaches (Bajoub, Bendini, Fernández-Gutiérrez & Carrasco-Pancorbo, 2018; Conte et al., 2019). However, it is known that the levels of some of these analytes change along EVOO shelf life (i.e. phenols and pigments) and others are related to olive oil quality/purity (i.e. volatile compounds), meaning that they can be affected by storage and processing factors (García-González & Aparicio, 2010). Other studies focused on the olive oil chemical fingerprint by stable Isotope Ratio Mass Spectrometry and Nuclear Magnetic Resonance (Alonso-Salces et al., 2015; Camin et al., 2016). Even though their results were promising by combining data from both analyses, they require smart instrumentation that is not often affordable for common control laboratories. For these reasons, we can state that there is room still for improvement in the development of EVOO and VOO geographical markers.

To develop efficient tools for the geographical authentication of EVOO and VOO, it is necessary to identify the most robust markers and analytical approaches. To be reliable, geographical markers of food products should depend mainly climatic and agronomic factors linked to a specific area, while keeping the influence of other

63 factors to a minimal degree (Vichi, Tres, Quintanilla-Casas, Bustamante & Guardiola, 2018). Additionally, the
64 determination of such markers for routine analysis should imply low cost, short times and automatable
65 procedures.
66 Recent studies reveal that sesquiterpene hydrocarbons (SHs) might act as valid markers to address the genetic
67 and geographical origin of EVOO and VOO (Bortolomeazzi, Berno, Pizzale & Conte, 2001; Zunin, Boggia, Salvadeo
68 & Evangelisti, 2005; Vichi, Guadayol, Caixach, Lopez-Tamames & Buxaderas, 2006; Vichi, Lazzez, Grati-Kamoun,
69 Lopez-Tamames & Buxaderas, 2010; Damascelli & Palmisano, 2013). SHs are semi-volatile plant metabolites
70 comprising an extremely wide number of compounds in nature. In EVOO and VOO, SH composition is highly
71 dependent on the olive trees' cultivar and growing area, and scarcely influenced by other factors such as oil
72 extraction conditions and storage (Vichi et al., 2018). The effect of agronomic and pedoclimatic conditions on
73 olive oil SHs has been proven by the fact that significant differences in the SH composition have been found
74 between samples from the same cultivar produced in different geographical areas (Ben Temime, Campeol, Cioni,
75 Daoud & Zarrouk, 2006; Youssef et al., 2011; Vichi et al., 2015) and also between EVOOs from different cultivars
76 grown in the same parcel did (Vichi et al., 2010). However, the suitability of SHs as geographical markers in a
77 realistic scenario should be tested with olive oils from different geographical areas under the usual production
78 practices, implying the use of monovarietal oils from typical olive cultivars as well as their usual market blends,
79 as addressed by some studies (Zunin et al., 2005; Damascelli & Palmisano, 2013).
80 In the last years, the analysis of SHs has evolved from time-consuming methods (Bortolomeazzi et al., 2001) to
81 simpler methods based on the analysis of the volatile fraction such as solid phase microextraction (SPME) (Vichi
82 et al., 2006), allowing further studies of these compounds in EVOOs and VOOs and considering their use as
83 possible authenticity markers.
84 Concerning the analytical approach, the traditional way to assess these semi-volatile compounds is based on a
85 target-type analysis to identify and determine the SH profile of samples. This approach involves a peak
86 identification step, which presents some difficulties because the mass spectra of these analytes contain the same

specific ions in different proportions, which causes that many SHs have not been identified yet. Consequently, under a targeted profiling approach, as defined by Ballin and Laursen (2019), part of the information is ignored. Nowadays, the emerging strategy in food authentication consists in finding specific patterns in highly dimensional analytical data, known as fingerprints, which might be based directly in raw analytical signals such as a chromatogram (Berrueta, Alonso-Salces & Heberger, 2007; Bosque-Sendra, Cuadros-Rodriguez, Ruiz-Samblas & de la Mata, 2012; Melucci et al., 2016; Ballin & Laursen, 2019). When these distinctive patterns are specific to a given food category (such as a particular geographical origin) and can be used to verify its authenticity. Under the fingerprinting approach, since peak identification and quantitation are not necessary, some of the drawbacks related with the targeted profiling approach mentioned above are overcome. Besides, since the full analytical data is used, more information is considered and misclassifications are revealed easier.

With the aim to evaluate the suitability of SHs as geographical markers for EVOO and VOO under real production conditions we carried out a prospective study on EVOOs from seven different geographical origins, comprising monovarietal oils as well as market blends of oils from various cultivars typically produced in these origins. The SHs were determined by HS-SPME and gas chromatography-mass spectrometry (GC-MS) and data was evaluated under targeted (profiling) and non-targeted (fingerprinting) analytical approaches with the aim to compare their discrimination-efficiency in the verification of the geographical origin.

2. Material and Methods

2.1. Sampling

A total of 82 authentic and traceable samples, declared as EVOO by the suppliers, were obtained in the framework of OLEUM project (EC H2020 Programme 2014-2020) from seven different EU and non-EU countries: Croatia (HRV) (n=11); Slovenia (SVN) (n=8); Spain (ESP) (n=17); Italy (ITA) (n=15); Greece (GRC) (n=6); Morocco (MAR) (n=15) and Turkey (TUR) (n=10). With the aim of reflecting the real production scenario, EVOO samples in this prospective study were obtained under usual production practices for commercial purposes, and thus

consisted of both monovarietal oils as well as market blends of olive cultivars typical of each geographical origin **(Supplementary material, Table S1)**.

2.2. Headspace-Solid Phase Microextraction (HS-SPME)

SHs present in EVOO were analyzed using a Triplus autosampler (Thermo Fischer Scientific, Bremen, Germany) at the conditions reported by Vichi et al. (2006). Shortly, 2 g of oil was weighed into a 10 mL vial fitted with a silicone septum and kept at 70 °C under agitation. After 10 min of sample conditioning, a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (2 cm length, 50/30 μm film thickness) was exposed during 60 min to the sample headspace and then desorbed for 10 min in the GC injection port (260 ° C). The thermal stability of SHs at these SPME conditions was previously verified (Vichi et al., 2006). During the desorption step, the injector was maintained in split-less mode during 5 min. Oil samples were previously spiked with a standard solution of indene to a final concentration of 10 μg/kg.

2.3. Gas Chromatography-Mass Spectrometry (GC-MS)

Separation and detection of volatile compounds was performed by GC coupled to an ion trap mass selective spectrometry using a ThermoFinnigan Trace GC equipped with an ITQ MS (Thermo Fisher Scientific, Waltham, MA) using helium as carrier gas at a constant flow of 1.3 mL min^{-1}. Analytes were separated on a Supelcowax-10 (Supelco, Bellefonte, PA) 60 m x 0.25 mm i.d., 0.25 μm film thickness. Column temperature was held at 40 °C for 3 min, increased to 75 at 4 °C min^{-1}, then to 200 at 8 °C min^{-1} and to 260 °C at 15 °C min^{-1}, holding the last temperature for 2 min. The temperatures of the ion source and the transfer line were 200 and 275 °C, respectively. Mass spectra were recorded with a scan event time of 0.37 s; electron energy was 70 eV. Acquisition in the complete scanning mode (SCAN) was in the range *m/z* 40-300, to allow the identification of compounds in EVOO samples.

