Changes in the Amino Acid Composition of the Different Nitrogenous Fractions during the Aging of Wine with Yeasts

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A study was made of the composition of free and total amino acids, amino acids in peptides, and amino acids in proteins in four base wines and the sparkling wines obtained from them following the Champenoise method. Samples were taken of the sparkling wines after 9, 12, 15, 18, 24, and 31 months of aging with yeasts. The results were evaluated using different multivariate analysis techniques. It was observed that the release of amino acids during aging does not occur in all wines at the same time. The free amino acid composition of sparkling wines depends on that of the base wines from which they originate, while the distribution of amino acids in peptides and proteins depends, at least partly, on the aging time with yeasts. The findings indicate the existence of a relationship between the aging time of the wine with yeasts and the amino acid composition of peptides with a molecular weight lower than 700 Da.

Keywords: *Amino acids; peptides; proteins; wine; sparkling wine*

INTRODUCTION

In the manufacture of sparkling wines by the Champenoise or traditional method, there is a period of aging with yeasts, the length of which varies depending on the legislation in each country and on the quality of the wine it wishes to produce. During this stage the yeasts undergo autolysis, and as a result an intense interchange of compounds between the yeasts and wine confer on the latter specific sensory characteristics, making it different from other wines.

In a previous work on peptides in musts and wines, and on the changes occurring in this nitrogenous fraction during the manufacture of sparkling wines in the presence of yeasts following the traditional or Champenoise method (Moreno-Arribas et al., 1996), we reported that peptides are released from the yeasts together with proteases that in turn hydrolyze these peptides into less polymerized forms. Later (Bartolomé et al., 1997), it was observed that individual peptides in sparkling wines of different varieties, but submitted to the same production process, seemed to be similar. It was also observed, with the use of capillary electrophoresis (Luguera et al., 1997), that during secondary fermentation and the first stages of the aging of wine with yeasts, the proteins in wine decrease and at the same time there is a release of polypeptide forms probably originating from the yeasts. In those papers, however, the characterization of the peptides and proteins themselves was not fully examined.

The free amino acid composition of wines and the changes that occur during manufacture and aging with yeasts have been the subject of numerous studies (Suárez et al., 1979; Feuillat and Charpentier, 1982; Margheri et al., 1983; Colagrande et al., 1984; Silva et al., 1987). There are few works on the study of the amino acid composition of the peptides and proteins in wine, among them those of Yokotsuka et al. (1975, 1991), Usseglio-Tomasset and Bosia (1990), Acedo et al. (1994), Waters et al. (1995), and Marchal et al. (1996). All of these papers refer to the composition of these compounds in still wines. Moreover, to our knowledge, there is no information on what changes occur in these fractions during the manufacture of sparkling wines.

This study was undertaken with the aim of characterizing these nitrogenous fractions and to discover the main cause of the variability existing in the amino acid composition of each of these fractions during the aging of wine with yeasts. To this end, four varietal sparkling wines were manufactured industrially, and samples were taken of the base wines and of the sparkling wines after 9, 12, 15, 18, 24, and 31 months of aging in the bottle with yeasts. For each of the wines, a study was made of their composition in free and total amino acids, amino acids in peptides with an approximate molecular weight lower and higher than 700, respectively, and amino acids in proteins. Results were analyzed using analysis of variance, principal component analysis, and cluster analysis. In order to predict the aging time from nitrogenous fraction, partial least-squares (PLS) regression was also used.

MATERIALS AND METHODS

Manufacture of Wines. Four varietal base wines were industrially manufactured from white grapes of the Macabeo, Xarel.lo, Parellada, and Chardonnay varieties of the Penedès region (Spain). Wines were obtained from sulfited must (80 mg of SO_2/L) in 100 000-L tanks at 16−18 °C by inoculation with a selected winery yeast (*Saccharomyces cerevisiae*). Clarification was carried out with the addition of 20 g of bentonite/hL and 1 g of gelatin/hL, and the wines were tartrate-stabilized (−4 °C, 48 h).

The sparkling wines were obtained by the Champenoise method from the varietal base wines. Sucrose (21 g/L) and bentonite (3 g/hL) were added to the clarified base wines, and

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10.1021/jf9803381 CCC: $15.00
Published on Web 09/24/1998

they were inoculated with a yeast culture from the winery's collection (*Saccharomyces bayanus*). Degorging was performed after 9, 12, 15, 18, 24, and 31 months of aging with yeasts. Six bottles at each degorging time were mixed and homogenized before sampling. All the analyses were conducted in duplicate on wines that had been centrifuged for 15 min at 5000*g*.

Isolation of the Peptide and Protein Fractions. Wines (200 mL) were concentrated under vacuum (to 10 mL) and then precipitated with five volumes of 95% ethanol in an acid medium (Usseglio-Tomasset and Castino, 1975). After centrifugation (10000*g*, 30 min), two fractions were obtained, the supernatant (ethanol-soluble fraction) and the precipitate (ethanol-insoluble fraction). Two peptide fractions were obtained from the ethanol-soluble fraction, as described previously (Moreno-Arribas et al., 1996), by chromatography on a Sephadex G-10 column (Pharmacia Fine Chemical, Uppsala, Sweden). Fractions corresponding to peptides with molecular weights higher and lower than 700, respectively, were collected.

Hydrolysis. For amino acid analysis, samples of wines and of each fraction containing proteins (ethanol-insoluble fraction) and peptides (fractions from the Sephadex G-10 column) were lyophilized. Aliquots of each lyophilized fraction were subjected, in duplicate, to acid hydrolysis with 6 M HCl and thioglycolic acid under vacuum atmosphere for 24 h at 110 °C.

Amino Acid Analysis. Prior to RP-HPLC analysis, all samples were diluted with 0.4 M borate buffer, pH 10. Amino acid analysis was carried out by RP-HPLC using a Waters liquid chromatograph controlled by the Maxima 820 program. Samples were submitted to an automatic precolumn double derivatization with *o*-phthaldialdehyde (OPA) to determine primary amino acids and with 9-fluorenylmethyl chloroformate (FMOC) to detect secondary amino acids (Einarsson, 1985). All separations were performed on a Waters Nova-Pak C_{18} column (150 × 3.9 mm i.d., 60 Å, 4 μm). Eluents and gradient conditions were as described by González de Llano et al. (1991). Detection was by fluorescence using wavelengths of excitation and emission at 340 and 425 nm, respectively, for OPA derivatives and at 250 and 335 nm, respectively, for FMOC derivatives. Samples were injected in duplicate onto the column after being filtered through a 0.22 μm filter. Variation coefficients of the amino acid determination, including the hydrolysis, were from 3.5% to 9.8%. Due to the partial conversion of asparagine and glutamine into aspartic acid and glutamic acid, respectively, during hydrolysis, the data for asparagine plus aspartic acid and glutamine plus glutamic acid are reported as Asx and Glx, respectively, in all the hydrolyzed samples. The absence of tryptophan and methionine in the hydrolyzed samples may be ascribed to the breakdown of these amino acids during hydrolysis.

Statistical Methods. The statistical methods used for analysis were as follows: Two-way analysis of variance was used to test the effects of variety and time factors; Studen−Newman−Keuls test was used for means comparisons; principal component analysis (from correlation matrix) was used to examine the relationship among the variables; cluster analysis (Ward's method, from standardized data) was used to discover natural groupings of the samples and partial least-squares (PLS) regression (Geladi and Kowalski, 1986) to predict the aging time of the samples from the nitrogenous fraction. Statistica (1996) and Unscrambler (1996) programs were used for data processing. These programs were run on a personal computer.

