Structural and chemical changes in cocoa (*Theobroma cacao* L) during fermentation, drying and roasting

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Abstract: Cocoa seeds and pulp were fermented for 144 h, followed by natural drying. The tegument was removed and the cotyledons were broken into nibs which were roasted at 150 °C for 30 min. Non-fermented material, material fermented for 24, 48 and 72 h, material fermented for 144 h and then dried, and also the roasted nibs, were all prepared for chemical and microscopic analyses. Light microscopy revealed the presence of anionic and cationic residues and of neutral sugars. During fermentation there was a reduction in the cytoplasmic content of phenolic compounds and in the number of protein bodies. The cell wall showed a reduction in anionic residues and a loss of crystallinity. These alterations were maximum after 72 h. Drying and roasting increased the number of damaged cells and reduced the amount of cytoplasmic material. The chemical analyses generally confirmed the microscopy results. The concentration of amino-terminal groups and total free amino acids increased during fermentation (up to 72 h), but returned to the initial values after roasting. The principal chemical changes were related to reducing sugars, free amino acids, proteins and phenols, and PCA was suggested as a useful tool to compare different samples. Microscopic analysis revealed the degradation of protein and phenolic bodies and cellular damage during roasting.

Keywords: cocoa; processing; structure; composition

INTRODUCTION

Genotype, soil, climate and harvest conditions, as well as processes such as fermentation, drying and roasting, have important effects on the characteristics of cocoa. The latter processes in particular are continuously being altered to improve the homogeneity and acceptability of the final product.[1]

Studies of the structural alterations that occur in cocoa cotyledons during fermentation have shown that numerous subcellular changes are important in the chemical reactions that occur during fermentation and drying.[2,3] Scanning electron microscopy is a useful method for monitoring the effects of fermentation, drying and roasting on the final quality of the product.[4,5]

During processing, proteolysis, especially that of the globulin fraction, produces peptides and amino acids that contribute directly to the flavour of cocoa[6] and to the Maillard reaction during roasting, the profile and the process conditions being more responsible for a good flavour than the total amounts of these components.[7,8] The oxidation, condensation and complexation of polyphenolics contribute to the astringency of the cocoa product, and these substances are involved in reactions leading to the formation of other compounds.[9,10] Additional substances, produced during fermentation and absorbed by the seeds,[2] include acetic acid which, in large amounts, may be detrimental to the quality of the cocoa product.[11]

In this study we evaluated some of the structural and chemical changes that occur in cocoa seeds during fermentation, drying and roasting, with emphasis on the free amino acids and sugars and the importance of these to the mechanisms of flavour formation in cocoa.

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MATERIALS AND METHODS

Cocoa samples

Cocoa fruit of the Forastero variety were harvested at the experimental station of the Instituto Agronômico in Pariquera-Açú, São Paulo, Brazil. Two hundred and fifty kg of seeds and placenta-free pulp were fermented in a wooden box. The beans were turned every 24 h, and after 144 h of fermentation they were dried naturally in the sun for 72 h. The tegument and germ tissue were removed and the cotyledons were broken into nibs. The material which passed through a 3.5-mesh screen but was retained by an 8-mesh screen was roasted (250 g nibs per roasting) in a PRE 1Z rotating electric oven (Probat-Werke, Germany) at 150 °C for 30 min. Samples were taken before fermentation (t_0), every 24 h during fermentation (t_{24}, t_{48} and t_{72}), after drying (t_D) and after roasting (t_R). They were immediately fixed for microscopic analysis. The remaining material was stored at −18 °C for subsequent chemical analysis. Parts of the samples were dried in an oven at 45 °C to constant weight and defatted with petroleum ether in a Soxhlet extractor. The above procedure was performed three times.

Preparation of samples for microscopy

The samples were fixed in a solution of chromic acid–formaldehyde (1:1, v/v) for 14 days, dehydrated in a series of ethanol solutions, cleared in xylol for 30 min, soaked in paraffin and cut longitudinally and obliquely into 7 μm thick sections. After deparaffinisation and hydration, part of the material was stained with 0.1% xylidine ponceau (XP) in 3% acetic acid, pH 2.5, to detect cationic residues.[12] Anionic residues were detected by staining with 0.025% toluidine blue (TB) in McIlvaine buffer, pH 4.0.[13] Carbohydrates were visualised by oxidation with 0.5% periodic acid for 10 min, followed by staining with Schiff reagent for 8 min (PAS). Control samples were immersed in the Schiff reagent without prior oxidation.[14] The stained sections were examined by light microscopy, using polarised light in some cases.

