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Determination of twenty eight biogenic amines and amino acids during wine aging by micellar electrokinetic chromatography and laser-induced fluorescence detection

G. Nouadje[a,b], N. Siméon[a], F. Dedieu[b], M. Nertz[a], Ph. Puig[b], F. Couderc[c,*]

[a]ZETA Technology, Parc Technologique du Canal, 31520 Ramonville-Toulouse, France
[b]Laboratoire de Chimie Analytique et Bromatologie, Université des Sciences Pharmaceutiques, 31062 Toulouse Cedex, France
[c]Laboratoire de Biologie Moléculaire Eucaryote, IBCG du CNRS 118 Route de Narbonne, 31062 Toulouse Cedex, France

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Abstract

28 biogenic amines and amino acids were quantitated in red French wines over a 14 year period by micellar electrokinetic chromatography separation and laser-induced fluorescence detection of fluorescein thiocarbamate derivatives. The grapes were grown in the same wine yard, under identical conditions, and the wines were made under fixed standard procedures. A pattern of evolution of amines and amino acids during wine aging is given. Principal component analysis indicates some correlations between the different amino acids and biogenic amines. A discussion on the behavior of amino acids during wine aging is presented.

Keywords: Wine; Laser-induced fluorescence; Micellar electrokinetic chromatography; Amines; Biogenic amines; Amino acids

1. Introduction

Amino acids are significant factors in the growth of yeast and bacteria that produce wine. They are present in the mature fruit of *Vitis vinifera* and accumulate in the berries during maturation [1]. In addition, in wine science, it is generally accepted that free amino acids contribute to the wine's aroma, taste and appearance [2].

The amount of each amino acid, in wine and must, varies widely according to variety [3,4], yeast and bacteria strain [5], region [6], treatment [7] and age [8].

Because amino acids are poorly affected by alcoholic fermentation they have often been chosen as labels for the different varieties of grapes [3,4]. Free alanine, glycine, valine, serine, proline, cysteine, methionine and tyrosine in the total amino acid fraction were especially affected by vintage year, in a 2 year period [8]. Yet, the evolution of the different amino acids found in wine during aging has been poorly studied over a long period and results are quite difficult to interpret [3]. The longest period studied for wine ripening has been 7 years in some Portuguese vineyards [3]. The use of this kind of analysis has been demonstrated to be useful in identifying a variety of different wines [3,4,9]. Otherwise, yearly treatment of wine and climatic change are the most influent parameters on amino acid composition. To our knowledge, no attempt has

*Corresponding author.

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been made to interpret amines or amino acid concentration evolution following the time of ripening.

Gas chromatography–mass spectrometry [3], high-performance liquid chromatography (HPLC) [9], thin layer chromatography [10] and capillary electrophoresis (CE) [2] have been used to quantitate some amino acids and amines in wines.

We have previously demonstrated that micellar electrokinetic chromatography (MEKC) associated with laser-induced fluorescence, allows a very quick, sensitive and selective determination of amino acids and amines in a very complex medium such as wine [2]. This previous study was limited to quantitating eight biogenic amines and amino acids in some different wines.

In this study we will present the first attempt to quantify 28 amino acids and amines, in a single CE run, during a 14 year period of ripening of a French red wine from the Cahors region. A principal component analysis (PCA) attempts to find some correlations between amino acids and biogenic amines quantity following time. Some attempts on amino acid (AA) or biogenic amines (BA) behaviors in wine aging are presented.

2. Experimental

2.1. Instrument and separations conditions

A modular injector and high voltage power supply Prime Vision 100 (Europhor Instruments (now Zeta Technology), Toulouse-Ramonville, France) equipped with a modular capillary-LIF detector (Zeta Technology, Toulouse-Ramonville, France) and a 488 nm wavelength laser (type 54225A, ILT, Salt Lake City, UT, USA) were used. A 75 cm×50 mm I.D. fused-silica capillary (Polymicro Technology, Phoenix, AZ, USA) has an effective length of 42 cm.

All chemicals were purchased from Aldrich (St Quentin Falavier, France).

