Ciência e Tecnologia de Alimentos
ISSN: 0101-2061
revista@sbcta.org.br
Sociedade Brasileira de Ciência e Tecnologia de Alimentos
Brasil

Fontes Moreau CRUZ, Jaqueline; Bacelar LEITE, Paula; SOARES, Sergio Eduardo; da Silva BISPO, Eliete

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Ciência e Tecnologia de Alimentos, vol. 35, núm. 2, abril-junio, 2015, pp. 279-284
Sociedade Brasileira de Ciência e Tecnologia de Alimentos
Campinas, Brasil

DOI: http://dx.doi.org/10.1590/1678-457X.6541

Bioactive compounds in different cocoa (*Theobroma cacao*, L) cultivars during fermentation

Jaqueline Fontes Moreau CRUZ[1] *in memoriam*, Paula Bacelar LEITE[1], Sergio Eduardo SOARES[1]*, Eliete da Silva BISPO[1]

Abstract

One component that contribute to the flavor and aroma of chocolate are the polyphenols, which have received much attention due to their beneficial implications to human health. Besides bioactive action, polyphenols and methylxantines are responsible for astringency and bitterness in cocoa beans. Another important point is its drastic reduction during cocoa processing for chocolate production and the difference between cultivars. Thus, the present study aimed to evaluate the modifications in monomeric phenolic compounds and methylxanthines during fermentation of three cocoa cultivars grown in southern Bahia. Cocoa beans from three cultivars were fermented and sun dried and monomeric phenolic compounds and methylxantines were determinated. The results showed that each cultivar have different amounts of phenolic compounds and the behaviour of them is different during fermentation. The amount of methylxantines varied but there was not a pattern for methylxantines behavior during process. In addition a huge reduction in phenolic compounds could be observed after drying. Differently of phenolic compounds, methylxantines did not have great modification after sun drying. So, the differences observed in this study between cultivars, take to the conclusion that the compounds studied in those cocoa cultivars have different behavior during fermentation and drying, which consequently, give to these cultivars differences in sensory characteristics.

Keywords: cocoa; fermentation; polyphenols; methylxantines.

Practical Application: Observe the behavior of bioactive substances in cocoa beans during preprocessing.

1 Introduction

Beyond the "witches' broom disease", caused by the fungus *Moniliophthora perniciosa*, which caused a great reduction in cocoa production in southern Bahia, other problem for cocoa treading is the low quality of the beans produced due to the poor control of fermentation and drying, obtaining a significant amount of beans with undesirable characteristics, that will not develop the characteristic flavor for high quality chocolate production (Cruz et al., 2013).

The cocoa pre-processing steps (harvest, breaking, fermentation and drying) are important to ensure the high quality of the beans. For Lagunes-Gálvez et al. (2007) fermentation is one of the post-harvest step that most affect the quality of the products obtained from cocoa.

During fermentation, the pulp is degraded by the successive action of microorganisms, promoting the rise of temperature to about 50 °C and the formation of ethanol and lactic acid, driving to the embryo death, which creates an environment for formation of flavor and aroma precursors of chocolate, developed through roasting process (Lopez, 1986; Leite et al., 2013).

Other components that contribute to the flavor and aroma of chocolate are the polyphenols, which have received much attention due to their beneficial implications to human health by preventing cancer, cardiovascular diseases and other pathologies (Keen et al., 2005; Hermann et al., 2006; Cooper et al., 2008). In this group of compounds, catechin and epicatechin are the most importante, since they are easily absorbed by the intestinal walls and brought the bloodstream, presenting a great potential for action in the body (Richelle et al., 1999). Besides bioactive action, polyphenols are responsible for the development of astringency and bitterness in cocoa beans, followed by alkaloids, some amino acids, peptides and pyrazines (Bonvehi & Coll, 2000).

Another important point to be observed for these compounds is its drastic reduction during cocoa processing for chocolate production. This reduction occurs during fermentation, drying and roasting. Furthermore, the concentration varies depending on the cultivar used and also the type of chocolate produced. Chocolates with higher concentrations of cocoa have higher levels of polyphenols (Miller et al., 2006; Payne et al., 2010).

