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## Chemometric Classification of Apulian and Slovenian Wines Using $^1\text{H}$ NMR and ICP-OES Together with HPICE Data

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High-performance ion chromatography exclusion, inductively coupled plasma emission spectroscopy, and nuclear magnetic resonance (NMR) measurements were carried out in combination with chemometrics on 33 wine samples coming from three Slovenian wine-growing regions and from Apulia (southern Italy). The chemometric classification of wines according to their geographical origin was obtained with a nearly 100% degree of achievement. The discriminating potential of the  $^1\text{H}$  NMR and of the other analytical determinations has been estimated separately. The best prediction of wines has been obtained with NMR data.

**KEYWORDS:** Wines; NMR; ICP-OES; HPICE; chemometrics; geographical origin

### INTRODUCTION

Wine is an important agricultural product which is, in many cases, a target of economic fraud, by, for example, the addition of exogenous sugars before or during the fermentation to increase the natural ethanol content in wine, the misleading of cheap wine, or the mixing of excellent and expensive wines with low-quality wines, often originating from other geographical regions or even countries. Therefore, the determination of authenticity is a problem of increasing importance in the wine industry.

Wine is a complex mixture consisting of several hundreds of compounds present at different concentrations. To carry out authenticity studies including determination of the chaptalization, geographical origin, and year of production, many compounds belonging to different chemical classes were tested using several different analytical techniques. For example, inorganic compounds were determined by flame emission spectrophotometry and atomic absorption spectrophotometry, and the determination of volatile compounds was done by gas chromatography–mass spectrometry (1). The analysis of trace elements was performed by inductively coupled plasma optical emission spectroscopy (2) and by electrothermal atomic absorption spectrometry (3). HPLC was used for the determination of amino acid composition, organic acids, sugars, and polyphenols (4–6). The use of a combination of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy and pattern recognition techniques was also reported (7). Good results concerning the characterization of wine authenticity and the

classification of wines according to the geographical origin were also obtained by means of stable isotope ratios determined by site-specific natural isotope fractionation NMR (SNIF-NMR) and isotopic ratio mass spectrometry (IRMS) (4, 9, 10, 11).

The application of different analytical methods for the determination of many components in wine results in large amounts of data. The use and the evaluation of so many parameters are common problems in food chemistry. To deal with this problem, chemometrics have been applied to different chemical or physical variables in the past decade.

Principal component analysis (PCA), soft independent modeling of class analogy (SIMCA), and Kohonen artificial neural networks (KANN) were used on typical variables determined in wines by classical wine analysis. KANN was employed for the classification of wines or wine vinegars using the concentration of trace elements (1, 2, 11, 12). PCA and discriminant analyses (DA) were applied on proton and  $^{13}\text{C}$  chemical shifts and signal intensities (7). PCA, linear discriminant analysis (LDA), KANN, and cluster analysis were used on SNIF-NMR and IRMS results (8–10).

In the present work, we compare the use of different analytical techniques for the determination of different compounds in wine in combination with chemometric methods for the classification of wines from Slovenia and from Apulia – a region in the south of Italy. The analytical techniques used to obtain the input parameters for chemometric analysis were high-performance ion chromatography exclusion (HPICE) for the determination of some organic acids, inductively coupled plasma emission spectroscopy (ICP-OES) for the determination of trace elements, and  $^1\text{H}$  nuclear magnetic resonance (NMR) for the semiquantitative determination of some amino acids, organic acids, and alcohols. The data obtained by classical analytical methods and

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**Table 1.** Variety and Origin of the Analyzed Italian and Slovenian Samples

