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Multivariate statistical analysis of two-dimensional NMR data to differentiate grapevine cultivars and clones

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Multivariate statistical methods were applied on two-dimensional NMR data (^{1}H-^{13}C) of polyphenol extracts from grapevine clones harvested in the Bordeaux region. An analysis of variance detected the most discriminating NMR spectral correlation volumes which were then used to perform principal component, hierarchical clustering and discriminant analyses. The results showed that clones are divided into three groups according to the cultivar and they can be differentiated inside each cultivar. This method was applied to polyphenol extracts from grape seeds and leaves. Nowadays, two-dimensional NMR is the only analytical tool which can differentiate grapevine clones. It could thus be used to check the identity of grapevine species, cultivars or clones as well as any other plants which produce polyphenols. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Nowadays, wine-growers are aware that selection of the cultivars which are the most suitable for each region and which have the highest potential quality is necessary in order to improve the final quality of wine. This selection aims to isolate individuals from each cultivar which have stable genetic characteristics and which give the best grapes; they may then be vegetatively multiplied to obtain clones. In fact, the identity of the best clones cannot be directly determined as there was until now no method to differentiate them. Therefore, it would be very useful to find a procedure to identify grapevine cultivars and clones.

For a long time, grapevine cultivars were identified by ampelography (Swanepoel & De Villiers, 1987; Lacroix & Posluszny, 1991), which is based on the morphological observation of the plant, but it is impossible to differentiate clones by this method because most of them have nearly the same morphological characteristics. It is, therefore, necessary to have another analytical tool.

Several wine-growing countries are working on this subject, using in particular genomic DNA fingerprinting of the vine (Bowers *et al.*, 1993; Thomas *et al.*, 1994; Jean-Jaques *et al.*, 1993) or by isozymes analysis (Walters *et al.*, 1989; Boursiquot & Parra, 1992) but none of these methods can, at the moment, differentiate clones.

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The present study describes a procedure to differentiate grapevine cultivars and clones by two-dimensional heteronuclear ^{1}H-^{13}C NMR analysis of their polyphenols, and the multidimensional statistical analysis of the data. The discriminant models produced may then be used to classify individuals of unknown or uncertain origin. The classification ability and robustness of the discriminant model was validated by Jackknifing (Tomassone *et al.*, 1993) and cross-validation (Esbensen *et al.*, 1994).

MATERIALS AND METHODS

Materials

Grapevine seeds and leaves were supplied by the Chambre d'Agriculture of Blanquefort and the Lycée Agricole of Montagne St-Emilion (Gironde, France) which certified the identity of the samples. Clones of three cultivars were collected; a list of these samples is given in Table 1 where the cultivar, the clone number and the geographical origin are specified.

Sample preparation

Grape seeds and leaves were dried at 40 °C and protected from light to prevent oxidation. Raw materials (50 g) were extracted using 500 ml of acetone–water (2:3,

Table 1. Grapevine samples and abbreviations

Cultivars	Clone numbers	Geographical origin
For grape seed extracts		
Cabernet Franc: CF or cf	332 331 330 312 187	Blanquefort: bt
	332 331 312	Montagne: mg
Cabernet Sauvignon: CS or cs	341 339 337 335 191	Blanquefort: bt
Merlot Noir: MN or mn	348 347 346 343 181	Blanquefort: bt
For leaf extracts		
Cabernet Franc: CF or cf	312 187	Blanquefort: bt
Cabernet Sauvignon: CS or cs	337 335	Blanquefort: bt
Merlot Noir: MN or mn	347 343	Blanquefort: bt

v/v) and each extract was concentrated under vacuum to eliminate acetone. The residual water solution was then filtered and directly extracted three times by ethyl acetate (600 ml, total amount) in the case of seeds and after a purification stage by diethyl ether (300 ml), in the case of leaves, to get rid of pigments and waxes. After concentration under vacuum, the ethyl acetate residues are dissolved in the minimum volume of water and freeze-dried in order to facilitate the quantitative analysis.

Three extracts were independently prepared from each grape seed clone and two from each leaf clone.

NMR analyses

Two-dimensional NMR spectra were acquired with a Bruker AMX-500 NMR spectrometer equipped with high current version of gradient-field facility and operating at 500.130 MHz for ^{1}H and 125 MHz for ^{13}C. All the NMR experiments were performed at 303 K on 100 mg of extract dissolved in 0.5 ml of DMSO-d_6 (99.96% D) and transferred to a 5 mm NMR tube. Solvent signals were used to frequency-calibrate the proton spectra at 2.49 ppm (residual ^{1}H) and at 39 ppm for carbon spectra. All two-dimensional NMR data were acquired in a reverse mode.

HMBC-GAS (Hurd & John, 1991)
Spectra were processed in a magnitude mode. For the seed extracts, a spectral width of 9.15 ppm in the ^{1}H dimension and 220 ppm in the ^{13}C dimension were used. For the leaf extracts, the ^{1}H spectral width is 14.5 and 220 ppm in the ^{13}C dimension. Prior to Fourier transformation, FIDs were multiplied by a squared sine-bell function in both dimensions. For each of the 512 t_1-values, 16 transients were accumulated. Total acquisition time was 3 h for grape seed extracts and 2 h for leaf extracts.

