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Characterization of Selected Spanish Table Wine Samples According to Their Biogenic Amine Content from Liquid Chromatographic Determination

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Pattern recognition techniques, such as principal component analysis, cluster analysis, and linear discriminant analysis, have been applied to samples of red, white, and rosé wines to determine whether some biogenic amines could be considered as chemical descriptors. Eight amines (tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) were determined by RP-HPLC, after derivatization with dabsyl chloride. However, only putrescine, cadaverine, histamine, tyramine, spermidine, and spermine were found in the wines analyzed. From the association between variables obtained by principal component analysis and clustering and from the relationship found by linear discriminant analysis, it can be deduced that the amines generated during malolactic fermentation (putrescine, histamine, and tyramine) could be used as chemical descriptors to characterize table wine samples.

KEYWORDS: Wines; biogenic amines; malolactic fermentation; multivariate analysis

INTRODUCTION

Biogenic amines are organic base compounds that occur in different kinds of food, such as fish products (*1*), cheese (*2*), wine (*3*), cured meats, and other fermented foods (*4*).

These compounds are produced during and after wine-making, although some are present in small amounts in grape juice. They can be present in the must or formed by yeasts during alcoholic fermentation. Chemically, the main factors involved in the generation of these compounds are malolactic fermentation, during which the main biogenic amines generated by decarboxylation of the corresponding amino acids are putrescine, histamine, and tyramine (*5*), and the pH of the wine. At high pH, biogenic amines are always produced in large amounts (*5*). Thus, red wines, which are generally less acidic, contain higher biogenic amine concentrations than white wines. Furthermore, in wine-making, malolactic fermentation usually has a greater importance in red wines than in white wines. There is also a third type of wine, rosé wine, made from red grapes or from a mixture of red and white grapes, of which the juices are fermented like white wines, that is, without their skins, showing properties between those of red and white wines (*6*).

Consequently, as a result of the extent of malolactic fermentation, red, rosé, and white wines can be expected to have different biogenic amine contents, which would permit the classification of the wines as well as to explain a misclassification related to the wine-making.

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An attractive possibility for this purpose of wines is based on unsupervised and supervised pattern recognition techniques (*7*−*9*), which make it possible to extract information from analytical parameters, allowing us (a) to verify associations among variables, (b) to group or to cluster samples (objects) with respect to comparable chemical descriptors, and (c) to search multivariate data classification on the basis of known class membership of those objects.

In parallel with these studies, data analysis can be carried out using analysis of variance and correlation studies and by establishing the discriminant capacities of the variables, one by one, through the Fisher index (*10*). This univariate approach implies that each variable has been studied separately from the others, by calculating and comparing mean values and standard deviations. It can be used as a first approach to establish the possibility of a pattern recognition study.

The aim of this work is to demonstrate that the content of those biogenic amines closely related to malolactic fermentation could be used as a chemical descriptor to differentiate between the types of wine and thus to discover if the wine has been irregularly processed or not.

The biogenic amine content was determined by RP-HPLC after derivatization with dabsyl chloride (*11*−*13*).

MATERIALS AND METHODS

Apparatus and Software. The liquid chromatograph consisted of a Hewlett-Packard 1050 series equipped with a UV−visible variable-wavelength detector, a 3396-A integrator, and a Rheodyne (Rheodyne, Inc., Cotati, CA) 7125 loop injector with a 20-μL sample loop. A

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Table 1. Biogenic Amine Content (Milligrams per Liter) of Table Wine Samples

