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Controlling protected designation of origin of wine by Raman spectroscopy

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Abstract: In this paper, a Fourier-Transform Raman spectroscopy method, to authenticate the provenience of wine, for food traceability applications was developed. In particular, due to the specific chemical fingerprint of Raman spectra, it was possible to discriminate different wines produced in the Piedmont area (North West Italy) in accordance with i) grape varieties, ii) production area and iii) ageing time. In order to create a consistent training set, more than 300 samples from tens of different producers were analyzed, and a chemometric treatment of raw spectra was applied. A discriminant analysis method was employed in the classification procedures, providing a classification capability (percentage of correct answers) of 90 % for validation of grape analysis and geographical area provenance, and a classification capability of 84 % for ageing time classification. The present methodology was applied successfully to samples without any preliminary treatment of the sample, providing a response in a short time.

## \*Highlights (for review)

- Designation of origin of wines can be controlled through Raman spectroscopy;
- Grape cultivar, provenience and ageing time of wines is determined through Raman;
- A rapid, sensitive and non-destructive method for wine analysis is proposed;
- Discriminant Analysis is applied to spectral data for wine classification.

# 1 Controlling Protected Designation of Origin of wine

## 2 by Raman Spectroscopy

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12  
13 **Keywords:** wine, raman spectroscopy, food traceability, chemometrics, fingerprint

### 14 Abstract

15 In this paper, a Fourier Transform Raman spectroscopy method, to authenticate the provenience of wine, for  
16 food traceability applications was developed. In particular, due to the specific chemical fingerprint of the Raman  
17 spectrum, it was possible to discriminate different wines produced in the Piedmont area (North West Italy) in  
18 accordance with i) grape varieties, ii) production area and iii) ageing time. In order to create a consistent training  
19 set, more than 300 samples from tens of different producers were analyzed, and a chemometric treatment of raw  
20 spectra was applied. A discriminant analysis method was employed in the classification procedures, providing a  
21 classification capability (percentage of correct answers) of 90 % for validation of grape analysis and  
22 geographical area provenance, and a classification capability of 84 % for ageing time classification. The present

23 methodology was applied successfully to raw materials without any preliminary treatment of the sample,  
24 providing a response in a very short time.

25

## 26 **Main text**

### 27 **1. Introduction**

28 In order to preserve the quality of food products from particular geographical areas, and to protect consumers  
29 against imitations and false information, the European Commission has defined, via Regulations 1151/2012, the  
30 designations: Traditional Specialty Guaranteed (TSG), Protected Designation of Origin (PDO) and Protected  
31 Geographical Indication (PGI) (Regulation (Eu) No 1151/2012 Of The European Parliament And Of The  
32 Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs). Quality labels have  
33 an important role in consumer behavior and give confidence about the origins and the quality of food. Label  
34 assignment is an important market claim and represents a valuable weapon to attest and justify the economic  
35 value of products. Traceability has become a very relevant concept in association with edible products and  
36 represents an essential tool to enhance traders and consumers' confidence in the safety, quality, and authenticity  
37 of food.

38 Unfortunately, most of food traceability procedures involve tedious administrative documents, while scientific  
39 methodologies that identify the authenticity of food objectively are preferable. Accordingly, scientific research is  
40 focusing on the development of analytical methods for traceability to authenticate the geographical origin of  
41 foods (Peres, Barlett, Loiseau and Montet, 2007), with the aim of linking food products with distinctive features,  
42 such as ingredients, physical properties and production methods. Food traceability analysis are usually  
43 performed by means of several analytical techniques, such as mass spectrometry for isotope ratio determination  
44 (Durante, Baschieri, Bertacchini, 2015), DNA based techniques, such as polymerase chain reaction (PCR)  
45 (Pardo, 2014) and nuclear magnetic resonance spectrometry (NMR) (Mazzei, Francesca, Moschetti, Piccolo,  
46 2010).

47 In the last two decades, stable isotope methodologies, based on gas chromatography-isotope ratio mass  
48 spectrometry (GC-IRMS) and GC-pyrolysis-IRMS (Fronza, Fuganti, Graselli, Reniero et al 1998; Adam,  
49 Bartels, Christoph, Stempf 1995; Misselhorn, Grafahrend, 1990), have been applied successfully in quality  
50 control of wine following the establishment of an official wine database for stable isotope parameters (EU

51 regulations 2670/90, 2347/91 and 2348/91) (Rossmann, 2001). As reported by Breas et al. (Bréas, Reniero,  
52 Serrini, Martin and Rossmann, 1994), a classification of wines from different European countries can be  
53 achieved with  $^{13}\text{C}/^{12}\text{C}$  analysis of ethanol and  $^{18}\text{O}/^{16}\text{O}$  determination of water, underlining the importance of the  
54 photosynthetic pathway as well as the environmental and climatological conditions of the vineyard. Even if  
55 stable isotope methods provided consistent results, which could be used for routine analysis of wines, it is not  
56 always simple to find a physical, chemical or biochemical explanation for variations of isotope ratios in natural  
57 substances or to establish a relevant database for statistical evaluation.

58 DNA based technologies have also been exploited in this field due to their specificity in analysis, which is  
59 strictly associated with genotype (the inherited instructions that an organism carries within its genetic code), but  
60 these technologies inevitably miss the stochastic significant epigenetic differences accumulating over time across  
61 cells (Petronis, 2010). Dordevic et al. (2013) highlighted the need for new methods and better geographical  
62 discrimination between samples, demonstrating that multivariate methods are superior to univariate approaches.

63 The NMR and vibrational spectroscopy techniques represent interesting alternatives or even complementary  
64 methods. Godelmann et al. (2013) analyzed about 600 German wines and demonstrated that  $^1\text{H}$ NMR coupled  
65 with statistical data treatment could provide individual “fingerprints” for wine samples, which include  
66 information about variety, origin, vintage, physiological state, technological treatment, and other factors. The  
67 fusion of NMR profiling and stable isotope data for wine analysis has been reported in literature with good  
68 results (Monakhova et al. 2014). However, the main drawbacks of the cited techniques (i.e. MS, NMR and DNA  
69 based techniques) are related to the cost of instruments, extensive sample pre-treatments, and the duration of  
70 analysis, which often reduce the accuracy and precision of measurements. Since simple and rapid analytical  
71 methods are needed to meet the demands of European labeling legislation, vibrational spectroscopy is emerging  
72 as a new and powerful tool in authenticating food provenance.