number of samples	wine-growing region	vine variety	color
I1	South Apulia	Negroamaro	red
I2	North Apulia	Sangiovese	red
I3	South Apulia	Sangiovese	red
I4	South Apulia	Primitivo	red
I5	South Apulia	Primitivo	red
I6	South Apulia	Primitivo	red
I7	North Apulia	Sangiovese	red
I8	North Apulia	Sangiovese	red
I9	North Apulia	Montepulciano	red
I10	Center Apulia	Uva di Troia	red
I11	Center Apulia	Aglianico	red
I12	Center Apulia	Montepulciano	red
I13	Center Apulia	Lambrusco	red
I14	Center Apulia	Aglianico	red
I15	Center Apulia	Castel del Monte	red
S1	Coastal	Welsch Riesling	white
S2	Drava	Welsch Riesling	white
S3	Sava	Welsch Riesling	white
S4	Coastal	Sauvignon	white
S5	Drava	Sauvignon	white
S6	Sava	Sauvignon	white
S7	Coastal	Chardonnay	white
S8	Drava	Chardonnay	white
S9	Sava	Chardonnay	white
S10	Coastal	Merlot	red
S11	Coastal	Cabernet Sauvignon	red
S12	Coastal	Merlot	red
S13	Coastal	Cabernet Sauvignon	red
S14	Coastal	Refosco	red
S15	Sava	Blauer Koelner	red
S16	Sava	Limberger	red
S17	Sava	Limberger	red
S18	Drava	Riesling	white

those obtained by  $^1\text{H}$  NMR were treated separately by multivariate analysis to compare the discriminating potential of each methodological approach.

The applied chemometric methods were PCA, hierarchical clustering analysis (HCA), and regularized discriminant analysis (RDA).

## MATERIALS AND METHODS

**Samples.** The variety and origin of the 33 analyzed wines are reported in **Table 1**. The Italian samples were supplied from some known and reliable wine maker associations situated in different areas of the Italian Apulia region. The Agricultural Institute of Slovenia collected samples from three Slovenian wine-growing regions: Coastal, Sava, and Drava. For each sample, analytical and NMR determinations were performed. All of the investigated wines were produced in the 1998 vintage year.

**Chemical Analyses.** Determinations of ash content were carried out according to official analytical methods (13).

**Chromatographic Analyses.** High-performance ion chromatography exclusion (HPICE) (5, 14) was employed for the tartaric, citric, and succinic acids determination. This analysis was performed on a Dionex 120 EX equipped with a conductometric detector and interfaced to a computer running PeakNet 5.1 software by Dionex located in the Department of Chemistry, University of Bari (Italy). The external standard method was applied for quantitative determinations. Samples were diluted 30 times and filtered before injection.

The separation was carried out using an ICE-AS6 column and an Anion-ICE MicroMembrane suppressor with an external regenerating. The mobile phase was 0.8 mN heptafluorobutyric acid at a flow rate of 1.00 mL/min; the regenerating phase was 5.00 mN tetrabutylammonium hydroxide, and its flow rate was 3–5 mL/min.

**Emission Spectroscopy Measurements.** These measurements were performed to determine Al, B, Ba, Cu, Fe, Mn, and Zn. The used instrument was an inductively coupled plasma optical emission spec-

trophotometer (ICP-OES) Varian Liberty 110 located in the Department of Materials Science, University of Lecce (Italy). Before analysis, samples were mineralized according to this procedure (15): 0.05 g of ashes, obtained from 50 mL of wine, were dissolved in 5 mL of 70%  $\text{HNO}_3$  and 2 mL of 40%  $\text{H}_2\text{O}_2$  and digested by a microwave system (Milestone 1200 MEGA).

The working conditions for each element are shown in **Table 2**.

**$^1\text{H}$  NMR Measurements.** All spectra were recorded on a Varian INOVA-600 NMR spectrometer located at the NIC (Slovenian National NMR Center, Ljubljana, Slovenia). The spectrometer was equipped with a 5.00 mm Indirect Detection Pulsed Field Gradients probe (ID-PFG), operating at 600.126 MHz for  $^1\text{H}$ .