HMQC (Bax & Subramanian, 1986) and HMQC-HOHAHA (Lerner & Bax, 1986)
Spectra were obtained in the tppi-phased (Marion & Wüthrich, 1983) mode and 256 t_1-increments were used with a ^{1}H spectral width of 7.5 ppm and a ^{13}C spectral width of 130 ppm with 32 transients for each experiment. Total acquisition time was 4 h. Prior to Fourier transformation, FIDs were multiplied by a squared sine-bell function in both dimensions. Zero-filling of the original 256×2048 data matrix gives a final digital resolution of 32.3 Hz per point for the ^{13}C dimension and 1.85 Hz per point for the ^{1}H dimension.

For each grape seed extract all the above experiments were recorded while, for the leaves three HMBC-GAS were recorded for the first extract to get enough data from each clone and only one HMBC-GAS for the second extract to ensure the reproducibility of the extraction method.

Volume integration of spectral correlations was calculated by a Bruker program (AURELIA[1]). This program adds together the intensity of the points located in a defined sector surrounding a correlation. These quantitative data were used as variables in the statistical analyses.

Multivariate statistical analyses

Analyses were performed using the S-Plus procedure (Sigma-Plus, Toulouse, France).

ANOVA (Schwartz, 1993; Box et al., *1978)*
This analysis was used to select the most discriminating variables by calculating an F factor which is proportional to the ratio of the 'inter-group' variance to the 'intra group' variance. The higher this ratio, the more the groups are significantly different from each other. The variables selected were then used to perform hierarchical clustering (HCA), principal components (PCA) and factorial discriminant (FDA) analyses.

Hierarchical clustering analysis (Bouroche & Saporta, 1989)
This method clusters the individuals into classes by calculating the Euclidean distance between the data which represent the individuals. A complete linkage clustering method was used. The results are represented by dendrograms which may be used to detect groups of similar individuals.

Principal component analysis (Bouroche & Saporta, 1989; Robert, 1989)
This analysis describes the individuals that were initially present in an n-dimensional space of the variables, in a

[1]AURELIA: Computer-aided analysis of two- and three-dimensional NMR data. Bruker Analytische meßtechnik GmbH.

space of many fewer dimensions, often only two or three. These new axes, or 'principal components' are linear combinations of the original variables, calculated in order to maximize the dispersion of the individuals. This exploratory method may also be used to detect groups of individuals.

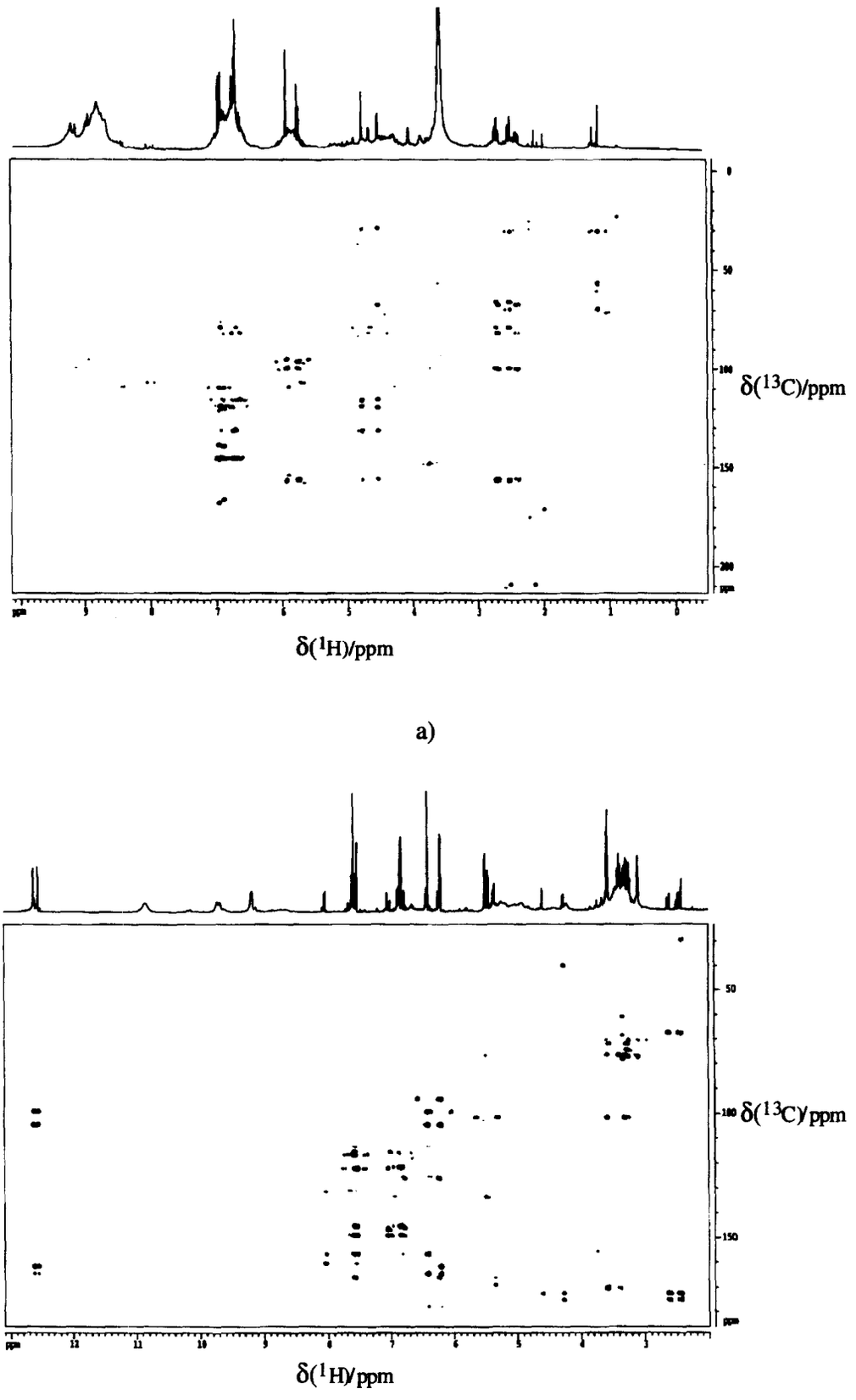


Fig. 1. HMBC-GAS spectra of (a) a grape seed extract and (b) a leaf extract.