2.3. Data processing

2.3.1. Profiling approach

Compounds were identified by comparing their mass spectra and retention times to those of the standard compounds, or the ones available in the NIST 2.0 mass spectrum library and in the literature. Non-isothermal

linear retention indices (LRI), using the definition of Van den Dool and Kratz (1963), were calculated and compared with those available in the literature (**Supplementary material, Table S2**).

Several common ions, only differing in their proportions, characterize the mass spectra of SHs. Therefore, a quantitative assessment of SHs was carried out in Extracted Ion Chromatogram (EIC) by selecting the following ions: *m/z* 69, 93, 107, 119, 135, 157, 159, 161, 189, 200, 202 and 204. The selection of quantification ions was done according to Vichi et al. (2006) and the confirmation ions were the molecular ions *m/z* 204, 202 or 200. Quantification was carried out by internal standard, considering a response factor equal to 1, and expressed as µg equivalents of IS/kg of oil **(Supplementary material, Table S2)**. According to Vichi et al. (2006), both SH and monoterpenes may be taken into consideration to be studied as genetic or geographic markers of virgin olive oil origin. However, monoterpene content suffers higher variability due to their low-boiling point compared with sesquiterpenes, introducing variability not related to the origin into the model (data not shown). Since models developed with sesquiterpene data were successful, it was not considered necessary to also include monoterpenes.

2.3.2. Fingerprinting approach

The EIC of specific SH ions (*m/z* 93, 107, 119, 135, 157, 159, 161, 189 and 204) were obtained from the Total Ion Current (TIC). The intensities of scans comprised from 18th to the 30th minute (2467 scans) were considered for each ion (2467 scans x 9 ions = 22,203 variables per sample). To solve the retention time shifting, for each selected ion the EICs of the 82 samples were aligned by *icoshift* algorithm in Matlab® (Tomasi, Savorani & Engelsen, 2011). Once aligned, the 9 matrices of the 9 aligned EICs were concatenated conforming a two-way unfolded matrix (82 samples x 22,203 variables).

2.3.3. Chemometrics

Univariate statistical analysis for the profiling approach was carried out with SPSS software v25© (IBM Corp., NY USA). A one-way ANOVA was applied: F test and Tukey multiple comparisons test were used when variances were equal between groups. Instead, Welch test and Games-Howell multiple comparisons test were applied when groups presented unequal variances. $P<0.05$ was considered significant.

Multivariate analysis of profiling and fingerprinting approaches was performed with SIMCA software v13.0© (Umetrics AB, Sweden). After data pre-processing (log10, mean centering and scaling for the target data; first derivative, log10, mean centering and scaling for the fingerprint data), a Principal Component Analysis (PCA) was developed for both profile and fingerprint data to explore the natural grouping of samples and detect potential outliers (according to Hotelling's T^2 range and distance to the model parameters). Partial Least Square-Discriminant Analysis (PLS-DA) classification models were built with data obtained by profiling (34 variables) and fingerprinting analysis (22,203 variables) to verify the geographical origin of EVOO samples coming from 7 different countries: HRV, SVN, ESP, ITA, GRC, MAR and TUR. PLS-DA is a supervised discriminant technique based on finding the maximum correlation between the data (the SH profile or the SH fingerprint) and each of the categories (each of the seven countries of origin). By doing this, PLS-DA finds the most different features between categories while minimizing those variables not related with a given category. The models were internally validated by leave 10% out cross-validation and the number of latent variables of PLS-DA models were selected according to the lowest RMSEcv value. Model successfulness was evaluated by their prediction power (Q^2 value) and the % of correct classifications. Random behavior and model over-fitting were assessed through the ANOVA on the cross-validated predictive residuals (p-value) and the permutation test, in which the prediction power (Q^2 value) of 20 models developed after randomizing sample categories (countries) was compared with that of the original model.

3. Results and Discussion

3.1. Profiling approach

The chromatograms obtained extracting typical SH ions from the TIC, showed an extremely complex fraction (**Figure 1**). As commented above, the identification of SHs is a challenging task because they present very similar mass spectra. Despite this fact, a total of 34 peaks were included in the SH profile; 23 of them were assigned to previously reported SH (Bortolomeazzi et al., 2001; Vichi et al., 2006) while the remaining ones were not found in literature but could be related to SH compounds based on their mass spectra. The quantitative data of these SHs, expressed as μg equivalents of IS/kg of oil, were used to perform the univariate statistical analysis by a one-

186 way ANOVA (**Supplementary material, Table S2**). Although some differences were found for some SHs, the high
187 intra-class and inter-class variability caused that this univariate approach was not successful in distinguishing the
188 various origins and that specific markers of origin could not be directly found.
189 Multivariate techniques under a profiling and a fingerprinting approach were assayed in order to better explore
190 the differences between samples from different countries. In the profiling approach, after data pre-treatment
191 and PCA exploration, no outliers were detected. Therefore, the PLS-DA classification model for the targeted data
192 was developed with all the samples (n=82) **(Figure 2a)**. After various pre-processing techniques assayed, the
193 model on the log10, mean centering and data scaling to unit variance was the most successful, and with 8 latent
194 variables it achieved the lowest global RMSEcv for most of the categories.
195 **Table 1** shows the classification results obtained from cross-validation by leave 10%-out and the respective
196 RMSEcv values for each class. The model rendered good percentages of correct classification for samples from
197 certain geographical origin, such as SVN (100%), TUR (100%) and MAR (93.3%). However, in the case of oils from
198 the rest of the countries, it generated some misclassifications, particularly in the case of HRV (45.5%), resulting
199 in a non-satisfactory model. This agrees with the fact that the global Q^2 score (0.351) was low, which indicates a
200 low prediction power of the present classification model. On the other hand, the ANOVA p-value (0.013) indicates
201 that the model is significant and thus, that the classification is not at random. Also, the Q^2 values of the
202 permutation test for each category were below 0 indicating the absence of a random classification and of model
203 overfitting.
204 As aforementioned, the target analysis is limited to the number of compounds that can be identified or
205 tentatively identified based on their mass spectrum and linear retention index (LRI). However, the
206 chromatograms obtained by extracting typical terpene fragment ions (**Figure 1**) show that the SH fraction is much
207 more complex, and that many SHs might have not been considered, meaning that the profiling approach might
208 have missed part of the information of the SHs profile.
209 ***3.2. Fingerprinting approach***

With the aim to extract exhaustive information from the SH fraction in EVOO, a non-target fingerprinting analysis was evaluated. All data points obtained from the selected region of each SH specific EIC were used as variables so that every signal related to SH was taken into account by the model.

The two-way unfolded matrix obtained (82 samples x 22,203 variables) was subjected to data pre-processing and PCA exploration, in which any outlier was detected. Then, a PLS-DA classification model was performed. The model leading to the lowest RMSEcv used 6 latent variables (**Figure 2b**). In this case, the sample grouping according to the origin was drastically improved compared to the profiling model. A 100% of correct classification (by leave 10%-out cross-validation) was obtained for each of the 7 countries of origin (**Table 1**). ANOVA p-value (1.6^{e-18}) indicated that the model was significant and excluded a random classification. Results from the permutation test were very satisfactory, with Q^2 values below 0.2, suggesting that the optimized classification model was not over-fitted.