RESULTS

Free and Total Amino Acids. Figure 1a,b shows the sum of free and total amino acids, respectively, in the base wines and the sparkling wines after 9, 12, 15, 18, 24, and 31 months of aging with yeasts for the four varieties. The free and the total amino acid contents of the Chardonnay variety base wine (1478 and 2121 mg/L, respectively) are much higher than those of the other three varieties studied (from 417 to 511 mg/L for free amino acids and from 538 to 865 mg/L for total amino acids). Millery et al. (1986) observed in two consecutive harvests that the free amino acid content of the Chardonnay variety musts was greater than that of the other two varieties studied, Pinot Noir and Pinot Meunier. The greater amino acid content of the musts, and consequently of the wines, of the Chardonnay variety than that of other grape varieties is probably a genetic characteristic of this variety.

The wines of the Macabeo, Xarel.lo and Parellada varieties, after 9 months of aging with yeasts, show a lower free and total amino acid content than the base wine, while that of the Chardonnay variety shows a free amino acid content higher than that of the base wine from which it originates. During aging with yeasts there is an increase in free amino acids (Figure 1a). This increase was not detected in all the wines at the same time, although the four wines were manufactured in the same winery and under the same conditions. In the Chardonnay variety wine, the increase in free amino acids was detected after 15 months of aging; in the Xarel.lo and Parellada wines, the increase was detected between 9 and 12 months and in the Macabeo variety, between 12 and 15 months. After 18 months in the Macabeo, Parellada, and Chardonnay variety wines and after 24 months in the Xarel.lo variety wine, there is a decrease in free amino acids. This may be due to deamination or decarboxylation reactions (Feuillat and Charpentier, 1982) or to the formation of esters (Herraiz et al., 1993). After 31 months of aging with yeasts, the Macabeo, Xarel.lo, and Chardonnay variety wines show a lower free amino acid concentration than the base wines from which they originate.

After secondary fermentation and 9 months of aging with yeasts, the Macabeo and Chardonnay variety wines show a higher concentration of total amino acids than the corresponding base wines (Figure 1b). The Xarel.lo variety wine, after 9 months of aging with yeasts, shows a lower concentration of total amino acids than its corresponding base wine, and the Parellada variety wine shows a total amino acid concentration similar to that of the base wine from which it originates.

The total amino acid/free amino acid ratio increases between 9 and 12 months in the Macabeo, Xarel.lo, and Parellada variety wines and decreases later. This indicates that during aging peptides and/or proteins are released and later hydrolyzed into amino acids.

To summarize the results obtained from the individual analysis of amino acids, Table 1 shows the mean and the standard deviation values of free amino acid content in the wines of the four varieties, grouped by time of aging with yeast. Because of the differences in the free amino acid concentrations in the wines of the different varieties, the values of the amino acids have been expressed as the percentage molar distribution, so as to facilitate comparison. The table also indicates the results of the application of the two-way analysis of variance (the interaction and the within error terms were pooled) to study whether significant differences existed between amino acid content due to the variety and/or aging time factors. For amino acids in which differences do not exist among varieties, the results of the application of the Student−Newman−Keuls test to

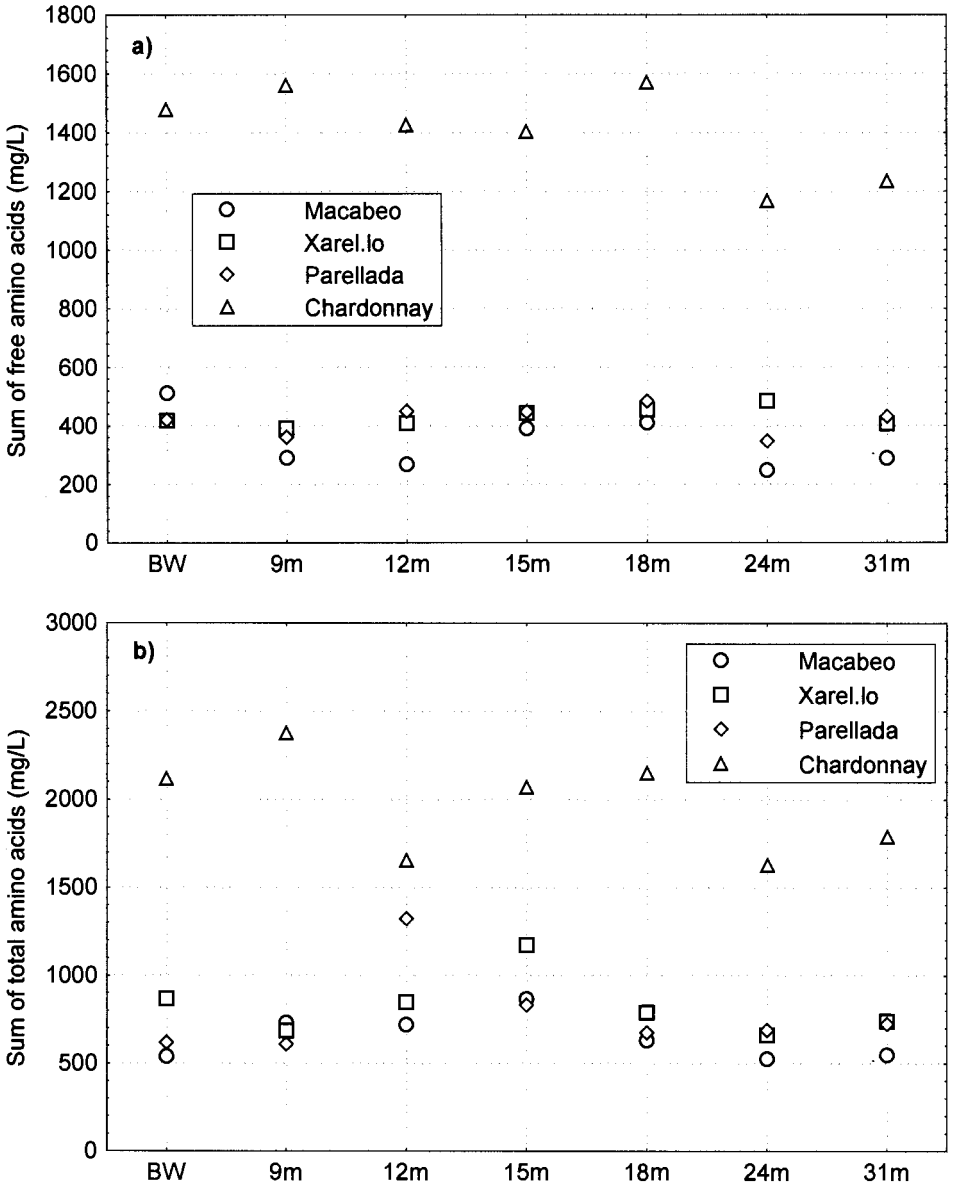

Figure 1. Sum of free (a) and total (b) amino acids (mg/L) in the base wines (BW) and in the sparkling wines after 9, 12, 15, 18, 24, and 31 months of aging with yeast.

compare the means at the different aging times are also indicated.

The major amino acid, in all the wines, is proline, which accounts for 57.20% of total amino acids in the base wine and for 49.4−66.7% of those in the sparkling wines. Of the other amino acids, those present in the greatest proportions in the base wines are α-alanine, γ-aminobutyric acid, glutamic acid, and arginine. These results are similar to those obtained by De la Presa-Owens et al. (1995) in base wines of the Macabeo, Xarel.lo, and Parellada varieties in a study of three consecutive vintages.

