Food Chemistry 216 (2017) 217–224

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

NMR spectroscopy and chemometrics to evaluate different processing of coconut water

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ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form 10 August 2016
Accepted 11 August 2016
Available online 12 August 2016

Keywords:
Cocus nucifera
Multivariate analysis
Sterilization
Sulphite
UHT

ABSTRACT

NMR and chemometrics was applied to understand the variations in chemical composition of coconut water under different processing. Six processing treatments were applied to coconut water and analyzed: two control (with and without sulphite), and four samples thermally processed at 110 °C and 136 °C (with and without sulphite). Samples processed at lower temperature and without sulphite presented pink color under storage. According to chemometrics, samples processed at higher temperature exhibited lower levels of glucose and malic acid. Samples with sulphite processed at 136 °C presented lower amount of sucrose, suggesting the degradation of the carbohydrates after harshest thermal treatment. Samples with sulphite and processed at lower temperature showed higher concentration of ethanol. However, no significant changes were verified in coconut water composition as a whole. Sulphite addition and the temperature processing to 136 °C were effective to prevent the pinking and to maintain the levels of main organic compounds.

1. Introduction

Coconut water is a popular isotonic beverage due to its refreshing, nutritional, and potential therapeutic properties (Tan, Cheng, Bhat, Rusul, & Easa, 2014), and moreover, is flavorful, sweet, slightly acidic, rich in phosphorus and potassium. It also contains proteins, fats, minerals, carbohydrates (glucose, fructose, and sucrose), and organic acids such as tartaric, citric and malic acids (Campbell-Falck, Thomas, Falck, Tutuo, & Clem, 2000; Santoso, Kubo, Ota, Tadokoro, & Maekawa, 1996). However, the water extracted from the nut spoils within a day after the exposure to air due to oxidation induced by the naturally presents enzymes as polyphenol oxidase (PPO) and peroxidase (POD) (Murasaki-Aliberti, Da Silva, Gut, & Tadini, 2009), as well as microbiological contamination (Reddy, Das, & Das, 2005). Those enzymes are thermophilic and induce the formation of yellow, brown and pink color of coconut water during the storage (Damar, Balaban, & Sims, 2009; Prades, Dornier, Diop, & Pain, 2012). Therefore, preservation processes are necessary to increase the shelf life of the product to enable long-term commercialization.

The UHT (Ultra High Temperature) sterilization processing is effective in the microbiological and enzymatic control (Awuah, Ramaswamy, & Economides, 2007). In general, the sterilization have to be planned in order to reach microbiological stability and safety with a minimum recommended process lethality (F_o) of 3 min (Holdsworth, 1997). However, sensory and nutritional changes are usually present, which compromises the quality and acceptance of the final product (Campos, Souza, Coelho, & Glória, 1996; Tan et al., 2014). Sterilization associated with the use of sulphite has also been adopted by the industries to increase the shelf life of the product. Sulphur dioxide, sulphites and meta-bisulphites is widely used to prevent browning caused by enzymatic or oxidative reactions on foodstuffs (Pereira, Faria, & Pinto, 2013). In addition, the sulphite species exhibit antiseptic properties (Martins et al., 2011) and help to stabilize the product color, while improve flavor and appearance of several products (Ruiz-Capillas & Jiménez-Colmenero, 2009). According to Damar and co-workers (Damar et al., 2009), the appearance of pink color in coconut water thermally processed and stored is possibly due to aeration and heat treatment, and the addition of ascorbic acid or sulphite stabilize the color. The appearance of this color due microbial or enzymatic activity is unlikely, since even the boiling did not avoid the pinking.

Nuclear magnetic resonance (NMR) spectroscopy is an important analytical tool for complex mixture analysis, such as in the quality control of food (Choze et al., 2013; Silva, Alves Filho, Choze, Lião, & Alcantara, 2012). Nevertheless, considering the fact that NMR data might generate highly complex matrices with a large inherent spectral similarity, the visual analysis may be

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http://dx.doi.org/10.1016/j.foodchem.2016.08.035
0308-8146/

unfeasible (Spraul et al., 2009). Together to NMR, chemometrics is adopted to employ mathematical tools in the chemical data (Alves Filho et al., 2012; Larsen, van den Berg, & Engelsen, 2006). Purkayastha et al. (2012) studied the effects of addition of L-ascorbic acid on the quality of micro-filtered coconut water by using ^{1}H NMR and observed that on average, signals from α-rhamnopyranosyl ainomeric proton, free sugars or sugar alcohols were mostly present (Purkayastha et al., 2012). Jagannathan, Govindaraju, and Raghunathan (1995) studied the mature coconut water through magnetic resonance imaging (MRI) and verified sugar regions (δ 5.1–5.5) (Jagannathan et al., 1995).

The aim of this work was the evaluation of the chemical composition of coconut water subjected to sulphite adding and/or ultra high temperature sterilization by using ^{1}H NMR and chemometrics in order to detect possible chemical changes from different processing methods.