Chemical analysis

The phenolic compounds were extracted with acetone–water (70:30, v/v) as described elsewhere,[15] and the total phenol content was determined using the Prussian blue method[16] and expressed in mg tannic acid g^{-1} sample. Amino-terminal groups were determined after extraction with a mixture of trichloroacetic acid–sodium acetate–acetic acid (0.11 M:0.22 M:0.33 M)[17] and reaction with *o*-phthalaldehyde.[18] The results were expressed in mg glycine g^{-1} sample. The residue obtained after extraction of the phenolic compounds was digested with 0.1 M NaOH at room temperature and the protein concentration was measured by the method of Bradford using BSA as a standard.[19] Reducing sugars were extracted with a mixture of methanol–chloroform–water (MCW; 12:5:3, v/v), after which soluble polysaccharides were extracted with ethanol–water (10:90, v/v). The residue was treated with 30% perchloric acid to extract the starch. The reducing sugars were quantified using a dinitrosallcylic acid (DNSA) procedure[20] and expressed in mg glucose g^{-1} sample. Soluble polysaccharides and starch were quantified by the anthrone method using glucose as a standard.[21]

Sugar analysis

Defatted cocoa powder (1 g) was extracted three times with ethanol–water (80:20, v/v) for 6 h at 37 °C. The extracts were combined and the ethanol was evaporated under reduced pressure. Samples were deionised through cation (Dowex-50W, Na^+ form) and anion (Dowex-1, Cl^- form) columns and eluted with deionised water. The eluted material was evaporated to dryness and resuspended in 1 ml of deionised water. For the sugar analysis, 25 μl of this suspension was injected into a high-performance anion exchange chromatograph with a pulse amperometric detector (HPAEC-PAD) in a Dionex DX300 system (USA) with a CarboPac PA-100 column (4 mm id, 250 mm length) and an isocratic programme using 16 mM NaOH as eluent at 1 ml min^{-1} for 30 min. The applied PAD potentials for E1 (300 ms), E2 (120 ms) and E3 (60 ms) were 0.04, 0.60 and −0.80 V respectively and the output range was 1000 nA. Standards of 0.89 mM glucose, 0.89 mM fructose and 0.93 mM sucrose (Sigma) were employed to calculate the proportion of each sugar.[22] Total sugars were determined by the phenol–H_2SO_4 method[23] and reducing sugars by the DNSA method.[20]

Free amino acid analysis

Fat-free cocoa powder (2 g) was extracted with 30 ml of sodium citrate (20 g l^{-1}, pH adjusted to 2.2 with HCl) for 3 h. The extract was centrifuged (4100 × *g*) and filtered and the volume was made up to 100 ml with the sodium citrate solution.[7] Before analysis the samples were filtered through Millipore membranes (0.22 μm) and 2 μl aliquots were injected. An automatic amino acid analyser (Thermo Separation Products, Riviera Beach, FL, USA) with a PCX3100 reactor (Pickering Laboratories, Mountain View, CA, USA) was employed. An Na^+ column (3 mm id, 250 mm length) was used at 55 °C. The detector temperature was 130 °C. The eluents were sodium citrate buffers at pH 3.15 and 7.4 and ninhydrin at a flow rate of 0.3 ml min^{-1}. The total analysis time including column regeneration was 72 min.

Polypeptide analysis

Polypeptides were extracted from 0.2 g of cocoa powder with 20 ml of trichloroacetic acid–sodium acetate–acetic acid (0.11 M:0.22 M:0.33 M) solution (TCA mixture).[17] The extract was centrifuged (4100 × *g*), filtered (0.45 μm) and freeze-dried. The material was suspended in phosphate buffer, pH 7.2, and 100 μl was applied to an FPLC apparatus (Pharmacia, Uppsala, Sweden) with an anionic column (Mono Q). The elution was done with

phosphate buffer in a linear gradient from 0.0 to 1.0 M NaCl in 20 min. The flow rate was 1 ml min^{-1} and detection was effected using a UV detector with $\lambda = 280$ nm. Aliquots corresponding to the peaks were collected manually and analysed for peptides by the method of Bradford using BSA as a standard.[19]

Statistics

The results were compared by analysis of variance using the software package Statistica (Tulsa, OK, USA). Significant differences amongst means were confirmed using the Tukey test for multiple comparisons at $P < 0.05$. Principal component analysis (PCA) was carried out to study the variation in sugars and free amino acids due to different processes. Computations were performed using the SAS statistical program (SAS Institute Inc, Cary, NC, USA).