The separation buffer consisting of 20 m*M* of sodium dodecyl sulfate (SDS) and 100 m*M* boric acid, pH 9.3, was adjusted by addition of sodium hydroxide. The capillary was rinsed with 0.1 *M* NaOH for 3 min, with water for 2 min, and then with separation buffer for 3 min. Samples were injected by hydrodynamic injection for 2 s (15 nl).

Separations were carried out by applying a separation potential of +20 kV resulting in an electrophoretic current of 43μA.

2.2. Derivatization procedure

A $2.1 \cdot 10^{-4}$ *M* solution of fluorescein isothiocyanate (FITC) isomer I in acetone was prepared by dissolving 2.5 mg of FITC in 30 ml of acetone. Then 2 mg of each AA or BA were dissolved in 2 ml of 0.2 *M* carbonate buffer at pH=9.0. One ml of each AA or BA solution was allowed to react with 1 ml of FITC solution for 2 h in the dark. At the same time, 1 ml of a $2.1 \cdot 10^{-4}$ *M* solution of the FITC in acetone was mixed with 1 ml of 0.2 *M* carbonate buffer to obtain a blank and kept in darkness for 2 h. Then, both the FITC solution and the amino acid plus FITC solution were diluted 10 000 times by step of 10 in water.

50 μl of each wine yearly vintage was mixed with 50 μl of $2.1 \cdot 10^{-4}$ *M* FITC solution, and kept to react for 2 h in darkness. The resulting solution was diluted 2000 times prior to injection.

2.3. Data collection and analysis

Data collection, processing and analysis were performed using an integrator (Merk, Darmstadt, Germany). Data were collected at a sampling rate of 10 Hz. Peaks were identified by spiking wine samples with known quantities of standards solutions of AA or BA. To obtain the quickest, unambiguous identification procedure with a minimum of runs, we made four standard solutions, where each AA or BA were well separated: Solution 1= Putrescine, arginine, histamine, ethylamine, spermidine, threonine, phenylalanine–valine, serine, taurine, glutamic acid, aspartic acid, cysteic acid. Solution 2= Lysine, ethanolamine, ammonia, spermine, isoleucine–leucine, tryptophane, alanine, glycine. Solution 3= Ornithine, β-phenylethylamine, proline, asparagine, cysteine. Solution 4= Histidine, tyramine, tyrosine. The identification of the 28 AA or BA necessitated at least four runs for each yearly wine sample. Previously we found that the derivation yield is roughly identical for AA or BA and the calibration curves are quite similar [2,11,12]. We calculated the ratio of each AA or BA prior to total amount of AA or BA

by measuring the ratio of peak area of AA or BA to total identified peak area.

Repeatability of injections and derivation of AA and BA by FITC are quite good and R.S.D. is below 6%, either for standards or wine samples.

2.4. Principal component analysis

Each wine sample was regarded as an assembly of features. The normalized percent amino acid or amine composition was calculated as the mean of three replicates for each amino acid or amine derivative. Principal component analysis [3,13,14] was performed by means of the statistical software package Statview II SE+ Graphic ([ABACUS concept] Alfa Système Diffusion-Alsyd, Grenoble, France).

2.5. Wine samples

Grapes were obtained from the same vineyards cultivated under the same conditions to eliminate the possible variations due to different soils, climatic conditions and fertilization procedures. All grapes were vinified according to controlled and yearly identical procedures and treated identically over the 14 year period. Wines were stocked at room temperature in years 1978–1990 and stocked at constant temperature (18°C) for the following years. We previously indicated that AA and BA in the wines could be easily identified in very diluted solutions [2]. Prior to analysis, we diluted our sample 2000 times, which allows us to obtain very high separation power and to avoid the influence of the native fluorescence of molecules like flavonoids.

3. Results and discussions

Table 1 presents the efficiencies of analyses which are obtained from a standard mixture of AA and BA in a concentration range of 10^{-9} *M*. A very high number of theoretical plates was achieved in most of the cases varying from 2 890 000 to 520 000 theoretical plates. Leucine and isoleucine were not separated, and phenylalanine and valine were difficult to separate. Methionine was not easily identified and quantitated (results not reported). For our analysis we will consider valine–phenylalanine and leucine–isoleucine and the other independent AA and BA.

