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CRedit Author statements

Rosa Palagano: Investigation, Data curation, Writing - Original Draft.

Enrico Valli: Conceptualization, Writing- Reviewing and Editing

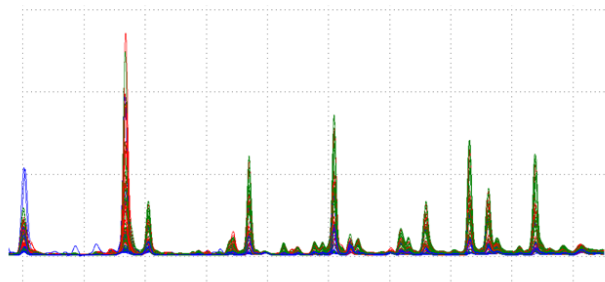
Chiara Cevoli: Methodology, Formal analysis, Visualization

Alessandra Bendini: Conceptualization, Writing- Reviewing and Editing

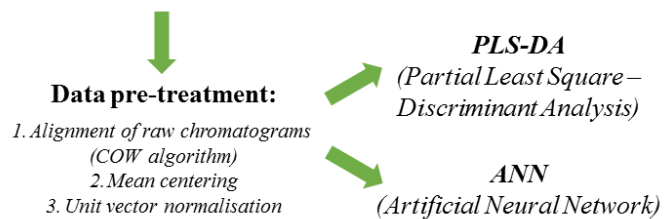
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210 SAMPLES (EVOOs and VOOs)



Extraction of raw data



Journal Pre

1 **Compliance with EU vs. extra-EU labelled geographical provenance in virgin olive oils:**
2 **a rapid untargeted chromatographic approach based on volatile compounds**

3
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Abstract

13 Many studies have shown that geographic origin is one of the most influencing factors in
14 consumers' choice of olive oil. To avoid misleading consumers, European regulation has
15 established specific rules to report the geographical origin of extra virgin (EVOOs) and virgin olive
16 oils (VOOs) on the product label, even if an official analytical procedure to verify the origin has not
17 been yet defined. In this work, a flash gas chromatography (FGC) untargeted approach based on
18 volatile compounds, followed by a chemometric data analysis, is proposed for discrimination of
19 EVOOs and VOOs according to their geographical origin (EU and extra-EU). A set of 210 samples
20 was analyzed and two different classification techniques were used, one linear (Partial Least
21 Square-Discriminant Analysis, PLS-DA) and one non-linear (Artificial Neural Network, ANN).
22 The two models were also validated using an external data set. Satisfactory results were obtained
23 for both chemometric approaches: with PLS-DA, 89% and 81% of EU and extra-EU samples,
24 respectively, were correctly classified; for ANN, the percentages were 92% and 88%, respectively.
25 These results confirm the reliability of the method as a rapid approach to discriminate EVOOs and
26 VOOs according to their geographical provenance.

27

Key words

29 Virgin olive oil; Geographical origin; Chemometric analysis; Flash Gas Chromatography; Volatile
30 compounds.

31 **1. Introduction**

32 Over the last 40 years many investigations have focused on understanding what attributes are
33 important determinants in consumer choice, which have highlighted that geographic origin is one of
34 the most influencing factors for olive oil (Dekhili, Sirieix, & Cohen, 2011; Del Giudice, Cavallo,
35 Caracciolo, & Cicia, 2015). In order to ensure that consumers are not misled, the fourth article of
36 the EU Reg. 29/2012 establishes that “*Extra virgin and virgin olive oil shall bear a designation of*
37 *origin on the labelling*”. This means that for extra virgin (EVOOs) and virgin olive oils (VOOs)
38 commercialized within the EU, it is mandatory to specify the geographical provenance of the
39 product on the label following specific rules. If an oil comes from an EU Member State or third
40 country, a reference to the EU Member State, to the EU, or to the third country must to be reported.
41 In the case of blends of oils originating from more than one EU Member State or third country, one
42 of the following phrases must be used: ‘blend of olive oils of European Union origin’ or a reference
43 to the EU; ‘blend of olive oils not of European Union origin’ or a reference to origin outside the
44 EU; ‘blend of olive oils of European Union origin and not of European Union origin’ or a reference
45 to origin within the EU and outside the EU. An exception is the case where the olives were
46 harvested in an EU Member State or third country other than that in which the mill where the oil
47 was extracted is located. In this case, the designation of origin shall contain the following wording:
48 ‘*(extra) virgin olive oil obtained in (the Union or the name of the Member State concerned) from*
49 *olives harvested in (the Union or the name of the Member State or third country concerned)*’.

50 However, the regulation does not specify an official analytical procedure to verify the conformity of
51 the label-declared geographical origin, and this has raised the interest of researchers to develop a
52 reliable and effective method for the purposes of authentication (Conte et al., 2019). During recent
53 years, different analytical techniques have been applied in order to find potentially useful markers
54 and efficient instrumental approaches that are able to discriminate olive oils according to their
55 geographical origin.