More significant differences ($p < 0.05$) due to the variety than to the aging time were observed (Table 1). The only difference between the base wines and the wines after secondary fermentation and 9 months of aging with yeasts occurs for the amino acid glutamine which decreases. Between 9 and 31 months of aging with yeasts, small variations were observed in the amino acids glutamine, β-alanine, and tryptophan.

Amino Acid Composition of Peptides. The data of the molar distribution (mean and standard deviation of the four varieties) of the amino acids of peptides with a molecular weight lower than 700 in the base wines and the sparkling wines, together with the results of the application of the two-way analysis of variance and the Student−Newman−Keuls test, are shown in Table 2.

It should be pointed out that the proline content of the peptides (9.16−15.15%) is much lower than the content of this amino acid in the free form (49.40−66.70%). The major amino acids in the base wines are lysine, glycine, γ-aminobutyric acid, proline, threonine, serine, and aspartic acid plus asparagine. Glycine, aspartic acid plus asparagine (Asx), serine, and proline are also the major amino acids in the peptides of still wines studied by other authors (Yokotsuka et al., 1975; Usseglio-Tomasset and Bosia, 1990; Acedo et al., 1994).

No differences have been found in amino acids from peptides with molecular weights lower than 700 due to the variety. Peptides with a molecular weight lower than 700 in the sparkling wines after 9 months of aging with yeasts show a distribution of amino acids very similar to that of the corresponding base wines (Table

Table 1. Mean ± Standard Deviation Values of Free Amino Acid Molar Distribution (%) in the Base Wines and Sparkling Wines ($n = 4$) and Results of Two-Way Analysis of Variance When the Interaction and the Within Error Terms Were Pooled

amino acids	factor effect								
	variety	time	base wine	9 months	12 months	5 months	8 months	24 months	31 months
Asp	*	ns	2.25 ±1.09	1.65 ± 0.13	2.14 ± 0.45	1.87 ± 0.38	1.84 ± 0.33	2.36 ± 0.60	2.08 ± 0.47
Glu	*	*	4.27 ± 1.49	2.60 ± 0.31	3.12 ± 0.40	2.99 ± 0.44	2.02 ± 0.23	2.65 ± 0.25	2.53 ± 0.44
Asn	*	*	2.09 ± 0.63	3.73 ± 0.50	2.94 ± 0.51	3.30 ± 0.95	1.95 ± 0.20	2.39 ± 0.41	1.41 ± 0.18
Ser	*	ns	1.33 ± 0.33	1.09 ± 0.38	0.91 ± 0.24	1.14 ± 0.27	0.98 ± 0.17	1.32 ± 0.29	0.91 ± 0.22
Gln	ns	*	0.50^{b} ± 0.12	0.08^{a} ± 0.02	0.03^{a} ± 0.04	0.00^{a} ± 0.00	0.65^{b} ± 0.12	0.98^{c} ± 0.51	0.00^{a} ± 0.00
His	*	*	1.73 ± 0.79	1.41 ± 0.23	0.86 ± 0.26	2.22 ± 0.48	0.39 ± 0.14	1.37 ± 0.70	1.37 ± 0.19
Gly	*	*	2.62 ± 1.33	3.36 ± 0.86	3.02 ± 0.95	2.01 ± 0.23	2.58 ± 0.71	2.52 ± 0.77	2.26 ± 0.72
Thr	ns	ns	0.73^{a} ± 0.25	1.32^{a} ± 0.43	1.05^{a} ± 0.32	2.70^{a} ± 2.39	0.89^{a} ± 0.19	1.39^{a} ± 0.39	1.12^{a} ± 0.25
Arg	*	*	3.13 ± 1.07	3.67 ± 2.16	3.40 ± 1.69	4.83 ± 2.37	2.75 ± 1.42	2.85 ± 1.43	2.72 ± 1.14
β-Ala	ns	*	0.61^{ab} ± 0.08	0.68^{ab} ± 0.12	0.49^{ab} ± 0.15	1.58^{c} ± 0.48	3.10^{d} ± 0.64	1.13^{bc} ± 0.67	0.02^{a} ± 0.03
α-Ala	*	*	6.99 ± 3.75	7.33 ± 4.94	7.06 ± 3.87	9.17 ± 4.25	5.93 ± 2.81	6.40 ± 3.59	5.87 ± 3.23
GABA	*	*	5.46 ± 2.10	5.84 ± 3.03	5.30 ± 2.22	6.30 ± 2.42	4.05 ± 1.96	3.66 ± 1.57	3.24 ± 1.46
Tyr	*	ns	1.27 ± 0.47	1.26 ± 0.19	1.25 ± 0.19	1.41 ± 0.28	1.05 ± 0.22	1.38 ± 0.34	1.29 ± 0.33
Met	*	*	0.45 ± 0.19	0.12 ± 0.02	0.02 ± 0.02	0.42 ± 0.10	0.26 ± 0.04	0.63 ± 0.29	0.54 ± 0.18
Val	*	*	0.98 ± 0.34	0.98 ± 0.15	0.79 ± 0.13	1.28 ± 0.12	0.81 ± 0.04	1.31 ± 0.29	1.34 ± 0.27
Trp	ns	*	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.09^{a} ± 0.19	0.74^{b} ± 0.20	0.00^{a} ± 0.00	0.00^{a} ± 0.00
Phe	*	*	1.36 ± 0.67	1.08 ± 0.24	0.78 ± 0.20	1.35 ± 0.32	1.57 ± 0.37	1.43 ± 0.40	1.51 ± 0.45
Ile	*	*	0.44 ± 0.21	0.27 ± 0.05	0.09 ± 0.08	1.65 ± 0.95	1.52 ± 0.33	0.55 ± 0.23	0.78 ± 0.26
Leu	*	ns	2.29 ± 1.25	1.90 ± 0.35	2.01 ± 0.62	2.10 ± 0.68	1.58 ± 0.41	1.96 ± 0.52	2.27 ± 0.72
Orn	*	*	1.92 ± 1.32	2.07 ± 1.13	1.63 ± 0.98	1.75 ± 0.96	3.76 ± 1.42	2.25 ± 1.22	0.44 ± 0.15
Lys	*	*	2.37 ± 1.12	3.58 ± 1.06	3.25 ± 1.26	2.48 ± 0.54	2.98 ± 0.82	2.71 ± 1.11	1.59 ± 0.56
Pro	*	*	57.21 ± 12.44	55.95 ± 7.89	59.85 ± 5.39	49.35 ± 3.79	58.59 ± 2.78	58.76 ± 7.26	66.69 ± 5.04

$^{a-d}$ Mean values in the same row with the same superscript indicate that there are no significant differences between them. *, significant differences ($p < 0.05$); ns, no significant differences; GABA, γ-aminobutyric acid.

