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^{1}H NMR, a Rapid Method to Monitor Organic Acids during Cupuassu (*Theobroma grandiflorum* Spreng) Processing

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The development of an analytical method using ^{1}H nuclear magnetic resonance (^{1}H NMR) spectrometry to monitor cupuassu (*Theobroma grandiflorum* Spreng) bean fermentation, drying, and roasting processes is reported. The analysis of organic acids and alcohols of crude water extracts of cupuassu ground kernels were monitored by HPLC and ^{1}H NMR spectroscopy. The residual protein signals caused deleterious effects on acid and alcohol quantifications. Therefore, the analytical procedures were optimized by sample cleanup and water suppression pulse sequences in order to obtain compatible data using HPLC and ^{1}H NMR. The quantification of lactic acid, acetic acid, and 2,3-butanediol by NMR is 5- to 10-fold faster than by HPLC, with the advantage of providing the identification of several chemical species in a single experiment. Application of these analytical conditions to some cupuassu samples revealed that this methodology can be applied to the quality profiles of fermentation and roasting processes.

KEYWORDS: **^{1}H NMR spectroscopy; cupuassu; WET-CPMG; PRESAT; fermentation; roasting processes, microwave**

INTRODUCTION

Cupuassu is a commercially important tree, naturally occurring in eastern Amazon (Brazil). The fruits have a white-yellow pulp with a pleasant acidic taste and a strong fragrance. The production of "cupulate" from cupuassu seeds (a product similar to chocolate) is a multistep process: after harvesting, the seeds are separated from the fruits and fermented. The dry fermented seeds are roasted, sieved, and finely ground. The resulting solid product ("liquor") is the raw material for the "cupulate" industries (*1*−*3*).

Flavor attributes such as flavor intensity, bitterness, astringency, and acidity are assigned to kernel fermentation, drying, and roasting and caused by several reactions occurring during post-harvest processing (*4*). Roasting leads to development of specific aromas and formation of volatile organic compounds with desirable flavor properties (*5*, *6*), such as esters (ethyl and butyl butyrate, ethyl and butyl 2-methylbutyrate), terpenoids (β-linalool), and heterocyclic compounds (pyrazines, piperazine, 2,5-dihydro-2,5-dimethoxyfuran). Moreover, some undesirable volatile fermentation compounds are eliminated, such as organic acids, resulting in flavor enhancement (*7*, *8*). The fermentation process is characterized by the production of organic acids (acetic acid and lactic acid) (*9*, *10*), which are particularly important in defining cupuassu quality in the manufacture of "cupulate". It was reported that beans with high levels of acetic and lactic acid provided cocoa beans with low chocolate flavor (*11*).

The bacterial production and degradation of acetic acid during fermentation are important to cupuassu curing and flavor precursor development. However, an excess of acetic acid causes an adverse effect on cupuassu flavor. Additionally, under anaerobic glucose fermentation there is formation of large amounts of lactate and traces of acetate, ethanol, formate, and 2,3-butanediol, which is markedly different from the composition of end products under aerobic fermentation when a mixture of lactate, acetate, and acetoin-diacetyl is produced (**Figure 1**) (*12*).

Thus rapid methodologies to monitor fermentation and roasting are important in optimizing cupuassu manufacture, and ^{1}H nuclear magnetic resonance is one of the most adequate nondestructive, multinuclear, and noninvasive techniques, successfully employed for the simultaneous detection of several substances present in one sample. Additionally the spectrum acquisition time, requiring minimal sample preparation, enables the examination of many samples in a reduced time interval, as required for most food composition, authenticity, and quality control applications (*13*, *14*). Notwithstanding these properties,

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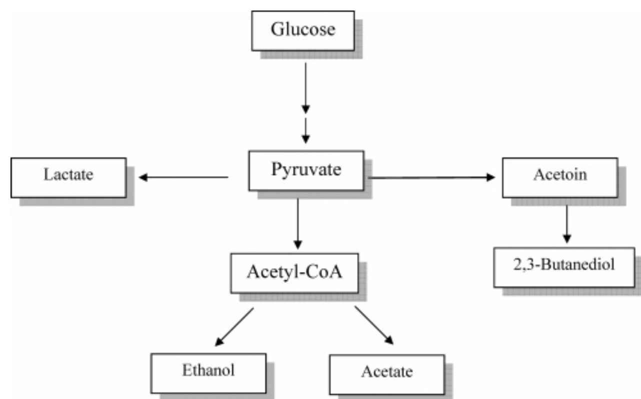

Figure 1. Pathways proposed for glucose metabolism (*11*).

this technique was never applied to cupuassu processing while HPLC is usually applied in a time-consuming protocol (*15*).