56 In this regard, traditional chromatographic techniques, analyzing both major and minor compounds
57 either individually or in a combined way, coupled or not with specific statistical chemometric tools,
58 have been investigated. A study in 2009 (García-González et al., 2009) proposed the application of
59 artificial neural network (ANN) models for different levels of geographical classification (country,
60 region, province, PDO) on a set of 687 EVOOs and VOOs from Spain, Italy, and Portugal, which
61 were chemically characterized for the content of fatty acids, hydrocarbons, sterols, and alcohols.
62 Other researchers have evaluated the triacylglycerol (TAG) content and composition to discriminate
63 Moroccan oils (Bajoub et al., 2016) and Croatian samples (Peršurić, Saftić, Mašek, & Kraljević
64 Pavelić, 2018). In addition, the stereospecific distribution of fatty acids in TAGs was reported to be
65 useful in discriminating olive oils from different areas of North-Eastern Italy (Vichi, Pizzale, &
66 Conte, 2007). Specific metabolites such as sterols and phenolic compounds have been investigated
67 to identify the optimal markers, which may be a promising approach to discriminate oils according
68 to geographical origin (Giacalone, Giuliano, Gulotta, Monfreda, & Presti, 2015; Ben Mohamed et
69 al., 2018; Ghisoni et al., 2019). Interesting findings have also been recently reported on
70 sesquiterpene hydrocarbons as geographical markers (Quintanilla-Casas et al., 2020). Moreover,
71 volatile compounds have been amply studied by applying different instrumental techniques
72 combined with chemometric data analysis (Kosma et al., 2017; Bajoub et al., 2018; Lukić, Carlin,
73 Horvat, & Vrhovsek, 2019).

74 Rapid and innovative instrumental approaches have also been developed and tested to satisfy the
75 need for simple, rapid, and environmentally friendly techniques (Valli et al., 2016). This critical
76 review (Valli et al., 2016) overviews the principal applications of optical techniques (UV-Vis, NIR,
77 MIR, RAMAN, NMR, and fluorescence spectroscopy), methods based on electrical characteristics,
78 and instruments equipped with electronic chemical sensors (electronic nose and tongue) for
79 discrimination of EVOOs and VOOs according to their geographical provenance. In addition to
80 these approaches, other promising techniques include stable isotopes analysis (Angerosa et al.,
81 1999; Chiocchini, Portarena, Ciolfi, Brugnoli, & Lauteri, 2016; Bontempo et al., 2019), multi-

82 element fingerprint (Sayago, González-Domínguez, Beltrán, & Fernández-Recamales, 2018),
83 differential scanning calorimetry (Mallamace et al., 2017), and GC-IMS (Gerhardt, Birkenmeier,
84 Sanders, Rohn, & Weller, 2017).

85 Melucci and co-workers (Melucci et al., 2016) proposed the application of flash gas
86 chromatography (Heracles II) and a multivariate data analysis to control the compliance of
87 information on geographic origin declared in the label (“100% Italian” vs “non-100% Italian”) for
88 the first time. This instrumental approach allows to carry out headspace analysis in a short time and
89 the results are processed by chemometric tools following an untargeted approach. For this reason, it
90 can be considered as a fingerprint method, since the data can be elaborated for sample classification
91 that is not aimed towards identification and quantification of specific analytes. Following these
92 preliminary results and the actual need for a rapid and effective method for geographical
93 authentication of VOOs, the aim of this work was the application of FGC for rapid discrimination
94 of 210 EVOOs and VOOs according to geographical provenance. In this case, the categories
95 considered for samples classification were EU member states vs extra-EU countries, and the data
96 obtained were analyzed by applying two different classification techniques, one linear (Partial Least
97 Square-Discriminant Analysis, PLS-DA) and one non-linear (Artificial Neural Network, ANN).

98

99 **2. Materials and methods**

100 **2.1 Samples**

101 A total of 210 EVOOs and VOOs with a different geographical origin were collected directly from
102 companies that were also asked to provide, when available, information about location of the mill,
103 type of plant used, olive variety, and commercial category (Table S1, Supplementary material).
104 Samples were collected during three crop years: 84 samples were collected during the crop season
105 2016-2017, 90 during the crop season 2017-2018, and 36 during the crop season 2018-2019.
106 Samples belonging to both the EVOO and VOOs commercial categories were included in this

107 study, considering that the indication of the geographical origin on the product label is mandatory
108 for both these grades.

109 According to geographical provenance, samples were distributed in 3 classes (Table 1): “EU” for
110 oils coming from EU member states; “extra-EU” for oils coming from extra-EU countries (outside
111 EU); “blends” for samples obtained by mixing oils coming from different EU state members or oils
112 coming from EU state members and third countries.

113 Aliquots of each sample (50 mL) were stored at -18 °C in dark plastic bottles.

114 Oils were defrosted for at least 12 h and stored at 12°C before analysis.

115

116 **2.2 Volatile compounds analysis by FGC**

117 The analysis of volatile compounds was carried out using the FGC Electronic Nose Heracles II
118 (Alpha MOS, Toulouse, France). The instrument was equipped with two metal capillary columns
119 (MXT-5: 5% diphenyl, 95% methylpolysiloxane; MXT-1701: 14% cyanopropylphenyl, 86%
120 methylpolysiloxane, for both columns: 10 m length, 180 µm internal diameter, 0.4 µm film
121 thickness) working in parallel mode with a different polarity of the stationary phase. This permits
122 slight differences in the separation capability of molecules detected by a FID applied at the end of
123 each column.

