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Influence of Yeast Strain and Aging Time on Free Amino Acid Changes in Sparkling Ciders

BELÉN SUÁREZ VALLES,* NOEMÍ PALACIOS GARCÍA, ROBERTO RODRÍGUEZ MADRERA, AND ANNA PICINELLI LOBO

Área de Tecnología de los Alimentos, Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), 33300 Villaviciosa, Asturias, Spain

An analytical method for the determination of free amino acids in ciders is reported. It is based on high-performance liquid chromatography with an automatic precolumn derivatization with *o*-phthaldehyde and 3-mercaptopropionic acid and diode array detection. The method was applied to monitor the amino acids during second fermentation of sparkling ciders. This paper reports the influence of yeast strains and aging time on the amino acid composition of sparkling ciders. The application of principal component analysis enables the ciders to be differentiated on the basis of the two factors considered (yeast strain and aging time). The first principal component, which accounts for 58% of the total variance, achieved the separation according to aging time with serine, glycine, alanine, valine, ornithine, leucine, and lysine as the most important variables. The second principal component, accounting for 28% of the explained variance, is closely related to aspartic acid and asparagine and separates the samples according to the yeast strain.

KEYWORDS: Amino acids; HPLC; cider; yeast; aging time

INTRODUCTION

The analytical determination of free amino acids in fruit derivatives has been studied for several reasons: as chemical markers (authenticity/adulteration and geographic origin), to determine the optimum ripening time or sensorial quality, as precursors of aroma or toxicological compounds (biogenic amines and ethyl carbamate), and to determine fermentation kinetics (*1*−*5*).

Free amino acid contents in apple juices and ciders are reported by several authors (*6*−*9*). In general, amino acids are important compounds for yeasts and their contents in ciders are closely related to cider apple varieties and almost all of the steps in the cider-making process.

In Spain, sparkling cider is an alcoholic beverage made by complete fermentation of the apple juice; the final product is obtained by injection of carbon dioxide and addition of sugars after fermentation. However, a sparkling cider elaborated by the "Champenoise method" and characterized by a secondary fermentation in the bottle is protected by the Spanish Appellation of Origin "Sidra de Asturias" (*10*).

The manufacture of sparkling wines by the traditional method or in a refrigerated/pressurized tank (Charmat method) is influenced by the autolysis of yeasts. During the autolysis process, yeasts release to the extracellular medium different compounds that could modify the sensorial proprieties of wine (*11*−*15*). The main changes consist of variations in the nitrogen fractions (*16*−*19*). The free amino acid composition of sparkling wines and the changes that occur during aging time have been the subject of numerous studies (*20*−*22*).

Moreover, to our knowledge, there is no information on the changes that occur in free amino acid composition during the elaboration of sparkling cider (Champenoise method). In this work, under industrial conditions and using the same base cider, two sparkling ciders were elaborated with two different yeast strains (*Saccharomyces cerevisae* and *Saccharomyces bayanus*). The purpose of this study was to examine the influence of the yeast strain and aging time on the evolution of free amino acids in sparkling ciders.

MATERIALS AND METHODS

Cider Production. The apple juice was obtained with an automatic hydraulic Bucher-Guyer press from a mixture of cider apples belonging to the Origin Appellation in Asturias (Spain). The apple juice was fermented in a 20000 L steel tank in the cellar "El Gaitero S.A.". Prior to the second fermentation, the base cider was clarified and sterilized by cross-flow filtration, with 0.22 μm ceramic membranes from Millipore Corporation (Bedford, MA).

Sparkling ciders were produced from the same base cider using the "traditional method". Sucrose (18 g/L) and bentonite (3 g/HL) were added, and two different yeasts strains were used to inoculate the base cider: *S. cerevisiae* Levuline CHP (Groupe Oeno, France), a commercial dry wine yeast strain selected because of its appropriate characteristics to produce Champagne wines, and *S. bayanus* C6, a native cider strain from the Asturias region, belonging to the collection of pure cultures of the SERIDA (Asturias, Spain). The base cider was

* To whom correspondence should be addressed. E-mail: mbsuarez@serida.org.