In the present study, we have compared several clean-up procedures and NMR methodologies in order to obtain comparable results by HPLC and by NMR, aiming for a faster analytical procedure to be applied to cupuassu process monitoring.

MATERIALS AND METHODS

Sample Preparation. Unfermented cupuassu beans constituted sample A. The cupuassu bean fermentation was performed at 33 °C and 65% relative humidity using wooden boxes with three compartments endowed with draining facilities (*16*). Soon after fermentation, the kernels were sun dried and roasted. The roasting process was carried out in an adapted laboratory scale microwave oven. The microwave process was set up at two conditions of time and internal kernel temperature, which were 16 min and 105 °C (sample B) and 14 min and 103 °C (sample C) (*7*). The cupuassu liquor samples were obtained by grinding in a refrigerated analytical laboratory mill (IKA - UNIVERSAL) to prevent volatilization of some components, and the water-soluble components of the liquor (2 g) were extracted with 20 mL of Nanopure water using screw-capped tubes in a laboratory tube agitator for 3 min. The samples were then filtered through Whatman N*o*. 1 paper, a 45 μm Millipore filter, and then a 20 μm Millipore filter.

For the high-resolution ^{1}H NMR measurements the samples were prepared using 400 μL of the aqueous cupuassu extract and 200 μL of D_2O containing 2.5 mmol L^{-1} sodium d_4-trimethylsilylpropionate (TSP). The latter had the double function of internal reference and quantification standard.

^{1}H NMR Measurements. The experiments were performed with an NMR spectrometer (Varian, INOVA-500), operating at a magnetic field of 11.7 T and equipped with a standard *Z*-axis-PFG triple-resonance (H{C/N}) probe (maintained at 25 °C) and VNMR 6.1c software.

Method 1. The water signal suppression was obtained by applying the PRESAT (WATER) pulse sequence (*17*), and the ^{1}H NMR (PRESAT) optimized acquisition parameters were as follows: relaxation delay (1.5 s), acquisition time (2.7 s), 32 scans, spectral width of 12 000 Hz. The water presaturation was obtained by setting the decoupler channel to phase coherence with the transmitter and irradiating for 1.5 s at 6 dB at the HDO frequency. The processing optimization was obtained by exponential function multiplication of the 65 536 complex points (lb = 0.3 Hz) and zero filling for better digital resolution prior to Fourier transformation.

Method 2. The water and protein signals in the analyzed samples were removed by the WET-CPMG (*18*) pulse train that exploits the shorter transverse relaxation rate (T_2) of macromolecules. Optimized acquisition parameters of all ^{1}H NMR (WET-CPMG) spectra were as follows: relaxation delay (5 s), acquisition time (3.7 s), 64 scans, spin−echo delay (100 μs), and number of cycles (350) with a spectral width of 10 000 Hz. The 65 536 complex points were optimized by applying an exponential function (lb = 0.2 Hz) and zero filling for better digital resolution. A selective pulse of 9 dB and 32 ms of length was used for water suppression in the WET pulse sequence.

Component quantitative analyses (acetic acid, lactic acid, and 2,3-butanediol) were carried out by first selecting a signal without overlapping and comparing the integrated values to that of the TSP signal.

Commercial samples of acetic acid, lactic acid, and 2,3- butanediol were obtained from Aldrich and Fluka.

Chromatography. The organic acid HPLC analyses were carried out with a HP liquid chromatograph (model HP 1050) equipped with a model VDC 3390A temperature controller (Waters), UV detector (Waters 486), and an Aminex HPX-87H cationic column (9 μm, 300 × 7.8 mm i.d.) coupled with a similar precolumn (30 × 4.6 mm). A 50 mmol L^{-1} sulfuric acid solution was used as mobile phase. The injected sample volume was 20.0 μL (crude cupuassu aqueous extract prepared as described above), and the separation was performed at a flow rate of 0.6 mL min^{-1} at 70 °C for 25 min. All the analyses were performed in triplicate.