124 Each sample was analyzed in triplicate, weighing 2 ± 0.1 g of oil in a 20 mL vial sealed with a
125 magnetic plug. For analysis, the vial was placed in a shaker oven for 20 min at 40 °C and 500 rpm.
126 Next, 5 mL of the headspace was collected, introduced in a splitless injector (injector temperature
127 200 °C, injection speed 100 µL/s, carrier gas flow, to ensure a fast transfer of the sample from the
128 inlet to the trap, 30 mL/min), and adsorbed on a Tenax[®] TA trap maintained at 40 °C for 60 s to
129 concentrate the analytes. The syringe temperature was set at 70 °C. Subsequently, desorption was
130 obtained by increasing the trap temperature to 240 °C in 93 s and the sample was injected (pressure
131 of the carrier gas at the column head was 40 kPa.) and split (split flow 5 mL/min) into the two
132 columns. The thermal program started at 40 °C (held for 2 s), increased up to 80 °C at 1 K/s, and

133 then to 250 °C at 3 K/s. Hydrogen was used as the carrier gas with a pressure from 40 kPa to 64
134 kPa, increasing with a rate of 0.2 kPa/s. At the end of each column, a FID detector (detector
135 temperature 260 °C) was placed and the acquired signal was digitalized every 0.01 s. The software
136 used to control the instrument was AlphaSoft version 14.5.

137

138 **2.3 Data processing**

139 For data analysis, the full chromatograms were processed by applying chemometric data analysis
140 with an untargeted approach. The raw data from each chromatogram (intensity values for each point
141 of the chromatogram considering that the signal was digitalized every 0.01 s, Palagano et al., 2019
142 [Dataset]) were exported from the software of the instrument and the data set with all the samples
143 was imported into MatlabR2018a[®]. As data pre-treatment, chromatograms were aligned by COW
144 (Correlation Optimized Warping) algorithm (Tomasi, Van Den Berg, & Andersson, 2004) and
145 autoscaled (mean-centering followed by division of each column (variable) by the standard
146 deviation of that column). Preliminary tests showed that chromatograms obtained from the MXT-5
147 column had a discriminant power higher than the other one (MXT-1701) and for this reason the
148 classification models were developed considering only this column. Considering the reduced
149 number of samples for the classes “blend EU” and “blend EU/extra EU”, these oils were grouped
150 together with “EU” and “extra-EU” samples, respectively. While this can be considered to be
151 simplification, the decision was based on the fact that the percentage of extra-EU oils was higher
152 than EU ones in these EU/extra-EU mixtures. This means that for the data elaboration only two
153 sample categories were considered: “EU” and “extra-EU”.

154 Two different statistical techniques were used to classify samples according to geographical origin,
155 the first (PLS-DA) based on a linear approach, and the second (ANN) on a non-linear approach.

156 In particular, the PLS-DA model was built using the PLS Toolbox for Matlab2018a[®]: intensity
157 values of each point of the chromatogram, for a total of 19,900 data points, were used as variable X
158 (matrix X), while the origin (“EU” and “extra-EU”) was implemented as variable Y (binary

159 variables, 0 - 1). The sample data set was split into a calibration/full-cross validation set (75% of the
160 sample) and an external validation set (25% of the samples) using the Kennard-Stone method
161 (selects samples that best span the same range as the original data, but with an even distribution of
162 samples across the same range) (Daszykowski, Walczak, & Massart, 2002). The threshold value
163 useful to define the category of each sample was defined using a probabilistic approach based on
164 Bayes's rule.

165 The ANN model was performed by using the Neural Net Pattern Recognition tool for
166 Matlab2018a[®]. Specifically, a Multi-Layer Perceptron (MLP) neural network was built to predict
167 the specific class to which samples belong using a non-linear method. For input and hidden layers,
168 linear and logistic activation functions, respectively, were used, while for output layer the SoftMax
169 function was applied. From a statistical point of view, with the SoftMax activation function and
170 cross entropy error, the output is interpretable as posterior probabilities for categorical target
171 variables (Bishop, 1995). One nominal output variable is returned, assuming that the target output is
172 1.0 in the correct class output, and 0.0 in the non-correct class. Looking for the best classification
173 ability, different node numbers in the hidden layer and combinations were tested. The convergence
174 of ANN was ruled by a back propagation algorithm.

175 The original data set was divided into training (55%), validation (20%), and external validation test
176 (25%) sets. The latter was composed of the same samples used for the external validation of the
177 PLS-DA model and selected using the Kennard-Stone method. The training set was used to
178 calculate the transfer function parameters of the network, the validation set to measure network
179 generalization and to halt training when generalization stops improving, and the test set was treated
180 as an unknown, the correct classification of which indicates that the neural network is performing
181 well.

182

183 **3. Results and discussion**

184 A set of 210 EVOOs and VOOs were analyzed for their volatile profile by FGC. Considering the
185 large amount of data and aim of this work, chemometric data analysis following an untargeted
186 approach was carried out.

187 For elaborations, samples were grouped into two categories: “EU”, which included oils from single
188 EU state members and blends of oils from different EU countries, and “extra-EU” that consisted of
189 oils from single countries outside the European Union and blends of oils from the EU and third
190 countries.

191 In Fig. 1-a the mean chromatogram of “EU” and “extra-EU” categories, obtained averaging the
192 intensity of each variable for all “EU” or “extra-EU” samples, is reported: even if almost all peaks
193 are concentrated in the initial part of the chromatogram (between 2000 and 10000 variables), a clear
194 difference, in terms of variable intensities, exists between the two groups, thus confirming the
195 discriminating power of the volatile profile with respect to the geographical origin (Melucci et al.,
196 2016; Lukić, Carlin, Horvat, & Vrhovsek, 2019).