sample	class	code	TRY	PHE	PUT	CAD	HT	TYR	SPD	SP
1	R	R1	nd[a]	nd	6.90 (6.8)[b]$_5$	1.45 (6.3)$_5$	4.74 (8.4)$_5$	5.91 (4.3)$_5$	nd	nd
2	R	R2	nd	nd	7.33 (5.3)$_5$	3.15 (9.0)$_5$	3.95 (9.5)$_5$	3.58 (4.1)$_5$	nd	nd
3	R	R3	nd	nd	19.10 (5.3)$_5$	2.20 (8.6)$_6$	2.31 (6.5)$_5$	3.17 (8.0)$_6$	nd	nd
4	R	R4	nd	nd	13.67 (3.4)$_7$	1.88 (4.3)$_6$	3.21 (7.2)$_6$	2.55 (8.4)$_6$	nd	0.57 (8.6)$_6$
5	R	R5	nd	nd	4.67 (3.2)$_6$	nd	0.39 (10.3)$_5$	2.24 (8.3)$_6$	nd	0.38 (7.0)$_5$
6	W	W6	nd	nd	1.62 (7.3)$_7$	nd	1.35 (5.5)$_7$	0.23 (12.6)$_5$	nd	nd
7	W	W7	nd	nd	12.61 (3.7)$_7$	0.86 (5.0)$_6$	2.85 (4.4)$_7$	1.22 (7.8)$_6$	nd	nd
8	W	W8	nd	nd	3.43 (1.7)$_7$	0.70 (4.2)$_5$	0.37 (11.2)$_5$	nd	nd	nd
9	W	W9	nd	nd	4.39 (5.7)$_6$	1.16 (3.8)$_6$	1.10 (9.8)$_6$	nd	nd	nd
10	W	W10	nd	nd	2.34 (5.2)$_6$	0.53 (4.7)$_7$	nd	nd	nd	nd
11	C	C11	nd	nd	3.25 (1.5)$_6$	0.39 (6.6)$_5$	2.92 (4.3)$_6$	0.86 (10.7)$_6$	nd	nd
12	C	C12	nd	nd	4.20 (3.7)$_6$	nd	0.45 (16.4)$_6$	0.33 (13.5)$_6$	nd	nd
13	C	C13	nd	nd	9.33 (0.5)$_6$	0.41 (6.9)$_6$	3.24 (3.1)$_6$	1.49 (4.9)$_6$	nd	nd
14	C	C14	nd	nd	3.63 (0.7)$_5$	2.87 (1.3)$_6$	nd	1.23 (7.0)$_6$	0.64 (5.1)$_5$	nd
15	C	C15	nd	nd	3.58 (3.2)$_6$	0.26 (15.7)$_7$	1.62 (5.3)$_7$	1.63 (3.0)$_6$	nd	nd
16	C	C16	nd	nd	9.60 (2.2)$_6$	nd	3.10 (7.6)$_6$	2.59 (6.8)$_6$	nd	nd
17	R	R17	nd	nd	5.91 (3.1)$_7$	nd	1.95 (6.8)$_7$	nd	nd	nd
18	C	C18	nd	nd	8.67 (2.9)$_7$	nd	1.36 (4.7)$_7$	1.05 (3.7)$_6$	nd	nd
19	W	W19	nd	nd	1.48 (7.9)$_6$	0.70 (7.0)$_6$	1.39 (6.7)$_7$	1.44 (5.5)$_6$	nd	nd

[a] nd, not detected. [b] Relative standard deviation in percent. Subscript indicates the number of replicates.

Lichrospher 100 RP-18 (244 × 4.4 mm i.d., 5 μm) column (Merck, Darmstadt, Germany), linked to a Lichrospher guard column (10 × 4.6 mm i.d.), was used for all separations.

A vortex mixer, model Reax 2000 (Heidolph Elektro GmbH & CoKG, Helheim, Germany), a thermostated bath, Precisterm model s-137 (Selecta, Barcelona, Spain), and a centrifuge model BHG Fixette 2 (Comerimsa, Madrid, Spain) were also used.

All pH measurements were made with a Crison 2000 pH-meter (Insulab, Valencia, Spain), equipped with a combined AgCl−glass electrode assembly.

The statistical packages CSS:Statistica (14) and Statgraphics Plus for Windows (*15*) were used for data manipulation.

Chemical and Reagents. *Amine Standard Solutions.* All amine standards were purchased as hydrochloride salts of the highest purity available. 3-(2-Aminoethyl)indole (tryptamine, TRY), 2-phenylethylamine (PHE), *N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine (spermine, SP), and *N*-(3-aminopropyl)-1,4-butanediamine (spermidine, SPD) were obtained from Fluka (Neu-Ulm, Germany); 2-[4-imidazolyl]ethylamine (histamine, HT), 1,5-diaminopentane (cadaverine, CAD), 1,4-diaminobutane (putrescine, PUT), and 4-hydroxyphenethylamine (tyramine, TYR) were from Sigma (St. Louis, MO); and 1,7-diaminoheptane (as internal standard) was from Aldrich (Steinheim, Germany). Stock solutions of the biogenic amines containing 0.5 or 1.0 g/L were prepared by dissolving them in 0.1 mol/L HCl containing 0.2% (w/v) 3,3′-thiodipropionic acid (TDPA) (Fluka) as an antioxidant. They were kept refrigerated at −20 °C.