2. Experimental

2.1. Sample and UHT sterilization process

Green coconuts (*Cocos nucifera*, L.) with maturation ages between 6 and 7 months were harvested in Ceará state, Brazil. The coconuts were initially rinsed in tap water, followed by 15 min sanitization in chlorinated water (100 mg.L^{-1} of sodium hypochlorite) and then, cut for water extraction, filtered, and frozen at −17 ± 2 °C before the processing.

Six treatments were obtained with different heating temperatures and addition of sulphite, as described: control; control with sulphite; 110 °C (sample with pink color); 110 °C with sulphite; 136 °C; 136 °C with sulphite. These temperatures were chosen in a randomized experimental design and also based on previously sterilization studies in order to verity the appearance of pink color at 110 °C without sulphite addition. The thermal treatment of the samples was performed with retention time of 8 s using an Armfield tubular heat exchanger (model FT74), cooling with chiller Armfield FT63, filled under aseptic conditions in 210 mL glass bottles and closed with plastic screw cap. The packages were sterilized with 0.5% peracetic acid solution and rinsed with sterile water before filling. The sulphite (Vetec™) addition at 40 mg.L^{-1} was performed to prevent browning caused by enzymatic or oxidative reactions in the processed coconut water, and to compare the possible composition changes. The control samples were kept frozen until the NMR analysis. Samples submitted to heat treatment were stored at room temperature until the occurrence of the color changes (same time of storage for all processed samples) and then, aliquots were also frozen before the NMR analysis. All these processing were performed in triplicate.

2.2. NMR spectroscopy and molecular identification

An aliquot of 3.0 mL of the resultant samples was transferred to tubes and centrifuged at 605g for 15 min. Then, 130 μL of the samples were transferred to vials and mixed with 14 mM of EDTA in 350 μL of CD_3OD-d4 (tetradeutered methanol 98%) containing 1% of sodium-3-trimethylsilylpropionate (TMSP-2,2,3,3-d4 98% purity), and transferred to 5 mm NMR tubes. The EDTA was added to minimize the ionic strength effect on frequency shifts in the NMR spectra.

The NMR experiments were performed on Agilent 600-MHz spectrometer equipped with 5 mm (H-F/^{15}N-^{31}P) inverse detection One Probe™ and actively shielded Z-gradient. The NMR probe was frequency tuned and impedance matched before each acquisition. The ^{1}H NMR spectra were acquired in quadruplicate using the PRESAT pulse sequence for the water suppression (δ 4.98), since this pulse program presented less effect in the surrounding region according to the saturation profile of the non-deuterated water signal. The data were acquired under quantitative conditions using 90° hard pulse (providing maximum signals intensity) determined by the annulment of the most ^{1}H signals after 360° pulse (90° = ¼ × 360°) (Alves Filho et al., 2015). A total of 64 scans were acquired with 64 k of time domain points for a spectral window of 12 ppm, and receiver gain adjusted to 16 for all ^{1}H NMR measurements. The acquisition time of 5.0 s and relaxation delay of 10.0 s used were more than 5 times the longest T_1 observed in the signals. The temperature was controlled at 298 K and the TMSP-d4 was used as an internal standard (0.0 ppm). The spectra were processed applying an exponential multiplication of the FIDs by a factor of 0.3 Hz before Fourier transformation of 64 k points. Phase corrections were performed manually and the baseline corrections were applied over the entire spectral range. The manual mode was used also for the signals integration process choosing the same width for each compound (Winning, Larsen, Bro, & Engelsen, 2008).

Two-dimensional (2D) NMR experiments were acquired using the standard spectrometer library pulse sequences. ^{1}H-^{1}H COSY experiments were obtained with spectral width of 18,028.1 Hz in both dimensions; 1442 × 200 data matrix; 32 scans per t1 increment and relaxation delay of 1.0 s. One-bond ^{1}H-^{13}C HSQC experiments were acquired with an evolution delay of 1.7 ms for an average ^1J(C,H) of 145 Hz; 1442 × 200 data matrix; 80 scans per t1 increment; spectral widths of 9615.4 Hz in f2 and 30,165.9 Hz in f1, and relaxation delay of 1.0 s. The ^{1}H-^{13}C HMBC experiments were recorded with an evolution delay of 50.0 ms for ^{LR}J(C,H) of 10 Hz; 1442 × 200 data matrix; 180 scans per t1 increment; spectral widths of 9615.4 Hz in f2 and 30,165.9 Hz in f1, and relaxation delay of 1.0 s.

The identification of the constituents within the coconut water samples was performed through ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC experiments. The results were compared to the existing data in open access databases and literature reports (see Supplementary Information).

2.3. Chemometric analysis

The ^{1}H NMR spectra were utilized as input data for Amix™ program to Principal Component Analysis (PCA) in order to create an overview, showing grouping trends and outliers in the data with confidence level of 95% (Hotelling, 1933). Chemometric analyses were performed using the quadruplicate of the six treatments of coconut water, as described in item 2.1. PCA analyses were performed using two regions of the ^{1}H NMR spectra: whole spectra – δ 0.84 to δ 8.54; and aliphatic region – δ 0.84 to δ 3.02.

