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

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Alessandra Bendini: Conceptualization, Writing- Reviewing and Editing

Tullia Gallina Toschi: Funding acquisition, Supervision

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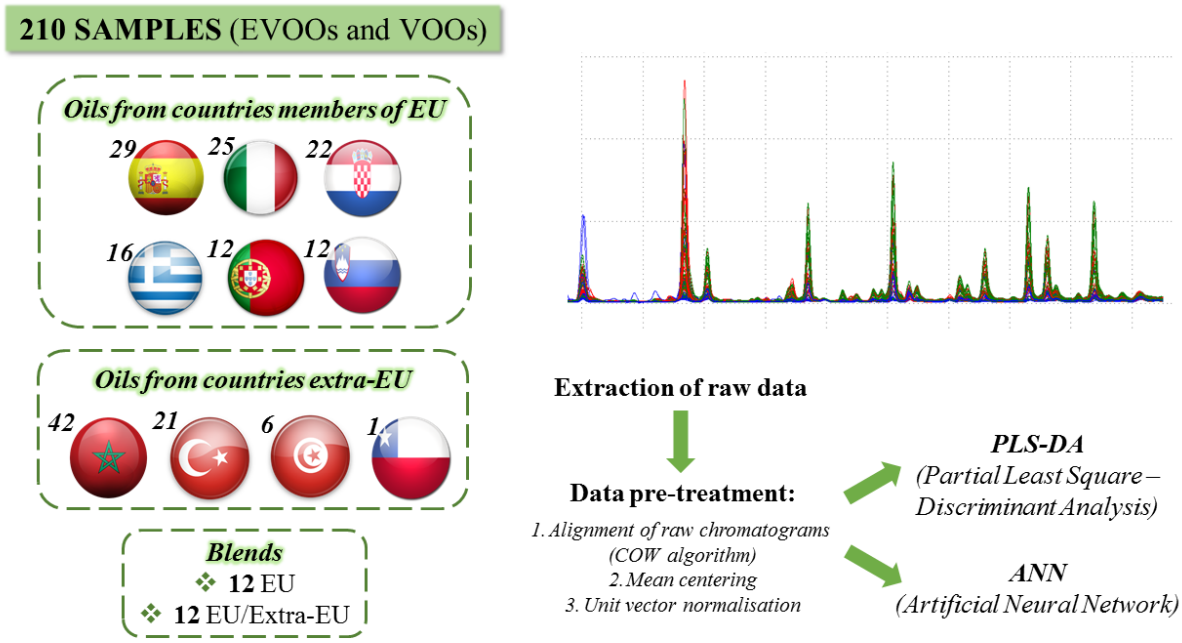
210 SAMPLES (EVOOs and VOOs)
Oils from countries members of EU
29
25
22
16
12
12
Oils from countries extra-EU
42
21
6
1
Blends
12 EU
12 EU/Extra-EU
Extraction of raw data
Data pre-treatment:
1. Alignment of raw chromatograms (COW algorithm)
2. Mean centering
3. Unit vector normalisation
PLS-DA
(Partial Least Square – Discriminant Analysis)
ANN
(Artificial Neural Network)

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

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Abstract

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

Key words

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

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1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1 Samples

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

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

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

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

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

2.2 Volatile compounds analysis by FGC

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

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

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

2.3 Data processing

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

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

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

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

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

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

3. Results and discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Conclusions

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

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

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

The results obtained herein sustain the use of multivariate chemometrics with untargeted detection of volatile compounds as a powerful tool to discriminate EVOOs and VOOs of different origin, leading to the possibility to verify compliance with the labelled geographical provenance (EU vs extra-EU). The identification of the specific country of origin, both in blended and not blended oils, may be of significant relevance, but to obtain a robust and satisfactory classification model a specific sampling, comprising a very high number of oils coming from each country, is still needed. Other studies have already reported that the analysis of volatile compounds is suitable for tracing the geographical origin of VOOs. It is, therefore, of utmost importance to identify and quantify the main volatile compounds involved in the discrimination with other analytical techniques. Nonetheless, the methodology proposed herein presents some advantages over other techniques generally applied for this analysis, as it is very rapid (only 200 s are needed for each chromatographic run) and easy to use since no sample treatment is required. In particular, for the effective application of the proposed analytical methodology, it would be worthwhile for each

