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Differentiation of Wine Vinegars Based on Phenolic Composition

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Phenolic composition of 92 wine vinegars produced from different wines from the south of Spain (Jerez, Montilla, El Condado) is determined by HPLC with diode array detection. Pattern recognition techniques were applied to distinguish between different methods of elaboration (slow traditional methods with surface culture or quick methods carried out in bioreactors with submerged culture) or wines employed as substrate. Multivariate analysis of data includes principal component analysis, cluster analysis, and linear discriminant analysis (LDA) as well as artificial neural networks trained by back-propagation (BPANN). The classification depending on the acetification process leads to good recalling rates in both LDA (mean = 92.5) and BPANN (mean = 99.6). With respect to the classification on the basis of the geographical origin, the obtained recalling rates were 88.8 for LDA and of 96.5 for BPANN (mean values).

Keywords: *Vinegar; phenols; discriminant analysis; artificial neural network; HPLC*

INTRODUCTION

Vinegar is a liquid for human consumption obtained, exclusively from agricultural raw materials containing starchs or sugars, by a double-fermentation process, the first one alcoholic and the second one acetic (FAO/WHO, 1982). In Mediterranean countries, vinegar is mainly produced using wine as raw material, whereas in non-wine-producing countries other substrates are employed: malt, cider, fruits, or even diluted acetic acid (Adams, 1985). With regard to wine vinegars, the different wines employed as substrates and the different technological procedures result in a great variety of products of diverse quality and organoleptic properties.

In general, methods of making vinegar can be divided into two kinds: slow methods in which the culture of acetic acid bacteria is placed on the surface of a wood barrel and quick processes involving submerged culture where the oxygenation has been increased so that the process is achieved at faster rates of acetification. The most widespread method of submerged acetification is the "acetator", arisen from the work of Hromatka and Ebner and marketed by Frings GmbH & Co. of Bonn (Hromatka and Ebner, 1959). Wine vinegars obtained from table wine by a quick acetification process in steel tanks with submerged culture constitute the largest production. However, traditional vinegars elaborated by a slow acetification process with surface culture, which usually involves aging in wood, are the most appreciated due to their extraordinary sensorial characteristics (González-Viñas et al., 1996). Among them, sherry wine vinegar is outstanding for its brilliant flavor acquired due to a elaboration accomplished throughout the "solera" system and has been awarded recently the first D.O. trademark (Checked Denomination and origin) for vinegars in Spain (Consejería de Agricultura y Pesca, 1995). A solera system consists of a series of butts arranged in steps, the number of which may vary from three to eight. The substrate arrives at the step on the top of the system and the final product is withdrawn from the step at the bottom, which is the most aged, but the volume taken will never exceed one-third of the total volume. Barrels in stage 1 are immediately filled with vinegar from stage 2, which, in turn, are filled with the content of barrels in step 3. In this way, a great homogenization throughout the whole system is accomplished. This is a dynamic method of production in contrast with the static method in which vinegar is produced in a single butt. From an economical point of view, the importance of sherry wine vinegar is increasing, and it is a worthy subject to study. Sherry wine vinegar from Jerez in Spain and Aceto Balsamico from the Italian city of Modena have the greatest reputations and are of the highest recognized value (Llaguno and Polo, 1991); the need for their protection as typical national products has been pointed out (Carnacini and Gerbi, 1992).

The final quality of vinegar is determined by the acetification system used, the raw material used as substrate, and eventually the period of aging in wood as chemical and physicochemical composition and organoleptic properties are influenced by these factors. Indeed, each type of vinegar contains chemical compounds remaining from the type of raw material employed. One of the topics that remains unsolved is to determine which is the factor that most contributes to the final quality of a certain vinegar: the acetification process involved or the raw material employed. However, the limits for chemical composition and analytical parameters useful to characterize the product may be quite wide (Carnacini and Gerbi, 1992), which is the main difficulty to be overcome in reaching reliable characterization.