RESULTS AND DISCUSSION

High-Resolution NMR Experiments. The main problem in establishing ^{1}H NMR as a technique to quantify the organic acid content in cupuassu crude water extracts and obtaining an overall picture of the water-soluble components produced during the fermentation and roasting process is the water signal, which has to be reduced by several orders of magnitude for adequate digitalization of the millimolar solute resonance.

Water suppression was obtained by applying the PRESAT pulse sequence to the ^{1}H NMR data acquisition. The spectrum was complex with signal overlapping (**Figure 2**), and the identity of the peaks in the mixture was confirmed by comparing the ^{1}H NMR spectra with those of the pure standard compounds (acetic, lactic, citric acids, and 2,3-butanediol) and by fortification of the mixture with these standards.

The peak assignments were performed on the basis of chemical shift comparisons with data in the literature (*12*), and those with no visible overlapping were selected for quantification of acetic acid (singlet, 3H, 1.95 ppm), lactic acid (doublet, 3H, 1.34 ppm), 2,3-butanediol (doublet, 6H, 1.14 ppm), citric acid (doublet, 2H, 2.59), (doublet, 2H, 2.74 ppm), acetoacetate (singlet, 3H, 2.24 ppm), and alanine (doublet, 3H, 1.48 ppm). For a complete evaluation of the mixture components, 2D NMR (H,H-COSY and H,C-HSQC) might have been useful and will be considered for investigation in the future. However these 2D NMR techniques consume longer equipment time, and those including water signal suppression pulse sequences have to be optimized to avoid suppression of signals very close to water resonance.

To minimize spectral acquisition, a recycle time of 4.5 s was chosen, taking into consideration that no detectable integral deviation was observed by decreasing the recommended recycle time ($5T_1$ + acquisition time = 30 + 2 s) to the above-mentioned value.

Quantification was obtained by comparing the integrated values of the selected signals with those of the internal reference sodium d_4-trimethylsilylpropionate (TSP, 2.5 mmol L^{-1}), each corrected to a single hydrogen value. The weighted percentages of the different components in the liquor ($\%_{com/liq}$) were obtained by eq 1:

$$\%_{comp/liq} = \frac{\dfrac{A_{comp}/n(^1H)}{A_{TPSA}/9} \cdot 1.25 \times 10^{-3} \cdot MM_{comp} \times 10^{-3}}{W_{liq.ext.}} \cdot 100 \quad (1)$$

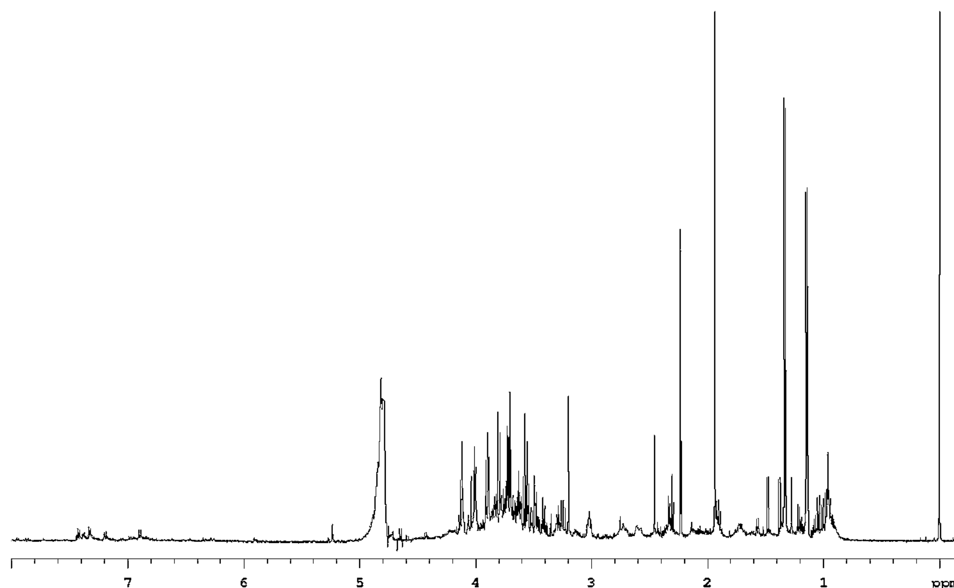

Figure 2. ^{1}H NMR (499.88 MHz, H_2O/D_2O; water PRESAT) spectrum of cupuassu liquor aqueous extract.