Table 2. Mean ± Standard Deviation Values of the Amino Acid Molar Distribution (%) in Peptides with Weights Lower than 700 Da in the Base Wines and Sparkling Wines ($n = 4$) and Results of Two-Way Analysis of Variance When the Interaction and the Within Error Terms Were Pooled

amino acids	factor effect								
	variety	time	base wine	9 months	12 months	15 months	18 months	24 months	31 months
Asx	ns	*	8.16^{b} ± 0.80	7.31^{ab} ± 0.73	7.69^{ab} ± 0.22	8.28^{b} ± 1.17	6.17^{a} ± 0.59	10.90^{c} ± 1.75	12.20^{c} ± 0.37
Glx	ns	*	5.58^{b} ± 0.43	6.80^{c} ± 0.22	6.79^{c} ± 0.63	6.85^{c} ± 0.54	5.87^{bc} ± 0.51	9.70^{d} ± 0.23	4.72^{a} ± 0.84
Ser	ns	*	8.43^{bc} ± 0.73	8.51^{bc} ± 1.84	12.23^{d} ± 1.63	10.74^{cd} ± 2.85	6.77^{ab} ± 0.22	4.73^{a} ± 2.85	3.72^{a} ± 0.91
His	ns	*	2.23^{abc} ± 1.11	3.16^{cd} ± 0.35	1.27^{ab} ± 0.68	0.45^{a} ± 0.27	0.90^{ab} ± 0.57	4.45^{d} ± 1.66	2.72^{bc} ± 1.10
Gly	ns	ns	12.66^{a} ± 2.63	9.45^{a} ± 1.46	11.72^{a} ± 2.45	12.12^{a} ± 1.47	11.30^{a} ± 0.39	9.50^{a} ± 1.97	11.15^{a} ± 1.73
Thr	ns	*	8.85^{c} ± 1.35	11.22^{cd} ± 2.32	13.20^{d} ± 1.42	11.60^{cd} ± 3.17	6.70^{b} ± 0.35	4.77^{ab} ± 2.05	3.05^{a} ± 0.34
Arg	ns	ns	2.07^{a} ± 0.27	1.88^{a} ± 0.62	2.25^{a} ± 0.60	2.37^{a} ± 0.12	2.62^{a} ± 1.08	3.12^{a} ± 0.52	3.61^{a} ± 2.53
β-Ala	ns	*	0.51^{a} ± 0.36	0.63^{a} ± 0.35	1.02^{a} ± 0.61	1.23^{a} ± 1.33	4.32^{c} ± 1.16	2.95^{b} ± 0.89	0.02^{a} ± 0.04
α-Ala	ns	*	1.46^{a} ± 0.42	8.51^{bc} ± 1.02	10.37^{d} ± 1.26	9.38^{cd} ± 1.43	7.58^{b} ± 0.13	11.72^{e} ± 0.64	13.65^{f} ± 0.71
GABA	ns	*	10.98^{b} ± 0.59	0.44^{a} ± 0.32	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.19^{a} ± 0.26
Tyr	ns	*	0.74^{ab} ± 0.61	0.21^{a} ± 0.21	2.16^{c} ± 0.92	1.69^{bc} ± 0.96	0.63^{ab} ± 0.10	1.90^{c} ± 0.75	0.17^{a} ± 0.14
Val	ns	*	6.74^{b} ± 0.41	7.36^{b} ± 0.42	6.23^{ab} ± 1.32	7.26^{b} ± 0.36	6.89^{b} ± 0.79	6.00^{ab} ± 1.21	4.79^{a} ± 0.91
Phe	ns	*	0.84^{b} ± 0.22	0.25^{a} ± 0.33	0.75^{ab} ± 0.06	0.86^{ab} ± 0.18	1.21^{b} ± 0.35	1.74^{c} ± 0.55	0.49^{ab} ± 0.12
Ile	ns	*	4.36^{bc} ± 0.32	5.14^{c} ± 0.17	4.06^{b} ± 0.49	4.82^{bc} ± 0.28	4.78^{bc} ± 0.28	3.93^{bc} ± 0.95	2.67^{a} ± 0.56
Leu	ns	ns	3.91^{a} ± 0.36	4.32^{a} ± 2.00	4.13^{a} ± 0.41	4.19^{a} ± 0.45	3.54^{a} ± 0.30	5.06^{a} ± 1.03	3.09^{a} ± 0.56
Orn	ns	*	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.87^{a} ± 1.75	3.32^{b} ± 1.87	0.00^{a} ± 0.00	3.31^{b} ± 1.29
Lys	ns	*	12.90^{ab} ± 2.56	15.65^{b} ± 5.51	6.45^{a} ± 3.63	7.95^{a} ± 2.53	12.38^{ab} ± 3.38	8.20^{a} ± 0.73	18.23^{b} ± 2.78
Pro	ns	ns	9.58^{a} ± 4.04	9.16^{a} ± 2.32	9.69^{a} ± 2.76	9.33^{a} ± 6.04	15.01^{ab} ± 3.14	11.32^{a} ± 3.54	11.77^{a} ± 5.14

$^{a-f}$ Mean values in the same row with the same superscript indicate that there are no significant differences between them. *, significant differences ($p < 0.05$); ns, no significant differences; GABA, γ-aminobutyric acid.

2). The exceptions to this are glutamic acid plus glutamine and α-alanine, which increase, and γ-aminobutyric acid and phenylalanine, which decrease. Between 9 and 31 months of aging in the bottle, there are significant differences in the amino acids aspartic acid plus asparagine, α-alanine, and ornithine, which increase, and glutamic acid plus glutamine, serine, threonine, valine, and isoleucine, which decrease.

Table 3 summarizes the data of the molar distribution (mean and standard deviation of the four varieties) of the amino acids in peptides with an approximate molecular weight of more than 700, for each stage studied, and the results of two-way analysis of variance. For amino acids in which there are no differences among varieties, the results of the application of the Student−Newman−Keuls test to compare the means at the different aging times are also shown. The amino acids found in the highest proportions in the peptides of the base wines are serine, glycine, proline, γ-aminobutyric acid, and threonine. It should be pointed out that in these peptides, the lysine content (4.85%) is much lower than the content of the same amino acid in the peptides below 700 Da (12.90%).

From the application of the two-way analysis of variance, only significant differences due to the variety have been found for the amino acids α-alanine and tyrosine. The peptides of the sparkling wines after 9 months of aging with yeast show a distribution of amino acids similar to that of the base wine, with the exception of the higher percentage of threonine and the lower percentage of γ-aminobutyric acid, which decrease after secondary fermentation, as in the peptides with a molecular weight below 700. Between 9 and 31 months

Table 3. Mean ± Standard Deviation Values of the Amino Acid Molar Distribution (%) in Peptides with Weights Higher than 700 Da in the Base Wines and Sparkling Wines ($n = 4$) and Results of Two-Way Analysis of Variance When the Interaction and the Within Error Terms Were Pooled

