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Effect of fermentation time on phenolic content and antioxidant potential in Cupuassu (*Theobroma grandiflorum* (Willd. ex Spreng.) K.Schum.) beans

Efecto del tiempo de fermentación sobre el contenido fenólico y el potencial antioxidante en granos de Copoazú (*Theobroma grandiflorum* (Willd. ex Spreng.) K.Schum.)

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Abstract

Cupuassu (*Theobroma grandiflorum* (Willd. ex Spreng.) K.Schum.) is an evergreen tree in the family Malvaceae, with nutritional qualities of interest in the food and cosmetic industry. It is necessary for its processing, in addition to other processes, to perform a fermentation, affecting its chemical composition. Therefore, the effect of fermentation time on the phenolic content and antioxidant activity of Cupuassu (*T. grandiflorum*) beans, was determined. During this process, the chemical properties of the beans and the phenolic content were evaluated every two days; also quantifying the secondary metabolites Catechin, Epicatechin, Theobromine and Caffeine by high performance liquid chromatography (HPLC). The antioxidant activity was analyzed using the ABTS, DPPH, and FRAP assays. Analysis of phenolic content and antioxidant activity showed a decrease after 6 days of fermentation. Therefore, it is not recommended to continue fermentation after this period due to a negative influence of the process on the bioactive substances (Catechins) content, and the reduction of the ability to inhibit free radicals, exhibited by Cupuassu beans.

Key words: Bromatological analysis, phenols, fermentation, free radicals.

Resumen

El árbol de Copoazú (*Theobroma grandiflorum* (Willd. ex Spreng.) K.Schum.) es una planta de la familia Malvaceae con cualidades nutritivas de interés en la industria de alimentos y cosmética. Para su procesamiento se requiere realizar entre otros procesos el de fermentación que afecta su composicion quimica. Por lo anterior, se determinó el efecto del tiempo de fermentación sobre el contenido fenólico y la actividad antioxidante de los granos de Copoazú (*Theobroma grandiflorum*). Durante este proceso, se evaluaron cada dos días las propiedades químicas del grano y el contenido fenólico; cuantificando también los metabolitos secundarios Catequína, Epicatequina, Teobromina y Cafeína por cromatografía líquida de alta eficiencia (HPLC). La actividad antioxidante se analizó empleando los métodos de ABTS, DPPH, FRAP. Los análisis del contenido fenólico y la actividad antioxidante, evidenciaron una disminución a partir del día 6 de fermentación. Por ello, no se recomienda continuar con la fermentación después de dicho periodo, debido a la influencia negativa que presenta el proceso sobre el contenido de sustancias bioactivas (Catequinas), y a la reducción de la capacidad de inhibir radicales libres, exhibida por parte del grano de Copoazú.

Palabras claves: análisis bromatológico, fenoles, fermentación, radicales libres.

Introduction

Cupuassu (*Theobroma grandiflorum* (Willd. ex Spreng.) K.Schum.) is an evergreen tree in the Malvaceae family, common throughout the Amazon basin, which is found in the wild southern and southeastern part of the eastern Amazon. (Venturieri & Lopes, 1988). Cupuassu fruit is an ellipsoid-oblong drupaceous berry, with rounded ends, and has a woody and breakable epicarp (shell). The pulp (endocarp) enveloping the seed is edible, yellow, creamy and acid taste. The seed contains an aromatic fat similar to cocoa butter (Criollo *et al.*, 2010). In Colombia, this is a little-known fruit, hence its consumption is limited. However, there are institutions, companies and craftsmen who seek to give added value to the food or cosmetic products, taking advantage of the fruit organoleptic potential, in order to offer the consumer a new type of chocolate with similar nutritional qualities to those of commercial Cacao (Criollo *et al.*, 2010).

