^{1}H NMR spectroscopy applied to the cocoa evaluation of *Theobroma cacao* clones

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Abstract: *Major constituents of aqueous cocoa extracts from ten Theobroma cacao clones resistent to witches' broom disease (Moniliophthora perniciosa) were evaluated by ^{1}H NMR. This analytical tool provided the simultaneous monitoring of nine secondary metabolites (ethanol, acetic acid, lactic acid, succinic acid, frutose, glucose, sucrose and caffeine). These analyses were faster than those by HPLC.*

Resumo: *Os principais constituintes do extrato aquoso das sementes fermentadas de dez clones de Theobroma cacao resistentes à doença da vassoura de bruxa (Moniliophthora perniciosa) foram avaliados por ^{1}H RMN. Esta ferramenta analítica permitiu o monitoramento simultâneo de nove metabólitos secundários (etanol, ácido acético, ácido láctico, ácido succínico, frutose, glicose, sacarose e cafeína). Estas análises foram realizadas mais rapidamente do que por CLAE.*

Introduction

Cacao (*Theobroma cacao*), a small tree native to the Americas, as recently shown by genetic studies, originated in the Amazon region then was distributed throughout Central America and Mesoamerica. Its seeds are used to make cocoa and chocolate[1].

Nowadays the witches' broom disease is one of the main problems in cacao cultivation in Bahia Brazil, due to the increasing propagation of the pathogenic fungus *Moniliophthora perniciosa* (former *Crinipellis perniciosa*), leading to the devastion of 85% of cacao and trees causing a large economical loss to the Brazilian economy. This has motivated several research centers (such as the Center for Cacao Research, CEPEC) to select new varieties more resistant to this disease[1,2].

Moniliophthora perniciosa resistent clones are agriculturally important. However, only clones that produce cocoa beans of good quality are of real economical value. The quality is directly related to the development of chocolate flavour during the fermentation process and to the indigenous metabolites, theobromine and caffeine[3]. Major flavour differences have been assigned to the presence of organic acids (lactic, acetic and succinic acids) in the final product, as monitored by analytical tools that quantify not only these organic acids but also cafeine, theobromine, sugars and alcohols. Among the most used analytical tools are high performance liquid chromatography (HPLC) [4,5] and, alternatively, high-field ^{1}H and ^{13}C NMR methodologies[6,7,8,9] requiring less analytical time and providing access to both qualitative and

quantitative information. Indeed 1 and 2D NMR experiments were used to study the localization and identification of phenolic compounds in *Theobroma cacao*[10].

The present investigation was apreliminary evaluation of the cocoa quality by monitoring sugars, cafeine, theobromine, ethanol and organic acids produced during cacao fermentation and the roasting process of *Moniliophthora perniciosa* resistent *Theobroma cacao* clones by ^{1}H NMR.

Experimental

Sample preparation.

Theobroma cacao beans (50 kg) were fermented for 6 days in square perforated wooden boxes (50 cm x 50 cm x 50 cm)[11]. The boxes was maintained without motion for 48 h and then shaken every 24 h up to 144 h. Fermentation monitoring samples were drawn at 0, 48, 96 and 144 h. After a 6 day fermentation period, the kernels were sun dried to 8% content and then roasted. Aqueous cacao extracts for the NMR experiments were obtained by grinding 2 g of kernels in a refrigerated analytical laboratory mill (IKA – Universal) to prevent volatilization of some components. The water soluble components were extracted with 20 mL of Nanopure water using screw capped tubes in a laboratory tube agitator for 3 minutes. The samples were then filtered through Whatman n°1 paper, a 45 μm Millipore filter and then through a 20 μm Millipore filter[7].The water content was measured according to Horwitz´s method 31.1.03 with Titroline Alpha Shott equipment, model TZ1282[12].

^{1}H NMR samples were prepared using 200 μL of the aqueous cacao extract and 400 μL of D_2O containing 0.75 mmolL^{-1} sodium d_4-trimethylsilylpropionate. The latter had the double function of acting as the internal reference and as the quantification standard.

^{1}H NMR measurements.