Solutions for Dabsylation Reaction. Dabsyl chloride solution, 12.4 mmol/L, was prepared by dissolving 40 mg of dabsyl chloride (Fluka) in 10 mL of acetone (Merck, Darmstadt, Germany), following ultrasonic treatment for 15 min and filtering through an ANOTOP filter (Merck) into a brown glass vial. This solution was stored at −20 °C. Reaction buffer medium consisted of 1.06 g of Na_2CO_3 (Panreac, Barcelona, Spain), in 50 mL of water. Dilution solution was a mixture of 50 mL of acetonitrile (Panreac), 25 mL of ethanol (Panreac), and 25 mL of elution buffer A (see Chromatographic Solutions).

Chromatographic Solutions. Eluent A, consisting of 4.0×10^{-2} mol/L sodium acetate (Panreac), 10% (v/v) dimethylformamide (DMF) (Fluka), and 0.23% (v/v) triethylamine (TEA) (Carlo Erba, Milan, Italy),was adjusted to pH 5.0 with diluted acetic acid (Panreac). Eluent B consisted of 87.5% acetonitrile (Panreac), 10% *tert*-butyl methyl ether (Fluka) and 2.5% (v/v/v) water.

All glassware was rinsed thoroughly with 70% ethanol and water and dried before use. Glass vials for standards were heated at 500 °C for 3 h to remove any organic contaminants. Highly purified water (Milli-Q, Millipore) was used throughout for the preparation of buffers and reagents.

Procedure. *Sample Preparation.*The samples were filtered through a 0.20-μm membrane Millipore filter before the derivatization process.

Dabsylation Reaction. An aliquot of 1.5 mL of diluted wine [if necessary, diluted with 0.1 mol/L HCl 0.2% (m/v) TDPA solution] was transferred into a vial and adjusted to pH ∼8.2 with reaction buffer, and water was added to 3.8 mL. After thorough mixing on a vortex mixer, 1.6 mL of dabsyl chloride solution was added and it was mixed again. The mixture was heated in a water bath for 21 min at 70 °C, with shaking at 1 and 15 min. Then, 4.6 mL of the dilution solution was added and allowed to stand (for ∼20 min) in the water bath, shaking from time to time.

Chromatographic Analysis. The C_{18} column was equilibrated at 40 °C with a mobile phase constituted by 45% eluent A/55% eluent B. An aliquot of 20 μL of the dabsyl derivatives solution was injected, and eluted, at a flow rate of 1.0 mL/min, using the following gradient profile: 55% eluent B for 3 min, then 75% B in 13 min, then 100% B in 10 min, at 100% B for 10 min, then back to 55% B in 5 min, and finally 55% B for 15 min. The detection wavelength was 446 nm.

Samples. Nineteen samples of commercially available Spanish table wines, obtained from local supermarkets, were studied. The samples belonged to three wine types: red (six), identified as R; rosé (seven), identified as C; and white wines (six), identified as W. Wines from the regions of Granada, Murcia, Ciudad Real, and Badajoz were analyzed.

RESULTS AND DISCUSSION

Biogenic Amine Content. The amines in each sample were determined using a chromatographic method based on their derivatization by treatment with dabsyl chloride and separation by RP-HPLC with gradient elution and spectrophotometric detection at 446 nm (*12*, *13*). As this method has been previously validated for red wines (*13*), we also considered it necessary to validate it for rosé and white wines. Because the samples were not standard reference materials, the validation study was carried out on the basis of a statistical protocol (*16*, *17*) based on three calibration procedures (i.e., standard calibration, standard-additions calibration, and Youden calibration) with different sample sizes. In all cases, the trueness tests indicated that the method was reliable for the samples analyzed. **Table 1** shows the biogenic amine contents found in the samples analyzed.