In some beans such as Cacao and Cupuassu, fermentation is a prerequisite for the generation of flavor precursors (Hue *et al.*, 2016), leading to desirable changes in the chemical and sensory attributes of the product (Rodríguez-Campos *et al.*, 2011). Different chemical reactions take place during this operation, where a great amount of substances are produced. Among them, phenolic compounds, which play an important role in human health through digestive stimulation, anti-inflammatory and antimicrobial effects, and antimutagenic and anticancer activity (Yeo & Ewe, 2015) are highlighted.

Different plant species are potential sources of antioxidant phenolic compounds, including Cacao (Lee *et al.*, 2003) and the promising Cupuassu species (Fantinelli *et al.*, 2017; Galeano, 2011). Research on Cupuassu is scarce and mainly focused on fruit pulp (de Souza *et al.*, 2010). Therefore, the possible health benefits of the chocolate analog, obtained from Cupuassu seeds, have never been reported. Research on Cupuassu beans shows vitamin C and flavonoids content, including 5,7,8,4-tetrahydroxyflavone, catechin, epicatechin, isoscutellarin-8-O-β-D-glucoronide, hypolaetine-8-O-β-D-glucoronide, quercetin, and two new glycosylated flavonoids, theograndin I and II (Yang *et al.*, 2003; Pugliese, 2013). These compounds are characterized by their antioxidant properties, perhaps related as in traditional cocoa, with the organoleptic properties of final product.

Despite having botanical similarities with Cocoa, and being processed the same way at commercial level, Cupuassu beans present differences in the contents of some components, such as linoleic acid, which has a higher concentration in the Cupuassu fat (8.3%), Compared to cocoa (1.1%). Linoleic acid is an essential fatty acid, necessary for the arachidonic acid synthesis. The major precursor for prostaglandins biosynthesis, which are physiologically and pharmacologically active compounds (Pugliese, 2013). In addition, while cocoa fat has a 69.3% of saturated fatty acids, Cupuassu fat has about 48.9% (Pugliese, 2013). Therefore, the aim of this research was to determine the phenolic content and antioxidant activity of Cupuassu beans Cupuassu (*T. glandiflorum*) considering the effect of fermentation time as an important variable on these attributes.

Materials and methods

Collection and processing of plant material

Cupuassu (*T. grandiflorum*) beans were selected from mature fruits from commercial crops, located at the Centro de Investigaciones Macagual Cesar Augusto Estrada Gonzales at the Universidad de la Amazonía, Florencia- Caquetà, Colombia, Located at 1°37' N and 75°36' W, 300 m.a.s.l., with an AF climate according to koppen classification.

Cupuassu collected beans were fermented, according to the method previously used and described by Hernández *et al.* (2006). Raw material was extracted and pooled for subsequent analyses every 48 hours, over a 10 days period. In fact, sample material was grounded and extracted by percolation with methanol. The last day of fermentation, a sample was taken and subsequently dried and roasted (Krysiak, 2006), then it was used to compare itself with fermented Cupuassu beans in the previous days.

Bromatological analysis

Each sample was conducted in triplicate as follows: fermentation index (ICONTEC, 2003), moisture, total acidity, pH, protein content and lipid concentration (AOAC, 1990). Also, the reducing sugars were quantified according to the NTC 440 (ICONTEC, 2004) standard, expressed as glucose percentage.

Phenolic content

Total phenols concentration was determined with the Folin Ciocalteu reagent (Galeano, 2011); and total anthocyanins were determined using the pH differential method proposed by Lee *et al.* (2005). For the flavonoids total quantification, the aluminum chloride colorimetric method (Zhishen *et al.*, 1998), was used.

Antioxidant activity

The free-radical trapping activity of the Cupuassu methanolic extracts of fermented beans, was evaluated using the DPPH assay carrying out absorbance readings at 517 nm (Galeano, 2011). Also, the reaction with the ABTS + radical by the oxidation of ABTS (3.5 mM) with potassium persulfate (1.25 mM), was determined. The absorbance was measured at a wavelength of 732 nm (Galeano, 2011). The DPPH and ABTS + results, were expressed in TEAC (Antioxidant Capacity in trolox equivalents) values, by constructing a standard curve using the antioxidant TROLOX®. The reducing capacity FRAP (Ferric reducing ability of plasma) was measured. For instance, a calibration curve with ascorbic acid was carried out at a wavelength of 590 nm.

