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Bioactive amines and phenolic compounds in cocoa beans are affected by fermentation

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ABSTRACT

Cocoa is the target of increased scientific research as it is one of the richest source of bioactive compounds. The formation of bioactive amines and their changes in cocoa beans during seven days of traditional fermentation was investigated for the first time. In addition, total phenolic compounds, anthocyanins contents and the scavenging capacity against ABTS radical were determined to monitor the fermentation process. Only two biogenic amines (tryptamine and tyramine) and two polyamines (spermidine and spermine) were detected in cocoa beans during fermentation. Fermentation was characterized by three stages: i) high levels of tryptamine, phenolics, and scavenging capacity; ii) high contents of spermine, total biogenic amines and total polyamines; and iii) the highest spermidine levels and total acidity, but the lowest total phenolic compounds and anthocyanins contents. The scavenging capacity of cocoa beans during fermentation correlated with total phenolic compounds and anthocyanins contents.

1. Introduction

Cocoa beans constitute a basic raw material for chocolate production; they must undergo technological processes, including fermentation, which is a very important step in the development of cocoa flavor precursors. During fermentation, cocoa beans are exposed to the action of various microorganisms and enzymes on carbohydrates, proteins, lipids and phenolic compounds, which may determine the quality of chocolate and cocoa-based products (Schwan & Wheals, 2004). The world leaders in cocoa bean production are Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon, Brazil, Ecuador, Dominican Republic and Malaysia, supplying about 90% of the world production (Bordiga et al., 2015; Fowler, 2009; Jahurul et al., 2013).

Cocoa has recently become the target of increased scientific research due to its health promoting properties. Indeed, cocoa is one of the highest natural sources of phenolic compounds (mainly epicatechin, catechin and procyanidins) and it has high antioxidant potential, even compared to tea or red wine (Ellam & Williamson, 2013; Lee, Kim, Lee, & Lee, 2003). In addition, cocoa can be a source of polyamines, which also contribute to cocoa's antioxidant activity. Furthermore, biogenic amines can be formed during fermentation, which can be detrimental to cocoa quality and human safety. Both polyamines and biogenic amines belong to the bioactive amines family. These compounds play relevant roles in plant development and human health. In fact, there are very few reports on bioactive amines during cocoa fermentation, whereas there are several comprehensive studies on phenolic compounds profile during fermentation (Albertini et al., 2015; Di Mattia et al., 2013; Suazo, Davidov-Pardo, & Arozarena, 2014).

Polyamines are ubiquitous in all living cells where they play roles in development, growth, and resistance to stress (Kalac, 2014). These compounds have antioxidant activity, preventing damage to cell membranes and DNA. Moreover, polyamines are efficient scavengers of hydroxyl radicals at physiological concentrations. However, in the presence of free iron ions and hydrogen peroxide, spermidine and spermine may exhibit pro-oxidant properties (Mozdzan, Szemraj, Rysz, Stolarek, & Nowak, 2006). Some biogenic amines are naturally present in plants and are important precursors of hormones and in plant protection against predators. Other biogenic amines can be produced and accumulate during fermentation processes, mostly through amino acid decarboxylation by microbial enzymes (Glória, 2005; Ordonez, Callejon, Morales,

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& Garcia-Parrilla, 2013). Low levels of phenylethylamine in cocoa products are desirable due to its associated aphrodisiac effects (Afoakwa, 2008). Moreover, mood lifting and heightened sensitivity were also associated due to the presence of phenylethylamine and N-acylethanolamine in cocoa and chocolate (Afoakwa, 2008; Glória, 2005). At low levels, bioactive amines are relevant to human health; however, some amines, at high levels, may cause adverse effects to human health. According to Glória (2005) and EFSA (Hazards, 2011), high levels of histamine are associated with allergic type reactions and headache, whereas high levels of tyramine, tryptamine and phenylethylamine can cause hypertensive crisis and migraine.

Cocoa beans are widely known to contain proteins that can be hydrolyzed by microorganism (yeasts, filamentous fungi, lactic acid and acetic acid bacteria) releasing free amino acids, which can undergo decarboxylase activity by some bacterial enzymes to form amines (Granvogl, Bugan, & Schieberle, 2006; Shukla, Park, Kim, & Kim, 2010). Decarboxylase activity is favored by low pH values during fermentation, as a protection mechanism of bacteria against the acid medium (Glória, 2005; Shukla et al., 2010; Oracz & Nebesny, 2014). Furthermore, contaminating bacteria can also decarboxylate amino acids and form amines. Therefore, fermentation of cocoa beans and the sanitary conditions prevalent during cocoa processing can affect the levels and profiles of biogenic amines.

Very few studies investigated amines in cocoa and products. Cocoa beans were reported to contain low levels of phenylethylamine and tyramine (Coutts, Baker, & Pasutto, 1986). 2-Phenylethylamine, tyramine, tryptamine, serotonin, dopamine and histamine have been found in cocoa and chocolate (Guillen-Casla, Rosales-Conrado, Leon-Gonzalez, Perez-Arribas, & Polo-Diez, 2012; Kosman, Stankevich, Makarov, & Tikhonov, 2007; Pastore et al., 2005; Smit, 2011). However, as far as our knowledge is concerned, the changes in bioactive amines during the fermentation process of cocoa beans were not yet reported.