amino acids	factor effects								
	variety	time	base wine	9 months	12 months	15 months	18 months	24 months	31 months
Asx	ns	*	$7.54^{ab} \pm 1.44$	$7.33^{a} \pm 1.46$	$8.29^{ab} \pm 0.63$	$7.86^{ab} \pm 0.59$	$7.01^{a} \pm 0.40$	$8.88^{ab} \pm 0.83$	$9.51^{b} \pm 0.58$
Glx	ns	*	$5.79^{a} \pm 0.75$	$6.37^{a} \pm 0.94$	$6.83^{ab} \pm 0.38$	$6.16^{a} \pm 0.43$	$5.82^{a} \pm 0.22$	$10.11^{c} \pm 0.51$	$7.55^{b} \pm 0.66$
Ser	ns	*	$11.15^{a} \pm 1.08$	$10.75^{a} \pm 1.67$	$14.63^{b} \pm 1.73$	$13.49^{b} \pm 1.42$	$9.84^{a} \pm 1.30$	$9.15^{a} \pm 1.00$	$10.33^{a} \pm 1.02$
His	ns	*	$4.66^{b} \pm 1.20$	$3.93^{ab} \pm 4.36$	$2.16^{ab} \pm 0.85$	$1.72^{ab} \pm 0.12$	$0.39^{a} \pm 0.09$	$3.57^{ab} \pm 0.69$	$2.08^{ab} \pm 0.59$
Gly	ns	ns	$10.77^{a} \pm 1.92$	$8.44^{a} \pm 1.27$	$10.35^{a} \pm 1.88$	$9.32^{a} \pm 0.66$	$9.61^{a} \pm 0.56$	$7.85^{a} \pm 2.13$	$9.02^{a} \pm 0.33$
Thr	ns	*	$9.32^{a} \pm 0.79$	$13.40^{b} \pm 1.56$	$14.55^{b} \pm 1.19$	$13.10^{b} \pm 1.93$	$10.02^{a} \pm 1.34$	$10.29^{a} \pm 1.39$	$9.68^{a} \pm 1.39$
Arg	ns	ns	$1.71^{a} \pm 0.45$	$1.63^{a} \pm 0.59$	$1.65^{a} \pm 0.43$	$1.46^{a} \pm 0.29$	$1.56^{a} \pm 0.21$	$2.12^{a} \pm 0.24$	$2.40^{a} \pm 1.81$
β-Ala	ns	*	$1.89^{ab} \pm 2.84$	$0.18^{a} \pm 0.33$	$2.82^{ab} \pm 0.63$	$2.16^{ab} \pm 0.48$	$3.37^{b} \pm 1.63$	$2.08^{ab} \pm 0.60$	$0.00^{a} \pm 0.00$
α-Ala	*	*	1.87 ± 1.18	8.41 ± 3.03	11.64 ± 0.71	9.95 ± 1.32	9.52 ± 0.72	10.29 ± 1.76	11.08 ± 1.59
GABA	ns	*	$9.84^{b} \pm 6.74$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.14^{a} \pm 0.29$
Tyr	*	*	3.08 ± 1.13	2.76 ± 1.34	3.27 ± 0.97	3.58 ± 0.49	2.93 ± 0.46	2.20 ± 0.39	1.04 ± 0.35
Val	ns	ns	$5.09^{ab} \pm 0.83$	$5.80^{ab} \pm 0.86$	$5.39^{ab} \pm 1.13$	$4.54^{a} \pm 0.58$	$5.16^{ab} \pm 0.45$	$5.56^{ab} \pm 0.36$	$6.45^{b} \pm 0.37$
Phe	ns	*	$2.13^{a} \pm 1.07$	$1.24^{a} \pm 0.64$	$1.08^{a} \pm 0.30$	$1.02^{a} \pm 0.11$	$1.75^{a} \pm 0.41$	$2.23^{a} \pm 0.64$	$1.35^{a} \pm 0.29$
Ile	ns	*	$3.68^{ab} \pm 0.51$	$4.34^{b} \pm 0.45$	$3.82^{ab} \pm 0.63$	$3.28^{a} \pm 0.51$	$3.80^{ab} \pm 0.25$	$4.24^{b} \pm 0.28$	$4.39^{b} \pm 0.31$
Leu	ns	ns	$5.45^{a} \pm 0.52$	$5.77^{a} \pm 0.65$	$5.34^{a} \pm 0.66$	$5.01^{a} \pm 0.78$	$4.99^{a} \pm 0.47$	$5.50^{a} \pm 0.79$	$5.51^{a} \pm 1.19$
Orn	ns	*	$0.71^{a} \pm 0.62$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$2.12^{b} \pm 1.41$	$0.00^{a} \pm 0.00$	$0.48^{a} \pm 0.72$
Lys	ns	*	$4.85^{ab} \pm 1.88$	$5.96^{ab} \pm 0.89$	$3.73^{a} \pm 3.08$	$2.04^{a} \pm 0.44$	$2.17^{a} \pm 1.78$	$4.12^{ab} \pm 0.86$	$7.67^{b} \pm 2.34$
Pro	ns	*	$10.47^{bc} \pm 5.05$	$13.68^{b} \pm 4.19$	$4.45^{a} \pm 2.35$	$15.29^{b} \pm 7.09$	$19.95^{b} \pm 4.28$	$11.84^{ab} \pm 3.34$	$10.63^{ab} \pm 0.65$

$^{a-c}$ Mean values in the same row with the same superscript indicate that there are no significant differences between them. *, significant differences ($p < 0.05$); ns, no significant differences; GABA, γ-aminobutyric acid.

Table 4. Mean ± Standard Deviation Values of the Amino Acid Molar Distribution (%) in Proteins in the Base Wines and Sparkling Wines ($n = 4$) and Results of Two-Way Analysis of Variance When the Interaction and the Within Error Terms Were Pooled

amino acids	factor effects								
	variety	time	base wine	9 months	12 months	15 months	18 months	24 months	31 months
Asx	*	*	10.28 ± 1.36	9.19 ± 0.36	12.24 ± 0.77	8.80 ± 0.44	9.52 ± 0.88	9.33 ± 1.75	10.49 ± 1.31
Glx	ns	*	$6.52^{bc} \pm 0.73$	$2.17^{a} \pm 1.82$	$7.74^{c} \pm 1.15$	$5.78^{bc} \pm 0.52$	$7.60^{c} \pm 0.58$	$11.46^{d} \pm 1.00$	$4.65^{b} \pm 1.57$
Ser	*	*	9.83 ± 1.94	9.42 ± 0.94	14.70 ± 2.38	10.05 ± 2.24	7.85 ± 2.10	8.92 ± 4.10	3.53 ± 1.97
His	ns	*	$6.18^{d} \pm 1.35$	$0.27^{a} \pm 0.55$	$5.81^{d} \pm 0.81$	$8.31^{e} \pm 1.35$	$0.43^{a} \pm 0.07$	$1.94^{b} \pm 0.85$	$3.32^{c} \pm 0.25$
Gly	*	*	8.32 ± 0.71	10.04 ± 0.89	7.56 ± 1.36	7.56 ± 0.53	7.37 ± 0.87	5.26 ± 1.15	7.60 ± 0.82
Thr	*	*	7.28 ± 1.50	7.50 ± 0.62	8.21 ± 1.21	6.54 ± 1.32	5.10 ± 1.15	6.13 ± 2.42	2.51 ± 0.95
Arg	*	*	5.02 ± 0.81	5.91 ± 2.89	6.16 ± 3.88	7.92 ± 5.54	7.63 ± 6.86	6.09 ± 4.51	9.22 ± 8.95
β-Ala	ns	ns	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$1.24^{ab} \pm 1.70$	$1.88^{b} \pm 0.84$	$0.38^{ab} \pm 0.11$	$1.45^{ab} \pm 0.78$	$0.00^{a} \pm 0.00$
α-Ala	ns	*	$11.64^{d} \pm 1.26$	$6.45^{a} \pm 0.76$	$10.53^{cd} \pm 0.86$	$8.51^{b} \pm 0.28$	$7.68^{b} \pm 0.42$	$9.94^{c} \pm 0.98$	$11.01^{cd} \pm 0.55$
GABA	ns	*	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$1.88^{c} \pm 0.33$	$0.67^{ab} \pm 0.39$	$0.58^{ab} \pm 0.34$	$0.00^{a} \pm 0.00$	$1.00^{c} \pm 0.67$
Tyr	ns	*	$0.46^{ab} \pm 0.56$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.58^{ab} \pm 0.06$	$0.53^{ab} \pm 0.02$	$0.00^{a} \pm 0.00$	$0.59^{ab} \pm 0.33$
Val	*	*	3.13 ± 1.00	6.73 ± 0.95	0.70 ± 0.46	2.60 ± 0.35	2.47 ± 0.45	3.39 ± 0.84	2.36 ± 0.89
Phe	ns	*	$1.02^{ab} \pm 0.21$	$2.50^{b} \pm 2.05$	$0.27^{a} \pm 0.16$	$0.65^{a} \pm 0.09$	$0.46^{a} \pm 0.02$	$1.35^{ab} \pm 0.36$	$1.03^{ab} \pm 0.32$
Ile	*	*	1.87 ± 0.72	4.40 ± 0.52	1.00 ± 0.54	1.39 ± 0.10	1.21 ± 0.24	1.85 ± 0.36	1.38 ± 0.47
Leu	*	*	2.35 ± 0.74	5.47 ± 0.75	1.15 ± 0.67	1.49 ± 0.05	1.37 ± 0.35	2.24 ± 0.15	1.96 ± 0.60
Orn	*	*	5.07 ± 2.07	10.37 ± 2.30	0.62 ± 0.27	4.58 ± 1.28	6.14 ± 1.80	6.67 ± 1.57	10.98 ± 4.65
Lys	*	*	9.06 ± 1.63	10.18 ± 0.83	9.83 ± 1.79	8.29 ± 0.34	11.30 ± 1.21	6.48 ± 2.44	12.10 ± 0.57
Pro	*	*	10.20 ± 2.51	9.39 ± 2.94	10.36 ± 3.54	14.39 ± 0.47	22.38 ± 1.10	17.49 ± 5.77	16.25 ± 2.69