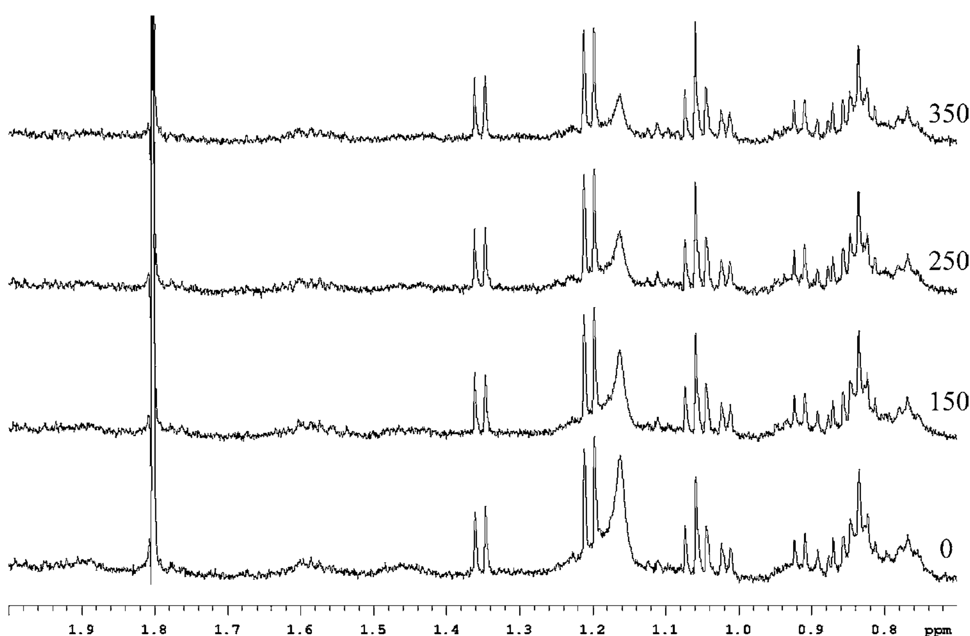

Figure 3. WET-CPMG spectra with $\tau = 200$ μs and ncyc arrayed (0, 150, 250, and 350) to show the effect of increasing the spin–echo time on the signals originating from protein. Arrows indicate main regions where protein signal depletion occurred.

Table 1. Metabolite Concentrations (g 100 mL^{-1}) from Model Samples Obtained by ^{1}H NMR (WET-CPMG, PRESAT)

	mixture (sample 1 - ref values)		mixture + BSA (sample 2)		filtered mixture (sample 3)	
technique	A Ac[a]	L Ac[b]	A Ac	L Ac	A Ac	L Ac
PRESAT	0.058	0.063	0.100	0.098	0.059	0.067
WET-CPMG[c]	–	–	0.108	0.082	–	–
HPLC	0.050	0.060	–	–	0.050	0.060

[a] A Ac: acetic acid. [b] L Ac: lactic acid. [c] ncyc = 250.

where A_{comp} is the signal area of the considered compound, normalized to one proton, and MM_{comp} is its molar mass (90.07 and 60.05 g mol^{-1} for lactic and acetic acids, respectively). A_{TSP} is the area of the TSP signal, normalized to one proton, whose concentration in the samples its equal to 1.25 mmol L^{-1} (8.33 $\times$ 10^{-4} $\times$ 1.5), and $W_{liq.ext.}$ is the cupuassu liquor weight used to prepare the sample.

Of the previously selected signals only lactate and acetate were integrated, and a significant acid concentration variation was observed when comparing the NMR results with those obtained by HPLC. This and the signal line broadening indicated the interference of soluble proteins. Removal of the protein envelope was achieved by applying the WET-CPMG program (**Figure 3**). This method exploits the short transverse relaxation rate (T_2) of macromolecules in order to minimize the protein signals without affecting the overall signals from the metabolites, increasing the accuracy of the measurements. No comments have been found with respect to the quantitative accuracy of this methodology, and comparison of the HPLC and the ^{1}H NMR results for acetic acid and lactic acid were not coherent.