The experiments were performed with a NMR spectrometer (Varian, INOVA-500) operating at a magnetic field of 11.7 T, and equipped with a 1H{13C/15N/31P} 5 mm z PFG Penta Probe**,** maintained at 25°C, and using VNMR 6.1c software. Water signal suppression was obtained by applying the PRESAT (WATER) pulse sequence and the ^{1}H NMR (PRESAT) optimized acquisition parameters were: relaxation delay: 18.0 s, based on the average T_1 value obtained with water saturation; acquisition time: 3.0 s; number of scans: varied from 8 to 32 according to the samples; spectral width: 12000 Hz. The water presaturation was obtained by setting the decoupler channel to phase coherence with the transmitter and irradiating for 18.0 s at -4 dB at the HDO frequency. The processing optimization was obtained by exponential function multiplication of the 72008 complex points (lb = 0.3 Hz) and zero filling for better digital resolution prior to Fourier transformation.

Quantitative component analyses (ethanol, acetic acid , lactic acid, succinic acid, frutose, glucose, sucrose and caffeine) were carried out by first selecting a signal with no overlapping and comparing the integrated values to that of the trimethylsilylpropionic acid sodium salt d4 (TSPNa) signal (0.5 mmol/L). The signal

integrals were optimized by signal deconvolution.

Results and discussion

High resolution NMR experiments.

To obtain the picture and the quantitative analysis of water-soluble components by ^{1}H NMR the water signal had to be canceled applying PRESAT, Watergate or WET water suppression techniques. PRESAT provided better quantitative results (Figure 1) eliminating the water signal without perturbing the sucrose (5.41 ppm) and glucose (5.23 and 4.64 ppm) anomeric signals that were diagnostic in our analyses. Standard solutions of sucrose, glucose and fructose were used to select -17 dbm as the optimum water saturation power. Figure 2 depicts the ^{1}H NMR sugar signal region with H-5 frutose signal at 3.99 ppm standing alone allowing quantification. Main metabolites were identified by chemical shift comparison with data in the literature and confirmed by fortifying the samples with commercial standards (acetic acid, succinic acid, lactic acid)[7], Figure 3 displays a representative ^{1}H NMR spectrum of *Theobroma cacao* extract and the assignment of signals: ethanol (1.28 ppm), succinic acid (2.50 ppm), acetic acid (1.96 ppm), lactic acid (1.33 ppm) that were used in quantification. Caffeine is not visible (3.20 ppm) due to signal overlapping. The metabolic profiles of cacao produced by the 10 selected clones from Bahia – Brazil were monitored at different fermentation times (0, 48, 96 e 144 h) and liquors (Figure 4).

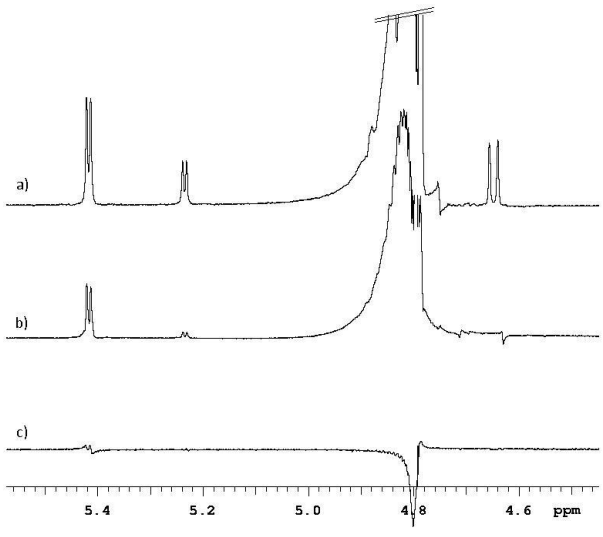

Figure 1. ^{1}H NMR (499.88 MHz, H_2O/D_2O; water PRESAT) spectra of common cacao aqueous extract after 48 h of fermentation, applying different water suppression techniques. a) PRESAT b) 1H WET-CPMG c) WATERGATE.