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inoculated with 2% of each of the pure cultures. The second fermentation and aging time in bottle were carried out at the cellar temperature (13−15 °C).

Sampling Times. Samples were taken from the cider base (time 0) and then after 22 days (end of the second fermentation) and 2, 5, 8, 11, 14, 17, 20, 23, and 26 months of aging with yeasts. For analytical determinations, three bottles were thawed and centrifuged at 5000*g* (15 min, 10 °C) at each sampling point. Cider samples were frozen at −20 °C until analysis. All of the analyses were performed in duplicate.

Microbiological Analysis. *Populations of Yeasts.* Aliquots of several dilutions were spread onto yeast malt extract agar supplemented with 25 mg/L penicillin G potassium salt (Calbiochem, United Kingdom) and 100 mg/L streptomycin sulfate (Acofarma, Spain) to inhibit bacterial growth. Plates were incubated at 28 °C for 2 days.

Characterization of Yeast. Plates containing between 30 and 300 colonies were counted, and 50 colonies from each of the samples were picked. The isolated yeasts were preserved on yeast extract peptone D-glucose medium. Genetic characterization of yeast strains was done according to the method based on the restriction fragment analysis of mitochondrial DNA (*23*).

Populations of Lactic Bacteria. Aliquots of samples were spread onto MRS supplemented with the antifungal antibiotic pimaricin (50 mg/L). Plates were incubated in anaerobiosis jars at 28 °C for 5 days.

Populations of Acetic Bacteria. Several dilutions were spread onto basal medium (apple juice concentrate diluted at 10° Brix, 2.5% agar, and 1% yeast extract) supplemented with 25 mg/L penicillin G potassium salt (Calbiochem) and 50 mg/L pimaricin (Calbiochem). Plates were incubated at 28 °C for 2 days.

Enological Parameters. Density, pH, total (g sulfuric acid/L) and volatile (g acetic acid/L) acidities, alcoholic proof (% v/v), residual sugars (g/L), and free and total sulfur dioxide (mg/L) were done by following the methodology described in the Official Analytical Methods (*24*).

Amino Acid Analysis. Twenty-three L-amino acids and the internal standard (norvaline) were all from Sigma (St. Louis, MO). Triethylamine (TEA), tetrahydrofurane (THF), sodium acetate, *o*-phthaldialdehyde (OPA), 3-mercaptopropionic acid (3-MPA), boric acid, sodium hydroxide, and methanol were purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q purification system from Millipore Corporation.

Standard solutions were prepared in 0.1 N HCl. Norvaline was used as the internal standard at 50 mg/L in 0.1 N HCl. The derivatizing reagent was prepared as follows: OPA (100 mg) was dissolved in methanol (9 mL) and sodium borate buffer (0.4 M; pH 10.00) (1 mL). Finally, 100 μL of 3-MPA was added. This acid was periodically added (10 μL) to keep a proper excess. The reagent solution was stable at 4 °C up to 1 week. The standards and samples were filtered through a disposable 0.45 μm filter prior to injection onto the chromatographic column.

Amino acid analysis was carried out by reversed phase high-performance liquid chromatography (RP-HPLC) using a liquid chromatograph (Waters, United States), equipped with two 510M pumps, a 717 automatic injector, and a 996 diode array detector. The system was controlled by Millenium v.3.1 software. Samples were submitted to an automatic precolumn derivatization with OPA/3-MPA, and a diode array detector was used to detect the primary amino acids at 338 nm. The separation was performed at 40 °C on a Waters X-Terra MS C_{18} column (150 mm × 4.6 mm, 5 μm), protected with a guard column μ-Bondapak C_{18} (Waters), using a flow rate of 1 mL/min. The mobile phase consisted of a 40 mM sodium acetate buffer containing 0.018% (v/v) TEA and 0.3% (v/v) THF adjusted to pH 7.0 with dilute acetic acid (eluant A) and methanol (eluant B). The linear gradient used was as follows: in 15 min from 0 to 31% B, hold for 4 min at 31% B, in 13 min to 57% B, return to 100% A in 6 min, and hold for 10 min to condition column for next analysis. The injected volume was 20 μL.