73 Vibrational spectroscopy techniques usually provide non-destructive analysis of samples, rapid collection times  
74 with no or minimal sample pre-treatment, which reduce the total time of analysis and could support the  
75 development of reliable control procedures and screening methods for food traceability. Moreover, new modern,  
76 portable instruments with smart accessories have been developed, making these techniques more suitable for *in*  
77 *line* process monitoring and *in situ* analysis (Gallego, Guesalaga, Bordeu and González, 2011). These methods  
78 encompass absorption spectroscopy in the mid-infrared (MIR) and the near-infrared (NIR) for studying  
79 fundamental molecular vibrations and their harmonics (Bauer et al., 2008; Cozzolino, Damberg, Janik, Cynkar,

80 & Gishen, 2006; Cozzolino, McCarthy, & Bartowsky, 2012, Cozzolino D., 2014), and absorption spectroscopy in  
81 the ultra-violet and visible (UV-vis) regions for probing electronic transitions (Acevedo, Jiménez, Maldonado,  
82 Domínguez, & Narváez, 2007; García-Jares & Médina, 1995; Harbertson & Spayd, 2006; Roig & Thomas,  
83 2003; Urbano, Luque de Castro, Pérez, García-Olmo, & Gómez-Nieto, 2006). Raman spectroscopy, which is  
84 based on the inelastic scattering of a monochromatic light, also provides a characteristic spectroscopic pattern  
85 (i.e. “molecular fingerprint”) of organic compounds based on the vibrational modes of chemical bonds (Li-Chan,  
86 Griffiths and Chalmers, 2010; Thygesen, Løkke, Micklander and Engelsen, 2003). Moreover, Raman analysis  
87 can be easily done in aqueous media and through glass containers, because signals from both water and glass are  
88 very weak in the Raman spectrum (Schulz and Baranska, 2007; Yang, Irudayaraj 2001) and do not overlap with  
89 those from food components, such as proteins (Li-Cha, Nakai, Hirotsuka, 1994), lipids (Yang, Irudayaraj and  
90 Paradkar, 2005) and carbohydrates (Mathlouthi, Koenig, 1986), which are sensitive and specific.

91 Raman spectroscopy has demonstrated its value in food traceability for olive oil provenance and composition  
92 (Bernuy, Meurens, Mignolet and Larondelle, 2008), honey provenance (Özbalçia, Hakkı Boyacıa, Topcu,  
93 Kadırlar, Tamerc, 2013; Paradkar and Irudayaraj, 2001) and the authenticity of beers (Downey, 2009). As  
94 regards alcoholic beverages, Raman spectroscopy has been used for the quantification of the alcohol content in  
95 whisky, vodka and other spirituous beverages (Nordon, Mills, Burn, Cusick and Littlejohn 2005). The feasibility  
96 of exploiting Raman scattering to analyze white wines has also been investigated (Meneghini et. al., 2008). In  
97 particular, a recent work by Coralie et al. (2014) demonstrated that resonance condition of some chemical  
98 species present in wine, such as phenolic compounds, hydroxycinnamic acids and sugars, can be analyzed  
99 selectively using lasers at different wavelengths.

100 In this work, we evaluated the potential to use Raman spectroscopy, coupled with a chemometric data treatment,  
101 to discriminate different wines from the Piedmont area (North West Italy) in accordance with grape varieties,  
102 production area and ageing time. In particular, tests were performed on Nebbiolo, Dolcetto and Barbera wines,  
103 which were chosen for their wide distribution and their productive and economic relevance to the Italian wine  
104 market. The purpose of the work was to provide a statistically substantial classification method, based on a set of  
105 known responses (training set) through the chemometric treatment of data. The work scheme was structured on  
106 three levels: classification of wines in accordance with the (1) grapes used, (2) production area, and (3) age.

107

## 108 **2. Material and Methods**

109 *2.1 Samples*

110 315 commercial wines were obtained from different winemakers using Nebbiolo, Barbera and Dolcetto grapes.  
111 For each grape variety, wines from the different area and age were selected (Table 1). More than 10 Protected  
112 Designation of Origin (PDO) wines were examined. The number of samples for each PDO wine was different  
113 based on the winemaker and commercial dissemination and, inevitably, limited by the availability of samples.  
114 All the samples were furnished directly by the producers, and stored at +4°C until analysis.

115 **Tab.1 – Distribution of wines examined in accordance with grape, PDO and production area**

116

117 *2.2 Raman measurements*

118 Raman spectroscopy was performed with a Thermo Scientific NXR FT-Raman Module Nicolet Series™  
119 equipped with an InGaAs detector (ThermoFisher Scientific, Waltham, USA), a CaF<sub>2</sub> beamsplitter and a 1064  
120 nm laser line. Raman spectra were collected using a laser (power 0.9 W) in the spectral range 200 to 4000 cm<sup>-1</sup>  
121 with a resolution of 4 cm<sup>-1</sup>. 256 scans were collected to obtain S/N ratio higher than 15. Samples were analyzed  
122 in 4 ml glass vials positioned vertically on a motorized stage.

123 *2.3 Multivariate Analysis.*

124 The raw Raman spectra were subjected to discriminant analysis using TQ Analyst™ 8.0 software (ThermoFisher  
125 Scientific, Waltham, USA). Spectra were pre-processed using the Savitzky-Golay smoothing filter (Savitzky,  
126 Golay, 1964) to remove of as much noise as possible without unduly degrading the spectral information. The  
127 spectral range to be analyzed was selected in such a way that interference from random variability of spectra was  
128 minimized and did not generate spurious information in the classification model. Seven restricted spectral  
129 regions around Raman peaks were selected to optimize the classification result. The frequency regions of spectra  
130 that did not contain Raman peaks (e.g. 800-600 cm<sup>-1</sup> and 2800-1800 cm<sup>-1</sup>) were excluded. In this way,  
131 worthless information was ignored and the best class separation was obtained. The number of PCs selected was a  
132 compromise between explained variance for each PC and the predictive capability of the model: when the  
133 cumulative variance reaches the *plateau*, further components do not provide any useful information and should  
134 be excluded so variables that represent only noise are not considered. Variables that explain only a small portion  
135 of the variability are not excluded if they improve significantly the classification capability of the model (% of



136 samples correctly classified). The chemometric models presented for wine classification were first validated  
137 through a leave-one-out cross-validation procedure during model optimization (mathematical pretreatment  
138 choice, selection of significant PCs, etc.). Finally, the optimized models were validated through a cross  
139 validation procedure using exclusion sets made up of five samples chosen randomly; the number of exclusion  
140 sets was proportional to the total number of calibration samples. This classification technique permits unknown  
141 distance to a class center in terms of Mahalanobis distance (Mahalanobis, 1936) to be calculated and each  
142 unknown samples to be assigned correctly. Md is based on the idea that it contains an auto-scaling process in and  
143 overcomes assumption about the spherical distribution of sample points around the center of mass; thus, non-  
144 spherical distributions can be described as well as spherical ones. In the generalized formula for Md, the  
145 observation are represented by  $x=(x_1, x_2, \dots, x_n)$  while  $\mu=(\mu_1, \mu_2, \dots, \mu_n)$  represents the observations' mean.  
146 The apex  $T$  indicates the transposed matrix  $(x - \mu)$ .  $S^{-1}$  is the inverse of the covariance matrix of the observations.

$$147 \quad Md(x)=\sqrt{(x-\mu)^T S^{-1} (x-\mu)}$$

148 If an ellipsoidal distribution is considered, we would expect that the probability a test point belongs to the set  
149 depends not only on the distance from the center of mass but also the direction. (De Maesschalck, Jouan-  
150 Rimbaud, Massart, 2000).