Therefore, the objective of this study was to investigate the profile and contents of bioactive amines during cocoa beans fermentation, using the most traditional process prior to chocolate production. Moreover, since phenolic compounds are also important bioactive compounds in cocoa beans, total phenolics and anthocyanins as well as the associated scavenging capacity were investigated throughout the fermentation process. Furthermore, two multivariate exploratory techniques were used to describe the main characteristics attributed to each fermentation day.

2. Material and methods

2.1. Chemicals

Bioactive amine standards – spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, agmatine sulfate, cadaverine dihydrochloride, 5-hydroxytryptamine – serotonin, histamine dihydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride and tryptamine, as well as *o*-phthalaldehyde, (+)-catechin and 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Acros Organics (Morris Plains, NJ, USA). Folin-Ciocalteu's phenol reagent, potassium persulfate, ethanol, methanol, acetone, sodium carbonate, acetic acid, hexane, sodium hydroxide and hydrochloric acid were from Merck (Darmstadt, Germany). Ultrapure water was from Milli-Q System (Millipore Corp., Milford, MA, USA). Mobile phases for HPLC analysis were filtered through 0.45 μm membranes (Millipore Corp., Milford, MA, USA).

2.2. Cocoa bean samples

Amazonian cocoa fruits (*Theobroma cacao* L.) (Forastero hybrid cultivar, n = 420) were harvested in Combu Island, Belém, PA, Brazil (01°31′S, 48° 29′W) and shipped immediately to the laboratory. The ripe fruits were opened with stainless steel knives; the beans were removed from the fruits and immediately submitted to the fermentation process.

2.3. Cocoa bean fermentation

Three fermentations were carried out in wooden boxes (45 kg capacity) containing three compartments. The beans were placed into the first compartment, covered with banana leaves (natural yeast source) and by burlap bags to keep the generated heat. After 48 h, the resulting fermented mass was transferred to the second compartment, where it remained for another 48 h, and afterwards, it was turned over daily until the end of the process. After 96 h, the mass was transferred to the third compartment, where it was allowed to ferment for up to 168 h, always covered by banana leaves and burlap bags.

The samples were collected over time in sterile low-density polyethylene bags at 0 (fresh cocoa beans) and after 24, 48, 72, 96, 120, 144 and 168 h of fermentation (0–7 days), from different areas within the box compartment (middle, bottom, and surface – central and diagonal regions), assuring a representative sampling. The temperature was measured at three different heights of the box (top, middle and bottom). Part of the samples were used for analysis of pH, total acidity and bioactive amines. The other part was freeze-dried (Liobras, Liotop L101, Brazil) to about 6.0 g/100 g of moisture content, ground in a food processor, sieved through a 40-mesh sieve and analyzed for total phenolic compounds, total anthocyanins and scavenging capacity. All the analysis were carried out in triplicate.

2.4. Preparation of extracts for phenolic compounds and scavenging activity assays

Sample extraction was carried out according to Ioannone et al. (2015) with some modifications. Prior to extraction of the phenolic compounds, 1 g freeze-dried sample was defatted with 5 mL of n-hexane, and this step was repeated four times. The residue was dried to remove residual n-hexane. Then, 0.1 g of the defatted sample was weighed in test tubes followed by the addition of 5 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v). The sealed tubes were stirred in a vortex for 5 min, centrifuged at 18000 *x g* for 15 min at room temperature and the supernatant was filtrated through cellulose filters. The filtered cocoa extract was used for determination of both total phenolic content and scavenging capacity.

2.5. Total phenolic compounds determination

Total phenolic compounds were determined by the colorimetric Folin-Ciocalteu assay (Singleton, Orthofer, & Lamuela-Raventos, 1999). The quantification was performed using a UV–Visible spectrophotometer (Thermo Scientific, Evolution 60S, MA, USA) at 750 nm by using seven-point analytical curves (measurements in duplicate), with concentrations varying from 1 to 8 μg/mL of catechin standard ($r^2 > 0.99$). The results were expressed as mg of catechin equivalent/g defatted freeze-dried cocoa beans (mg CE/g).

2.6. Scavenging capacity against ABTS radical

The $ABTS^{\cdot+}$ scavenging capacity assay was carried out by spectrophotometry (Re et al., 1999). From each extract, four dilutions

were prepared and then, 30 μL of each diluted extract were added to 3 mL of the $ABTS^{\cdot+}$ solution (7 mM ABTS + 140 mM potassium persulfate) prepared on the previous day. After homogenization and rest for 6 min, the absorbance was measured at 734 nm. Ethanol was used as blank. The results were expressed as μM trolox equivalent/g of defatted freeze-dried cocoa beans, as determined by comparison to the five-point analytical curves of trolox (100–2000 μM, $r^2 \geqslant 0.99$).

2.7. Total anthocyanins determination

The determination of total anthocyanins was carried out according to Fuleki and Francis (1968). Briefly, 0.5 g of the freeze-dried sample was added to 4 mL of 95% ethanol/1.5 N HCl (85:15, v/v) and manually stirred for 2 min before standing overnight at room temperature (30 °C) protected from light exposure. The extracts were filtered through Whatman® No. 1 paper and the residue was submitted to exhaustive extraction with the same ethanolic solution until colorless. The filtered solution was transferred to a volumetric flask; it was left to stand for 90 min at room temperature and protected from light exposure and the total anthocyanins were quantified by spectrophotometry at 535 nm ($E^{1\%}_{1cm} = 98.2$). The results were expressed as mg anthocyanins/g of defatted freeze-dried cocoa beans.