**Procedure.** For one-dimensional proton spectra, 0.5 mL of wine was mixed with 0.1 mL of  $\text{D}_2\text{O}$  (Aldrich) in a 5 mm NMR tube. The purity of the  $\text{D}_2\text{O}$  used was 99.9 atom % D. 3-(Trimethylsilyl)-1-propane-sulfonic acid, sodium salt (TMSF) (Cambridge Isotope Laboratories), served as an internal chemical shift standard. For all spectra, 64 transients were accumulated with 30 272 data points over an 8000.0 Hz bandwidth in the absolute intensity mode. The used pulse width was 5.6  $\mu\text{s}$  (90°). Measurements and processing of spectra of all of the samples were carried out under the same conditions. The signal-to-noise ratio of the spectra was improved by multiplying each free induction decay with an additional exponential factor corresponding to 1 Hz. The signal of water was suppressed with presaturation, and the temperature during all experiments was set to 298 K. Baseline correction was applied to all spectra. Semiquantitative data were obtained after performing a peak-picking procedure and taking the intensities of the selected signals. The  $^1\text{H}$  NMR spectrum of sample I12 showed bad homogeneity, probably due to the presence of paramagnetic ions. Therefore, it was not considered in this study.

**Statistical Treatment of Data.** Statistical elaboration of the data was done using SCANWIN software (Minitab Inc., USA). Analytical and NMR data were analyzed in two different data sets. The applied chemometric methods were PCA, HCA, and regularized discriminant analysis (RDA). PCA calculates orthogonal linear combinations of a starting set of variables on the basis of a maximum variance criterion. Such linear combinations are called principal components (PC). The coefficients, by which the original variables must be multiplied to obtain the PCs, are referred to as loadings. The numerical value of a given variable loading on a principal component indicates how much the variable has in common with that component. Hence, the loadings can be interpreted as a correlation between the variables and the components (16). HCA classifies objects in clusters on the basis of interobject distances in high dimensional space. The results are shown in a dendrogram, which may be used to detect groups of similar individuals (17).

RDA obtains discriminant functions calculated to maximize distances between predefined groups (16). Its purpose is to calculate class models and boundaries, giving a rule of classification based on a set of known objects (*training set*). This rule can be applied to define the classification of unknown objects (*test set*), but it needs to be validated. In this work, two validation approaches were applied: the cross-validation and the test set method. For the cross-validation test, one individual at a time is removed from the initial population, and RDA is performed on the remaining individuals. This method gives an indication of the influence of each individual. The coordinates of the removed individual are calculated, and the individual's group assignment is verified. For the test set method, samples were randomly split into a training set, formed by 66% of the samples, to develop a discriminant model and a validation set, formed by the remaining 33% of the samples, on which the model could be tested. Such a division allows one to have a sufficient number of samples in the training set as well as a representative number of wines in the test set. To get a good estimate for the stability of the model, this validation test has been repeated three times, each time selecting a different subset for the validation set. Summary statistics of results are shown in **Tables 3** and **4**.

## RESULTS AND DISCUSSION

**Analytical Determinations.** PCA was applied to the data matrix formed by 11 analytical parameters, described in the

**Table 2.** Working Conditions for the Elements Analyzed with ICP-OES

parameters	Al	B	Ba	Cu	Fe	Mn	Zn
length-wave (Nm)	396.604	208.959	455.403	324.754	259.940	257.610	206.200
power (KW)	1.00	1.50	1.00	1.00	1.50	1.50	1.50
plasma (L/min)	15.0	15.0	15.0	15.0	15.0	15.0	15.0
auxiliary gas (L/min)	1.50	1.50	1.50	1.50	1.50	1.50	1.50
pump rate (rpm)	25.0	25.0	25.0	25.0	25.0	25.0	25.0
sprayer (KPa)	150	150	150	150	150	150	150
viewing height (mm)	6	6	6	4	6	6	6
PMT (V)	800	800	650	650	800	800	650

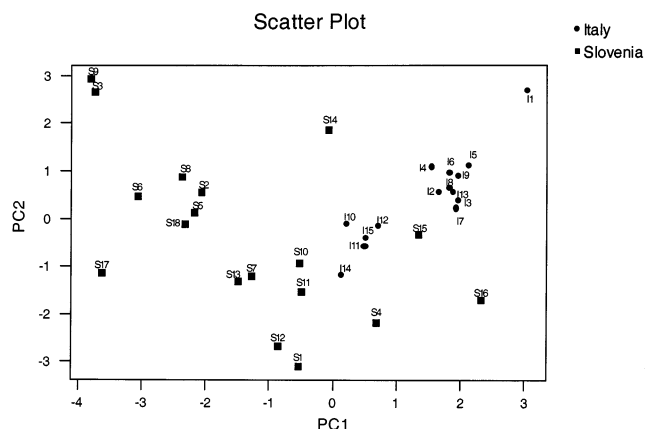
**Table 3.** Mean, Median, and Standard Deviations of the Determined Analytical Parameters for the Two Groups<sup>a</sup>