Statistical Analysis. The statistical methods used for data analysis were analysis of variance to test the main effects of the two factors studied (yeast strain and aging time), Duncan's test for mean comparisons, and principal component analysis (PCA) to examine the relationships among the variables. A matrix with 36 rows (ciders) and 13 columns (amino acids) was constructed. The SPSS for Windows (version 11.5) program was used for data processing.

RESULTS AND DISCUSSION

Validation of the Amino Acid Methodology. First, we achieve the optimization of the automatic precolumn derivatization procedure by testing the ratio of sample to reactants. In this way, the best conditions were found to be the following: the autosampler was programmed to add 75 μL of sodium borate buffer and 50 μL of OPA/3-MPA to 25 μL of sample. The mixture was shaken during one cycle and finally injected. The injection volume was 20 μL, and the autosampler temperature was set at 12 °C, to avoid precipitation of the borate buffer.

The initial HPLC working conditions were selected on the basis of previously published works (*8*, *25*, *26*), and the different parameters (pH, temperature, gradient, ionic strength, and analytical column) were optimized to obtain good reproducibility and precision in the determination of the amino acids studied. The optimized chromatographic conditions (Material and Methods) showed the best resolution and faster separation of the 24 primary amino acids in 37 min. In **Figure 1** is presented a chromatogram of a sparkling cider, where the ability of the chromatographic method to separate the amino acid derivatives can be envisaged.

The linearity and precision of the method were determined with amino acid standard mixtures. Five points of calibrations for each pure amino acid, with concentration levels included in the expected working range, and three injections by point were used for performing the linear regression analysis. The linearity was determined by the square correlation coefficients of the calibration, and the limit of detection was estimated from the residuals of the calibration graph (*27*).

All compounds showed a good linearity, with regression coefficients >0.998 and detection limits ranging from 0.63 mg/L for methionine to 1.83 mg/L for lysine. The repeatability for peak areas and retention times was calculated by injecting the same standard five times. The relative standard deviation (RSD) obtained was less than 0.4% for retention times and less than 6.3% for peak areas. The reproducibility, measured on different days, was determined by injecting the same standard solution for a period of three consecutive days. The RSD obtained in this case was less than 8% for peak areas and less than 2.5% for retention times. Resolution values between peaks were higher than 1.0 in all of the cases. Additionally, the peak purity was checked by means of the spectral contrast facilities of the PDA Millenium software.

Recovery experiments were performed in order to study the accuracy of the method. Known amounts of pure standards were added to a cider sample at three different concentration levels, in duplicate. The average recoveries ranged from 90% for aspartic acid to 110% for ornithine. These results suggest that the proposed HPLC method has an adequate degree of accuracy and precision for the determination of amino acids in ciders. This method was applied to quantify the major free amino acids in base cider and their evolution throughout the second fermentation in bottle and aging time with yeasts.

Chemical Analyses and Cellular Viability. Base cider had an alcoholic proof of 5.6% (v/v), 0.94 g acetic acid/L volatile acidity, 3.06 g sulfuric acid/L total acidity, pH 3.65, 8 g/L residual sugar (fructose), and free and total sulfur dioxide contents of <10 and 49 mg/L, respectively. The mtDNA restriction patterns of the isolated yeasts during the second fermentation were identical to those of the two inoculated yeast strains. These results determined that the inoculated yeasts were