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

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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

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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)

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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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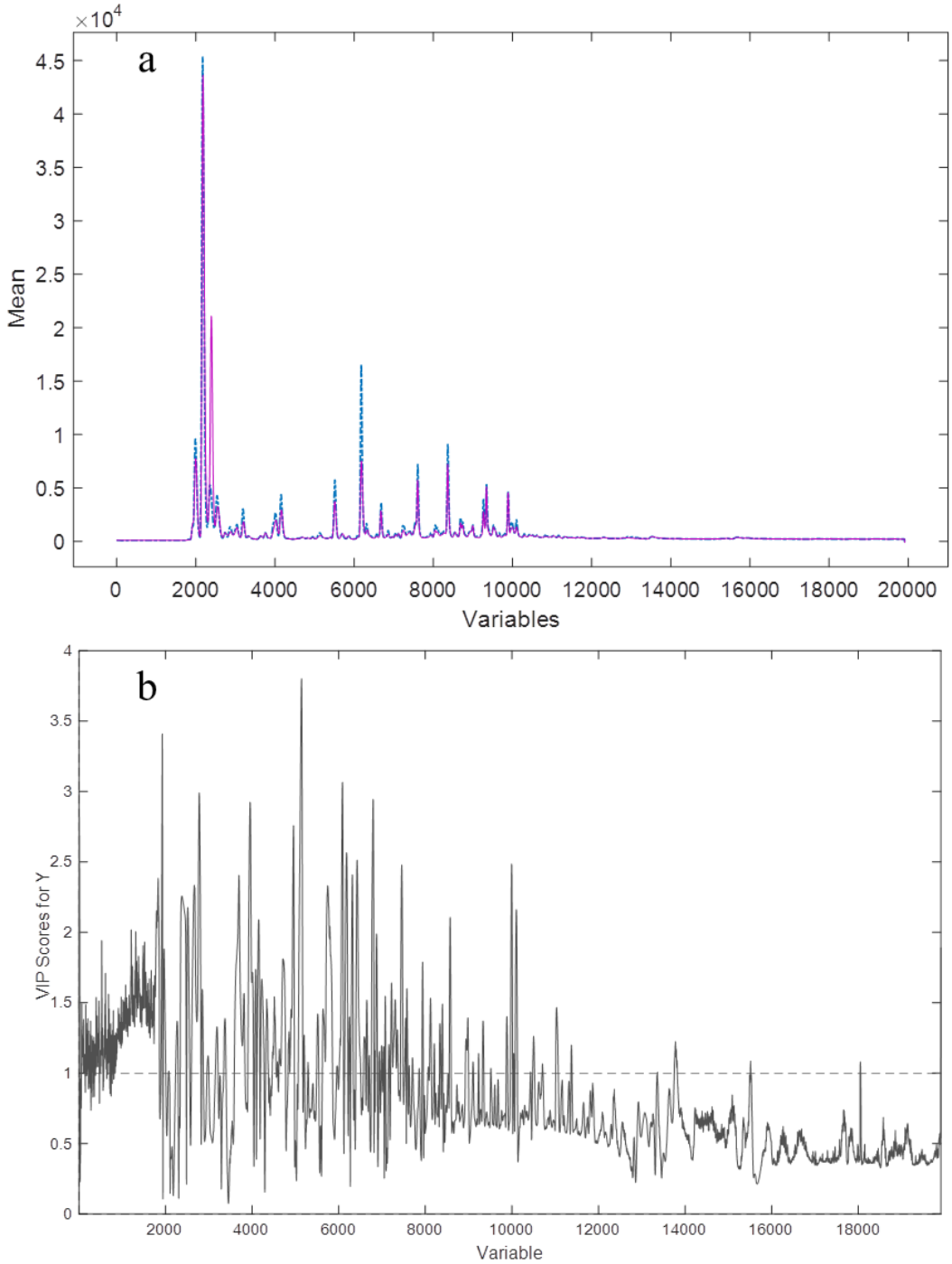
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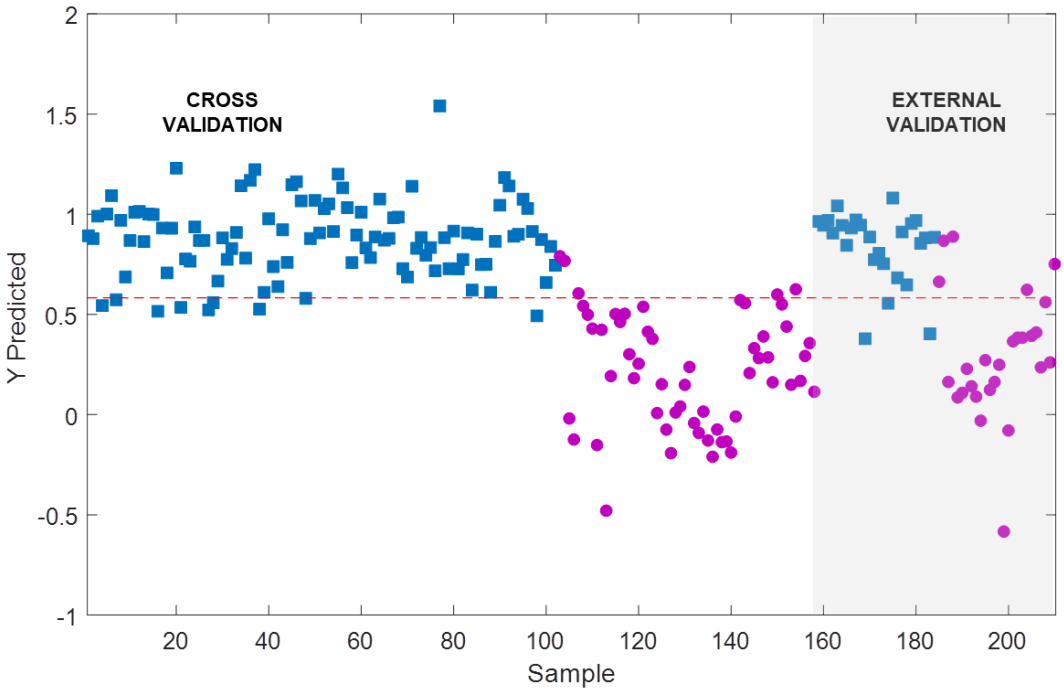
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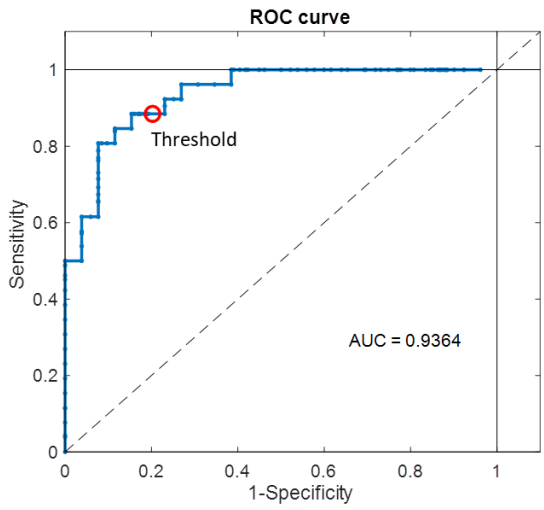

