Influence of drying methods on cocoa (*Theobroma cacao* L.): antioxidant activity and presence of ochratoxin A

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Abstract
This work aimed to evaluate the performance of different drying methods in the development of phenolic compounds, methylxanthines, and antioxidant activity in cocoa bean and to evaluate the presence of ochratoxin A (OTA). Antioxidant activity was determined using DPPH, FRAP, ORAC and β-carotene-linoleic acid assays. Phenolic compounds and methylxanthines were determined using high-performance liquid chromatography with an ultraviolet detector (HPLC-UV). OTA was determined by ultra-performance liquid chromatography with fluorescence detection (UPLC-FLR). It was found that during cocoa drying, there was a reduction in antioxidant activity with reduced phenolic compound content (0.04 mg g⁻¹ of catechin before drying to 0.02 mg g⁻¹ after drying) and reduced methylxanthines (19.44 mg g⁻¹ of theobromine before drying to 11.71 mg g⁻¹ after drying). Only one sample showed contamination by OTA (7.1 μg kg⁻¹). The traditional drying method showed the highest conservation of antioxidant activity and methylxanthine and phenolic compound content.

Keywords: catechin; epicatechin; caffeine; theobromine; mycotoxin.


1 Introduction
Cocoa has a characteristic and complex chemical composition. Chocolate is its principal product and is generally consumed for its sensory qualities. It has been recognized however, as a source of bioactive compounds with potential health benefits.

In cocoa preprocessing after harvest, cocoa beans are fermented and then dried to reduce moisture content and water activity. The drying process interferes with biochemical reactions initiated during fermentation, leading to a reduction in the bitterness, astringency, and acidity of cocoa beans. Darkening cotyledons contribute to the formation of precursors for desirable flavor in the final product (Beckett, 2009).

Drying may be carried out naturally using solar or artificial energy in greenhouses. Drying conditions can promote reactions that form new compounds that are responsible for the development of the distinctive chocolate color. There is evidence that during drying, there is a reduction in polyphenol content due to enzymatic browning caused by a polyphenoloxidase reaction, followed by non-enzymatic browning resulting from the quinone polymerization due to the accumulation of insoluble compounds.

Drying is also responsible for reducing the acidity of cocoa beans and should be conducted in such a way as to obtain a moisture content of approximately 7%. Excessive drying can make the shell brittle, whereas excess moisture favors fungal development. The number of phenolic compounds present in cocoa beans and chocolate depends not only on genetic characteristics but also on other factors, such as climate, soil properties, growing region, and cocoa processing (Jalil & Ismail, 2008). Kyi et al. (2005) determined the oxidation reaction kinetics of polyphenols in cocoa beans during drying at temperatures varying between 40 and 60 °C and moisture content between 50 and 80%. The authors found that the higher the temperature and moisture content, the higher the oxidation of polyphenols in cocoa.

Ochratoxins are secondary metabolites produced by fungi from the genera *Aspergillus* and *Penicillium*, usually present in cereals, coffee, grapes, peppers, and cocoa (Amézqueta et al., 2009; Duarte et al., 2010). Ochratoxin A (OTA) in tropical climates is produced by *Aspergillus ochraceus* and *Aspergillus* from the Nigri group, which includes *Aspergillus carbonarius* (Kara et al., 2015).

OTA is known for its carcinogenic, nephrotoxic, teratogenic, genotoxic, and immunotoxic properties in animal cells (International Agency for Research on Cancer, 1993). Humans are more sensitive to its nephrotoxic effects. Classified as Group 2B (possible human carcinogen) by the International Agency for Research on Cancer (1993), OTA has been found in cocoa powder coming from the Ivory Coast, Guinea, Nigeria, and Cameroon (Serra Bonvehí, 2014).
2.1 Materials

It was used a blend of cocoa fruits (*Theobroma cacao* L.) Forastero from a farm in the south of Bahia located in the Northeast of Brazil (14° 41' 96" S and 39° 12' 109" W) that has a tropical climate. The seeds were derived from natural fermentation and were subjected, without light, to reaction with 3.9 mL of the DPPH radical (0.06 mM) and homogenized in a shaker tube. The radical reduction was measured by absorbance continuously monitored at 515 nm against the reduction in the blank using a UV spectrophotometer for 30 min at room temperature in the dark. The values were calculated using a linear regression, and the results are presented as averages. A reduction of absorbance in the sample ($A_{red}$) was calculated in comparison with a reduction of control absorbance ($A_{blank}$) and expressed as a free radical seizure percentage (% FRS) according to the Equation 1 below:

$$\% \text{FRS} = \left(\frac{A_{blank} - A_{red}}{A_{blank}}\right) \times 100$$  \hspace{1cm} (1)

