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Application of One- and Two-Dimensional NMR Spectroscopy for the Characterization of Protected Designation of Origin Lambrusco Wines of Modena

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ABSTRACT: Lambrusco is a Protected Designation of Origin (PDO) red wine of Modena (Italy) produced according to the production regulation (Decreto Ministeriale (DM) July 27, 2009; GU no. 184-187-188, 13/08/2009). Here the use of ^{1}H NMR spectroscopy as molecular fingerprints of several PDO Lambrusco wines was proposed to serve as indicators of authenticity and quality control. Application of partial least squares discriminant analysis (PLS-DA) revealed a good varietal discrimination by analyzing the low-frequency spectral region. This model explains 68.8% of the variance for the *Y* vector (classification factor: varietal source). In particular, the signals of 2,3-butanediol, lactic, succinic and malic acids, and threonine were found to be the most statistically significant variables in the model. These findings seem to be very promising in the attempt to extend the study to geographical discrimination.

KEYWORDS: *Lambrusco wine, ^{1}H NMR spectroscopy, multivariate statistical methods, PLS-DA, varietal classification*

INTRODUCTION

Lambrusco wines are Protected Designation of Origin PDO red wines produced in the province of Modena (Italy) according to their production regulations (DM July 27, 2009; GU no. 184-187-188, 13/08/2009). They have been classified in four distinctive wines: Lambrusco di Sorbara, Lambrusco Salamino di Santa Croce, Lambrusco Grasparossa di Castelvetro, and Lambrusco di Modena. For each of these wines the production code establishes the ampelographic composition; at least 60% Lambrusco di Sorbara, up to 40% Lambrusco Salamino, up to a maximum 15% of other Lambrusco grapes either of one variety or in combinations for Lambrusco di Sorbara; for Lambrusco Salamino di Santa Croce and Lambrusco Grasparossa di Castelvetro at least 85% of grapes from vines of the same name, respectively, and the remaining 15% of other Lambrusco (Ancellotta, Fortana, and Malbo Gentile) grapes; at least 85% of Lambrusco grapes of different varieties, up to a maximum 15% of grapes from Ancellotta, Malbo Gentile, and Fortana vines for Lambrusco di Modena. All of these wines are characterized by marked organoleptic qualities, full flavors, and intense and fruity bouquets, which contribute to making them pleasing, versatile, and easy to combine with different foods.

The PDO designation of origin constitutes a further confirmation of the strong bond that Lambrusco has with the land around Modena, where natural and human factors have concurred to determine the origin and to consolidate the tradition of this product. A number of various elements demonstrate the importance of Modena as a wine-growing and -producing area: the average annual production of PDO Lambrusco of Modena, which is about 450,000 hL in the past 10 years; over 10,000 ha of vineyards (updated 2010 data); the oldest wine produced in the entire region of Emilia-Romagna; the presence of the oldest wine cooperative still operating in Italy; the 11 wineries currently part of the Consortium for the Historic Mark of Modenese Lambrusco, which account for about 85% of the PDO Lambrusco production.[1,2]

In general, the chemical composition of a wine is closely related to the territorial characteristics and the environmental conditions that occur in vineyards from which the wine is produced.[3] In particular, the relationship between wine quality and its specific site of production is one of the most important elements that characterize a product with designation of origin. The PDO represents a "treasure" that must be preserved from possible sophistications and adulterations. This fraud may compromise the quality and the salubrity of those products having peculiarities, such as varietal and regional connotations, that make them unique in their kind and influence the consumer's choice. All of this justifies the attempt to obtain new analytical approaches to ensure the varietal origin of these products. In recent years, several analytical methods have been used to investigate the bond between the chemical composition of red wines and environmental factors: ultrahigh-pressure liquid chromatography (UHPLC),[4] headspace-solid phase microextraction (HS-SPME) coupled with gas chromatography−mass spectrometry (GC-MS),[5] direct-infusion pneumatic spray (DIPS) electrospray ionization tandem mass spectrometry (ESI-MS/MS),[6] Fourier transform-IR spectroscopy (FT-IR), and two-dimensional IR (2D-IR) correlation spectroscopy.[7]