151 The statistical reliability of results will be discussed case by case to assess the effective classification capability  
152 of the proposed Raman method, even if an external set dedicated to test set validation was not available. The  
153 work scheme of this study was divided into three consecutive steps: discrimination according to (i) grape, (ii)  
154 production area, and (iii) age.

155

### 156 **3. Results and Discussion**

157 Food systems are dynamic, chemically complex and, generally, heterogeneous matrices containing large  
158 numbers of biological molecules. The chemical specificity, ease of sampling, speed, and non-destructive nature  
159 of FT-Raman spectroscopy makes it an attractive tool for food analysis. Chemical specificity of the Raman  
160 technique relies on the fact that different molecular bonds or groups of chemical bonds are identified by  
161 characteristic frequency-shifts in incident light (Figure1). For this reason, the first step in compositional analysis  
162 of wine using FT-Raman is attribution of characteristic frequency shifts to vibrational modes of molecular bonds  
163 observed in spectra (Table 1S in supplementary information).

164 As Figure 1 shows, a large band ascribed to OH stretching at  $3350\text{ cm}^{-1}$  was clearly visible in all the spectra  
165 analyzed. In addition, a minor band related to OH bending at  $1700\text{-}1500\text{ cm}^{-1}$  was observed. The group of peaks  
166 between  $3000\text{-}2800\text{ cm}^{-1}$  is due to symmetric and asymmetric stretching of  $\text{CH}_x$  bonds. Several other  
167 characteristic peaks of ethanol are present at frequencies less than  $1500\text{ cm}^{-1}$ . These are associated with several  
168 deformation modes of  $\text{CH}_x$  as reported in Tab 1S (Mammone, Sharma, Nicol, 1980). All peaks in the wine were  
169 shifted slightly in comparison with the pure ethanol peaks; this is due to the simultaneous presence of different  
170 organic species, such as glycerol, acetaldehyde, organic acids, and polyphenols including flavonoids and non-  
171 flavonoids. At  $1630\text{ cm}^{-1}$  a low intensity band was present in the wine spectra. This band is characteristic of C=O  
172 stretching, a relatively inactive Raman vibration. The C=O peak observed could be attributed to several species  
173 present in the matrix (e.g. organic acids and flavonoids) the carbonyl groups of which are characterized by  
174 slightly different vibration frequencies. This, a quite broad signal was registered in this spectral region.

175 The samples analyzed were chosen with the aim of representing a wide selection of the wines, which were  
176 purchased from different producers. Numerous samples were requested to capture the variability in the system  
177 and obtain a representative dataset for multivariate calibration. Raman spectra of the different wines were very  
178 similar to each other, as it can be seen in Figure 1 where the spectra of Dolcetto, Barbera and Nebbiolo are  
179 compared. This explains why a univariate analysis would not be effective. It was decided a multivariate  
180 approach would be employed to ensure a more complete interpretation of characteristic patterns in the spectra.

#### 181 **Fig. 1**

182 From an oenological point of view, the specific features of wine are the result of synergic effects involving  
183 several factors. The wine composition is very complex and the final organoleptic features are produced by the  
184 interaction of many chemicals, such as sugar, alcohol, acids and tannins; e.g. *total acidity* refers to the sour  
185 attributes of the wine, which are evaluated in relation to how well the acidity balances out sweetness. During the  
186 course of winemaking and in the finished wines, tartaric, malic, citric, acetic and lactic acids can have significant  
187 roles and together define the characteristic acidity of the wine (Bellman et al., 1979). In the same way, from a  
188 spectroscopic point of view, the final wine spectrum is the result of a synergic interaction of many factors and  
189 none can be regarded in isolation. The literature is poor in respect of interpretative analysis of Raman spectra  
190 from wine because of the complexity, and only chemometric analysis permits extraction of the more interesting  
191 information and selective parameters to distinguish and attest to the authenticity of wine products.

192 The chemometric approach used for the classification was a supervised classification method, which groups a set  
193 of objects in such a way that objects in the same group (called a class) are more similar to each other than those  
194 belonging to other classes. Training data were given as sets of spectra partitioned as suggested by the supervising  
195 method (Finley and Joachims, 2005). Different distance functions were used to evaluate distances between  
196 objects in the same class or the assignment of an unknown object to the correct class. In this case, Md was used,  
197 as described in detail in Materials and Methods. Applying this concept to the spectral data of wine, several  
198 classification models with good classification capability were obtained.

### 199 *3.1 Discrimination in accordance with grape*

200  
201 Three classes of grapes (Nebbiolo, Barbera, Dolcetto) were selected. 185 Nebbiolo, 75 Barbera and 45 Dolcetto  
202 wine samples were subjected to Raman analysis to create a substantial training set. The *eigen\_analysis* attested  
203 that the selected 305 calibration standards contain sufficient variability for the method calibration. The spectral  
204 range was optimized as reported in Materials and Methods section. The optimized chemometric model shows a  
205 total variability of 99.34 % explained using 20 principal components (PCs); the number of principal components  
206 was optimized by considering the classification capability (%) (number of correctly classified samples during  
207 cross-validation) as a function of the PCs number. In particular, leave-one-out cross validation was performed  
208 reiteratively raising the number of PCs considered during each run, and the percentage of correctly classified  
209 samples was plotted as a function of PCs number (Figure 1S) as well as the variance explained corresponding to  
210 each PC. The plot reported in Figure 1S was used to determine the ideal number of PCs, which corresponded to  
211 20. In order to avoid the over-fitting of data, components that did not contribute significantly to cumulative  
212 variance, and did not provide useful information for classification, were excluded because they dealt exclusively  
213 with experimental noise.