Quantification of secondary metabolites

The quantification of (+)-Catechin, (-)-Epicatechin, Theobromine and Caffeine, was performed on a liquid chromatograph (SHIMADZU, LC-2010HT®) equipped with autosampler, an LC-2010CHT pump and a UV detector (calibrated at 280 nm). The separation of (+)-Catechin and (-)-Epicatechin was carried out on a RESTEK-PINNACLE RP-18® (15µm) column. For both, (+)-Catechin and (-)-Epicatechin, the mobile phase Acetonitrile (A) with 3% acetic acid (B) was used, in ratio 90:10. The mobile phase flow rate was 1.0 $ml.min^{-1}$. The peaks identification was compared with (+)-Catechin and (-)-Epicatechin HPLC standards (Fantinelli *et al.*, 2017). For both, Theobromine and Caffeine, methanol (A) and water (B) were used as the mobile phase, in ratio 40:60. The mobile phase flow rate was 0.7 $ml.min^{-1}$. The peaks identification was compared with (+)-Catechin and (-)-Epicatechin HPLC standards (Fantinelli *et al.*, 2017).

Statistical analysis

A randomized split-plot design with three replicates was performed for physical-chemical analysis, and the study on the effect of the fermentation time on the phenolic content and the antioxidant potential in Cupuassu beans. A descriptive statistics test was performed for the results. In fact, Fisher's LSD method was used to compare the mean values of treatments with a significance level of 0.05. Analyses were performed using the InfoStat software ® (Di Rienzo *et al.*, 2017), through a friendly R platform interface, version 3.4.0 (R Development Core Team, 2017).

Results

Bromatological characterization

According to NTC 1252 standard, Cupuassu beans fermentation was not adequate due to the quantity of beans classified as insufficiently fermented, slaty, mouldy and germinated (Table 1).

Table 1. Fermentation index of processed Cupuassu beans

	Fermentation days					
% of beans	**0**	**2**	**4**	**6**	**8**	**10**
Well fermented	0	15	33	64	67	71
insufficiently fermented	100	85	65	26	9	2
Slaty	0	0	0	0	1	3
Mouldy	0	0	0	5	8	9
Germinated	0	0	2	5	15	15

The pH, acidity, moisture, lipids, reducing sugars and protein were different during all fermentation evaluated days (P <0.05) (Table 2).

Table 2. Physico-chemical analysis of the fermented Cupuassu beans

Days	pH*	Acidity* (citric acid %)	Moisture* (%)	Fat* (%)	Reducing Sugar* (%)	Protein* (%)
0	6.14 ±0.01^{e}	0.16±0.01^{e}	61.23±0.65^{a}	19.76±0.20^{a}	0.22±0.00^{a}	0.75±0.02^{a}
2	5.90±0.02^{f}	0.20±0.00^{b}	60.47±0.78^{a}	14.31±0.71^{b}	0.45±0.02^{b}	0.74±0.05^{a}
4	5.21±0.01^{g}	0.27±0.00^{a}	55.72±0.31^{b}	11.95±0.22^{c}	0.59±0.06^{c}	0.73±0.02^{ab}
6	6.72 ±0.01^{d}	0.21±0.00^{b}	51.75±0.18^{c}	9.87±0.26^{d}	0.70±0.02^{d}	0.70±0.03^{bc}
8	6.82 ±0.01^{c}	0.19±0.00^{c}	47.47±0.33^{d}	6.82±0.25^{e}	1.10±0.01^{e}	0.67±0.06^{c}
10	6.94 ±0.01^{b}	0.17±0.00^{d}	46.38±0.15^{e}	6.00±0.48^{f}	1.44±0.03^{f}	0.58±0.00^{d}
FST**	6.98 ±0.02^{a}	0.12±0.00^{f}	39.04±0.67^{f}	3.98±0.21^{g}	1.52±0.05^{g}	0.56±0.01^{d}

*Reported mean values ± standard deviation. Values with different letters within the same column, are significantly different based on the results of the LSD Fisher test (P<0.05).

