

Recognition of vinification technology through gas chromatographic data on enantiomeric purity of free amino acids

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Abstract. Analysis of D-amino acids in food, a matter of growing interest due to nutritional implications, has been used as a tool for food evaluation, detection of falsifications and as a genuinity parameter. The evolution of free D-amino acids in wines of a portuguese *Vitis vinifera* variety (Roupeiro white), bottled during the period 1978–1989 is studied by means of chiral high resolution gas chromatography. D-Ala, D-Val, D-Thr, D-Leu, D-Ser, D-Asx, D-Met, D-Phe, and D-Glx are present in most of the analyzed wines. It was not possible to correlate the evolution of the D/L ratio to the age of the wine. However, a clear correlation between enantiomer purity profiles and the vinification technique could be established. The results show that free D-amino acids may be used as markers for the biotechnological process used in vinification.

Introduction

The occurrence of D-amino acids in foods is undesirable for nutritional reasons, since they do not contribute to the biological value of food. Biochemically, the uptake of large amounts of D-amino acids may lead to an overloading of the D-amino oxidase system. They may originate from food processing [1–3] and are generally found in fermented foods [4] and fermented beverages, including wines [5], where some D-amino acids occur in high relative enantiomeric concentrations, probably as a consequence of the action of microorganisms [3, 4, 6]. D-Ala, D-Asx, and D-Glx are the most frequently reported D-amino acids.

In order to determine if the occurrence of D-amino acids in wines is a time related event or rather a fermentation related one, the free D-amino acids profiles were studied in bottled wines from a portuguese *Vitis vinifera* variety (Roupeiro white) produced from grapes originated from the same vineyard along the period of 1978–1989.

Dedicated to Professor Dr. Dr. h.c. mult. J.F.K. Huber on the occasion of his 70th birthday

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The results show that the relative enantiomeric contents on free D-amino acids are not time dependent. The use of endogenous yeast or inoculation with selected strains in the process of vinification is reflected in the profiles of the free D-amino acids in the wines.

Experimental

Reagents

Standard amino acids were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pentafluoropropionic anhydride (PFPA) was from Pierce Chemical Co. (Rockford, Ill., USA) and distilled prior to use. All solvents were obtained from E. Merck (Darmstadt, Germany). The 3 mol/l solution of HCl in isopropanol was prepared daily by adding the corresponding amount of acetyl chloride to 25 ml of isopropanol under ice-cooling.

Gas chromatography

Capillary gas chromatography of the pentafluoropropyl amino acid isopropyl esters was carried out with a Carlo Erba instrument, model GC 6000 Vega series 2, equipped with a split-splitless injector, a flame ionisation detector and a 15 m × 0.25 mm i.d. fused silica capillary coated with Chirasil-val[®], $d_f = 0.25 \mu\text{m}$. Analysis was performed under the following conditions: split ratio, 1:25; injector and detector temperatures, 250°C; initial oven temperature, 70°C, hold isothermally for 3 min followed by linear temperature programming at a heating rate of 3°C/min until the final temperature of 200°C was reached; carrier gas, hydrogen, $P_i = 45 \text{ kPa}$; peak integration was made with a Shimadzu, model CR3-A integrator.

Wine samples

The grapes of the *Vitis vinifera* variety Roupeiro were grown in the same vineyard each year at Reguengo do Alentejo (Alentejo, South Portugal). Elementary wines were produced at the winery of the Universidade de Évora, where the destemmed grapes were vinified without skin under two different techniques: a) without yeast inoculation and no temperature control (wines before 1983) and b) inoculated with selected yeasts, (*S. uvarum*, *S. ellipsoides*, *S. Chevallerii*) under temperature control at 18–20°C (wines after 1983). The wines were bottled after one year in corks. As bottling time malolactic fermentation was accomplished. Missing years (1979) are due to grape spoilage caused by unfavourable climatic conditions.