The successful classification results obtained under this approach agreed with the fact that the sub-models for each geographical origin found patterns of the SH fingerprint that were characteristic of each of them, as revealed by the regression coefficient plots **(Supplementary material, Figure S1)**. To illustrate this, a section of EIC for *m/z* 119 of TUR samples (**Figure 3a**) is plotted against the corresponding regression coefficients of the SHs fingerprint of TUR sub-model (**Figure 3b**). It reveals that some of the highest regression coefficients corresponded to peaks (i.e. peaks 7, 9, 13 and 17) that had been quantified with the *m/z* 119 and included in the profiling model. Nevertheless, other significant regression coefficients were related with parts of the EIC that had not been included in the profiling approach, such as minor SHs or not well-resolved peaks. Thus, this explains the higher discrimination power of the fingerprinting approach compared to the profiling approach.

This prospective study sets SHs as successful EVOO geographical markers because even if various monovarietal EVOOs and EVOO cultivar blends were included for each geographical origin (Supplementary material, Table S1), the country of origin was correctly verified. This is because PLS-DA was supervised per geographical origin (country), and thus the model was addressed to focus on the SHs features more related to the geographical area, beyond the cultivar. This means that the PLS-DA model finds features that are common between samples from

235 the same region even if they are from different cultivars. In this way, even if in some cases the same cultivar was
236 present in different countries ['Arbequina', 'Leccino' and 'Istrska belica' (**Figures 4a, 4b and 4c**, respectively)],
237 the model correctly classified the samples into the country of origin. This is especially relevant because it is known
238 that genetic factors influence EVOO's SH profile (Guinda, Lanzon & Albi, 1996; Osorio-Bueno, Sanchez-Casas,
239 Montaño García & Gallardo González, 2005; Vichi et al., 2010). However, here, thanks to the sampling design
240 and to the ability of PLS-DA to extract information from the fingerprint correlated with the discriminated
241 characteristic (origin in this case), the influence of pedoclimatic aspects on SHs could be exploited.
242 On the other hand, it is noteworthy that although the model was supervised per country of origin, it naturally
243 grouped samples into smaller sub-regions within the same country (although the sub-region information had not
244 been provided to the model). **Figure 5** illustrates this behavior by exemplifying the case of Italian and Turkish
245 oils, where samples from Tuscany, Sicily and Apulia (Figure 5a), and samples from North Aegean, Germencik and
246 Antakya (Figure 5b), respectively, conform independent clusters within each class. This entails that the SH
247 fingerprint holds similar traits among samples from regions smaller than a country and sets a promising scenario
248 for downscaling the model to verify the geographical origin of EVOO produced in smaller regions of interest such
249 as those from protected designations of origin (PDO) or protected geographical indications (PGI).

250 ***4. Conclusions***

251 This prospective study focused on the suitability of SHs as EVOO geographical markers and the evaluation of the
252 best approach for data processing, allowed us i) to confirm that SH can be successfully used for the verification
253 of EVOO geographical origin, ii) to state that the fingerprinting approach provided a model with a higher
254 discrimination capacity (100% correct classification) with respect to the targeted profiling one (from 46 to 100%
255 correct classification, depending on the country). It is remarkable that this classification rate was achieved under
256 a real scenario of EVOO global production, which implied the use of various monovarietal and blends of oils from
257 cultivars typically produced and marketed in each country. Also, samples from the same olive cultivar coming
258 from different countries were correctly classified according to the geographical origin Moreover, as the SH
259 fingerprint holds similar traits among samples from sub-regions within a country, it sets a promising scenario for

downscaling the model to smaller regions of interest such as PDO or PGI oils, as well as for challenging model robustness with samples for various harvest years. Actually, evaluating the effect of the harvest year has been shown to be crucial for some authentication models developed for EVOO verification, because as reviewed by Tres et al. (2013) the differences in the climatic conditions might affect EVOO composition.

Overall, we can conclude that the successfulness of the model is the result of a conjunction of factors: i) sesquiterpenes are suitable geographical markers, ii) the use of the sesquiterpene fingerprint permits to exploit all the information obtained during the analysis in contrast of the target approach, and iii) PLS-DA finds features in the sesquiterpene fingerprint that are common between samples from the same region even if they belong to different cultivars. Although we are aware that an increment of samples (with more samples from these and other origins, and from different harvest years) and external validation are still necessary to develop a more robust and elaborated model for the classification of samples according to their geographical origin, these preliminary results confirm the suitability of SHs as geographical markers and set the basis for the most efficient approach for the treatment of SH analytical data with this purpose up to date.

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Conflict of interest statement

Authors declare no conflict of interest.

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Figure captions

Figure 1. Extracted ion chromatograms of sesquiterpene hydrocarbons: a) Quantification ions; b) Confirmation ions (molecular ions), obtained by analysing an extra virgin olive oil from Spain by HS-SPME-GC-MS.

Figure 2. Score scatter plot (first 3 latent variables) of classification models (PLS-DA) developed by country of origin, based on extra virgin olive oil sesquiterpene data by applying a) profiling approach (34 variables); b) fingerprint approach (22,203 variables). HRV: Croatia, SVN: Slovenia, ESP: Spain, ITA: Italy, GRC: Greece, MAR: Morocco and TUR: Turkey.

Figure 3. a) Section of *m/z 119 EIC* (from 23.8 to 27 min) of Turkish extra virgin olive oils by HS-SPME-GC-MS; b) PLS regression coefficients of the fingerprinting classification model, resulting from each data point in Figure 3a vs. 'Turkey' category (the highest coefficients are in red). Peaks considered in the profiling approach are: 7: α-bergamotene; 8: β-gurjunene; 9: β-caryophyllene; 13: non-identified sesquiterpene; 17: α-zingiberene; 18: germacrene D; 21: (E,E)- α-farnesene; 24: δ-cadinene.

Figure 4. Score scatter plot (first 3 latent variables) of sesquiterpene fingerprint classification model (PLS-DA) supervised by geographical origin, showing how extra virgin olive oils (EVOO) from the same olive cultivar cluster according to the country of origin: a) 'Arbequina' EVOOs produced in Italy (ITA), Spain (ESP) and Morocco (MAR); b) 'Leccino' EVOOs produced in Italy (ITA) and Croatia (HRV); c) 'Istrska belica' EVOOs produced in Croatia (HRV) and Slovenia (SVN).

Figure 5. Score scatter plot (first 3 latent variables) of sesquiterpene fingerprint classification model (PLS-DA) supervised by country of origin, exemplifying the grouping of extra virgin olive oils into sub-regions of origin: a) samples from Italy (ITA); b) samples from Turkey (TUR).