The attempts to differentiate vinegars have been based either on the type of raw material employed (Acosta-Artiles et al., 1993) or on the kind of process involved (Guerrero et al., 1994); however, literature concerning both criteria at the same time is scarce. In these studies, samples were analyzed by Official Methods to determine their acidity, total extract, ash content, glycerol, alcohol, and sulfates. Besides, proline content

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Table 1. Description of Samples

group	no. of samples	acetification procedure	origin (substrate wine)
SJ	42	slow	Jerez
SC	18	slow	Condado (Huelva)
SM	8	slow	Montilla-Moriles
QJ	7	quick	Jerez
QM	4	quick	Montilla-Moriles
QX	13	quick	not known

has already been proposed to distinguish quality vinegars from adulterated ones (Polo et al., 1976). On the other hand, volatile compounds and organic acids proved to be useful to follow the acetification process (Nieto et al., 1993).

The purpose of the present work is to find criteria to differentiate wine vinegars considering the following as principal sources of variation: different origins of the substrate wines employed and the type of acetification process (quick or slow) applied.

Phenols are of major interest in the chemotaxonomic differentiation of vegetal species as they are widespread among a wide number of plants (Harbone, 1975). Some phenolic groups are powerful tools for pattern recognition in different fruits, such as flavonoid content in the characterization of apricot (García-Viguera et al., 1994) and citron (Mouly et al., 1994) and anthocyanin content in the characterization of grape varieties (Roggero et al., 1988). Indeed, evidence revealing that phenols may constitute a fingerprint to differentiate wines from pure varieties has been found (Archier et al., 1992). Moreover, many studies regarding the evolution of phenols during common processes in enology have been carried out. They have been used to follow must fermentation (Roggero et al., 1992) or aging (Puech et al., 1984) with the aim of control operations. The influence of phenolic composition on the quality of vinegars was formerly pointed out by some authors (Díez de Bethencourt et al., 1980), and more recently HPLC techniques have been set up for its assay (Carrero Gálvez et al., 1994; García Parrilla et al., 1994, 1996). Phenols are present in wine vinegar due to their natural content in grapes or as a result of contact with wood during the aging process. As phenols seem to be a very significant group of substances to accomplish the differentiation by origin and technology involved, they have been selected to match the above-mentioned purposes.

MATERIALS AND METHODS

Samples. Ninety-two vinegar samples derived from both slow (S) and quick (Q) acetification techniques were used to perform this study (Table 1). Among the samples obtained by slow traditional methods, three groups according to their different origins (all of them placed in the south of Spain) were made: sherry (SJ), El Condado de Huelva (SC), and Montilla-Moriles (SM). Sherry vinegars and vinegars from Montilla were obtained by dynamic methods (solera systems), while vinegars from El Condado were obtained by static ones. The sherry group is significatively the largest as its production and the number of wineries producing them are greater.

With regard to samples obtained by quick acetification methods, they were also divided into three groups: sherry wine vinegar obtained from an industrial acetator (QJ), Montilla wine vinegar produced in an experimental bioreactor in the laboratory (QM), and commercial wine vinegars purchased in the market (QX); the substrate wines used were from very different origins; they are produced in large amounts by Frings acetators and represent the most widely consumed vinegar in Mediterranean countries.

Apparatus. Samples were filtered through a Millex-GV_{13} of 0.22 μm filters, which incorporates the low-extractable Durapore poly(vinylidene fluoride) membrane; these filters proved to be useful as they did not retain compounds under study. This was the single treatment of the sample before injection onto the column. The HPLC apparatus was a Waters 600E system controller connected to a Waters 996 photodiode array detector. Data treatment was performed in a Waters Millennium 2.0 data station. The column was a Merck Superspher 100 RP-18 (250 $\times$ 4 mm) protected by a guard cartridge Nova-Pack C_{18} module from Waters. The volume injected was of 50 μL.

Procedure. The chromatographic conditions were originally described for the analysis of simple phenols and flavonols in wines (Roggero et al., 1990). Recently, the method was enhanced by changing the acetic content of the solvents used in the gradients (Roggero et al., 1991). This method has been successfully applied to sherry wine vinegars (García Parrilla et al., 1996). The solvents are as follows: A, acetic acid/water (1/99); B, acetic acid/water (6/94); C, acetic acid/watee/acetonitrile (5/65/30). The gradient profile is as follows:

time (min)	%A	%B	%C
0	100	0	0
15	0	100	0
30	0	100	0
50	0	90	10
60	0	80	20
80	0	70	30
120	0	0	100

The flow was 0.5 mL/min, and the temperature was set at 22.5 °C. Solvent is heated as it travels through the heater before entering the column, and an internal cover mantains thermal stability during operation.