For the PCA, each spectrum was divided into 0.04 ppm wide buckets, using simple rectangular bucket, sum of intensities in integration mode and scaled to total intensity in scaling process. The spectra were divided into 145 buckets for PCA using total spectra, and into 44 buckets using only the aliphatic region. The area influenced by water suppression according to the saturation profile (δ 4.62 to δ 5.15) was excluded of the bucketing process. The bucket tables were pre-processed by mean-centered, with the mean value of each column subtracted from individual elements since this pretreatment provided better differences between the samples and it did not allow that noises affect negatively the distribution (Beebe, Pell, & Seasholtz, 1998). The chemical shift values in the loadings plots refer to the center position of each bucket.

2.4. Quantification analysis

The compounds that stood out in the multivariate evaluation were quantified in order to corroborate the chemometric results. Therefore, sucrose, fructose, glucose, ethanol, and malic acid were

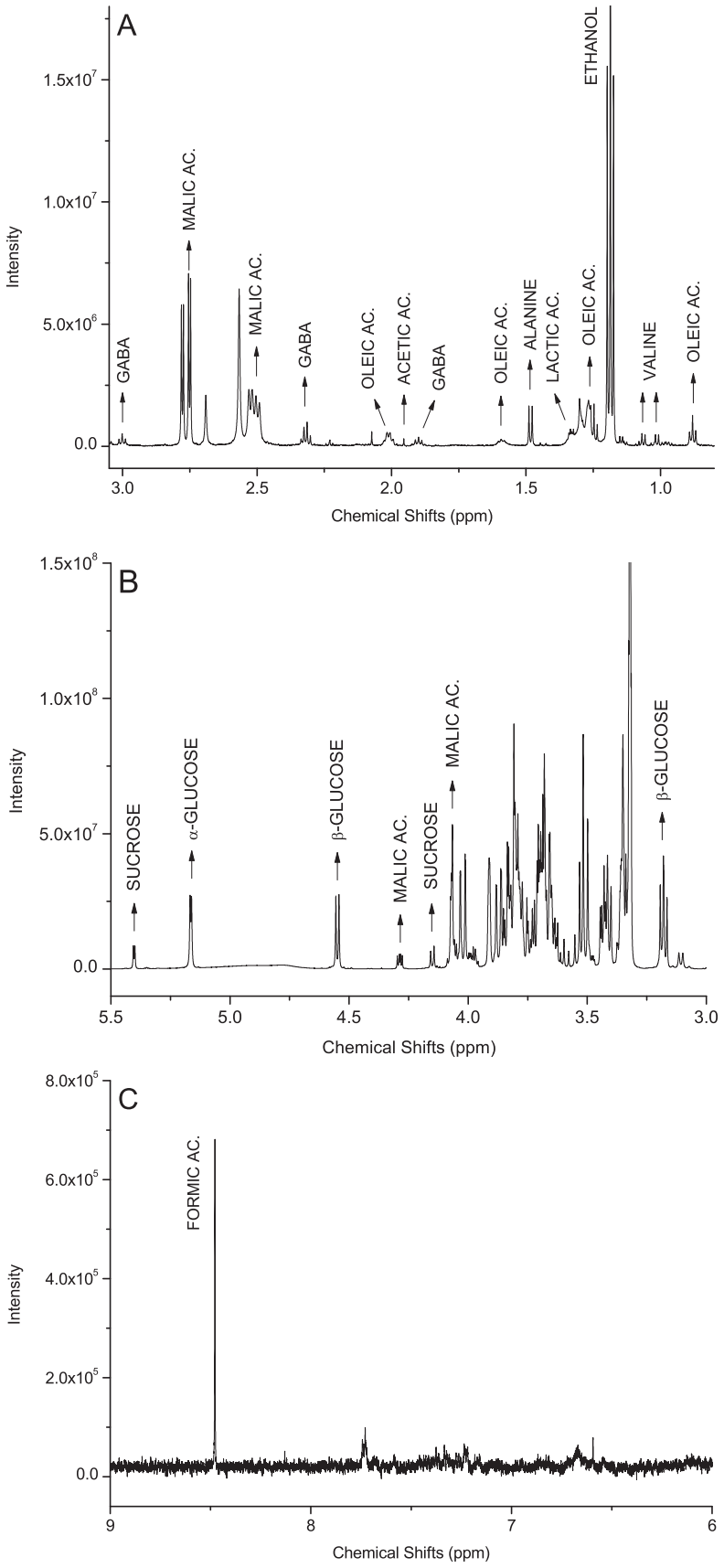

Fig. 1. Expansions of a representative ^{1}H NMR spectrum from coconut water: δ 0.8 to δ 3.0 (A); δ 3.0 to δ 5.5 (B); δ 6.0 to δ 9.3 (C).

quantified using an external reference method. A standard solution of sucrose (5.0 mg.L^{-1}) was used to calibrate the equipment, and the probe file was updated with all the parameters required for determination of the concentrations in unknown samples (Alves Filho et al., 2016; Malz, 2008). When necessary, the deconvolution process was performed in order to remove problems as overlapped signals. The combined uncertainty was estimated based on the analytical errors and the standard deviations from the three replicates of sampling (Section 2.1) and quadruplicate of ^{1}H NMR acquisitions. The results were evaluated using the analysis of variance ANOVA single factor analysis (with significance level of 0.05; means comparison using Tukey test; and Levene to test the homogeneity of variance) to statistically certify the differences in the concentrations.