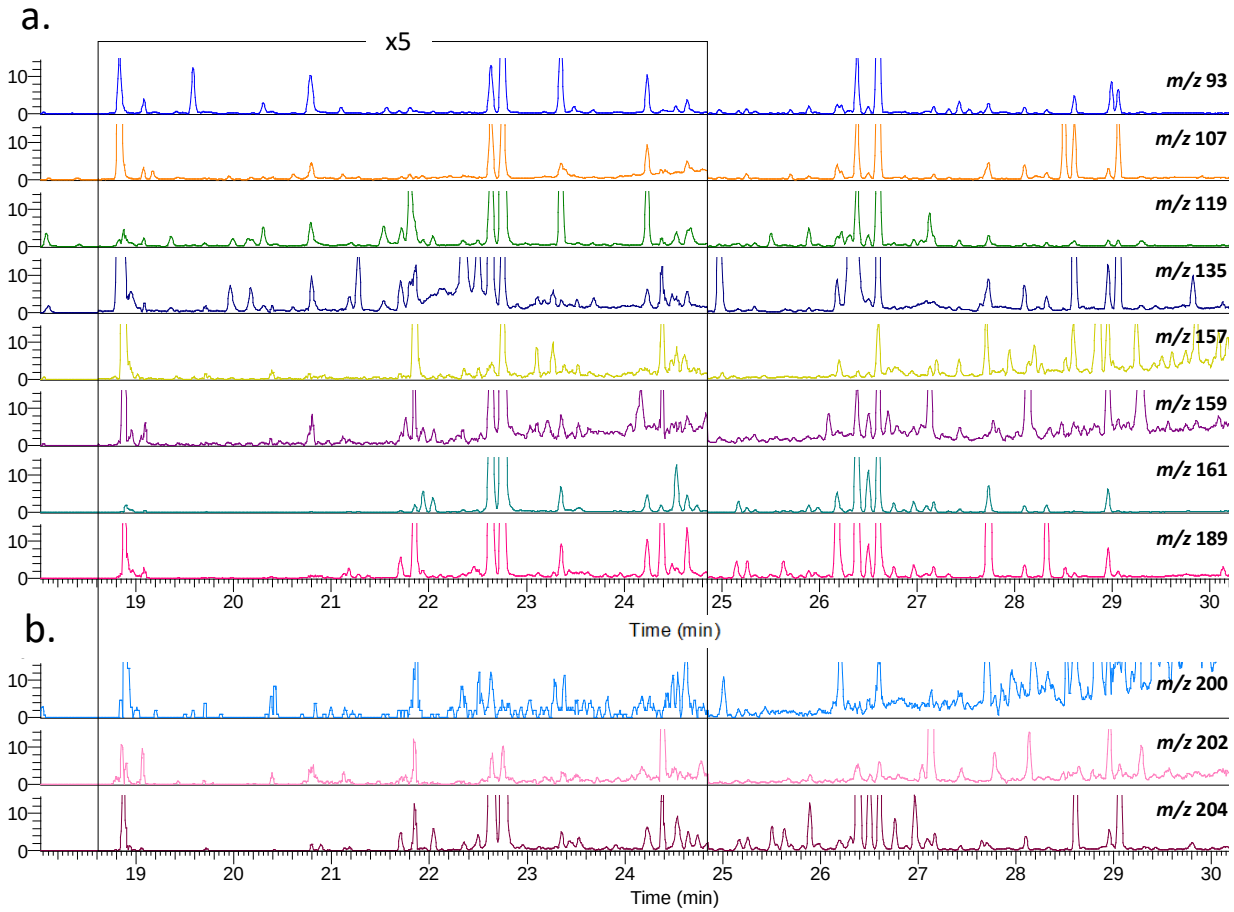
a.
x5
m/z 93
m/z 107
m/z 119
m/z 135
m/z 157
m/z 159
m/z 161
m/z 189
10
0
19
20
21
22
23
24
25
26
27
28
29
30
Time (min)
b.
m/z 200
m/z 202
m/z 204
Time (min)

Figure 1.

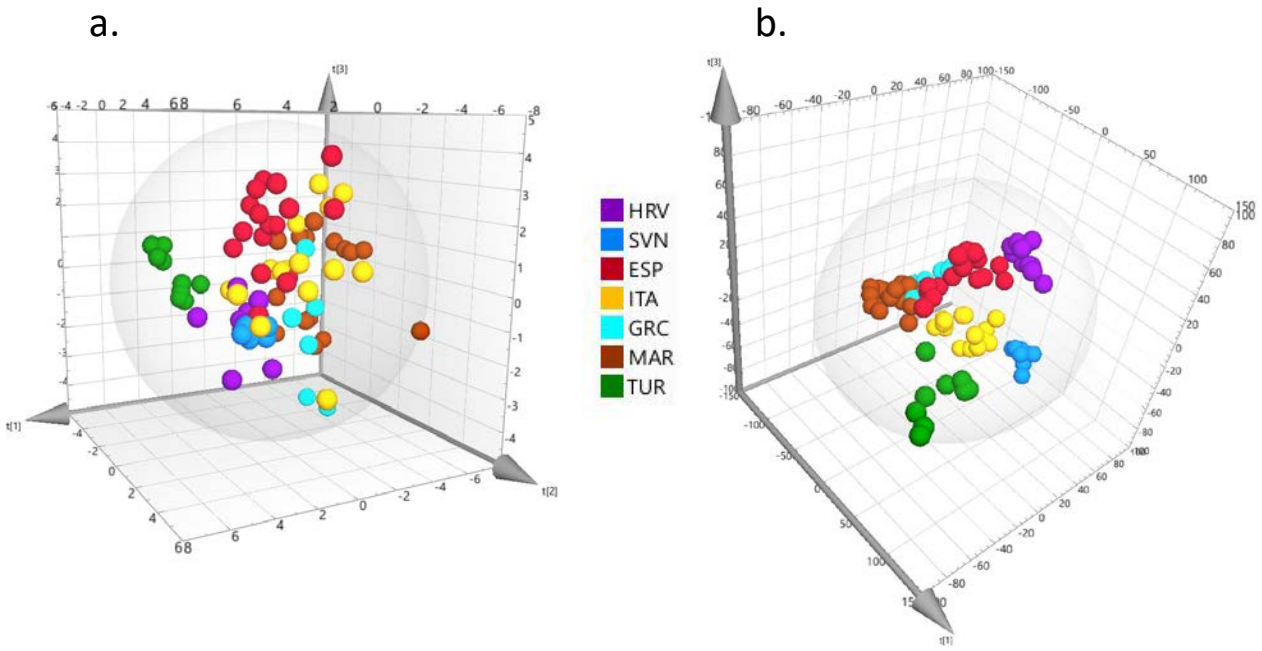

Figure 2.

