



# Quality Parameters, Caffeine and Theobromine Contents and Antioxidant Activity of Artisan and Commercial Chocolate from Brazil

Maria Cristina Marcucci<sup>1\*</sup>, Andiara Gonçalves Ferreira da Silva<sup>2</sup>, Carolina Passarelli Gonçalves<sup>2</sup>, Alexandra Christine Helena Frankland Sawaya<sup>3</sup>, Roberta Caroline Bruschi Alonso<sup>4</sup>, Marciano Marques de Oliveira<sup>5</sup>, Douglas Fernandes Barbin<sup>5</sup>

<sup>1</sup>Institute of Science and Technology, UNESP, São Jose dos Campos, São Paulo, Brazil

<sup>2</sup>Postgraduate Program in Pharmacy and Biotechnology Anhanguera University of São Paulo, Pirituba, Avenida Raimundo Pereira de Magalhães, Pirituba, São Paulo, SP, Brazil

<sup>3</sup>Department of Plant Biology and Faculty of Pharmaceutical Science, State University of Campinas, UNICAMP, Campinas, SP, Brazil

<sup>4</sup>Metropolitan University of Santos, UNIMES, Santos, SP and Nucleus of Technological Research (NPT), University of Mogi das Cruzes, UMC, SP, Brazil

<sup>5</sup>Faculty of Food Engineering, State University of Campinas, UNICAMP, Campinas, SP, Brazil

Email: \*cris.marcucci@yahoo.com.br

**How to cite this paper:** Marcucci, M.C., da Silva, A.G.F., Gonçalves, C.P., Sawaya, A.C.H.F., Alonso, R.C.B., de Oliveira, M.M. and Barbin, D.F. (2021) Quality Parameters, Caffeine and Theobromine Contents and Antioxidant Activity of Artisan and Commercial Chocolate from Brazil. *Open Access Library Journal*, 8: e7377.

<https://doi.org/10.4236/oalib.1107377>

**Received:** April 1, 2021

**Accepted:** May 28, 2021

**Published:** May 31, 2021

Copyright © 2021 by author(s) and Open Access Library Inc.

This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

## Abstract

Several types of chocolate were analysed: bitter, half-bitter, milk chocolate, 70% cocoa, white chocolate and cocoa seeds. Analysis of fat, insoluble solids, total phenols, theobromine and caffeine contents and antioxidant capacity were carried out to evaluate the quality of artisan and commercial chocolate products in Brazil. Fat contents and insoluble residues were similar to those reported in the literature. We develop a simple method by HPLC-DAD (High-Performance Liquid Chromatography with Diode-Array Detection) to quantify methylxanthines whose identification has been confirmed by UPLC-MS (Ultra performance liquid chromatography—mass spectrometer). The levels of phenols, caffeine and theobromine varied between types. White chocolate had no phenolics nor caffeine or theobromine. The ones with the highest phenolic contents were those with the highest cocoa mass content. The same happened with the antioxidant activity, which is correlated to the amount of phenolics, caffeine and theobromine, shown by the statistical analysis. It is the presence of cocoa that determines the antioxidant activity of chocolates. This work contributed to the confirmation of the properties of the chocolates, highlighting the importance of the artisan product. Chocolates are important for human health and are considered functional foods.

---

## Subject Areas

Food Science & Technology

## Keywords

Artisan and Commercial Chocolates, HPLC/UPLC, Theobromine, Caffeine, Antioxidant Activity

---

## 1. Introduction

Chocolate is a solid dispersion of cocoa seeds (*Theobroma cacao*), sugar particles, additives, cocoa butter, lecithin and flavourings, giving an average of 568 kcal/100g (Leite, 2012 [1]; Godočíková *et al.*, 2019 [2]). The Mayan and Aztec people, in South America, were the first to document its use. Chocolate and cocoa seeds have several medicinal applications, such as: stimulating the nervous system, the stomach and kidneys activity, treating fever, tuberculosis, treating low virility and anaemia, against mental fatigue, including cardiovascular benefits, among others (Efraim *et al.*, 2011 [3]; Vicentim & Marcellino, 2012 [4]; Kerimi & Williamson, 2015 [5]; Magrone *et al.*, 2017 [6]; Anwar *et al.*, 2018 [7]; Kardum & Glibetic, 2018 [8]; Stanley *et al.*, 2018 [9]; Coutinho *et al.*, 2019 [10]; Godočíková *et al.*, 2020 [11]). Chocolate is known to contain polyphenols and flavonoids (Counet *et al.*, 2006 [12]; Alvarez-Barreto *et al.*, 2018 [13]; Muhammad *et al.*, 2018 [14]) and these compounds are in higher concentrations in bitter chocolate, presenting greater health benefits and antioxidant activity (Lee *et al.*, 2003 [15]; Hu *et al.*, 2016 [16]; Mudenuiti *et al.*, 2018 [17]; Tafurt *et al.*, 2021 [18]).

Chocolate is a good source of polyphenols (Torres-Moreno *et al.*, 2012 [19]; Hu *et al.*, 2016 [16]) such as epicatechin and catechin, along with the proanthocyanin oligomers (Gu *et al.*, 2004 [20]; Gu *et al.*, 2006 [21]; Vinson *et al.*, 2006 [22]; De Oliveira & Genovese, 2013 [23]; Cambrai *et al.*, 2017 [24]; Martini *et al.*, 2018 [25]; Fayeulle *et al.*, 2019 [26]) and alkaloids, such as theobromine and phenethylamine (Gopalakrishnan *et al.*, 2021 [27]). These molecules can function as primary antioxidants, directly reducing the formation of free radicals (Hu *et al.*, 2016 [16]; Mudenuiti *et al.*, 2018 [17]). Chocolate also contains vitamins A, B, C, D, and potassium, sodium, iron, calcium, magnesium, phosphorous, manganese, copper, zinc and selenium (Leite, 2012 [1]; Torres-Moreno, Torrescasana *et al.*, 2015 [28]; Oliveira *et al.*, 2021 [29]). In milk chocolate, the cocoa mass is partly replaced by milk powder, giving it a sweeter taste. Milk chocolate or half-bitter chocolate contain between 40% and 53% cocoa butter. A bar of chocolate (30 g) contains an average of 165 calories and between 2 to 3 g of protein. Bitter chocolate, has a high concentration of cocoa mass and low sugar content. It is made with cocoa paste and cocoa butter. It is less refined, having a characteristic dark colour and bitter taste, are due to the reduced sugar content. Half-bitter

chocolate is slightly refined with a dark colour. It does not contain milk, but contains sugar, and has a less bitter taste, presenting and about 550 kcal/100g. Some people use chocolate as a form of self-medication to compensate for nutrient deficiency, probably magnesium. Chocolate and cocoa have an exceptionally high concentration of this mineral (100 mg/100g and 250 mg/100g, respectively) and in some Mg cases supplementation reduces chocolate compulsions and decreases the symptoms of premenstrual tension (Del Rei & Medeiros, 2011 [30]; Grassia *et al.*, 2019 [31]).