2.8. Bioactive amines determination

The bioactive amines of cocoa bean samples (5 g) were extracted with 7 mL 5% trichloroaceatic acid (TCA) mixed in a shaker for 5 min followed by centrifugation at 11180 *x g* at 4 °C for 10 min (Adão & Glória, 2005). Extraction was repeated twice and the supernatants were combined in a volumetric flask, and filtered through qualitative paper. Ten free bioactive amines (spermidine, spermine, putrescine, agmatine, cadaverine, serotonin, histamine, tyramine, tryptamine, and phenylethylamine) were determined by ion-pair reverse phase high-pressure liquid chromatography (HPLC) (Adão & Glória, 2005). A Shimadzu LC-10AD with SIL-10AD VP automatic injector (Shimadzu, Kyoto, Japan) was used, and the amines were separated using a Novapak C_{18} column (3.9 × 300 mm, 4 μm, 60 Å, Waters, MA, USA) and a gradient elution of 0.2 mol/L sodium acetate and 15 mmol/L sodium octanesulfonate with pH adjusted to 4.9 (mobile phase A) and acetonitrile (mobile phase B). The amines were tentatively identified by comparison of retention times and co-elution with the standards. Quantification was carried out by fluorimetry (340 and 445 nm of excitation and emission, respectively), after post-column derivatization with *o*-phthalaldehyde, by using analytical curves (measurements in duplicate) for each amine ($r^2 > 0.99$). The results were expressed mg/100 g of cocoa beans.

2.9. Statistical analysis

All the results (means ± standard deviation) were submitted to analysis of variance using one-way (ANOVA) and the means were compared by Tukey's test at 95% significance ($p < 0.05$), using Statistica 7.0 software. Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied for characterization of cocoa beans at each fermentation day, using Statistica 7.0. For PCA, pH, temperature, total acidity, total phenolic compounds, total anthocyanins, bioactive amine contents, the total sum of biogenic amines, the total sum of polyamines and the total sum of bioactive amines were used as active variables in the derivation of the principal components, and the supplementary variable (scavenging capacity against ABTS) was projected onto the factor space. PCA analysis was performed using the covariance matrix. For HCA, the hierarchical tree was obtained considering the same active variables applied to PCA and cocoa beans from each fermentation day were joined by unweighted pair-group average as the linkage rule, considering the Euclidian distances as the coefficient of similarity.

3. Results and discussion

3.1. Temperature, pH and total acidity alterations during fermentation of cocoa beans

As indicated in Table 1, during natural fermentation of cocoa beans, there was a significant ($p < 0.05$) increase in both temperature and total acidity; however, there was a decrease in pH values. Based on these results, cocoa fermentation proceeded as expected in a typical natural cocoa fermentation process.

The temperature increased from 22 °C, at the beginning of fermentation, reaching a maximum of 41 °C on the 5th fermentation day. It decreased afterwards to 34 °C at the end of fermentation. These changes are due to biochemical reactions that occur during cocoa fermentation, which are associated with the activities of some osmo- and acid-tolerant microorganisms naturally found in cocoa beans (Schwan, Pereira, & Fleet, 2014). The increased pH, temperature and oxygen availability favor acetic acid bacteria, which require 30 °C and pH 5–6.5 to metabolize ethanol into acetic acid through a highly exothermic process, leading to a fast temperature increase, with the fermenting mass reaching temperatures above 45 °C (Nielsen, Crafack, Jespersen, & Jakobsen, 2013; Pereira, Miguel, Ramos, & Schwan, 2012).

The sugars present in cocoa beans pulp are metabolized into ethanol by the yeasts, which are the prevalent microorganisms at the beginning of fermentation. Consequently, pectinolytic enzymes are excreted and they break down the pulp leading to an increase in the oxygen availability. Lactic acid bacteria act by the citric acid metabolism leading to a pH increase (Schwan & Wheals, 2004). The pH values decreased along fermentation, from 6.56 to ~5.00 and total acidity increased from 2.85 mEq NaOH/100 g reaching a maximum on the 3rd fermentation day (15.25 mEq NaOH/100 g), followed by a slight decrease at the end of the fermentation process (13.78 mEq NaOH/00 g).

3.2. Total phenolic compounds, total anthocyanins and free radical scavenging capacity changes during cocoa beans fermentation

The contents of total phenolic compounds, total anthocyanin, as well as free radical scavenging capacity of cocoa beans during fermentation are described in Table 2. Total phenolic compounds content decreased by ≈31% after 7 days of fermentation, whereas total anthocyanins showed a 79% reduction. The phenolic compounds, including anthocyanins, can be lost from cocoa beans during fermentation by lixiviation with the fermentation exudate and also

Table 1
Temperature, pH and total acidity during the fermentation of cocoa beans.