Free amino acid and biogenic amine composition of each wine sample is presented in Table 2. Fig. 1 presents an electropherogram of a sample of 1985 wine diluted 2000 times and labelled by FITC. Some peaks remained unidentified, and may correspond to unusual amino acids or other amines.

The relative percent concentration of AA and BA in the wines from the grape variety in the different years shows high relative deviations and falls outside the parameters of a normal distribution. Some AA and BA are present occasionally: spermidine, putrescine, histamine, taurine, arginine, aspartic acid, cysteic acid. The very high relative concentration of proline varies and increases with ages. It confirms the unreliability of taking the proline test for general wine authenticity [3]. These results confirm that, on the basis of the AA and BA composition alone, no typification of wine can be done. Fig. 2 presents the variation of the normalised total amount of the identified AA (without proline), BA and the concentration of proline during wine aging. Surprisingly, we see that the total concentration of AA and BA increases with age (Fig. 2), which means that the proteases from the yeasts or bacteria which are still in the wine [15] are quicker to degrade the proteins contained in the wine [16] to produce AA, than the decarboxylases that degrade AA into BA. Finally the increase of AA with age is quicker than the increase of BA. The total amount of amino acid and amines decreased sharply in 1985, 1988 and 1989 because of very bad climatic conditions during these two periods.

Because we have a large number of variables (14 yearly wines) we tried to correlate the variation pattern of each AA and BA by using a multivariate analysis as principal component analysis.

Principal component analysis (PCA) calculates linear combinations of variables (here, each relative concentration of AA or BA) describing as much of the variance of the original data as possible. This allows us to simplify an original multidimensional matrix (which comprises a rectangular contingency table showing the occurrences of combinations of relative abundance of BA and AA and the year) to be simplified without substantial loss of information,

Table 1
Efficiencies for FITC labeled amino acid and biogenic amine standard solutions

Standard solution	Migration time (min)	Number of theoretical plates $\times 10^3$
Amino acids		
Arginine	8.77	652
Lysine	9.05	659
Threonine	13.21	1405
Isoleucine–Leucine	13.67	392
Proline	13.92	567
Phenylalanine	14.11	341
Tryptophane	14.21	581
Asparagine	14.41	596
Tyrosine	14.68	1730
Serine	14.99	1810
Alanine	15.22	1040
Cysteine	15.97	1960
Glycine	16.15	1180
Glutamic acid	21.99	2890
Aspartic acid	23.01	1070
Biogenic amines or unusual amino acids		
Putrescine	7.08	672
Histamine	9.45	740
Ornithine	9.68	1710
Ethanolamine	9.99	803
Ethylamine	10.13	1870
Tyramine	10.18	834
β-Phenylethylamine	10.54	894
Ammonia	10.79	525
Spermidine	11.11	532
Taurine	15.97	2050

and eases interpretation of complex data matrices. The results of PCA can be multiple. In this study, we display a plot of the correlation between the different AA, BA and the total amount of amino acid. Principal component 1 (PC1) accounted for 26% of the total variability, while principal component 2 (PC2) accounted for 25%. Since it is very difficult to describe what the biological factors which influence PC1 and PC2 are, AA and BA were classified in six different pools as indicated in Fig. 3. In each pool AA or BA have approximately the same PC1 and PC2, showing the same evolutions with time of these components in the wine.

Pool 1 (negative PC 1 weigh, positive PC2 weigh): tryptophane, phenylalanine and valine, leucine and isoleucine, lysine and total amino acid amount.

Pool 2 (low PC1 weigh, positive PC2 weigh): arginine, histamine, spermine, β-phenylethylamine.

Pool 3 (positive PC1 weigh, positive PC2 weigh): glycine, serine, aspartic acid, tyrosine, cysteic acid, alanine.

Pool 4 (positive PC1 weigh, positive PC2 weigh): ornithine, asparagine.

Pool 5 (positive PC1 weigh, low positive PC2 weigh) threonine, ethanolamine, glutamic acid.

