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Article

Evaluation of Chemical Changes during *Myrciaria cauliflora* (Jabuticaba Fruit) Fermentation by ^{1}H NMR Spectroscopy and Chemometric Analyses

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Ácidos orgânicos, açúcares, álcoois, compostos fenólicos, propriedades de cor, pH e acidez titulável foram monitorados durante fermentação comercial de jabuticaba (*Myrciaria cauliflora*) através da espectroscopia de ressonância magnética nuclear (NMR) de ^{1}H, ensaios espectrofotométricos e métodos usuais de análise. Os dados coletados foram analisados por meio de análise de componentes principais (PCA), agrupamentos (HCA) e correlação canônica (CCA). Dois grupos de amostras foram reconhecidos, e as variáveis responsáveis pela separação foram açúcares, antocianinas, álcoois, tonalidade e ácidos acéticos e succínico. A análise de correlação canônica confirmou a influência dos álcoois (etanol, metanol e glicerol), ácidos orgânicos (ácidos cítrico, acético e succínico), pH e acidez titulável na extração e estabilidade das antocianinas e copigmentos. Consequentemente, as propriedades de cor também foram afetadas pelas variações nos níveis de compostos fenólicos durante o processo de fermentação.

Organic acids, sugars, alcohols, phenolic compounds, color properties, pH and titratable acidity were monitored during the commercial fermentation of jabuticaba (*Myrciaria cauliflora*) by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, spectrophotometric assays and standard methods of analysis. Data collected was analyzed by principal component (PCA), hierarchical cluster (HCA) and canonical correlation (CCA) analyses. Two sample groups were distinguished and the variables responsible for separation were sugars, anthocyanins, alcohols, hue and acetic and succinic acids. The canonical correlation analysis confirmed the influence of alcohols (ethanol, methanol and glycerol), organic acids (citric, succinic and acetic acids), pH and titratable acidity on the extraction and stability of anthocyanins and copigments. As a result, color properties were also affected by phenolic variation throughout the fermentative process.

Keywords: jabuticaba, phenolic compounds, sugars, organic acids, NMR, chemometrics

Introduction

Jabuticaba (*Myrciaria cauliflora* Berg.) is a Brazilian fruit that bears an edible purplish-black sweet fruit. It has a slightly acid taste and is rich in phenolic compounds, such as anthocyanins, flavonoids, tannins and depsides, which are responsible for antioxidant and anti-inflammatory activities, as well as for colon cancer cell cytotoxicity.[1] High sugar content of jabuticaba makes it suitable for jam, juice, liqueur and wine production.[2] Wines made from jabuticaba have shown great acceptance in sensory analysis and a higher antioxidant activity than grape wines in a β-carotene/linoleic acid system.[3,4]

Several fruit wines have been made from Brazilian tropical fruits such as cashew,[5] cacao, gabiroba, umbu and cupuaçu.[3] Such industrial processing is an alternative to prevent post-harvesting losses and to promote exotic fruit production, allowing a more rational use of Cerrado and Amazonian biomes (Brazil). Like most beverages, fruit wines not only have to be in accordance with the current legislation but also achieve high-quality standards to compete in the market.[2] For this purpose, recent studies have determined jabuticaba chemical composition in relation to soil culture and the degree of maturity for winemaking.[6,7] In addition, volatile compounds, alcohols and organic acids found in jabuticaba wines and spirits have been characterized.[3,8] However, there are as yet no reports regarding the chemical changes that occur during jabuticaba fermentation process.

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The evolution of metabolites during alcoholic fermentation is of crucial importance to the final product.[9] During the fermentative maceration stage, sugars are consumed and ethanol, glycerol and higher alcohols are produced. Organic acids originated from the fruit and produced by yeasts undergo a change in their concentration. A variety of volatile compounds is produced and extracted from the fruit and phenolic compounds (anthocyanins, flavonoids, phenolic acids and tannins) are extracted from the skins and seeds in this process.[10] All these compounds together play an important role in the organoleptic characteristics of wine. Therefore, monitoring the levels of these compounds during the fermentative process may reveal possible defects which could affect the quality of the final product.[11]