214 As Figure 2 shows, the best optimized method misclassified 13.1 % of 305 standards during leave-one-out cross  
215 validation. The clouds of points representing these three classes were dense, suggesting high homogeneity within  
216 each class. The three clouds were also very close to each other and overlapped partially, which was the cause of  
217 a misclassification percentage greater than 10 %. However, it should be taken into account that the discipline of  
218 some wine production allows a small percentage of other wines to be introduced (e.g. Barbera wine can contain  
219 up to 15 % of Nebbiolo grape); this might explain the closeness of sample classes, which also caused

220 misclassification. A cross validation test was performed (and repeated five times) to attest the real ability of the  
221 calibrated model to distinguish wines according to grape. 100 spectra (one third of the number of calibration  
222 standards per each class chosen randomly) were used in groups of five for cross validation of the model. During  
223 this leave-five-out validation,  $86 \pm 2$  % of unknown samples provided a correct answer. Among the misclassified  
224 samples, 9 % belong to Barbera, 2 % belong to Dolcetto and 3 % belong to Nebbiolo, on average. It should also  
225 be noted that the percentage of misclassified samples during leave-five-out cross validation method was  
226 comparable with leave-one-out cross validation results (14 % of misclassified with 20 PCs) achieved during  
227 model optimization. Subsequently, 10 unknown Nebbiolo samples were used as a small test set that provided 90  
228 % correct answers.

## 229 **Fig. 2**

230 The loadings profiles corresponding to PCs 1 to 10, which were the most interesting for a qualitative description,  
231 are shown in Figure 3. From careful analysis of them, it is possible to determine which organoleptic and  
232 compositional features were responsible for classification. However, it must be taken into account that a synergic  
233 interaction of variables led to the class separation and none can be considered separately. For example, the  
234 alcohol content of a wine is a key parameter for its oenological characterization and plays an important role in  
235 the spectroscopic analysis in order to depict a faithful portrait of each sample. The ethanol Raman peaks are the  
236 easiest to be identified in Raman spectra and these can be identified in most calculated PCs as well. This aspect  
237 plays a crucial role in wine classification.

238 Sugar content is another important feature that can help in classification. Since the sugar content of a wine  
239 depends on the advancement of the alcoholic fermentation, a well-founded hypothesis is the negative correlation  
240 between sugar and the alcohol contents. PC8 and PC9 revealed a significant variability in data observed around  
241  $3500\text{-}500\text{ cm}^{-1}$ , where carbohydrates peaks are typically found. The scores plot, built in accordance with these  
242 PCs, revealed the carbohydrate content varied from sample to sample without any correlation with the Dolcetto,  
243 Barbera or Nebbiolo classes. The difficulty of defining coherent variability in this case lies in the fact that all the  
244 samples considered were dry wines.

245 Another important parameter in the Raman characterization of a biological matrix is the effect of fluorescence.  
246 The colored substances in wine, such as anthocyanins and polyphenols in general, are directly related to the  
247 fluorescence effect observed during spectra acquisition. Fluorescence is, generally, an undesirable effect in  
248 Raman analysis because of the risk of disguising interesting signals in the spectrum. It can also influence the

249 statistical analysis of wine spectra during classification. Indeed, the baseline slope of PC1, and the wide band  
250 around 2000 and 1200  $\text{cm}^{-1}$  in PC6 and PC7, attest to the fact that fluorescence represents a significant variable  
251 in the system examined. This behavior is even more evident when looking at the disposition of data clouds as a  
252 function of PCs influenced by fluorescence, where it can be seen clearly that fluorescence is a significant  
253 variable. However, the classification of wines is not impaired by fluorescence, the success of which is not only in  
254 satisfactory modeling of training sets but also by external validation sets.

### 255 **Fig. 3**

256 Our data revealed that synergic interactions among variables represented the key to solving an apparently  
257 complicated problem. It was not possible to describe the data if the variables were considered independently but  
258 taken together good separation was achieved.

259 Also, dual class models were optimized and, as it turned out, the most difficult wines to separate were Barbera  
260 and Dolcetto whereas Nebbiolo sets formed a specific well separated class.

### 261 *3.2 Discrimination in accordance with production area*

262 We also demonstrated the capability of Raman spectroscopy to separate wines according to grape, and developed  
263 a method to attest to geographical provenance within the same grape class. In order to understand the importance  
264 of geographical area of produced, it is good to know that if a technical expression describing particular  
265 combination of elements, such as climate, soil and regional knowhow of winemaker, which defines the  
266 uniqueness and unrepeatability that characterize a labeled wine (*Terroir*) exists. The study focused on two wines  
267 in particular, Dolcetto and Nebbiolo. Within the Dolcetto class (i) Dolcetto d'Alba Doc and (ii) Dolcetto di  
268 Dogliani Docg were chosen. The production area of Dolcetto di Dogliani is situated in the southernmost part of  
269 Piedmont whereas the Dolcetto d'Alba region is situated in a northern part of the Langhe territory as shown in  
270 Figure 4 a. Dolcetto is highest in the Langhe territory (from 250 to 700 m above sea level) and characterized by a  
271 fresh climate because of its proximity to the Appenino Ligure and Alpi Marittime mountains ranges. This  
272 represents the best climate condition for Dolcetto wine production because it slows the grape maturation process.

273  
274 In this geographic area, the soil varies from generous red soil to sandy and dry soil ([www.regione.piemonte.it](http://www.regione.piemonte.it));  
275 the best soil type for the Dolcetto production is white, deep, clayey and calcareous. Dolcetto di Dogliani and

276 Dolcetto d'Alba wines are produced according to a strict discipline that declares, in a very precise way, the  
277 mandatory geographical area and the variety of grape permitted. Also, the winemaking procedure and the final  
278 organoleptic features are usually controlled through a qualified panel test. Dolcetto d'Alba and Dolcetto di  
279 Dogliani wines have strong sensory features and even an expert sommelier might find it difficult to distinguish  
280 the geographic origins of the two by taste. The Raman analysis coupled with chemometrics provided a good  
281 identification method for classification of the wines according to the area of production, as shown in the  
282 Cooman's plot in Figure 4 a.

283 For Nebbiolo wine, two classes were also set: (i) Langhe (including Nebbiolo d'Alba, Barolo, Barbaresco); (ii)  
284 Novara&Carema (including Colline Novaresi, Coste della Sesia, Ghemme, Gattinara and Carema). The  
285 geographical areas involved are shown in the Piedmont map in the inset of Fig 4 b. Nebbiolo wine is an ancient  
286 red mono-vine wine. Its history in Piedmont region predates the 17<sup>th</sup> century, and it has thrived because of  
287 adaptation to cold climates ([www.langhevini.it](http://www.langhevini.it)). The geographic area designated for production of Nebbiolo is  
288 also clearly specified. The soil should be clayey, calcareous and acidic or a combination of the three; the territory  
289 must be hilly (at least 650 m above sea level) and sunny ([www.regione.piemonte.it](http://www.regione.piemonte.it)). The chemometric analysis  
290 of Nebbiolo spectra enables classification of Nebbiolo from Langhe and from Novara & Carema, as shown in the  
291 Cooman's plot in Figure 4b.