CEPLAC (Executive Committee for Cacao Crops), in Ilhéus, Bahia, Brazil, has the mission of promoting the competitiveness and sustainability of the agricultural, agroforestry and agroindustry segments for the development of cocoa producing regions, producing artisan chocolates with high cocoa content. It is an organ of the Brazilian Ministry of Agriculture, created on February 20, 1957, when the cocoa economy was going through a serious crisis, initially focusing on supporting cacao farmers. It succeeded in increasing the Brazilian cocoa production by 310%; from 220 kg/ha in 1962 to 740 kg/ha in 2016, among others. The current priority is the recovery of the regional economy, with emphasis on fighting a disease that has been decimating the cacao plantations known as "witch-broom" and aiming to develop sustainable agroeconomic activities and preserving the remaining forest fragments of two strategic ecosystems in Brazil, the Atlantic Forest and the Amazon Forest (Ceplac, 2021) [32].

Brazilian laws determine that chocolate must have at least 25% cocoa. One in three chocolate-based products in Brazil does not contain this amount of cocoa and therefore cannot be considered chocolate (Lima *et al.*, 2011) [33]. It is estimated that roughly 35% of the common chocolates on the shelves, produced by large food companies, are really chocolate candy. Many products claim to have a high percentage of cocoa, which is not always the case, since there is no supervision to guarantee the information on the label. Furthermore, it is not compulsory by law to declare the amount of cocoa in the product. Instead, these candies contain high amounts of sugar and fat (Leite, 2012 [1]; Seem *et al.*, 2019 [34]). Countries in Europe and the United States have informative legislation on the composition of chocolates (European Union, 2000 [35]; Rodriguez-Negrette *et al.*, 2019 [36]).

The present work aimed at analyzing the properties of chocolates, especially that produced by CEPLAC, determining, among other parameters, the fat content, caffeine, theobromine and antioxidant activity, as well as the correlations between the evaluated parameters.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

All reagents employed were at least analytical grade: ethyl ether, ethanol, 16N HCl, sodium carbonate and sodium tartrate (Synth, Brazil), barium oxide (Vetec), universal indicator, Folin-Ciocalteu reagent, Merck (Darmstadt, Germa-

ny), 0.45  $\mu\text{m}$  filters (Sartorius, USA). Gallic acid, caffeine and DPPH (radical diphenylpicrylhydrazyl) were from Sigma-Aldrich (St. Louis, USA). Methanol and formic acid were HPLC (High-Performance Liquid Chromatography) grade from (Merck, Darmstadt, Germany), deionized water (18.2 M $\Omega$ /cm) was prepared using a Barnsted apparatus (VWR, USA).

## 2.2. Chocolate Samples

The following nine samples of commercial chocolates were analyzed: bitter 70% cocoa (AD70), white C (BC), white D (BD), Belgian milk (BLC), milk 20% cocoa (CS20), milk 70% of cocoa (CS70), milk H (LH), milk HE (LHE), bitter medium (MAH); as well as artisan chocolate (80% bitter CEPLAC (AC80)) and cocoa powder CEPLAC (SCC).

## 2.3. Fat Extraction

An aliquot of 15 g of each sample was packed in a pre-weighed cartridge. 200 mL of ethyl ether was used in a soxhlet apparatus for two hours. After extraction, the solvent was evaporated leaving only the fat, which was quantified. The fat-free dry matter, called insoluble solids, was also quantified. The procedure was performed in triplicate.

## 2.4. Preparation of Solutions

The dried fat-free samples were ground and exactly about 0.1 g of the sample powder was weighed and dissolved in ethanol to give the 0.1% solutions used in tests for antioxidant activity and total phenolics. For the chromatographic analysis, the solutions were prepared as follows: 1.25 g of barium oxide (BaO) were weighed for each chocolate sample plus 2.5 g of the chocolate powders, then 1.25 mL of water was added, and boiled on the heating plate. After 10 minutes the mixture was removed and the pH adjusted to 7 with concentrated HCL. It was then transferred to a 250 mL volumetric flask completed with water and filtered through paper.

## 2.5. Determination of the Total Phenolic Content

An aliquot of 0.1 g of the fat-free sample powder was dissolved in a beaker with a little water, transferred to a 50 mL volumetric flask and the volume was made up with water (stock solution: 2 mg/mL). The procedure was performed from this point in triplicate. An aliquot of 200  $\mu\text{L}$  was transferred to a 10 mL volumetric flask (1:50) containing approximately 5 mL of distilled water. 800 mL of the Folin-Ciocalteu reagent was added and stirred for a few seconds and in an interval of 1 to 8 minutes 1.2 mL of the 20% sodium carbonate-tartrate solution was added. The volume of water was completed until near the meniscus. The solution was kept in a 20°C bath. After 2 hours, the final volume was completed at 20°C. It was shaken for a few seconds and read at 760 nm (in a Cary 50, Varian Inc spectrophotometer, USA). The value of total phenolics was determined by

comparison to a calibration curve with gallic acid. The same procedure was used for the quantification of phenolics in chocolates extracted with BaO. The procedure was performed in triplicate.

## 2.6. HPLC-DAD Conditions

The samples were stored in vials for HPLC analysis using LaChrom chromatograph, model D-7100, L-7100 pump, L-7200 automatic sampler and L-7455 diode array detector (Merck, Darmstadt, Germany). To determine theobromine and caffeine in the chocolate samples, a reverse phase C18 column (Gemini 00F-4435-E0, 110A RP 18, 12.5 × 0.4 cm, 5 μm particle diameter Merck, Darmstadt, Germany) with pre-column (Safety Guard Cartridges Kit KJO-4282, Merck, Darmstadt, Germany) and a isocratic system (1 mL/min flow) composed of water acidified with 0.5% formic acid (85%) and methanol (15%), was used. The volume injected was 50 μL. The maximum time of analysis was 10 minutes and detection performed at a wavelength of 272 nm. The program used for data analysis was the Merck-Hitachi model D-7100 (Chromatography Data Station - DAD Manager, Merck). The samples (1% chocolate ethanolic solution) were dissolved in methanol, filtered on a 0.45 μm filter and injected into HPLC-DAD (Matsume *et al.*, 2000 [37]; De Maria & Moreira, 2007 [38]).