The compounds in the samples were identified both by retention time and by spectra matching as described in the above-mentioned reference, while quantitative assay was performed in duplicate thanks to external calibration curves.

Pattern Recognition Techniques. Multivariate analysis of data included principal component analysis, cluster analysis, and stepwise discriminant analysis. Data were processed on a PC-compatible computer using CSS software (Statsoft, 1991). Artificial neural networks (ANN) trained by back-propagation (BPANN) were performed by means of the program WINN.97 (Danon, 1996).

RESULTS AND DISCUSSION

Table 2 contains the means and standard deviations for a total of 23 phenolic compounds analyzed according to this method. Notice that 5-(hydroxymethyl)furfural and furfural have been included despite their nonphenolic structure since they appear as large peaks in the chromatogram obtained at 280 nm (Figure 1) and their spectra are quite similar to those observed for phenols. Their presence in vinegar may be explained either by wood contact or by must caramel addition, which is a legal practice in vinegar making (Quesada-Granados et al., 1996; Consejería de Agricultura y Pesca, 1995).

A number of phenolic compounds have been found in some kinds of vinegar, while it was impossible to detect them in others, which is promising for establishing differentiation criteria. That is the case of aldehydes: benzaldehyde, syringaldehyde, and vanillin, which are more likely to be found in vinegars elaborated by slow traditional methods than in quick vinegars as the former are aged in wood and the latter are made in steel tanks. On the other hand, catechin, epicatechin, and quercetin are present in only the QX group (quick vinegars, unknown origin). This result is in accordance with the values obtained for the procyanidin index determined by a spectrophotometric method described in a previous work (García Parrilla et al., 1997), the raw

Table 2. Means and Standard Deviations (SD) of Measured Substances[a] (in Milligrams per Liter)

	SJ		SC		SM		QJ		QM		QX	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
1	27.1	18.43	29.7	18.47	60.3	33.66	36.8	16.29	6.1	4.77	6.0	7.66
2	0.4	1.20	2.6	5.1	0.6	1.11	0.9	0.69	0.6	1.20	0.0	0.00
3	0.03	0.13	1.5	2.76	0.4	0.82	0.0	0.00	0.0	0.00	0.0	0.00
4	2.1	1.85	0.2	0.57	1.0	0.80	2.6	1.35	0.3	0.22	0.4	0.47
5	1.7	1.70	0.8	0.89	0.7	0.79	1.4	0.56	0.3	0.29	0.3	0.40
6	16.5	13.72	1.9	4.21	7.3	7.44	28.3	12.77	3.4	3.93	7.1	7.88
7	5.4	6.42	0.1	0.45	0.9	1.84	9.1	5.30	1.2	0.80	1.4	1.80
8	3.9	7.63	0.0	0.00	1.0	1.8	6.6	5.15	1.38	2.55	3.4	4.2
9	0.4	0.49	0.1	0.41	0.2	0.15	0.4	0.24	0.03	0.05	0.1	0.1
10	0.5	0.66	0.2	0.36	0.2	0.06	0.3	0.15	0.03	0.05	0.1	0.1
11	1.6	2.66	2.4	2.44	5.6	4.9	0.5	0.56	0.4	0.51	0.3	0.81
12	0.5	1.20	0.4	0.92	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
13	0.2	0.65	2.1	5.15	0.1	0.21	0.0	0.00	0.0	0.00	0.0	0.00
14	0.1	0.33	0.2	0.58	0.4	1.02	0.0	0.00	0.0	0.00	0.0	0.00
15	0.6	0.92	2.3	2.13	0.2	0.42	0.66	1.74	0.0	0.00	0.0	0.00
16	0.1	0.32	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
17	0.7	1.37	0.0	0.00	3.1	3.41	0.6	1.47	1.8	1.33	2.3	4.02
18	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.7	2.41
19	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	1.6	5.72
20	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.3	1.00
21	8.7	8.33	47.9	37.35	12.6	12.93	2.3	6.16	13.6	5.86	1.9	3.8
22	22.6	21.69	18.2	16.38	12.9	8.47	0.5	1.32	2.3	1.01	0.7	1.18
23	2.5	5.40	13.0	14.36	0.0	0.00	0.0	0.00	0.0	0.00	0.01	0.055