variable	group	mean	median	std dev
ashes	Italy	2.8	2.9	0.3
	Slovenia	2.1	2.0	0.7
Ba	Italy	0.1	0.08	0.1
	Slovenia	0.2	0.09	0.2
Cu	Italy	0.06	0.05	0.02
	Slovenia	0.2	0.1	0.09
Al	Italy	0.8	0.9	0.4
	Slovenia	2.4	1.8	2.0
Zn	Italy	0.3	0.3	0.2
	Slovenia	0.9	0.8	0.6
B	Italy	2.5	2.4	0.2
	Slovenia	3.8	3.6	1.6
Mn	Italy	0.9	0.6	0.6
	Slovenia	1.2	1.0	0.6
Fe	Italy	3.0	2.5	1.2
	Slovenia	4.9	4.6	3.6
tartaric acid	Italy	2230	2243	407.6
	Slovenia	1392	1200	682.4
citric acid	Italy	293.7	241.9	130.6
	Slovenia	78.2	73.8	54.6
succinic acid	Italy	661.3	655.9	97.4
	Slovenia	771.5	655.2	372.2

<sup>a</sup> Ashes are in g/L, metals are in ppm, and organic acids are in mg/L.**Table 4.** Mean, Median, and Standard Deviations of the Determined NMR Parameters for the Two Groups

signal (ppm)	group	mean	median	std dev
3.63	Italy	111.1	111.1	7.5
	Slovenia	108.3	109.1	10.6
3.26	Italy	2.1	2.3	0.8
	Slovenia	1.9	2.1	0.9
3.13	Italy	0.6	0.6	0.2
	Slovenia	0.2	0.2	0.1
3.05	Italy	0.7	0.6	0.3
	Slovenia	0.4	0.3	0.4
2.66	Italy	34.1	34.1	6.9
	Slovenia	57.4	55.2	25.0
2.34	Italy	2.4	2.0	1.1
	Slovenia	1.2	0.9	0.8
2.00	Italy	4.2	3.5	2.0
	Slovenia	2.3	1.8	1.3
1.49	Italy	1.4	1.5	0.5
	Slovenia	1.7	1.5	1.3
1.13	Italy	19.9	19.5	4.0
	Slovenia	9.2	11.2	2.6
1.05	Italy	0.4	0.3	0.1
	Slovenia	0.5	0.4	0.4

Materials and Methods section, of 32 wines to visualize origin differences. On the score plot of the first two PCs (**Figure 1**), that account for 55% of the total variance, a rough clustering of wines can be observed according to the different geographical origin. Slovenian samples are more spread than Italian wines, and they are separated into wines originating from the Coastal wine-growing region and wines from the Drava and Sava wine-

**Figure 1.** Scatter plot of the scores from the first two principal components PC1 and PC2 obtained using analytical data.**Table 5.** Loadings Values of the Variables Associated with the First Two Principal Components Calculated Using Analytical Data

variable	PC1	PC2
ash	0.071	0.527
Ba	-0.281	0.269
Cu	-0.377	0.155
Al	-0.315	0.225
Zn	-0.404	-0.094
B	-0.364	-0.010
Mn	-0.212	-0.364
Fe	-0.258	0.213
tartaric acid	0.452	0.050
citric acid	0.249	0.358
succinic acid	0.016	-0.508

growing regions. Two samples from the Sava region fall into the group of Italian wines. Italian wines are divided in two groups: wines from north and south of the Apulia cluster and wines coming from the center. However, one sample from the center falls into the first group. The loading values of the variables associated to the first two principal components are reported in **Table 5** and show the contribution of the variables to the more significant principal components. According to these values, Italian wines were described as rich in organic acids, while Slovenian wines have a higher content of heavy metals.