During fermentation, the polyphenol content decreases about 70%, and the (-) - epicatechin, the main substrate of polyphenoloxidase, suffers a reduction of 90% of its initial concentration. At the same time, catechins form complex tannins and anthocyanins are hydrolysed to anthocyanins which polymerise themselves (Efraim et al., 2011).

Methylxanthines, such as caffeine and theobromine are another group of bioactive compounds also found in cocoa beans.

Received 28 Oct., 2014
Accepted 19 Mar., 2015
[1]*Universidade Federal da Bahia – UFBA, Salvador, BA, Brasil*
*Corresponding author: ssoares.ssa@gmail.com

While caffeine has a stimulating effect on the brain, increasing the psychomotor reactions and blood pressure, the theobromine acts as a muscle relaxant, diuretic and blood pressure reducer (Van den Bogaard et al., 2010; Bruinsma & Taren, 1999).

The theobromine has low detection threshold (10 $mg.L^{-1}$), which contributes to generate a bitter taste to foods. After roasting the beans, theobromine can form chemical adducts with diketopiperazine, which have some relationship with the bitter notes attributed to roasted cocoa (Pickenhagen et al., 1975).

In order to achieve the knowledge of dynamics involving the compounds that may influence the sensory characteristics of chocolate during cocoa pre-processing, the present study aimed to evaluate modifications in monomeric phenolic compounds and methylxanthines amounts during fermentation of three cocoa cultivars grown in southern Bahia.

2 Materials and methods

2.1 Cocoa beans

Cocoa beans from cultivars resistant to "witches' broom disease", "SR162" and "PH16", and a blend of cultivars, composed by *Pará, Parazinho* and *Maranhão*, all of them belonging to *Forastero* group, no resistant to the disease were used. These *Forastero* cultivars is grown from decades in Bahia, considered cultivars of high productivity and high fruit quality, however, no resistant to the disease (Pinto & Pires, 1998).

The "PH16" is a cultivar originated from a selection made in a commercial area. They have unknowing parents and the plant was originally identified in 1996 in a population of cocoa hybrids (crosses between *Amazônico* group and *Trinitario*) from "Porto Híbrido" farm, in São José da Vitória County, Bahia, Brazil.

The "SR162" is a cultivar derived from genetic mutation of the common cocoa (*Alto Amazônico* group), with white seeds. The name came from the farm where there were identified, "São Roque" farm, in Itagibá County, Bahia, Brazil.

2.2 Fermentation and drying process

The fermentation was carried out in 70 × 70 × 75cm wooden boxes, with around 20 holes with 1.27cm each in the bottom and sides of the boxes, in order to flow the liquefied pulp. A total mass of 400 kg in each box were processed. Turnings were performed for oxygenation and mixing of the mass, 48 hours from the beginning of each fermentation and after the decreasing of temperature, until the end of the process. To cover the mass, banana leaves were used. After fermentation, the seeds were dried in the sun-roof surfaces with mobile timber for 5 to 7 days up to 8.0% of moisture.

2.3 Samples collection and data treatment

Samples from two fermentations were taken at the beginning and after 72, 108 and 120 hours. Cocoa beans were collected from the surface, middle and bottom of the boxes, mixed and freeze dried in a LIOTOP Freeze Dryer, L108 model in order to perform the analysis. Data were subjected to analysis of variance (oneway ANOVA) using the Tukey test at 5% significance for means comparison .

2.4 Extraction of Phenolic Compounds and Methylxanthines

Methanolic extracts were obtained according to Fantozzi & Montedoro (1978). Ten grams of cocoa mass and chocolate were weight and defatted with petroleum ether, under shacking during 30 minutes for three times. From defatted samples, five grams were weight and 80% methanol in water (v/v) solution were added and stirring during one hour. After filtration, the methanolic extracts were stored in dark flask under nitrogen atmosphere at low temperature.