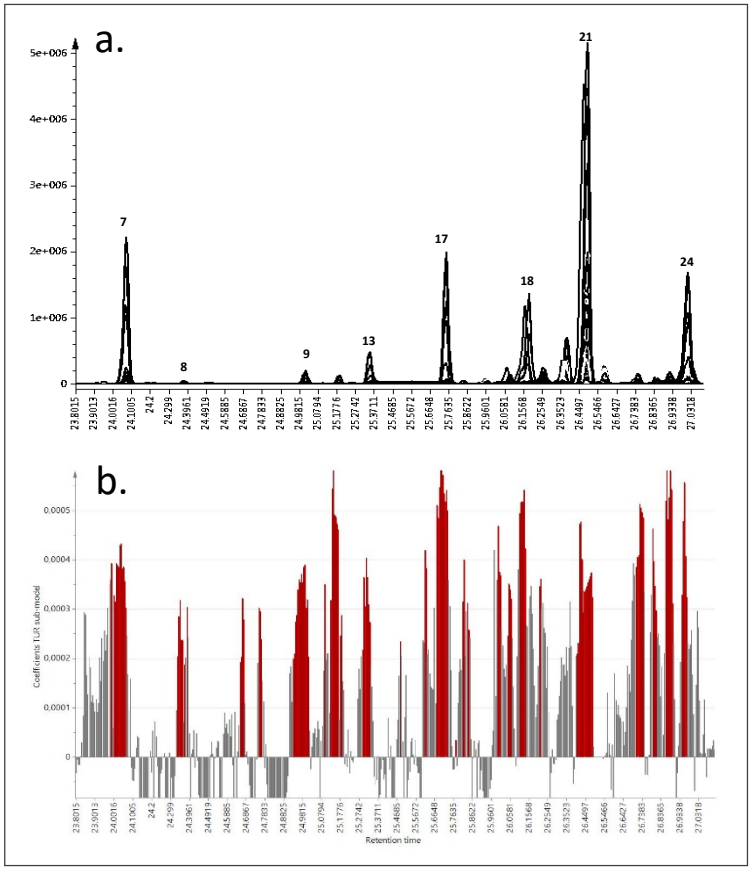
a.
5e+006
4e+006
3e+006
2e+006
1e+006
0
7
8
9
13
17
18
21
24
238015
239013
240016
241005
242
242399
243961
244919
245885
246867
247833
248825
249815
250734
251776
252742
253711
254685
255672
256648
257635
258622
259601
260581
261568
262549
263523
264497
265466
266427
267383
268365
269338
270318
b.
0.0005
0.0004
0.0003
0.0002
0.0001
0
Coefficients (Lift sub-model)
Retention time

Figure 3.

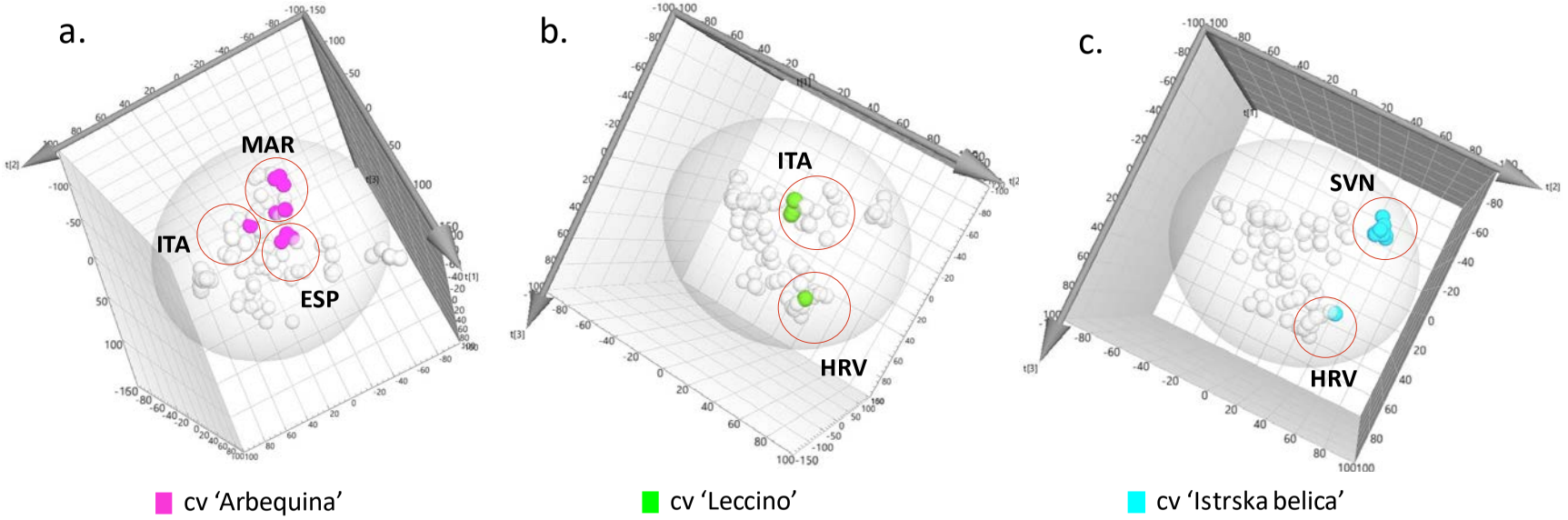
a.
MAR
ITA
ESP
b.
ITA
HRV
c.
SVN
HRV
cv 'Arbequina'
cv 'Leccino'
cv 'Istrska belica'

Figure 4.

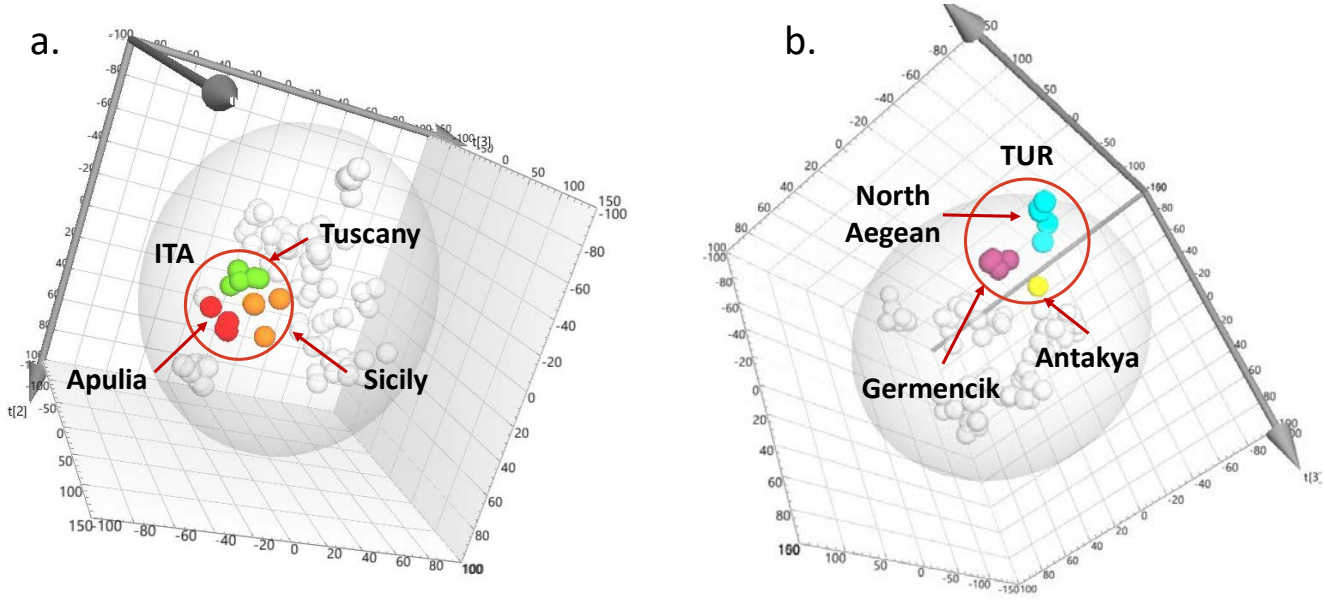
a.
ITA
Tuscany
Apulia
Sicily
b.
TUR
North
Aegean
Germencik
Antakya

Figure 5.