Afterward, a hierarchical clustering analysis applying a Ward linkage procedure on Manhattan distances on the first five PC's scores of the studied samples, considered as variables, was applied. The first five principal components have been considered, because they explain 82% of the variance. In **Figure 2**, a hierarchical clustering dendrogram resulting from this investigation is displayed. It shows that sample S14 falls in the group of Italian wines.

At the same time, RDA was performed on the first five PC's scores of the analyzed samples to classify the wines according to their geographical origin. The model gave 100% of clas-

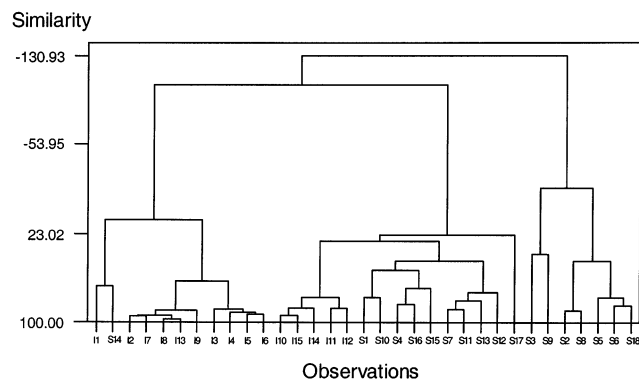


Figure 2. Dendrogram of wines obtained using analytical data.

sification ability, and the cross-validated assignments were 100% correct. The results of the test set method showed that 88% of the samples were assigned correctly. For Italian samples, the percentage of prediction was about 90%, while 87% of the Slovenian samples were correctly assigned.

**NMR Determinations.** The resonances observed in the range of 0.5–5 ppm of the  $^1\text{H}$  NMR spectrum (Figure 3) were submitted to multivariate statistical analysis. The intensities of signals showing neither overlapping nor correlation with other signals have been evaluated. The signals of highly volatile compounds were not considered because it is difficult to control all conditions during the sample preparation and measurements, which could effect their concentration. The signals present in

Table 6.  $^1\text{H}$  Chemical Shift and Assignment of the Signals Chosen as Variables for the Statistical Analysis

peak	$\delta$ (ppm)	group	multiplicity	compound
1	3.63	$-\text{CH}_2$	doublet	glycerol
2	3.26	$-\text{CH}_2$	triplet	arginine
3	3.13	$-\text{CH}_2$	triplet	citrulline
4	3.05	$-\text{CH}_2$	triplet	$\gamma$ -aminobutyric acid
5	2.66	$-\text{H}_2$	singlet	succinic acid
6	2.34	$-\text{CH}_2$	multiplet	proline
7	2.01	$-\text{CH}$	multiplet	isoleucine
8	1.49	$-\text{CH}_3$	doublet	alanine
9	1.13	$-\text{CH}_3$	doublet	butylenglycol
10	1.05	$-\text{CH}_3$	doublet	valine

these regions were submitted to the analysis of variance (ANOVA) that has been used to select the most discriminating signals to be used as variables for the statistical analysis. This means that signals showing more pronounced intensity differences among wines of different origin have been chosen. The chemical shift assignment of the selected resonances is reported in Table 6 (18).

Results of PCA for selected resonances show that Slovenian wines are separated from Italian wines on the first PC (Figure 4). Loadings are reported in Table 7. Variables influencing mostly PC1 are peaks at 2.01, 2.34, and 3.13 ppm, which are respectively due to the amino acids isoleucine, proline, and citrulline. The obtained result was confirmed by a hierarchical clustering analysis performed on the first five PCs applying a