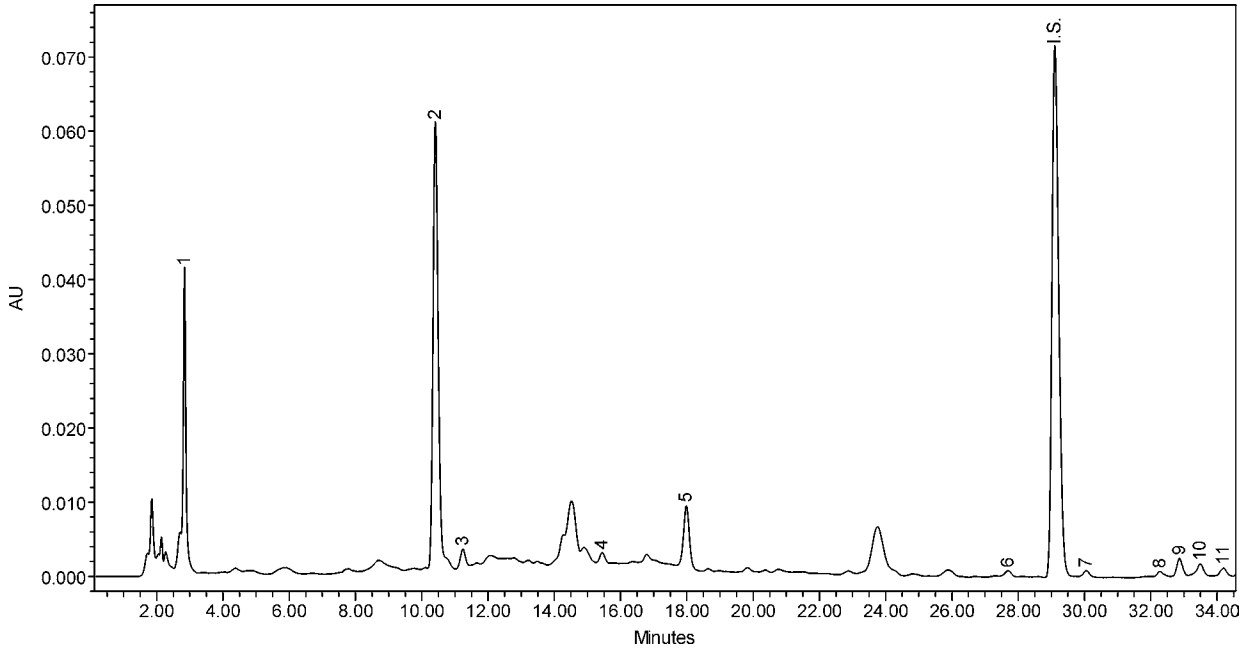

Figure 1. Chromatogram of free amino acids detected in a sparkling cider using OPA/3-MPA at 338 nm. Peak identification: 1, aspartic acid; 2, asparagine; 3, serine; 4, glycine; 5, alanine; 6, valine; I.S., norvaline (internal standard); 7, phenylalanine; 8, isoleucine; 9, ornithine; 10, leucine; and 11, lysine.

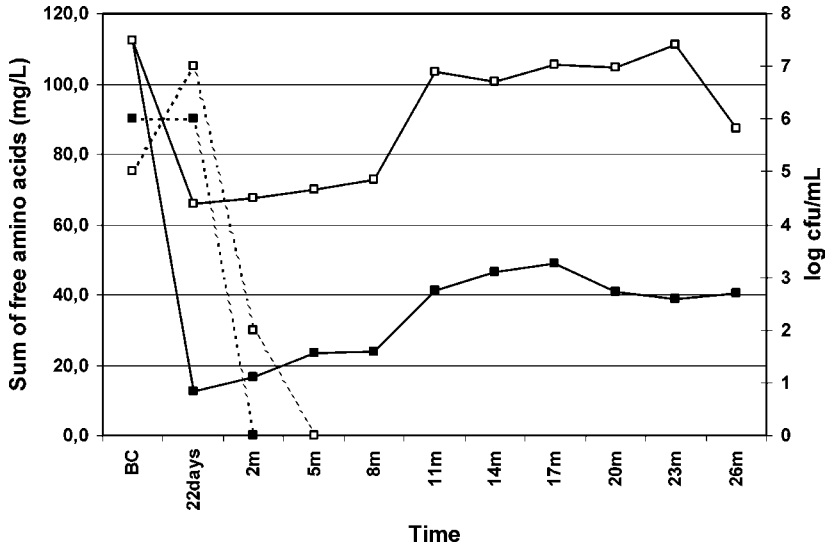

Figure 2. Changes in the total amino acid contents (—) and number of viable cells (- - -) during fermentation and aging in bottle of ciders. Legend for yeast strains: ■, Levuline; □, C6.

responsible for the second fermentation. Lactic and acetic bacteria were not detected in any of the sparkling ciders.

