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**FT-IR extra virgin olive oil classification based on ethyl ester content**

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17 **ABSTRACT**

18 According to the Regulation (EU) 2016/2095, extra virgin olive oils (EVOO) must contain a  
19 maximum of 35 mg kg<sup>-1</sup> of fatty acid ethyl esters (FAEE). The official method for FAEE  
20 quantification is time-consuming and requires the use of a large amount of toxic solvents.  
21 Thus, the aim of this work was the application of FT-IR spectroscopy for the development of  
22 classification models (i.e. Linear Discriminant Analysis, LDA; Soft Independent Modelling of  
23 Class Analogy, SIMCA) able to discriminate EVOO from non-EVOO based on FAEE  
24 content. To the aim, 113 EVOO and 46 non-EVOO samples were analysed. Since the  
25 Principal Component Analysis revealed that the whole FT-IR spectral range (both raw or pre-  
26 treated) was not promising in EVOO and non-EVOO distinction, a variable selection strategy  
27 was applied (i.e. SELECT algorithm). All the classification models were validated both by  
28 cross validation and with three different external test sets. The best and more robust LDA  
29 model was obtained with the raw FT-IR selected variables, reaching 96-100% of correct  
30 classification in prediction. SIMCA models resulted less reliable. In particular, the low  
31 specificity values (40-67%) revealed that there is a high probability of assignment of non-  
32 EVOO to the EVOO class. In conclusion, FT-IR spectroscopy coupled with a discriminant  
33 classification approach is a useful tool for a rapid and fast discrimination of EVOO and non-  
34 EVOO based on FAEE content. Since the variable selection strategy was effective, the  
35 development of simplified and cheap instruments can boost the FT-IR spectroscopy  
36 application also in small enterprises, giving the opportunity to acquire many important  
37 information about olive oils.

38

39 **KEYWORDS:** FT-IR; LDA; SIMCA; Fatty acid ethyl esters; Extra virgin olive oil;  
40 Authentication.

## 41 1. Introduction

42 Extra-virgin olive oil (EVOO) is a premium vegetable oil obtained from fresh fruits only by  
43 means of physical and mechanical processes (Council Regulation (EC) No 1513/2001). It has  
44 a great market value due to its appreciated features. Over the years, several European  
45 Regulations have modified the quality and purity characteristics of virgin olive oils (VOO) for  
46 their commercial classification and labelling. In 2011, the European Commission introduced a  
47 limit to the content of fatty acid alkyl esters (FAAE) in extra virgin olive oils (Commission  
48 Regulation (EU) No 61/2011). According to the mentioned Regulation, a virgin olive oil  
49 labelled as EVOO must contain a maximum of 75 mg kg<sup>-1</sup> for the sum of ethyl esters (FAEE)  
50 and fatty acid methyl esters (FAME) or their sum could be between 75 and 150 mg kg<sup>-1</sup> in  
51 case their ratio (FAEE/FAME) is guaranteed to be  $\leq 1.5$ .

52 FAAE are neutral lipids originating from the esterification of free low-weight alcohols with  
53 free fatty acids. Among involved alcohols, the most important are methanol and ethanol,  
54 yielding to FAME and FAEE, respectively. Among fatty acids, palmitic and oleic acids are  
55 the most common. Methanol and ethanol rise respectively from the progressive degradation of  
56 drupe cell walls and from fermentation processes mainly occurring during olive and/or oil  
57 storage in improper conditions. Fatty acids, instead, are commonly found in VOO to some  
58 extent, depending on the maturation stage of olives but, mainly, on their quality and integrity.  
59 FAAE content could be also affected by the extraction process (Alcalá et al., 2017; Caponio  
60 et al., 2018; Squeo, Silletti, Summo, Paradiso, Pasqualone, & Caponio, 2017). The  
61 Commission Regulation (EU) No 61/2011 has been finally modified by the Commission  
62 Delegated Regulation (EU) 2016/2095 focusing only on the FAEE content and setting the  
63 maximum value for EVOO at 35 mg kg<sup>-1</sup> of oil.

64 According to the official method (Commission Regulation (EU) No 61/2011), FAAE  
65 determination requires their separation from triacylglycerols and other oil constituents by

66 chromatography on a hydrated silica gel column using Sudan 1 (1-phenylazo-2-naphthol) as  
67 indicator for the elution. Then, the FAAE fraction is collected, dried and re-suspended in n-  
68 heptane or iso-octane. Alkyl esters are finally separated by capillary gas-chromatography.  
69 Quantification is achieved by the addition of a proper internal standard. Overall, around 5  
70 hours are needed to complete the analysis, without considering the preparation steps such as  
71 silica conditioning. Besides, a large amount of organic solvents is used for the determination,  
72 mainly n-hexane. Indeed, approximately 250 mL are required for one determination.  
73 Considering that the analysis should be performed at least in duplicate in order to obtain  
74 reliable results, around 1 L of solvent is required for the analysis of only two oil samples.

75 **Hexane is toxic for humans as well as for the environment as extensively reported in hexane**  
76 **safety data sheet, according to the Regulation (EC) No 1272/2008.** Thus, the possibility to  
77 significantly reduce the use of this solvent, together with the other organic solvents required  
78 for the analysis (diethyl ether, isooctane), matches the sustainability goals desired from  
79 Institutions all over the world (United Nations, 2016). The possibility of overcoming these  
80 issues (time-consuming analysis, health and environment hazards) lies in green approaches  
81 like the use of spectroscopic techniques that is one of the most promising.