292 As stated previously, the whole spectra for the different wines are responsible for class separation. The number  
293 of PCs considered (6 for Dolcetto classification and 14 for Nebbiolo classification) represented the best  
294 compromise between explained variance and classification capability, as discussed in Section 3.1 (Figure 1S b  
295 and Figure 1S c, available in supplementary information). Again, the only way to achieve the desired results was  
296 to use multivariate approach. Appreciable classification capability (> 90 %) was obtained for the two  
297 classification models, and the low number of misclassified standards suggests Raman spectroscopy is able to  
298 discriminate wine provenance when a consistent calibration is performed.

299 **Fig. 4**

300 The cross validation test provided satisfactory results for both models. Ten samples were chosen randomly (ca.  
301 30 % of the calibration samples from each class) and used in pairs to validate the Dolcetto model with an error of  
302 8 %; all of the misclassified samples belonged to "Dolcetto d'Alba". The leave-five-out cross-validation for  
303 Nebbiolo was performed using 65 spectra, five-by-five chosen randomly with respect to the total in each class. In

304 this case, 7 % were misclassified. In particular, one of them was from Alba, while five were from the northern  
305 part of Piedmont (Novara&Carema class). The validation procedure was repeated five times for both DA  
306 methods attesting a standard deviation of classification capability of 1 % and 2 % respectively.

307

### 308 *3.3 Discrimination in accordance with age*

309 As a third step, the potential to ‘recognize’ aged from non-aged oenological products was investigated. Many  
310 wines improve in quality during barrel and bottle storage. Left too long, however, such wines begin to  
311 deteriorate. During the ageing period, acidity decreases, and further clarification and stabilization occur as well  
312 as the precipitation of undesirable substances, and complex compounds affecting flavor and aroma are formed.  
313 Wines are usually aged in wooden barrels made of oak, allowing oxygen to enter but preventing water and  
314 alcohol from escaping. Simple phenols are transformed during ageing into complex molecules formed by the  
315 condensation of proanthocyanidins and anthocyanins, which also explains the change of color of aged wines. As  
316 the wine ages, anthocyanins react with other acids and compounds, such as tannins, pyruvic  
317 acid and acetaldehyde, which change the color of the wine to "brick red" hues.

318 One of the most interesting comparisons that can be performed on Piedmont’s wines concerns Barolo and  
319 Barbaresco wine. They are both produced with the Nebbiolo grape and follow a mono-grape strict production  
320 protocol. What makes a Barolo wine different from a Barbaresco wine is essentially the ageing time: Barbaresco  
321 is aged for at least 26 months whereas Barolo is aged for at least 38 months. In this study, 56 samples of Barolo  
322 and 24 samples of Barbaresco were analyzed using Raman spectroscopy and the data collected were processed  
323 by discriminant analysis, as previously described. The statistical separation of the two wines produced positive  
324 results when 9 PCs were considered, as shown in Figure 5.

### 325 **Fig. 5**

326 A cross validation of the calibrated model was performed. Spectra from unknown samples (30) were subjected to  
327 analysis in groups of five. The validation procedure was repeated five times and provided  $84 \pm 4$  % correct  
328 answers, on average. Among the 16 % wrongly classified, 80 % were Barolo and 20 % were Barbaresco.

## 329 **4. Conclusions**

330 In this paper, it was shown that Raman spectroscopy coupled with chemometric analysis can play a role in the  
331 authentication of wine, providing positive results in the recognition of mono-vine wines in terms of grape

332 (validation test provided reliability of 93%), geographical provenance (reliability higher than 90%) and ageing  
333 time (reliability higher than 80%). One of the biggest advantages of the proposed method is the direct analysis of  
334 wine, through the glass container, without any pretreatment and purification process. These advantages, together  
335 with the speed of data collection, make Raman Spectroscopy particularly interesting for the prevention fraud and  
336 control of quality labels. The common drawbacks of Raman spectroscopy in analysis of food matrices, such as  
337 problems with interpretation, were overcome with user-friendly software that allow sophisticated chemometric  
338 methods to be elaborated using large amounts of data. The chemometric identification of variability between the  
339 different classes meant wines could be differentiated in accordance with grape, geographical origin, and ageing  
340 time using Raman spectrometry. A dedicated test set consisting of external samples was subjected to analysis in  
341 order to demonstrate the classification capability of the proposed method; this proof of principle aimed to show  
342 that a multivariate calibration procedure could provide consistent classification results when a substantial  
343 calibration set was subjected to spectroscopic analysis, even in a complex matrix. The more specific and user-  
344 friendly Raman analysis is, the more likely it is to be exploited by wine producers for certification. The  
345 application of Raman spectroscopy to distinguish a single producer will be the next challenge, with a higher  
346 impact in commercial field.

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350 |



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471 **FIGURE CAPTIONS**

472 **Figure 1**— Dolcetto d’Alba PDO (100% Dolcetto grape) (green spectrum), Barbera d’Alba PDO (minimum 85% Barbera  
473 grape) (red spectrum) and Barolo PDO (100% Nebbiolo grape) (black spectrum).

474 **Figure 2**– Cooman’s plot for Nebbiolo, Barbera, Dolcetto classification model calculated using Discriminant  
475 Analysis.

476 **Figure 3**– Loadings profiles of the first 10 PCs of the Nebbiolo, Barbera, Dolcetto classification model  
477 calculated through discriminant analysis.

478 **Figure 4**– a) Geographical representation of Dolcetto d’Alba and Dolcetto di Dogliani wine production areas.  
479 Cooman’s plot and statistical data of DA calibration. b) Geographical representation of Nebbiolo d’Alba and  
480 Nebbiolo di Novara & Carema wine production areas. Cooman’s plot and statistical data of DA calibration.

481 **Figure 5**– Cooman’s plot of Barolo and Barbaresco classification model and statistical results of calibration.

482

483

484

Table 1

<b>Grape</b>	<b>Denomination</b>	<b>Ampelographic origin</b>	<b>Production Area</b>	<b>Number of samples</b>	
Nebbiolo	Barbaresco	100% Nebbiolo	Langhe	24	
Nebbiolo	Barolo	100% Nebbiolo		56	
Nebbiolo	Nebbiolo d'Alba	100% Nebbiolo		27	
Nebbiolo	Nebbiolo Langhe	100% Nebbiolo			
Nebbiolo	Colline Novaresi Nebbiolo	100% Nebbiolo		33	
Nebbiolo	Coste della Sesia Nebbiolo	100% Nebbiolo		2	
Nebbiolo	Ghemme	100% Nebbiolo		North Piedmont (Novara)	10
Nebbiolo	Gattinara	100% Nebbiolo			12
Nebbiolo	Carema	100% Nebbiolo			25
Nebbiolo	Lessona	100% Nebbiolo			3
Nebbiolo	Canavese	100% Nebbiolo	Canavese	3	
Barbera	Barbera d'Alba	85-100% Barbera 0-15% Nebbiolo	Langhe	50	
Barbera	Barbera d'Alba Superiore	85-100% Barbera 0-15% Nebbiolo	Langhe	14	
Barbera	Various (Asti, Pinerolo, Novara)	85-100% Barbera 0-15% Nebbiolo	North Piedmont	11	
Dolcetto	Dolcetto d'Alba	100% Dolcetto	Langhe	16	
Dolcetto	Dolcetto di Dogliani	100% Dolcetto	Dogliani	11	
Dolcetto	Dolcetto di Diano d'Alba	100% Dolcetto	Langhe	18	