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Over the past few years, many works based on high resolution-nuclear magnetic resonance spectrometry (HR-NMR) coupled with multivariate data analysis have been conducted concerning the study of the usability of this technique as a fingerprint analysis tool in food chemistry,[8−17] and in particular in enology, either for its short sample preparation and for the wide range chemical compounds detected.[18−25] NMR in particular can rapidly provide spectra with high reproducibility describing the main molecular profile without laborious sample pretreatments. Here the use of a ^{1}H NMR technique as a molecular fingerprint of wines produced from three PDO Lambrusco varietals of Modena (Lambrusco di Sorbara, Lambrusco Salamino di Santa Croce, and Lambrusco Grasparossa di Castelvetro) was proposed to serve as an indicator of authenticity and quality control. This work is part of an extensive Italian cooperative project between grant-making foundations aimed at the development of the agro-food sector by boosting scientific research. The study of new analytical approaches able to characterize and assess the authenticity of Lambrusco wine is certainly an element that strengthens the bond with the territory of origin and promotes the consumer's perception of greater protection and quality of PDO Lambrusco of Modena.

MATERIALS AND METHODS

Materials and Sample Preparation. A total of 110 samples of different PDO Lambrusco wines of Modena, representative of the diversity of each category, were analyzed; 34 dry Lambrusco di Sorbara (SOR), 38 Lambrusco Salamino di Santa Croce (SAL), and 38 Lambrusco Grasparossa di Castelvetro (GRA), made in both sweet and dry styles. These samples were provided by several local wineries joined to the research project AGER. All of the wines were produced in 2009. Each wine was used immediately after bottle uncorking and degassed to avoid the formation of bubbles, which could interfere with the lyophilization process. From a first exploratory ^{1}H NMR analysis, significant variations in the chemical shifts of several metabolites were observed in the wine samples, mainly due to large pH variations between 2.90 and 3.50. The pH values of wine samples were therefore adjusted to 2.00 by the dropwise addition of 1 M HCl to standardize the difference in acidity of the samples and minimize the chemical shift variations of pH-dependent signals.[23] Finally, each sample was diluted 1:10 with water to improve the lyophilization process, reducing the concentration of solid matter, in particular, sugars, and lyophilized overnight to reduce the residual water and ethanol signals. After lyophilization and taking up the residue with 600 μL of deuterium oxide (Sigma-Aldrich, Milan, Italy; 99.9%, D_2O), the equivalent of 2 mL of wine was transferred into the Wilmad NMR tube (5 mm, Ultra-Imperial grade, 7 in. L, 526-PP purchased from Sigma-Aldrich, Milan, Italy), and sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (Sigma-Aldrich) (98%, TSP) was added as reference compound.

NMR Spectroscopy. ^{1}H NMR spectra were acquired with a Bruker FT-NMR Avance 400 spectrometer (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany) operating at 400.13 MHz for ^{1}H. All of the experiments were performed at 300 K and nonspinning. ^{1}H NMR data were acquired using the Bruker spin−echo sequence "cpmgpr.fb" (Carr−Purcell−Meiboom−Gill, Bruker Library) with water presaturation, applied to suppress broad resonance signals.[26] Acquisition parameters were as follows: time domain (number of data points), 32K; dummy scans, 4; acquisition time, 3.4210 s; delay time, 3.0 s; number of scans, 64; spectral width, 5592.841 Hz; fidres, 0.1461. Total acquisition time was 7 min and 47 s. The assignments of the metabolites have been carried out on the basis of the ^{13}C NMR, ^{1}H−^{13}C heteronuclear multiple-bond correlation (HMBC), and ^{1}H−^{13}C heteronuclear single-quantum coherence (HSQC) analyses. The acquisition parameters of the ^{13}C NMR experiments were as follows: number of scans, 8K; dummy scans, 4; time domain (number of data points), 32K; spectral width, 22075.055 Hz; acquisition time, 0.7422 s; delay time, 1.5 s; fidres, 0.6737 Hz. Total acquisition time was 5 h, 14 min, and 59 s. The acquisition parameters of the HMBC experiments were as follows: number of scans, 32; dummy scans, 16; time domain, 3K in the acquisition or direct HMBC dimension F2 (^{1}H) and 100 in indirect HMBC dimension F1 (^{13}C); spectral width, 5592.841 Hz in F2 (^{1}H) and 20124.465 Hz in F1 (^{13}C); digital resolution, 1.8206 Hz in F2 (^{1}H) and 201.245 Hz in F1 (^{13}C); acquisition time, 0.2747 s; delay time, 0.5 s; HMBC delay time, 62.5 ms. Total acquisition time was 82 min and 11 s. The acquisition parameters of the HSQC experiments were as follows: number of scans, 4; dummy scans, 12; time domain, 1K in the acquisition or direct HSQC dimension F2 (^{1}H) and 256 in indirect HSQC dimension F1 (^{13}C); spectral width, 5995.204 in F2 (^{1}H) and 19118.721 F1 (^{13}C); digital resolution, 5.855 Hz in F2 (^{1}H) and 74.682 Hz in F1 (^{13}C); acquisition time, 0.0854 s; delay time, 1.5 s. Total acquisition time was 27 min and 50 s. The chemical shifts were reported as δ_H (ppm) relative to TSP.