The second fermentation in the bottle was completed (22 days) in both sparkling ciders. Ciders at the end of the fermentation showed an alcoholic proof ranging from 7.2 to 7.3%, and no differences were detected among ciders neither for total and volatile acidities nor pH.

Figure 2 shows the number of viable yeast cells throughout the sampling time. The numbers of yeast cells in base cider after inoculation of the Levuline and C6 yeast strains were 2.5×10^6 and 5.2×10^5 cfu/mL, respectively. Twenty-two days after inoculation, the number of viable cells was substantially higher in the cider elaborated with C6 (1.3×10^7 cfu/mL) and only slightly higher in the cider produced with Levuline (5.2×10^6 cfu/mL). Two months after the inoculation, no viable cells were detected in the cider elaborated with Levuline, whereas in those ciders made with the C6 strain, this decline (<10 cfu/mL) was not observed until 5 months after inoculation.

Amino Acid Analyses. The nitrogen content of fermented beverages is determined by the balance between the consumption of amino acids for yeast growth and the release of those compounds from autolysis and from viable cells. In this sense, during the second fermentation and aging time, differences in the free amino acid profiles were influenced by the yeast strain used in the tirage.

Figure 2 represents the sum of amino acids and the numbers of viable yeast at each sampling time. Concentrations of free amino acids at the end of the second fermentation were always lower than those found in the base cider; this decrease is due to the utilization of amino acids for yeast growth at this stage. However, the selected cider yeast C6 had a minor consumption of amino acids (41%) in comparison with the selected wine yeast

Table 1. Amino Acid Content (mg/L) in Base and Sparkling Ciders during Second Fermentation and Aging in Bottle[a]