Caffeine and theobromine (methylxanthines) were quantified using the HPLC method described by comparison to an external calibration curve of caffeine standard, using the least squares method (triangle equation) and averaged, standard deviation, coefficient of variation (%) and uncertainty. In the linearity, the residuals were also calculated (Ribeiro *et al.*, 2008) [39].

## 2.7. UHPLC-MS Conditions

UHPLC-MS (Ultra-high performance liquid chromatography mass spectrometry) chromatography was performed on a Waters Acquity UPLC instrument using a Waters C-18 column (50 mm × 2 mm, 1.7 μm particle) with oven at 30 °C. Solvent A = milli-Q water with 0.1% formic acid and solvent B = methanol, 3 μL injection, 0.2 mL/min flow, gradient starting with 90% A, ramping to 0% A in 5 minutes, held until 5.50 minutes, then back to 90% A at 5.51 minutes, and equilibrated until 6.00 minutes, with a total run time of 6.00 minutes. Samples were compared to the caffeine, theobromine and theophylline standards by UHPLC-MS (Waters Acquity TQD mass spectrometer (USA)). The mass spectrometry conditions were: electrospray ionization, positive ion mode, capillary 3.0 kV, cone 30 V, source temperature 150 °C, desolvation gas temperature 300 °C, collision energy 25V (in MS/MS mode). The presence of caffeine and theobromine were identified due to peaks in the sample with the same retention time as the standards, and confirmed by MS/MS.

The use of the method described above for the UHPLC was carried out to capture data that require more sensitive devices, than the conventional analysis performed by HPLC. Both methods developed in this study for UHPLC-MS and

HPLC-DADUV were separately validated.

## 2.8. Antioxidant Activity

The method was based on a free radical scavenging reaction using the diphenylpicrylhydrazyl radical (DPPH). Wells were numbered from 0 to 10 in a 24-well plate. Corresponding ethanol volumes were added into each of the various tubes and various volumes of the 0.01% chocolate ethanolic solution. A volume of DPPH (0.15 mM) was then added, with a one-minute interval between tubes, and these were shaken sporadically. The absorbance was read at 517 nm on a Cary 50, Varian Inc spectrophotometer (USA) 30 minutes after addition of DPPH in the first tube, and so on. The absorbance vs. chocolate concentration plot was built and the ED<sub>50</sub> (dose eliminating 50% free radicals) calculated by the least squares method. The procedure was performed in triplicate (Veiga *et al.*, 2017) [40].

## 2.9. Statistical Analysis

The fat content, insoluble solids, phenol content, theobromine content, caffeine content and antioxidant activity (ED<sub>50</sub>) data were submitted to the Shapiro-Wilk normality test. Once the normality of the distribution was verified, the data were submitted to analysis of variance (ANOVA) and Tukey test with significance level of 5%, considering the type of chocolate. PAST software Version 3.22 was used (Hammer, Harper & Ryan, 2001) [41].

A matrix containing the data from the various tests, with the lines representing the samples and the columns the variables, was analyzed by Principal Component Analysis. In the present work the software Usrambler X10.4, 2016 (CAMO AS, Trondheim, Norway) was used.

## 3. Results and Discussion

The following parameters of chocolate quality were evaluated in the present work: fat content, total phenolics, theobromine and caffeine (methylxanthines) content and antioxidant activity. **Table 1** shows the values obtained for chocolate samples and cocoa bean powder.

It can be seen (**Table 1**) that SCC presented a significantly higher fat concentration (% w/w) than all the chocolate samples. The AC80 presented significantly lower fat concentration than SCC and the others, with the exception of MAH and BLC. Among the chocolate samples, AD70, CS70 and CS20 presented the highest concentrations of fat (% w/w). Cocoa bean powder showed the highest fat content, 33.40%. Leite (2012) [1] evaluated the fat content in toasted cocoa beans, finding values between 32.60% and 36.52%, similar to our result. For the cocoa mass, the same author found values from 52.95% to 58.17% and for chocolates, values between 37.87% and 45.50% were found. De Melo *et al.* (2020) [42] reported that the percentage of fat varies between 35.1% to 41.8%. The fat content found for chocolates in our study was between 15.51% to 25.51%, values

**Table 1.** Amount of fat (% w/w), dried residue (DR) (% w/w) and total phenolic content (% w/w) in chocolate samples and cocoa beans.

Chocolate sample	Fat*		Dried residue (DR) after extraction with ether*			Total polyphenol content*			
	% (w/w)	CV%	Unc.	% (w/w)	CV%	Unc.	% (w/w)	CV%	Unc.
AC80	15.54 ± 0.11 <sup>e</sup>	0.73	0.07	80.50 ± 0.41 <sup>b</sup>	0.51	0.24	4.21 ± 0.01 <sup>a</sup>	0.21	0.01
AD70	25.51 ± 0.25 <sup>b</sup>	0.99	0.15	76.27 ± 0.54 <sup>d</sup>	0.71	0.31	3.22 ± 0.03 <sup>c</sup>	0.84	0.02
BC	18.35 ± 0.17 <sup>d</sup>	0.90	0.10	81.50 ± 0.50 <sup>ab</sup>	0.61	0.29	0.06 ± 0.01 <sup>e</sup>	15.71	0.01
BD	21.52 ± 0.46 <sup>c</sup>	2.14	0.27	77.25 ± 0.61 <sup>cd</sup>	0.79	0.35	0.31 ± 0.00 <sup>e</sup>	0.78	0.00
BLC	16.40 ± 0.44 <sup>e</sup>	2.70	0.26	81.50 ± 0.50 <sup>ab</sup>	0.61	0.29	1.87 ± 0.05 <sup>d</sup>	2.64	0.03
CS20	23.29 ± 0.30 <sup>bc</sup>	1.30	0.17	74.63 ± 0.40 <sup>d</sup>	0.54	0.23	2.03 ± 0.06 <sup>d</sup>	2.81	0.03
CS70	24.50 ± 0.35 <sup>b</sup>	1.43	0.20	71.49 ± 0.38 <sup>e</sup>	0.54	0.22	3.60 ± 0.06 <sup>b</sup>	1.75	0.04
LH	19.71 ± 0.03 <sup>cd</sup>	0.16	0.02	81.58 ± 0.55 <sup>ab</sup>	0.67	0.31	2.70 ± 0.04 <sup>d</sup>	1.41	0.02
LHE	19.62 ± 0.31 <sup>d</sup>	1.58	0.18	78.06 ± 0.39 <sup>c</sup>	0.49	0.22	2.00 ± 0.07 <sup>d</sup>	3.71	0.04
MAH	15.51 ± 0.12 <sup>e</sup>	0.76	0.07	82.60 ± 1.11 <sup>a</sup>	1.34	0.64	3.17 ± 0.17 <sup>c</sup>	5.29	0.10
SCC	33.40 ± 0.29 <sup>a</sup>	0.85	0.16	59.53 ± 0.50 <sup>f</sup>	0.84	0.29	3.70 ± 0.01 <sup>b</sup>	0.32	0.01