Spectral Calculation. Because the peak intensities are directly proportional to compound concentrations, the ^{1}H NMR spectra were used as intensity without performing any type of signal integration, using the absolute intensity value for each spectral points. The application of the ^{1}H NMR technique to wine samples generates very complicated spectra that need to be previously processed and subsequently analyzed by chemometric methods. Before analysis, all ^{1}H NMR spectra were phased and calibrated using the TSP signal by the XWinNMR software package (Bruker Biospin GmbH Rheinstetten). To reduce the inhomogeneous proton NMR chemical shift, primarily concerning pH-dependent signals, all spectra were aligned using the toolbox Icoshift 1.0 for MATLAB (Mathworks Inc., Natick, MA, USA).[27] Finally, the spectra were baseline corrected by PLS_Toolbox version 5.2.2 for use with MATLAB (eigenvector Research Inc., Wenatchee, WA, USA). Each spectrum generated a 16K data file; these files were collected in a data set consisting of 16K spectral variables and 110 samples. No zones of the spectra without signals were considered. The region from 1.175 to 1.228 ppm, related to the residual ethanol CH_3 group, due to incomplete removal during lyophilization, was cut to reduce the effect of ethanol on the multivariate data analysis. The resulting data set refers to the complete spectral region. Three other data sets have been prepared, the first one referred to the extended spectral region between 0.65 and 5.50 ppm, which contains the signals readily distinguishable from background noise; the second one referred to the low-frequency spectral region between 0.65 and 3.15 ppm, which principally contains the signals of acidic and aliphatic compounds; and the third one, which contains the signals of the midfrequency region, between 3.15 and 5.50 ppm, is related to the anomeric region. The spectral region of the aromatic compounds (over 5.50 ppm) has not been taken into account in this work because the corresponding signals are characterized by poor resolution and low absolute intensity; the relative peaks indeed are often not distinguishable from background noise, and it is therefore not possible to remove misalignment of these NMR signals using the toolbox Icoshift.

Spectral Preprocessing and Multivariate Data Analysis. Before the analyses on NMR spectra were performed, all data were normalized, mean-centered, and scaled by the pareto-scaling method, which is used when noise is expected to be proportional to the square root of the standard deviation of the variables. In addition, the pareto-scaling technique reduces the relative importance of large values while keeping the data structure partially intact.[28] To achieve a reliable classification of the different wine samples, unsupervised and supervised pattern recognition procedures were applied to the data sets. Principal component analysis (PCA) was performed to verify the intrinsic variation in the data sets. To maximize the separation between samples, partial least squares discriminant analysis (PLS-DA) models were calculated.[18,24,25] The PLS-DA can be described as the regression extension of PCA, giving the maximum covariance between measured data (NMR spectral intensities distribution) and the response variable (represented in this case by the classification of samples based on the varietal source).[29] Before the classification methods were applied, each data set was randomly divided into a training-test set for modeling by