3. Results and discussion

3.1. Identification of the primary metabolites

A detailed identification of the primary metabolites in coconut water is presented in three expansions of the regions from a representative ^{1}H NMR spectrum (Fig. 1): "A" for α-carbonylic/alkyl residue hydrogen (δ 0.8 to δ 3.0); "B" for carbinolic hydrogen (δ 3.0 to δ 5.5); and "C" for aldehydic and aromatic protons (δ 6.0 to δ 9.3). In general, the ^{1}H NMR spectra presented compounds in two different regions (A and B) showing that the coconut water comprises a high level of sugar and aliphatic structures. The presences of amino acids, organic acids, and sugars in coconut water have been reported earlier by different groups (Vigliar, Sdepanian, & Fagundes-Neto, 2006; Yong, Ge, Ng, & Tan, 2009). Basically, one compound was certainly identified in the aldehydic and aromatic chemical shifts (expansion C) – the formic acid at δ 8.43. The sucrose, glucose, fructose, ethanol, and malic acid were the major organic compounds identified in coconut water. Ethanol is not a contaminant from the processing and its presence was previously verified in fresh coconut water during the development of this study. The structures of the compounds, ^{1}H and ^{13}C chemical shifts, multiplicity, correlations and constant coupling are presented in the Supporting Information.

3.2. Chemometric analysis

Initially, it was observed that the sub-processed sample (at 110 °C) without sulphite presented visual changes with the occurrence of pinking during the storage at room temperature for all replicates (see Fig. 1 in the Supporting Information). Other samples did not show visual changes. Additionally, after two weeks the color faded and apparently being agglomerated and deposited on the package bottom. It was also observed that during the storage period, the enzymatic activity within the coconut water did not regenerate (data not shown) showing that the color changing on the coconut water may be due to chemical oxidation. The addition of sulphite and the increase of temperature from 110 °C to 136 °C prevented the formation of pink color in coconut water. Therefore, the chemometric analysis was performed to investigate the differences in the composition of coconut water processed at two different temperatures and to evaluate the addition of sulphite as an anti-oxidant.

The bucket tables from the ^{1}H NMR spectra were analyzed using box-and-whisker graphic (Fig. 2) to observe the overall trends of the major compounds within the most significant regions for PCA. The Fig. 2 also shows a ^{1}H NMR spectrum from coconut water to illustrate the regions of the buckets that presented higher variation. Therefore, this analysis highlighted the compounds with higher variation between the samples with carbohydrates as the most important variables for the chemometric model. It was also observed the variables considered outliers at 95% confidence level.