2.5 Determination of monomeric phenols and methylxanthines by HPLC

The determination of monomeric phenolic compounds (galic acid, catechin, caffeic acid and epicatechin) and methylxantines (caffeine and theobromine) was performed according to the method described by Elwers et al. (2009). Ten microlitres of each sample solution was analyzed by HPLC system (Perkin Elmer Model Flexar) equipped with VI Flow injector, C_{18} column (100 mm × 4.6 mm O.D.S.-2, 3 μm) and using the solvents (A): 2% acetic acid in water and (B): a mixture of acetonitrile, water and acetic acid (400:90:10 v/v/v). Elution was performed with a linear gradient showed in Table 1. The compounds were monitored by UV detection at 280 nm wavelength. The total run time was 20 min and the temperature was 26 °C. All standards used for quantitative determinations were from Sigma-Aldrich, St. Louis, MO.

3 Results and discussion

3.1 Phenolic compounds and methylxantinesin cocoa beans during fermentation process

In Table 2 are showed the phenolic profile during fermentation of three cocoa cultivars. First of all, it can be observed that the cultivar have different amounts of phenolic compounds. The non-resistant blend have the highest amount of phenolic compounds in the beginning of fermentation process, 18.9$mg.g^{-1}$, while PH16 have 15.3$mg.g^{-1}$ and SR162 5.7$mg.g^{-1}$. It was already expected, since non-resistant cultivars were composed by healthy and "witches' broom disease" infected fruits and the fungal infection enhance the synthesis of phenolic compounds (Soares, 2002). When a plant is attacked by a potential pathogen, there is activation of the defense responses complex, with expression of various genes, resulting in synthesis and accumulation of secondary metabolites

Table 1. HPLC gradient used for separation of phenolic compounds and methylxanthines in cocoa mass and chocolates.

Time (min)	Flow rate ($mL.min^{-1}$)	A (%)	B (%)
2	0.4	90	10
3	0.3	88	12
3	0.4	86	14
2	0.4	84	16
2	0.5	82	18
10	0.5	90	10

Table 2. Phenolic compounds amounts (mg.g^{-1}) during fermentation of three cocoa cultivars*.

Cultivars	Phenolic Compound	Fermentation time (hours)			
		0	72	108	120
Non-resistant blend	Galic acid	0.14 ± 0.00^{a}**	0.05 ± 0.01^{b}	0,18 ± 0,03^{a}	0,13 ± 0.03^{a}
	Catechin	2.46 ± 0.01^{a}	2.13 ± 0.04^{a}	3.11 ± 0.07^{b}	2.48 ± 0.04^{a}
	Caffeic acid	0.00 ± 0.00^{a}	1.66 ± 0.06^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Epicatechin	16.35 ± 1.41^{a}	2.96 ± 0.25^{b}	12.38 ± 0.22^{c}	9.37 ± 0.63^{d}
SR162	Galic acid	0.10 ± 0.03^{a}	0.18 ± 0.09^{a}	0.18 ± 0.09^{a}	0.10 ± 0.02^{a}
	Catechin	1.71 ± 0.11^{a}	2.77 ± 0.31^{b}	2.37 ± 0.04^{b}	2.92 ± 0.92^{b}
	Caffeic acid	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}
	Epicatechin	3.88 ± 0.20^{a}	7.25 ± 1.07^{b}	5.53 ± 0.21^{c}	7.00 ± 0.79^{b}
PH16	Galic acid	0.11 ± 0.01^{a}	0.10 ± 0.00^{ab}	0.08 ± 0.01^{b}	0.04 ± 0.01^{c}
	Catechin	1.48 ± 0.04^{a}	1.02 ± 0.00^{b}	1.20 ± 0.03^{c}	1.24 ± 0.01^{c}
	Caffeic acid	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Epicatechin	13.71 ± 0.84^{a}	11.20 ± 1.19^{b}	5.39 ± 0.36^{c}	6.65 ± 0.00^{c}

*The values shown are the average of 2 fermentation replicates and expressed in dry defatted basis. **Same letter in the same line shows no statistical difference ($p \leq 0.05$).

such as phenolic compounds (Leite et al., 2013). On the other hand the SR-162 cultivar have the lowest levels of monomeric phenolic compounds, showing that this cultivar is not only poor in anthocyanins, but also in other phenolic compounds.