[a] **1**, gallic acid; **2**, *p*-hydroxybenzoic acid; **3**, vanillic acid; **4**, caffeic acid; **5**, *p*-coumaric acid; **6**, caffeoyltartaric acid; **7**, *p*-coumaroyltartaric acid glucosidic ester; **8**, *p*-coutaric acid; **9**, caffeic ethyl ester; **10**, *p*-coumaric ethyl ester; **11**, gallic ethyl ester; **12**, protochualdehyde; **13**, benzaldehyde; **14**, vanillin; **15**, syringaldehyde; **16**, resveratrol; **17**, isoquercetrin; **18**, quercetin; **19**, catechin; **20**, epicatechin; **21**, tyrosol; **22**, 5-(hydroxymethyl)-2-furaldehyde; **23**, 2-furaldehyde.

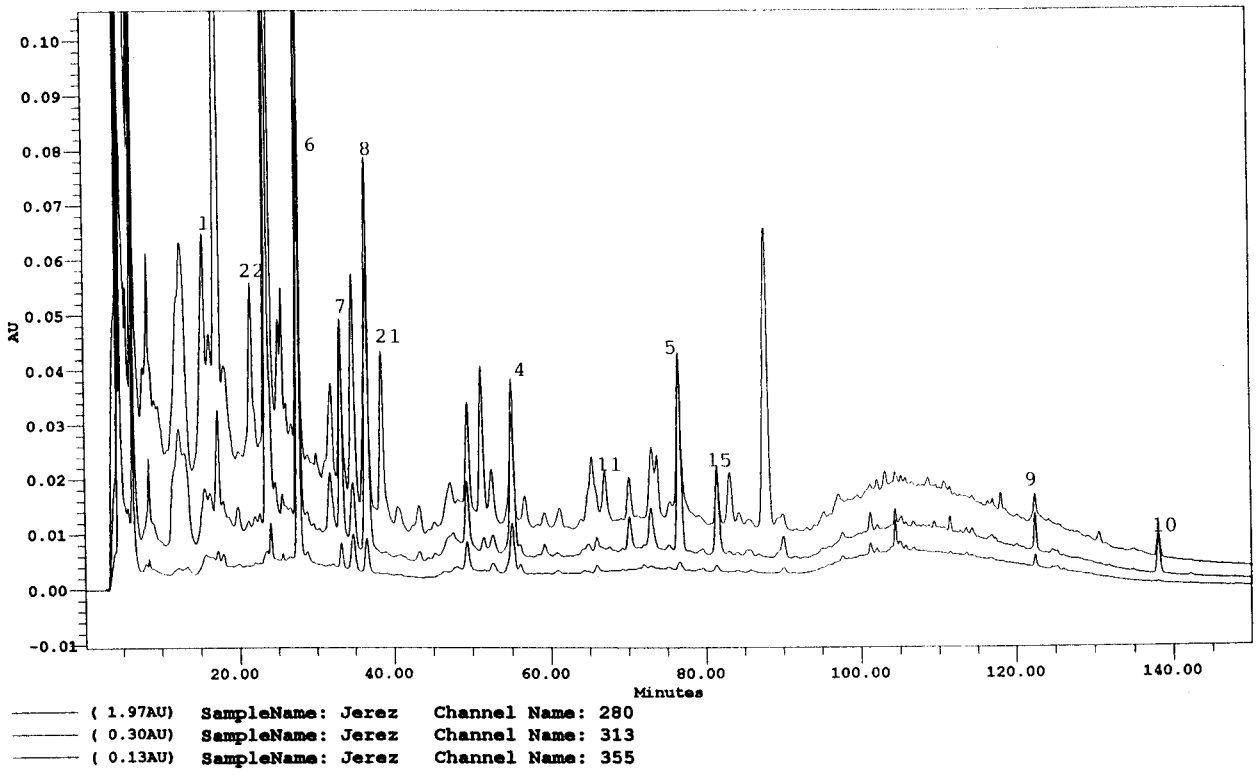

Figure 1. Chromatogram corresponding to a Jerez sample obtained by slow acetification procedure (number of peaks corresponding to Table 2).

material employed being the major factor responsible for this fact. Other compounds offer larger figures for some groups being scarce in others; for instance, caffeic acid and caffeoyltartaric acids, which are present in a lesser quantity in the SC group (slow vinegars from El Condado). Gallic acid ethyl ester presents higher values in those vinegars obtained by traditional means, while in quick vinegars its concentration is lower (Table 1). There are differences enough in both quantitative and qualitative analysis to find differentiation criteria.