\*Average of three measurements. CV% is the percentile variation coefficient and Unc. the uncertainty of the measurements, calculated by the standard deviation/square root of the number of measurements. The same letters in each column indicate that there is no statistical difference at a significance level of 5%, according to the Tukey Test results.

slightly lower than those reported by Leite (2012) [1] and De Melo *et al.* (2020) [42]. As expected, the bitter and half-bitter chocolates presented the lowest fat contents. In the same extraction process with ethyl ether, the solid residue content was measured after extraction. The values are also presented in **Table 1**. These values ranged from 59.53% to 82.60%. SCC presented insoluble solids concentration (% w/w) which was significantly lower than all chocolates. AC80 showed a significantly higher concentration of insoluble solids than SCC and AD70, CS70, LHE, CS20 and BD chocolate samples. The HAM sample showed a higher concentration of insoluble solids than the AC80 sample. The CS70 sample, on the other hand, had the lowest concentration of insoluble solids.

The total phenolic content was initially measured in the dry extract, after extraction with ethyl ether however, only five samples were evaluated: AD70 (total phenolic content of 2.48% ± 0.11%), BD (0.00% ± 0.00%), BLC (0.31% ± 0.17%), LH (0.36% ± 0.01%) and MAH (0.77% ± 0.36%). After this initial evaluation, the use of BaO for defatting the samples was implemented (Alves & Bragagnolo, 2002) [43] and all the samples were extracted. Total phenolic, caffeine and theobromine contents were quantified in these BaO extracted samples. **Table 2** shows the total phenolic content for the samples extracted with BaO. AC80 total phenolic content significantly higher than SCC and all other samples. SCC presented significantly higher total phenolic content than all samples, with the exception of CS70 and AC80. The bitter (AD70 and CS70) and the half-bitter (MAH) samples presented significantly higher total phenolic content than the milk chocolate samples (LH, LHE BLC and CS20) and white chocolate samples (BD and BC). The white chocolate samples BC and BD presented significantly

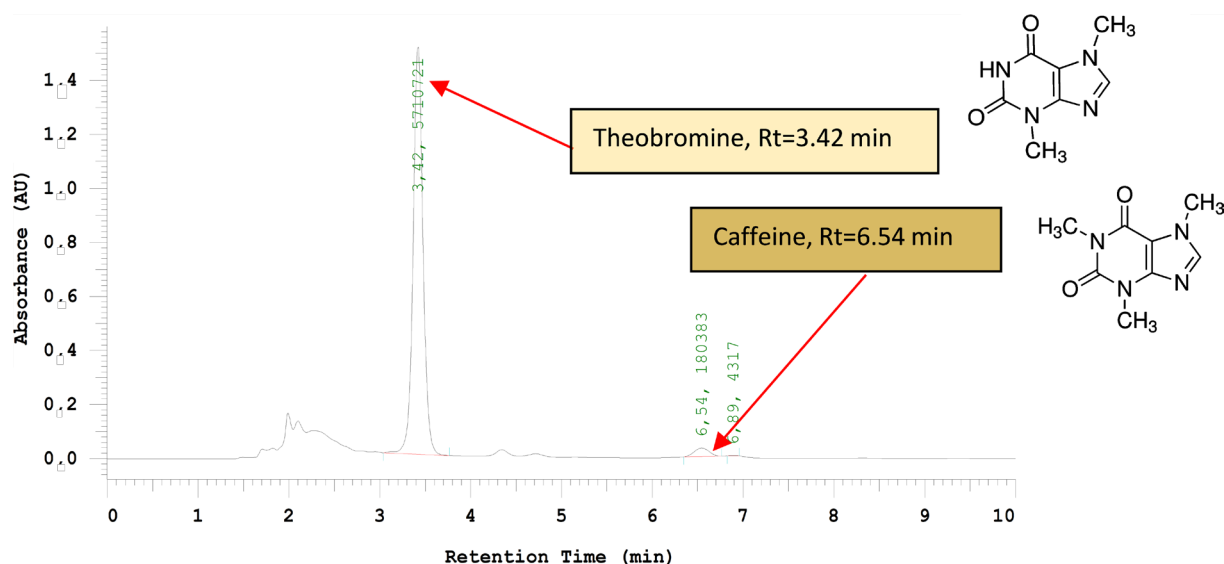
lower total phenolic content than all the other chocolate samples tested. The samples that presented the highest total phenolic content were cocoa seed powder (3.70%) and those with higher cocoa content, which was expected (from 3.17% to 4.21%). The milk chocolates presented intermediate values and the whites almost no phenolic content. White chocolates do not have cocoa mass in their composition, only cocoa butter, which explains a virtually zero content of polyphenols. Ramli *et al.* (2001) [44] determined the total phenolic content in different samples of cocoa from Malaysia and Ghana, finding for beans, from 3.49% and 6.02%. Vertuani *et al.* (2014) [45] found values between 2.0% to 3.5% for cocoa. In the present study, the amount found is approximately the same. The values presented in **Table 1** are in accordance with the data of these researchers, except as previously mentioned, for white chocolate samples, which do not have the cocoa mass.