197 Concerning the PLS-DA results (4 latent variables), the values of the estimated Y variable
198 (geographical category) by the model in cross and external validations are shown in Fig. 2. The
199 dotted line identifies the threshold value used to define the attribution of samples to different
200 classes. Regarding the location of each sample, a greater distance from the threshold line can be
201 interpreted as a better classification capacity of the model.

202 The results, in terms of percentage and number of samples correctly classified, are reported in Table
203 2. The percentage ranged from 80.8% to 91.2%. The values obtained for the “EU” category were
204 higher, likely because of the greater number and variability of samples used to build the model. The
205 external validation percentages were lower compared to those obtained for the cross-validation as
206 expected, but the results can be considered more robust since they were obtained considering the
207 25% of samples that were not used to build the model.

208 The ROC (Receiver Operating Characteristic) curve, reported in Fig. 3, summarizes the trade-off
209 between sensitivity (number of samples predicted to be in the class divided by number actually in

210 the class) and specificity (number of samples predicted to not be in the class divided by the actual
211 number not in the class) for the PLS-DA classification model (external data set). The area under the
212 curve (AUC=0.9365) and the height values of sensitivity (0.89) and specificity (0.8) suggest that the
213 model was characterized by a good degree of discrimination. The specificity and sensitivity as
214 threshold variation is reported on the right. The dotted line identifies the threshold value used in the
215 model and is shown in Fig. 2.

216 The VIP (Variable Importance in Projection) score obtained by the PLS-DA confirmed that the
217 section of the chromatogram ranging from 2000 to 10,000 variables has a major contribution to
218 sample discrimination (VIP values greater than 1) according to geographical origin (Fig. 1-b).

219 Focusing on incorrectly classified samples, a specific trend as a function of characteristics that
220 could usually affect the volatile profile of the oil (such as the commercial category, olive cultivar, or
221 specific country of origin) was not seen, thus not justifying the observed misclassifications.

222 Results related to the probabilistic approach are shown in Fig. 4. The graph refers to the category
223 "EU": this means that the higher a sample is located, the higher the probability for which it is
224 classified as member of the "EU" category. As a consequence, oils classified as members of the
225 other category (extra-EU) are located in the bottom area of the graph. In this case, the threshold
226 value is fixed at 0.5, corresponding to a probability of 50%: a sample classified with a probability
227 lower than this is considered as not correctly grouped. It is also interesting to note that most samples
228 were correctly classified with a probability between 90% and 100%.

229 Regarding ANN, an early stopping technique was used to select the number of training cycles
230 (epochs) to avoid over-fitting, using the validation set to monitor the prediction error. An example
231 of this procedure is reported in Fig. 5, where the best ANN training was characterized by 47 epochs.
232 Above this point, the error increased further indicating that the ANN tends to overfit. Consequently,
233 the results of ANN are related to these iterations.

234 The best prediction results were obtained with a three layers network (input, hidden and output),
235 having 5 nodes in the hidden one; a larger number of nodes did not increase the network
236 performance.

237 The classification results, in terms of number and percentage of samples correctly classified
238 (according to the highest class probability), are summarized in Table 3, for the validation and
239 external data sets. The external data set was composed of the same samples selected to validate the
240 PLS-DA model. As reported for the PLS-DA model, even in this case higher percentages (from
241 92.3% to 97.5%) were achieved for the “EU” category in all three data sets.

242 In particular, comparing the results of the external validation data (the same for both classification
243 methods), it is possible to note that higher percentages were obtained using the ANN for both the
244 “EU” and “extra-EU” categories. In particular, an increment of 3.8% and 7.6% of correctly
245 classified samples was obtained, respectively. The ROC curve (specificity vs sensitivity) and AUC
246 value (0.9526) of the ANN model (external data set) confirms the higher classification power of this
247 techniques compared to PLS-DA (Fig. 6). This is probably due to the fact that the ANN model is
248 based on a non-linear approach.

249 In general, the percentages obtained were slightly lower than those reported by other studies based
250 on volatile compounds and chemometric untargeted data analysis (Gerhardt, Birkenmeier, Sanders,
251 Rohn, & Weller, 2017; Bajoub et al., 2018; Lukić, Carlin, Horvat, & Vrhovsek, 2019). This aspect
252 can be explained by the large variability, in terms of geographical origin, olive variety, and
253 commercial category of the samples analyzed, which represents a strong point of this work.

254 The results described herein confirm the suitability of FGC for verifying geographical traceability
255 of EVOOs and VOOs, even using untargeted chromatographic signals of the volatile fraction as
256 variables for multivariate analysis (Melucci et al., 2016). An in-house validation of this analytical
257 method, performed to verify that a repeatable and reproducible signal, with sufficient sensitivity to
258 collect the valuable information from the samples, has been carried out which underlined the good
259 performance of the technique; this will be discussed in more detail in a subsequent publication.