Pool 6 (high positive PC1 weigh, low negative PC2 weigh): ethylamine, taurine, spermidine, histidine, cysteine.

Pool 7 (low positive PC1 weigh, negative PC2 weigh): tyramine, ammonia.

Proline (high negative PC1 weigh, high negative PC2 weigh) and putrescine (low negative PC1 weigh, high positive PC2 weigh) cannot be pooled. Total amino acid amount belong to the pool 1.

Biological pathways of taurine and cysteic acids shows that they are synthesized from cysteine [17],

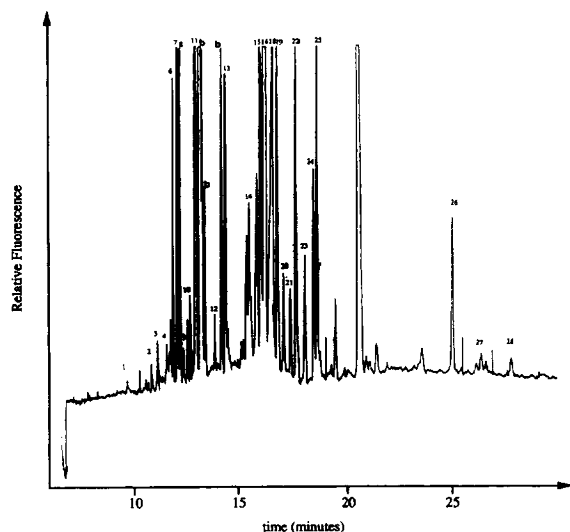

Fig. 1. CE-LIF separation of FITC labelled 1985 wine diluted 2000 times after derivation. Buffer 20 m*M* SDS, 100 m*M* Boric acid, pH=9.3. Hydrodynamic injection 2 s (15 nl). +20 kV. 1=putrescine; 2=arginine; 3=lysine; 4=histamine; 5=ornithine; 6=histidine; 7=ethanolamine; 8=ethylamine; 9=tyramine; 10=β-phenylethylamine; 11=ammonia; 12=spermidine; 13=spermine; 14=threonine; 15=leucine–isoleucine; 16=proline; 17=phenylalanine–valine; 18=tryptophane; 19=asparagine; 20=tyrosine; 21=serine; 22=alanine; 23=cysteine; 24=taurine; 25=glycine; 26=glutamic acid; 27=aspartic acid; 28=cysteic acid. b: shows FITC peaks, identified in the blank sample. Peaks 5 and 17 are not identified in the 1985 wine.

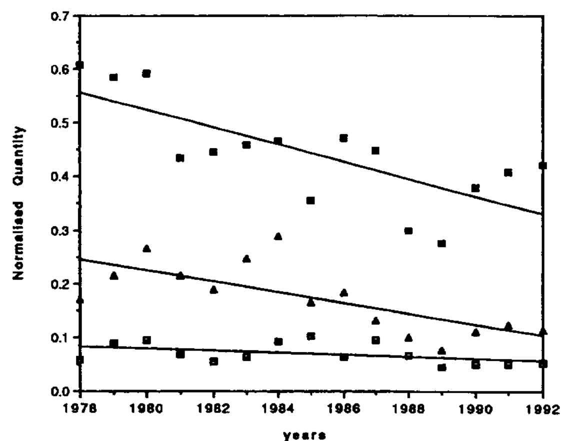

Fig. 2. Evolution of: (■) normalised (prior to total AA and BA concentration) concentrations of AA (without Proline), (□) BA and (▽) Proline during wine aging.