Time (days)	Temperature (°C)	pH	Total acidity (mEq NaOH/100 g)
0	22.1 ± 2.36^{f}	6.56 ± 0.08^{a}	2.85 ± 0.35^{d}
1	27.7 ± 2.00^{e}	6.47 ± 0.08^{b}	3.32 ± 0.32^{d}
2	30.3 ± 2.31^{d}	6.21 ± 0.16^{c}	5.08 ± 0.86^{c}
3	34.2 ± 3.34^{c}	5.53 ± 0.55^{d}	15.25 ± 0.33^{a}
4	38.3 ± 1.86^{b}	5.39 ± 0.57^{e}	$14.95 \pm 0.67^{a,b}$
5	41.4 ± 1.02^{a}	5.10 ± 0.22^{f}	15.28 ± 3.85^{a}
6	35.3 ± 4.10^{c}	4.98 ± 0.27^{g}	15.04 ± 4.60^{a}
7	34.0 ± 1.73^{c}	5.13 ± 0.26^{f}	13.78 ± 3.91^{b}

Means ± standard deviation (wet basis) with different superscripts in the same column are significantly different (Tukey test, $p < 0.05$).

Table 2
Total phenolic compounds, total anthocyanins and scavenging capacity against ABTS radicals during the fermentation of cocoa beans.

Time (day)	Total phenolic compounds (mg CE/g)	Total anthocyanins (mg/g)	Scavenging capacity (μmol TE/g)
0	77.31 ± 2.25^{a}	3.01 ± 0.15^{a}	1296.57 ± 52.50^{a}
1	72.20 ± 1.86^{b}	2.80 ± 0.12^{b}	1122.09 ± 103.10^{e}
2	67.45 ± 12.30^{c}	2.35 ± 0.29^{c}	1140.90 ± 184.49^{d}
3	69.19 ± 6.70^{c}	2.26 ± 0.77^{d}	1209.29 ± 37.52^{b}
4	66.66 ± 7.50^{c}	1.16 ± 0.75^{e}	1155.81 ± 365.23^{c}
5	55.06 ± 1.51^{d}	0.86 ± 0.29^{f}	959.69 ± 242.02^{f}
6	53.03 ± 2.12^{d}	0.70 ± 0.18^{g}	620.01 ± 143.79^{h}
7	53.26 ± 10.72^{d}	0.63 ± 0.05^{h}	789.85 ± 212.58^{g}

CE = Catechin equivalent; TE = Trolox equivalent. Means ± standard deviation (defatted dry weight basis) with different superscripts in the same column are significantly different (Tukey test, $p < 0.05$).

due to the polyphenol oxidase activity, which can be easily noticed by the brown aspect of fermented cocoa beans (Wollgast & Anklam, 2000; Camu et al., 2008; Luna, Crouzillat, Cirou, & Bucheli, 2002). In addition, the major anthocyanins in cocoa beans are cyanidin 3-*O*-arabinoside and cyanidin 3-*O*-galactoside, and they may be hydrolyzed by glycosidases into anthocyanidins during fermentation, resulting in the brightening of cotyledons (Oracz, Nebesny, & Żyżelewicz, 2015). In our study, the contents of anthocyanins decreased by 62% after 4 days and 80% after 7 days (Table 2). According to the literature, anthocyanin contents decreased in a fast rate during fermentation process (loss of 93% after 4 days) and they are considered as a good fermentation degree index during cocoa beans fermentation (Emmanuel, Jennifer, Agnes, Jemmy, & Firibu, 2012).

As a result of the significant decrease on total phenolic compounds and total anthocyanin contents, the scavenging capacity of cocoa beans against ABTS radical also decreased, resulting in loss of 39% after 7 days of fermentation. Suazo et al. (2014) also observed a reduction in the phenolic compounds (63%) and scavenging capacity (83%) in Nicaraguan cocoa beans (Trinitario cultivar) after fermentation, which was higher when compared to this work. Despite the loss of scavenging capacity during the fermentation of cocoa beans, the remaining activity is still significant and may be responsible for the high antioxidant potential reported for fermented cocoa products (Aikpokpodion & Dongo, 2010; Othman, Ismail, Ghani, & Adenan, 2007). Moreover, other bioactive compounds with antioxidant capacities may remain or they can be formed during fermentation, such as bioactive amines.

3.3. Bioactive amines changes during cocoa beans fermentation

Among the ten bioactive amines investigated, only two biogenic amines (tryptamine and tyramine) and two polyamines (spermidine and spermine) were detected during the fermentation of cocoa beans (Table 3, Fig. 1). Importantly, as far as our knowledge is concerned, this is the first time that spermidine and spermine were detected and quantified in cocoa beans during the fermentation process. In fact, the profile and contents of bioactive amines during cocoa fermentation were not reported in the literature until now. The presence of tyramine and tryptamine in fresh cocoa beans were already reported (Oracz & Nebesny, 2014) and should be highlighted. These compounds, at low concentrations modulate vaso- and neuro-activities relevant to man; however, high concentrations of tyramine can be detrimental to human health: they may induce headache and hypertension to susceptible individuals (Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2008). According to EFSA (Hazards, 2011), sensitive individuals and those under classical monoamine inhibitor drugs should not be exposed to any tyramine.