Table 1. Misclassification results of classification models (PLS-DA) developed with Extra Virgin Olive Oil sesquiterpene profile (34 variables; log10, mean centering and scaling to unit variance; 8 latent variables) and extra virgin olive oil sesquiterpene fingerprint (22,203 variables; 1st derivative, log10, mean centering and scaling to unit variance; 6 latent variables), cross-validated by leave 10%-out.

	Members	**Correct classification**	**HRV**	**SVN**	**ESP**	**ITA**	**GRC**	**MAR**	**TUR**	**No class (YPred < 0.5)**	**RMSEcv**
Profiling[a]											
HRV	11	45.5%	**5**	0	0	0	0	0	0	6	0.28
SVN	8	**100%**	0	**8**	0	0	0	0	0	0	0.22
ESP	17	58.8%	0	0	**10**	0	0	0	0	7	0.38
ITA	15	53.3%	0	0	*1*	**8**	0	0	0	5	0.39
GRC	6	50%	0	0	0	0	**3**	0	0	3	0.25
MAR	15	**93.3%**	0	0	0	0	0	**14**	0	1	0.26
TUR	10	**100%**	0	0	0	0	0	0	**10**	0	0.17
Total	82	73.7%	5	8	11	8	3	14	10	22	
Fingerprinting[b]											
HRV	11	**100%**	**11**	0	0	0	0	0	0	0	0.25
SVN	8	**100%**	0	**8**	0	0	0	0	0	0	0.23
ESP	17	**100%**	0	0	**17**	0	0	0	0	0	0.32
ITA	15	**100%**	0	0	0	**15**	0	0	0	0	0.33
GRC	6	**100%**	0	0	0	0	**6**	0	0	0	0.23
MAR	15	**100%**	0	0	0	0	0	**15**	0	0	0.26
TUR	10	**100%**	0	0	0	0	0	0	**10**	0	0.19
Total	82	**100%**	11	8	17	15	6	15	10	0	

Abbreviations used: HRV: Croatia, SVN: Slovenia, ESP: Spain, ITA: Italy, GRC: Greece, MAR: Morocco; TUR: Turkey; RMSEcv: Root Mean Square Error of cross-validation.

[a] Profiling PLS-DA model: Q^2: 0.351; ANOVA p-value: 0.013;

[b] Fingerprinting PLS-DA model Q^2: 0.561; ANOVA p-value: 1.6^{e-18}.

Highlights

- Geographical authentication models developed with virgin olive oil sesquiterpene (SH) data
- The suitability of SH as virgin olive oil geographical markers was confirmed
- Better classification by SH fingerprinting (100%) than by profiling (46-100%)
- SH fingerprinting set a promising scenario for downscaling the model to smaller regions
- The efficiency of the model by geographical origin was independent from the cultivar

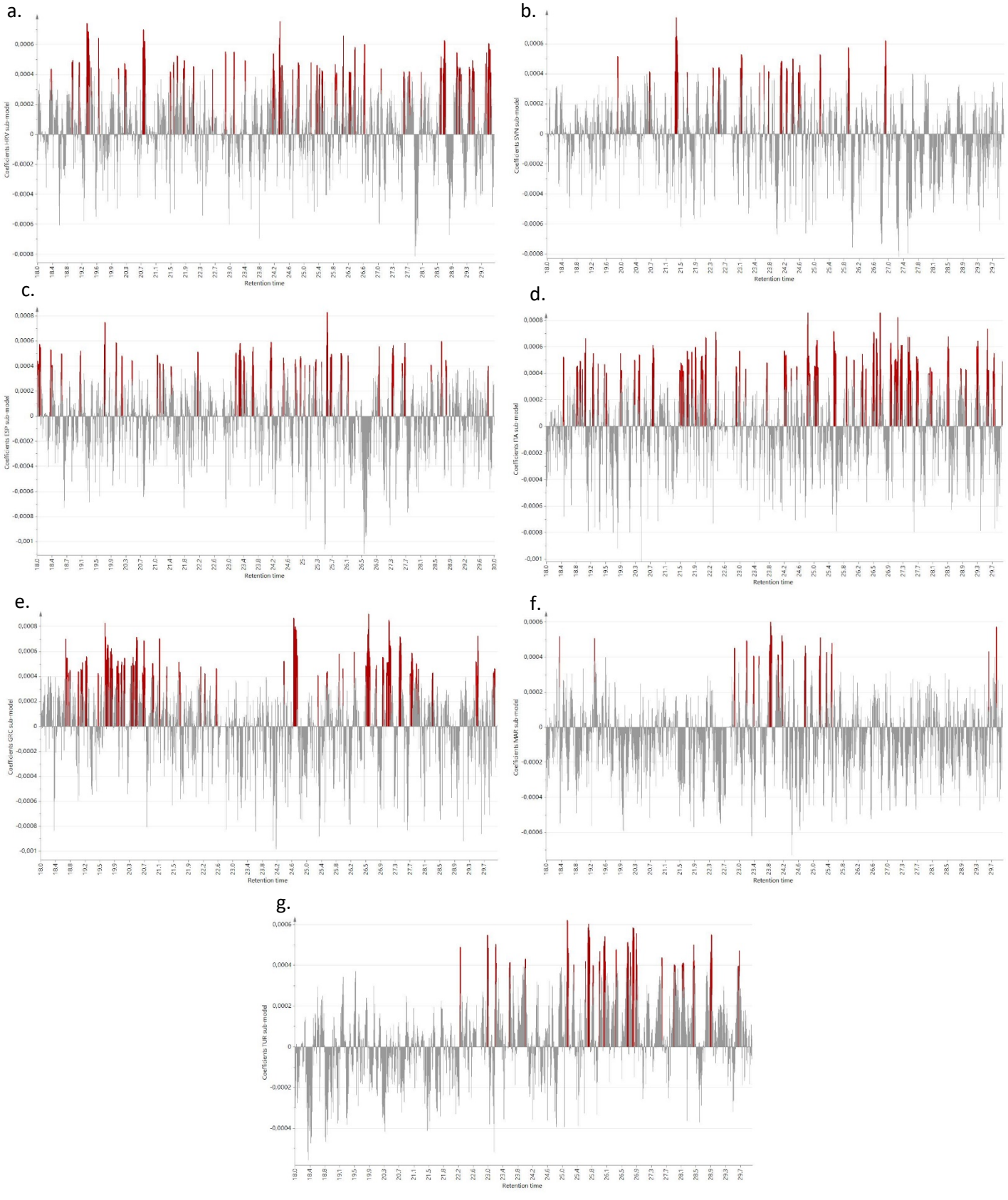

Figure S1. PLS regression coefficients of fingerprinting PLS-DA classification model, resulting from each data point of the *m/z* 119 EIC vs each country of origin: a) Croatia (HRV); b) Slovenia (SLO); c) Spain (ESP); d) Italy (ITA); e) Greece (GRC); f) Morocco (MAR) and g) Turkey (TUR). The highest coefficients are in red.

Table S1. List of EVOO cultivars per country included in the sampling of monovarietal and cultivar blends.