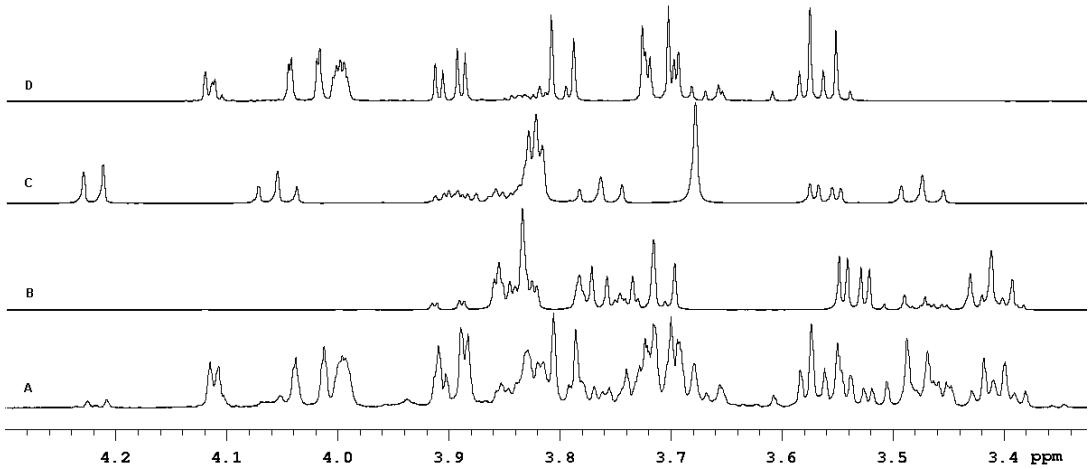

Figure 2. 1) Expanded ^{1}H NMR spectra of sugars 3.32 – 4.30 ppm A) common cacao, B) sucrose, C) glucose and D) fructose.

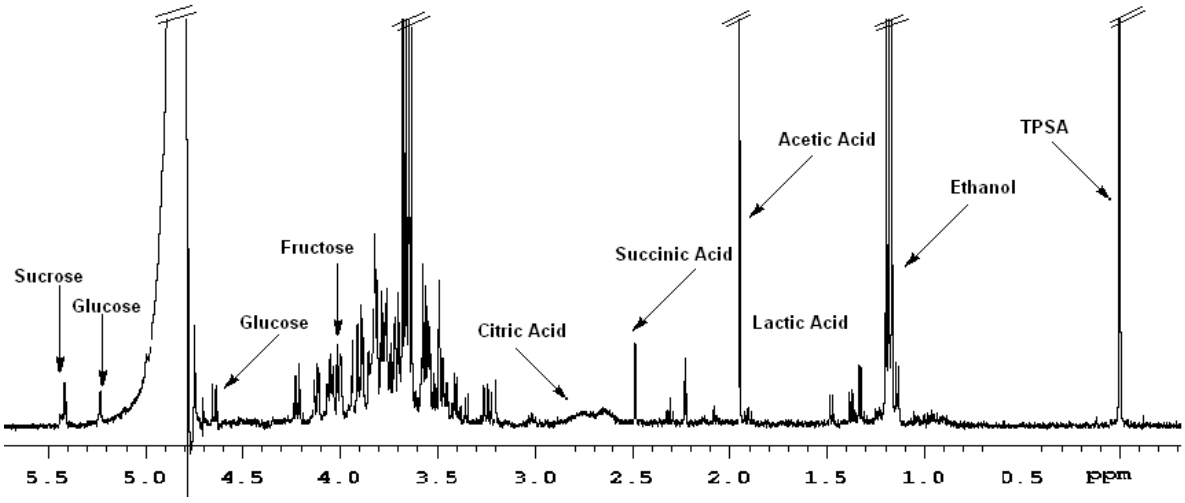

Figure 3. Secondary metabolities signals on 1H NMR presat spectra of common cacao aqueous extract before 48 h of fermentation.