Nuclear magnetic resonance spectroscopy (NMR) has been applied in the analysis of fruit juices and wine,[12,13] as well as in monitoring organic acids, sugars and alcohols during fermentation.[14-16] ^{1}H NMR spectroscopy is useful to simultaneously quantify diverse compounds in a complex mixture, such as must and wine, without previous laborious sample treatment.[14,16] Spectral techniques are also used in wine analysis to quantify the total amounts of phenolics,[17] monomeric anthocyanins,[18,19] tannins,[20] and for measuring color intensity and hue.[19]

This study reports, for the first time in the literature, the evolution of organic acids, phenolic compounds, alcohols and sugars during fourteen days of a commercial fermentation of jabuticaba using ^{1}H NMR spectroscopy and spectrophotometric assays. Multivariate statistical techniques were applied to detect pattern distributions of variety during the fermentative period, to identify which parameters distinguish the natural groups and to study the influence of organic acids and alcohols on phenolic compound levels and chromatic parameters.

Experimental

Chemicals

Tannic acid and iron (III) chloride were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterium oxide (99.9%) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other chemicals used were of analytical grade.

Must samples

Commercial fermentation of *Myrciaria cauliflora* took place in 2010 at Jabuticabal Winery (S 16° 55' 25.9", W 49° 21' 41.0"), located in the outskirts of Hidrolândia City, Goiás State, Brazil. Ripe fruits from the same orchard were washed, crushed and divided into three 200 L stainless steel tanks. Sodium metabisulphite was added (16.2 g 100 kg^{-1} of jabuticaba) and sugar concentration was adjusted to 22 ^{o}Bx with sucrose. The same amount of wild (indigenous) yeasts, previously prepared with jabuticaba fruits, was inoculated in each tank. Fermentation was conducted at 27 ºC and the caps were immersed five times a day. Seed and skin contact lasted for four days, after which the musts were pressed at 1.5 bar in stainless steel tanks and stored at room temperature. Samples were daily collected for 14 days and kept frozen at −18 ºC. Prior to all analyses, they were defrosted and centrifuged at 3000 rpm for 10 min.

Determination of total acidity, pH and ^{o}Brix

Must samples (1.0 mL) were titrated against 0.1 mol L^{-1} NaOH solution to pH 8.2. Results were expressed as g L^{-1} of tartaric acid. The pH values were measured by pH-meter Ingold pH-206 and soluble solid (^{o}Bx) determination was performed by Abbe DR-A1 refractometer (Atago Corp., LTD, Japan). All measurements were performed in triplicate.

Color evaluation

Color measurements were made with a Beckman DU-70 spectrophotometer (Beckman Instruments, Inc., CA, USA) with a 1.0 mm optical path length glass cell. Must color intensity was determined as the sum of the absorbance intensities at 420, 520 and 620 nm and the hue was the ratio of A_{420}/A_{520}.[19]

Determination of phenolic compounds

Total phenols were quantified by the Folin-Ciocalteu method described by Escarpa and Gonzalez.[21] Tannins were measured by protein precipitation assay with the use of BSA (Hagerman-Butler method described by Waterman and Mole).[22] Results from both assays were expressed as g L^{-1} of tannic acid. Anthocyanin content was determined by the pH differential method.[18,23] Pigment concentration was expressed as g L^{-1} of cyanidin 3-glucoside. All measurements were performed in triplicate.

NMR determinations

Must samples (0.95 mL) were mixed with 0.05 mL of D_2O and placed in a 5 mm NMR tube.[14] NMR spectra were recorded on a Bruker Avance III 500 spectrometer,

operating at 500.13 MHz for ^{1}H. The field frequency was locked with D_2O. The following parameters were applied: the spectral window was 16 ppm and data were collected into 65 k data points after 48 scans; the recycle delay was 1 s and had a flip angle of 90º, with an acquisition time of 4.06 s at a fixed temperature of 25 ºC. Data analysis was performed by TopSpin 2.1 software (Bruker BioSpin Corp., MA, USA), following the methodology developed by Clark *et al.*[14] In this methodology, all integrations were normalized to the water peak. The integration values were then divided by the number of hydrogens contributing to the respective peak, this furnished the relative number of moles for each compound. The actual mass was obtained by correction for relative molecular mass. Finally, in order to determine the concentrations in g kg^{-1}, the masses of all compounds were summed. Calculations are shown in Table S1 (in the Supplementary Information section, SI).