$^{a-e}$ Mean values in the same row with the same superscript indicate that there are no significant differences between them. *, significant differences ($p < 0.05$); ns, no significant differences; GABA, γ-aminobutyric acid.

of aging with yeasts, aspartic acid plus asparagine and glutamic acid plus glutamine increase and threonine decreases.

Amino Acid Composition of Proteins. The mean and standard deviation values of the amino acids in the protein fraction of the base wines and the sparkling wines at different times of aging with yeasts are shown in Table 4. The table also shows the results of the two-way analysis of variance. The amino acids found in the highest proportions in the base wine proteins are α-alanine, aspartic acid plus asparagine, proline, serine, lysine, and glycine. These findings are similar to those observed by Yokotsuka et al. (1991), Waters et al. (1995), and Marchal et al. (1996) in other still wines. Only the amino acids glutamic acid plus glutamine, histidine, β-alanine, α-alanine, γ-aminobutyric acid, and tyrosine are not influenced by the variety. For the rest of the amino acids significant differences have been found after secondary fermentation and 9 months of aging with yeasts. The proteins of the wines show higher values of glycine, valine, leucine, isoleucine, and ornithine and lower values of glutamic acid plus glutamine, histidine, and α-alanine. After 31 months of aging with yeasts, the proteins in the wines show higher significant percentages of glutamic acid plus glutamine, histidine, and α-alanine.

Principal Component Analysis. To gain greater knowledge of the causes of the variability of the values found for the amino acid composition of the different fractions, principal component analysis, from the correlation matrix, was performed on the data of the molar distribution of free amino acids, the amino acids in peptides with a molecular weight lower and higher than 700, respectively, and the amino acids in proteins. The number of principal components was selected by cross-validation. To aid in the interpretation, the principal components selected were rotated (varimax method). From the analysis of the free amino acids, it was observed that about 52.1% of the variation in these values could be explained by the first two principal

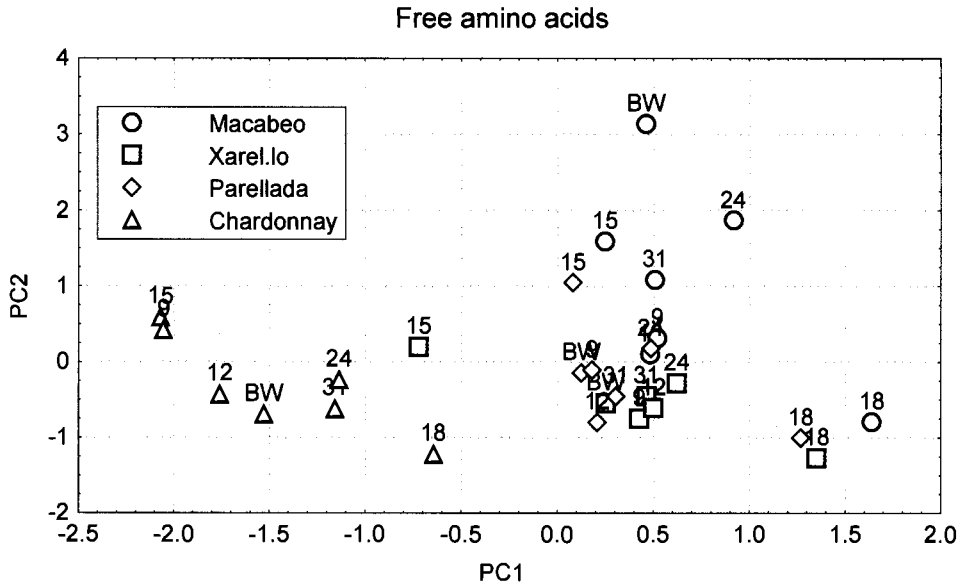

Figure 2. Plot of the 28 samples of wines on the plane defined by the two first principal components from data of free amino acids.

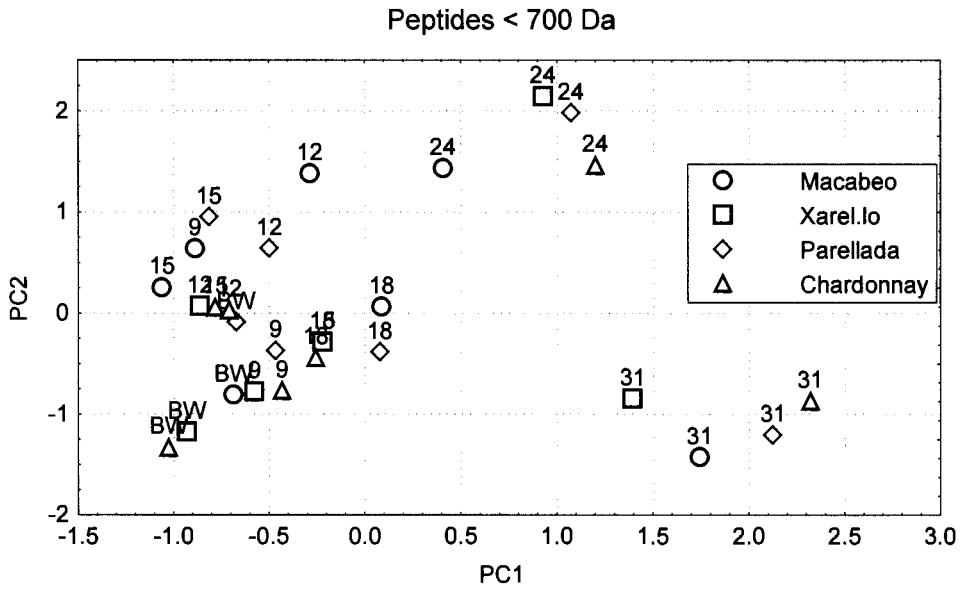

Figure 3. Plot of the 28 samples of wines on the plane defined by the two first principal components from data of amino acids in peptides with weights lower than 700 Da.

components. Arginine (−0.906), α-alanine (−0.906), γ-aminobutyric acid (−0.891), and ornithine (0.744) were strongly correlated with the first principal component, while histidine (0.858), tyrosine (0.786), valine (0.778), leucine (0.749), and aspartic acid (0.722) contributed more strongly to the second principal component.