It can be observed that TRY and PHE were not detected in the analyzed samples, and SPD and SP were found in very few wines. The biogenic amines found in almost all of the samples

Table 2. Basic Statistics for the Table Wines Analyzed

	red wines ($n = 6$)		rosé wines ($n = 7$)		white wines ($n = 6$)	
biogenic amine	mean[a]	SD[b]	mean	SD	mean	SD
putrescine	9.59	5.61	6.04	2.98	4.31	4.21
cadaverine	1.45	1.25	0.56	1.03	0.66	0.39
histamine	2.75	1.54	1.81	1.31	1.17	0.99
tyramine	2.91	1.92	1.31	0.71	0.48	0.67
spermidine			0.09	0.24		
spermine	0.16	0.25				

[a] mg/L. [b] Standard deviation.

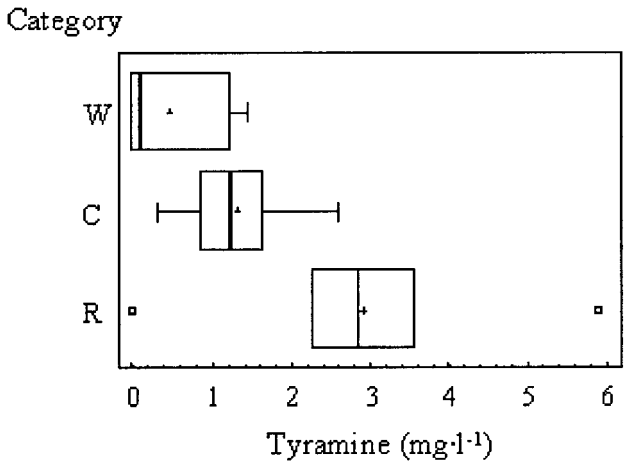

Figure 1. Multiple box-whisker plot for the content of tyramine.

were PUT, HT, TYR, and CAD, the average content being higher in red wines than in the others. This agrees with reported data (*18*).

Univariate Approach. The basic statistics for the three types of wines are given in **Table 2**.

A one-way ANOVA (analysis of variance) study showed that of the studied amines, only TYR, PUT, and HT have significance or indication of significance. The biogenic amine with the highest *discriminant ability* should be TYR, which has the lowest *P* value (0.8%). This is shown in **Figure 1**, where the values of TYR are represented in a box-whisker plot, in which the box collects 50% of the data (those between the first and the third quartiles) and the whisker is extended up to a maximum of 1.5 times the interquartile range.

The variables correlation study performed using Spearman rank order correlation (*19*) indicated that the most strongly correlated pairs of variables were PUT−HT, HT−TYR, and PUT−TYR.

Both the correlation between the variables and the ANOVA results are in accordance with those that can be directly inferred from the fermentation (mainly malolactic fermentation) that occurs during wine-making.

Finally, to establish the discriminant capacities of the variables one by one, the Fisher index (*10*) was calculated. The TYR, with a weight value >1.0, has the best discriminant capacity, whereas the rest of the amines have a Fisher weight of <0.5. It seems that among the biogenic amines analyzed, the content of TYR may be suitable for use as a simple criterion to discriminate among wines.

Unsupervised Methods. *Principal Component Analysis (PCA).* PCA is a projection method that reduces the dimensionality in a data matrix while retaining most significant information. PCA has been used for processing the biogenic amine contents to extract principal components (significant

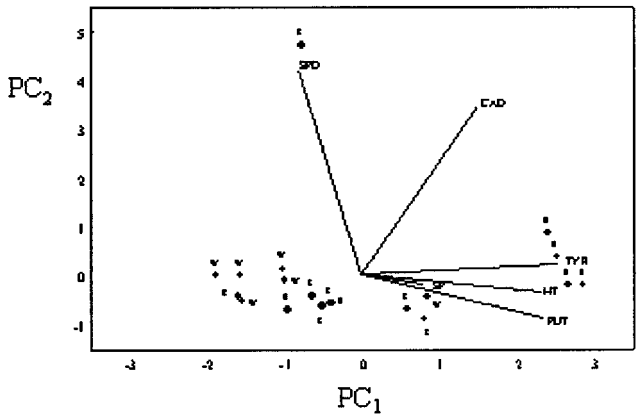

Figure 2. Principal component biplot on the first and second PCs.

variables). The six biogenic amines found are reduced to two new variables, PC_1 and PC_2 (first and second principal components, respectively).