PCA-Based Display Methods and Cluster Analysis. When data matrix was subjected to PCA, seven significant PCs arose according to both Kaiser's criterion (1960) and the assurance of suitable communalities for variables (>0.5). With these factors, 76% of total variance is explained. It is noticeable that some PCs have a significant physical meaning as indicated in the following: The first PC, PC1 (which explains 22.25% of total variance), mainly contains the descriptors caffeic acid, caffeoyltartaric acid, coumaroyltartaric acid, cou-

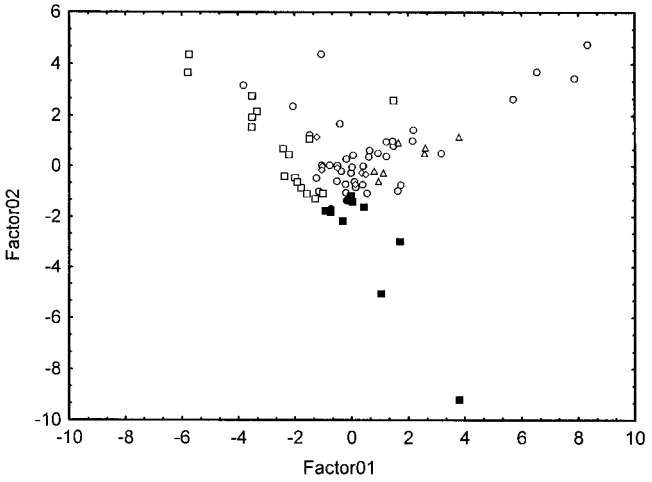

Figure 2. Plots of the two first principal components issued from PCA: (○) SJ; (□) SC; (◇) SM; (△) QJ; (●) QM; (■) QX. The notation following the sample groups' name corresponds to sample numeration.

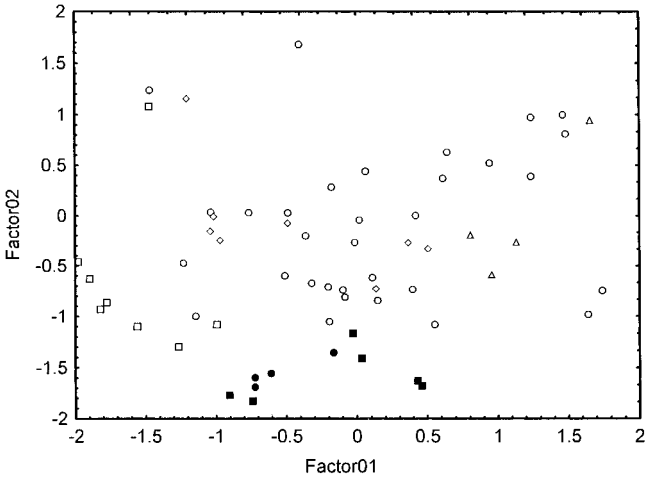

Figure 3. Zoom of the plot of the two first principal components: (○) SJ; (□) SC; (◇) SM; (△) QJ; (●) QM; (■) QX.

maric acid, caffeic acid ethyl ester, and ethylcoumaric ester, which present a hydroxycinnamic structure. The third PC, PC3 (explaining 12.85% of total variance), is contributed to mainly by compounds of flavonoid structure such as quercetin, isoquercetrin, catechin, and epicatechin. PC2 (which explains 15.84% of total variance), however, did not show any structure relationship. Hydroxycinnamic acids have already proved their utility in characterization of white wines of the same origin elaborated in different cellars (De la Pressa-Owens et al., 1995a,b), in our case being an important contributor to PC1.

Plots of the two first principal components issued from PCA may be of interest to visualize data trends. The corresponding scores plot for the studied vinegars is shown in Figure 2. At first glance, a jungle of samples is observed. After a zoom of the thicket, the distribution of vinegars belonging to different classes can then be observed (Figure 3). A quasi-linear separation between vinegars elaborated by quick and slow methods may be considered. However, a linear separation of classes based on geographical origin was not found.