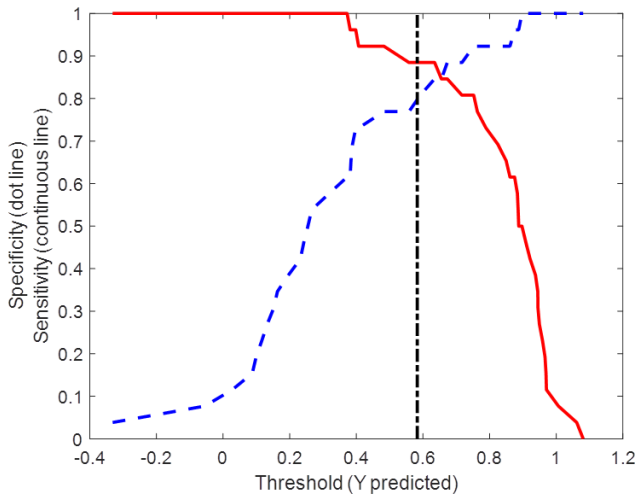

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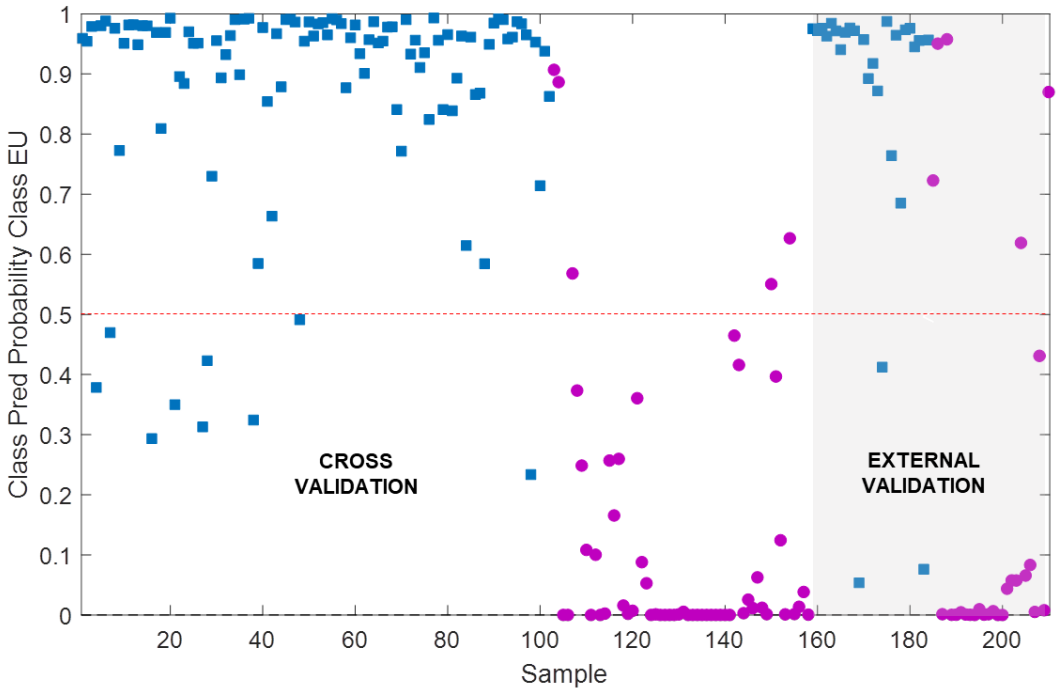
Class Pred Probability Class EU
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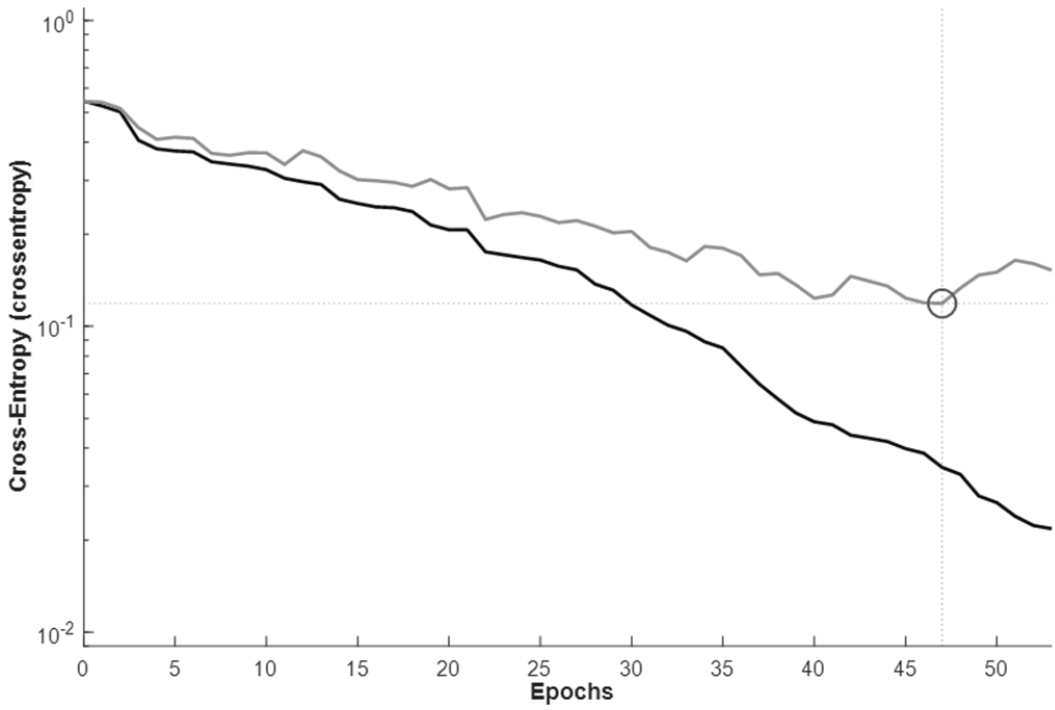
Journal Pre