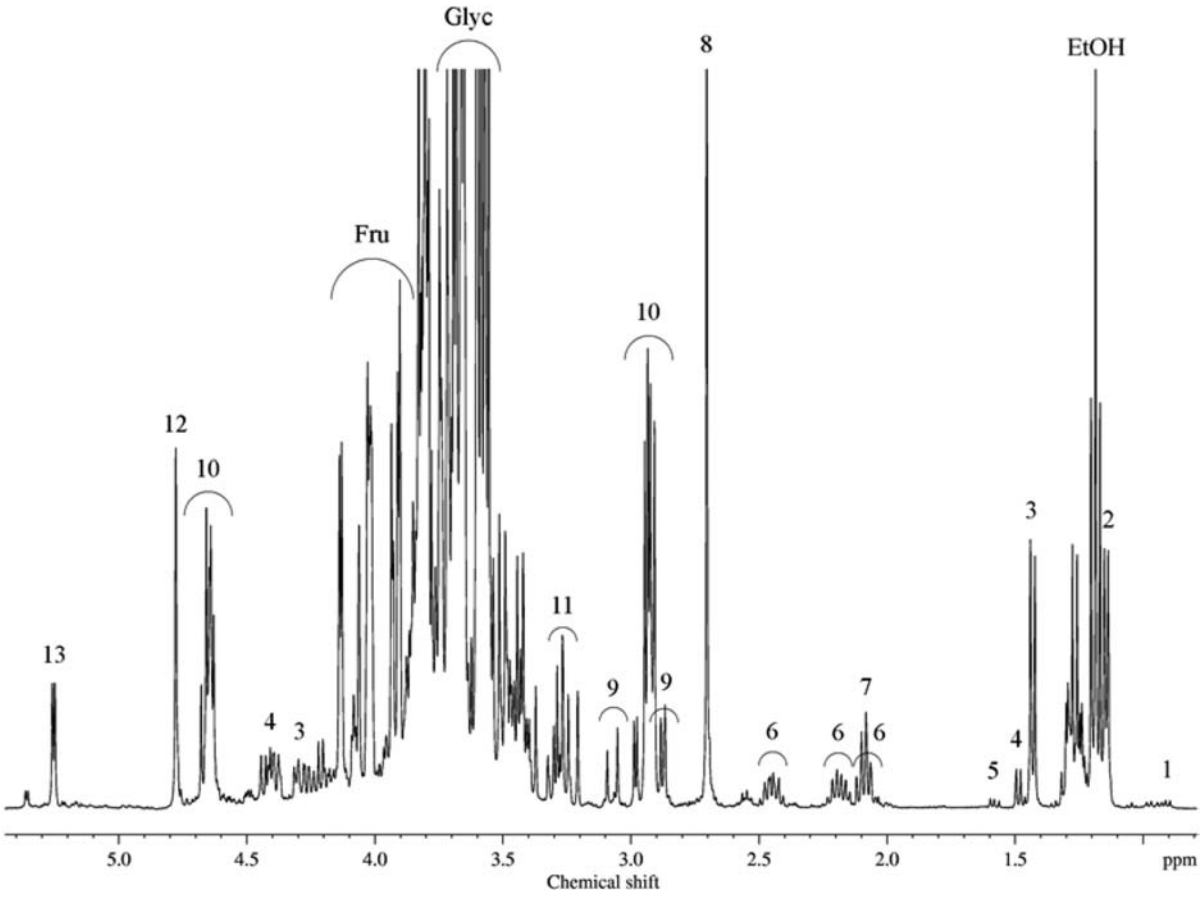

Figure 1. [1]H NMR spectrum of PDO Lambrusco wine of Modena (Lambrusco Salamino di Santa Croce); vertical expansion of the aliphatic and anomeric region with principal metabolites assignments (see Table 1).