To assess this preliminary study, unsupervised pattern recognition methods were applied for searching natural groupings among the samples. Thus, the data matrix was then subjected to a hierarchical agglomerative cluster analysis of cases. Taking the euclidean distance as metric and the Ward method as amalgamation rule (Ward, 1963), the dendrogram was obtained (Figure 4). Some comments may be easily derived from a simple inspection: Quick X vinegars cluster together (with two exceptions) at a distance <10% of the maxi-

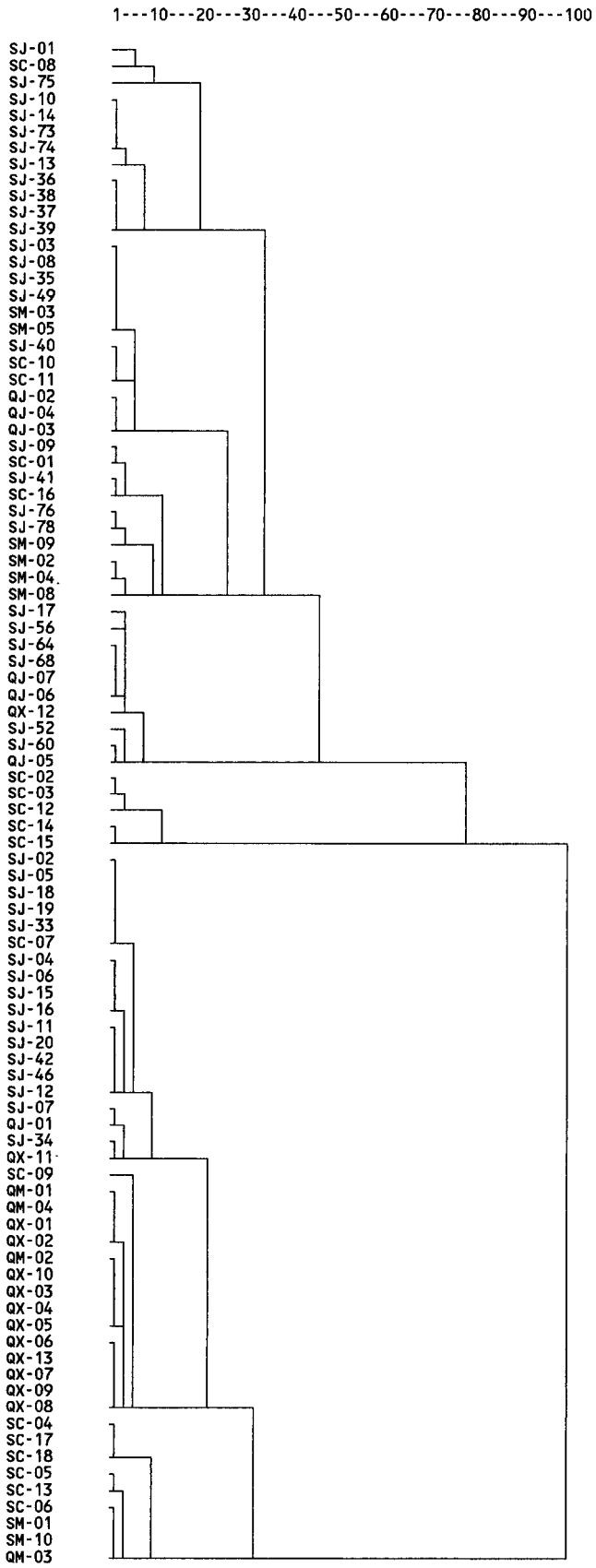

Figure 4. Dendrogram obtained after hierarchical agglomerative cluster analysis of cases.