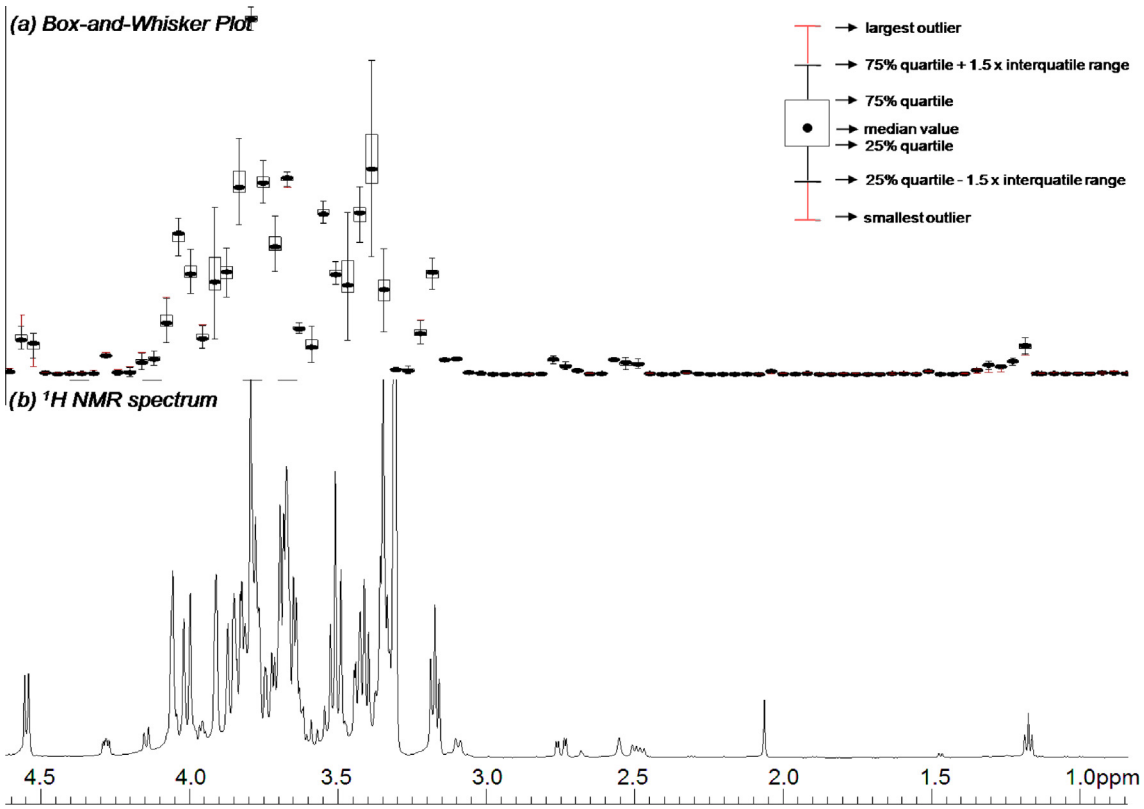

Fig. 2. Box-and-whisker analysis of the bucket tables from the ^{1}H NMR spectra.

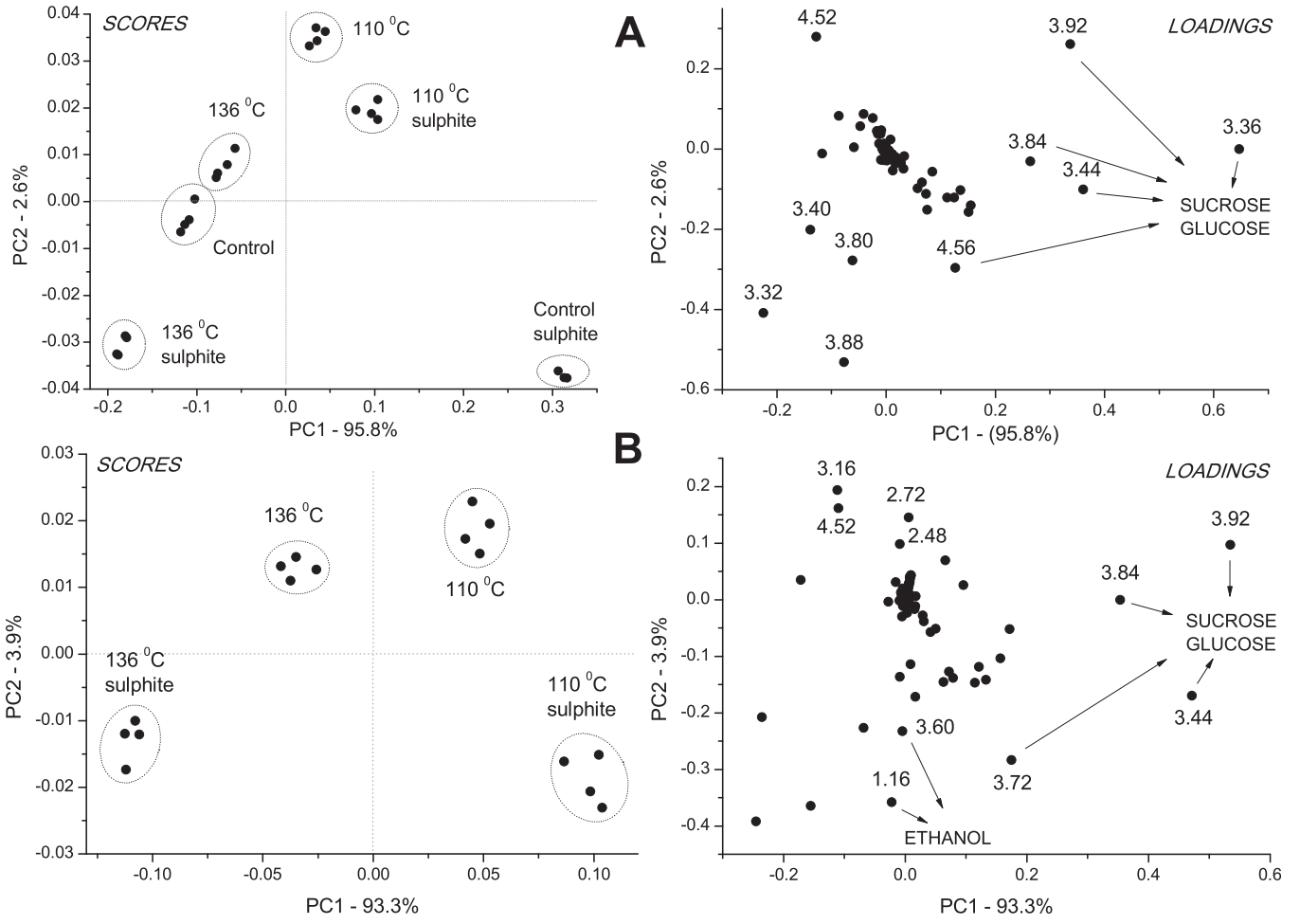

Fig. 3. PC1 and PC2 scores (left side) and loadings (right side) coordinate system for coconut water: (A) using all spectra; (B) thermally processed.

The PCA was applied to reduce the dimensionality of the original data in two PCs and to assist the interpretation of the multivariate data. Fig. 3A shows the PCA scores plot for the control, samples thermally processed and the samples with addition of sulphite. An additional PCA was developed only for thermal processed and the samples with addition of sulphite (Fig. 3B) in order to detail the effect of these treatments in the composition of the coconut water.