82 Spectroscopic techniques are non-destructive, green, fast and easy to use. Among them, mid-  
83 infrared (MIR) spectroscopy is one of the most used, having an illustrious history in lipid  
84 chemistry, and it has experienced growing interest and applications thanks to the introduction  
85 of the Fourier transform instruments (FT-IR) (Dobson, 2001). The MIR range goes from  
86 around 2.5 to 25  $\mu\text{m}$  or, as most commonly reported, from 4000 to 400  $\text{cm}^{-1}$ . Absorption of a  
87 MIR photon typically excites one of the fundamental vibrations, associated with a change of  
88 the dipole moment of an oscillating molecule (Sikorska, Khmelinskii, & Sikorski, 2014).  
89 Despite the complexity of spectra collected along the food systems, the association of MIR  
90 spectroscopy with chemometrics allows the extraction of the significant and valuable

91 information (Gómez-Caravaca, Maggio, & Cerretani, 2016). Indeed, when spectra are  
92 recorded from real food samples, they contain information about different components of the  
93 sample matrix together with their interactions, and multivariate methods are successfully used  
94 in interpreting the spectra signals for analytical purposes (Bro, 2003; Kjeldahl & Bro, 2010,  
95 Sikorska, Khmelinskii, & Sikorski, 2014). Several chemometric approaches might be used,  
96 falling in two main classes: qualitative and quantitative methods. As regard to alkyl esters, in  
97 a previous study by Valli et al. (2013), Partial Least Square (PLS) regression models were  
98 tentatively developed for the quantification of FAAE based on VOO FT-IR spectra. However,  
99 they were aimed at the quantification of the sum of ethyl and methyl esters as well as their  
100 ratio, parameters that are no longer considered for the EVOO classification.

101 After the introduction of the Commission Delegated Regulation (EU) 2016/2095, few authors  
102 have taken interest in FAAE determination by green methods. Indeed, near infrared (NIR) and  
103 Vis-NIR spectroscopy has been used to develop regression models for measuring total FAAE  
104 content, as well as FAEE and FAME content separately (Cayuela, 2017; Garrido-Varo,  
105 Sánchez, De la Haba, Torres, & Pérez-Marín, 2017). However, even though chemometric  
106 approaches can overcome the overlapping NIR signals resulting from first and second  
107 overtones and combinations of the fundamental vibrations, more accurate assignments of  
108 absorption bands can be reached by MIR spectroscopy. This is particularly relevant when  
109 assessing differences among molecules having similar bonds that scatter in a complex matrix  
110 such as oil. As far as we know, despite the importance of FAEE, no other attempts have been  
111 carried out by IR spectroscopy to develop a rapid procedure for their analysis. **Starting from**  
112 **these considerations, the aim of this work was the application of IR spectroscopy to the**  
113 **development of classification models (based on Linear Discriminant Analysis and Soft**  
114 **Independent Modelling of Class Analogy) able to discriminate between EVOO and non-**  
115 **EVOO based on FAEE content.** Though FAEE is a continuous variable, a classification

116 approach was chosen instead of quantification since, by a practical point of view, the  
117 proposed method should address a discrimination issue regarding the authentication of  
118 EVOO, where authentication is intended as the compliance of a food with its label description  
119 (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016). A similar approach based on  
120 discriminant classification techniques has already been applied in the literature in order to  
121 develop fast sorting tests for olive oils, based on the content of  $\alpha$ -tocopherol or squalene  
122 (Cayuela & Garcia, 2018; Cayuela & Garcia, 2017). Supervised classification techniques use  
123 the information about the known class membership of training samples in order to create  
124 classification rules able to assign new unknown samples to one of the defined classes, based  
125 on their fingerprint measurement (Berrueta, Alonso-Salces, & Héberger, 2007). Thus, these  
126 chemometric techniques perfectly fit in authentication issues where the goal is to verify if a  
127 sample belongs to a predefined class, such in the case of EVOO and non-EVOO differentiated  
128 by the FAEE content.

129

## 130 **2. Materials and methods**

### 131 *2.1. Sampling*

132 A set of 159 VOO (113 extra virgin and 46 virgin) from Apulia region (southeast Italy) were  
133 collected during 2016/17 and 2017/18 production seasons directly from olive mills located in  
134 different provinces (i.e., 100 samples from Bari province; 15 from Brindisi province; 13 from  
135 Barletta-Andria-Trani province; 11 from Foggia province; 11 from Lecce province; 9 from  
136 Taranto province). All samples were bulk oils, blends of the principal Apulian olive cultivars,  
137 all extracted by continuous plants equipped with decanter centrifuge.

138

### 139 *2.2. Fatty acid ethyl esters determination*

140 The analysis of FAEE was carried out according to the official method (Commission  
141 Regulation (EU) No 61/2011). Briefly, for each determination, 15 g of pre-conditioned silica  
142 gel was suspended in n-hexane and introduced in a glass column for liquid chromatography.  
143 Samples were prepared by adding to 500 mg of oil 250  $\mu\text{L}$  of internal standard (methyl  
144 heptadecanoate 0.02% w/v in iso-octane) and 50  $\mu\text{L}$  of Sudan I solution (1% w/v). In order to  
145 remove impurities, 30 mL of n-hexane were percolated through the column before loading the  
146 sample. About 230 mL of n-hexane/ethyl ether mixture (99:1) was percolated through the  
147 column, with an elution flow of about 15 drops every 10 s, till the Sudan dye reached the  
148 bottom of the column. After the elution, solvents were evaporated and the remaining fraction  
149 containing the methyl and ethyl esters was diluted with 2 mL of iso-octane. The iso-octane  
150 solution (1  $\mu\text{L}$ ) was then injected directly on-column in the GC-FID system composed by an  
151 Agilent gas chromatograph (7890B, Agilent Technologies, Santa Clara, CA, USA) equipped  
152 with a FID detector (set at 350  $^{\circ}\text{C}$ ) and a DB-5HT (15 m  $\times$  0.32 mm, 0.1  $\mu\text{m}$  film thickness)  
153 nonpolar capillary column (Agilent Technologies, Santa Clara, CA, USA). The temperature  
154 gradient was programmed as follows: 80  $^{\circ}\text{C}$  for 1 min; from 80 to 140  $^{\circ}\text{C}$  at 20  $^{\circ}\text{C min}^{-1}$ ; from  
155 140 to 335  $^{\circ}\text{C}$  at 5  $^{\circ}\text{C min}^{-1}$ ; 335  $^{\circ}\text{C}$  for 20 min. Helium was the carrier gas at a flow rate of 2  
156 mL  $\text{min}^{-1}$ .