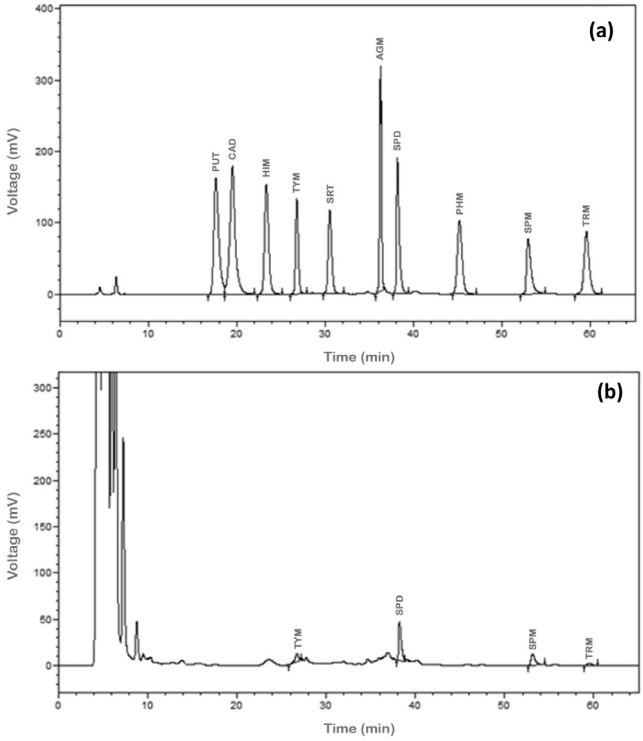

Fig. 1. Typical HPLC chromatograms of (a) bioactive amines standard solution and (b) sample of cocoa beans fermented on the 5th fermentation day. Peak identity: Putrescine = PUT, Cadaverine = CAD, Histamine = HIM, Tyramine = TYM, Serotonin = SRT, Agmatine = AGM, Spermidine = SPD, Phenylethylamine = PHM, Spermine = SPM, Tryptamine = TRM.

Table 3
Bioactive amine contents during the fermentation of cocoa beans.

Time	Bioactive amines (mg/100 g)						
(days)	Spermidine	Spermine	Tryptamine	Tyramine	Polyamines[1]	Biogenic[2]	Total[3]
1	0.33 ± 0.01^{c}	0.25 ± <0.01^{b,c}	0.29 ± 0.01^{a}	0.40 ± <0.01^{c,d}	0.58 ± <0.01^{c}	0.70 ± < 0.01^{c}	1.28 ± <0.01^{e}
2	0.65 ± 0.59^{b,c}	0.59 ± 0.13^{a,b}	0.24 ± < 0.01^{b,c}	0.22 ± 0.01^{d}	1.25 ± 0.51^{ab}	0.45 ± < 0.01^{d}	1.70 ± 0.52^{d}
3	0.98 ± 0.46^{a,b,c}	0.44 ± 0.01^{a,b,c}	0.28 ± 0.03^{a,b}	0.32 ± 0.10^{d}	1.42 ± 0.32^{abc}	0.59 ± 0.05^{cd}	2.01 ± 0.37^{cd}
4	1.91 ± 0.12^{a}	0.68 ± 0.12^{a}	0.19 ± < 0.01^{c}	1.18 ± 0.03^{a}	2.58 ± <0.01^{a}	1.37 ± 0.02^{a}	3.96 ± 0.02^{cd}
5	1.59 ± 0.17^{a,b}	0.36 ± 0.02^{a,b,c}	0.23 ± < 0.01^{bc}	0.75 ± 0.03^{b}	1.94 ± 0.11^{ab}	0.98 ± 0.02^{b}	2.93 ± 0.13^{a}
6	1.94 ± 0.22^{a}	0.48 ± 0.14^{a,b,c}	0.26 ± 0.01^{a,b}	0.83 ± 0.12^{b}	2.42 ± 0.06^{ab}	1.08 ± 0.07^{b}	3.51 ± 0.01^{abc}
7	1.23 ± 0.16^{a,b,c}	0.22 ± 0.05^{c}	0.19 ± 0.01^{c}	0.59 ± 0.02^{b,c}	1.45 ± 0.07^{abc}	0.78 ± 0.02^{c}	2.23 ± 0.10^{bcd}

Means ± standard deviation (wet basis) with different superscripts in the same columns are significantly different (Tukey test, $p < 0.05$).
[1] Spermidine + spermine.
[2] Tryptamine + tyramine.
[3] Total biogenic amines + polyamines.

From the second day until the end of fermentation (7th day), spermidine (polyamine) become the predominant bioactive amine, contributing from 38 to 55% to the total of bioactive amines in cocoa beans during the fermentation. The presence of spermidine and spermine in the cocoa beans is expected since these amines are inherent to plants and all living organisms as they are involved in cell growth, renewal and metabolism. The increase on spermidine contents during fermentation is desirable considering that this compound is an effective antioxidant, which is beneficial for products shelf life and human health (Glória, 2005; Kalac, 2014).

According to Table 3, the contents of tryptamine and spermine varied throughout fermentation, but remained below 0.30 and 0.70 mg/100 g, respectively. The concentration of tyramine increased up to the 4th fermentation day, decreasing afterwards to initial contents. Spermidine concentrations increased up to the 3rd fermentation day, maintaining the contents throughout fermentation. It is well known that spermidine is synthesized and metabolized by microorganisms during growth; however, no information is available regarding its change during cocoa fermentation. It is also known that bacteria produce some amines, e.g. tyramine, as a protection against the acidic environment (Shukla et al., 2010).