Cross-Entropy (crossentropy)
10^0
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20
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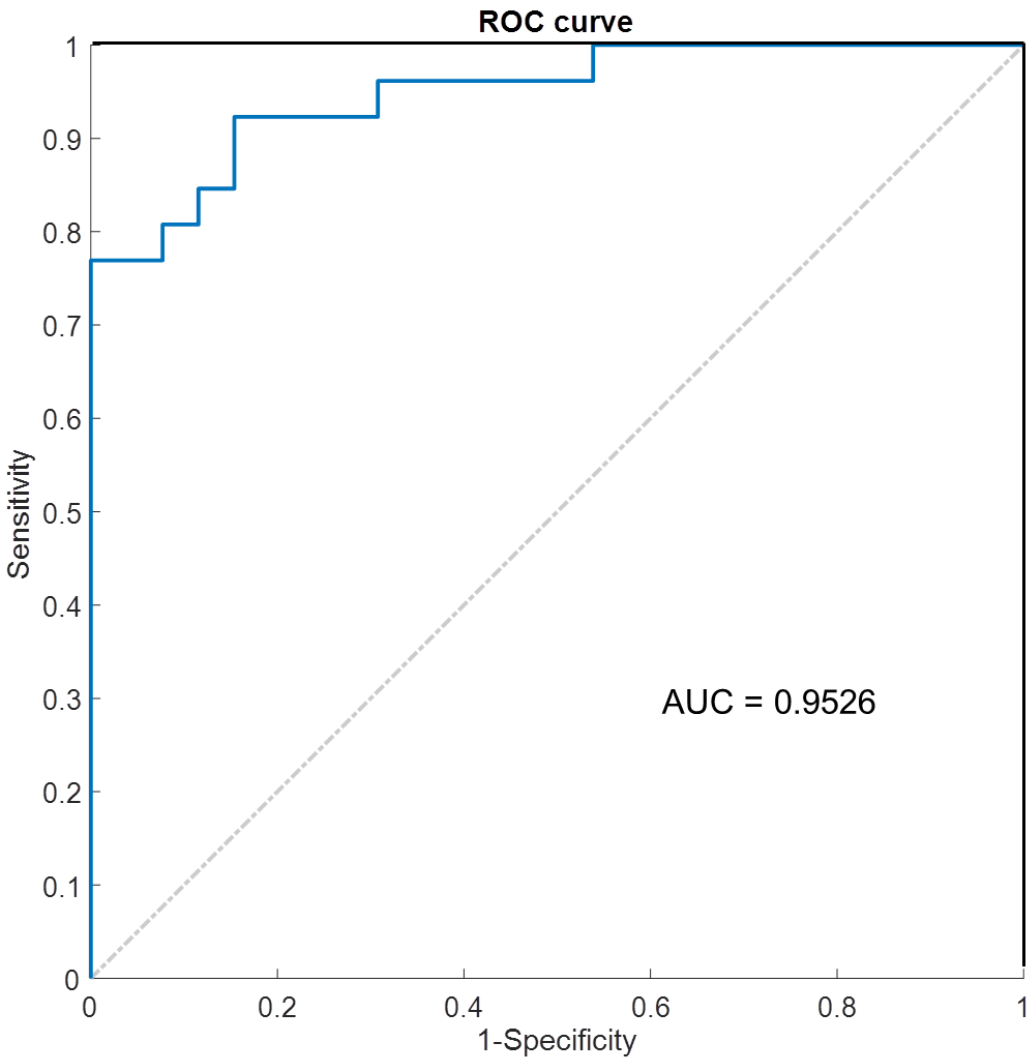
Epochs

Journal P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1

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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

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Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

- o 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.

- o This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

- o The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

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