### Ferric Reducing Antioxidant Power (FRAP)

The FRAP method was used to evaluate reduction power based on the method described by Pulido et al. (2000) with some modifications. FRAP reagent was prepared as follows: 2.5 mL of TPTZ (2,4,6-Tripyridyl-s-Triazine) solution (10 mM) in 40 mM HCl added to 2.5 mL of FeCl$_3$·6H$_2$O and 25 mL of acetate buffer (0.3 mM pH 3.6). The solution was incubated at 37 °C for 30 minutes. For antioxidant activity evaluation, 2.7 mL of the FRAP reagent was mixed with 270 μL of distilled water and 90 μL of sample or standard. Samples and standards were homogenized and incubated at 37 °C for 30 minutes, and the reading was performed at 595 nm on the spectrophotometer. Ferrous sulfate solution was used as a standard. Results are expressed as μM ferrous sulfate·g$^{-1}$ of the sample.

### Oxygen Radical Absorbance Capacity (ORAC)

An ORAC assay was carried out according to the method described in Prior et al. (2005). The fluorescein and AAPH (2,2’-azobis (2-methypropionamidine) dihydrocholoride) solutions and Trolox standard were prepared on the day of analysis. A microplate reader was used (absorbance and fluorescence) with 96-well plates. Analysis was performed in phosphate buffer (pH 7.4) at 37 °C. Fluorescein was used as the substrate. The protective antioxidant effect was verified by calculating the area under the curve (AUC) of the sample fluorescence when undergoing oxidative damage versus time compared to the blank. Fluorescence conditions...
were as follows: excitation at 485 nm and emission at 520 nm. Results are expressed as mM of Trolox equivalent g\(^{-1}\) of sample.

β-carotene–linoleic acid system

A β-carotene linoleic acid assay was carried out according to the method of Duarte-Almeida et al. (2006). To prepare the reactive mixture, 20 μL of linoleic acid, 200 mg of Tween 40, 25 μL of β-carotene solution (2.0 mg.mL\(^{-1}\) in chloroform) and 500 μL of chloroform were added to an Erlenmeyer flask. Subsequently, the mixture was subjected to complete evaporation of the chloroform, and 25 mL of water saturated with oxygen was added to the mixture with the chloroform evaporated. To the test tube, 250 μL of reaction mixture and 10 μL of extracts or methanol (control sample) were added. The tubes were heated at 50 °C for approximately 2 minutes in order to accelerate oxidation reactions and initiate β-carotene bleaching after cooling. Readings were taken for initial absorbance and final absorbance (after two hours) in a spectrophotometer at 470 nm. The sample absorbance decrease (SAD) and the control absorbance (Ca) were used to yield the percentage of oxidation inhibition (%I) by the Equation 2:

\[
%I = \left(\frac{(Ca - SAD)}{Ca}\right) \times 100
\]  

Monomeric phenols and methylxanthines determination

Ten microliters of each sample were analyzed with a high-performance liquid chromatography (HPLC) system (Perkin Elmer Model Flexar) equipped with an injector Flow VI and a C-18 column (250 × 4.6 millimeters / 5 μ). The column was maintained at 30 °C, and the wavelength used for detection was 280 nm. The standards (Sigma Chemical Co., USA) used for this study were phenolic compounds (Catechin and Epicatechin) and methylxanthines (Theobromine and Caffeine). The standard solutions were prepared in methanol, and calibration curves were obtained from five injections in duplicates of five concentrations. Elution was carried out along the gradient as shown in Table 1, wherein the mobile phases used were water acidified with 1% phosphoric acid (A) and methanol (B). The total run time was 75 minutes.