Statistical analysis

Principal component (PCA) and hierarchical cluster (HCA) analyses were applied to assess the interrelationships between the metabolites produced and extracted during fermentation. For this, the SPAD software package was used.[24] Nearest neighbor complete linkage technique by Benzécri algorithm was used as an index of similarity.[25] Hierarchical clustering was performed according to Ward's variance minimizing method.[26] Relationships between organic acids, alcohols and phenolic compounds were obtained via canonical correlation analysis (CCA) using the SAS CANCORR procedure.[27] The magnitude of the structure correlation coefficients (canonical loadings) was used to explain canonical variates. Prior to the multivariate analysis, the data were processed by means of auto-scaling and mean centering.

Average multiple comparisons were established by one-way analysis of variance (ANOVA) using SAS GLM analyses. All data were checked for homoscedasticity with Hartley's test.[28] Whenever heteroscedasticity was observed, the variable was angular or rank-transformed. In addition, whenever a difference in one-way ANOVA was established, a Tukey's *post-hoc* test was performed. Results are shown as mean values and are joined by the standard deviation of independent measurements in some cases. *p*-Values below 0.05 were regarded as significant.

Results and Discussion

The time-course evolution of sugars, alcohols and organic acids across the jabuticaba fermentative process was monitored by ^{1}H NMR, and the results are shown in Figures 1 and 2. Sucrose, glucose and fructose concentrations decreased to levels below the limit of detection by the sixth day, whereas ethanol reached a plateau on the same day (Figure 1a). The soluble solids showed the same trend, decreasing from 22 to 6.91 ºBx (day 1 to 6), which confirms that alcoholic fermentation with indigenous jabuticaba yeasts lasted about six days. This was a shorter period compared with that of indigenous gabiroba yeasts, which showed an average of 14 days for total sugar consumption.[29] Fermentative by-products glycerol and methanol were detected only after the sixth day due to the overlap of sugar signals before this day (Figures 1 and 2). Methanol was produced during the fermentative maceration of the skins by the enzymatic hydrolysis of methoxy groups from the pectin moieties; its concentration (0.21 g kg^{-1}) did not reach the legal limit of 0.35 g L^{-1}.[2] Glycerol was produced in relatively low concentrations (3.27 to 3.88 g kg^{-1}), which is a negative aspect, as this compound provides the wine with a soft and sweet taste when in the range of 7-9 g L^{-1}.[30]

Citric acid was the most abundant organic acid in the must (Figures 1a and 2). This finding differs from that

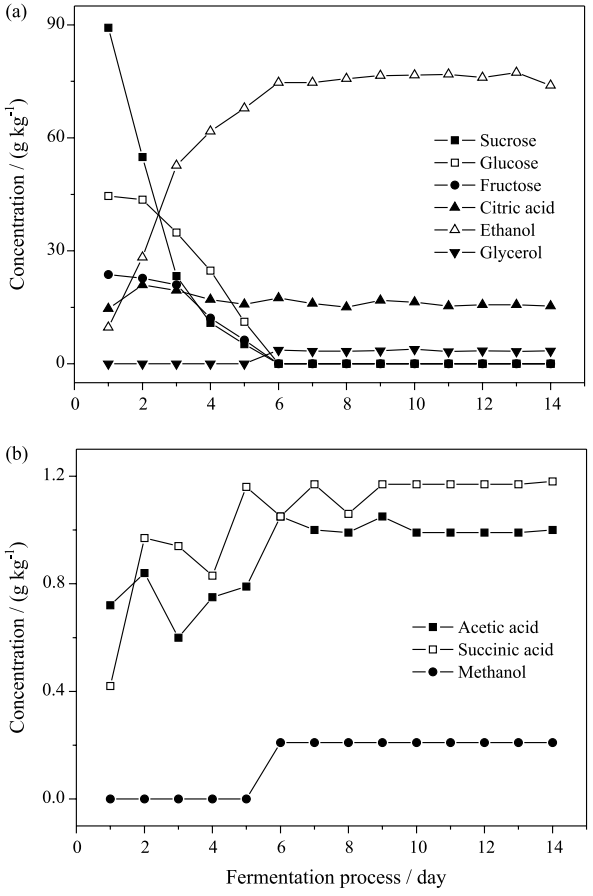

Figure 1. Changes in the metabolite concentration during the jabuticaba must fermentation: (a) sucrose, glucose, fructose, citric acid, ethanol and glycerol, (b) methanol, acetic and succinic acid.