260

261 **4. Conclusions**

262 In this work, the application of FGC for volatile compounds analysis combined with untargeted
263 chemometric data analysis (PLS-DA and ANN) to discriminate EVOOs and VOOs with different
264 geographical origin was presented. For both elaborations, satisfactory results, in terms of
265 percentages of correctly classified samples, were obtained: PLS-DA (external validation) allowed
266 classification of 89% and 81% of “EU” and “extra-EU” samples, respectively; for ANN (external
267 validation), the percentages were 92% and 88%, respectively.

268 It is important to highlight that these promising results were achieved by analyzing a set of samples
269 that are representative of the large variety of parameters (olive cultivar, country of origin,
270 commercial category) that can describe olive oil and affect its chemical characteristics.

271 It cannot be excluded *a priori* that other factors could also affect this discrimination, but the results
272 clearly showed that the EU vs. extra-EU origin of the oils played a key role.

273 The results obtained herein sustain the use of multivariate chemometrics with untargeted detection
274 of volatile compounds as a powerful tool to discriminate EVOOs and VOOs of different origin,
275 leading to the possibility to verify compliance with the labelled geographical provenance (EU vs
276 extra-EU). The identification of the specific country of origin, both in blended and not blended oils,
277 may be of significant relevance, but to obtain a robust and satisfactory classification model a
278 specific sampling, comprising a very high number of oils coming from each country, is still needed.

279 Other studies have already reported that the analysis of volatile compounds is suitable for tracing
280 the geographical origin of VOOs. It is, therefore, of utmost importance to identify and quantify the
281 main volatile compounds involved in the discrimination with other analytical techniques.

282 Nonetheless, the methodology proposed herein presents some advantages over other techniques
283 generally applied for this analysis, as it is very rapid (only 200 s are needed for each
284 chromatographic run) and easy to use since no sample treatment is required. In particular, for the
285 effective application of the proposed analytical methodology, it would be worthwhile for each

286 laboratory to build its own model on a large dataset of samples of interest in order to achieve, year
287 after year, a historical memory that is useful to control the conformity of oils coming from the same
288 areas and sold by the same or different suppliers. Inter-laboratory tests should be also carried out to
289 verify the comparability of the results among different laboratories, allowing the establishment of a
290 shared data set to increase overall reliability, on which the predictive models should be based.

291

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302

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Table 1. Number of samples for each class of origin considered and geographical origin. EU: oils from EU state members. Extra-EU: oils from countries outside the European Union. Blends: oils obtained mixing EU oils or EU and extra-EU oils.

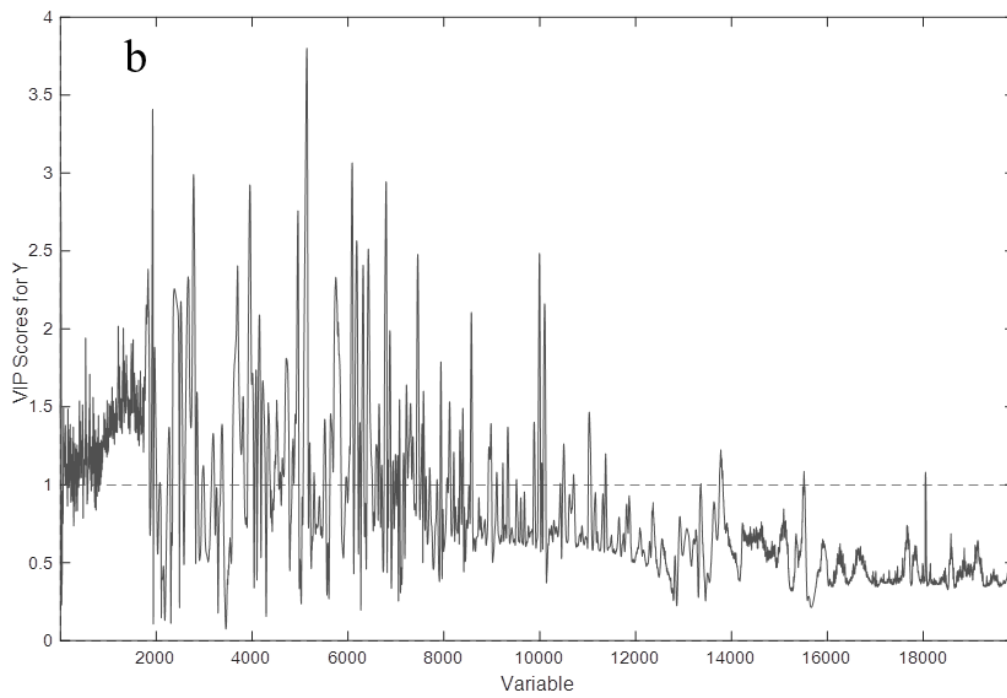
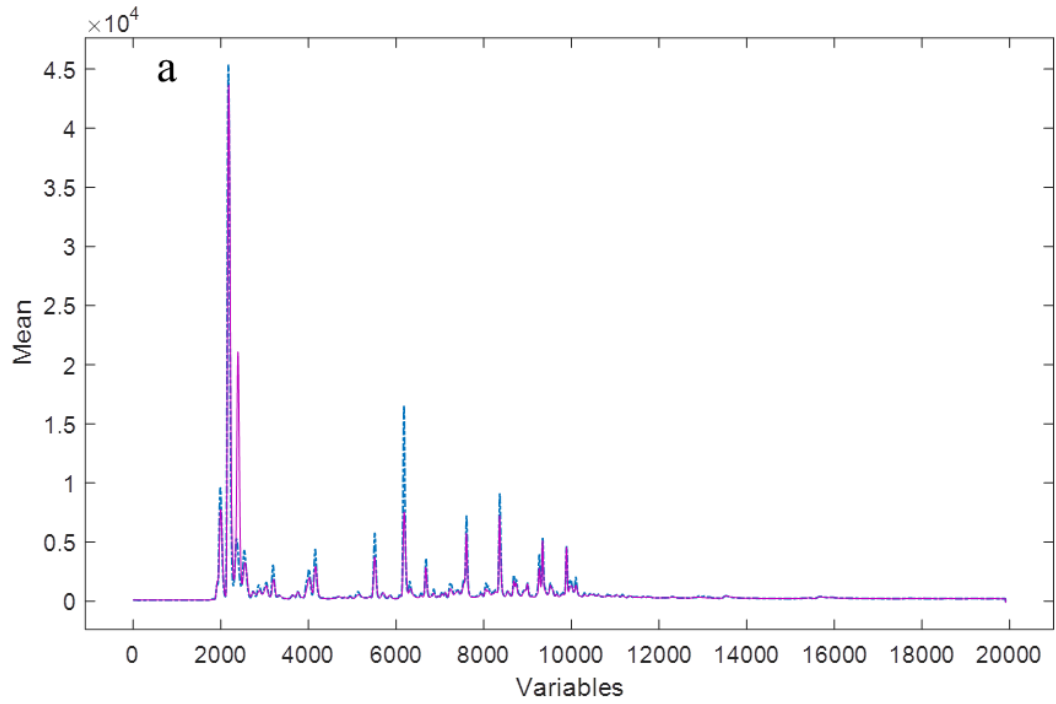
Origin class	N	Country of origin
EU	116	29 Spain, 25 Italy, 22 Croatia, 16 Greece, 12 Portugal, 12 Slovenia
Extra-EU	70	42 Morocco, 21 Turkey, 6 Tunisia, 1 Chile
Blends	24	12 EU blends, 12 EU/extra-EU blends