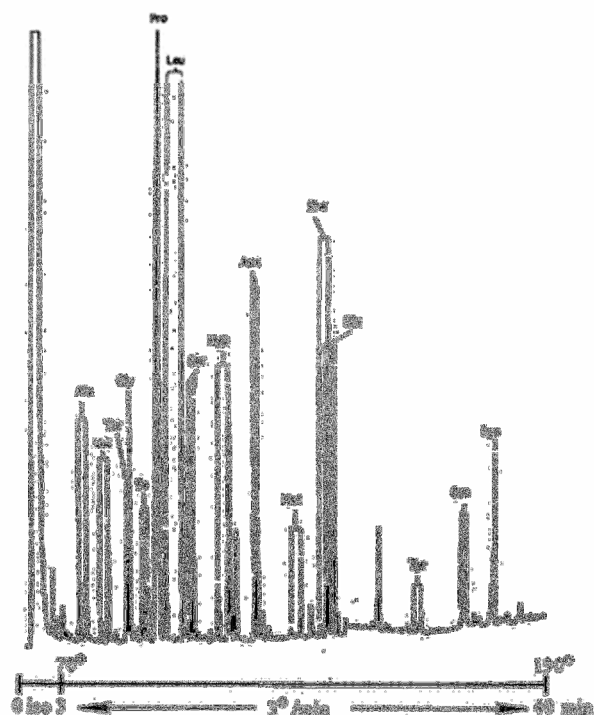


Fig. 1. Chiral capillary gas chromatography of free amino acids in an 11 years elementary wine of the *Vitis vinifera* variety Roupeiro. For conditions see Experimental

Sample preparation

The wine sample (20 ml) was deproteinized by addition 80 ml of 95% ethanol and the mixture left at -10°C for 20 min. After that time the sample was centrifuged at 3,500 rpm for 10 min. The supernatant was transferred to a 50 ml round-bottom flask and concentrated to a final volume of 10 ml in a rotary evaporator. The amino acids were isolated by ion exchange over Dowex SPW-X2, by elution with 4 mol/l NH_4OH , according to standard procedures. The eluate was concentrated to a final volume of approximately 2 ml in a rotary evaporator. A 250 μl aliquot was transferred to a derivatization vial and evaporated to dryness under a light stream of nitrogen. The residue was left overnight over P_2O_5 in vacuo. To the dry residue was added 100 μl of a 3 mol/l NCS /acetone solution. The solution was heated at 110°C for 30 min in the closed vial. After cooling to room temperature, the solvent was evaporated under a light stream of nitrogen and 50 μl of HPPA and 100 μl of CCl_4 was added to the residue. This solution was heated at 130°C for 15 min. The cooled solution was directly used for gas chromatography.

Results and discussion

Figure 1 shows a typical gas chromatogram from an 11 years old Roupeiro wine. The D-enantiomer is present for almost all the amino acids. D-Ala, D-Leu, D-Asp, and D-Glu are present in high relative enantiomeric concentrations confirming previous observations [4, 5].

The average percent enantiomeric concentrations for the D-enantiomers in each wine corresponding to the 1978–1989 vintages is represented graphically in Fig. 2. The most striking features in Fig. 2 are the high values for D-Ala observed in the 1978, 1980, and 1981 vintages which exclude the possibility of natural isomerization.