The PC1 *vs* PC2 scores show the tendency of separation of the samples with 98.4% and 97.2% of the total variance (Fig. 3A and B, respectively) at the first two axes. The two samples processed at 110 °C and the control sample with sulphite addition were positioned in positive values of PC1, and the two samples processed at 136 °C and the control sample without sulphite addition in negative values of the same PC (Fig. 3A). In general, the control coconut water with sulphite presented higher concentration of sugars, which can be attributed to the anti-oxidative effect of the sulphite. The Fig. 3B showed more detailed the separation between the processed samples. It is observed two trends: in PC1 the samples agglomerates regarding the thermal treatment – positive scores are located the samples processed at 110 °C, while in negative scores are located the samples processed at 136 °C; and in PC2 the samples agglomerated regarding the sulphite addition – in positive scores are located the samples without sulphite, while in negative scores are located the samples with sulphite.

A careful inspection of the loadings plots from PC1 × PC2 provided important evidences regarding the different treatments. Overall, the loadings graph shows the sucrose, glucose, ethanol and malic acid as the compounds that presented the highest variation in concentration after the processing of coconut water. Therefore, the samples processed at 136 °C presented less glucose and sucrose content, which showed that the harshest thermal treatment resulted in the carbohydrates degradation. The thermal processing of foodstuffs may induces non-enzymatic reactions between amino acids and reducing sugars, know as Maillard reaction that lead to color and flavor changes (Mottram, 2007). Hence, the reduction detected in this analysis can be associated with the Maillard mechanism. However, the decreasing on carbohydrates content was not as sharp as shown in the quantitative analysis. For PC2, it was observed that the samples with sulphite addition presented highest amount of ethanol. This fact occurred because the sample that gives risen to it was the control coconut water with addition of sulphite, which presented also highest amount of ethanol as shown in quantification graph (Fig. 5).

In order to get better understanding of the effect of processing on the particular components of the coconut water, chemometric model was performed only for the aliphatic region, as described below. Fig. 4A presents the PCA analysis performed to detailed evaluation of the aliphatic region (δ 0.84 to δ 3.02), and a specific PCA analysis was developed only to the processed samples to detect specific behaviors among them (Fig. 4B).

The most important trends on PCA scores from aliphatic region (Fig. 4A and B) were found according to the sulphite addition and temperature during the processing. It is observed more clearly in the Fig. 4B, with the coconut water samples processed without sulphite addition in negative values of PC1, and the samples processed at higher temperature (136 °C) located in positive scores of PC2.