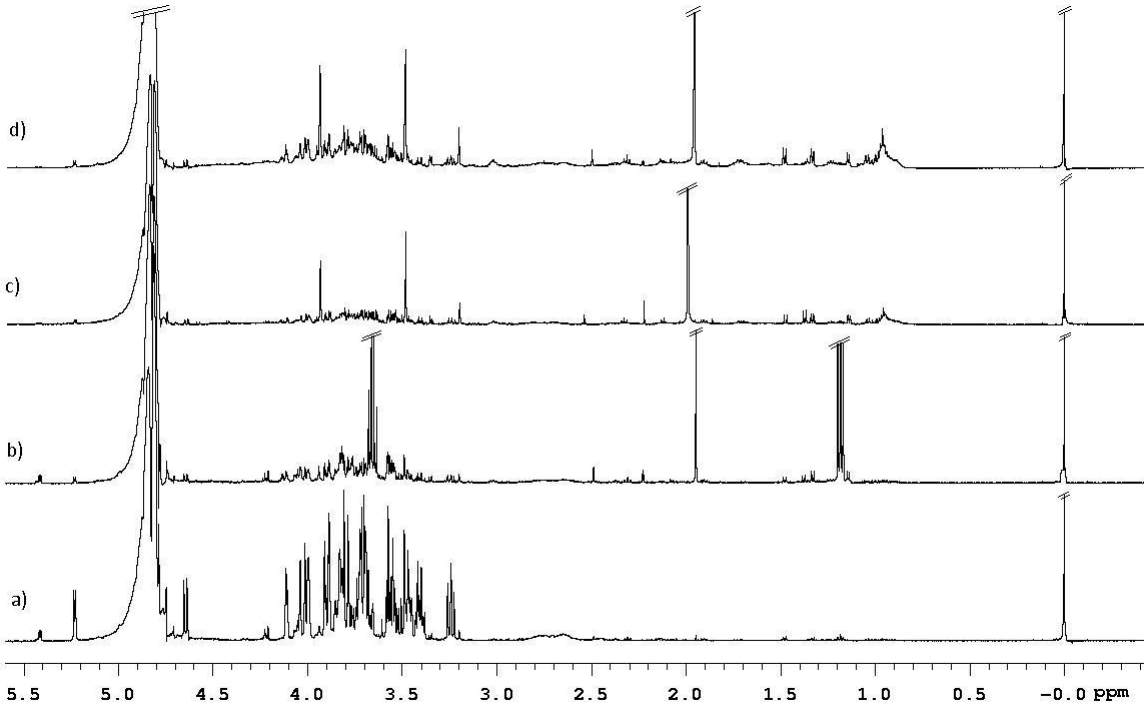

Figure 4. Expanded ^{1}H NMR spectra of common cacao –0.4 - 5.6 ppm a) before fermentation b) after 48 h of fermentation c) after 96 h of fermentation and d) after 144 h of fermentation.

Quantification was obtained by comparing the integrated area values of the selected signals with those of the internal reference sodium *d*$_4$-trimethylsilylpropionate (0.5 mmol L^{-1}), each corrected to a single hydrogen value and water content (equation 1):

$$\%_{comp/liq} = \frac{\frac{A_{comp}/n(^1H)}{A_{TPSA}/9}.0.5.10^{-3}.3.M_{comp}.10^{-3}}{W_{liq.ext.}}.\frac{100}{(100-H)} \qquad \textbf{Equation 1}$$

where A_{comp} is the signal area of the considered compound, normalized to one proton, and M_{comp} is its molar mass, A_{TSPA} is the area of the TSP sodium salt signal, normalized to one proton, whose concentration in the samples its equal to 0.50 mmol L^{-1} , $W_{liq.\ ext.}$ is the cacao weight used to prepare the sample and *H* is the water content of each sample. The correction factor (H) was introduced in equation 1 due to the large water content differences of the samples at each fermentation times (**Table 1).**