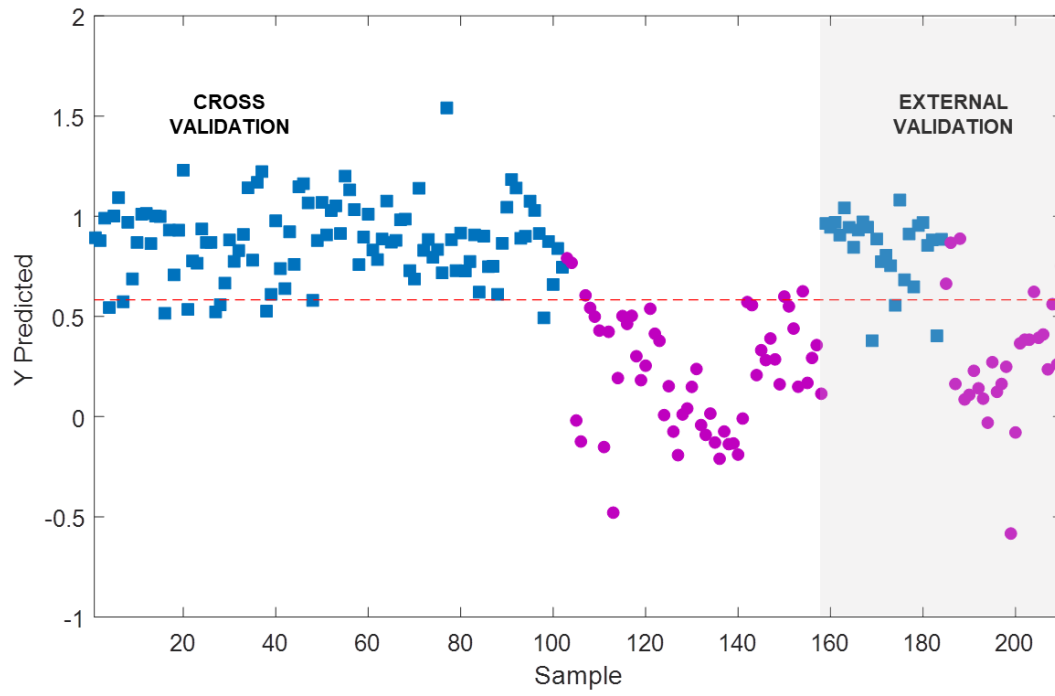
Table 2. Percentages and number (in parentheses) of correctly classified samples for each category using the PLS-DA model. EU: oils from a single state member of European Union and oils obtained by mixing EU oils. Extra-EU: oils from a single country outside the European Union and oils obtained by mixing EU and extra-EU oils.