Other point to be observed is the predominance of (-) epicatechin in all cultivars when fermentation started, and once more, the non-resistant blend showed highest levels of this compound, 16.3mg.g^{-1}, while for SR162 and PH16 were 3.9mg.g^{-1} and 13.7mg.g^{-1}, respectively. Many authors reported the highest levels of (-) epicatechin in cocoa beans, mass and chocolate (Miller et al., 2006; Payne et al., 2010; Leite et al., 2013).

In addition, the non-resistant blend showed more varied phenolic profile with small amounts of galic and caffeic acids, followed by SR162 that had galic acid and PH16 that had just catechin and epicatechin. This fact can occur since factors as genetic, soil, growing conditions and weather characteristics can affect the profile and concentration of these compounds (Niemenak et al., 2006; Meng et al., 2009; Elwers et al., 2009; Leite et al., 2013).

A significant reduction in (-) epicatechin was observed for non-resistant and PH16 cultivars during fermentation. At the end of the process, the reduction observed for non-resistant cultivar was 58% and for PH16 was 48%. Gil et al. (2011) described higher reduction of epicatechin (80%) in colombian cocoa after fermentation and drying and it was due to this compound be the principal substrate of existing polyphenoloxidase.

Other authors suggest that the reduction of (-) epicatechin may be due to an epimerizarion phenomena caused by changes in pH during fermentation process (Seto et al., 1997).

On the other hand, the SR162 cultivar showed an increase in (-) epicatechin during fermentation. This behavior is related to the hydrolysis of tannins and procyanidins and the action of polyphenoloxidase, processes that generate and oxidize, respectively, smaller phenolic compounds in the medium. Under this aspect, it can be assumed that the phenolic compounds had lower oxidation in this cultivar than the other cultivars while had more hydrolytic activity, probably due to the fermentation conditions achieved by this cultivar during the process as described by Cruz et al. (2013).

Concerning (+) catechin, the amounts remained almost the same during the fermentation process for all cultivars, as observed by Seto et al. (1997), but SR162 showed slightly higher amounts than the other cultivars, which is a goal for this cultivar, since (+)-catechin have a well-known healt benefits (Maskarinec, 2009; Khawaja et al., 2011).

The three cultivar studied showed different amounts of theobromine in the beginning of fermentation (Table 3), the non-resistent blend showed the highest value with 7.96mg.g^{-1}, followed by PH16 and SR162 with 6.50mg.g^{-1}and 5.45mg.g^{-1}, respectively. The same phenomena was observed for caffeine (Table 3), the non-resistant blend showed the highest amount, 4.35mg.g^{-1}, while PH16 had 3.65mg.g^{-1}and SR162 1.32mg.g^{-1}. This fact was already demonstrated by Aneja & Gianfagna (2001) in their studies, showing that the plant tissue infected by fungus can generate some defence biochemical mechanisms, evidenced by higher content of methylxantines and phenolic compounds. Besides, some authors also reported the existing difference between different cocoa genotype and methylxantine content (Figueira et al., 1997).

On the other hand, higher levels of methylxantines can affect the sensory characteristics of the cocoa beans, since sensory attributes like bitterness and astringency were strongly correlated with methylxantines and phenolic compounds (Efraim et al., 2011).

During fermentation, a significative variation in methylxantines levels was found only for non-resistant blend and PH16. The theobromine content in non-resistant blend was 6.18mg.g^{-1} at the end of fermentation, showing a reduction of 22% when compared with the beginning of the process. PH16 showed a reduction of 26% while SR162 showed an increase of theobromine content around 9%, which was not significative (Table 3). For caffeine the reduction at the ending of fermentation was around 50% for non-resistant blend and 14% for PH16 while for SR162 was an increase of 36%.