From the plot of the 28 wines on the plane defined by these two principal components (Figure 2), the Chardonnay variety wines were grouped on the left side of the plane and were separate from the other variety wines. The Xarel.lo variety wines were similar to those of the Parellada variety and are grouped closely together with them in the graph. This result reveals that the major cause of variation in the free amino acid values of sparkling wines is the variety of grape from which they originate.

Figure 3 shows the principal component analysis representation of wines according to the molar distribution of amino acids in peptides lower than 700 Da. The first two principal components account for 47.8% of the variance. Isoleucine (−0.791), threonine (−0.787), valine (−0.751), aspartic acid plus asparagine (0.735), and serine (−0.717) are the amino acids that contribute most toward explaining the first component, whereas the second principal component is more correlated with glutamic acid plus glutamine (0.827), lysine (−0.774), and tyrosine (0.767). In Figure 3 it is observed that the base wines of the four varieties and the wines after 9, 12, 15, and 18 months of aging form one grouping and appear separate from the rest, which were related to the first principal component. The wines with 24 and 31 months of aging have different characteristics from each other and are also different from those wines with shorter aging times. In general, wines are grouped more by aging time than by variety.

When principal component analysis was performed on the values of the molar distribution of the amino acids in peptides with weights higher than 700 Da, nine principal components were selected which explain 91%

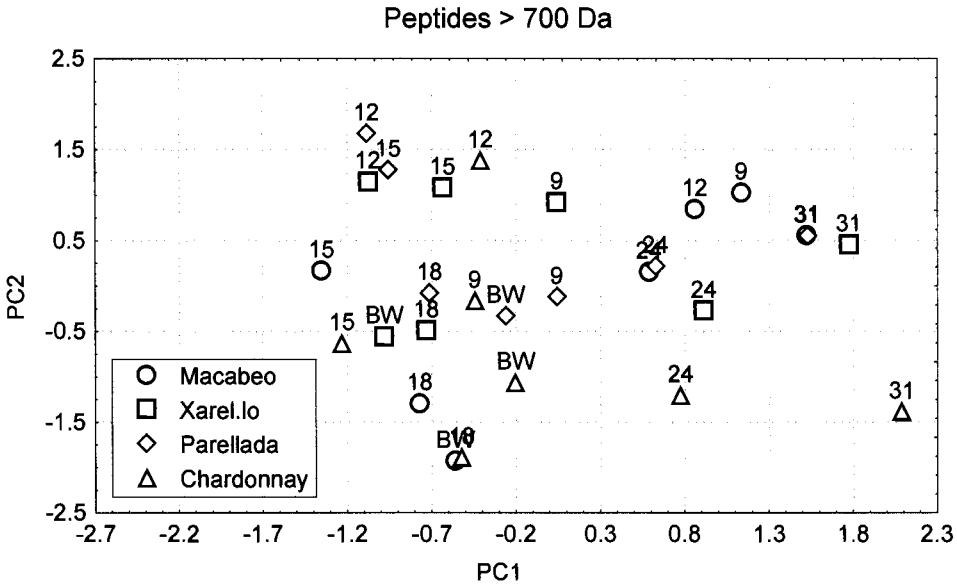

Figure 4. Plot of the 28 samples of wines on the plane defined by the two first principal components from data of amino acids in peptides with weights higher than 700 Da.

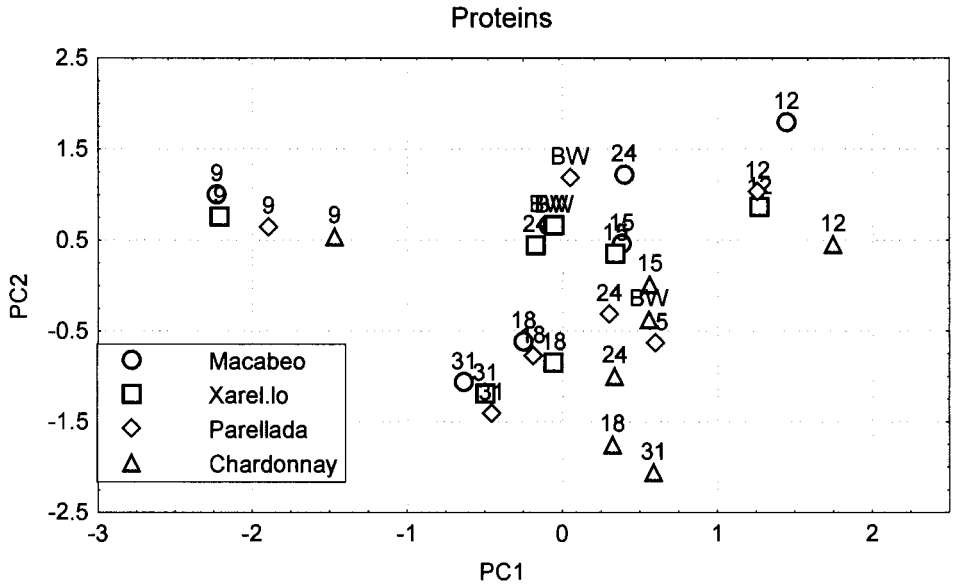

Figure 5. Plot of the 28 samples of wines on the plane defined by the two first principal components from data of amino acids in proteins.

of the total variance. The first two principal components accounted for 42.6% of the variation. The amino acids valine (0.815), tyrosine (−0.782), isoleucine (0.753), and lysine (0.752) are more correlated with the first principal component, whereas threonine (0.872) is more correlated with the second principal component.

Again there are no differences to be observed in this case due to variety (Figure 4), but the wines were grouped, in most cases, in accordance with the time they have been aging with yeasts. The greatest differences were to be found between the wines with 24 and 31 months of aging, which appeared close together and separated from the rest of the wines.

When the amino acids in proteins were analyzed by principal component analysis, 52% of the total variance could be explained by the first two principal components. The variables that contribute most to explaining the first principal component are the amino acids valine (−0.904), isoleucine (−0.866), leucine (−0.889), and ornithine (−0.811). The second principal component is highly correlated with the amino acids threonine (0.968), serine (0.886), and proline (−0.759). In this case also, as in that of the amino acids in the peptides with a molecular weight higher than 700, it can be observed that the wines are grouped according to the aging time (Figure 5).