		second fermentation		aging (months)								
	strain	base cider	22 days	2	5	8	11	14	17	20	23	26
aspartic acid	Levuline	11.06	2.87	3.26 a	4.70 b	5.05 bc	7.13 e	8.54 f	9.90 g	5.95 d	6.41 de	5.62 cd
	C6		8.05	9.18 a	11.58 b	10.60 b	17.56 c	18.87 d	22.25 f	21.08 e	23.04 f	19.53 d
asparagine	Levuline	91.98	5.53	5.54 a	5.71 a	5.80 a	9.02 b	10.30 b	11.85 c	10.11 b	9.63 b	9.87 b
	C6		54.50	53.47 a	53.26 a	51.28 a	68.17 c	62.98 bc	60.11 b	63.35 bc	67.36 c	51.36 a
serine	Levuline	1.23	ND	ND	0.96 a	0.91 a	1.15 b	1.36 c	1.55 d	1.41 cd	1.30 bc	1.39 cd
	C6		ND	ND	ND	0.91 a	1.38 b	1.41 b	1.59 b	1.62 b	1.77 b	1.53 b
glycine	Levuline	1.45	1.88	2.15 a	2.18 a	2.16 a	2.29 ab	3.40 de	3.72 e	3.17 cd	2.71 bc	3.13 cd
	C6		1.57	1.57 a	1.59 a	1.30 a	2.15 bc	2.19 bc	2.30 c	2.23 bc	2.62 d	1.96 b
alanine	Levuline	6.53	2.29	2.73 a	3.39 b	3.41 b	5.25 d	5.83 ef	6.28 fg	6.47 g	4.76 c	5.78 e
	C6		2.29	2.41 a	2.54 a	3.09 b	5.03 c	4.94 c	5.63 d	5.73 d	5.84 d	5.00 c
valine	Levuline	ND	ND	ND	0.75 a	0.94 b	1.29 b	1.47 b	1.50 b	1.35 b	1.50 b	1.40 b
	C6		ND	ND	ND	ND	ND	0.75 b	0.84 c	0.84 c	0.90 d	0.68 a
phenylalanine	Levuline	ND	ND	ND	ND	ND	2.06 bc	2.16 c	2.25 c	2.12 c	1.86 ab	1.78 a
	C6		ND	ND	ND	ND	1.68 ab	1.64 ab	1.77 b	1.82 b	1.77 b	1.52 a
isoleucine	Levuline	ND	ND	ND	0.78 a	0.78 a	1.56 d	1.48 cd	1.45 bcd	1.35 bc	1.38 bc	1.31 b
	C6		ND	ND	ND	0.94 c	0.64 a	0.95 c	1.07 d	0.95 c	0.92 c	0.75 b
ornithine	Levuline	ND	ND	1.21a	1.26 ab	1.43 ab	2.87 c	3.42 d	3.39 d	1.24 ab	1.56 b	1.26 ab
	C6		ND	ND	ND	1.12 a	2.54 b	2.92 c	2.98 c	2.63 b	2.65 b	1.29 a
leucine	Levuline	ND	ND	1.15a	1.51 b	1.50 b	2.82 c	2.88 cd	2.73 c	3.15 de	3.22 e	4.55 f
	C6		ND	0.99a	1.13 ab	1.41 c	1.46 c	1.94 de	1.79 d	2.08 e	1.98 de	1.33 bc
lysine	Levuline	ND	ND	ND	2.15 a	1.97 a	5.62 c	5.62 c	4.11 b	4.52 b	4.64 b	4.49 b
	C6		ND	ND	ND	2.03 a	2.73 d	2.06 a	2.24 b	2.49 c	2.43 c	2.43 c

[a] Results are the means of two determinations. Mean values in the same row with the same letter are not significantly different at the 5% confidence level (Duncan's test); ND, not detected.

Levuline, which utilized about 89% of the amino acids during the second fermentation in the bottle. Likewise, between 22 days and 8 months, a small increase in the total amino acid content was observed; subsequently, a major release of those analytes into the medium was found. The greatest increase was observed for the C6 strain (31 mg/L) followed by Levuline (18 mg/L). A small enrichment of ciders by nitrogen compounds could be explained in two ways: in the presence of viable cells in the medium, by a physiological response of yeasts to nutrient deficiency; after the decline of viable cells, the autolysis process plays an important role (*15*, *28*, *29*). At the end of the aging time, there is a differentiate reduction in free amino acids according to the yeast strain. Thus, in the sparkling ciders elaborated with Levuline, free amino acids decreased after 17 months, whereas in the cider produced with C6, they decreased after 23 months to reach amino acid levels similar to those of the base cider (**Figure 2**). This fact could be explained by deamination reactions of amino acids or participation in the formation of different compounds (*21*, *30*). Trends in total free amino acid (sum) profiles are in agreement with those reported by different authors in sparkling wines (*19*, *21*, *22*, *31*).

Table 1 summarizes the data of concentration of free amino acids in base and sparkling ciders at different aging times. Asparagine (82%) was the predominant amino acid in base cider, followed by aspartic acid (10%) and alanine (6%). Serine and glycine were minority compounds (1%). These data were in accordance with previous works about the composition of amino acids in ciders (*6*). During the second fermentation process (22 days), asparagine and aspartic acid experimented the greatest reduction. As said before, this reduction was influenced by the yeast strain inoculation. Thus, asparagine and aspartic acid metabolized by Levuline represented 94 and 71%, respectively, whereas the uptake by C6 was 41% for asparagine and 13% for aspartic acid. However, at the end of fermentation, the inoculated yeasts did not differ in their pattern of uptake of glycine, alanine, and serine. Yeasts consumed about 65% of alanine and 100% of serine and excreted glycine (data not shown). Differences between yeast strains in their ability to use amino acids have been previously reported (*32*−*34*). After fermentation, there is an increase in amino acids but aging and yeast factors had a significant effect on the concentration of these analytes.