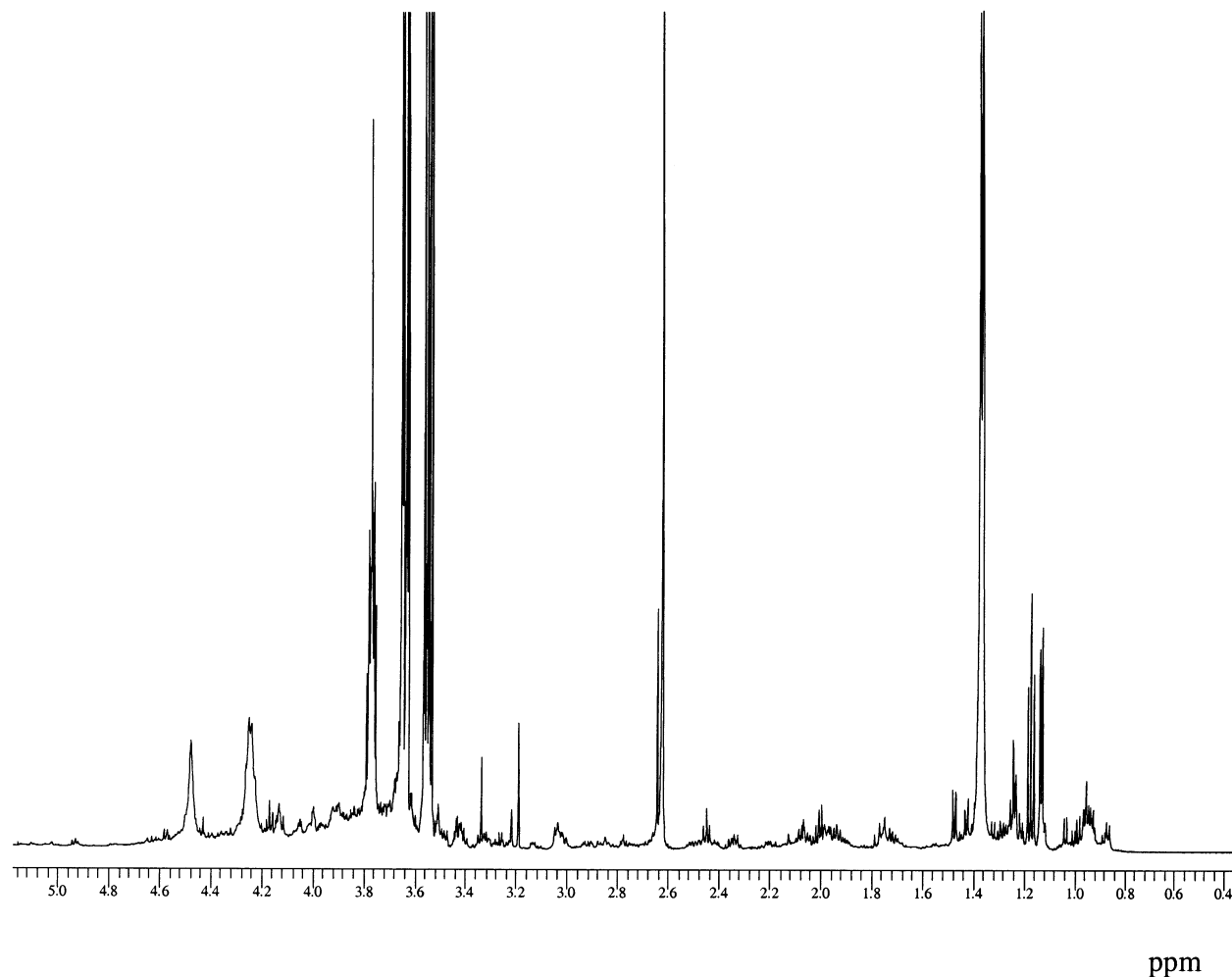
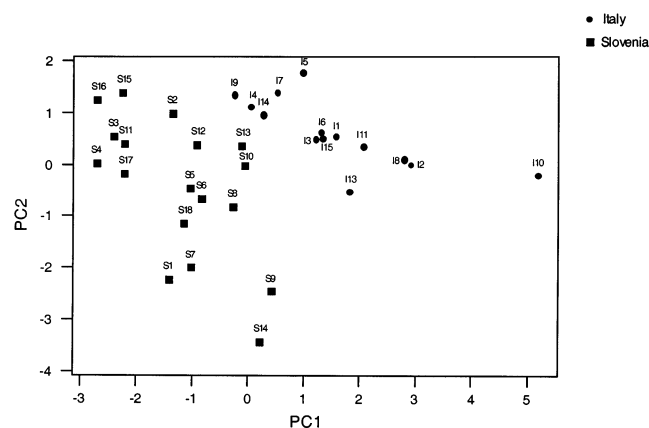


Figure 3.  $^1\text{H}$  NMR spectrum of one of the investigated red wines.