Table 1. Moisture content in cacao samples

	MOISTURE (%)				
	Fermentation time (hours)				**sun dried**
	0	**48**	**96**	**144**	
CEPEC 42	58.15 ± 1.09	55.38 ± 1.01	54.46 ± 0.15	44.24 ± 0.57	6.10 ± 0.02
COMMOM	53.83 ± 0.88	52.50 ± 0.25	47.74 ± 0.09	44.02 ± 0.64	6.60 ± 0.36
EET 397	56.21 ± 1.16	51.61 ± 0.89	51.29 ± 0.95	46.81 ± 0.76	5.80 ± 0.01
TSA 654	52.95 ± 0.55	52.98 ± 0.61	48.79 ± 0.21	41.90 ± 0.71	6.63 ± 0.08
TSA 656	53.68 ± 0.63	53.13 ± 0.79	50.18 ± 2.16	46.13 ± 3.08	6.09 ± 0.03
TSAN 792	57.29 ± 0.84	50.63 ± 1.13	50.47 ± 0.77	44.98 ± 0.47	6.48 ± 0.02
TSH 516	54.73 ± 0.59	52.90 ± 1.05	52.73 ± 0.45	42.87 ± 0.26	6.74 ± 0.54
TSH 565	57.19 ± 1.11	51.18 ± 0.93	51.55 ± 0.34	47.22 ± 0.80	6.78 ± 0.16
TSH 774	57.78 ± 0.52	55.91 ± 1.12	55.18 ± 0.90	44.88 ± 0.73	5.92 ± 0.04
TSH 1188	56.21 ± 0.97	54.14 ± 0.59	52.54 ± 1.87	40.12 ± 1.09	6.28 ± 0.01

Table 2 shows the quanitfication results for sacarose, glucose and fructose, at each fermentation stage for the analyzed cacau varieties. Sugar quantification in the liquor of the dry and roasted samples was not possible due to the low signal to noise ratio.

During the first 48 hours of fermentation fructose and glucose are rapidly depleted in the glycolysis process while the sucrose content remains almost unaltered. The glucose and fructose contents decrease by about five and two times, respectively.

These observations can be rationalized as a preferencial metabolization of glucose and/or glucose isomerization by phosphohexose isomerase, converting glucose 6-phosphate into fructose 6-phosphate.

Phosphorilated and non phosphorilated species diplay similar chemical shifts thus are not easily discriminated by ^{1}H NMR[14]. Therefore we are indeed monitoring fructose and/or fructose-6-phosphate.

Sucrose is only cleaved by yeast fermentation after 96 hours, producing more fructose and glucose finally after 144 hours of fermentation and sun drying the sugar content is rather low, sucrose is not detected and the fructose and glucose contents are insignificant when compared to the values detected at fermentation time zero.

Table 2. Quantitative analyses by ^{1}H NMR of *Theobroma cacao* kernel aqueous extracts, monitoring sugars (mg.g^{-1}).

Fermentation process (hours)															
Samples	**Sucrose**					**Glucose**					**Fructose**				
	0 h	48 h	96 h	144 h	after drying	0 h	48 h	96 h	144 h	after drying	0 h	48 h	96 h	144 h	after drying
TSH 516	8.68	8.77	2.22	1.00	0.40	31.3	5.41	5.73	4.62	1.32	38.6	14.3	28.4	9.17	3.92
TSH 565	12.7	12.0	3.38	2.33	1.38	49.6	9.46	4.64	4.70	1.84	50.2	13.1	7.53	7.29	8.32
TSA 654	ND	9.04	1.29	0.88	ND	20.7	4.10	4.02	3.48	0.63	16.6	7.66	6.46	8.28	2.77
TSA 656	6.74	6.36	1.50	0.54	ND	37.5	16.9	8.23	4.34	1.99	45.1	20.1	16.8	8.02	7.55
TSH 774	7.46	6.36	3.41	1.14	0.60	47.7	4.25	9.46	4.39	3.60	43.0	13.2	14.9	6.17	12.2
TSA 792	10.8	10.7	2.04	1.16	ND	48.7	12.2	7.73	4.34	ND	47.5	15.8	17.3	6.05	4.20
TSH 1188	12.6	12.3	1.56	0.88	ND	55.7	6.06	3.10	3.61	2.20	46.7	11.5	8.03	5.18	5.72
EET 397	8.45	6.63	1.87	ND	ND	59.1	10.3	7.82	5.68	2.60	63.5	17.5	17.10	9.91	6.86
Cepec 42	9.63	9.38	ND	0.91	ND	58.2	13.2	5.16	4.89	2.00	63.9	23.7	5.33	13.0	7.67
Commom	9.14	5.83	0.76	1.11	ND	50.7	6.65	4.04	3.39	3.58	49.6	12.9	5.93	5.52	9.43