RESULTS AND DISCUSSION

Microscopic analysis

Anionic residues, mainly in pectin-like substances from the cell walls, were abundant at t_0 (Fig 1(a)) but decreased after 72 h of fermentation, perhaps because of complexation with Ca^{2+} ions,[24] association with polyphenols or degradation during processing. Dried and roasted material still contained a large number of ruptured cells as compared to the material at the start of fermentation (Figs 1(a) and 1(b)).

The presence of polyphenol-rich cells in cocoa cotyledons was first described in the 19th century.[25] In the present study the non-fermented material had a high number of phenolic bodies (Fig 1(a)), which were present even after 24 h of fermentation. As the fermentation progressed, polyphenols diffused throughout the whole cotyledon up to 48 h. After 72 h, no phenolic material could be detected (Fig 1(b)). These results, based on natural fermentation, agree with those of laboratory fermentation and other cocoa types.[2,3]

Prior to fermentation, the proteinaceous material was shown to be quite dispersed in the seed cytoplasm (Fig 1(c)). As fermentation progressed, this material diffused, similarly to that observed following germination, when degradation and mobilisation of proteins take place.[13] After 72 h of fermentation, almost no protein material was detected (Fig 1(d)), the same being observed for dried and roasted seeds. The decrease in staining intensity was probably related to the formation of smaller, more soluble peptides, not detectable by the assay. Similar results were reported