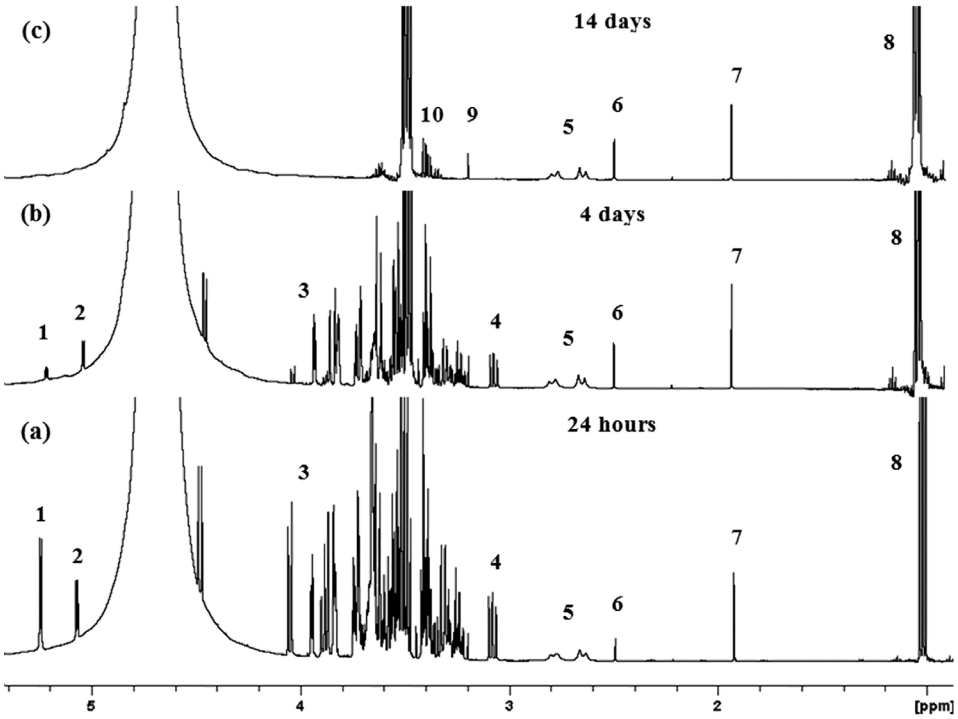

Figure 2. Metabolite evolution during jabuticaba must fermentation by ^{1}H NMR spectra (500 MHz). Peaks: 1, sucrose; 2, α-glucose; 3, fructose; 4, β-glucose; 5, citric acid; 6, succinic acid; 7, acetic acid; 8, ethanol; 9, methanol; 10, glycerol.

of Jham *et al.*,[31] who found succinic acid to be the main acid in two jabuticaba varieties. Organic acid levels in fruits are influenced by abiotic factors such as climate and fertilization.[32] As regards jabuticaba, a correlation between fruit acidity and soil chemical composition was confirmed.[6]

Succinic acid was mainly formed during fermentation. It reached a plateau (1.17 g kg^{-1}) after the eighth day (Figure 1b). In grape wines, this acid varies from 0.5 to 1.5 g L^{-1},[33] and high amounts of it give the wine a salty bitter taste.[3] Acetic acid, mostly produced by non-*Saccharomyces* yeasts, increased up to the sixth day (Figure 1b); the maximum concentration (1.0 g kg^{-1}) fell short of the 1.2 g L^{-1} limit.[2] This acid is responsible for volatile acidity, which is an important physicochemical parameter in wines and needs to be controlled during the entire winemaking process.

Jabuticaba must pH varied from 3.32 to 3.61, which is in accordance with previous reports.[34] The range of titratable acidity (11.9-16.1 g L^{-1}) was higher than that usually conferred to *Vitis vinifera* and *Vitis labrusca* wines, which is 5.0-8.6 g L^{-1}.[13,35] Such high acidity is mainly due to the high contents of citric acid, which contains three carboxylic groups. This is usually a problem for most jabuticaba wines.[2]