**FTS: Fermented, dry and toasted bean.

For instance, the influence time had achieved a negative relationship between fermentation and moisture content (R = -0.95, P <0.05), reporting a 7% mean moisture loss per hour (y = 62.21 - 0.07 H r^2 = 0.96 P <0.05), and a similar trend showed by the fat content, which translates in a decreasing of 6% for every hour of fermentation (y = 18.12 - 0.06h r^2 = 0.94 P <0.05). For sugars, there was an increasing of 0.5% per hour (y = 0.17 + 0.005h r^2 = 0.95 P <0.05). Conversely, there was a quadratic decreasing trend for protein content (y = 0.74 + 0.0002h - 0.000004h r^2 = 0.88 P <0.05).

Phenolic content

Differences in total phenol content, total anthocyanins, total flavonoids, catechin and epicatechin, were found among Cupuassu beans submitted to different fermentation times (P <0.05) (Table 3). The total phenols content varied between 190 and 1030 mg.g^{-1}, showing the highest peak on day 6. The anthocyanins concentration in fermented Cupuassu beans decreased as a function of the fermentation hours (P <0.05).

Table 3. Phenolic content of fermented Cupuassu beans

Days	Phenols Total* (mg.g^{-1})	Anthocyanins Total* (mg.g^{-1})	Flavonoids Total* (mg.g^{-1})	Catechin* (mg.g^{-1})	Epicatechin* (mg.g^{-1})
0	840.14±8.50*	12.64±0.39*	363.73±3.76^a	10.06±0.11^a	5.74±5.83^a
2	923.99±25.67^b	10.69±0.29^b	296.70±8.37^b	9.28±0.26^b	3.74±3.56^b
4	950.14±27.53^b	1.45±0.19^c	214.17±6.37^c	8.8±0.34^c	3.07±3.05^c
6	1030.13±8.69^c	0.78±0.10^d	200.43±2.70^d	7.83±0.08^d	2.80±3.01^c
8	386.50±1.04^d	0.50±0.00^{de}	86.73±0.31^e	5.40±0.14^e	2.75±2.84^c
10	325.46±2.02^e	0.33±0.00^e	74.90±0.42^f	3.58±0.05^f	2.72±2.57^c
FST**	190.70±0.57^f	0.17±0.00^e	62.40±0.30^g	2.25±0.04^g	1.26±1.29^d

* Reported mean values ± standard deviation. Values with different letters within the same column, are significantly different based on the results of the LSD Fisher test (P<0.05).

**FTS: Fermented, dry and toasted Cupuassu beans.

Antioxidant activity

The entrapment of the DPPH radical was found in a range between 1687.72 μmol Trolox g^{-1} and 564.10 μmol Trolox g^{-1}, showing a higher increase in antioxidant activity on day 6 (Table 4).

Table 4. Antioxidant activity of fermented Cupuassu beans

Days	DPPH*[1] (μmol Trolox.g^{-1})	ABTS*[2] (μmol Trolox.g^{-1})	FRAP*[3] (μmol aa.g^{-1})
0	1438.17±13.00^a	925.45±9.98*	624.67±6.49^a
2	1495.92±41.24^b	949.66±25.52*	747.67±19.44^b
4	1571.50±48.78^c	996.66±20.96^b	790.33±24.34^c
6	1687.72±10.40^d	1096.10±4.13^c	818.67±5.59^d
8	1152.31±7.22^e	991.32±10.81^b	518.67±1.22^e
10	736.08±3.93^f	691.79±5.01^d	363.00±2.62^f
FST**	564.10±0.45^g	487.58±7.45^e	117.33±0.37^g

* Reported mean values ± standard deviation. Values with different letters within the same column, are significantly different based on the results of the LSD Fisher test (P<0.05).