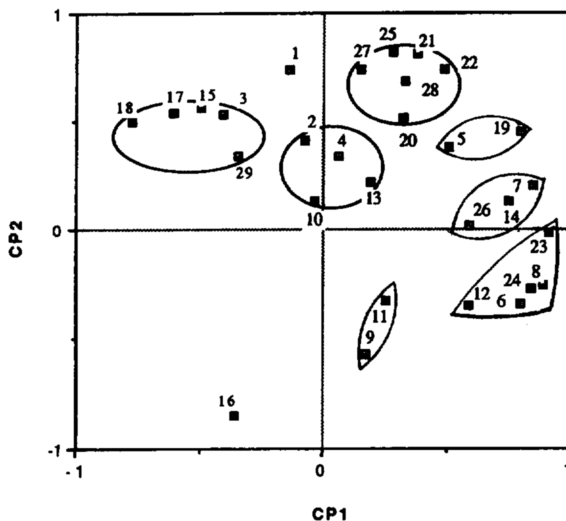

Fig. 3. Plot of component weights in first principal component (CP1) versus component weights in second principal component (CP2) from PCA analysis of free AA and BA concentrations in elementary wines from *V. vinifera* from Cahor Region (France) from 1978 to 1990. Sample labels as in Fig. 1. 29=Total (AA+BA).

and that spermidine and spermine come from arginine and ornithine [18]. Interestingly, CPA does not find any correlation during wine aging between, β-phenylethylamine and phenylalanine, histamine and histidine, spermidine and arginine–ornithine, ethylamine and alanine, cysteic acid and cysteine, tyramine and tyrosine. The only correlations following the biosynthetic pathways of biogenic amines are taurine and cysteic acid (pool 6), arginine and spermine (pool 2). The low number of correlations could be due to the rate of degradation of the wine proteins into amino acid quicker than the rate of degradation of amino acids into biogenic amines. Such poor aging correlations between the evolution of concentrations of the precursor amino acids and their related biogenic amines, have been found earlier. For example, correlations between glutamic acid and γ-butyric acid [3] or between ornithine and putrescine [19] were found elsewhere, even if some studies found the contrary [13], in this last study, the wine aging is quite short (3 years).

Proline seems to have a particular behavior (Fig. 3), as registered previously [3], it cannot be correlated with other studied components. Proline has a

Table 2
Normalised percent free amino acids and biogenic amines of yearly wines from *V. vinifera* from the Cahors region (France) from 1978 to 1992

Year	putrescine	Arg	Lys	histamine	His	ornitine	ethanolamine	ethylamine	tyramine	ß-phenylethylamine	ammonia	spermidine	spermine
1992	0.1	0.0	0.3	0.3	0.7	0.2	3.1	0.5	0.3	0.3	2.1	0.0	2.3
1991	0.1	0.0	0.3	0.3	0.9	0.3	2.7	0.5	0.3	0.3	2.1	0.0	2.3
1990	0.0	0.0	0.2	0.1	1.4	0.1	2.7	0.7	0.3	0.4	2.7	0.0	2.3
1989	0.0	0.0	0.1	0.0	1.5	0.3	4.3	1.5	0.3	0.4	2.3	0.4	2.2
1988	0.0	0.2	0.2	0.3	2.3	0.3	4.2	1.4	0.2	0.3	3.4	0.3	3.9
1987	0.1	0.1	0.3	0.2	0.5	0.2	2.0	0.3	0.2	0.4	7.7	0.0	3.2
1986	0.1	0.2	0.2	0.2	1.1	0.2	3.2	0.4	0.2	0.4	0.8	0.1	3.5
1985	0.1	0.1	0.2	0.2	1.4	0.3	5.2	2.1	0.2	0.3	5.0	0.3	3.3
1984	0.9	0.8	0.3	0.7	0.7	0.3	3.9	0.4	0.2	0.3	1.6	0.0	2.9
1983	0.3	0.1	0.8	0.1	0.5	0.2	2.9	0.4	0.1	0.3	1.1	0.0	3.0
1982	0.2	0.0	0.2	0.1	0.6	0.2	3.2	0.4	0.2	0.4	0.8	0.0	3.0
1981	0.2	0.1	0.6	0.1	0.8	0.5	3.5	0.4	0.2	0.7	1.8	0.0	2.6
1980	0.1	0.7	0.2	0.0	0.9	0.2	4.2	0.4	0.1	0.2	1.2	0.1	3.6
1979	0.2	0.2	0.5	0.2	0.8	0.2	4.1	0.4	0.2	0.3	1.6	0.2	3.2
1978	0.2	0.9	0.5	0.1	0.3	0.1	1.8	0.2	0.1	0.3	0.7	0.3	3.3