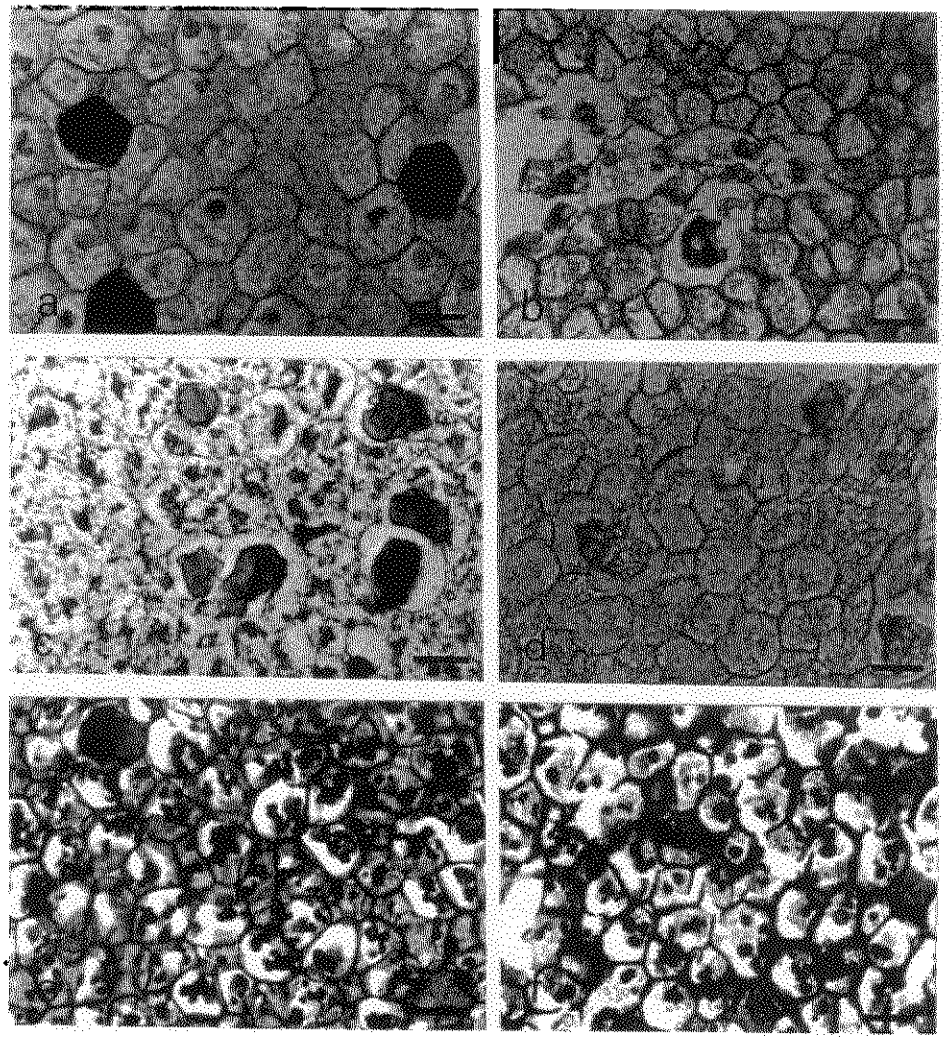

Figure 1. Cocoa cotyledons at different times of fermentation and processing. (a) At t_0, stained with 0.025% toluidine blue. Note the presence of phenolic bodies. (b) Roasted cocoa, stained with 0.025% toluidine blue. Note the presence of damaged cells and the absence of phenolic bodies. (c) At t_0, stained with xylidine ponceau. Note the proteinaceous material dispersed in the cytoplasm. (d) At t_{72}, stained with xylidine ponceau. Note the general lack of proteinaceous material. (e) At t_0, PAS-positive staining of cytoplasmatic material (starch and glycoproteins) and cell walls (cellulose). (f) Dried cocoa, PAS-positive staining for starch grains. Bar = 20 μm.

	Fermented			
	0h	72h	Dried	Roasted
Total phenol (mg tannic acid g^{-1})	231±5a	213±5a	157±6b	131±6c
Protein (mg g^{-1})	220±8a	157±9b	118±8b	138±7b
Amino-terminal groups (mg glycine g^{-1})	21.6±0.2b	35.6±1.2a	32.6±1.9a	22.1±1.1b
Free amino acids (mg g^{-1})	25.7±0.7a	32.1±1.8b	35.3±1.3b	24.1±2.1a
Reducing sugar (mg glucose g^{-1})	50.1±0.3b	63.0±0.5a	28.0±0.5c	14.6±0.1d
Oligosaccharide (mg glucose g^{-1})	7.9±1.4a	6.7±1.9a	7.9±0.7a	7.7±1.1a
Starch (mg glucose g^{-1})	140±5a	127±5a	136±9a	162±4a

Values are mean±SD.
Means with different letters within a row are significantly different ($P<0.05$).
All values are expressed on a defatted and dry weight basis.

Table 1. Effect of fermentation, drying and roasting on cocoa composition

in a previous electron transmission microscopy study of cocoa.[2]

Seeds stained positive for neutral sugars by the PAS method (Fig 1(e)). Besides cellulose, grains of starch and glycoproteins were seen in the cytoplasm (Fig 1(e)), indicating the presence of protein reserves in cocoa seeds as reported for other seeds.[12,13]

Drying and roasting increased the number of damaged and disrupted cells (Fig 1(f)). PAS staining and polarised light microscopy showed the presence of starch at the beginning of fermentation. With the loss of protein material and cell disruption the starch grains became increasingly evident, particularly after roasting. Similar phenomena have been observed for almonds and peanuts.[26,27]

Chemical analysis

Although cytochemical results had shown that phenols diffused during fermentation, the phenol content at t_0 was no different from the value obtained after 72h of fermentation (Table 1). Nevertheless, after drying and roasting, the level of phenolic compounds decreased markedly to approximately 131 mg g^{-1} (Table 1). According to Forsyth,[28–30] who extensively studied phenol transformations in cocoa, losses of total phenol are due to diffusion out of the cotyledons and can be calculated as 24% after 60h of fermentation, reaching 58% after 8 days. Phenolic compounds also complex with cocoa proteins and polysaccharides.[10,15,31]

In agreement with the XP staining, a gradual decrease in protein content up to 72h of fermentation (Table 1) was observed. Concomitantly, the amount of amino-terminal groups increased with time, as well as that of total free amino acids (Table 1), indicating proteolysis and the formation of smaller peptides and free amino acids. The rate of production of free amino acids during fermentation has been related to the rate of flavour and aroma development as established by Rohan and Stewart.[32,33] Similar findings have been reported for cocoa from Malaysia and Ecuador.[34,35] In one of these studies the nitrogen content at the end of fermentation accounted for approximately 57% of that at the beginning of the process.[35] A similar value was observed here for the protein content in dried samples (54%). According to Voigt *et al*,[6] fermentation provides the conditions for the action of endoproteases that produce specific peptides and amino acids, which are of extreme importance to cocoa flavour.