Two-way analysis of variance was used to study differences between free amino acid concentrations due to yeast strain and time aging (the interaction and the within error terms were pooled). The results obtained revealed that the concentrations of all amino acids were significantly influenced by aging time ($p < 0.01$). The main term of yeast strain factor ($p < 0.01$) affected the concentration of all free amino acids, with the exception of alanine, glycine, phenylalanine, and serine. In **Table 1** are also shown data of mean values of amino acids according to yeast strains and aging time. Different letters indicate the existence of significant differences among ciders (Duncan's test).

PCA was carried out with the free amino acids studied in sparkling ciders. First, the correlation matrix was computed to reject variables with high correlations. Correlation coefficients higher than 0.95 in absolute value were obtained between phenylalanine and alanine and between isoleucine and lysine. So, the data matrix was reduced to nine variables (aspartic acid, asparagine, serine, glycine, alanine, valine, ornithine, leucine, and lysine) and 36 objects. Two factors with eigenvalues greater than 1 were computed, accounting for 86.4% of the variance. **Figure 3** shows the 36 ciders projected onto the plane defined by the two principal components.

The first principal component (PC1) accounts for 58.2% of the total variance and separates ciders by the time aging factor. The second principal component (PC2) gives rise to two clearly differentiated groups on the basis of the yeast strain inoculation. Serine (0.782), glycine (0.877), alanine (0.900), valine (0.928), ornithine (0.695), leucine (0.847), and lysine (0.915) have the highest contribution to the first component, whereas the second principal component is more correlated with aspartic acid (0.985) and asparagine (0.878) contents. On one hand, leucine, isoleucine, phenylalanine, and lysine are typical yeast autolysis products, as reported before (*7*), thus explaining their contribu-

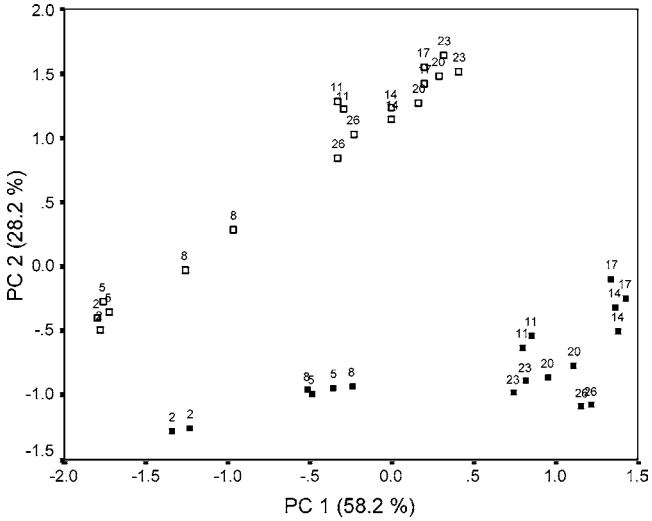

Figure 3. Two factorial axes projection of sparkling ciders. Numbers correspond to aging time (months). Legends for yeast strains are as in **Figure 2**.

tion to the first principal component. On the other hand, yeast strains exhibited different consumption patterns for amino acids (*34*). In this sense, the cider yeast, perfectly adapted to the nutritional conditions of apple musts, showed minor and slower consumption of amino acids than the wine one.

A feasible method for analyzing amino acids in ciders was validated. The amino acid composition of sparkling ciders is significantly influenced by the yeast that conducts the fermentation process and the aging time in the bottle. The results from PCA showed two principal components explaining 86.4% of the variance. The first component, associated with aging time, has serine, glycine, alanine, valine, ornithine, leucine, and lysine as the main contributors, whereas asparagine and aspartic acid were more correlated with the second component, which is associated with yeast strains.

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