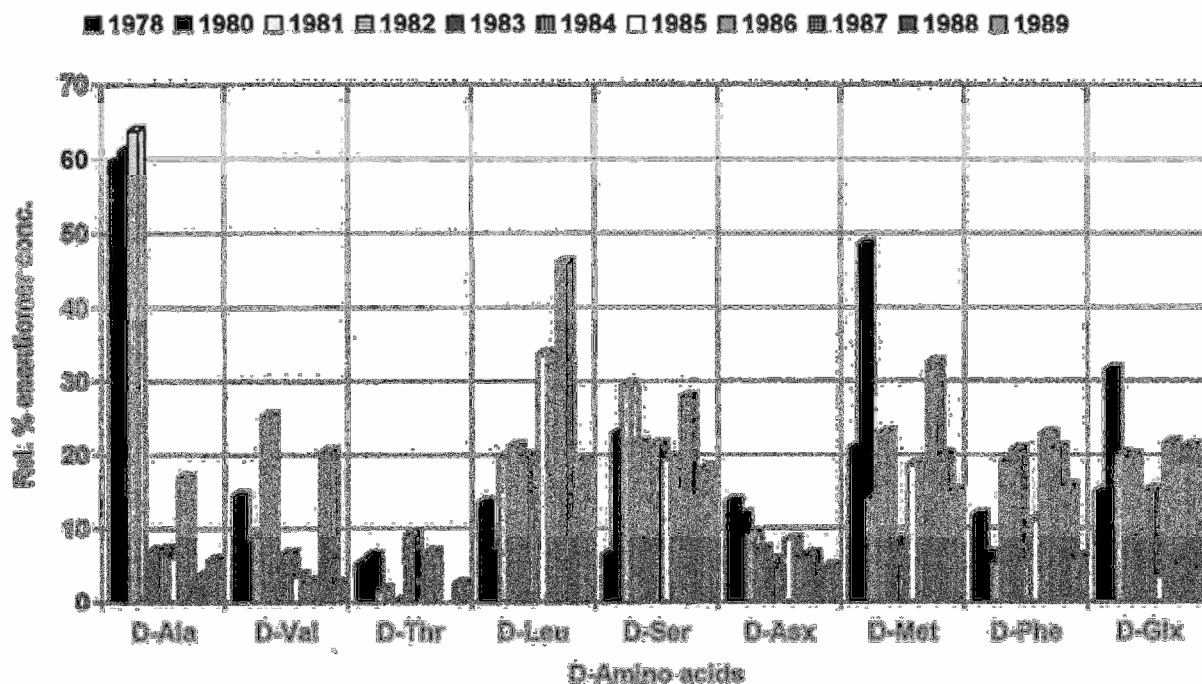


Fig. 2. Relative enantiomeric percent concentrations of free D-amino acids in elementary wines of the variety Roupeiro corresponding to the vintages from 1978 to 1989

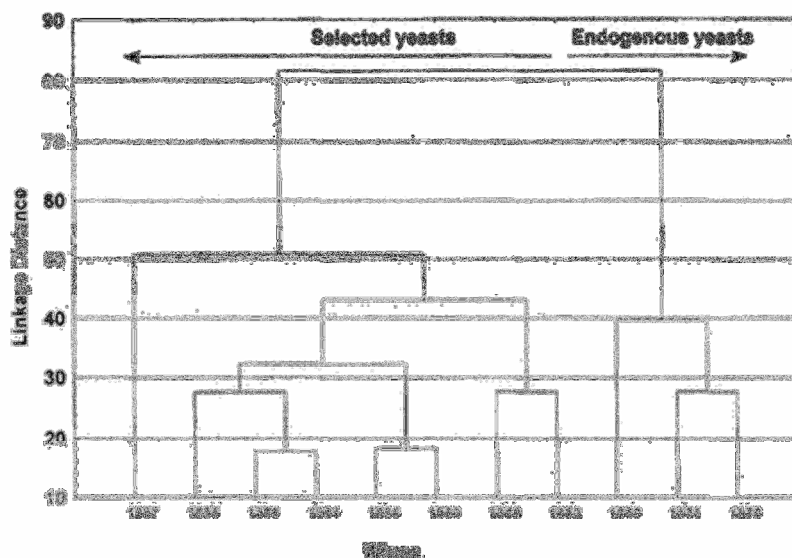


Fig. 3. Dendrogram of complete hierarchical clustering of elementary wines of the variety Roupeiro corresponding to the vintages from 1978 to 1989. The relative percent enantiomeric concentrations of free D-amino acids are used as variables. The values are grouped in two separated main clusters, corresponding to the different vinification techniques used.

There is an outlying high value for D-Met in the 1980 wine. D-Leu and D-Phe tend to be present in higher relative amounts in the vintages after 1982, while the relative content of D-Asx tend to lower values. For the other amino acids, the percent enantiomeric compositions do not show to manifest time dependences and oscillate with expected yearly variances around average values.

These data suggest that, in what concerns the relative enantiomeric composition of free amino acids, we are dealing with two different populations. However, the numerical data alone present a complex picture where the global features are difficult to apprehend. Thus, a technique for data deconvolution may be used in order to emphasize any relevant information concealed in the collected data.