Leite (2012) [1] analyzed the phenolic content in two varieties of cocoa mass from the region of Ilhéus-BA and chocolates prepared with this same mass. For the cocoa mass, the author phenolic content in the range of 2.39% to 2.58% and for chocolates between 1.55% to 1.91%. Several authors have reported between 2.00% and 8.37% of phenolic content in natural cocoa powder (Waterhouse *et al.*, 1996 [46]; Serra-Bonvehí & Ventura Coll, 1997 [47]; Kealey *et al.*, 2000 [48]; Natsume *et al.*, 2000 [49]; Lee *et al.*, 2003 [15], Vinson *et al.*, 2006 [22]; Efraim *et al.*, 2011 [3]; Vertuani *et al.*, 2014 [45]). For chocolates, from 0.26 to 4.85% (Hu *et al.*, 2016) [16] and 1.00% to 4.50% (Mudenutti *et al.*, 2018) [17] have been reported; while for bitter chocolate, between 0.246% and 2.39% (Waterhouse *et al.*, 1996 [46]; Natsume *et al.*, 2000 [49]; Vinson *et al.*, 2006 [22]; Counet *et al.*, 2006 [12]), and for milk chocolate from 0.192 to 0.990% (Waterhouse *et al.*, 1996 [46]; Gu *et al.*, 2006 [21]; Vinson *et al.*, 2006 [22]). For cocoa liquor, values of 0.367% to 8.52% (Adamson *et al.*, 1999 [50]; Kealey *et al.*, 2000 [48]; Luna *et al.*, 2002 [51]) and  $2.84 \pm 0.24$  (De Oliveira & Genovese, 2013) [23] were reported. Natsume *et al.* (2000) [49] found 6.04% of phenolic content in Brazilian cacao liquor, but its geographic origin was not reported.

The range of total polyphenol content for milk chocolate and bitter chocolate found by Efraim *et al.* (2011) [3] are lower than the values found in the present work for the same types of chocolates. However, the reported value for cocoa is within the range reported by the same authors for this product. Efraim *et al.* (2011) [3] citing Ramirez-Sanchez *et al.* (2010) [52] reported that phenolic content in cocoa may vary according to its geographical origin, plant variety, climate, soil type and planting area. The different steps of the transformation of cocoa into chocolate may also influence the phenolic content in the final product. With respect to the quantification of theobromine and caffeine, **Figure 1** shows the chromatogram of one of the samples analyzed.

After using barium oxide (BaO) as a fat extracting agent, caffeine and theobromine were quantified using a calibration curve based on caffeine (**Figure 1**). Theobromine has the same UV-Vis spectrum of caffeine (in the HPLC spectra



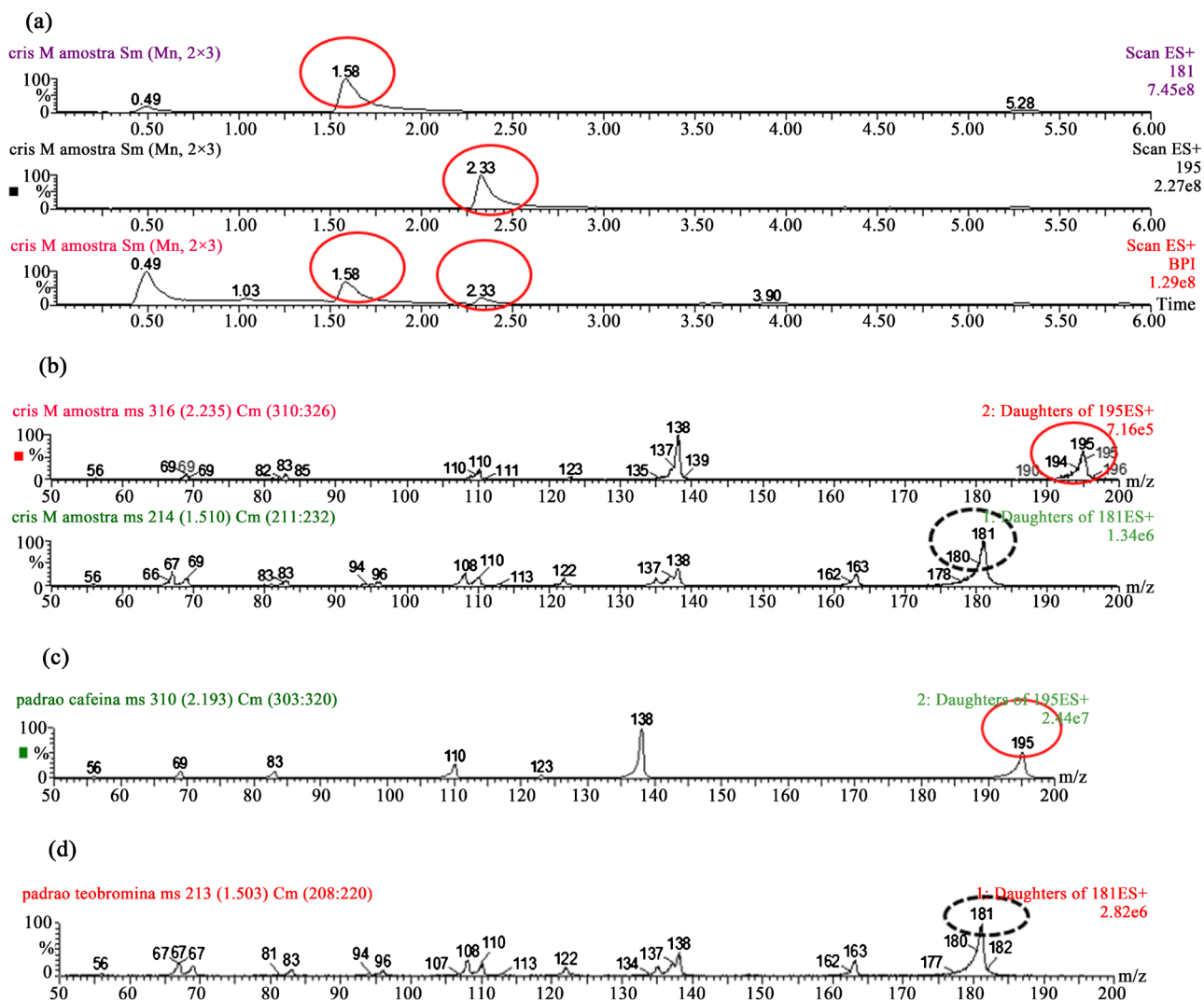


**Figure 1.** Chromatogram of the 80% cocoa chocolate sample from CEPLAC. The arrows indicate the peaks for methylxanthines (theobromine and caffeine) and their retention times in minutes (Rt).

library), but with different retention time, therefore, the proposal was to quantify theobromine by the caffeine area. **Figure 2(a)** shows the UHPLC-MS that confirm the presence of both theobromine and caffeine in the samples of chocolates, in comparison with standards. In **Figure 2(b)** are the mass spectra (MS/MS) of the chocolate sample compared to the caffeine (**Figure 2(c)**) and theobromine (**Figure 2(d)**) standards. Theobromine has a lower retention time than caffeine in reverse phase HPLC because the former has a methyl group less than the latter, facilitating its elution from the chromatographic column (**Figure 1**).