Figure 1  
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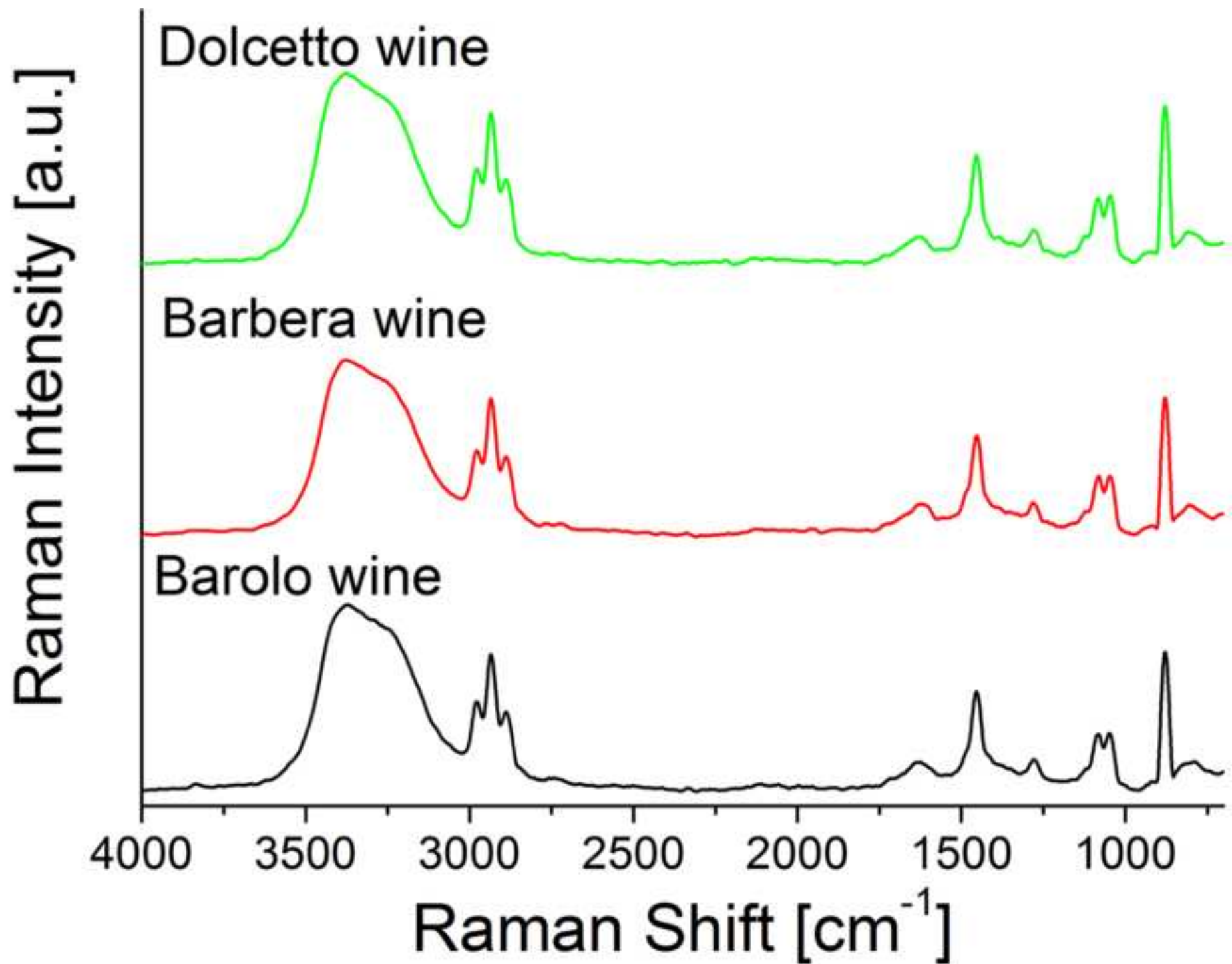
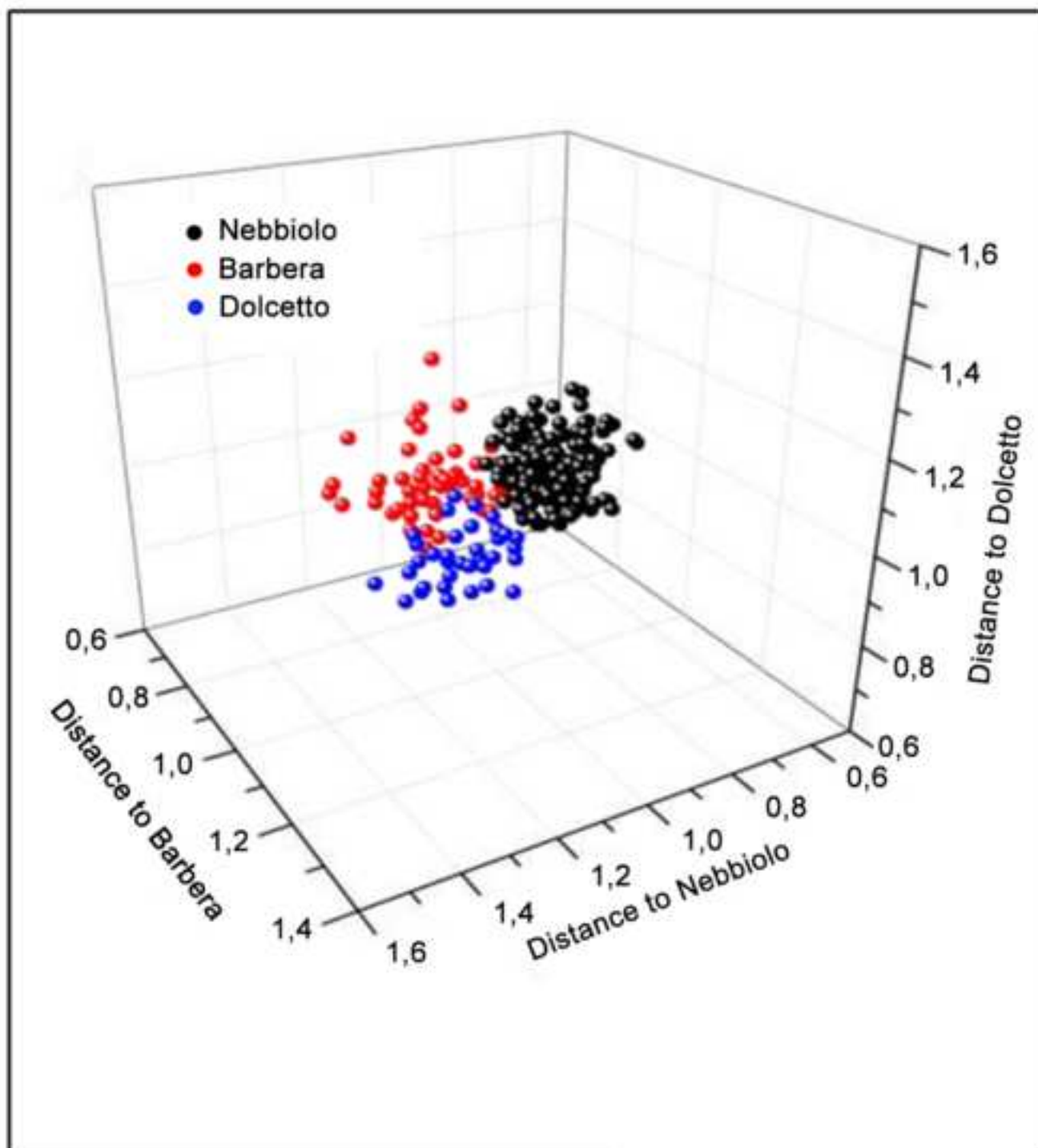