Factorial discriminant analysis (Bouroche & Saporta, 1989; Robert, 1989)

The aim of this method is to obtain discriminant axes, or discriminant functions, which, as in PCA, are linear combinations of the original variables but this time calculated to maximize distances between predefined groups. The discriminant functions may be used to class new individuals.

Jackknifing (Tomassone et al., *1993) and cross-validation (Esbensen* et al., *1994)*

To validate the discrimination of the grapevine cultivars and clones, it is necessary to statistically test the robustness and the classification ability of the discriminant functions.

For the Jackknife test, one individual at a time is removed from the initial population, and an FDA is performed on the remaining individuals. The mean and standard deviation of the coefficients of the discriminant functions are calculated. This allows one to calculate more robust discriminant functions, to have an indication of the influence of each individual and to test the stability of the coefficients of the functions. The co-ordinates of the removed individual in the space of the discriminant functions are calculated and the individual's group assignment is verified. The method is validated when the percentage of correctly classified individuals is high and the discriminant functions are stable whatever the removed individual.

For the cross-validation, five individuals at a time are successively removed; otherwise the method is the same as for the Jackknifing.

Those methods were applied to the quantitative data from the three NMR experiments (HMBC-GAS, HMQC, HMQC-HOHAHA) for the grape seed extracts and to the HMBC-GAS data for the leaf extracts.

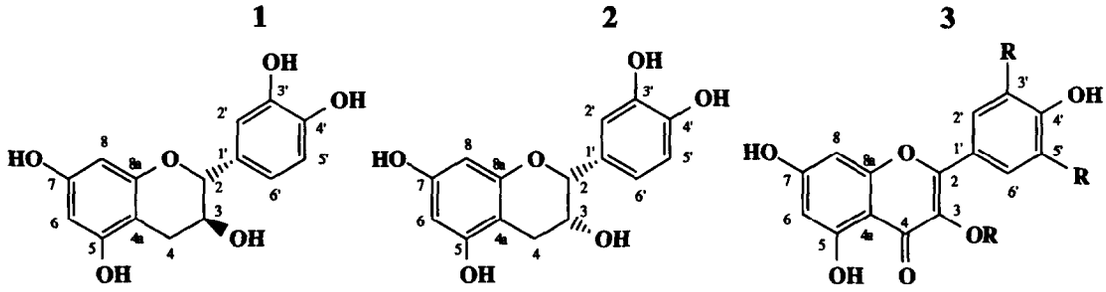
RESULTS AND DISCUSSION

Analysis of NMR maps

Each two-dimensional NMR map has about 50–150 correlations. Figure 1 shows two HMBC-GAS spectra recorded for a grape seed extract and a leaf extract, respectively. Main signals are those of the more concentrated compounds which are the catechin **1**, epicatechin **2** and oligomers of these monomers for grape seed extracts (Balas & Vercauteren, 1994; Balas *et al.*, 1995), and flavonol glycosides **3** for leaf extracts (Diaz Lanza *et al.*, 1989; Egger *et al.*, 1976) (see Scheme 1).

Analysis of variance

As variables which differentiate cultivars were not the same as those that differentiate clones inside each cultivar, two different ANOVA models were used, one to take into account cultivars, the other one for clones nested inside each cultivar.



Scheme 1

Table 2. Variables selected by the ANOVA to differentiate cultivars

Number of peaks of correlations integrated for each analysis		
For grape seed extracts:	n = 48 for the HMBC-GAS n = 75 for the HMQC n = 93 for the HMQC-HOHAHA	
For leaf extracts	n = 120 for the HMBC-GAS	
Results of the ANOVA Analysis	*F* selected	Number of variables
For grape seed extracts:		
HMBC-GAS	$F > 130$	12
HMQC	$F > 50$	10
HMQC-HOHAHA	$F > 100$	13
For leaf extracts:		
HMBC-GAS	$F > 20$	6

Differentiation of cultivars

Analysis of grape seeds extracts

The variables selected by ANOVA are shown in Table 2, this reduction in the number of variables gives a ratio of number of samples to number of variables between 4 and 20.

Figure 2 shows the results of the PCA for data from each NMR experiment (HMBC-GAS, HMQC, HMQC-HOHAHA) for the three cultivars. The variance explained by the first two principal components is 97% for the HMBC-GAS and 98% for the other analyses; therefore samples are shown only in the plane of the first two Principal components and, sometimes, in the space defined by the first three principal components.

For all three experiments, clones are clustered according to the cultivar to which they belong. The PCA performed on the HMQC data gives the best separation of cultivars on the first two principal components. Nevertheless, for the other two NMR experiments, clones which are badly placed on the first principal plane are well located along the third principal component (Fig. 3).

To confirm the differentiation of the clones according to cultivars, a hierarchical clustering analysis was performed. Results for each NMR experiment are shown in Fig. 4.

At level 5×10^6 of the dendrogram, one can see the following.