These results showed that there was not a pattern for methylxantines behavior during fermentation process, since this process is composed by many complex biochemical reactions involving lipids, proteins, methylxantines and phenolic compounds, driving all these compounds to many chemical changes (Efraim et al., 2006). Specifically for methylxantines, probably their complexation with some peptides and the decompartmentalisation of structures within the cell, related to fermentation conditions like temperature and pH during the process, can explain that difference observed for these three cultivars (Cruz et al., 2013).

3.2 Phenolic compounds and methylxantinesfrom fermented and dried cocoa beans

A huge reduction in phenolic compounds can be observed after drying as shown in Table 4. The reduction of epicatechin was higher in SR162 cultivar (68%), for non-resistant and PH16 the reduction was around 55%. For catechin the reduction was 50% for non-resistant cultivar, 45% for SR162 and 35% for PH16. This reduction was reported for other authors and they attributed the reduction of polyphenols during sun drying mainly to enzymatic browing, followed by non-enzymatic reactions due to quinones polymerization (Payne et al., 2010; Gil et al., 2011). The polyphenol oxidase would not have appropriate conditions to react during fermentation, but during sun drying, with the increase in pH and high O_2 uptake, the conditions will be appropriate to phenolic oxidation. These can also explain the reduction differences between cultivars, since each of them presented different genotypic characteristics and consequent specific behavior during fermentation, and the sun drying process were extremely influenced by climactic conditions (Niemenak et al., 2006; Cruz et al., 2013). Although losses in phenolic compounds were observed, sun drying allows to obtain products with better sensory quality in relation to those artificially drying (Faborode et al., 1995; Efraim et al., 2010).

In health aspects, this reduction is not a goal, since phenolic compounds like catechin and epicatechin play an important part in preventing some degenerative diseases through their high antioxidant activity in biological systems (Keen et al., 2005; Cooper et al., 2008; Mostofsky et al., 2010).

The methylxantines content in fermented and dried cocoa beans are shown in Table 5. Differently of phenolic compounds, methylxantines did not have great modification after sun drying, the amounts of either caffeine or theobromine were almost the same before and after the sun drying process. However, for all cultivars theobromine always appears as the main methylxantine in beans, around three-fold the caffeine content for non-resistant and SR162 cultivars and two-fold for PH16, but in all cultivar de theobromine content is almost the same, varying from 5.53 $mg.g^{-1}$ in PH16 cultivar to 6.13$mg.g^{-1}$ in non-resistant blend, with no statistical difference between them. For caffeine higher variation was found, the content varied from 1.89$mg.g^{-1}$ for SR162 cultivar to 3.02$mg.g^{-1}$ for PH16 cultivar, with significative difference between them.

Table 3. Methylxantines amounts ($mg.g^{-1}$) during fermentation of three cocoa cultivars*.

Cultivars	Methyl-xantine	Fermentation time (hours)			
		0	72	108	120
Non-resistant blend	Caffeine	$4.35 \pm 0.03^{a**}$	3.58 ± 0.17^{b}	3.86 ± 0.23^{b}	2.16 ± 0.02^{c}
	Theobromine	7.96 ± 0.05^{a}	6.94 ± 0.26^{b}	8.29 ± 0.45^{a}	6.18 ± 0.36^{b}
SR162	Caffeine	1.32 ± 0.70^{a}	0.77 ± 0.11^{ab}	0.77 ± 0.12^{ac}	1.79 ± 0.27^{abc}
	Theobromine	5.45 ± 0.85^{a}	6.47 ± 0.52^{a}	6.42 ± 0.59^{a}	5.97 ± 0.48^{a}
PH16	Caffeine	3.65 ± 0.46^{ac}	3.82 ± 0.01^{a}	2.19 ± 0.15^{b}	3.13 ± 0.15^{c}
	Theobromine	6.50 ± 0.36^{a}	5.62 ± 0.44^{ab}	5.00 ± 0.82^{b}	4.81 ± 0.21^{b}

*The values shown are the average of 2 fermentation replicates and expressed in dry defatted basis. **Same letter in the same line shows no statistical difference ($p \leq 0.05$).