157

### 158 2.3. FT-IR spectra acquisition

159 FT-IR spectra were collected by means of an ATR module on a Nicolet iS50 spectrometer  
160 (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: 4000-  
161 600  $\text{cm}^{-1}$  spectral range, 4  $\text{cm}^{-1}$  resolution, 32 scans for both sample and background. The  
162 equipment was controlled by OMNIC software (Thermo Fisher Scientific Inc., Waltham, MA,  
163 USA). Six (6) spectra per each sample were collected at room temperature (around 25  $^{\circ}\text{C}$ ),  
164 recording a new background every 3 spectra. Before each measurement, the ATR crystal was

165 cleaned with alcohol by a cotton wipe. Spectral acquisition was concomitant with the alkyl  
166 esters determination.

167

#### 168 2.4. Data analysis

169 Dataset descriptive statistics were calculated by means of Minitab 17 software (Minitab Inc.,  
170 State College, PA, USA). The replicates of FT-IR spectra collected from each sample were  
171 averaged before data elaboration. Spectral range was reduced in order to eliminate the noisiest  
172 and the least informative regions (600-650  $\text{cm}^{-1}$ ; 1890-2600  $\text{cm}^{-1}$ ). The resulting dataset (159  
173 samples x 5478 spectral variables) was pre-treated with smoothing (moving average, 15  
174 wavenumbers window size; SMOOTH), eventually followed by standard normal variate  
175 (SNV) or SNV coupled with first derivative (second polynomial order, 15 wavenumbers  
176 window size; d1), and explored by Principal Component Analysis (PCA). Then, a thirty-  
177 variable selection was performed by SELECT algorithm (Forina, Lanteri, Casale, & Cerrato  
178 Oliveros, 2007; Kowalski, & Bender, 1976) that searches for the variable with the largest  
179 Fisher classification weight (FW), defined for the variable  $v$  and the two categories 1 and 2 as:

$$180 \quad FW = \frac{(\bar{x}_{v1} - \bar{x}_{v2})^2}{\sum_{i=1}^{I_1} \frac{(x_{iv1} - \bar{x}_{v1})^2}{I_1} + \sum_{i=1}^{I_2} \frac{(x_{iv2} - \bar{x}_{v2})^2}{I_2}}$$

181 where  $\bar{x}_{vc}$  is the mean of variable  $v$  in category  $c$ ;  $I_c$  is the number of objects in category  $c$   
182 (Fisher, 1936). The variable is selected and decorrelated from the remaining predictors and  
183 then the process continues to iterate until all the given variables are selected.

184 The discrimination of EVOO (class 1; FAEE  $\leq 35 \text{ mg kg}^{-1}$ ) from non-EVOO (class 2; FAEE  
185  $> 35 \text{ mg kg}^{-1}$ ) samples was performed by applying two classification techniques on the  
186 reduced spectral datasets, i.e. Linear Discriminant Analysis (LDA), a supervised pattern  
187 recognition technique based on discriminant canonicals, and Soft Independent Modelling of  
188 Class Analogy (SIMCA), a supervised class-modelling technique. Classification models were

189 validated both in cross-validation by 5 cancellation groups (CV) and in prediction using three  
190 different external test sets, randomly created, each containing about 30% samples of the  
191 whole dataset.

192 Since LDA maximises the separation among classes by the construction of an optimal *a-*  
193 *posteriori* classification rule that assigns every object to one unique class, the LDA models'  
194 performance was evaluated by correct classification percentage, i.e. by the percentage of  
195 samples correctly assigned by the *a-posteriori* rule to the *a-priori* defined class. In the case of  
196 SIMCA, sensitivity (true positive rate) and specificity (false positive rate) were also  
197 calculated to evaluate the effectiveness of the classification models. Indeed, in a class-  
198 modelling technique, such as SIMCA, a sample can be assigned: (1) correctly and exclusively  
199 to the actual class; (2) exclusively to one class which differs from the actual class; (3) to no  
200 classes; or (4) to more than one class. Thus, while evaluating a SIMCA model, it comes the  
201 need to consider the latter three cases, sources of classification errors, through the calculation  
202 of the above-mentioned figures of merit (sensitivity and specificity).

203 Data elaboration was performed by The Unscrambler X software (v. 10.2, Camo Software  
204 AS, Oslo, Norway) and the V-Parvus package (Forina, Lanteri, Armanino, Casolino, Casale,  
205 & Oliveri, 2008).

206

### 207 **3. Results and discussion**

#### 208 *3.1. Fatty acid ethyl ester content of olive oil samples*

209 Table 1 reports the descriptive statistics of the olive oil samples, divided by classes: EVOO  
210 (class 1), non-EVOO (class 2). The number of objects was quite different between the two  
211 classes since no previous information about the amount of FAEE in the collected samples  
212 were provided by producers. In any case, this situation is representative of a real scenario,  
213 because during the authentication of EVOO a low number of non-EVOO should be expected.

214 Class 2 (non-EVOO), although made up of a lower number of samples, was characterised by  
215 a higher variability, covering a wide range of FAEE content, from 37.7 to 298 mg kg<sup>-1</sup>. On the  
216 contrary, the variability of class 1 (EVOO samples) was quite low, as most samples had a  
217 FAEE content in the range 1-10 mg kg<sup>-1</sup>.