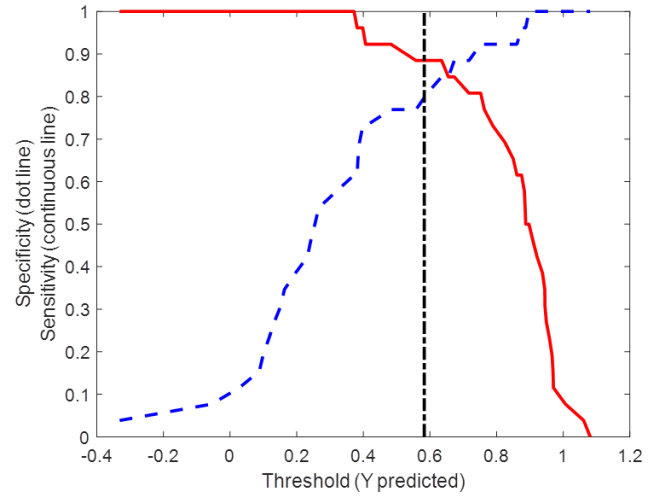
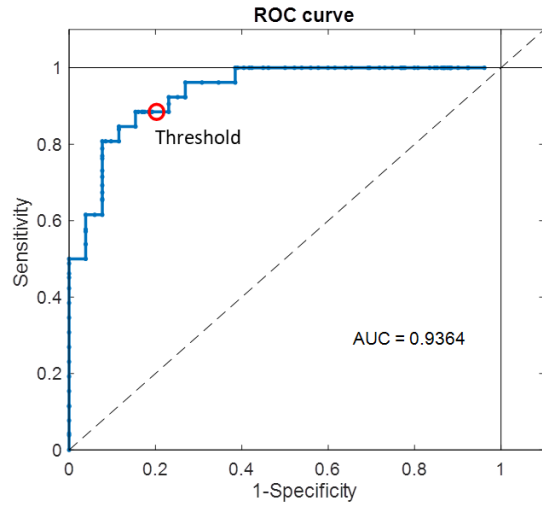
Category	Cross validation	External validation
EU	91.2% (93/102)	88.5% (23/26)
Extra-EU	91.1% (51/56)	80.8% (21/26)

Table 3. Percentages of samples correctly classified for each category using the ANN model. EU: oils from a single state member of the European Union and oils obtained by mixing EU oils. Extra-EU: oils from a single country outside the European Union and oils obtained by mixing EU and extra-EU oils.

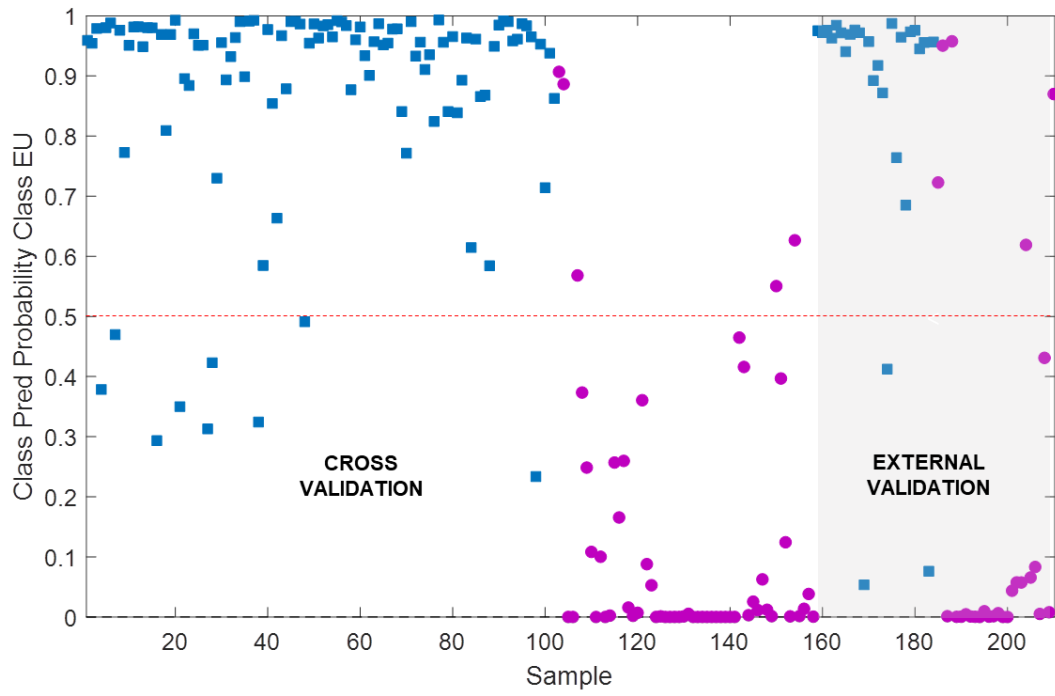
Category	Training (%)	Verification (%)	External validation (%)
EU	97.5 (80/82)	95.0 (19/20)	92.3 (24/26)
Extra-EU	93.2 (41/44)	91.7 (11/12)	88.4 (23/26)