As shown in Table 1, the protein levels remained constant during drying and roasting, whereas the concentration of amino-terminal groups decreased during roasting. The same was observed for total free amino acids (Table 1), which represents nearly a 32% reduction. This reduction can be attributed mainly to the Maillard reaction that led to consumption of amino acids during cocoa roasting.[7,36] This depends, among other factors, on the variety, the roasting time and the temperature and can vary from 24.1 to 71.8%.[7,37,38]

Table 2 shows the results of the fractionation of the polypeptides obtained by the TCA mixture. It was observed that the number of peaks decreased markedly during drying and roasting. On the other hand, the total peptide bonds detected increased after 72h of fermentation and decreased in the dried beans and roasted nibs. These results indicated that polypeptide changes are related to fermentation and drying, the decrease being related not only to proteolysis but also to polyphenol complexation.

The free amino acid profile is presented in Table 3. After 72h of fermentation there was an increase in all the free amino acids except tyrosine and lysine. In dried beans, higher values were detected when compared to those of the unfermented seeds, except for glutamic acid and proline. Seiki[39] also reported a reduction in glutamic acid for cocoa fermented in trays (similar to the method used here), but not in heap fermentation. A different pattern was observed by other authors, especially for aspartic and glutamic

Table 2. Number of peaks eluted from FPLC with peptide bonds detected by the method of Bradford,[19] and total protein concentration calculated from the sum of the peaks

Sample	Number of peaks	μg protein ml^{-1}
Unfermented	7	46.4
Fermented 72h	6	64.5
Dried	3	14.1
Roasted	2	13.2

Compound	Fermented 0h	Fermented 72h	Dried	Roasted
Amino acids				
Aspartic acid	9.8	13.0 (32.7)	15.7 (20.8)	6.5 (−58.6)
Glutamic acid	7.4	11.7 (58.1)	4.3 (−63.2)	5.8 (34.9)
Leucine	7.4	14.8 (100.0)	17.6 (18.9)	12.3 (−30.1)
Alanine	9.7	18.1 (86.6)	21.2 (17.1)	12.0 (−43.4)
Phenylalanine	3.3	11.7 (254.5)	10.8 (−7.7)	5.9 (−45.4)
Tyrosine	3.6	1.8 (−50.0)	11.0 (511.1)	3.0 (−73.0)
Valine	5.6	11.0 (96.4)	12.5 (13.6)	6.4 (−48.8)
Isoleucine	4.1	9.0 (119.5)	8.8 (−2.2)	3.3 (−62.5)
Lysine	72.6	49.4 (−32.0)	73.0 (47.8)	69.7 (−4.5)
Arginine	18.0	23.5 (30.6)	23.5 (—)	19.1 (−18.7)
Histidine	2.0	4.9 (145.0)	5.4 (10.2)	0.7 (−87.0)
Threonine	4.6	11.8 (156.5)	8.8 (−25.4)	3.6 (−59.1)
Serine	15.8	27.8 (75.9)	18.8 (−32.4)	14.5 (−22.9)
Glycine	4.3	8.6 (100.0)	8.4 (−2.3)	2.9 (−65.5)
Methionine	2.9	7.2 (148.3)	5.6 (−22.2)	1.4 (−75.0)
Proline	11.3	14.0 (23.9)	6.4 (−54.3)	5.6 (−12.5)
Cystine	1.3	2.7 (107.7)	2.6 (−3.7)	0.4 (−84.6)
Sugars				
Glucose	89.6	90.5 (1.0)	28.3 (−68.7)	27.4 (−3.2)
Fructose	188.7	259.5 (37.5)	127.2 (−51.0)	49.7 (−60.9)
Sucrose	184.2	146.5 (−20.5)	81.9 (−44.1)	42.7 (−47.9)