If we consider each wine as an object described by a number of properties such as the relative enantiomeric composition in free D-amino acids, it is possible to assess the similarities among them by means of cluster analysis. The term cluster analysis encompasses a number of different algorithms with the purpose of organizing data into meaningful structures in the absence of any a priori hypothesis by means of some measures of similarity distance. The objective is to deconvolute a great amount of data from which no clear information can be obtained into manageable and meaningful sets of data. The purpose of the joining tree cluster technique of multivariate analysis is to join together objects (wines) described by a number of features (variables) into successively larger clusters. If the data contain information in terms of objects that are similar or dissimilar to each other, in function of distances in the multidimensional space defined by the variables, this structure may be reflected in an hierarchical representation as distinct branches from a given distance on.

The results of similarity evaluation by means of Euclidean distances — a measure of the geometrical distances in the multidimensional space — are shown in a complete linkage hierarchical clustering graphically expressed in

the dendrogram represented in Fig. 3. The dendrogram is a way of visualizing the relationships between objects represented in the horizontal axis as a function of the linkage distances. The universe of analyzed wines is split into two main different clusters. It can be seen that there are great similarities between the 1978–1982 vintages on one side and the 1983–1989 vintages on the other, suggesting that the objects may possibly be classified according to two different populations, in function of the composition in D-amino acids. These correspond to the wines produced with endogenous yeast, no inoculation, and no temperature control, and to the wines obtained by fermentation with inoculation of selected yeasts and temperature control.

A correlation between free D-amino acid profiles and the technology of vinification is apparently reflected on the hierarchical clustering. Whether such a correlation

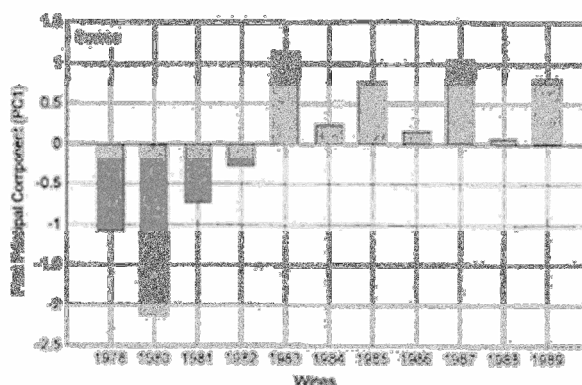


Fig. 4. PC1 scores of the individual elementary wines of the variety Roupeiro produced by two different vinification procedures during the period 1978–1989, in principal component analysis. The relative percent enantiomeric concentrations of free D-amino acids are used as variables.

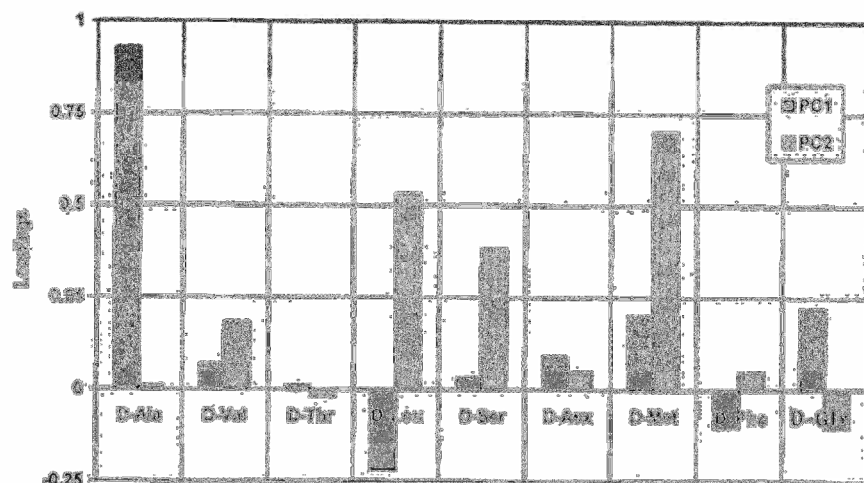


Fig. 5. Principal component analysis of the elementary wines of the variety *Roupeiro* produced in the period 1978–1989. Loadings of the free D-amino acids in the first two principal components