1. Four groups for the data obtained with the HMBC-GAS: the first is composed of clones from

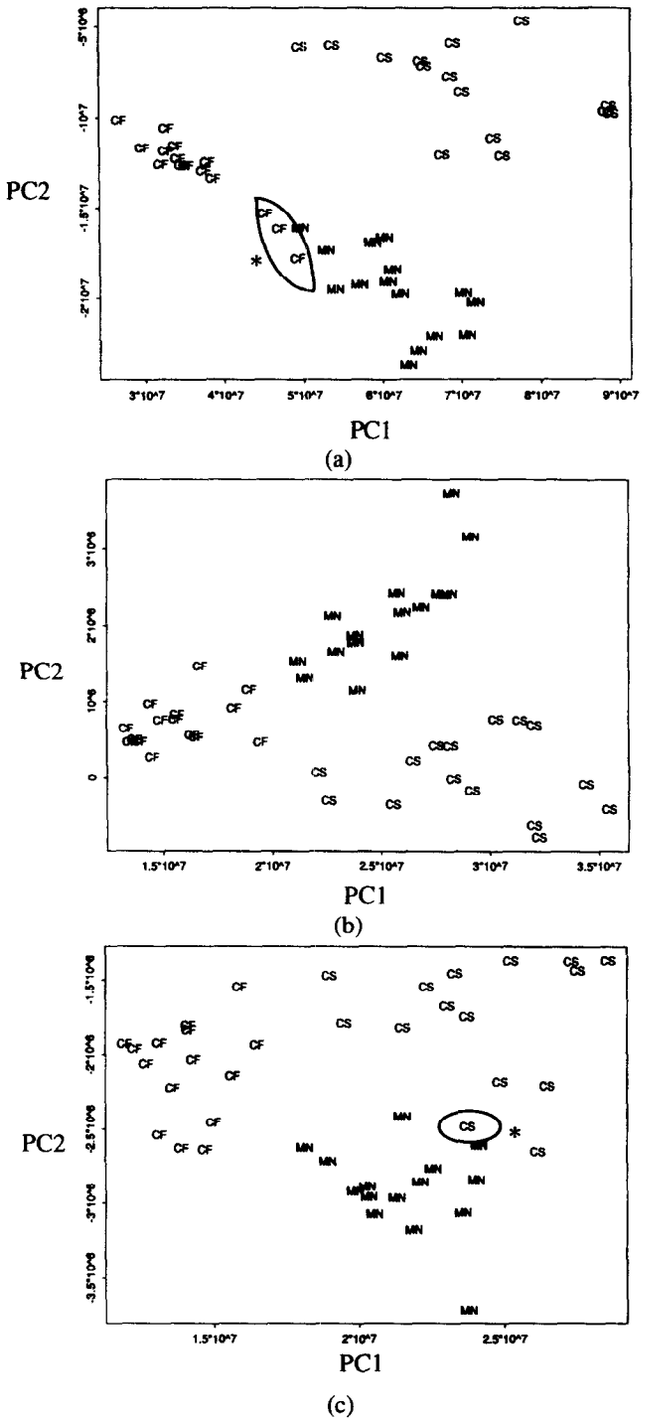


Fig. 2. Representations of the PCA of the three cultivars for grape seed extracts for (a) the HMBC-GAS, (b) the HMQC and (c) the HMQC-HOHAHA. (*Clones badly placed on first two PCs.)

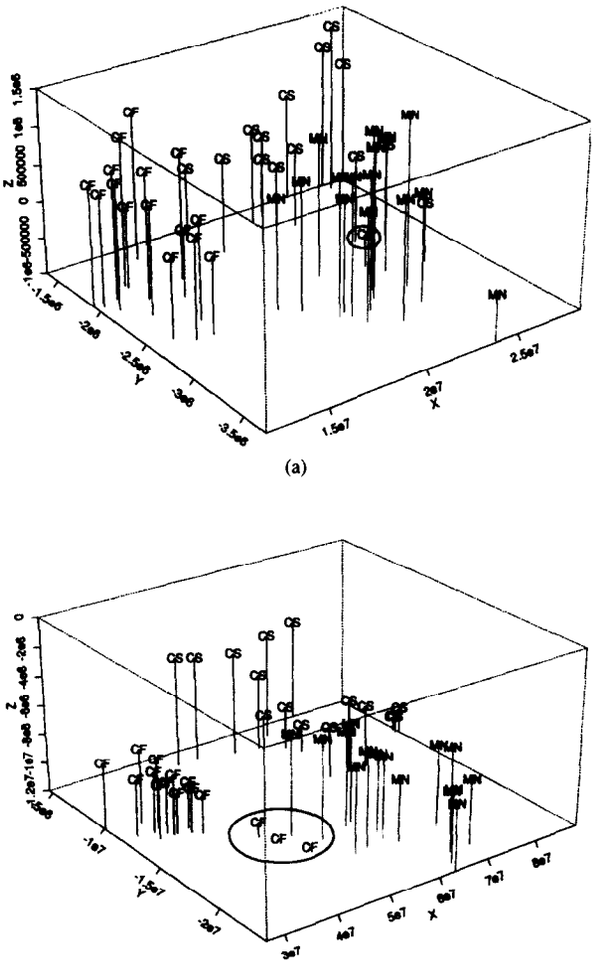


Fig. 3. Representations of the first three principal components of the three cultivars for grape seed extracts for (a) the HMBC-GAS and (b) the HMQC-HOHAHA.