Country	EVOO variety (monovarietal and cultivar blends)
Croatia (HRV, n=11)	Buza puntoza
	Istrska belica
	Oblica
	Picholine
	Ascolana tenera / Itrana / Frantoio blend
	Buza puntoza / Rosijnola / Bova blend
	Leccino / Pendolino blend
	Picholine / Leccio del Corno blend
	Plominka/Simjaca
Slovenia (SVN,n=8)	Istrska belica
	Istrska belica / Leccino / Maurino blend
	Istrska belica / Leccino / other varieties blend
Spain (ESP, n=17)	Arbequina
	Hojiblanca
	Manzanilla
	Picual
	Arbequina / Hojiblanca blend
	Hojiblanca / Picual blend
Italy (ITA, n=15)	Arbequina
	Biancolilla
	Castiligionese
	Coratina
	Frantoio
	Coratina / Ogliariola blend
	Leccino / Frantoio / Moraiolo blend
	Leccino / Frantoio / Pendolino blend
	Nocellara del Belice
	Nostrana di Brisighella
Greece (GRC, n=6)	Arbequina
	Koroneiki
	Manaki
Morocco (MAR, n=15)	Arbosana
	Arbequina
	Koroneiki
	Picholine
	Picholine / Hojiblanca blend
Turkey (TUR, n=10)	Ayvalik
	Domat
	Memecik
	Ayvalik / Domat blend
	Karamani / Hasebi blend
	Memecik / Gemlik blend

Table S2. Characterization of sesquiterpene hydrocarbons in samples: quantification and confirmation ions, Linear Retention Index (LRI) of identified compounds in comparison to those reported in literature and mean values with standard deviation calculated by country between parentheses. Significant statistical differences between groups (by ANOVA) are shown. Compounds have been tentatively identified by mass spectra and retention indices.

	Compound	Ion[a]	MW[b]	LRI Calc.[c]	LRI Liter.[d]	HRV (n=11)	SVN (n=8)	ESP (n=17)	ITA (n=15)	GRC (n=6)	MAR (n=15)	TUR (n=10)	Sig.[e]
		m/z	*m/z*			µg eq. IS/kg	µg eq. IS/kg	µg eq. IS/kg	µg eq. IS/kg	µg eq. IS/kg	µg eq. IS/kg	µg eq. IS/kg	
1	α-cubebene	161	204	1481	1461[g] 1481[h]	2.6 (2.5) ^{y}	2 (0.8) ^{xy}	3 (1.9) ^{y}	1.7 (2.8) ^{xy}	0.5 (0.4) ^{xy}	0.4 (0.4) ^{x}	1.5 (1.0) ^{xy}	**
2	Cyclosativene[f]	161	204	1512	1485[g]	63.6 (48.8) ^{y}	131 (33.2) ^{z}	31.6 (37.7) ^{xy}	39.6 (37.1) ^{y}	11.8 (8.7) ^{xy}	4.1 (4.4) ^{x}	63.6 (68.5) ^{xyz}	**
3	α-copaene	161	204	1519	1496[g] 1497[i]	540.7 (419.2)	1144.8 (296.2)	889.4 (2990)	330.6 (369.3)	61.5 (44.9)	30.5 (38.9)	549.5 (562.7)	
4	α-cedrene[f]	119	204	1551	1542[g]	1.1 (1) ^{x}	1.2 (0.4) ^{y}	7.9 (5.3) ^{z}	3.2 (4.4) ^{xyz}	0.2 (0.4) ^{x}	8.4 (9.3) ^{z}	201.4 (246.4) ^{xyz}	**
5	ni1[f]	161	204	ni	ni	0.7 (0.7) ^{xy}	1.5 (0.4) ^{y}	0.2 (0.5) ^{x}	0.3 (0.5) ^{x}	0.1 (0.2) ^{x}	0.1 (0.2) ^{x}	0.5 (0.3) ^{x}	**
6	β-cubebene[f]	161	204	1495	1521[j]	1.5 (0.6) ^{z}	1.2 (0.6) ^{yz}	0.2 (0.4) ^{x}	0.5 (0.7) ^{xy}	0.1 (0.1) ^{x}	0.1 (0.1) ^{x}	3.4 (6.4) ^{xyz}	**
7	α-bergamotene[f]	119	204	1604	1585[j] 1592[g]	4.2 (3.1) ^{xy}	3.4 (1.5) ^{y}	9.9 (6) ^{z}	5.7 (7.2) ^{xyz}	1 (0.4) ^{x}	2.1 (2.5) ^{xy}	117.5 (139.3) ^{xyz}	**
8	β-gurjunene[f]	161	204	1627	1600[g]	13.9 (9.7) ^{xz}	25 (6.7) ^{z}	6 (8.4) ^{xy}	7.6 (8.3) ^{xy}	0.8 (0.6) ^{xy}	0.6 (0.3) ^{x}	2.7 (1.8) ^{y}	**
9	β-caryophyllene[f]	119	204	1634	1592[j] 1612[i]	3.2 (2.8) ^{xyz}	1.7 (0.3) ^{y}	1.7 (0.6) ^{xy}	1.7 (0.9) ^{y}	2 (1.3) ^{xyz}	0.7 (0.5) ^{x}	3.0 (0.8) ^{z}	**
10	ni2[f]	161	204	ni	ni	1 (2.1) ^{xy}	0.4 (0.1) ^{y}	0.2 (0.5) ^{xy}	0.2 (0.1) ^{x}	0.1 (0.1) ^{x}	0.1 (0.0) ^{x}	0.4 (0.1) ^{y}	**
11	(Z)-β-farnesene[f]	69	204	1649	1652[g]	2.9 (1.9)	3.4 (1.3)	3 (1.4)	2.6 (1.5)	7.5 (6.0)	2.4 (2.0)	4.5 (1.6)	
12	(E)-β-farnesene[f]	69	204	1673	1644[j] 1672[g]	1.5 (1) ^{x}	1.2 (0.3) ^{x}	3.5 (1.6) ^{yz}	2.2 (1.2) ^{xy}	9.1 (13.1) ^{xyz}	1.8 (1.5) ^{xy}	5.7 (2.1) ^{z}	**
13	ni3[f]	119	204	ni	ni	5.8 (2.4) ^{y}	3.6 (0.5) ^{y}	5 (2.5) ^{y}	3.5 (1.6) ^{y}	1.6 (0.6) ^{x}	2.4 (2.2) ^{xy}	27.3 (34.0) ^{xy}	**
14	γ-gurjunene[f]	189	204	1696	1675[g]	1.2 (1.5) ^{xy}	2.8 (0.7) ^{y}	0.7 (0.6) ^{x}	0.7 (0.6) ^{x}	0.7 (0.7) ^{x}	0.2 (0.2) ^{x}	2.2 (0.8) ^{y}	**
15	β-acoradiene[f]	161	204	1712	1693[i]	1.4 (0.7) ^{y}	0.9 (0.3) ^{xy}	0.8 (0.5) ^{xy}	0.8 (0.5) ^{xy}	1.0 (0.6) ^{xy}	0.5 (0.7) ^{x}	35 (29.3) ^{y}	**
16	γ-muurolene[f]	161	204	1721	1692[h]	7.9 (5.7) ^{yz}	12.7 (3.2) ^{z}	3.3 (3.7) ^{vwxy}	4.1 (4.0) ^{wxy}	0.6 (0.6) ^{vwx}	0.3 (0.4) ^{v}	1.3 (0.7) ^{w}	**
17	α-zingiberene[f]	119	204	1715	1721[j] 1728[h]	5.2 (3.9)	4.6 (1.1)	3.6 (3.7)	3.3 (2.5)	4.5 (4.6)	2.9 (3.8)	16.8 (12.3)	
18	Germacrene D[f]	161	204	1736	1718[h] 1726[i]	1.8 (1.4) ^{x}	1.2 (0.2) ^{x}	1.8 (2.0) ^{x}	1.1 (1.1) ^{x}	3.4 (3.8) ^{xy}	2.0 (2.5) ^{x}	17.2 (12.3) ^{y}	*
19	Valencene[f]	161	204	1751	1757[g]	23.1 (26.7) ^{xy}	16.9 (7.5) ^{y}	5.4 (2.7) ^{x}	13.9 (10.0) ^{xy}	27.5 (20.0) ^{xy}	11.9 (13.2) ^{xy}	26.8 (17.3) ^{y}	**
20	α-muurolene[f]	161	204	1736	1721[j]	146.8 (101.5) ^{yz}	230 (94.3) ^{z}	51 (65.6) ^{xy}	71.2 (75.2) ^{xy}	6.4 (3.9) ^{x}	5.2 (4.4) ^{x}	121 (124.9) ^{xyz}	**
21	(E,E)- α-farnesene[f]	93	204	1760	1751[j] 1757[g]	80.8 (91.8) ^{xy}	30.2 (14.4) ^{xy}	68.9 (84.3) ^{xy}	56 (68.9) ^{xy}	17 (18.4) ^{x}	26.5 (25.6) ^{x}	371.8 (295.3) ^{y}	**