It is important to point out that the detection limit was 149 x 10^{-6} molL^{-1} and the probe signal to noise ratio is 908. Organic acids, ethanol and caffeine content of the 10 different cacau varieties are in **Table 3.** Ethanol is produced after 48 hours of fermentation as a consequence of glucose and fructose consumption, probably due to the action of *Saccharomyces cerevisiae* in the alcoholic fermentation. At this stage lactic and succinic acids are detected, indicating the metabolic contribution of anaerobic and aerobic processes, respectively[15]. Acetic acid is usually produced by microorganisms from the genus *Acetobacter*, and was detected after 48 hours of fermentation, reaching its maximum content after 96 hours of fermentation while the succinic and lactic acid contents decreased[16]. From then on acetic acid production decreased and its evaporation further decreased its content in the samples.

Table 3. Quantitative analyses by ^{1}H NMR of *Theobroma cacao* kernel aqueous extracts, monitoring organic acids, ethanol and caffeine (mg.g^{-1}).

Fermentation process (hours)																
Samples	**Acetic acid**					**Lactic acid**					**Succinic acid**				**Ethanol**	**Caffeine**
	48 h	96 h	144 h	after drying	Liquor	48 h	96 h	144 h	after drying	Liquor	48 h	96 h	144 h	after drying	48h	Liquor
TSH 516	3.88	11.8	6.27	3.68	2.87	2.38	0.57	0.73	0.47	0.51	0.36	0.34	0.26	0.20	5.56	1.08
TSH 565	3.21	22.3	10.7	6.94	3.47	3.07	0.76	1.14	1.05	ND	0.43	0.29	0.23	0.21	8.34	0.85
TSA 654	14.7	13.0	5.33	3.36	2.23	1.02	0.90	0.70	0.32	0.32	0.34	ND	0.17	0.11	7.17	0.55
TSA 656	7.21	11.5	6.79	3.82	2.95	2.90	1.04	1.56	1.11	0.65	0.40	0.24	0.22	0.27	5.05	0.86
TSH 774	6.99	7.29	8.05	7.60	3.27	0.67	1.47	0.63	0.79	0.51	0.42	0.31	0.16	0.22	2.37	1.07
TSA 792	3.26	19.3	8.18	3.52	5.32	1.36	0.91	0.73	0.92	0.57	0.45	0.32	0.18	0.11	6.35	1.24
TSH 1188	2.68	17.5	7.78	4.44	4.22	0.74	1.47	1.23	0.68	1.46	0.41	0.29	0.22	0.19	9.33	1.45
EET 397	7.44	11.8	6.28	4.28	1.47	4.98	0.80	2.14	1.40	0.19	0.33	0.31	0.38	0.30	5.66	ND
Cepec 42	3.02	17.5	11.5	8.68	0.54	1.37	0.33	1.38	0.80	0.76	0.02	0.15	0.23	0.21	7.69	1.27
Comum	2.27	15.2	7.91	6.56	3.62	0.74	0.75	0.86	0.68	0.84	0.40	0.23	0.20	0.21	8.27	1.17

Citric acid is present in low yield and was monitored before fermentation, its ^{1}H NMR signals are rather broad probably due to a complexation phenomenom with other components of the mixture.

Caffeine methyl signals resonate in the sugar region (3.96; 3.55 and 3.36 ppm). Thus signal overlapping prevented quantitation in most samples except in the liquor when the sugar content is low.

Concluding remarks

^{1}H NMR was an efficient analytical tool providing the simultaneous monitoring of nine secondary metabolites from cacao of 10 clones of different *Theobroma cacao* varieties which are *Moniliophthora perniciosa* resistents.

These analyses were about four times faster than those by HPLC. Finally the cacau profiles from the 10 clones were rather similar but the higher caffeine content in the TSH 1188 clone could be indicative of a better quality cacao.

Notwithstanding this observation we have to conclude that the main factor for the agricultural choice of the best variety would be their resistance to the *M. perniciosa,* while sensorial analysis would provide final answers as to the best candidate to produce good quality cacao.

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