218

### 219 3.2. FT-IR spectra and data exploration

220 Fig. 1 depicts the average FT-IR spectra for the two olive oil classes (i.e. EVOO and non-  
221 EVOO). Overall, spectra were similar to those reported for edible oils and olive oils (Dobson,  
222 2001; Sikorska et al., 2014). Although the spectra were almost overlapping, EVOO (class 1)  
223 presented slightly higher band intensities roughly along the whole spectral range. More  
224 evident differences in intensity were observed for particular bands, namely in the regions  
225 from around 3030 to 2800 cm<sup>-1</sup>, corresponding to different stretching vibrations, around 1238  
226 and 1160 cm<sup>-1</sup>, corresponding to -C-O, -CH<sub>2</sub>- stretching and bending, and around 1118, 1097  
227 and 1030 cm<sup>-1</sup>, corresponding to -C-O stretching (Sikorska et al., 2014). No considerable  
228 differences were observable for those bands reported as typical of the ester linkage (2730,  
229 2677 cm<sup>-1</sup>), with the exception of the peak around 1746 cm<sup>-1</sup> (highlighted in Fig. 1), which  
230 might be potentially influenced by the amount of alkyl esters. The little differences observed  
231 between the spectra of the two oil classes confirm the necessity of chemometric tools to  
232 extract useful information.

233 As a first step, an exploratory analysis was performed by PCA on both the raw and the  
234 different pre-treated data. Fig. 2a reports the score plot resulting from the raw data analysis.  
235 Few samples belonging to EVOO class resulted isolated from the sample cloud. However, no  
236 sample subsets were identified; EVOO and non-EVOO classes appeared confused. Sample  
237 distribution in the space of the first two principal components (PC) was very similar for any  
238 of the pre-treated dataset explored (data not shown).

239 The PC1 loadings plot (Fig. 2b) showed that the most informative variables affecting the  
240 sample distribution, i.e. the ones farer from zero, corresponded to the most evident differences  
241 in the absorbance observed in the raw spectra. PC2 loadings trend (Fig. 2b) remarked a strong  
242 negative influence in sample separation of the band from 3770 to 3070  $\text{cm}^{-1}$ , likely due to -  
243 OH group of oxidation products (Sikorska et al., 2014), and a strong positive influence of  
244 variables around 3030 - 2800  $\text{cm}^{-1}$  and 1743  $\text{cm}^{-1}$ . Those regions were highlighted also in the  
245 VOO spectra recorded by Valli et al. (2013) and pre-treated by multiplicative scatter  
246 correction and mean centering.

247

### 248 3.3. Classification models

249 As the whole spectral range did not look promising in EVOO and non-EVOO discrimination,  
250 a variable selection strategy was investigated. The SELECT algorithm allowed to identify the  
251 30 most informative variables, among the original 5478, to be used in the following  
252 classification procedures. Variable selection and classification model development were  
253 performed on both raw and pre-treated data (SMOOTH, SMOOTH-SNV, and SMOOTH-  
254 SNV-d1).

255 The 30 variables selected from the raw dataset are reported in Fig. 3a. Half of the selected  
256 variables belongs to the high informative fingerprint region, i.e. between 1500 and 650  $\text{cm}^{-1}$ .  
257 The signals from 750 to 679  $\text{cm}^{-1}$  can be linked to the  $\text{CH}_2$  rocking vibrations occurring at 723  
258  $\text{cm}^{-1}$  (Guillen & Cabo, 1997; Lerma-García, Simó-Alfonso, Ramis-Ramos, & Herrero-  
259 Martínez, 2011). The variables selected between 1000 and 900  $\text{cm}^{-1}$  could be linked to C-H  
260 out of plane deformation occurring at 968  $\text{cm}^{-1}$  due to isolated *trans* double bonds presence  
261 (Guillen & Cabo, 1997). The 1119  $\text{cm}^{-1}$  variable could originate from signal due to the C-O  
262 stretching vibration (Guillen & Cabo, 1997) as well as the variables selected in the range  
263 1300-1270  $\text{cm}^{-1}$ , where also the C-H bending vibration is expected to occur. The fourth range

264 of selected variables includes wavenumbers 1474-1472  $\text{cm}^{-1}$  that are related to  $\text{CH}_2$  and  $\text{CH}_3$   
265 scissoring vibrations (Guillen & Cabo, 1997). The other selected variables lay outside the IR  
266 fingerprint region. Between 1626  $\text{cm}^{-1}$  and 1580  $\text{cm}^{-1}$  the selected signals can be related to C-  
267 C vibration in aromatic structures. The absorption of carbonyl aldehydes and ketones are in  
268 the selected range 1730-1645  $\text{cm}^{-1}$  (Van de Voort, Sedman, & Russin, 2001). The C=O  
269 stretching vibration characterises the variables selected between 1775 and 1713  $\text{cm}^{-1}$ ; indeed,  
270 these are related to an intense peak that is normally observed at 1746  $\text{cm}^{-1}$  and known to be  
271 related to carbonyl group vibration in ester linkage between fatty acid and glycerol (Sikorska  
272 et al., 2014). Other four variables were selected in the range 4000-3600  $\text{cm}^{-1}$ ; normally, the  
273 vibration of C=O stretching belonging to triacylglycerol ester bonds occurs also in this range  
274 (Sikorska et al., 2014).