Determination of ochratoxin A

An Acquity ultra-performance liquid chromatography (UPLC) system (Waters\textsuperscript{®}, USA) was used with fluorescence detection at 335 nm excitation and 478 nm emission for detection of ochratoxin A. The system consists of a temperature-controlled auto sampler set at 10 °C, a binary pump, oven temperature controlled at 40 °C and an online degassing system. An Ethylene Bridged Hybrid (BEH, Waters\textsuperscript{®}) column of 10 cm with a portion of 1.7 μM was used. The mobile phase consisted of water acidified with phosphoric acid (H\(_3\)PO\(_4\)) pH 2: acetonitrile (40:60 v.v\(^{-1}\)), isocratic run, with a 3 μL injection volume and flow of 0.3 ml.min\(^{-1}\).

Statistical analyses

The results were statistically analyzed using univariate analysis of variance (ANOVA) and Tukey’s mean test (p <0.05) using SAS version 6.11.

3 Results and discussion

After fermentation, 52.18% of the cocoa bean total weight was moisture. The sample showing the lowest moisture content after drying was the sample subjected to the oven drying process (6.90%). The next lowest moisture content was found in the sample subjected to the traditional drying method (7.76%), followed by the samples dried using the stainless steel dryer with plastic roof, with UV protection (8.08%) and without UV protection (8.03%).

We may be able to explain these results owing to temperature differences experienced by the cocoa beans from the different drying methods. Artificial drying in the oven produced the highest temperature, followed by drying with direct sun light. Dryers with plastic roofs had the mildest temperatures. Were monitored the temperatures with thermometer and the drying that depended on solar light varied quite the temperatures but never exceeding 60 °C, temperature of the oven. All samples were considered acceptable for dry cocoa beans. Drying should be carried out until cocoa beans reach 7 to 8% water content, which requires 2-10 days depending on the method used or climatic conditions.

Figure 1 shows the chromatogram of phenolic compounds and methylxanthines standards, theobromine, (+) - catechin, caffeine, and (-) - epicatechin, respectively, and Figure 2 shows the OTA A standard chromatogram.

The chromatograms showed good resolution without the presence of interference peaks. The retention times for the analytes in the samples were similar to the peaks for the standard solution. The identification of the interesting analytes occurred by analysis of retention time compared to the respective standards and spectra.

Phenolic composition and methylxanthine composition in dry and defatted cocoa extracts before and after drying and presence of OTA (n of the sample, positive sample, and range) are shown on Table 2.

Results showed higher epicatechin content than catechin and higher theobromine content than caffeine, as was also reported by Leite et al. (2013) who studied cocoa mass and Carrillo et al. (2014) who studied sun-dried cocoa beans.

Cocoa beans prior to drying showed higher theobromine (19.44 ± 0.34 mg.g\(^{-1}\)) and caffeine (2.74 ± 0.03 mg.g\(^{-1}\)) content

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL.min(^{-1}))</th>
<th>Mobile phases A (%)</th>
<th>Mobile phases B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>0.6</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>0.6</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>30</td>
<td>0.6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>45</td>
<td>0.6</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>55</td>
<td>0.6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>75</td>
<td>0.6</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Gradient utilized for separating phenolic compounds and methylxanthines by HPLC.
than cocoa beans after drying, regardless of the dryer used, confirming that there is a methylxanthine content reduction during drying. The findings of this study were similar to those of Belscak et al. (2009), who found theobromine (16 mg·g⁻¹) and caffeine (2 mg·g⁻¹) in the dry cocoa analysis.

Theobromine levels were higher than those for caffeine in all samples. This fact is very important from a health perspective because caffeine acts as a stimulant and vasoconstrictor, whereas theobromine acts as a vasodilator and lowers blood pressure. Furthermore, theobromine acts as a muscle relaxant and diuretic (Van den Bogaard et al., 2010).

The traditional drying method was the one that most retained the methylxanthine content in dry seeds, showing that these chemical compounds are susceptible to degradation due to drying conditions.
Table 2. Phenolic compounds, methylxanthines, antioxidant activity and presence of ochratoxin A in cocoa subjected to different drying methods.