Other bioactive amines, which were not found in our study, were reported in the literature for fresh cocoa beans, such as phenylethylamine, serotonin, dopamine (Oracz & Nebesny, 2014) and histamine (Guillen-Casla et al., 2012; Pastore et al., 2005; Smit, 2011). The difference in the profile of bioactive amines of fresh cocoa beans in the literature could result from differences on cultivars, region of cultivation, growing conditions, degree of ripening, post-harvest processes and storage conditions (Bandeira, Evangelista, & Gloria, 2012; Oracz & Nebesny, 2014).

3.4. Multivariate analysis of changes on cocoa beans during fermentation

In the PCA, the first two components accounted for 84% of the explained variance, taking into consideration temperature, pH, total acidity, total phenolic compounds, total anthocyanins, bioactive amines contents, total biogenic amines, total polyamines and the total bioactive amines. The cocoa beans from each fermentation day were mainly located in PCA and HCA plots (Fig. 2a, b) according to their different yields of phenolic compounds and bioactive amines and the tree diagram from HCA (Fig. 2c) evidenced three groups considering their similarities.

Considering the classification of cocoa beans during the fermentation, the first group, which was formed by cocoa beans from days 1, 2 and 3 (beginning of fermentation), was characterized by the highest contents of total phenolic compounds, total anthocyanins, tryptamine and the highest pH values. The cocoa beans from the first group also exhibited the highest scavenging capacity against ABTS radicals (Table 2). A second group, formed by cocoa beans from days 5, 6 and 7 (end of fermentation) can be seen on the lower right side of the PCA plot (Fig. 2b). This group was characterized by highest values of total acidity, spermidine content and temperature during the fermentation process; however, the cocoa beans from this group presented the lowest total phenolic compounds, total anthocyanins, low spermine values and the lowest scavenging capacity. Finally, the last group (day 4, middle of fermentation) was discriminated from the others (Fig. 2b and c) by the highest contents of spermine, total biogenic amines (sum of tryptamine + tyramine), total polyamines (sum of spermidine + spermine) and total bioactive amines (sum of biogenic amines + polyamines). The cocoa beans from this last group (day 4) also

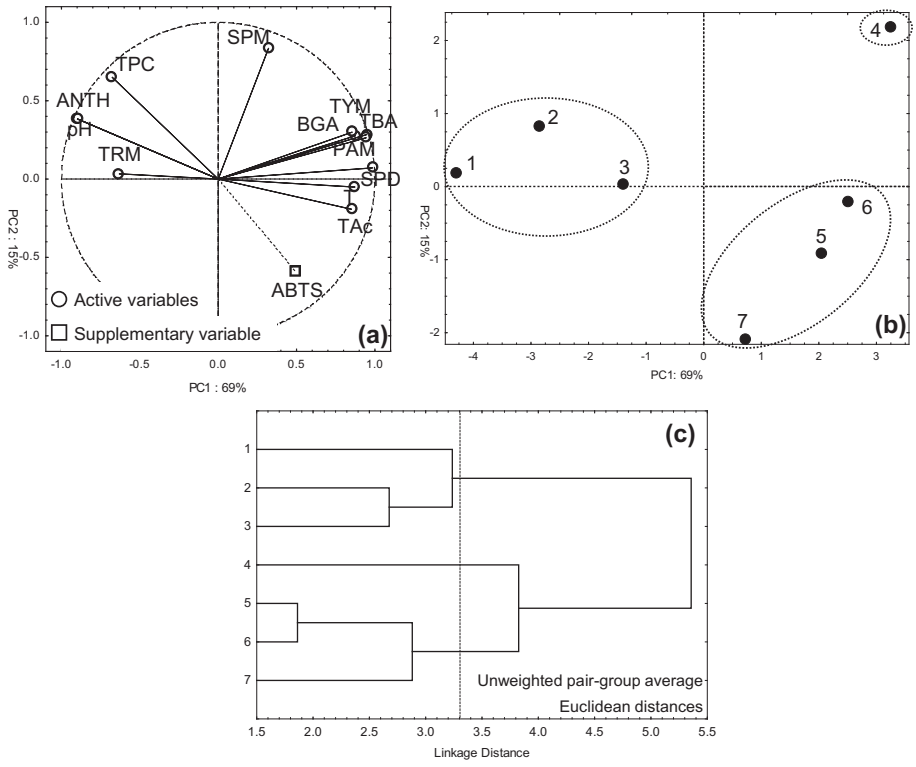

Fig. 2. Classification of cocoa beans by changes on composition and physicochemical characteristics during each fermentation day. (a) Variable projection by Principal Component Analysis (PCA), (b) Scatterplot for cocoa beans during each fermentation day by PCA with suggested grouping, in accordance with Hierarchical Cluster Analysis (HCA), and (c) dendrogram by HCA analysis. Abbreviations: TPC = total phenolic compounds, ANTH = total anthocyanins, pH = pH values, TRM = tryptamine, TYM = tyramine, SPM = spermine, SPD = spermidine, TAc = total acidity, T = temperature, BGA = total biogenic amines, PAM = total polyamines, TBA = total bioactive amines, ABTS = scavenging capacity against ABTS radical.

exhibited a notable scavenging capacity against ABTS radical, compared to cocoa beans from the first group (days 1, 2 and 3) (Table 2).