A calibration curve of the caffeine standard was constructed for quantification of caffeine and theobromine by HPLC-DAD. From the confirmation of the theobromine and caffeine peaks and also of the caffeine calibration curve, the contents of these compounds were calculated in the chocolate samples. **Table 2** shows the levels of theobromine and caffeine (g/100g) obtained in samples after the BaO extraction.

The concentration of both theobromine and caffeine were higher in the samples when the barium oxide extraction method was used, indicating that the barium oxide method is much more efficient in extracting these methylxanthines than when ethyl ether is used. Regarding the theobromine content, SCC presented a significantly higher percentage than all the chocolate samples. AC80 had significantly lower theobromine content than MAH, similar to CS70 and superior to AD70, and to all milk chocolate samples (LH, LHE, BLC and CS20). Theobromine was not detected in white chocolate samples (BD and BC). Regarding caffeine content, SCC presented significantly higher caffeine content than all the other chocolate samples. The AC80 showed significantly higher theobromine and caffeine content than all the other chocolate samples. Milk chocolates (LH, LHE, BLC and CS20) had significantly lower caffeine content



**Figure 2.** Positive ion mode UHPLC-MS chromatograms of the CEPLAC chocolate sample, showing retention times of theobromine  $[M + H]^+$  ion ( $m/z$  181) at 1.58 minutes and caffeine  $[M + H]^+$  ion ( $m/z$  195) at 2.33 minutes (circles). (a) MS/MS spectra of theobromine ( $m/z$  181) and caffeine ( $m/z$  195) in the chocolate sample (b) and of caffeine standard (c) and theobromine standard (d). Full circles indicate the precursor ion of caffeine and dashed lines the precursor ion of theobromine.

than all bitter chocolate samples (AC80, AD70, CS70 and MAH). Caffeine was not detected in white chocolate samples (BD and BC). Cacao seeds (SCC) presented higher caffeine and theobromine contents, higher than all evaluated chocolates.

The results of theobromine and caffeine content presented a strong positive correlation ( $R = 0.9297$ ). Ramli, Yatim, Said & Hok (2001) [44] evaluated the theobromine and caffeine content in different cocoa products: for cocoa liquor from Malaysia and Ghana, they found 1.720% to 2.530% (w/w) of theobromine and from 0.310% to 0.380% caffeine. For the cocoa beans from the same sites, between 1.620% and 2.660% of theobromine and from 0.250% to 0.500% of caffeine were found. The same authors evaluated these levels in cocoa powder in Malaysia, reporting between 1.960% to 2.940% of theobromine and 0.330% to

**Table 2.** Theobromine and caffeine contents (%) in chocolate samples and cocoa beans extracted with BaO.

	Theobromine content * % (g/100g)			Caffeine content* % (g/100g)		
	Average	CV (%)	Unc.	Average	CV (%)	Unc.
AD70	1.73 <sup>d</sup> ± 0.04	2.19	0.02	0.25 <sup>c</sup> ± 0.01	2.34	0.00
AC80	1.92 <sup>c</sup> ± 0.02	0.80	0.01	0.33 <sup>a</sup> ± 0.00	3.46	0.01
BC	0.00 <sup>f</sup> ± 0.00	0.00	0.00	0.00 <sup>i</sup> ± 0.00	0.00	0.00
BD	0.00 <sup>f</sup> ± 0.00	0.00	0.00	0.00 <sup>h</sup> ± 0.00	0.00	0.00
BLC	0.36 <sup>e</sup> ± 0.01	2.78	0.01	0.02 <sup>d</sup> ± 0.00	1.33	0.00
CS20	0.39 <sup>e</sup> ± 0.01	2.99	0.01	0.10 <sup>c</sup> ± 0.01	2.97	0.00
CS70	1.90 <sup>b</sup> ± 0.02	0.80	0.01	0.20 <sup>b</sup> ± 0.01	2.84	0.00
LH	0.47 <sup>e</sup> ± 0.02	3.27	0.01	0.06 <sup>e</sup> ± 0.01	2.19	0.00
LHE	0.40 <sup>e</sup> ± 0.01	1.46	0.00	0.07 <sup>f</sup> ± 0.01	2.66	0.00
MAH	2.82 <sup>b</sup> ± 0.02	0.71	0.01	0.32 <sup>ab</sup> ± 0.01	3.13	0.01
SCC	4.41 <sup>a</sup> ± 0.10	2.24	0.06	1.10 <sup>a</sup> ± 0.01	0.91	0.01

\*Average of three measurements. CV% is the percentile variation coefficient and Unc. the uncertainty of the measurements, calculated by the standard deviation/square root of the number of measurements. The same letters in each column indicate that there is no statistical difference at a significance level of 5%, according to the Tukey Test results.

0.55% of caffeine; for Malaysian chocolate with cocoa butter (brand I) from 0.080% to 0.100% theobromine and from 0.006% to 0.009% caffeine. Chocolates from the same place with cocoa butter (brand II) presented theobromine and caffeine contents from 0.036% to 0.065% and 0.003% to 0.006%, respectively. The results presented in the present study are in line with those reported by Ramli, Yatim, Said & Hok (2001) [44] regarding the theobromine content in cocoa beans. However, the caffeine concentration for the same product was much lower in Malaysian chocolate than in the present study. De Oliveira & Genovese (2013) [23] reported a caffeine content of 0.27% ± 0.01% for cocoa liquor. For the chocolates prepared with cocoa butter, the values mentioned by Ramli *et al.* (2001) [44] were much lower than those found in the present study. If, as reported by Efraim *et al.* (2011) [3], the levels of polyphenols in cocoa may vary according to their origin, the same may occur for the methylxanthines: theobromine and caffeine.