275 In Fig. 3b the 30 variables selected for the SMOOTH-SNV-d1 dataset are reported. Apart  
276 from the variables selected in the regions already discussed above, relevant variables appear  
277 between 3500 and 2600  $\text{cm}^{-1}$ . In the region 3000-2800  $\text{cm}^{-1}$ , C-H stretching vibrations are  
278 present, such as the stretching of  $\text{CH}_3$  in the range 2962-2873  $\text{cm}^{-1}$  (Vlachos, Skopelitis,  
279 Psaroudaki, Konstantinidou, Chatzilazarou, & Tegou, 2006) and two intense bands at 2924  
280 and 2853  $\text{cm}^{-1}$  linked to  $\text{CH}_2$  stretching of the fatty acid chains in triacylglycerols (Guillèn &  
281 Cabo, 1997). Also C=O Fermi resonance of ester groups occurs at 2677 and 2730  $\text{cm}^{-1}$   
282 (Guillèn & Cabo, 1997; Lerma-García et al., 2011). The last variables selected from the  
283 transformed dataset belong to the 4000-3500  $\text{cm}^{-1}$  range, where the relevant bands are related  
284 to C=O overtone and stretching vibrations (Vlachos et al., 2006).

285 Concerning LDA, good classification performances were obtained using both raw and  
286 SMOOTH-SNV-d1 pre-treated data, while less promising results were achieved with  
287 SMOOTH or SMOOTH-SNV data (Table 2). In details, for both raw and SMOOTH-SNV-d1  
288 datasets the correct classification rate in calibration was 100% no matter the external test set

289 used, whereas the SMOOTH-SNV model resulted affected by the data excluded from the  
290 calibration sets, ranging the correct classification from 98.9 to 99.1% depending on the  
291 considered test set. Smoothed data also provided very good classification rate in calibration  
292 even though less accurate than those of raw and SMOOTH-SNV-d1 datasets. With internal  
293 validation (cross-validation), the correct classification rates remained high for the SMOOTH-  
294 SNV-d1 dataset (97.3-100%), while decreased for raw data (96.4-97.3%) as well as for  
295 SMOOTH-SNV data (90.1-94.6%). Data smoothing gave the best results just after those of  
296 SMOOTH-SNV-d1 dataset (95.8-99.1%). The prediction step confirmed the reliability of the  
297 models developed with raw and SMOOTH-SNV-d1 datasets; indeed the correct classification  
298 rates resulted between 95.8 and 100%, whereas the SMOOTH and SMOOTH-SNV data  
299 reached as high as 97.9 and 93.7% of correct classification, respectively.

300 Considering that the dearth or soft mathematical pre-treatment should be preferred (Grassi,  
301 Casiraghi, & Alamprese, 2018), LDA results based on the selected variables of raw spectra  
302 looked the more promising among the developed models. In addition, the cross-validation  
303 results of raw data, being more stable when changing the external test set, revealed a more  
304 robust model. These results look much more promising than those obtained by Cayuela  
305 (2017) using NIR (100-2300 nm) spectra collected on a huge set of virgin (extra, virgin and  
306 lampante) olive oils. The author's main purpose was to perform PLS regression models to  
307 quantify FAAE, FAEE, and FAME. Furthermore, an oil classification based on each  
308 parameter was performed, but no information about the classification algorithm was reported.  
309 Moreover, the performance evaluation was based on the correspondence between the  
310 regression predicted values and the defined classes of the samples from reference  
311 measurements. In any case, correct classification rates of the models based on FAAE (two  
312 classes with 75 mg kg<sup>-1</sup> threshold), FAEE (two classes with 35 mg kg<sup>-1</sup> threshold), and FAME  
313 (two classes with 35 mg kg<sup>-1</sup> threshold) were 78.65-94.8%, 70.0-88.7%, and 95.2%,

314 respectively. Thus, it is difficult to deeply compare results by Cayuela (2017) with the LDA  
315 models obtained in this work.

316 Class-modelling techniques are often more appropriate in food authentication than  
317 discriminant methods, because they aim at answering to the essential question in food  
318 authenticity problems: if a product is sold with a specific label, it is important to verify if it is  
319 really consistent with the claimed characteristics (Oliveri, Di Egidio, Woodcock, & Downey,  
320 2011). Moreover, this approach does not force a sample to belong to one specific class,  
321 resulting in a more severe but realistic scenario in real-life applications when a large number  
322 of non-target classes is possibly present. Thus, SIMCA models for olive oil discrimination  
323 based on FAEE classes were developed and the related results are provided in Table 3.  
324 Differently from what observed with LDA, the best classification performances were obtained  
325 on pre-treated data instead of raw ones. In details, models obtained with SMOOTH-SNV-d1  
326 treated spectra gave higher percentages of classification ability than those developed with  
327 SMOOTH or SMOOTH-SNV data, ranging from around 91 to slightly less than 96%.  
328 Looking at the prediction performances of the models, the correct classification ability was,  
329 on average, higher when smoothing was applied to spectral data although the absolute best  
330 performance was observed applying first derivative in combination with smoothing and SNV  
331 (up to 81.25%). Sensitivity of all the models was promising, showing a percentage of true  
332 positive assignments ranging from 75 to about 92%, being again, on average, SMOOTH  
333 model the best. Indeed, when comparing the Cooman's plot (Fig. 4) of the best performing  
334 SIMCA models, it can be noticed that a similar and low number of samples is distributed  
335 outside the 95% model thresholds for SMOOTH and SMOOTH-SNV-d1 datasets (Fig. 4a and  
336 4c). It means that those samples were not accepted by any of the class models (false  
337 negatives). However, the most important issue concerned the low specificity affecting models.  
338 The average specificity was around 50% (Table 3) considering the different pre-treatments,