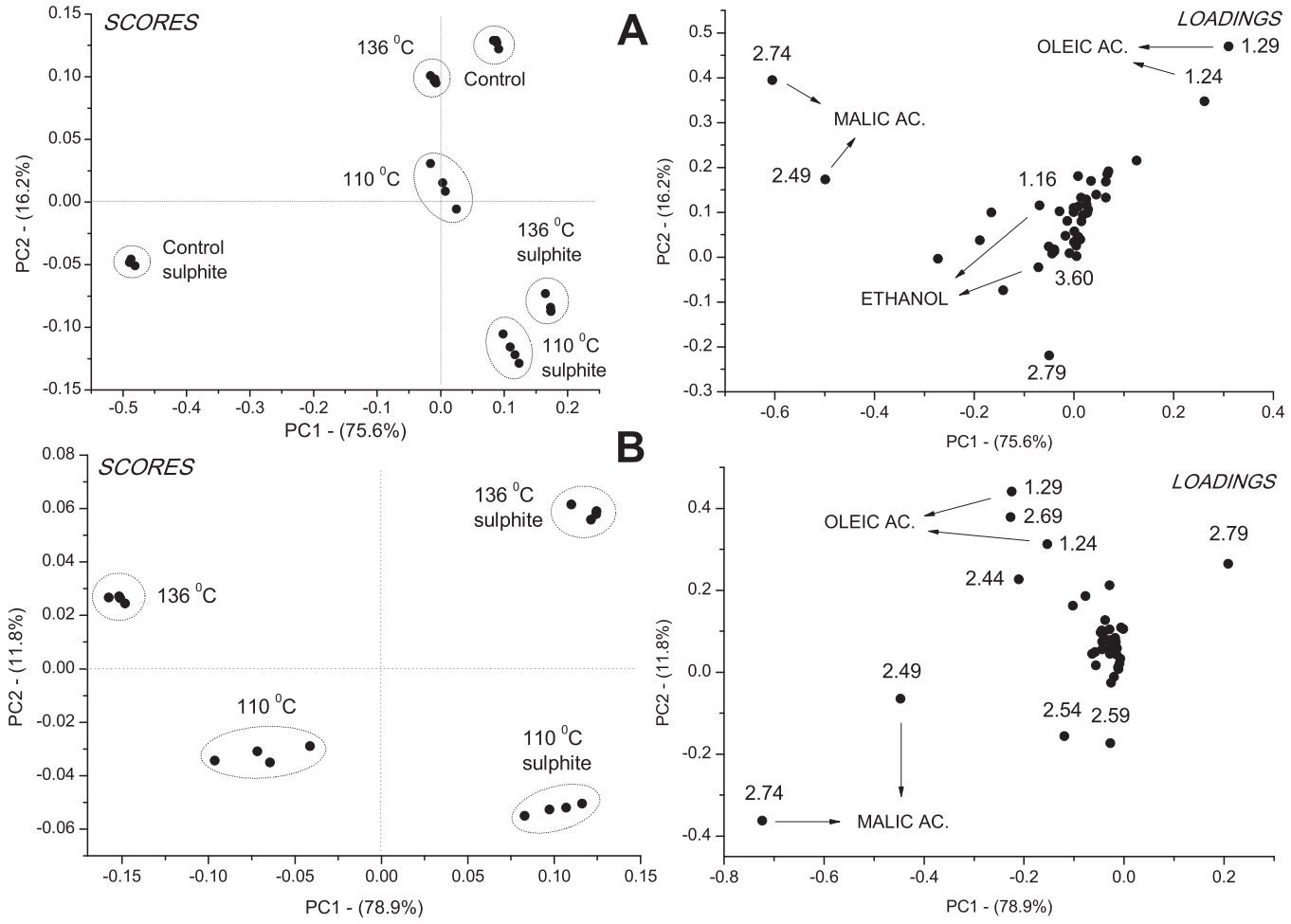

Fig. 4. PC1 *vs.* PC2 scores coordinate system for aliphatic region of the coconut water: A) using all spectra; B) thermally processing.

The loadings show that the fatty acids (manly oleic acid) were the most important variable for the clustering, and the samples without sulphite presented higher amount of the aforementioned substances. This may occurred because the sulphite is an anti-oxidant agent (Ruiz-Capillas & Jiménez-Colmenero, 2009), which decrease the oxidation on the unsaturated fatty acid. The harshest thermal treatment results in higher amount of oleic acid and less amount of malic acid. The low content of oleic acid on the sample processed at 110 °C may be due to oxidation process since this temperature is an underprocessing or not commercial for sterilization. On the other hand, the low content of malic acid in the sample processed at 136 °C may be due to its degradation at higher temperature. The decrease in the malic acid content in harshest thermal treatments, although significant when compared to control or treatment at 110 °C, is still small decreasing from 3.2 to 2.9 g.L^{-1}, as shown in Section 3.3.

The classes of compounds were affected differently according to the thermal processing and the sulphite addition, as shown by the PCA results. The temperature was the parameter that presented more effect in the amount of aforementioned components.

3.3. Quantitative analysis

The compounds that presented variations in chemometric analysis and did not exhibit overlapping resonances were quantified and the results are shown in Fig. 5. Based on ANOVA single factor analysis, the tendencies observed in PCA results were detected for the concentrations of sucrose, α and β-glucose, fructose, ethanol, and malic acid, corroborating the multivariate analysis. Although the sucrose, α and β-glucose signals (at δ 5.42, δ 5.21, and δ 4.64 respectively) are near of the pre-saturation region of the non-deuterated water (δ 4.98), the evaluation of the saturation profile did not show significant influence on that signals. Additionally, deconvolution process of the signals was performed in order to remove any residual water effect.

Glucose was found to be the major organic compound in coconut water (37.60 g.L^{-1}). In general, the lower temperature and the sulphite addition preserved all the quantified compounds, except α and β-glucose. The concentration of malic acid decreased 8% after processing at 136 °C and did not change at 110 °C. The concentrations of ethanol were higher around 14% in all the samples with sulphite addition, showing its preservation regardless the applied temperature. During the coconut water processing, it was achieved the optimal conditions in order to prevent microbiological fermentation through environmental control (aseptic conditions) and storage under refrigeration. The processing at 136 °C with sulphite addition decreased 12.6% the concentration of sucrose and did not affect the glucose amount. The sucrose decreasing is related to its degradation induced by the thermal treatments. In addition, a slight increase was observed in the concentration of sucrose and glucose after the processing at 110 °C. This feature might be related to the increase of their availability in the samples, which may be due to their extraction from suspended endosperm (Chen, Sharma, & Mudhoo, 2012), as well as