Figure 2 shows the biplot corresponding to our data, explaining 66.2% of the total information. The first principal component, PC_1, explains 41.2%, and the second, PC_2, explains 25.0% of the total variance. It can be observed that PUT, HT, and TYR, which appear during fermentation (*20*), show similar loadings and contribute greatly to PC_1. These appear to be the most discriminating features and seem to give the same information. CAD shows intermediate loading and the other two variables, SPD and SP, are less important.

Red wines have positive scores of PC_1 except for two red wines, which have negative scores. White and rosé wines have negative scores for this component except three samples, two rosés and one white, that have positive scores. White wines, which have very negative scores, have smaller amounts of biogenic amines than red wines.

The biplot did not lead to a complete separation of the studied samples, although TYR, HT, and PUT seem to be the variables contributing the most to explain the data variance.

These findings confirm that it is possible to establish a relationship between the type of wine and the biogenic amine content, closely related to malolactic fermentation (TYR, HT, and PUT).

Cluster Analysis. Cluster analysis (*7*, *20*), an unsupervised method for pattern recognition, was applied to search for natural grouping among samples. A hierarchical agglomerative cluster analysis of samples was performed using the contents of the biogenic amines detected as variables on one side, and, on the other, the tyramine content, which showed the best Fisher weight. The squared Euclidean was used as the similarity measurement and Ward's method as the amalgamation rule. **Figure 3** shows the resulting dendograms. When the six variables are used (**Figure 3A**), two clusters appear. The first mainly contains red wines, whereas the second contains rosé and white wines, together with a misgrouped red wine. It could be suggested that red wines are clearly distinguished from the other two types of wines and that white and rosé wines have similar contents of these biogenic amines.

When only TYR is used (**Figure 3B**), two clusters can be distinguished. The first contains red wines, with a misgrouped rosé wine, whereas, in the second, the white and rosé wines are grouped. This last cluster can be split into two clusters, one with mainly white wines and the other with rosé wines.

Using the content of the six amines, a clear distinction between red wines (with malolactic fermentation) and white and rosé wines (without or with less malolactic fermentation) can

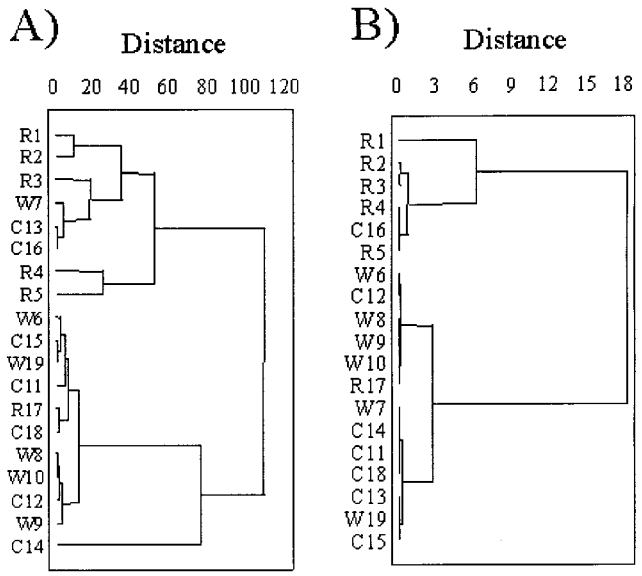

Figure 3. Dendogram constructed with Ward's method showing the results of cluster analysis: (A) using all variables; (B) using only tyramine. Wines: R, red; C, rosé; W, White. For sample labels, see **Table 1**.

Table 3. Classification Matrix for the Table Wines Analyzed

	LDA			step LDA		
category	red	rosé	white	red	rosé	white
red (6)	5 (83.3)[a]	1		5 (83.3)		1
rosé (7)		6 (85.7)	1	1	4 (57.1)	2
white (6)		1	5 (83.3)		2	4 (66.7)

[a] Percentage of cases correctly classified.

be observed. Nevertheless, using only TYR, it is also possible to differentiate between white and rosé wines.

When a white or rosé sample is misgrouped, it could indicate that it has undergone a malolactic fermentation, whereas a misgrouped red wine could indicate that it comes from pressed grapes or has undergone low malolactic fermentation.