Phenolic compounds, such as anthocyanins, tannins, flavonoids and phenolic acids are important for organoleptic properties such as chromatic features, flavor, body and structure of red wines.[10,36] These compounds are present in jabuticaba seeds and skins and are extracted during fermentative maceration,[7] which lasted the first four days. Total phenol concentration rapidly increased to its maximum in the third day and then decreased to a plateau. The same was observed for color intensity, whereas hue increased on day 5 (Figure 3). This fact could be explained by the concomitant reduction in monomeric anthocyanin levels, from 0.20 to 0.06 g L^{-1} (Figure 3), as well as by the polymerization and precipitation of some phenols complexed and/or adsorbed with citrates, proteins and dead yeasts.[37] A similar tendency was verified in the vinification of *Vitis vinifera* var. Monastrell,[38] as the

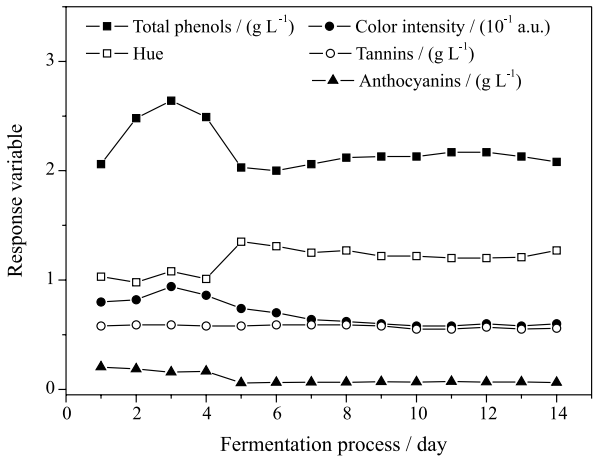

Figure 3. Variations in the levels of total phenols, anthocyanins, tannins, hue and color intensity during the jabuticaba fermentation.

maximum anthocyanin extraction occurred in the first days of maceration, followed soon after by polymerization.

An outstanding difference between grape and jabuticaba fermentation refers to the levels of extracted phenols. Whereas in the maceration of *Vitis vinifera* anthocyanins and total phenols reached maximum levels of about 0.85 and 5.0 g L^{-1},[19] respectively, the maximum concentrations of these compounds in jabuticaba amounted to only 0.20 and 2.64 g L^{-1}, respectively. As a result, color intensity levels were very low (5.8-9.4) and hue values (0.98-1.35) were too high compared with those of young red wines.[10,19] The low extrability of phenolic compounds from jabuticaba may be due to the rigid and thick structure of the skins, which contain high amounts of cellulose and hemicellulose (340 and 80 g kg^{-1}, respectively).[39] Skin cell walls may hinder the extrability of anthocyanins and other phenols by forming a mechanical barrier which prevents their complete release.[10,40]

Tannin levels ranged from 0.55 to 0.59 g L^{-1} (Figure 3). A different trend was observed for catechins and proanthocyanins from grapes, which reached a maximum level in the first days and decreased afterwards.[41] However, jabuticaba tannins are mainly composed of ellagitannins, whose specific features differ from those of condensed tannins.[42]

PCA was applied to reduce the dimensionality of the data (42 samples × 17 variables = 714 pieces of data) and to assess the relationships between samples and variables. In addition, HCA via Ward's technique was used to detect groups of similar individuals. According to PCA results (Figure 4), the first two principal components accounted for 78.73% of total variance. PC-1 revealed a time-course

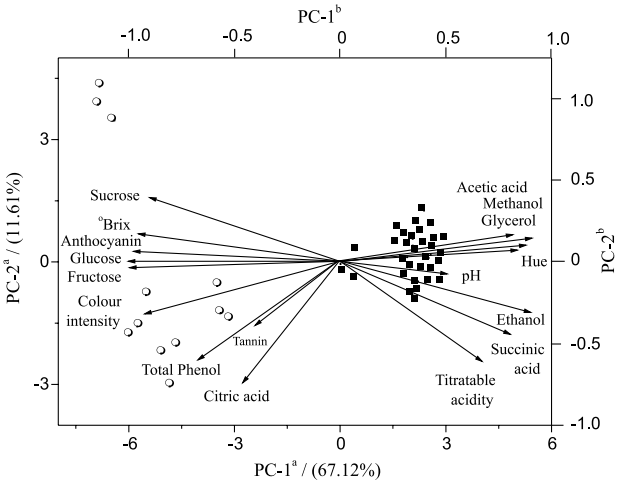

Figure 4. PCA ordination of organic acids, sugars, alcohols, phenolics, color parameters, pH and titatrable acidity variables, and must samples according to the clusters they belong to: I (○) and II (■). [a]Axes refer to ordination scores obtained from must samples. [b]Axes refer to ordination loadings obtained for the variables which are represented as vectors from the origin.

dependent separation between the variables; sugars and anthocyanins with high negative loadings represent the beginning of the fermentative process, whereas alcohols, acetic and succinic acids and hue amount to the end of alcoholic fermentation. On PC-2, the variables with high negative values were total phenols, citric acid and titratable acidity, contrasting with sucrose positive loadings.