Table 3. Free amino acids and sugars ($\mu g g^{-1}$) in cocoa, with the percentage change relative to the previous column in parentheses

acids.[32,34,40,41] Since accumulation of free amino acids is dependent on the action of endogenous proteases, and the activity of these enzymes is dependent on the conditions of fermentation,[40] the differences detected in free amino acid content could be explained on this basis. As mentioned before, roasting promoted a decrease in all the free amino acids except glutamic acid. The smallest decrease was in the lysine content and the greatest in histidine (Table 3). A different pattern was observed by other authors, who demonstrated a selective but incomplete destruction of amino acids during cocoa roasting, the rates of destruction varying among them.[37–39,42,43]

In agreement with the PAS results, the levels of starch did not change significantly during cocoa processing. However, a significant increase in the content of reducing sugars during fermentation was observed, followed by a significant decrease during drying and roasting (Table 1). These findings are in agreement with those shown in Table 3 for sucrose, glucose and fructose. There was a continuous decrease in sucrose content and an initial increase in fructose and glucose at t_{72}, followed by a decrease at t_D and fructose reduction at t_R. The increase in reducing sugars during fermentation is due to sucrose inversion.[34,36,41] The difference between fructose and glucose contents can be attributed to the preferential metabolism or polymerisation of glucose following sucrose hydrolysis, which is known to occur during fermentation.[44] The high decrease in reducing sugar content, especially fructose, during roasting is attributed to the Maillard reaction.[7] This reduction can reach 100% and, as mentioned for free amino acids, is related to the time and temperature of roasting.[7,42] Nevertheless, fructose presented the greatest reduction after roasting, probably owing to its higher content and the high reactivity of ketoses in the Maillard reaction. Complete glucose

Table 4. Principal component factor loadings for free amino acids and sugars of cocoa

Compound	PC1	PC2	PC3
Aspartic acid	0.875	−0.226	0.428
Glutamic acid	0.401	0.860	−0.317
Leucine	0.754	−0.591	−0.287
Alanine	0.893	−0.446	−0.053
Phenylalanine	0.935	−0.204	−0.289
Tyrosine	0.300	−0.811	0.502
Valine	0.930	−0.367	−0.004
Isoleucine	0.993	−0.088	0.078
Lysine	−0.609	−0.591	0.529
Arginine	0.961	−0.230	−0.152
Histidine	0.960	−0.124	0.251
Threonine	0.983	0.162	−0.087
Serine	0.867	0.438	−0.238
Glycine	0.990	−0.035	0.130
Methionine	0.985	0.160	0.063
Proline	0.409	0.907	0.102
Cystine	0.966	0.025	0.258
Sugars			
Glucose	0.161	0.958	0.236
Fructose	0.616	0.751	0.240
Sucrose	−0.159	0.826	0.540
Eigenvalue	12.5	5.8	1.6
Proportion of total variance (%)	62.6	91.7	99.9

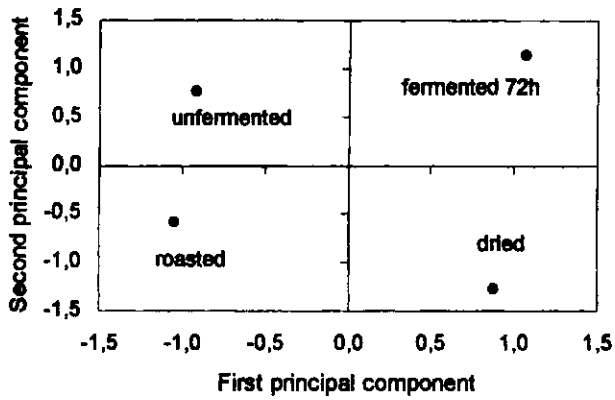

Figure 2. Score plot of the first two principal components from cocoa samples at different processing stages.

consumption or even no change in glucose content has already been reported.[42,44] The differences in composition and processing conditions could explain the distinctive results obtained here, but other characteristics such as pH, water activity and polyphenol content cannot be discounted. We also observed high sucrose reduction during roasting, which was not reported by other authors.[44]

Principal component analysis (PCA)