The antioxidant activity of chocolate samples was evaluated in the DPPH free radical discoloration method. **Table 3** shows the results for all evaluated chocolates; ED<sub>50</sub> values ranged from 4.54 to 285.83 µg/mL. Vertuani *et al.* (2014) [45] reported values of ED<sub>50</sub> between 140 to 470 µg/mL for cocoa. It was observed that the SCC and AC80 presented the highest antioxidant activity among the chocolate samples tested, being significantly superior to the other chocolates, with the exception of the MAH, which presented similar values. CS70 also had high antioxidant activity, higher than the other chocolate samples, but lower than SCC and AC80. Bitter chocolate samples (MAH, CS70 and AD70) showed

**Table 3.** Antioxidant activity of chocolate samples (ED<sub>50</sub> µg/mL).

Chocolate sample	ED <sub>50</sub> (µg/mL)*	CV%	Inc.
AD70	114.04 <sup>e</sup> ± 0.87	0.76	0.50
AC80	5.03 <sup>a</sup> ± 0.15	3.03	0.09
BC	285.83 <sup>i</sup> ± 0.15	0.05	0.09
BD	264.32 <sup>h</sup> ± 0.20	0.08	0.12
BLC	57.45 <sup>d</sup> ± 0.38	0.67	0.22
CS20	20.50 <sup>c</sup> ± 0.50	2.44	0.29
CS70	7.13 <sup>b</sup> ± 0.15	2.07	0.09
LH	120.54 <sup>g</sup> ± 1.66	1.38	0.96
LHE	117.94 <sup>f</sup> ± 0.08	0.07	0.04
MAH	5.55 <sup>ab</sup> ± 0.05	0.90	0.03
SCC	4.54 <sup>a</sup> ± 0.04	0.88	0.02

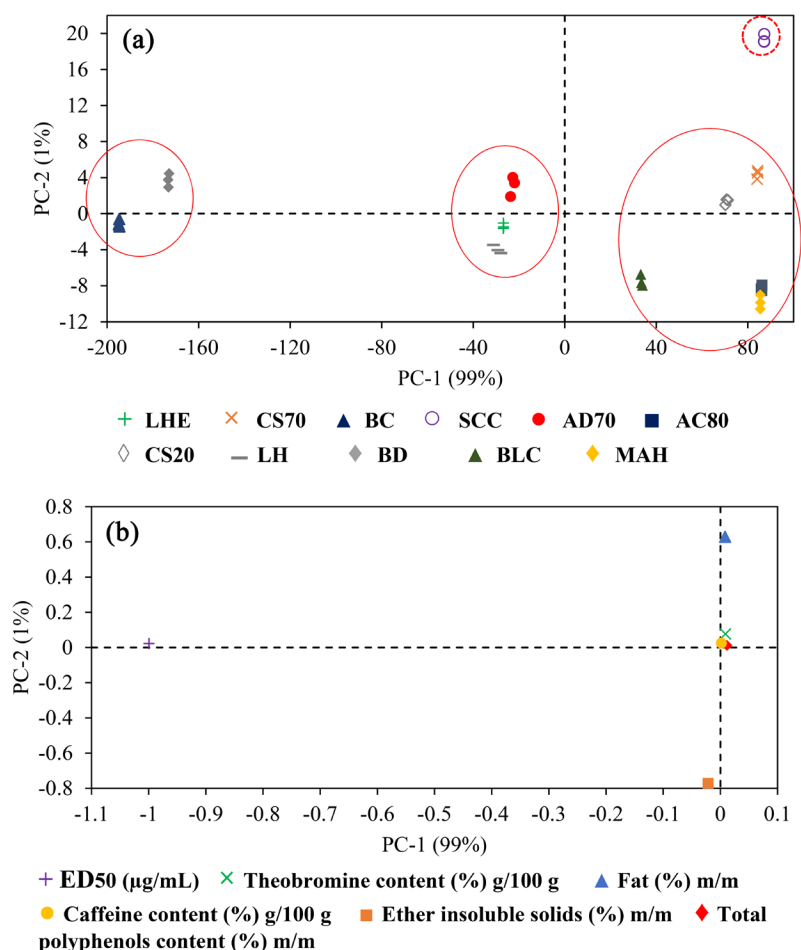
\*Average of three measurements. CV% is the percentile variation coefficient and Inc. the uncertainty of the measurements, calculated by the standard deviation/square root of the number of measurements. The same letters in each column indicate that there is no statistical difference at a significance level of 5%, according to the Tukey Test results.

significantly higher antioxidant activity than milk chocolates (LH, LHE, BLC and CS20). The white chocolate samples (BC and BD) had very low antioxidant activity, significantly lower than the other samples.

The data in **Table 3** confirms that the antioxidant activity is related to the phenolic content, as reported at the beginning of the present study. The bean powder and the chocolate samples with higher cocoa content are those that presented higher total phenolic content, consequently better antioxidant activity. Through the statistical analysis performed, the following correlations (R) were observed: very high between caffeine x theobromine (0.9274), moderated between ED<sub>50</sub> x theobromine (0.6441) and a weak ED<sub>50</sub> x caffeine (0.4854).

After collecting all the physical-chemical data and the antioxidant activity, they were submitted to a multivariate statistical analysis (Principal Component Analysis or PCA). According to this, factor 1 explained 99% of the variance and factor 2 plus 1% of the variance, in a total of 100%, which explains the distribution in two PCs (1 and 2). These results can be seen in **Figure 3**.

The samples were separated into three different groups. In Group 1, the white chocolate samples (BD and BC), which do not present antioxidant activity, total phenolic content is very low and lack theobromine and caffeine (they are in the negative quadrant of **Figure 3(b)**). Group 2 is the intermediate, with samples AD70, LH and LHE, with intermediate amounts of theobromine and caffeine and phenolic content and moderate antioxidant activity. In Group 3 are AC80, CS20, CS70, BLC and MAH, with better antioxidant activity, higher total phenolic and methylxanthine content. Group 3 stands out from all others for having the highest content of methylxanthines (caffeine and theobromine), consequently presenting the best antioxidant activity. The bean powder was separated



**Figure 3.** Principal component analysis of the (PCA) of the chocolate samples. PC1 xPC2 scores (samples) (a) and the loadings (variables) (b). For abbreviations see Materials and Methods.

from the rest because they had a high fat content. The AC80 and MAH chocolate samples have the highest content of ether insoluble solids (**Figure 3(a)**).

#### 4. Conclusion

The highest fat contents were found in the milk chocolate samples followed by the white chocolate samples. Bitter chocolate has a higher percentage of cocoa in its composition than other types of chocolates and therefore presented higher total phenolic contents than other types (such as white chocolate that has a very low or almost no polyphenols because there is no cocoa paste). To date, cocoa has been studied as a source of polyphenols, with its main components extracted, isolated and identified in bench processes, using organic solvents not allowed for use in food, such as methanol, for example. These solid-liquid extractions leave a residual content in the product, rendering it unsuitable for human consumption. Extractions using permitted organic solvents often promote degradation of the polyphenols during the process due to the temperature and oxygen exposure conditions to which the product is subjected. Polyphenols are

compounds that degrade easily in the presence of heat and oxygen. A method of extraction of the alkaloids using barium oxide was used, successfully separating of the methylxanthines, as confirmed by mass spectrometry. The literature describes the use of magnesium oxide for the same analyses. It was possible to evaluate and to prove that the antioxidant activity was closely linked to the phenolic content. Theobromine and caffeine content were also high in these samples, possibly due to the higher percentage of cacao paste used, confirmed by the multivariate statistical analysis. This work came to contribute to the confirmation of the properties of chocolates for human health, and these can be considered functional foods, such as CEPLAC chocolate with high phenol content, theobromine and caffeine, as well as a good antioxidant activity.