To optimize clean-up and NMR methodologies, two model samples were prepared, containing lactic (0.063 g 100 mL^{-1}) and acetic (0.068 g 100 mL^{-1}) acids, one without (sample 1, reference values) and the other with albumin (0.400 g 100 mL^{-1}) (sample 2). Quantification applying PRESAT to sample 2

Table 2. Acid Concentration in Cupuassu Water Extracts (0.20 μm Prefiltered) (g 100 mL^{-1}) by ^{1}H NMR (WET-CPMG and PRESAT) and HPLC

technique	sample A[a]		sample B[b]		sample C[c]	
	A Ac	L Ac	A Ac	L Ac	A Ac	L Ac
WET-CPMG	0.034 ± 0.003	0.029 ± 0.001	0.029 ± 0.002	0.029 ± 0.001	0.029 ± 0.001	0.038 ± 0.001
PRESAT	0.041 ± 0.002	0.042 ± 0.002	0.045 ± 0.003	0.065 ± 0.007	0.062 ± 0.001	0.066 ± 0.003
HPLC	0.040 ± 0.003	0.047 ± 0.003	0.047 ± 0.009	0.070 ± 0.004	0.063 ± 0.003	0.063 ± 0.004

[a] Sample A: unfermented. [b] Sample B: fermented and roasted for 16 min at 105 °C. [c] Sample C: Fermented and roasted for 14 min at 103 °C.