By applying a complete linkage procedure on Euclidean distances, HCA divided must samples into two main groups. The dendrogram (Figure S1 in the SI section) shows cluster I, comprised of samples from the first four days of fermentation which are characterized by high levels of sugars (sucrose, glucose, fructose and soluble solids), anthocyanins and color intensity (Table 1). In contrast, cluster II consisted of samples from days 5 to 14 with higher levels of alcohols (ethanol, methanol and glycerol), hue, acetic and succinic acids.

Table 1. Sugars, alcohols, organic acids, phenols, titratable acidity and color parameters in clustered jabuticaba must fermentation[a]

Variables	Clusters	
	I	II
Sucrose / (g kg^{-1})	44.68 ± 27.5 a	0.50 ± 0.9 b
Fructose / (g kg^{-1})	19.90 ± 3.9 a	0.63 ± 1.1 b
Glucose / (g kg^{-1})	36.95 ± 7.1 a	1.12 ± 2.0 b
Glycerol / (g kg^{-1})	–	3.12 ± 0.8
Methanol / (g kg^{-1})	–	0.19 ± 0.04
Ethanol / (g kg^{-1})	38.08 ± 19.1 a	75.02 ± 1.9 b
Citric acid / (g kg^{-1})	18.05 ± 2.2 a	15.96 ± 0.8 b
Succinic acid / (g kg^{-1})	0.79 ± 0.2 a	1.15 ± 0.1 b
Acetic acid / (g kg^{-1})	0.73 ± 0.1 a	0.98 ± 0.1 b
pH	3.41 ± 0.1 a	3.49 ± 0.1 b
Titratable acidity / (g L^{-1})	13.9 ± 1.0 a	15.4 ± 1.0 b
Soluble solids / °Bx	14.89 ± 2.5 a	6.87 ± 0.1 b
Total phenols / (g L^{-1})	2.42 ± 0.2 a	2.10 ± 0.05 b
Tannins / (g L^{-1})	0.58 ± 0.01 a	0.57 ± 0.02 b
Anthocyanins / (g L^{-1})	0.178 ± 0.002 a	0.065 ± 0.003 b
Color intensity / a.u.	8.6 ± 0.4 a	6.0 ± 0.2 b
Hue	1.03 ± 0.03 a	1.25 ± 0.04 b

Means followed by the same letter in the rows did not share significant differences at 5% probability by Tukey's test; [a]based on original data; –: not detected.

According to Pearson's correlation, the hue showed negative correlations with color intensity (r = –0.78, $p < 0.01$) and monomeric anthocyanins (r = –0.92, $p < 0.01$), whereas color intensity showed strong positive correlations ($p < 0.01$) with total phenols (r = 0.80) and anthocyanins (r = 0.65). Monomeric anthocyanins,

whose maximum absorbance values occur at 520 nm (red), underwent degradation and polymerization during fermentation. Consequently, color intensity decreased and hue increased due to higher absorbance at 420 nm (yellow). Phenolic compounds also act as copigments, aiding in color intensification and stabilization,[43] but their concentration declined from the third day onwards. As fermentation advanced, must color changed from strong red-violet to light red-yellowish, which confirmed the accumulation of polymeric pigments in the must.[18]

Canonical correlation (Table 2) was applied to assess the influence of acids, alcohols, titratable acidity and pH (set 1) on chromatic parameters and phenolic compounds (set 2). The method allows for the assessment of new variables called canonical variates (CV) so that, they show the highest correlation possible between two data sets. In fact, these two sets of variables were strongly correlated, thus the correlation coefficient was 1.000 and 0.998 for the first and second pairs of CV, which showed significant Wilks' lambda (1.1×10^{-7} and 0.0002, respectively); this accounts for a multivariate measure of group differences over both data sets. The data sets are statistically correlated as the *p*-values for the first and second CV were 1.7×10^{-6} and 0.004, respectively.