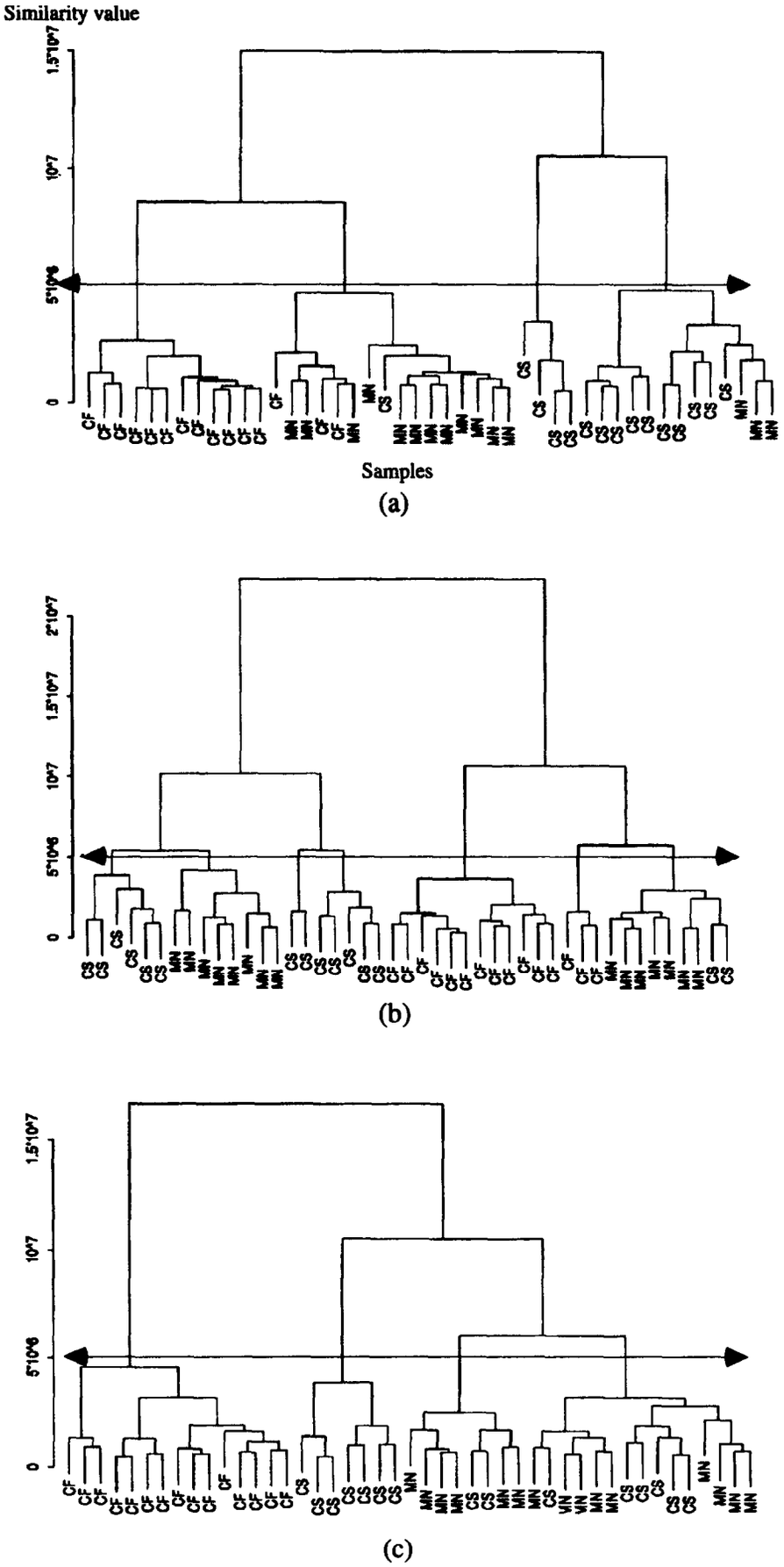


Fig. 4. Dendrogram obtained by hierarchical clustering analysis of the three cultivars for grape seed extracts for (a) the HMBC-GAS, (b) the HMQC and (c) the HMQC-HOHAHA.

the Cabernet Franc cultivar, the second of 75% of clones from the Merlot Noir cultivar, and the last two groups contain 82% of clones from the Cabernet Sauvignon cultivar. HCA results in Fig. 4a confirm PCA results in Fig. 2a.

2. Seven groups for the HMQC: three of them contain clones from the Cabernet Sauvignon cultivar; two others are composed of clones from the Cabernet Franc cultivar; another includes clones from the Merlot Noir cultivar, and the last is a mixture of the three cultivars. HCA results in Fig. 4b confirm PCA results in Fig. 2b.
3. Four groups for the HMQC-HOHAHA: one is composed of clones from the Cabernet Sauvignon, another consists of clones from the Cabernet Franc, and the other one contains 65% of clones from the Merlot Noir cultivar and 35% of clones from the Cabernet Sauvignon cultivar. The overlapping of MN and CS in Fig. 4c is confirmed by the PCA plot in Fig. 2c.

The best differentiation between cultivars was obtained with the data of the HMBC-GAS.

These two very different exploratory analyses (PCA and HCA) have revealed a definite differentiation of cultivars based on the NMR spectral correlation. Cultivars may, therefore, be used as the predefined groups for a FD analysis.

A very good separation between cultivars was given by the FDA of the data for all two NMR experiments (Fig. 5). The observation of the individuals in the space of the first three discriminant functions allowed us to perfectly distinguish the three cultivars.