Year	Thr	Ile Leu	Pro	PheVal	Trp	Asn	Tyr	Ser	Ala	Cys	taurine	Gly	Glu	Asp	cysteic acid
1992	0.0	4.2	71.1	4.3	3.6	1.1	0.3	0.3	1.7	0.3	0.3	1.5	1.0	0.1	0.0
1991	0.8	4.3	71.2	4.7	2.9	1.1	0.3	0.3	1.4	0.4	0.3	1.6	0.8	0.0	0.0
1990	0.7	4.7	71.0	3.7	2.4	1.1	0.4	0.3	1.4	0.7	0.6	1.3	0.8	0.0	0.0
1989	1.1	2.9	70.5	1.3	1.9	1.4	0.4	0.4	1.4	0.9	2.5	1.2	0.7	0.0	0.0
1988	2.0	2.7	64.1	3.1	1.7	1.7	0.7	0.3	2.1	0.6	1.6	1.3	0.7	0.1	0.1
1987	0.2	3.7	67.5	5.1	5.1	0.6	0.4	0.2	0.8	0.3	0.1	0.6	0.1	0.0	0.0
1986	0.5	6.1	66.8	4.4	4.4	1.2	0.4	0.3	2.0	0.5	0.6	1.9	0.1	0.2	0.1
1985	0.8	3.8	58.8	1.8	1.8	2.6	0.6	0.6	2.8	1.0	1.8	2.8	1.2	0.2	0.2
1984	1.1	5.1	56.5	7.4	4.6	1.9	0.8	0.9	3.0	0.6	0.7	3.2	0.7	0.2	0.1
1983	0.6	5.4	60.8	8.9	4.9	1.2	1.1	0.6	2.2	0.4	0.4	2.8	0.6	0.2	0.1
1982	0.4	4.9	66.0	9.4	4.5	0.8	0.3	0.2	1.1	0.2	0.5	1.7	0.4	0.2	0.3
1981	1.0	4.9	61.5	4.2	4.2	1.9	0.6	0.7	3.2	0.6	0.0	4.6	0.7	0.3	0.1
1980	1.1	4.2	63.7	4.9	3.0	2.0	0.5	0.6	3.2	0.7	0.7	3.2	0.2	0.0	0.0
1979	0.8	4.9	67.5	4.6	3.3	1.3	0.3	0.3	1.4	0.5	0.6	2.5	0.2	0.0	0.0
1978	0.3	3.9	73.6	4.1	4.1	1.2	0.4	0.3	1.9	0.3	0.0	0.8	0.2	0.0	0.0

particular evolution independent from the other amino acids. Its concentration following time is quite uncoordinated, and could be related to the wine stocking temperature. Proline concentration could not be correlated to glycine, serine, tyrosine as it has been done previously on a very short aging time [8] nevertheless glycine, serine, tyrosine are located in the same pool 3 of Fig. 3.

Total amino acid quantity is not correlated to proline quantity but to phenylalanine and valine, leucine and isoleucine, lysine and tryptophane, as partially advocated in [8]. Tryptophane, phenylalanine, valine, leucine and isoleucine are the most abundant AA after proline in the wine.

4. Conclusion

In this study we presented an CE-LIF analysis over 14 years of amino acids and biogenic amines in red wine coming from the same vineyard. Because of the very high number of theoretical plates obtained by using micellar electrokinetic chromatography, CE-LIF allowed us to identify and quantify 28 amino acid and biogenic amines in each run. We attempted to draw some conclusions on the behavior of the different AA or BA. It seems that proline has its own evolution independent of the other amino acid during ripening. Among the twenty eight biogenic amines or unusual amino acids, we found only two correlations (taurine/cysteic acid; arginine/spermine) during time of quantity evolution of amino acid and their corresponding degradation product. The poor number of correlations could correspond to the fact that the total quantity of amino acid increases with age, with a slow increase of biogenic amine quantity. The quickest degradation of wine protein into amino acid compared to the slowest degradation of these amino acids in biogenic amines, could be confirmed by the analysis of alcohols and acids (obtained by deamination of AA).

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