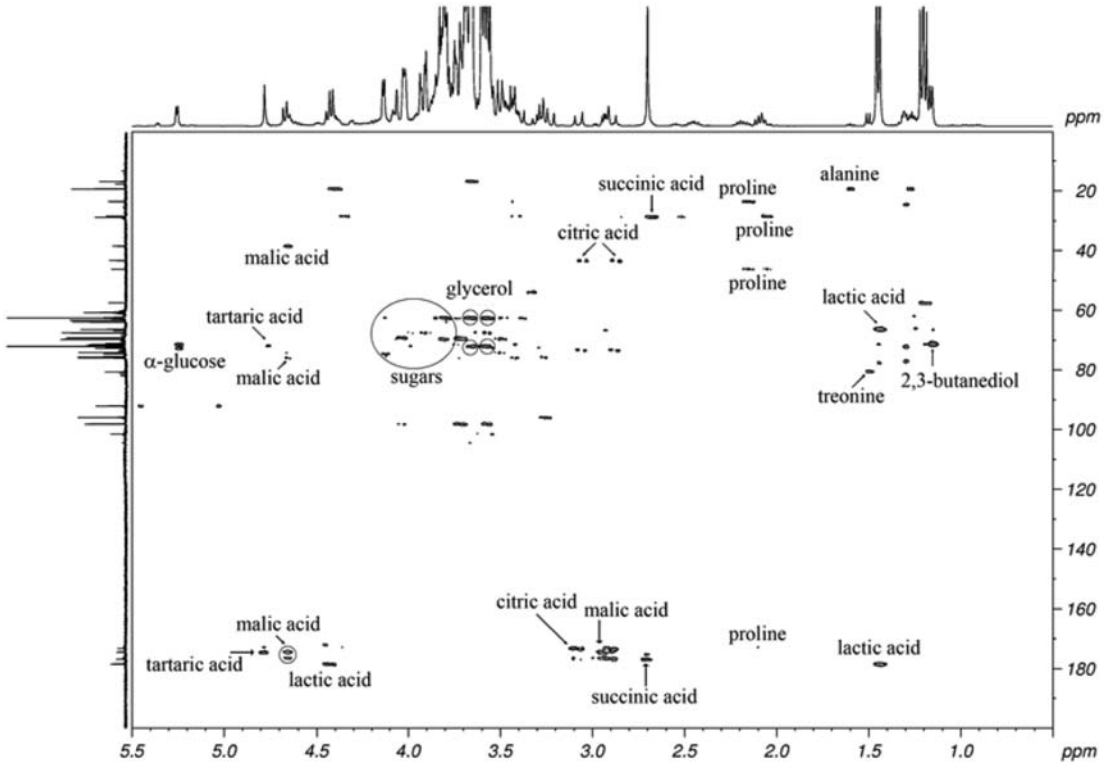

Figure 2. [1]H−[13]C HMBC spectrum of PDO Lambrusco wine of Modena (Lambrusco Salamino di Santa Croce) with principal spin systems assignment reported.

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cross-validation (CV) and an external test set for external validation. The training set was composed by 75 samples as follows: 28 GRA, 25 SAL, 22 SOR. The test set was composed by 35 samples as follows: 10 GRA, 13 SAL, and 12 SOR. For all of the calculated PLS-DA models venetian blind cross-validation was performed. The resulting classification performances were reported as percentage efficiency, which is the geometric mean of sensitivity (number of samples predicted to be in the class divided by number actually in the class, that is, the "in-class" samples accepted by the class model) and specificity (number of samples predicted as not in the class divided by actual number not in the class, that is, the "not-in-class" samples correctly rejected by the class model) values. All multivariate data analyses were performed using the software PLS_Toolbox version 5.2.2 for Matlab.

RESULTS AND DISCUSSION

A representative ^{1}H NMR spectrum acquired from Lambrusco Salamino di Santa Croce is shown in Figure 1. The metabolites were assigned on the basis of additional NMR experiments and literature data.[18−21] In Figure 2 the ^{1}H−^{13}C HMBC spectrum of PDO Lambrusco wine of Modena (Lambrusco Salamino di Santa Croce) with principal spin systems assignment is reported. The principal metabolites identified are reported in Table 1.

The ethanol content is significantly reduced by the lyophilization process. The CH_3 signal of ethanol (1.18 ppm), which is the most abundant compound of wine, has a much lower intensity than other metabolites, glycerol among them. The partial removal of this compound allows us to obtain high-resolution information also from this spectral region, owing to the fact that NMR signals of other compounds, which would otherwise be overlapped with ethanol, emerge from background noise. Tartaric acid is one of the most abundant organic acids of wine; however, this is not evident in the spectrum shown Figure 1, in which succinic (peak 8) and malic (peak 10) acids are more abundant. The chemical shift of tartaric acid (peak 12) is assigned to 4.71 ppm, and it is partially overlapped with the broad water signal; therefore, also the signal of tartaric acid is partially suppressed during presaturation and the corresponding peak intensity is significantly reduced. Another intriguing finding observed in the ^{1}H NMR spectrum is the large signal from malic acid compared to the signal from lactic acid. Lactate derives mainly from malolactic fermentation (MLF) through the decarboxylation of malate; nevertheless, this transformation represents a critical point in old vintage wine production.[30] Lambrusco wines, conversely, are meant to be consumed young and are characterized by a lack of MLF. As previously reported, the aromatic region (>5.50 ppm) was not considered in the present study.