As can be seen in Fig. 2a, high positive correlation was observed between total phenolic compounds and total anthocyanins (r = 0.91), and both bioactive compounds showed positive correlation with the scavenging capacity against ABTS (r = 0.89 and r = 0.75, respectively). On the other hand, regarding the bioactive amines, spermine and tryptamine showed a very week correlation with the scavenging capacity (r = 0.31 and r = 0.19, respectively), while spermidine and tyramine exhibited negative correlation (r = −0.51 and r = −0.31, respectively). Considering the correlations obtained by PCA analysis, total phenolic compounds and total anthocyanins were the bioactive compounds that contributed to the scavenging capacity of fermented cocoa beans, as determined by ABTS assay. These tendencies are supported by the results obtained in another study with fermented cocoa beans from Ecuador (Arriba Nacional variety), where the antioxidant activity (FRAP and ABTS assays) was attributed due to the presence of phenolic compounds, specially catechins and epicatechins (Albertini et al., 2015).

Overall, during fermentation of cocoa beans, three moments could be depicted according to changes in physicochemical characteristics and bioactive compounds composition. At the beginning of fermentation, during the first three days, the highest contents of total phenolic compounds, total anthocyanins, tryptamine and the highest pH values prevailed in cocoa beans. In the second moment (day 4 – middle of fermentation), the fermented cocoa beans exhibited the highest contents of spermine, total biogenic amines (tryptamine + tyramine), total polyamines (spermidine + spermine) and total bioactive amines (biogenic amines + polyamines). Finally, at the end of cocoa beans fermentation, the highest values of total acidity and highest contents of spermidine, but the lowest contents of total phenolic compounds, total anthocyanins, spermine and the lowest scavenging capacity were observed. Moreover, the scavenging capacity of cocoa beans against ABTS radicals during the fermentation was correlated with total phenolic compounds and anthocyanins, but not with the presence of any bioactive amines. Therefore, here we demonstrated, for the first time, the changes in the profile of bioactive amines, in consonance with other important bioactive compounds (total phenolic compounds and anthocyanins), as influenced by the fermentation of cocoa beans.

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References

Adão, R. C., & Glória, M. B. A. (2005). Bioactive amines and carbohydrate changes during ripening of 'Prata' banana (*Musa acuminata* × *M. balbisiana*). *Food Chemistry, 90*(4), 705–711.

Afoakwa, E. O. (2008). Cocoa and chocolate consumption – Are there aphrodisiac and other benefits for human health? *South African Journal of Clinical Nutrition, 21*(3), 107–113.

Aikpokpodion, P. E., & Dongo, L. N. (2010). Effects of fermentation intensity on polyphenols and antioxidant capacity of cocoa beans. *International Journal of Sustainable Crop Production, 5*(4), 66–70.

Albertini, B., Schoubben, A., Guarnaccia, D., Pinelli, F., Della Vecchia, M., Ricci, M., ... Blasi, P. (2015). Effect of fermentation and drying on cocoa polyphenols. *Journal of Agriculture and Food Chemistry, 63*(45), 9948–9953.

Ancín-Azpilicueta, C., González-Marco, A., & Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Reviews in Food Science and Nutrition, 48*(3), 257–275.

Bandeira, C. M., Evangelista, W. P., & Gloria, M. B. A. (2012). Bioactive amines in fresh, canned and dried sweet corn, embryo and endosperm and germinated corn. *Food Chemistry, 131*(4), 1355–1359.

Bordiga, M., Locatelli, M., Travaglia, F., Coïsson, J. D., Mazza, G., & Arlorio, M. (2015). Evaluation of the effect of processing on cocoa polyphenols: Antiradical activity, anthocyanins and procyanidins profiling from raw beans to chocolate. *International Journal of Food Science & Technology, 50*(3), 840–848.

Camu, N., De Winter, T., Addo, S. K., Takrama, J. S., Bernaert, H., & De Vuyst, L. (2008). Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *Journal of the Science of Food and Agriculture, 88*(13), 2288–2297.

Coutts, R. T., Baker, G. B., & Pasutto, F. M. (1986). Foodstuffs as sources of psychoactive amines and their precursors: Content, significance and identification. *Advances in Drug Research, 15*, 63.

Di Mattia, C., Martuscelli, M., Sacchetti, G., Scheirlinck, I., Beheydt, B., Mastrocola, D., & Pittia, P. (2013). Effect of fermentation and drying on procyanidins, antiradical activity and reducing properties of cocoa beans. *Food and Bioprocess Technology, 6*(12), 3420–3432.

Ellam, S., & Williamson, G. (2013). Cocoa and human health. *Annual Review of Nutrition, 33*, 105–128.

Emmanuel, O. A., Jennifer, Q., Agnes, S. B., Jemmy, S. T., & Firibu, K. S. (2012). Influence of pulp-preconditioning and fermentation on fermentative quality and appearance of Ghanaian cocoa (Theobroma cacao) beans. *International Food Research Journal, 19*(1), 127–133.

Fowler, M. S. (2009). Cocoa beans: From tree to factory. In *Industrial chocolate manufacture and use* (pp. 10–47). Wiley-Blackwell.

Fuleki, T., & Francis, F. J. (1968). Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanin in cranberries. *Journal of Food Science, 33*(1), 72–77.

Glória, M. B. A. (2005). Bioactive Amines. In H. Hui & L. L. Nollet (Eds.). *Handbook of Food Science, Technology and Engineering* (vol. 1, pp. 13–32). New York: Taylor & Francis.