Figure 2

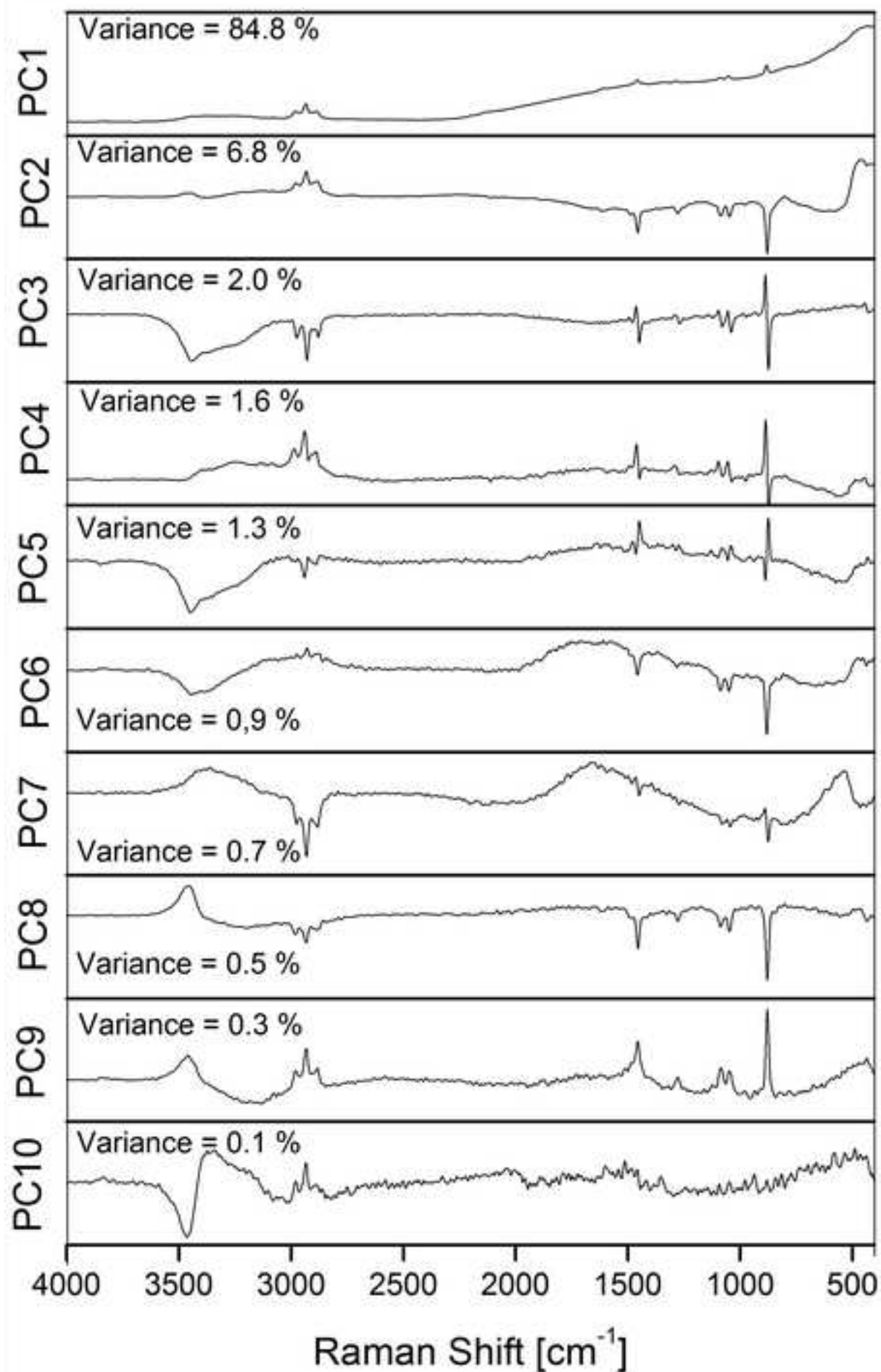
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<b>N° of samples per class</b>	Nebbiolo: 185 Barbera: 75 Dolcetto: 45
<b>N° PCs</b>	20
<b>Cumulative expl. Var.</b>	99.3 %
<b>Predictive capability</b>	86 ± 2 %