**FTS: Fermented, dry and toasted bean.

[1]DPPH: 2,2-diphenyl-1-picrylhydrazyl radical

[2]ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical

[3]FRAP: Ferric reducing ability of plasma

It is known that the secondary metabolites formation, which are precursors of the organoleptic characteristics, have allowed a decreasing in the antioxidant characteristic in the later fermentation days. Therefore, radical ABTS +, data varied with a range between 1096.10 and 487.58 μmol Trolox g^{-1}, respectively. Alternatively, DPPH radical, showed the highest value, which was reported on day 6 of fermentation; followed by a decreasing in the ABTS + radical trapping activity. Similarly, a decreasing in the extracts power was reported and ranged from 117.33 to 818.67 μmol aa.g^{-1}, respectively. Nevertheless, the highest decreasing power was obtained on day 6 of fermentation.

Methylxanthines content

The Theobromine and Caffeine content remained high during the first days of processing, even the lowest values of these alkaloids were present after 6th day of the processing (Table 5).

Table 5. Theobromine and Caffeine contents of fermented Cupuassu beans

Days	Theobromine* (mg.g^{-1})	Caffeine* (mg.g^{-1})
0	4.25±0.07^a	4.72±0.07^a
2	3.96±0.12^b	4.64±0.16^a
4	3.38±0.11^c	4.36±0.09^b
6	3.33±0.04^c	4.16±0.12^c
8	1.52±0.01^d	1.78±0.04^d
10	1.33±0.01^e	1.49±0.02^e
FST**	1.01±0.00^f	1.13±0.01^f

* Reported mean values ± standard deviation. Values with different letters within the same column, are significantly different based on the results of the LSD Fisher test (P<0.05).

**FTS: Fermented, dry and toasted Cupuassu beans.

Discussion

Bromatological characterization

According to results obtained from Cupuassu beans classification, fermentation process at second day was not adequate, due to the large amount of pulp covering the bean and the absence of an optimal temperature (44-47°C) (Criollo *et al.*, 2010). As the fermentation process progressed, the quantity of well fermented Cupuassu beans increased, exceeding the required minimum value by the standard between 6th and 10th day of processing. In fact, pH decreased after day 4, due to the cotyledon pulp sugars, suffered a conversion into acetic acid throughout microorganism action such as *Acetobacter rancens* and *Acetobacter melanogenum* (Camu *et al.*, 2008). This increasing in hydrogen-Ion potential in the cotyledon is also related to acidity, due to the increase of citric acid after day 6. During fermentation process, the acetic and lactic acids generated by microbial action in the pulp are diffused towards the cotyledon, increasing its acidity (Camu *et al.*, 2008). Similar results have been reported by other authors, where Cupuassu fermentation processes were carried out (Fantinelli *et al.*, 2017).

It was observed that the moisture loss during the monitoring period, is possibly due to the microbial action, which causes the released juices and aqueous exudates (Camu *et al.*, 2008), a decreasing in fat content is due to lipid oxidation and the oxygen action involved in the process.

Sensory attributes such as flavor and aroma, depend, among others, on the decreasing sugars content, due to the transformation of these compounds into glucose and fructose throughout the hydrolysis process (Cervantes *et al.*, 2011). Due to the invertase enzyme action, there was a sugar increasing of 0.5% per hour ($y = 0.17 + 0.005h$ $r^2=0.95$ $P<0.05$) (Rodríguez *et al.,* 2011). Changes were also found in the nitrogenous contents, favored by the Theobromine loss and proteins degradation in amino acids and peptides, under the action of proteolytic enzymes (Fantinelli *et al.*, 2017). These results are comparable in variability to the report by Hue (2016). There was a quadratic decreasing trend of the protein content ($y = 0.74 + 0.0002h - 0.000004h$ $r^2=0.88$ $P<0.05$).