Table 1. Metabolites and ^{1}H Chemical Shifts Identified[a]

peak	compound	group	δ_H[b] (J in Hz)
1	leucine	C_5H_3, C_6H_3	0.86 (d) (6.8)
2	2,3-butanediol	C_1H_3, C_4H_3	1.14 (d) (6.8)
EtOH	ethanol	C_2H_3	1.18 (t)
3	lactic acid	C_3H_3	1.43 (d) (6.8)
		C_2H	4.30 (m)
4	threonine	C_4H_3	1.48 (d) (6.8)
		C_3H	4.41 (m)
5	alanine	C_3H_3	1.57 (d) (7.3)
6	proline	C_4H_2	2.08 (m)
		C_3H	2.18 (m)
		$C_{3'}H$	2.44 (m)
7	acetic acid	C_3H_3	2.13 (s)
8	succinic acid	C_2H_2, C_3H_2	2.70 (s)
9	citric acid	C_2H, C_4H	2.87 (d) (15.7)
		$C_{2'}H$, $C_{4'}H$	3.07 (d) (15.7)
10	malic acid	C_2H	2.89 (dd) (16.5, 9.7)
		$C_{2'}H$	2.96 (dd) (16.5, 9.7)
		C_3H	4.63 (dd) (6.6, 4.9)
11	β-glucose	C_2H	3.26 (t) (9.6)
		βC_1H	4.65 (d) (8.2)
Glyc	glycerol	C_2H_2	3.57 (m)
		C_3H_2	3.67 (m)
		C_1H	3.81 (m)
Fru	fructose	C_3H, C_4H, C_5H[c]	3.90−4.15[c]
12	tartaric acid	C_2H, C_3H	4.71 (s)
13	α-glucose	αC_1H	5.25 (d) (3.9)

[a]Assignments were from HSQC and HMBC experiments. The chemical shifts were determined at pH 2.0 and expressed as relative values to those of TSP at 0 ppm. [b]Peak multiplicities in parentheses: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet. [c]Assigments of ^{1}H NMR signals of α-fructofuranose and β-fructofuranose.

The PCA was performed on the ^{1}H NMR complete spectral region to check possible sample grouping. This model resulted in four PCs explaining 81.6% of the total variance, and it was not possible to cluster the wines according to their varietal origin, demonstrating the considerable complexity of the system. The indicative result obtained by this model suggested to us the use of PLS-DA, which conversely to unsupervised PCA, uses preliminary information relating to the classification of the samples. The most relevant information resulting from PLS-DA is the correlation between the NMR data and the varietal source classification; therefore, PLS-DA was performed by considering samples belonging to three classes. Three models were developed, one for each data set of the spectral region containing well-resolved signals, readily distinguishable from background noise (0.65−5.50 ppm). The best model, selected by its CV efficiency, was obtained by analyzing the low-frequency region (0.65−3.15 ppm) and required five latent variables (LVs) (Table 2). This model explains 77% of X (NMR data) variance, whereas the explained variance for the Y vector (classification factor: varietal source) reached 68.8% of the total variance. These results show that the Lambrusco di Sorbara wines are differentiated from the other types. The variable importance in projection (VIP) scores plot (Figure 3), which estimates the importance of each variable in the projection used in a PLS-DA model, shows that this clustering is mainly due to five descriptors: 2,3-butanediol and lactic and succinic acids, which derive from fermentation processes, and threonine and malic acid, which originate from grapes. In addition to these well-known compounds, also the signal at about 1.3 ppm is responsible for PLS-DA classification. According to the sparse literature data available, this signal may be tentatively assigned to α-hydroxyisobutyrate.[19]

It may seem surprising that Sorbara wines were the most easily discriminated, because there can be up to 40% of Salamino varietal in Sorbara wines; therefore, it might be expected that these two varietal wines would be the most similar. In this regard, because the details of the steps followed during the winemaking process are unknown, we assumed that the Lambrusco di Sorbara wines analyzed contain <40% of Salamino varietal.