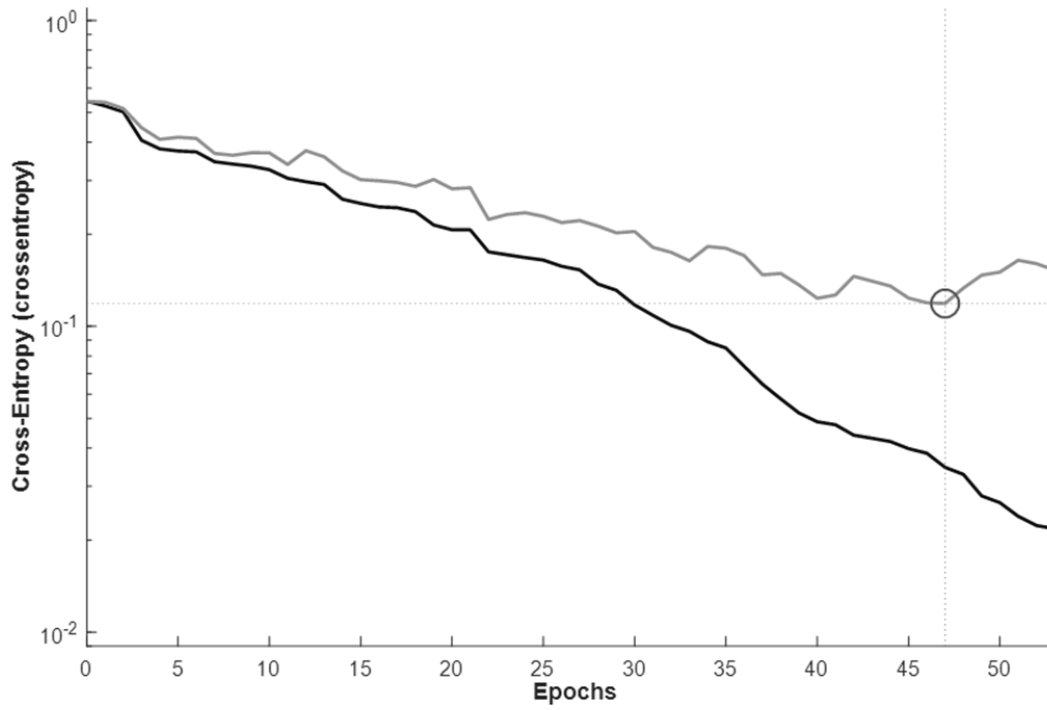


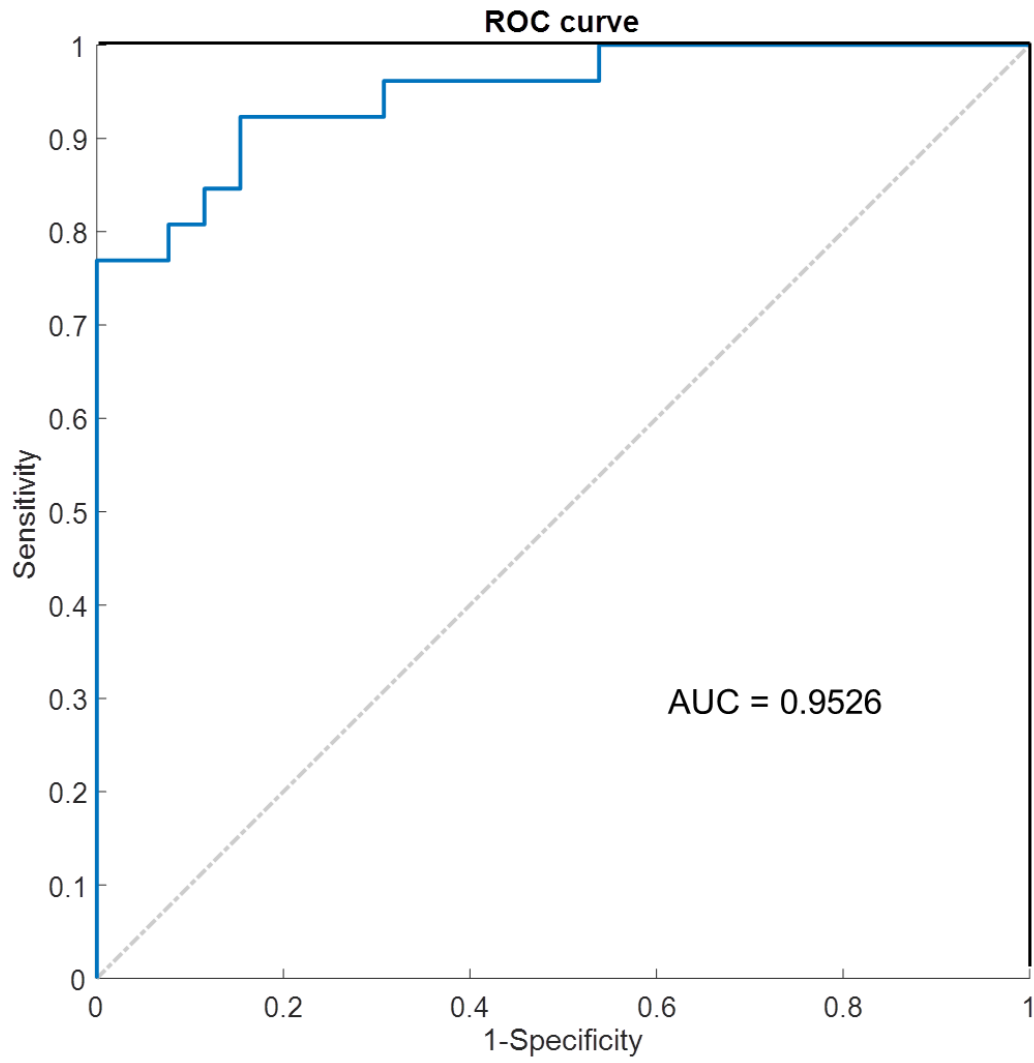




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Highlights

- FGC was applied to discriminate oils with different geographical origin
- Chemometric analysis with untargeted approach was applied
- PLS-DA and ANN classification models were built and validated
- Satisfactory percentages of samples correctly classified were obtained

Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

- All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
- This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
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