Figure 3

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**Figure 4**  
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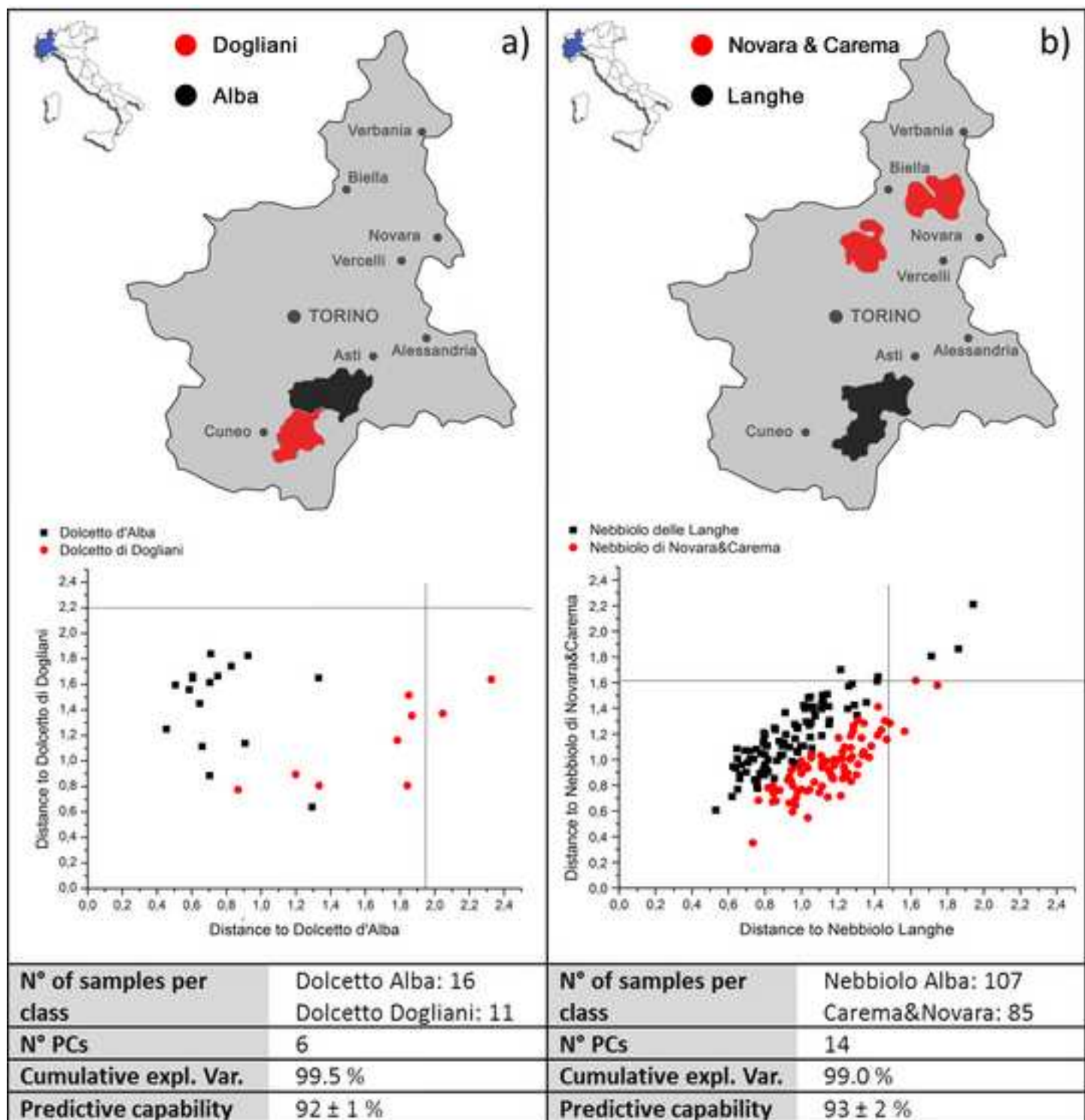
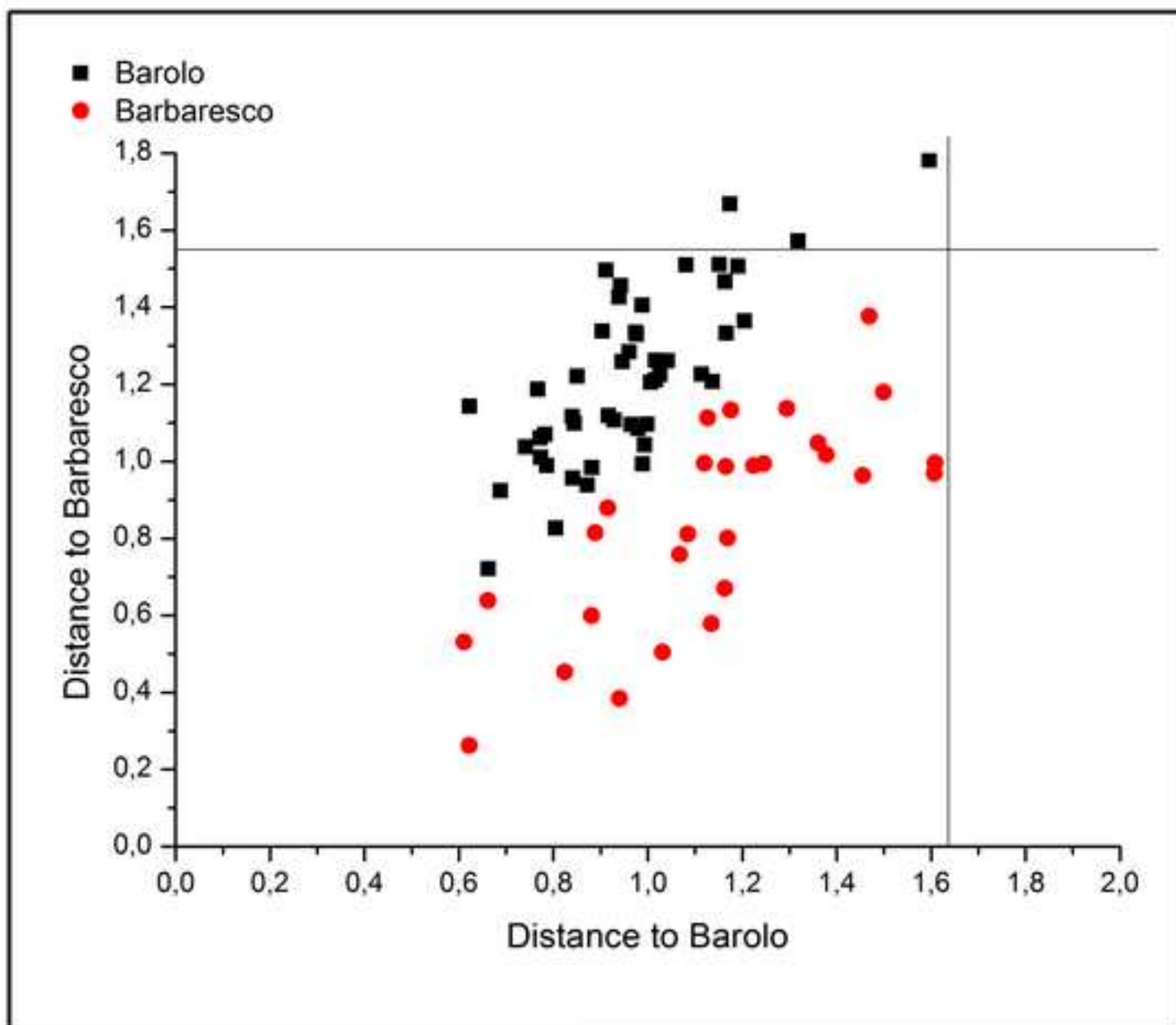


Figure 5  
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<b>N° of samples per class</b>	Barolo: 56 Barbaresco: 24
<b>N° PCs</b>	9
<b>Cumulative expl. Var.</b>	97.7 %
<b>Predictive capability</b>	84 ± 4 %