Analysis of leaf extracts

The variance explained by the first two principal components is 99.5%. The results of the PCA calculated with the HMBC-GAS data for the three cultivars showed that clones are separated according to the cultivar to which they belong. The factorial discriminant analysis gave a very good separation (Fig. 6).

These results show that it is possible to differentiate grapevine cultivars by analysing the NMR data of polyphenols extracted from grape seeds and leaves. The method was validated by the both Jackknifing and cross-validation; the percentages of correctly classified individuals are all greater than 99.7% and the discriminant models are stable. The averages and standard deviations of the coefficients of the discriminant functions for the HMBC-GAS experiment are given in Table 3.

Differentiation of grapevine clones

Analysis of the grape seed extracts

An ANOVA, a PCA and an FDA were done on the data of each of the three NMR experiments (HMBC-GAS, HMQC, HMQC-HOHAHA) for the clones of each cultivar, taken separately.

The variables selected by the ANOVA are shown in Table 4 where it can be seen that F values are not as

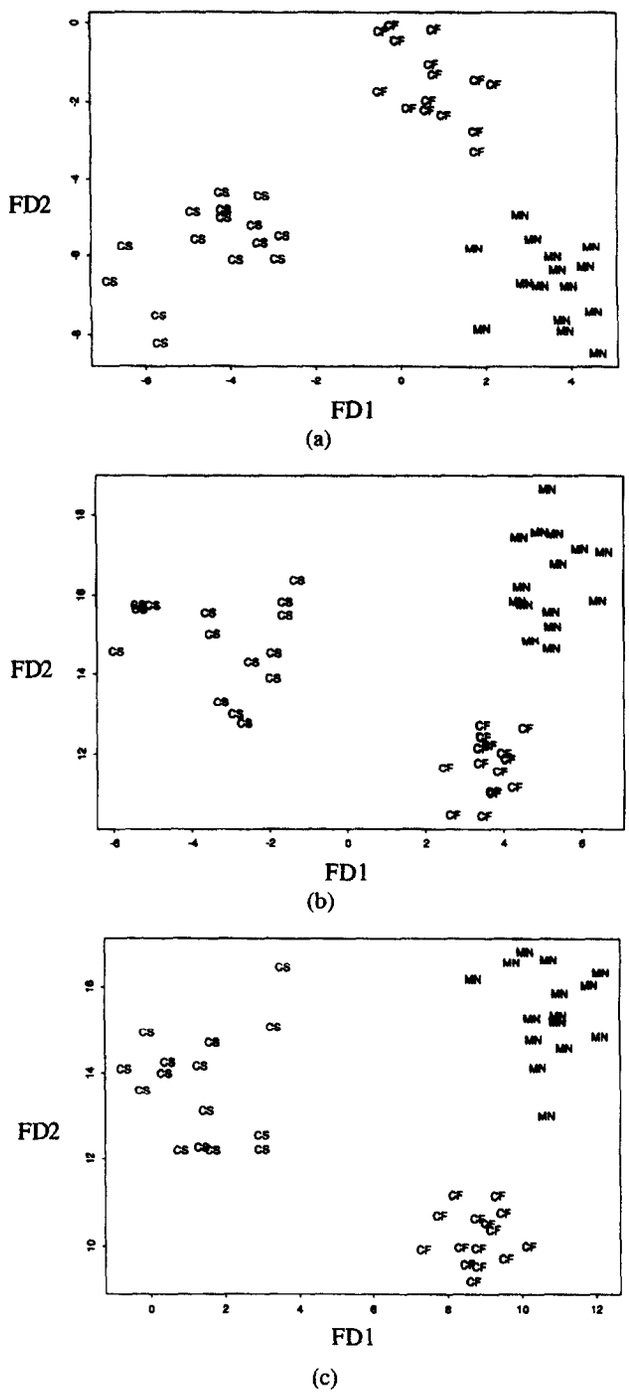


Fig. 5. Plot of the three cultivars for grape seed extracts on discriminant functions 1 and 2 for the FDA based on the cultivar for (a) the HMBC-GAS, (b) the HMQC and (c) the HMQC-HOHAHA.

high as those obtained for the variables discriminating the cultivars (Table 2). Therefore, as is to be expected, differences between clones are less significant than between cultivars.

A PCA based on these variables clearly separated the different clones within each cultivar and so the three repetitions for each clone were used as the predefined groups for factorial discriminant analysis. Nevertheless, clones 312 (cf312bt, cf312mg), 331 (cf331bt, cf331mg) and 332 (c332bt, cf332mg) of the Cabernet Franc cultivar, which were collected in

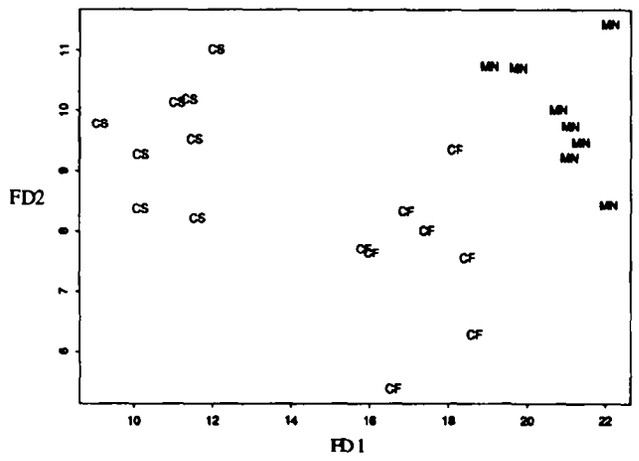


Fig. 6. Plot of the three cultivars for leaf extracts on discriminant functions 1 and 2 for the FDA based on the cultivar.