<table>
<thead>
<tr>
<th>Dryers</th>
<th>Bioactive Compounds</th>
<th>Antioxidant Activity</th>
<th>Mycotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenolics (mg.g⁻¹)</td>
<td>Methylxanthines (mg.g⁻¹)</td>
<td>Methods</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>Epicatechin</td>
<td>Caffeine</td>
</tr>
<tr>
<td>BD</td>
<td>0.04 ± 0.00 ²</td>
<td>1.05 ± 0.05 ³</td>
<td>2.74 ± 0.03 ³</td>
</tr>
<tr>
<td>DP</td>
<td>0.02 ± 0.00 ³</td>
<td>0.90 ± 0.05 ²</td>
<td>1.60 ± 0.06 ³</td>
</tr>
<tr>
<td>AD</td>
<td>0.021 ± 0.00 ³</td>
<td>0.635 ± 0.00 ³</td>
<td>1.61 ± 0.06 ³</td>
</tr>
<tr>
<td>TD</td>
<td>0.037 ± 0.00 ³</td>
<td>1.037 ± 0.02 ³</td>
<td>2.33 ± 0.02 ³</td>
</tr>
<tr>
<td>MD</td>
<td>0.020 ± 0.00 ³</td>
<td>0.756 ± 0.03 ³</td>
<td>1.81 ± 0.04 ³</td>
</tr>
</tbody>
</table>

BD: before drying; DP: Dryer with stainless steel platform and plastic roof with UV protection; AD: Artificial dryer using wooden platform with artificial heat source; TD: Traditional dryer in barge with wooden platform and direct sun light; MD: Mixed dryer with stainless steel platform and mobile plastic roof with UV protection for drying coverage and exposure to the sun; LOD: Limit of quantification; OTA: Ochratoxin; *Same letters in columns represent no significant differences between the samples by Tukey’s test (p <0.05).
to temperature variation. Changes may occur in polyphenol and methylxanthine content during cocoa and chocolate processing, particularly when high temperatures are reached (Kyi et al., 2005). Drying conditions can promote oxidative reactions that contribute to reactions that form new compounds, resulting in a decrease in sensory performance and the development of the “brown” color of chocolate. Depending on the adopted process, there is a reduction of methylxanthine and phenolic compounds during drying (Brito et al., 2000; Payne et al., 2010).

Epicatechin content was higher than catechin in all examined extracts. The values found in this study were similar to those of Belscak et al. (2009), who found 1.06 ± 0.16 mg·g⁻¹ of epicatechin and 0.38 ± 0.10 mg·g⁻¹ of catechin in cocoa mass.

Among cocoa bean drying methods, we found traditional drying to be the one that best retained phenolic compound content, showing no difference at 0.05% between epicatechin and catechin content before and after drying.

With the other drying methods, there was a reduction in phenolic compound content in the cocoa beans, especially when artificially oven-dried. These values were consistent with those found by Belscak et al. (2009) in cocoa powder extracts.

In traditional drying, with barge and direct sunlight, cocoa beans are exposed to heat, but the maximum temperature is cooler compared to covered and artificial dryers. In sun-dried samples, it is likely that cocoa beans have suffered less structural changes, with a limited effect on chemical and enzymatic reactions.

The tendency for reduction of monomers during drying was also observed in other epicatechin studies, such as those conducted by Kim & Keeney (1984), Kealey et al. (1998) and Camu et al. (2008).

In a study by Di Mattia et al. (2012), drying time and temperature affected epicatechin and catechin content. The authors concluded that this phenomenon may be due to rapid drying initially favoring condensation reactions. Other studies have shown that the drying rate is the most important factor in the preservation of a plant’s phenolic compound content (Mrkic et al., 2006).

There is little information in the literature regarding fermentation and drying effects on the procyanidin profile, on the polymerization of monomers to oligomers and polymers, and on some functional properties, such as antioxidant activity reduction (Ailkpokpodion & Dongo, 2010). However, the health aspects of these compounds should be considered because monomeric phenolic compounds are absorbed and utilized by the human body as exogenous antioxidants (Richelle et al., 1999; Holt et al., 2002).

A reduction in antioxidant activity was observed in cocoa beans after drying in all analysis methods. These results confirm those found by Efrain et al. (2010), who showed that there was antioxidant activity loss during cocoa bean drying.

Based on the DPPH (hydrazyl 2,2-diphenyl-1-picryl) method, which is an indirect method, there was a decrease in antioxidant activity in the sample after drying. There was no significant difference in dry cocoa bean antioxidant activity according to drying method, ranging from 94.09% of dry sample free radical scavenging (FRS) in the stainless steel dryer with plastic roof and UV protection to 94.35% of dry sample FRS using the traditional method. The value found for antioxidant activity in dried samples was similar to what Kowalska & Sidorczuk (2007) found when dry cocoa beans from the Ivory Coast were analyzed.