Cluster Analysis. Cluster analysis was used to obtain more information on the behavior of the samples according to the distribution of the amino acids in the nitrogenous compounds. The dendrogram shown in Figure 6 is the result of performing a cluster analysis on the data of the molar distribution of the free amino acids, the amino acids in the peptides, and the amino acids in the proteins of the base wines and of the sparkling wines. The greatest changes in the amino acid composition of the fractions studied occur between 24 and 31 months of aging and between the base wine and the wine after 9 months. However, no great changes occur between 12 and 15 months of aging with

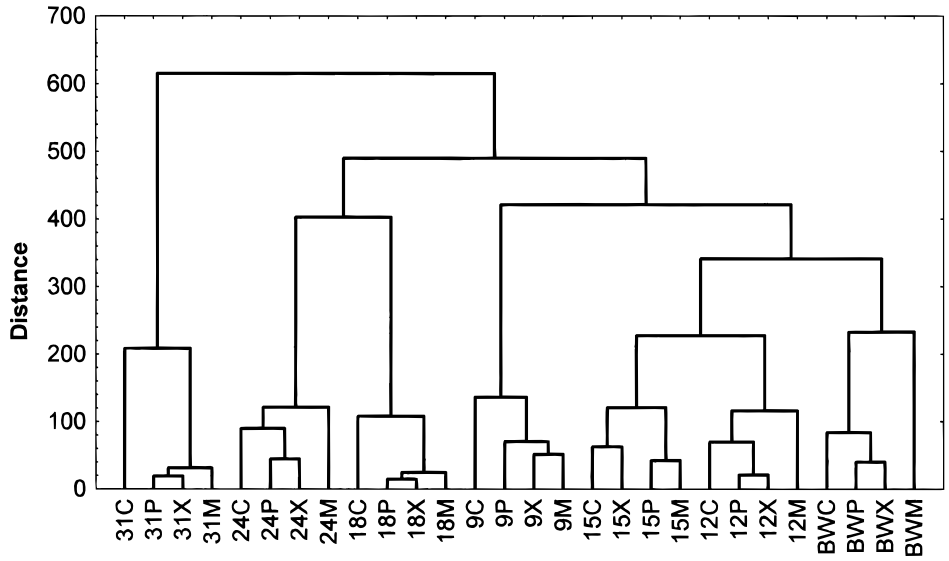

Figure 6. Dendrogram of the 28 wine samples according to the molar distribution (%) of the free amino acids, the amino acids in proteins, and the amino acids in peptides (M, Macabeo; X, Xarel.lo; P, Parellada; C, Chardonnay).

Table 5. Results of the Application of PLS Regression for Prediction of the Aging Time of Sparkling Wines Using Different Nitrogenous Fractions[a]

	NC	R^2	SD (months)	RMSEP (months)
free amino acids	5	0.885	3.59	5.43
amino acids in peptides < 700 Da	3	0.980	1.27	2.06
amino acids in peptides > 700 Da	1	0.733	5.02	6.10
amino acids in proteins	4	0.831	4.24	7.32

[a] NC, number of selected components by cross-validation; R^2, determination coefficient; SD, residual standard deviation; RMSEP, root-mean-square error of prediction by cross-validation.

yeasts. The wines of the four varieties are grouped, in all cases, by aging time.

Multivariate Regression. Table 5 shows the results of the application of PLS regression for prediction of the aging times from different nitrogenous fractions. An equation of the form, $\hat{t}_i = b_0 + \sum_{i=1}^{p} b_i X_i$, is assumed, where b_0 is the intercept of the model, b_i is the regression coefficient for the molar composition of the ith amino acid (X_i), p is the number of amino acids in the nitrogenous fraction, and $\hat{t}_i$ is the calculated aging time with the model. The aging time for the base wines was considered zero. In the table are included the number of selected components by cross-validation (NC), the determination coefficient (R^2), the residual standard deviation (SD), and the root-mean-square error of prediction by cross-validation (RMSEP) as an approximation of the prediction error. RMSEP is defined by the equation: $\text{RMSEP} = \sqrt{[\sum_{i=1}^{n}(t_i - \hat{t}_{(i)})^2]/n}$, where n is the number of samples (in this study $n = 28$), t_i is the true aging time, and $\hat{t}_{(i)}$ is the predicted time when the regression model is constructed without the sample i. The PLS prediction of the aging time from amino acids in peptides < 700 Da fraction (RMSEP = 2.06) was better than that obtained from the other fractions. From the absolute values of the 18 regression coefficients (b_i) for standardized predictor variables, it could be observed that the most important amino acids in peptides with a molecular weight lower than 700 Da for predicting the aging times were α-alanine (0.326), γ-aminobutiryc acid (−0.310), ornithine (0.178), aspartic acid plus asparagine (0.173), and threonine (−0.143). The fit for the predictions for the period between 9 and 31 months of aging was good, as shown by the higher value of R^2, when amino acids in peptides < 700 Da fraction was used.

DISCUSSION

The increase in free amino acids in wine has traditionally been considered as a sign of the beginning of autolysis (Suárez et al., 1979; Colagrande and Silva, 1981; Feuillat and Charpentier, 1982; Margheri et al., 1983; Kelly-Treadwell, 1988). From the data obtained on free and total amino acids, it is deduced that in some cases the increase in total amino acids is prior to the increase in free amino acids. This indicates, in accordance with Charpentier and Feuillat (1993) and Moreno-Arribas et al. (1996), that during the autolysis of the yeasts, first peptides and/or proteins are released into the wine and then these are later hydrolyzed, giving rise to free amino acids. There is no agreement in the literature on the length of time passing before the autolysis of yeasts in wines begins. Feuillat and Charpentier (1982) find an increase in free amino acids in wine from 6 months onward and Suárez et al. (1979) from 12 months onward. From the data of this study, it is deduced that the beginning of autolysis does not always occur at the same time, even if the wines are manufactured under the same conditions. There may be a difference of up to several months from some wines to others.

In the last stages of aging, a decrease was observed in the content of free amino acids. This may be due to deamination or decarboxylation reactions (Feuillat and Charpentier, 1982) or to the formation of esters (Herraiz et al., 1993).

In the case of most amino acids, the distribution of free amino acids is very different from the distribution of amino acids in peptides and in proteins. For example, proline, which accounts for 49.40−66.70% of free amino acids (Table 1), makes up only 4.45−22.38% of the other fractions studied (Tables 2−4). Serine, the major amino acid in peptides and proteins, is not the major amino acid in the free form. γ-Aminobutyric acid, the major

amino acid in the free form and in the peptides of the base wines, does not form part of the peptides in the sparkling wines or part of the proteins in any wine. The relatively high content of lysine in peptides of weights lower than 700 Da should be noted. This amino acid is also described as forming part of peptides in still wines (Daghetta et al., 1970; Acedo et al., 1994). As aging time increases, greater similarities are observed between the distribution of amino acids in each of the fractions studied, which may be interpreted as indicating that they have a common origin. This corroborates the hypothesis that the peptides in sparkling wines originate in the main from the breakdown of proteins in the yeasts (Charpentier and Feuillat, 1993; Moreno-Arribas et al., 1996).

Different authors (Frevert and Ballou, 1985; Orlean et al., 1991) describe the presence of serine and threonine in the glycosidic unions between proteins and the mannans of the cell wall of the yeasts, which explains the major presence of these amino acids in the peptides of the two fractions studied and in the proteins.

CONCLUSIONS

The release of free amino acids during the aging of sparkling wines, which is usually used as a sign of the beginning of the autolysis of yeasts, does not occur in all wines at the same time, even though they are manufactured under the same conditions. The increase in total amino acids was detected in some wines before the increase in free amino acids, which confirms that during autolysis, there is first a release of peptides and/or proteins that are then partially hydrolyzed into amino acids. It was also observed that the free amino acid composition of sparkling wines depends above all on the composition of the base wine and that the amino acid composition of peptides and proteins depends on the aging time with yeasts. From the results of multivariate regression, the amino acid compositions in peptides with a molecular weight lower than 700 Da of the base wines and sparkling wines could provide information on how long the wine has been aging with yeasts.

ACKNOWLEDGMENT

We are grateful to Castellblanch, S.A. (Sant Sadurní d'Anoia, Spain), for preparing the wines especially for the purpose of this research.

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Received for review April 2, 1998. Revised manuscript received August 3, 1998. Accepted August 5, 1998. This work has been supported by the Spanish Comisión Interministerial de Ciencia y Tecnología, Projects ALI94-0217-C02-02 and ALI97-0396-C02-02.

JF9803381