Supervised Methods. To obtain suitable classification rules, supervised pattern recognition methods were applied to the data matrix. These methods assume a prior knowledge of the number of classes as well as the class membership of each sample.

Linear Discriminant Analysis (LDA). LDA (*21*) is applied to the data set to obtain classification rules. A basic problem in LDA is to decide which variables should be included in the analysis, that is, the feature selection. There are two approaches: (a) to use all of the variables in the model or (b) to begin with all of the variables and then, at each step, to eliminate the variable that contributes least to the prediction of group membership (stepwise discriminant analysis backward procedure, SLDA) (*9*).

When the first option was applied to the data set described above, the discriminant functions produced good percentages of correct classification (**Table 3**). The values obtained were in the 83−86% range for the three classes, showing different contents of biogenic amines for the three types of wine. To study the prediction ability, the "leave-one-out" method was applied. As the studied wines were very different in quality, the validation was carried out by removing the three which were inferior wines. A prediction performance of 81% was obtained, verifying that the inferior wines were misclassified. This fact could be due to irregular wine-making. **Figure 4** shows the scatterplot of wine samples using the two discriminant functions

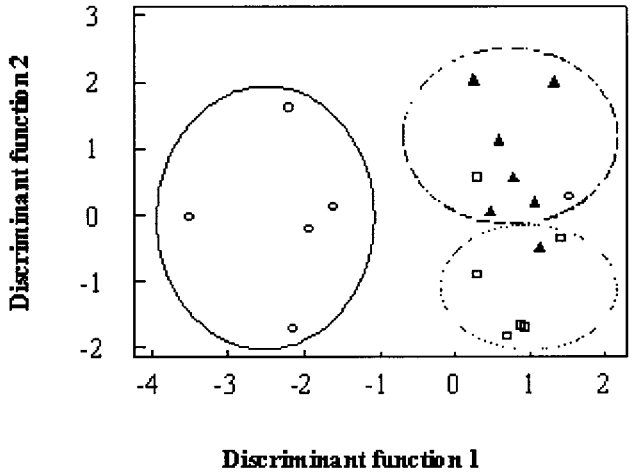

Figure 4. Representation of the discriminant functions found from biogenic amine content for all of the wines: (○) red wines; (▲) rosé wines; (□) white wines.

as axes. Red wines show negative values for function 1, whereas rosé and white wines show positive values. On the other hand, white wines show negative values for function 2, and rosé wines show positive values. However, to establish the model as a valid general one, more data should be included in the study.

The SLDA, based on the linear relationship between selected variables, was performed by calculating classification functions for the differentiation of wines. At each step the variable with the least discriminant power, measured by a Snedecor *F* statistic, was eliminated, until the elimination of more variables did not worsen the classification. The result was that only TYR remained in the model as the most discriminant variable. The following discriminant function (DF) was obtained:

$$\mathrm{DF} = -1.274 + 0.812C_{\mathrm{TYR}}$$

In this case, because only one discriminant variable was obtained, it would be possible to characterize each type of wine on the basis of its content of TYR. Thus, if centroids (average values of the DF for each type of wine) are known, cut marks can be calculated (*22*) and, by replacing them in DF, the limits of TYR content for the three types of wines are obtained. White wines show a TYR content of <0.90 mg/L, whereas red wines have a TYR content >2.11 mg/L. For rosé wines, the TYR content is between 0.90 and 2.11 mg/L.

Conclusions. The unsupervised methods used allowed us to establish the main biogenic amines for pattern recognition of wines. These are PUT, HT, and TYR, which, used together or using only TYR, allow the samples to be grouped according to the type of wine.

The LDA permits differentation, and, consequently, classification of the types of wine on the basis of the amines mentioned previously. The SLDA approach, using only the TYR content, also leads to linear separations among classes, with good discrimination, and permits characterization of these types of wines. The discriminant power of TYR was previously suggested by the univariate approach.

Finally, it has been shown that the amines related to malolactic fermentation, TYR, HT, and PUT, could be used as chemical descriptors to distinguish between the types of wines. Thus, it is possible to differentiate between wines in which malolactic fermentation is extensive (mainly red wines) and those with a limited or nonexistent malolactic fermentation (rosé and white wines). In addition, the TYR content can be used to

determine to what extent malolactic fermentation has taken place in wines in which this should not occurr, that is, irregular winemaking.

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