In the analysis using the FRAP method, in which the Fe²⁺ - TPTZ complex formation was spectrophotometrically monitored, 395.48 ± 0.46 μmol Fe²⁺·g⁻¹ was detected in the cocoa beans prior to drying. This antioxidant activity was reduced in all samples after drying, regardless of the methodology adopted. It was found that the sample that underwent the drying process in an oven suffered the most significant reduction in antioxidant activity, 371.78 ± 2.48 μmol Fe²⁺·g⁻¹, and the sample with the smallest reduction in antioxidant activity was the one dried using direct sun light, 378.15 ± 2.0707 μmol Fe²⁺·g⁻¹. Analysis by the FRAP method has the limitation that the system should be aqueous (Moon & Shibamoto, 2009).

In ORAC analysis, cocoa bean scavenging ability was checked in conjunction with a peroxyl radical induced by AAPH at 37 °C where the peroxyl radical reacted with fluorescein, forming a non-fluorescent product (Prior et al., 2005). Extracts rapidly reacted with radicals, donating hydrogen atoms and inhibiting loss of fluorescence intensity, forming a competition system. Fluorescein has high photostability and does not interact with antioxidants (Prior et al., 2005).

This analysis showed that there was no significant difference between types of dryers in conserving antioxidant activity, ranging from 617.88 ± 72.0 for the wooden dryer with direct sun drying (Traditional) to 570.06 ± 51.0 in the stainless steel dryer with plastic roof and UV protection. In this method, antioxidant activity reductions during cocoa drying were confirmed, with significantly lower values found in all cocoa beans after drying compared to the values recorded in the sample prior to drying, 736.41 ± 38.4.

The results of this study resemble those of Crozier et al. (2011) who analyzed cocoa mass, and antioxidant activity measured with the ORAC method was 634 ± 33 μMTE·g⁻¹. Similar values were found by Gu et al. (2006), ranging from 709 to 899 μMTE·g⁻¹, and by Miller et al. (2006), ranging from 720 to 875 μMTE·g⁻¹, in cocoa mass analysis.

In the β-carotene-linoleic acid method, cocoa bean extracts dried using the traditional method were the most protected from oxidation emulsion, 32.71 ± 0.09%. Samples artificially dried in an oven were the least likely to have contributed to system oxidation, and had lower antioxidant activity, 32.21 ± 0.09%. Extracts showed that they had compounds capable of interacting at the water/lipid interface where the oxidative process begins.

Othman et al. (2007) compared the antioxidant activity of cocoa beans from various producing countries and obtained values ranging from 26.1 ± 1.8% and 74.1 ± 2.5% for alcoholic extracts (ethanol). Thus, the results were consistent with those found in this study.

The study results demonstrate that antioxidant activity is not only related to monomeric phenol concentration, given that traditionally dried extract samples showed no significant...
differences with other samples in terms of catechin and epicatechin concentrations before drying. However, with all methods, cocoa bean antioxidant activity before drying was higher compared to that in dried samples.

Of the 32 samples examined, only one showed natural contamination by ochratoxin A (7.1 μg.kg$^{-1}$). The European Community in the EC Regulation No. 1881/2006 of the Commission of 19/Dec/2006 does not establish OTA limits for cocoa. The National Health Surveillance Agency of Brazil (ANVISA) establishes a value of 10 μg.kg$^{-1}$ of OTA in cocoa. The only positive sample showed a value lower than that established by ANVISA.

4 Conclusion

During cocoa drying, there is a reduction in antioxidant properties. This loss is not only related to phenolic compounds and methylyanthine content but is also related to interactions between bioactive compounds, thus decreasing their properties. The traditional drying method showed the best retention of cocoa antioxidant activity, possibly due to it being a slower drying method that uses milder temperatures. As only one sample of cocoa had OTA contents, it was not possible to infer the influence of the dryers on the occurrence of this mycotoxin.

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Belscak, A., Komes, D. E., Horzic, D., Ganic, K. K., & Karlovic, D. (2010). Effect of fermentation and drying method that uses milder temperatures. As only one sample of cocoa had OTA contents, it was not possible to infer the influence of the dryers on the occurrence of this mycotoxin.

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