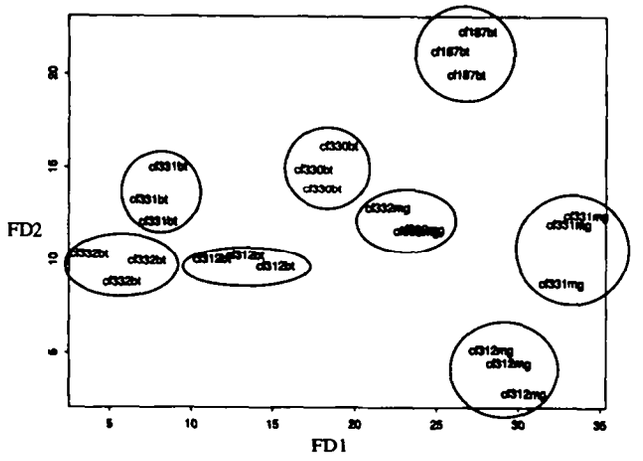


Fig. 7. Representation of the FDA of clones from the Cabernet Franc cultivar for the HMBC-GAS data for grape seed extracts.

Table 3. Averages and standard deviations (SD) for the coefficients (Coef.) of the discriminant functions

	Axe 1 Average	SD	Axe 2 Average	SD
For grape seed extracts: HMBC-GAS DATA				
Jackknifing:				
Coef. 1	24	14	29	12
Coef. 2	20	11	3	60
Coef. 3	14	30	42	9
Coef. 4	21	12	21	11
Coef. 5	3	80	2	105
Coef. 6	33	8	30	7
Coef. 7	11	33	23	11
Coef. 8	11	32	18	22
Coef. 9	14	15	6	38
Coef. 10	24	9	5	48
Coef. 11	4	32	13	14
Coef. 12	4	33	6	36
Cross-validation:				
Coef. 1	25	40	29	32
Coef. 2	21	31	7	35
Coef. 3	14	58	44	20
Coef. 4	22	22	21	22
Coef. 5	5	87	6	37
Coef. 6	33	16	31	10
Coef. 7	15	48	23	27
Coef. 8	11	81	17	54
Coef. 9	15	32	7	72
Coef. 10	23	34	9	63
Coef. 11	4	67	13	33
Coef. 12	4	30	7	53
For leaf extracts: HMBC-GAS data				
Jackknifing:				
Coef. 1	2	27	6	6.2
Coef. 2	1.9	9	0.8	19
Coef. 3	0.7	22	0.2	85
Coef. 4	0.2	75	0.2	96
Coef. 5	0.8	19	0.3	81
Coef. 6	2	15	2.5	15

Table 4. Variables selected by the ANOVA to differentiate clones within each cultivar

	F selected	Number of variables
For grape seed extracts:		
HMBC-GAS:		
Merlot Noir	$F > 10$	7
Cabernet Franc	$F > 30.5$	10
Cabernet Sauvignon	$F > 55$	7
HMQC:		
Merlot Noir	$F > 20$	7
Cabernet Franc	$F > 30$	7
Cabernet Sauvignon	$F > 25$	7
HMQC-HOHAHA		
Merlot Noir	$F > 20$	5
Cabernet Franc	$F > 20$	9
Cabernet Sauvignon	$F > 25$	6
HMBC-GAS		
For leaf extracts	$F > 20$	6

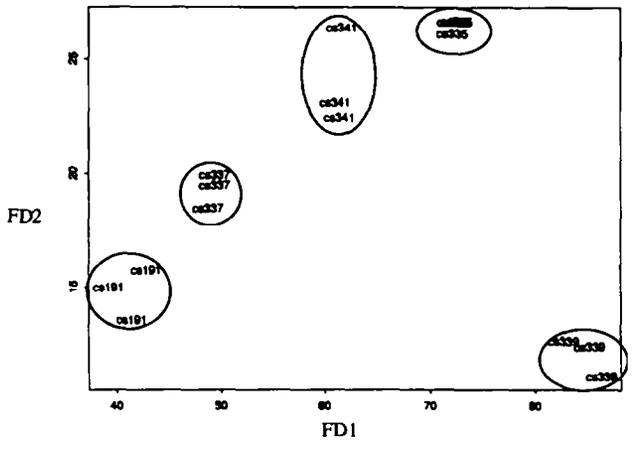


Fig. 8. Representation of the FDA of clones from the Cabernet Sauvignon cultivar for the HMBC-GAS data for grape seed extracts.

two different geographical places (bt, mg), were put in different groups.

Figure 7 shows the results of FDA of the HMBC-GAS data for clones of the Cabernet Franc cultivar. Clones are all differentiated and they tend to cluster by geographical origin.

Clones of the Cabernet Sauvignon cultivar are very well differentiated by the HMBC-GAS data as shown in Fig. 8. It is the same for clones of the Merlot Noir cultivar with the HMQC-HOHAHA data (Fig. 9).