Table 4. Phenolic compounds amounts ($mg.g^{-1}$) in fermented and dried cocoa beans of three cocoa cultivars*.

Phenolic Compound	Cultivars		
	Non-resistant blend	SR-162	PH-16
Galic acid	$0.14 \pm 0.03^{a**}$	0.06 ± 0.01^{b}	0.09 ± 0.02^{ab}
Catechin	1.25 ± 0.00^{a}	1.62 ± 0.16^{b}	0.82 ± 0.17^{c}
Caffeic acid	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.11 ± 0.05^{b}
Epicatechin	4.30 ± 0.34^{a}	1.26 ± 0.15^{b}	2.98 ± 0.51^{ac}

*The values shown are the average of 2 fermentation replicates and expressed in dry defatted basis. *Same letter in the same line shows no statistical difference ($p \leq 0.05$).

Table 5. Methylxantines amounts ($mg.g^{-1}$) in fermented and dried cocoa beans of three cocoa cultivars*.

Methyl-xantine	Cultivars		
	Non-resistant blend	SR-162	PH-16
Caffeine	$2.06 \pm 0.26^{a**}$	1.89 ± 0.21^{a}	3.02 ± 0.14^{b}
Theobromine	6.13 ± 0.34^{a}	5.95 ± 0.02^{a}	5.53 ± 0.25^{a}

*The values shown are the average of 2 fermentation replicates and expressed in dry defatted basis. **Same letter in the same line shows no statistical difference ($p \leq 0.05$).

These values found for methylxantines can be discussed in two points of views: first in technological aspect, since these compounds together with polyphenols are responsible for bitterness and astringency of the chocolate produced (Efraim et al., 2011). The other aspect is the effect of methylxantines in human body, since caffeine is well known for its action in central nervous system promoting a stimulating effect, increasing the sense of allarm, vitality, psychomotor reactions and rising the blood pressure. On the other hand, theobromine has no stimulating effect, because the action of theobromine in the central nervous system is very weak or almost non-existent, but it can act as a vasodilator, a muscle relaxant, diuretic and blood pressure reducer (Van den Bogaard et al., 2010; Mitchell et al., 2011).

4 Conclusions

A reduction in (-) epicatechin was observed for two cultivars (non-resistant and PH16) during fermentation. At the end of the process, the reduction observed was around 50% in these two cultivars and was attributed to (-)epicatechin be the principal substrate of existing polyphenoloxidase. Other hypothesis suggested was due to (-) epicatechinepimerizarion phenomena caused by changes in pH during fermentation process. On the other hand in SR162 showed an increase in (-) epicatechin during fermentation, assuming that the phenolic compounds had lower oxidation in this cultivar than the other cultivars, while had more hydrolytic activity, probably due to the fermentation conditions achieved by this cultivar during fermentation process.

During fermentation, an expressive variation in methylxantines contents was found. These results showed that there was not a pattern for methylxantines behaviour during fermentation process, and it is extremely related to fermentation conditions like temperature and pH during the process.

A huge reduction in phenolic compounds can be observed after drying, attributed mainly to enzymatic browing, followed by non-enzymatic reactions due to quinones polymerization with the increase in pH and high O_2 uptake during sun drying.

Differently of phenolic compounds, methylxantines did not have great modification after sun drying, the amounts of either caffeine or theobromine were almost the same before and after the sun drying process.

Under technological aspect, methylxantines and phenolic compounds are responsible for bitterness and astringency of the chocolate produced. So, the differences observed in this study between cultivars, take to the conclusion that the cocoa cultivars have different behavior during fermentation and drying considering phenolic and methylxantines contents, which consequently, give to these cultivars differences in sensory characteristics.

Acknowledgements

To Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) for the scholarship and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support.

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