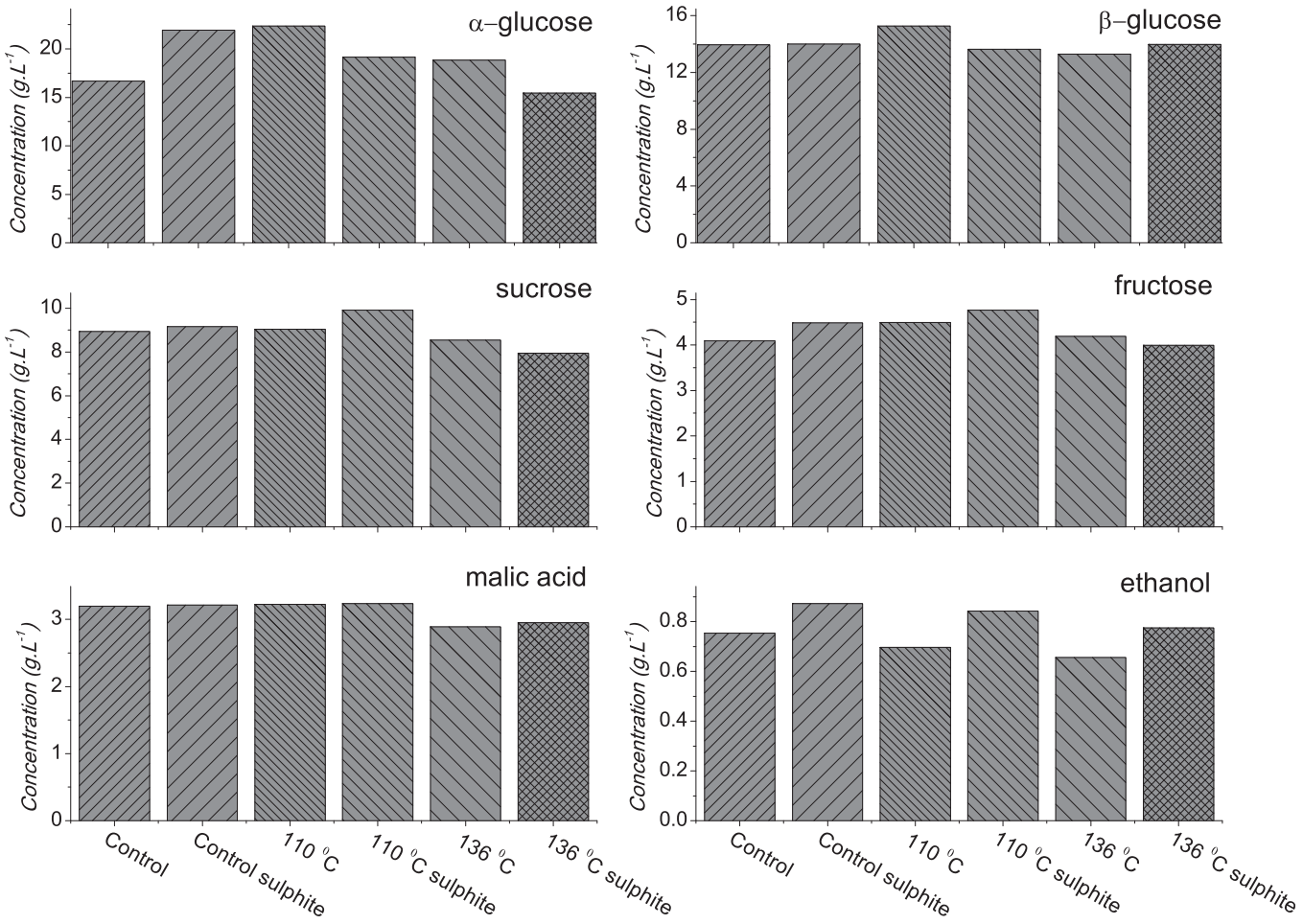

Fig. 5. Concentrations ($g.L^{-1}$) of sucrose, fructose, α and β-glucose, ethanol and malic acid in coconut water.

the use of lower temperatures that avoided the degradation of sugars in coconut water.

Despite the chemometrics have been able to distinguish the different processing, only minor changes were observed in the quantitative results. Moreover, the differences verified cannot be addressed to the pinking observed in the coconut water submitted to 110 °C and did not gives any reliable conclusions about pinking process. However, the use of sulphite in the sample processed at 110 °C and 136 °C were effective in preventing the appearance of the pink color and to avoid significant changes on the major compounds composition under storage at ambient temperature. Further analyses as HPLC-MS (high performance liquid chromatography coupled to mass spectrometer) are recommended to identify the chemical components that arise from the appearance of pink color or taste changes during UHT processing of coconut water. This advice is based on the higher sensitivity of the MS analysis compared to the NMR spectroscopy.

4. Conclusions

In this article, we demonstrate that it is possible to evaluate the variability of the primary metabolites from coconut water under different sterilization process. Chemometric analysis showed the coconut water sterilized at 136 °C with major changes in the organic composition, and lower temperature of processing (110 °C) with sulphite addition preserved the sucrose, glucose, ethanol, and malic acid. In other hand, according to the quantitative results these variations did not result in significant changes in coconut water composition as a whole, even under storage at ambient temperature.

The NMR analysis and chemometrics data were obtained quickly and non-destructively, providing quantitative information about the variation of the primary metabolites in the processed and non-processed coconut water. NMR spectroscopy has the advantage of requiring little sample preparation, which makes the sample more representative. This fact is important because to our knowledge, it was the first time that NMR combined to PCA were applied to evaluation of the composition of coconut water under different thermal processing and with addition of sulphite as an anti-oxidant.

Acknowledgments

The authors thank CNPq and CAPES for the financial support and for the scholarship; to EMBRAPA for supporting this research, and to NMR Laboratory of Universidade Federal de São Carlos (São Paulo, Brazil) for enabling the use of the chemometric program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.035.

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