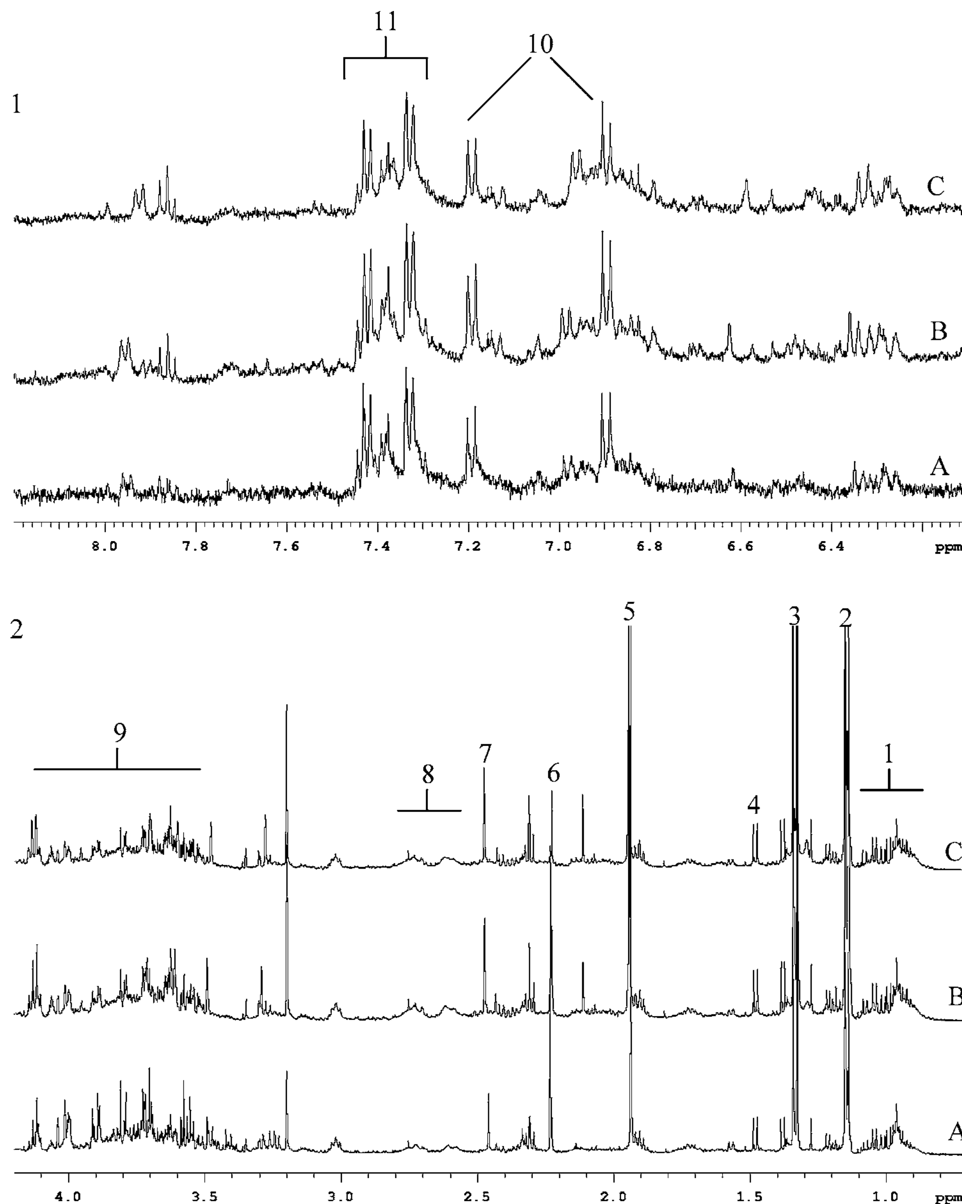

Figure 4. (**Panel 1**) Aromatic region of the ^{1}H NMR spectrum of cupuassu: (A) sample A, (B) sample B, and (C) sample C. (**Panel 2**) Aliphatic region of the ^{1}H NMR spectrum of cupuassu: (A) sample A, (B) sample B, and (C) sample C. Only the major resonances are labeled. Key: 1, leucine, isoleucine, and valine; 2, 2,3-butanediol; 3, lactic acid; 4, alanine; 5, acetic acid; 6, acetoacetate; 7, succinic acid; 8, citric acid; 9, carbohydrates; 10, tyrosine; 11, phenylalanine.

produced a set of results 40% above the reference values (sample 1), this was assigned to the presence of bovine serum albumin (BSA) protein signals. These were removed by applying WET-CPMG with 250 spin−echoes (100 ms); however, the quantification did not match those of the reference sample 1 (**Figure 3**, **Table 1**).

To overcome these distortions, BSA was eliminated by filtration using a 0.20 μm filter (Millipore) (**Table 1**, sample 3), and the experiments with sample 3 reproduced the expected values for sample 1. Thus it was concluded that sample filtration (0.20 μm) is an essential clean-up procedure to obtain good ^{1}H NMR quantitative analyses.

A critical analysis of **Table 2** indicated that the results obtained by applying PRESAT were more compatible with the HPLC data than those obtained by applying the CPMG pulse sequence, which were below the HPLC values. This was assigned to signal loss during the T_2 filter in the WET-CPMG method. Thus, PRESAT was the method of choice.

Table 3. Concentration of Some Components in Cupuassu Water Extracts (0.20 μm Prefiltered) (g 100 mL^{-1}) by ^{1}H NMR

	citric acid	alanine	acetoacetate	2,3-butanediol
sample A[a]	0.068 ± 0.001	0.016 ± 0.003	0.015 ± 0.002	0.039 ± 0.003
sample B[b]	0.049 ± 0.002	0.012 ± 0.002	0.012 ± 0.004	0.035 ± 0.002
sample C[c]	0.054 ± 0.002	0.011 ± 0.001	0.016 ± 0.001	0.031 ± 0.002

[a] Sample A: unfermented. [b] Sample B: fermented and roasted for 16 min at 105 °C. [c] Sample C: Fermented and roasted for 14 min at 103 °C.

This methodology was applied to cupuassu aqueous extracts: sample A (unfermented) and samples B and C (fermented and then microwave roasted by applying different internal kernel temperatures and processing times). The results, expressed in g 100 mL^{-1}, are in **Table 2** (**Figure 4**). Lactic and acetic acid concentrations (**Table 2**) were lower in sample B (compared to C) due to their volatilization during the roasting at a higher temperature for a longer processing time.

Additionally, a full profile of the water-soluble constituents was assessed. The total carbohydrate content in sample A, unfermented, is 50% higher than in samples B and C. Likewise, the content of some amino acids, like isoleucine, leucine, and valine, in sample A is 40% higher than in samples B and C. These unique 2 min ^{1}H NMR experiments further provided the total content of 2,3-butanediol, alanine, citric acid, and acetoacetate (**Table 3**), and the citric acid content was the most influenced by fermenting and roasting conditions. The ^{1}H NMR and HPLC values in Tables 2 and 3 were obtained by averaging triplicates which provided the standard deviation values. ^{1}H NMR spectroscopy represents a simple and quick technique to investigate the modifications in the cupuassu liquor before and after fermentation and during roasting, providing accurate information on the metabolic profile of the seeds and kernels in "cupulate" manufacture. This new analytical methodology has the advantage of providing the profile of all water-soluble metabolites and their abundance 10 times faster than the conventional HPLC methodology. This alternative is realistic mainly due to the increasing availability of NMR equipments, which are now gaining popularity in industries and in hospitals (*19*).

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