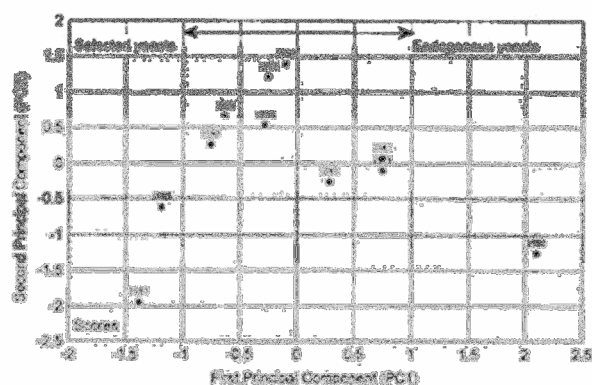


Fig. 6. Classification of the elementary wines of the variety *Roupeiro* produced in the period 1978–1989 with two different vinification techniques (see text), after data reduction. The variables used are the relative percent concentrations of D-Ala, D-Leu, D-Met, and D-Glx

may be used for classification purposes can be confirmed by means of principal component analysis (PCA). Application of principal component analysis to the data shows that 70% of the total variability between the samples is explained by the first two principal components (PCs) from which the first principal component (PC1, 58%) is responsible for the distinction of the samples according to fermentation. In Fig. 4, the scores of the individual wines in the first principal component are represented graphically. The wines are distributed along each side of the zero line showing that PC1 performs clear-cut differentiation of the samples into two different groups, encompassing the 1978–1982 vintages (negative scores) and the 1983–1989 vintages (positive scores) in agreement with the exploratory indications of hierarchical clustering.

The numerical values for variable loadings (Fig. 5) show that this differentiation is mainly due to the contents of D-Ala which has the highest loading in PC1, responsible for the classification of the wines in two groups in accordance with the vinification technology employed. D-

Leu, D-Met, and D-Glx are the variables with the highest expression in PC2, which accounts for separation of the individual wines within each group.

These observations lead to the conclusion that it is possible to detect a definite structure in the relationship between the variables as object descriptors, and that a classification of the wines according to the vinification technology used can still be achieved if only a few D-amino acids are used. Although D-Ala and D-Met alone are sufficient for a clear classification of the wine samples according to the type of yeasts used and the fermentation procedure, closer relationships within each group are obtained if the former four D-amino acids are used as descriptors.

Figure 6 shows the PCA classification of the wine samples as objects described by the four variables after data reduction. The object scores are projected in a plane defined by PC1 and PC2. The two groups are clearly separated in the projection plane, (1978–1982 wines on the left and 1983–1989 wines on the right), grouped in two distinct different zones according to the sample origin, along PC1. PC2 effects distinction between the different wines in each group due probably to the yearly variations. The first two PCs now account for 73% of the total variability among the objects. The 1982 sample lies somewhat in the border, probably due to its lower content in D-Ala.

It can be concluded that the use of endogenous yeast in the production of *Roupeiro* wines leads to a high content of D-Ala, as the most characterizing feature of this type of fermentation technique but other D-amino acids as D-Leu, D-Met and D-Glx are also common features that make the D-amino acid profiles independent of the yearly variations. Since it has been postulated that D-amino acids are related to the composition of cell membranes as a defence against exogenous enzymes, the results indicate that the presence of D-amino acids in fermented foods result mainly from cell autolysis. If the biological activity of some amino acids is considered as a potential toxicological risk of the high consumption of such foods, chiral gas chromatographic analysis of amino acid contents may be

come a very important tool for quality assessment in food chemistry for the future.

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References

1. Brückner H, Häusch M (1990) D-Amino acids in food: detection and nutritional aspects. In: Holsicht B, Frank H, Tejsa B (eds) *Chirality and biological activity*. Liss, New York, pp 129–136
2. Brückner H, Häusch M (1990) *Milchwissenschaft* 45: 337–360
3. Brückner H, Häusch M (1990) *Milchwissenschaft* 45: 421–425
4. Brückner H, Häusch M (1989) *Chromatographia* 28: 487–490
5. Chatain J, Naves H, Vayssières AMF, Guez H. (1990) *Determination of wine free amino acids as function of bottling age*. In: Holsicht B, Frank H, Tejsa B (eds) *Chirality and biological activity*. Liss, New York, pp 137–143
6. Brückner H, Lüpke M (1991) *Chromatographia* 31: 123–128