339 meaning that around the half of the test samples belonging to one of the class was not rejected  
340 from the assignment to the other class. Referring to the Cooman's plots (Fig. 4), these  
341 samples are located in the small square on the bottom resulting from the intersection of 95%  
342 threshold lines of class 1 and class 2 models. Those samples were accepted by both the model  
343 of EVOO and non-EVOO samples. In particular, the lower specificity values of class 2 (non-  
344 EVOO), which were on average between 40 and 50% for the three considered external sets  
345 (data not shown), implicates a high probability of assignment of non-EVOO to the EVOO  
346 class. Thus, it is clear that the application of SIMCA class-modelling could be hazardous for  
347 EVOO labelling based on FAEE content, and the discriminant strategy (LDA) should be  
348 preferred. Actually, when only two well-defined classes are considered, i.e.  $FAEE \leq 35 \text{ mg}$   
349  $\text{kg}^{-1}$  (EVOO class) and  $FAEE > 35 \text{ mg kg}^{-1}$  (non-EVOO class), a discriminant-based strategy  
350 as LDA is a reliable option. Indeed, a discriminant classification method divides the sample  
351 domain into a number of sub-areas corresponding to the spaces of the considered classes. A  
352 sample is always assigned to one of the considered classes, even if it is located very far from  
353 all the classes. However, when only two well-defined classes are considered, such in this  
354 study, the classification-discriminant method can give reliable results thanks to the low  
355 complexity of the dataset (Di Egidio, Oliveri, Woodcock, & Downey, 2011).

356 Future perspectives can consider the possibility to enlarge the VOO dataset by collecting  
357 samples from different Italian regions and covering different cultivars and storage strategies.  
358 Moreover, the non-EVOO class could be extended accordingly. From an instrumental point of  
359 view, a multichannel IR-instrument based on 30 or fewer wavenumbers could be developed  
360 from commercial prototypes and its reliability compared with the presented models.

361

#### 362 4. Conclusions

363 The potential of FT-IR spectroscopy coupled with chemometrics as a tool for a rapid and fast  
364 discrimination of extra virgin olive oils and virgin olive oils based on fatty acid ethyl ester  
365 content has been assessed. The legal limit for FAEE content enables to highlight two well-  
366 defined classes of products, thus a discriminant classification approach can be considered as  
367 the most suitable in this context, to be preferred over the SIMCA class-modelling algorithm.  
368 LDA models based on selected features of the raw spectra datasets gave good and robust  
369 results, with percentages of correct classification in prediction higher than 95%. Being the  
370 discriminant classification results so promising, the application of FT-IR spectroscopy should  
371 be encouraged in the olive oil sector. Moreover, since the variable selection strategy was  
372 effective, the development of simplified and cheap instruments can boost their use also in  
373 small enterprises. Such instruments can be calibrated in order to give also results about oil  
374 composition, thus providing producers and control bodies with many important information  
375 about olive oil samples.

376

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381

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483

484 **Figure captions**

485 **Fig. 1.** Average FT-IR spectra for extra virgin olive oils (Class 1-EVOO; black straight line)  
486 and non-extra virgin olive oils (Class 2-nonEVOO; grey dashed-line). In detail the peak  
487 around 1746  $\text{cm}^{-1}$ .

488  
489 **Fig. 2.** Principal Component Analysis results of raw FT-IR spectra collected on extra virgin  
490 (EVOO) and non-extra virgin (nonEVOO) olive oil samples: a) PC1 vs PC2 score plot and b)  
491 loading plot for PC1 and PC2.

492  
493 **Fig. 3.** FT-IR variables ( $\text{cm}^{-1}$ ) selected by SELECT algorithm before to perform classification  
494 of extra virgin and non-extra virgin olive oil samples: a) 30 variables selected from the raw  
495 dataset, b) 30 variables selected from the data pre-treated with smoothing, standard normal  
496 variate and first derivative.

497  
498 **Fig. 4.** Soft Independent Modelling of Class Analogy results for extra virgin (EVOO) and  
499 non-extra virgin (nonEVOO) olive oil samples. Cooman's plots obtained with the 30 FT-IR  
500 selected features and the external test set no. 3: a) smoothed data (SMOOTH; b) data pre-  
501 treated with smoothing and standard normal variate (SMOOTH-SNV); c) data pre-treated  
502 with smoothing, standard normal variate and first derivative (SMOOTH-SNV-d1).

Figure 1.

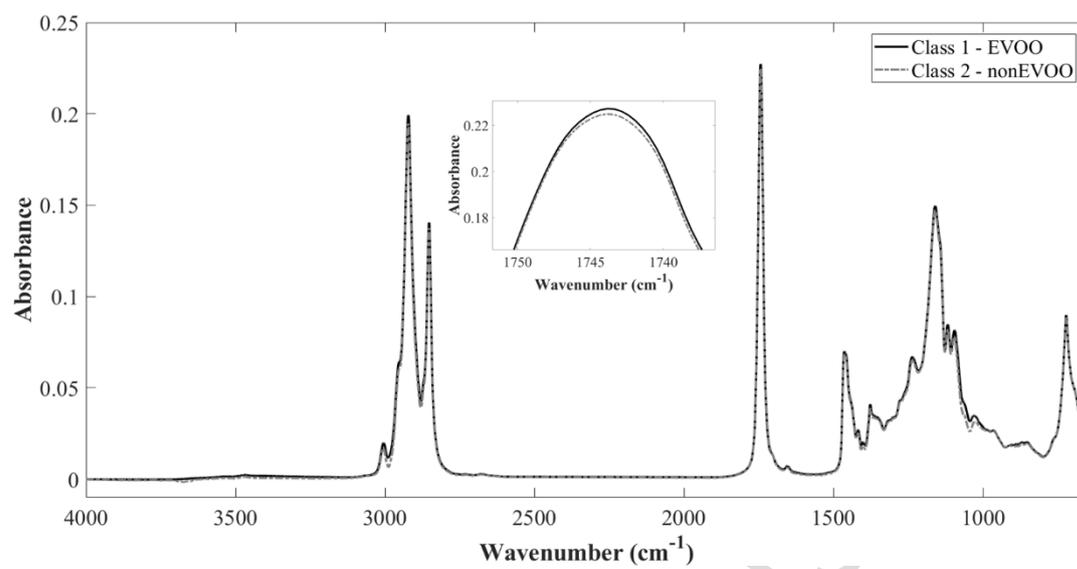


Figure 2.