Granvogl, M., Bugan, S., & Schieberle, P. (2006). Formation of amines and aldehydes from parent amino acids during thermal processing of cocoa and model systems: New insights into pathways of the Strecker reaction. *Journal of Agricultural and Food Chemistry, 54*(5), 1730–1739.

Guillen-Casla, V., Rosales-Conrado, N., Leon-Gonzalez, M. E., Perez-Arribas, L. V., & Polo-Diez, L. M. (2012). Determination of serotonin and its precursors in chocolate samples by capillary liquid chromatography with mass spectrometry detection. *Journal of Chromatography A, 1232*, 158–165.

Hazards, E. P. o. B. (2011). Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal, 9*(10), 2393.

Ioannone, F., Di Mattia, C. D., De Gregorio, M., Sergi, M., Serafini, M., & Sacchetti, G. (2015). Flavanols, proanthocyanidins and antioxidant activity changes during cocoa (Theobroma cacao L.) roasting as affected by temperature and time of processing. *Food Chemistry, 174*, 256–262.

Jahurul, M. H. A., Zaidul, I. S. M., Norulaini, N. A. N., Sahena, F., Jinap, S., Azmir, J., ... Omar, A. K. M. (2013). Cocoa butter fats and possibilities of substitution in food products concerning cocoa varieties, alternative sources, extraction methods, composition, and characteristics. *Journal of Food Engineering, 117*(4), 467–476.

Kalac, P. (2014). Health effects and occurrence of dietary polyamines: A review for the period 2005-mid 2013. *Food Chemistry, 161*, 27–39.

Kosman, V. M., Stankevich, N. M., Makarov, V. G., & Tikhonov, V. P. (2007). Biologically active substances in grated cocoa and cocoa butter. *Voprosy Pitaniia, 76*(3), 62–67.

Lee, K. W., Kim, Y. J., Lee, H. J., & Lee, C. Y. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *Journal of Agricultural and Food Chemistry, 51*(25), 7292–7295.

Luna, F., Crouzillat, D., Cirou, L., & Bucheli, P. (2002). Chemical composition and flavor of Ecuadorian cocoa liquor. *Journal of Agricultural and Food Chemistry, 50*(12), 3527–3532.

Mozdzan, M., Szemraj, J., Rysz, J., Stolarek, R. A., & Nowak, D. (2006). Anti-oxidant activity of spermine and spermidine re-evaluated with oxidizing systems involving iron and copper ions. *International Journal of Biochemistry & Cell Biology, 38*(1), 69–81.

Nielsen, D. S., Crafack, M., Jespersen, L., & Jakobsen, M. (2013). The microbiology of cocoa fermentation. In R. R. Watson, R. V. Preedy, & S. Zibadi (Eds.), *Chocolate in health and nutrition* (pp. 39–60). Totowa, NJ: Humana Press.

Oracz, J., & Nebesny, E. (2014). Influence of roasting conditions on the biogenic amine content in cocoa beans of different Theobroma cacao cultivars. *Food Research International, 55*, 1–10.

Oracz, J., Nebesny, E., & Żyżelewicz, D. (2015). Changes in the flavan-3-ols, anthocyanins, and flavanols composition of cocoa beans of different Theobroma cacao L. groups affected by roasting conditions. *European Food Research and Technology, 241*(5), 663–681.

Ordonez, J. L., Callejon, R. M., Morales, M. L., & Garcia-Parrilla, M. C. (2013). A survey of biogenic amines in vinegars. *Food Chemistry, 141*(3), 2713–2719.

Othman, A., Ismail, A., Ghani, N. A., & Adenan, I. (2007). Antioxidant capacity and phenolic content of cocoa beans. *Food Chemistry, 100*(4), 1523–1530.

Pastore, P., Favaro, G., Badocco, D., Tapparo, A., Cavalli, S., & Saccani, G. (2005). Determination of biogenic amines in chocolate by ion chromatographic separation and pulsed integrated amperometric detection with implemented

wave-form at Au disposable electrode. *Journal of Chromatography A, 1098*(1–2), 111–115.

Pereira, G. V., Miguel, M. G., Ramos, C. L., & Schwan, R. F. (2012). Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. *Applied and Environmental Microbiology, 78*(15), 5395–5405.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*(9–10), 1231–1237.

Schwan, R. F., Pereira, G. V. M., & Fleet, G. H. (2014). Microbial activities during cocoa fermentation. In R. F. Schwan & G. H. Fleet (Eds.), *Cocoa and coffee fermentations* (pp. 125–135). London: Taylor & Francis.

Schwan, R. F., & Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition, 44*(4), 205–221.

Shukla, S., Park, H. K., Kim, J. K., & Kim, M. (2010). Determination of biogenic amines in Korean traditional fermented soybean paste (Doenjang). *Food and Chemical Toxicology, 48*(5), 1191–1195.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.

Smit, H. J. (2011). Theobromine and the pharmacology of cocoa. In *Methylxanthines* (pp. 201–234). Berlin, Heidelberg: Springer, Berlin Heidelberg.

Suazo, Y., Davidov-Pardo, G., & Arozarena, I. (2014). Effect of fermentation and roasting on the phenolic concentration and antioxidant activity of cocoa from Nicaragua. *Journal of Food Quality, 37*(1), 50–56.

Wollgast, J., & Anklam, E. (2000). Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International, 33*(6), 423–447.