22	ni4	161	204	ni	ni	2.8 (3.4	1.7 (0.7	1.5 (4.3)	1.2 (0.8)	3.6 (1.1)	2.3 (2.3)	3.6 (2.7)	
23	ni5[f]	93	204	ni	ni	nd	nd	0.3 (0.2)	nd	0.2 (0.5)	0.1 (0.2)	8 (8.1)	
24	δ-cadinene[f]	161	204	1788	1757[j] 1771[g]	9.7 (4.8) ^{yz}	13.5 (4.1) ^{z}	5.6 (3.4) ^{y}	5.5 (4.0) ^{y}	1.6 (1.0) ^{x}	1.4 (1.4) ^{x}	5.2 (4.5) ^{xyz}	**
25	ni6[f]	161	204	ni	ni	1 (0.5) ^{xy}	1 (0.4) ^{xy}	1.7 (0.8) ^{y}	1.3 (1.1) ^{xy}	0.4 (0.4) ^{x}	0.6 (0.6) ^{x}	4.3 (2.1) ^{z}	**
26	ar-curcumene[f]	119	202	1798	1786[g]	5.8 (3.3) ^{yz}	2.7 (1.0) ^{y}	7.5 (4.2) ^{z}	4.3 (2.7) ^{yz}	1.4 (0.9) ^{xy}	2.8 (4.4) ^{xyz}	nd ^{x}	**
27	ni7[f]	161	204	ni	ni	1.5 (2.1) ^{xy}	0.6 (0.2) ^{y}	1.4 (1.7) ^{xy}	1 (1.3) ^{xy}	0.6 (0.3) ^{xy}	0.4 (0.5) ^{xy}	0.3 (0.1) ^{x}	**
28	ni8[f]	189	204	ni	ni	4.1 (3.6) ^{x}	nd ^{x}	4.9 (6.1) ^{xy}	3.7 (7.4) ^{xy}	5.8 (5.1) ^{xyz}	1.6 (2.3) ^{xy}	13.5 (4.2) ^{z}	**
29	(Z)-calamenene[f]	159	202	1875	1842[h] 1850[g]	15.8 (4.5) ^{z}	18.3 (2.8 ^{z}	8.9 (4.7) ^{y}	8.1 (5.5) ^{y}	2.2 (1.7) ^{x}	1.8 (1.4) ^{x}	13 (4.0) ^{yz}	**
30	ni9[f]	189	204	ni	ni	1.4 (1.3) ^{xy}	0.7 (0.2)^{x}	2.3 (1.7) ^{yz}	1.5 (2.1) ^{xy}	9.5 (14.9) ^{xyz}	4.2 (6.4 ^{xyz}	4.0 (1.7) ^{z}	**
31	ni10[f]	135	204	ni	ni	8.6 (10.1) ^{xy}	3.5 (1.0) ^{x}	8.2 (9.1) ^{xy}	6.3 (6.7) ^{xy}	2.2 (1.8) ^{x}	3.3 (4.1) ^{x}	26.4 (19.4) ^{y}	**
32	α-calacorene[f]	157	200	1930	1917[g]	3.0 (2.2) ^{xy}	1.8 (0.3) ^{xy}	3.4 (2.7) ^{y}	2.5 (1.5) ^{xy}	1.3 (1.1) ^{xy}	1.0 (1.2) ^{x}	11.5 (6.0) ^{z}	**
33	ni11[f]	135	204	ni	ni	10.8 (12.9) ^{xy}	4.3 (1.3) ^{x}	10.6 (12.6) ^{x}	8.2 (8.9) ^{x}	2.7 (2.4) ^{x}	3.8 (5.6) ^{x}	30.6 (15.6) ^{y}	**
34	β-calacorene[f]	157	200	1967	ni	2.8 (1.6) ^{yz}	3.5 (0.9) ^{z}	1.7 (1.0) ^{xy}	1.6 (1.2) ^{xy}	0.9 (0.6) ^{wx}	0.4 (0.2) ^{w}	2.9 (1.8) ^{xyz}	**

Abbreviations used: HRV: Croatia, SVN: Slovenia, ESP: Spain, ITA: Italy, GRC: Greece, MAR: Morocco; TUR: Turkey; ni: not identified compound; nd, not detected

[a] Ion used for quantification

[b] Molecular weight (confirmation ion)

[c] Calculated linear retention indices

[d] Literature linear retention indices

[e] Significance value, according to one-way ANOVA: *, P≤0.05; ** P≤0.01

[f] Unequal variances between groups: ANOVA performed with Welch test and multiple comparisons test carried out by Games-Howell test.

[g] Vichi, S., Guadayol, J. M., Caixach, J., López-Tamames, E., & Buxaderas, S. (2006). Monoterpene and sesquiterpene hydrocarbons of virgin olive oil by headspace solid-phase microextraction coupled to gas chromatography/mass spectrometry. *Journal of Chromatography A, 1125*, 117-123. https://doi.org/10.1016/j.chroma.2006.05.029

[h] Davies, N.W. (1990). Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. *Journal of Chromatography*, *503*, 1-24.

[i] Viljoen, A.M., Subramoney, S., van Vuuren, S.F., Başer, K.H.C., & Demirci. B. (2005). The composition, geographical variation and antimicrobial activity of Lippia javanica (Verbenaceae) leaf essential oils. *Journal of Ethnopharmacology, 96*, 271-277.

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[vwxyz] Values with different letters in a row indicate differences between countries according to post-hoc tests (P≤0.05).