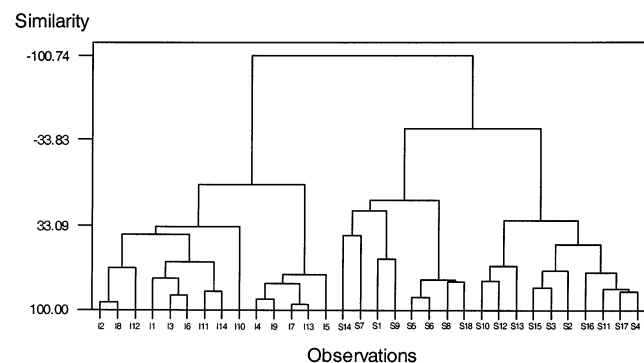




**Figure 4.** Scatter plot of the scores from the first two principal components PC1 and PC2 obtained using NMR data.

**Table 7.** Loadings Values of the Variables Associated to the First Two Principal Components Calculated on  $^1\text{H}$  NMR Data

peak (ppm)	PC1	PC2
3.63	0.169	0.031
3.26	-0.027	0.246
3.13	0.449	0.304
3.05	0.350	-0.263
2.66	-0.236	-0.200
2.34	0.466	-0.018
2.01	0.454	-0.056
1.49	0.156	-0.563
1.13	0.366	0.235
1.05	0.092	-0.601



**Figure 5.** Dendrogram of wines obtained using NMR data.

Ward linkage procedure on Manhattan distances. The dendrogram shown in **Figure 5** exhibits the clustering of the wines grouped on the basis of their geographical origin. After the PCA treatment, RDA was applied on the first five PC's scores to classify wines into separate groups according to Apulian or Slovenian origin. The model gave 100% of classification ability with and without cross validation. The prediction ability of the model, determined with the test set method, was about 97%.

In conclusion, the comparison of efficiency of two different methodologies for the classification of Slovenian and Apulian wines, using HPICE for determination of organic acids and ICP-OES for determination of heavy metals in combination with the chemometric methods PCA, RDA, and HCA, and  $^1\text{H}$  NMR signal intensities with the chemometric methods PCA, RDA, and HCA, has been explored. Very good separation between wines from the two countries was obtained using both methodologies in combination with PCA and HCA. In this work, the heavy metals are the most responsible for the discrimination

of wines when an analytical data set is used, while in the case of using an NMR data set, amino acids are the most responsible.

The models obtained from the application of RDA to the first five PC's scores calculated from the parameters obtained by both methodologies gave 100% correct classification, while the prediction ability of the built model, determined with the test set method, was 88% for the data set obtained with HPICE and ICP-OES results, and 97% for the  $^1\text{H}$  NMR data set. According to these results, better prediction of the origin of Apulian or Slovenian wines has been obtained with NMR data. Besides this, the advantage of proton NMR also is in the rapidity with which information can be obtained and because of the short sample preparation procedure required for analysis. Nevertheless, we have to stress that all techniques employed in our work are important because they give information about different compounds characterizing wines.

Differentiation between wine samples regarding different wine-growing regions inside Apulia and Slovenia is not satisfactory, probably due to the small differences in geoclimatic conditions between these regions.

## ABBREVIATIONS USED

DA, discriminant analysis;  $\text{D}_2\text{O}$ , deuterium oxide; HCA, hierarchical clustering analysis; HPICE, high-performance ion chromatography exclusion; HPLC, high-performance liquid chromatography; ICP-OES, inductively coupled plasma emission spectroscopy; ID-PFG, indirect detection pulsed field gradients probe; IRMS, isotopic ratio mass spectrometry; KANN, Kohonen artificial neural networks; LDA, linear discriminant analysis; NMR, nuclear magnetic resonance; PCA, principal component analysis; RDA, regularized discriminant analysis; SIMCA, soft independent modeling of class analogy; SNIF-NMR, site-specific natural isotope fractionation NMR; TMSP, 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt.

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