PCA was used to visualise the variation in the samples and to find correlations between the different variables. PCA generated two significant principal components (PCs) explaining 62.6 and 29.1% of the variation respectively (Table 4). The factor loadings are shown in Table 4. Loadings with an absolute value higher than 0.70 (shown in bold type) represent a strong influence. It can be seen that PC1 is heavily influenced by free amino acids, except glutamic acid, tyrosine, proline and lysine. On the other hand, PC2 is mainly influenced by glucose, fructose and sucrose. The loading plot (Fig 2) permits a better visualisation of the distribution. Free amino acids are positioned at the right side with similar values on PC1, and sugars are positioned at the top with similar values on PC2. The score plot of PC1 and PC2 (Fig 3) showed that cocoa samples could be separated into four groups, each in a different quadrant but with similarities in one of the PCs. Unfermented and t_{72} samples differ in PC1 (free amino acids) but have similarities in PC2 (sugars). On the other hand, t_{72} and t_D samples have similarities in PC1 but differ in PC2. The roasted sample differs from all the others in having negative values in PC1 and PC2, indicating a low content of free amino acids and sugars.

CONCLUSION

As the results presented in the literature are not in agreement with each other, we consider that the use of PCA could be useful to compare the transformations that occur during processing of different samples. Additional studies with amino acid and reducing sugar

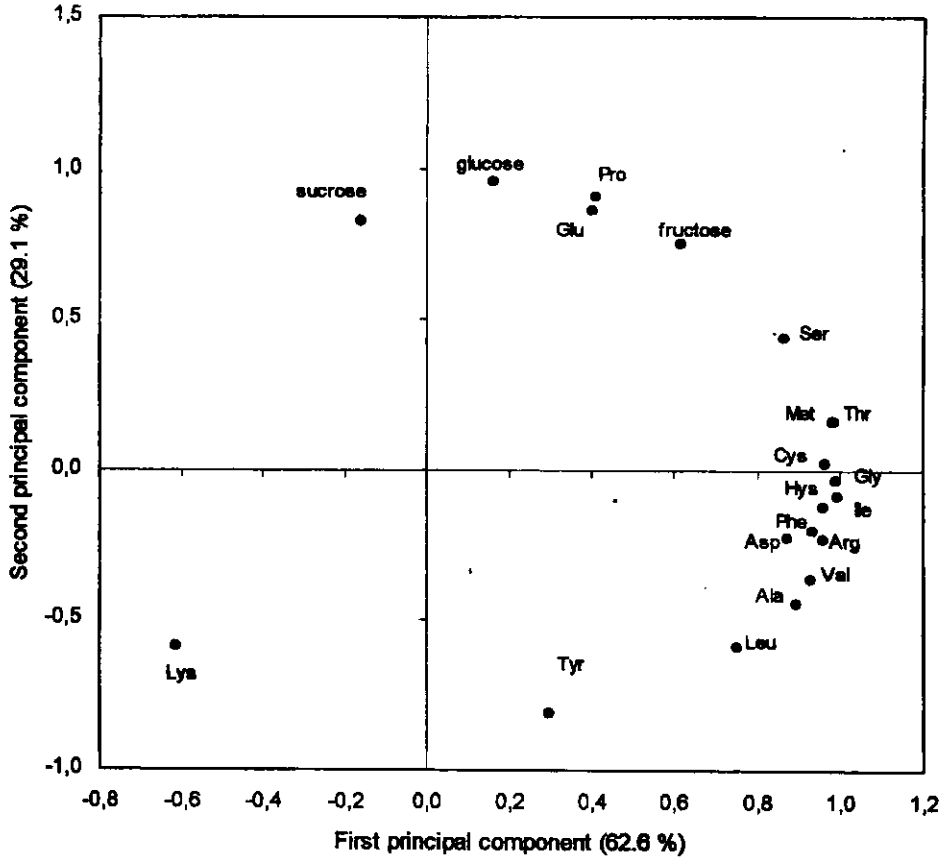

Figure 3. Loading plot of the first two principal components for free amino acids and sugars from cocoa at different processing stages.

supplementation prior to roasting, as well as sample characteristics such as pH and water activity, are needed to fully understand flavour formation in cocoa. The microscopic evaluation was useful to follow the degradation of protein and phenolic bodies. Cellular disruption, especially during roasting, was quite a common event.

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