Table 3. Classifications Obtained on the Basis of Acetification Procedure

	set	1	2	3	4	5	6	7	8	9	10
ANN	training	100	100	100	100	100	100	100	100	98	98
	test	78.3	69.6	73.9	69.6	82.6	87.0	91.3	78.3	69.6	95.7
LDA	training	92.8	89.9	91.3	95.7	92.8	88.4	94.2	94.2	92.8	92.8
	test	95.7	78.3	87.0	73.9	87.0	82.6	73.9	73.9	78.3	78.3

Table 4. Classifications Obtained on the Basis of Origin

	set	1	2	3	4	5	6	7	8	9	10
ANN	training	98	98	93	96	95	95	100	98	96	96
	test	84.2	68.4	89.5	89.5	94.7	73.7	68.4	73.7	84.2	89.5
LDA	training	86.7	90.0	85.0	81.7	91.7	88.3	86.7	96.7	88.3	93.3
	test	78.9	68.4	57.9	84.2	52.6	73.9	68.4	100	89.5	84.2

mum distance. Besides, three samples of Montilla elaborated from quick acetification are also included in the cluster. Slow vinegars, on the contrary, are rather disperse. One could conclude that quick vinegars are naturally very similar according to their phenolic composition, and this leads to more compact grouping when cluster analysis is applied.

Supervised Pattern Recognition. These methods assume an *a priori* knowledge of the number of classes and the sample class memberships. Two possible category classifications have been considered: from the acetification process of vinegars (quick and slow) and from their geographical origin (Jerez, Condado, or Montilla-Moriles). In the two instances, the samples studied were divided into two sets: the training set (75% of the whole set) and the evaluation set (25% of the whole set). To suitably validate the recalling rate (goodness of classification in the training set) and the prediction ability (goodness of classification in the prediction set), both training and prediction sets were repeated 10 times for different constitutions. The average of hits (percent) in the recalling and prediction obtained from these 10 runs is taken as a measurement of the discriminating procedure.

Linear discriminant analysis (LDA) is a widespread parametric method for classification purposes. Stepwise discriminant analysis (Powers and Keith, 1968) is a method for seeking out subsets of variables most useful to discriminate among classes. The forward selection approach was selected in our case. Variables are selected according to Wilk's lambda criterion (Wilks, 1960). For the classification according to elaboration procedures, the selected variables were (hydroxymethyl)furaldehyde, tyrosol, *p*-coumaric acid, isoquercetrin, gallic acid ethyl ester, furaldehyde, *p*-hydroxybenzoic acid, and coumaroyltartaric acid glycoside, whereas for classification according to geographical origin the chosen variables were (hydroxymethyl)furaldehyde, tyrosol, caffeoyltartaric acid, gallic acid ethyl ester, syringaldehyde, vanillic acid, vanillin, and caffeic acid.

For the sake of comparison, we call on another independent classification method based on BPANN (Zupan and Gasteiger, 1993). BPANN is very often used for classification because it is nonparametric and does not need to satisfy requirements of linear separation of classes. The architecture of the net is ($9 \times 3 \times 2$) and ($8 \times 3 \times 3$) (plus bias) for the classification according to the elaboration and origin, respectively. Thus, we chose as variables for the input layer the same ones previously selected by Wilk's lambda criterion. The output layer contains as neurons as classes are. The neurons of the hidden layer are selected empirically. The learning rate was set to 0.2 and the momentum at 0.5. The iterations were limited to 1000 epochs. Samples were taken randomly. Initial weights were taken randomly within the interval -0.1, 0.1.

Results obtained are shown in Tables 3 and 4. The classification depending on the acetification process leads to good recalling rates in both LDA (mean = 92.5) and BPANN (mean = 99.6) methods and discrete prediction abilities (mean = 81.0 for LDA and 78.3 for BPANN). This may be explained by the fact that vinegars from wines already aged in wood that have been submitted to quick acetification procedures have been included in the quick vinegars. These samples share features with both groups. The result is a product of intermediate quality difficult to classify according to the considered class models.

With respect to the classification on the basis of the geographical origin, the obtained recalling rates were88.8 for LDA and 96.5 for BPANN (mean values), and the prediction abilities were 75.8 for LDA and 81.6 for BPANN. The best results obtained from BPANN may easily be explained according the nonlinear separation among origin classes as was already indicated in the the study of the score plot. Thus, a method based on the assumption of lineal separation like LDA will give poor results compared with the BPANN, a procedure very often used in cases of nonlinear separation of classes.

Conclusions. Phenolic compounds of wine vinegars are useful to classify and predict the membership of samples according to the elaboration method applied or the geographical origin of the substrate wine. The classification depending on the acetification process leads to good recalling rates in both LDA and BPANN and discrete prediction abilities. With respect to geographical origin, BPANN proved to have better classification and prediciton abilities than LDA as there was a nonlinear separation among origin classes.

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