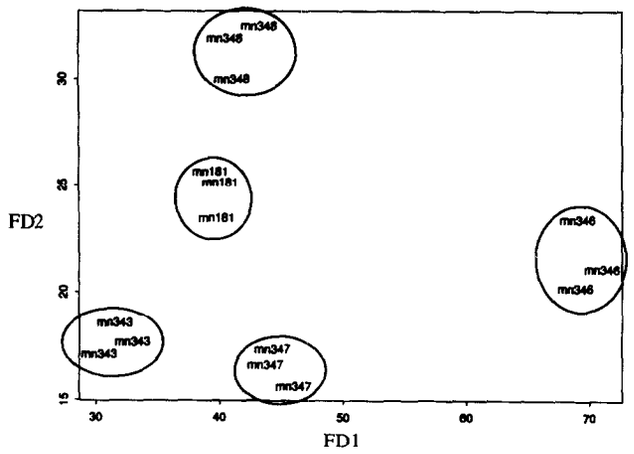


Fig. 9. Representation of the FDA of clones from the Merlot Noir cultivar for the HMQC-HOHAHA data for grape seed extracts.

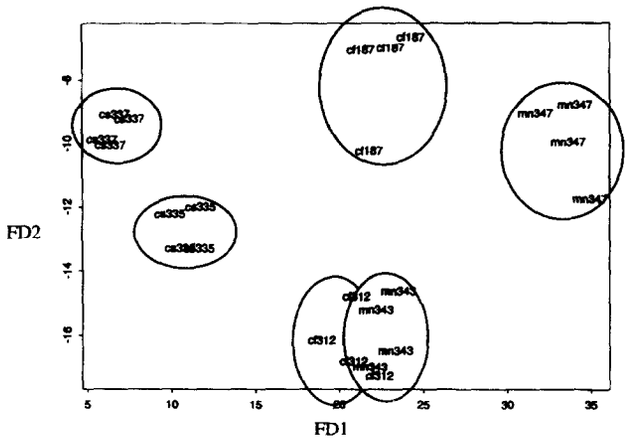


Fig. 10. Plot of the three groups of clones from leaf samples on discriminant functions 1 and 2 for the FDA based on the clone.

To conclude, it is possible to distinguish different grapevine clones of the same geographical origin by analysing the NMR data of polyphenol extracts from grape seeds. It was also shown that the geographical origin has a greater effect than the clonal origin. The models were validated by jackknifing. The percentages of correctly classified individuals are all greater than 99% and the models are stable. The more significant coefficients of the discriminant functions have a standard deviation ranging from 10 to 25%

Analysis of leaf extracts

For this analysis, as there are only two clones inside each cultivar, clones are taken together, whereas they were treated separately by cultivar for the grape seed extracts.

Fig. 10 shows that the clones are differentiated inside each cultivar even when the FDA is applied to all cultivars at the same time. The Cabernet Franc clone 312 and the Merlot Noir clone 343 do overlap slightly.

A hierarchical clustering of the data (Fig. 11) confirms that clones of the three cultivars are differentiated.

Therefore, it is also possible to differentiate grapevine clones by the analysis of polyphenol extracts from leaves. It should, however, be noted that, in this case, the discriminant functions could not be validated because there were not enough clones within each cultivar group.

CONCLUSION

The quantitative analysis by two-dimensional heteronuclear ^{1}H-^{13}C NMR of polyphenolic compounds extracted from different grapevine clones shows that it is possible to differentiate them according to the cultivar to which they belong and within each cultivar; clones are also clearly separated. In addition, identical clones which differ by their geographical origin are recognized.

The optimization of the experimental parameters should lead to a reduction in experiment time to as low as about 30 min, in which case this method could become routine. Moreover, the potential applications of these results are numerous. In the field of viticulture, the

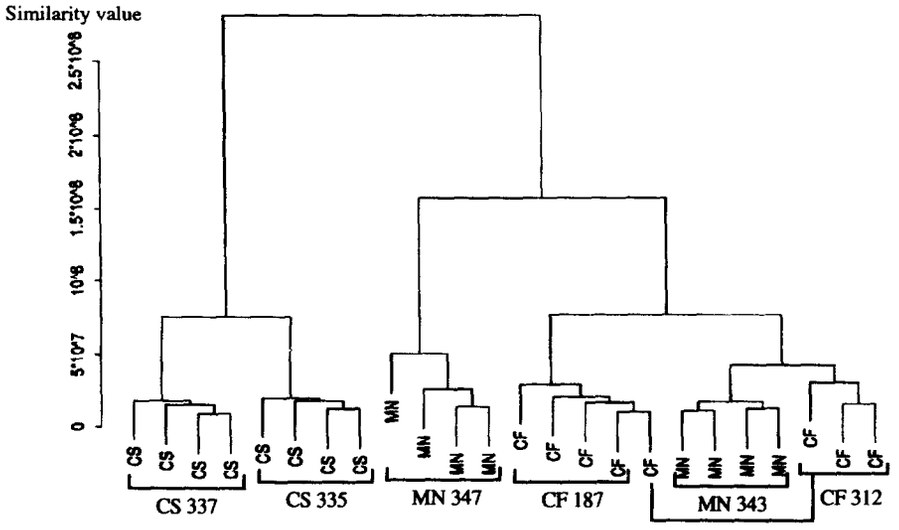


Fig. 11. Graph of the hierarchical clustering of the three groups of clones from leaf extracts.

creation of a sufficiently large two-dimensional NMR data base should allow one to use this technique to distinguish clones for plant selection, recognize oenological tannins (work in progress) according to their botanical origin and the extraction procedure (diethyl ether, ethyl alcohol, water) and even determine the cultivar by analysing the wine itself.

In the wider field of food and agriculture, the technique could be used for the certification of the origin of by-products from graminaceae (cereals) and the detection of frauds for fruit juice and fruit by-products, provided that they are derived from plants which produce polyphenols.

ACKNOWLEDGEMENTS

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