Another interesting result emerges by observing the model obtained by analyzing the anomeric region (Table 2). SOR wines are more reproducible compared to GRA and SAL wines;

Table 2. Results of the PLS-DA Models Obtained on ^{1}H NMR Spectra for Each Data Set

	extended spectral region (0.65−5.50 ppm)	acidic/aliphatic region (0.65−3.15 ppm)	anomeric region (3.15−5.50 ppm)
LVs[a]	6	5	5
training set[b]			
sensitivity			
GRA	96.4	80.0	89.3
SAL	92.0	76.9	84.0
SOR	95.5	91.7	95.5
specificity			
GRA	89.4	88.0	83.0
SAL	92.0	81.8	90.0
SOR	98.1	95.7	96.2
efficiency	**93.8**	**85.4**	**89.5**
CV[c]			
sensitivity			
GRA	85.7	75	57.1
SAL	72.0	80.0	60.0
SOR	95.5	90.9	90.9
specificity			
GRA	72.3	87.2	61.7
SAL	86.0	86.0	70.0
SOR	94.3	96.2	90.6
efficiency	**83.8**	**85.6**	**70.4**
test set[d]			
sensitivity			
GRA	90.0	92.9	70.0
SAL	76.9	92.0	69.2
SOR	91.7	95.5	91.7
specificity			
GRA	84.0	93.6	80.0
SAL	81.8	96.0	86.4
SOR	100	98.1	91.3
efficiency	**87.1**	**94.7**	**80.9**

[a]Latent variables. [b]Recognition ability. [c]Prediction ability in cross-validation. [d]Prediction ability in external validation.

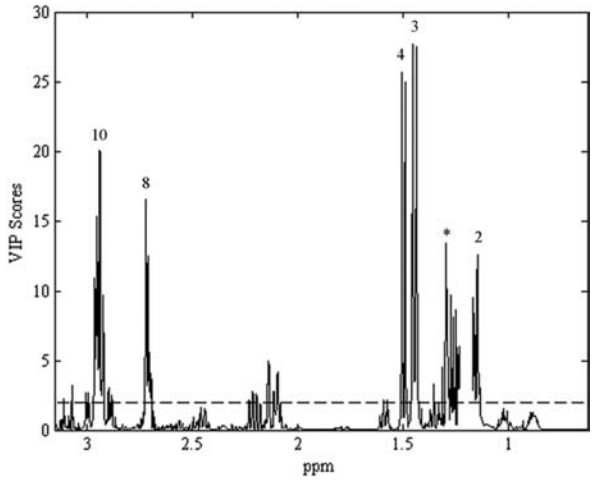

Figure 3. VIP scores for PLS-DA classification model obtained in the low-frequency spectral region (0.65−3.15 ppm). The horizontal dashed line indicates the threshold value. Peak numbers refer to the assignments of Table 1: 2, 2,3-butanediol; *, α-hydroxyisobutyrate (tentative assignment); 3, lactic acid; 4, threonine; 8, succinic acid; 10, malic acid.

the percentage efficiency values, in fact, are the highest both in CV and in test set. A possible reason is that SOR are dry wines, whereas GRA and SAL wines are made in both sweet and dry styles.

In conclusion, we have demonstrated the ability of ^{1}H NMR spectroscopy, coupled with multivariate statistical methods, to develop models that are effective and useful for the classification of PDO Lambrusco wines of Modena according to the varietal source. In fact, by means of PLS-DA of ^{1}H NMR spectra, a good discrimination was found by analyzing the low-frequency spectral region, revealing 2,3-butanediol, lactic and succinic acids, threonine, and malic acid to be important compounds for varietal discrimination.

These findings are highly promising, in particular, in the attempt to extend the study also to geographical discrimination, thus further confirming the relationship between wine quality and the specific area of production, one of the most important elements that characterize a product with a protected designation of origin.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

D.M, Decreto Ministeriale; SOR, Lambrusco di Sorbara; SAL, Lambrusco Salamino di Santa Croce; GRA, Lambrusco Grasparossa di Castelvetro; TSP, sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4; MLF, malolactic fermentation.

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