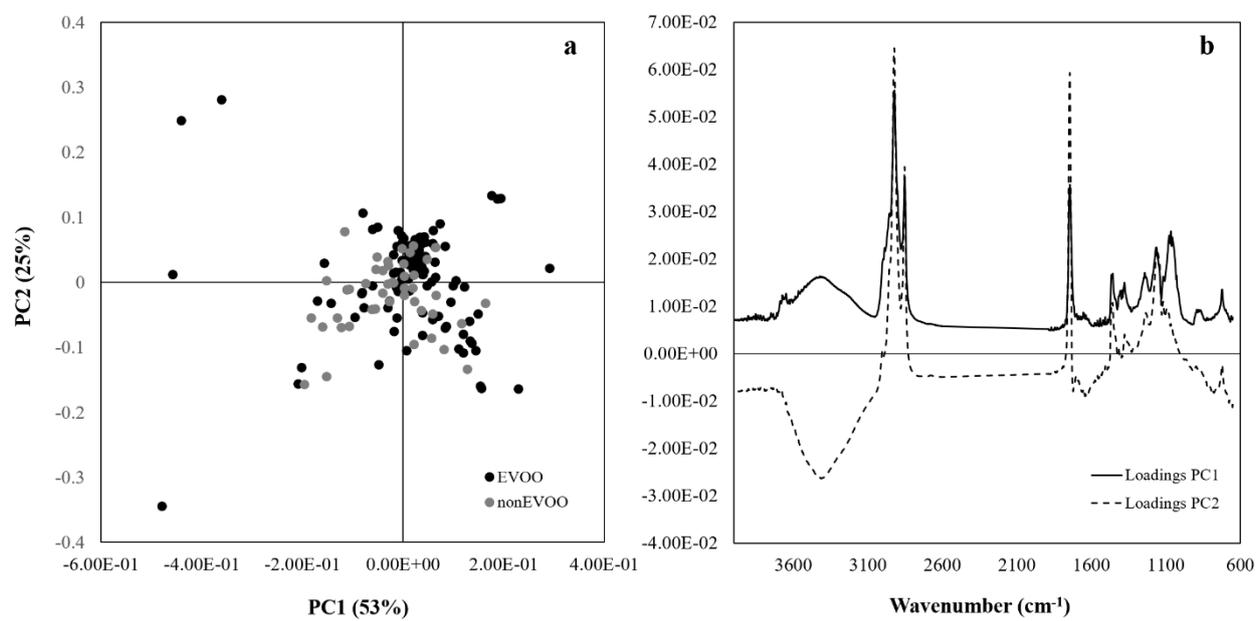


Figure 3.

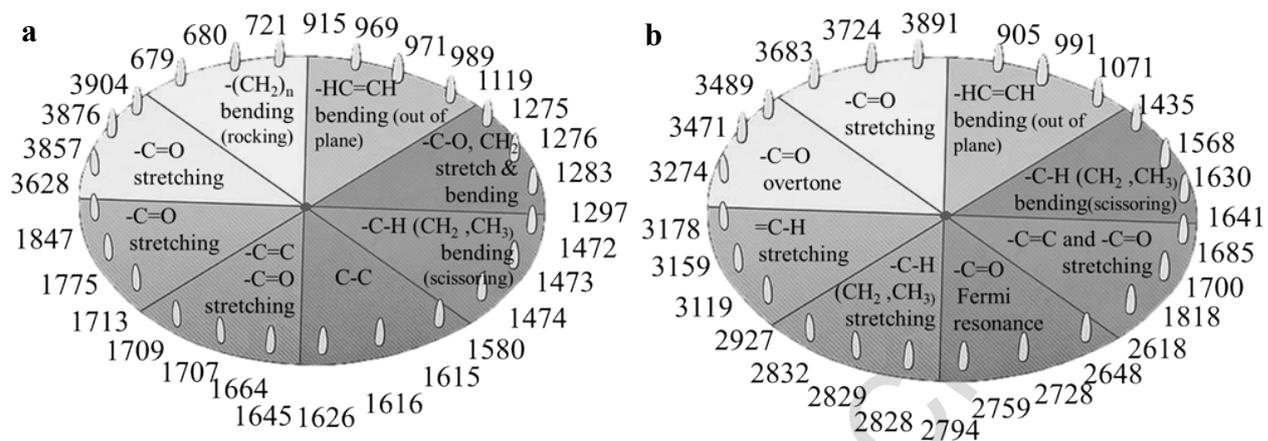
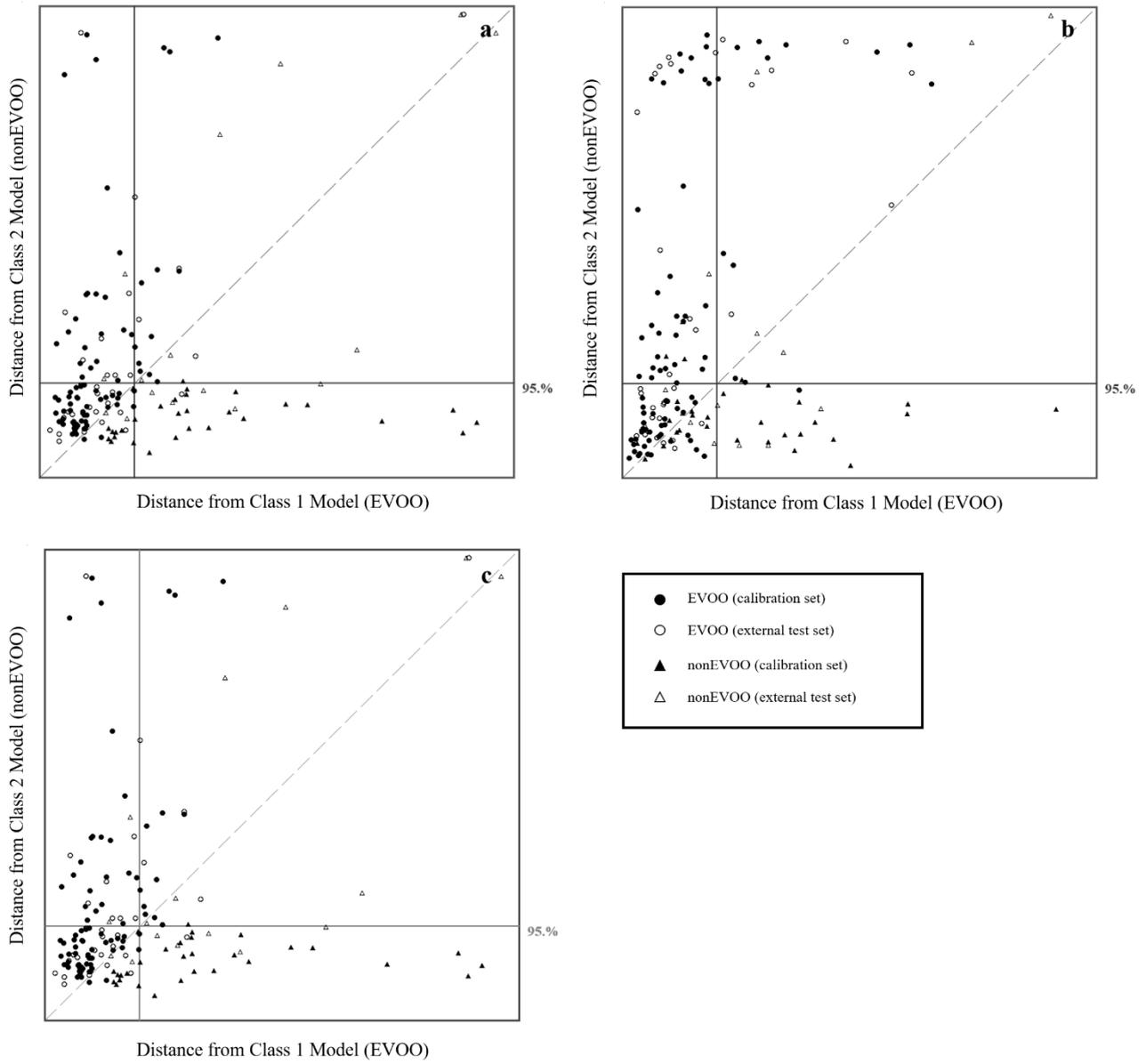