Phenolic content

During the fermentation process, the anthocyanins present in the cotyledon are hydrolyzed by the glycosidases action, which can lead to a loss of its purple color. These enzymes mainly produce sugars (galactose and arabinose), and aglycone, which causes a reduction in total anthocyanin content (Wollgast & Anklam, 2000). Therefore, secondary metabolites, specifically those involved in phenolic constitution, are linked to the antioxidant activity of the species containing them (Galeano, 2011). It was found that during fermentation process, the loss of most of the bean polyphenols, was similar to that reported by Zapata *et al.,* (2013). This, due to the liquids reduction, caused by the fermentation process (Wollgast & Anklam, 2000). Another mechanism of polyphenols loss, is the action of the polyphenoloxidase enzyme, EC 1.14.18.1, which is responsible for catalyzing the polyphenols oxidation to high-molecular-weight condensed polyphenols (Weisburger, 2001).

When comparing the results with the toasted sample, it was found that the phenolic content was lower, due to bean exposure to high temperatures (100°C) for a three days of fermentation period. These results are comparable in variability to the report by Krysiak, (2006), who controlled the mass changes to achieve an optimum bean toasting. Similar results have been reported by Arlorio *et al.* (2008), in relation to the drying and toasting incidence on the clovamide content in *Theobroma* cacao L. beans.

The researches of Arlorio *et al.* (2008), and Zapata *et al.* (2013), report the influence of some biotic and abiotic factors on the phenolic content of cacao as follows: physiological maturity of fruit, crop origin (conventional and ecological), fertilization scheme, soil fertility and genotype, respectively. In fact, through processing steps, operations or technologies, these aspects have allowed an increasing presence of desirable secondary metabolites in the matrix.

Antioxidant activity

The results of antioxidant activity presented for Cupuassu beans are consistent with those reported on fermentation of *Theobroma cacao* L. clones, in which the fermentation time, in addition to the genetic variety, influence the secondary metabolites production and a decreasing ability of cocoa beans (Zapata *et al.*, 2013). However, an increasing in the antioxidant activity is related to an increasing of some secondary metabolites. For example, sensorial attributes depend mainly on the tannins formation, which are responsible for the positive growth of the antioxidant activity as reported by Baba *et al.* (2007) and Camu (2008). In turn, the bioavailability of phytochemicals may be influenced by the type of processing performed in Cupuassu beans. During the fermentation process, the same phenomenon does not occur compared to what is occurring within the intestine, where food is further degraded by microorganisms present in the digestive system, in order to release the bioactive components and to take advantage of their nutritional content (Yeo & Ewe, 2015).

Methylxanthines content

It is important to note a decreasing after day 6 of processing, which is due to Theobromine and Caffeine diffusion content with the cellular liquids, which are released by exudation during the fermentation period (Brunetto *et al.*, 2007). Theobromine, Caffeine and its complexes are the major components of bitter taste of cocoa, which tend to diffuse out of the bean during fermentation. Therefore, it is important to ensure the fermentation initial conditions and to avoid acidity excess in the process, thus protecting the organoleptic characteristics of the processed bean, which is finally used for the elaboration of food products such as chocolate or confectionery (Othman *et al.*, 2005). The above statement is valid for Cupuassu, since the way in which is processed, and the presence of these common nitrogen compounds on both cocoas, have allowed the fermentation conditions similarly to the influence on these species belonging to the genus Theobroma (Camu *et al.*, 2008; Galeano, 2011).

Conclusion

The fermentation time directly influences the production, conservation and concentration of phenolic compounds in Cupuassu beans. This process promotes the appearance of different biochemical reactions occuring inside the cotyledon; some of the more significant aspects are as follows: acids production, anthocyanins degradation by the action of glycosidases, the polyphenoloxidases action in the conversion of polyphenols to quinones, respectively. Given these concerns, the formation of insoluble tannins, among others, are fundamental reactions for the production of compounds responsible for the flavor in the fully processed seed, which is used at industrial level for the production of commercial derivatives such as pastes, shortenings and liquor. The reduction of secondary metabolites, such as phenols, anthocyanins, flavonoids, epicatechin and catechin, is considerably affected by the fermentation time, which highlights the instability, loss and variation of these biocomposites during the process. Cupuassu is a food with high potential for inhibition of free radicals due to its high antioxidant ability.

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