The analysis revealed that alcohols, succinic and acetic acids, titratable acidity and pH from set 1 correlate negatively with all phenolics and color intensity and positively with hue (set 2), loading onto the first CV, which refers to samples from day 5 to day 14 (cluster II). In addition, there are moderate positive correlations between citric acid and all phenols, color intensity and low hue (loadings onto V1 and W1). In contrast, titratable acidity, citric and succinic acids, low acetic acid and ethanol reveal relationships with total phenols and color intensity in the second CV, which is associated to samples from days 1 to 4 (cluster I).

The diffusion rate of phenolic compounds from the cell into the must relies on several factors, such as temperature, concentration gradient, molecular weight, cell wall composition and ethanol concentration.[44] The increase in ethanol levels during fermentation usually facilitates the extraction of higher hydrophobic compounds, e.g., proanthocyanins with a high degree of polymerization, as well as small molecules such as flavan-3-ols.[19,41] At the beginning of jabuticaba fermentation, the concentration of ethanol together with succinic and citric acids had no influence in the extraction of tannins and anthocyanins; nevertheless, other phenols, possibly flavan-3-ols, phenolic acids and flavonoids increased during this period. These phenols probably act as copigments, given the fact that color intensity became stronger in day 3 even with less anthocyanins (Figure 3). Intermolecular copigmentation improves the stabilization of anthocyanin coloring and increases absorbance at the maximum absorption wavelength, thus producing a hyperchromic effect.[45]

Anthocyanins, color intensity and total phenols suffered a drastic reduction as fermentation came to an end (cluster II). This was strongly correlated with the increase in pH, titratable acidity, succinic and acetic acids and

Table 2. Canonical correlation summary of organic acids, alcohols, titratable acidity, pH, phenolics and color parameters with their canonical variates

Organic acids, pH, total acidity, and alcohol discriminants (set 1)	Canonical variate		Phenolic compounds and color variables (set 2)	Canonical variate	
	V1	V2		W1	W2
Titratable acidity / (g L^{-1})	0.694	0.389	Anthocyanin / (g L^{-1})	–0.998	0.022
pH	0.602	–0.094	Tannin / (g L^{-1})	–0.481	0.172
Citric acid / (g kg^{-1})	–0.475	0.450	Total phenol / (g L^{-1})	–0.587	0.691
Succinic acid / (g kg^{-1})	0.856	0.300	Color intensity / a.u.	–0.891	0.431
Acetic acid / (g kg^{-1})	0.849	–0.385	Hue	0.790	0.066
Glycerol / (g kg^{-1})	0.909	–0.250			
Methanol / (g kg^{-1})	0.912	–0.260			
Ethanol / (g kg^{-1})	0.928	0.327			
Eigenvalues				0.999	0.996
Canonical correlation				1.000	0.998
Wilks' lambda				1.1×10^{-7}	0.0002
Degrees of freedom				40	28
p-Value				1.7×10^{-6}	0.004
Cumulative variance / %:					
Of discriminant organic acids, pH, titratable acidity and alcohols				63.0	73.5
Of discriminant phenolic compounds and color variables				59.8	73.8

alcohols, as well as with the decrease in citric acid (Table 2). Anthocyanin stability is greatly dependent on pH and on the balance between the acids in the solution.[46] A previous study revealed that the type of acid was also important in jabuticaba anthocyanin extraction,[47] and that yield and color levels were significantly higher in citric acid than in an acetic acid medium.

Conclusions

Monitoring commercial jabuticaba alcoholic fermentation revealed important chemical changes. The evolution of sugar consumption and ethanol production showed the effectiveness of indigenous jabuticaba yeasts and provides valuable information regarding alcohols, organic acids, and phenolic compound variation. Through the multivariate statistical analysis, it was possible to confirm the significant influence of organic acids, acidity and alcohols on the levels of phenolic compounds and chromatic parameters.

Further studies on the composition of phenolic substances of jabuticaba skins and seeds will be crucial for a complete understanding of the stability of anthocyanins and copigments found in this fruit. Such knowledge will prove useful in the production of a jabuticaba wine of higher quality.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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