Figure 4.



**Highlights**

- FT-IR spectroscopy discrimination of olive oils based on fatty acid ethyl esters
- FT-IR variable selection improved olive oil discrimination
- Linear Discriminant Analysis models reached up to 100% of correct prediction
- Class modelling had high sensitivity but scarce specificity (<50%) in prediction

**Table 1.** Descriptive statistics about the fatty acid ethyl ester content ( $\text{mg kg}^{-1}$ ) of the olive oil samples under study divided by classes: extra virgin olive oils (class 1), non-extra virgin olive oils (class 2).

Class	N	Mean	SD*	CV*	Min	Median	Max	Range	IQR
1	113	6.68	4.95	74.21	1.81	5.34	31.36	29.54	3.93
2	46	106.90	77.20	72.25	37.70	72.50	298.80	261.10	114.60

N, number of samples; SD, standard deviation; CV, percent coefficient of variation; IQR, interquartile range.

\*Standard deviation and coefficient of variation are referred to the class distribution.

**Table 2.** Results of Linear Discriminant Analysis for olive oil discrimination based on fatty acid ethyl ester content: average correct classification percentages of models based on the 30 most informative variables of FT-IR spectra.

Pre-treatment	External test set	Correct classification (%)		
		Calibration	Cross-validation	Prediction
Raw	1	100	97.30	95.83
	2	100	96.40	95.83
	3	100	96.61	100
SMOOTH	1	100	97.30	97.92
	2	99.28	99.10	91.67
	3	99.49	95.76	97.56
SMOOTH-SNV	1	98.92	90.10	93.75
	2	99.10	94.60	87.50
	3	99.15	91.53	90.24
SMOOTH-SNV-d1	1	100	97.30	100
	2	100	100	95.83
	3	100	99.15	100

SMOOTH, moving average smoothing; SNV, standard normal variate; d1, first derivative.

**Table 3.** Results of Soft Independent Modeling of Class Analogy for olive oil discrimination based on fatty acid ethyl ester content: average classification, prediction ability, and sensitivity and specificity values in prediction of models based on the 30 most informative variables of FT-IR spectra after different mathematical pre-treatments.

Pre-treatment	External test set	Calibration	Prediction		
		Classification ability (%)	Prediction ability (%)	Sensitivity (%)	Specificity (%)
Raw	1	83.78	77.08	87.50	39.58
	2	88.29	75.00	91.67	45.83
	3	87.29	73.17	87.81	41.46
SMOOTH	1	72.07	79.17	91.67	45.83
	2	85.59	75.00	89.58	50.00
	3	88.98	80.49	90.24	53.66
SMOOTH-SNV	1	83.78	77.08	75.00	54.17
	2	88.29	75.00	79.17	66.67
	3	89.83	70.73	80.49	48.78
SMOOTH-SNV-d1	1	95.50	75.00	75.00	52.08
	2	90.99	81.25	70.83	62.50
	3	95.76	73.17	80.49	43.90

SMOOTH, moving average smoothing; SNV, standard normal variate; d1, first derivative.