### Acknowledgements

CEPLAC (Comissão Executiva de Planejamento da Lavoura Cacauera) Ilheus, Bahia, Brazil, which provided the samples of cocoa and chocolate seeds to carry out this work.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

### References

- [1] Leite, P.B. (2012) Caracterização de chocolates provenientes de variedades de cacau *Theobroma cacao* L. resistentes a vassoura de bruxa. Dissertação apresentada no Programa de Pós-graduação em Ciência de Alimentos da Faculdade de Farmácia. Universidade Federal da Bahia, Bahia, 170.
- [2] Godočíková, L., Ivanišová, E. and Kačániová, M. (2019) The Impact of Addition of Different Tea Powders on the Biological Value of White Chocolates. *Journal of Microbiology, Biotechnology and Food Science*, **9**, 396-399. <https://doi.org/10.15414/jmbfs.2019.9.special.396-399>
- [3] Efraim, C., Alves, A.B. and Jardim, D.C.P. (2011) Polifenóis em cacau e derivados: Teores, fatores de variação e efeitos na saúde. *Journal of Food Technology*, **14**, 181-201. <https://doi.org/10.4260/BJFT2011140300023>
- [4] Vicentim, A.L. and Marcellino, M.C.L. (2012) Efeito do pó do cacau (*Theobroma cacao*) e seus princípios ativos na pressão arterial de portadores de *diabetes mellitus* tipo II. *Salusvita*, **31**, 29-40.
- [5] Kerimi, A. and Williamson, G. (2015) The Cardiovascular Benefits of Dark Chocolate. *Vascular Pharmacology*, **71**, 11-15. <https://doi.org/10.1016/j.vph.2015.05.011>
- [6] Magrone, T., Russo, M.A. and Jirillo, E. (2017) Cocoa and Dark Chocolate Polyphenols: From Biology to Clinical Applications. *Frontiers in Immunology*, **8**, 677. <https://doi.org/10.3389/fimmu.2017.00677>
- [7] Anwar, N.Z.R., Jamaluddin, A., Shahidan, N. and Zakaria, Z. (2018) Nutritional Composition and Glycaemic Index of Milk Chocolate Using Different Sweeteners. *Journal of Agrobiotechnology*, **9**, 62-68.
- [8] Kardum, N. and Glibetic, M. (2018) Polyphenols and Their Interactions with 629

- Other Dietary Compounds: Implications for Human Health. *Advances in Food and Nutrition Research*, **84**, 103-144. <https://doi.org/10.1016/bs.afnr.2017.12.001>
- [9] Stanley, T.H., Van Buiten, C.B., Baker, S.A., Elias, R.J., Anantheswaran, R.C. and Lambert, J.D. (2018) Impact of Roasting on the Flavan-3-ol Composition, Sensory-Related Chemistry, and *in Vitro* Pancreatic Lipase Inhibitory Activity of Cocoa Beans. *Food Chemistry*, **255**, 414-420. <https://doi.org/10.1016/j.foodchem.2018.02.036>
- [10] Coutinho, N.M., Silveira, M.R., Fernandes, L.M., Moraes, J., Pimentel, T.C., Freitas, M.Q., et al. (2019) Processing Chocolate Milk Drink by Low-Pressure Cold Plasma Technology. *Food Chemistry*, **278**, 276-283. <https://doi.org/10.1016/j.foodchem.2018.11.061>
- [11] Godočiková, L., Ivanišová, E., Zaguła, G., Noguera-Artiaga, L., Carbonell-Barrachina, Á.A., Kowalczewski, P.Ł. and Kačániová, M. (2020) Antioxidant Activities and Volatile Flavor Components of Selected Single-Origin and Blend Chocolates. *Molecules*, **25**, 3648-3661. <https://doi.org/10.3390/molecules25163648>
- [12] Counet, C., Callemien, D. and Collin, S. (2006) Chocolate and Cocoa: New Sources of Trans-Resveratrol and Trans-Piceid. *Food Chemistry*, **98**, 649-657. <https://doi.org/10.1016/j.foodchem.2005.06.030>
- [13] Alvarez-Barreto, J.F., Quintero, D., Rodríguez, M., Rea, R. and Sosa, D. (2018) Evaluation of Phenolic Compounds, Lignin, Amino Acids and Carbohydrates in *Theobroma cacao* L. from Three Different Climate Regions in Venezuela. *Emirates Journal of Food and Agriculture*, **30**, 522-530.
- [14] Muhammad, D.R.A., Saputro, A.D., Rottiers, H., Van de Walle, D. and Dewettinck, K. (2018) Physicochemical Properties and Antioxidant Activities of Chocolates Enriched with Engineered Cinnamon Nanoparticles. *European Food Research and Technology*, **244**, 1185-1202. <https://doi.org/10.1007/s00217-018-3035-2>
- [15] Lee, K.W., Kim, Y.K., Lee, H.J. and Lee, C.Y. (2003) Cocoa Has More Phenolic Phytochemicals and a Higher Antioxidant Capacity than Teas and Red Wine. *Journal of Food Chemistry*, **51**, 7292-7295. <https://doi.org/10.1021/jf0344385>
- [16] Hu, Y., Pan, Z.J., Liao, W., Li, J., Gruget, P., Kitts, D.D., et al. (2016) Determination of Antioxidant Capacity and Phenolic Content of Chocolate by Attenuated Total Reflectance-Fourier Transformed-Infrared Spectroscopy. *Food Chemistry*, **202**, 254-261. <https://doi.org/10.1016/j.foodchem.2016.01.130>
- [17] Mudenuiti, N.V.R., De Camargo, A.C., Shahidi, F., Madeira, T.B., Hirooka, E.Y. and Grossmann, M.V.E. (2018) Soluble and Insoluble-Bound Fractions of Phenolics and Alkaloids and Their Antioxidant Activities in Raw and Traditional Chocolate: A Comparative Study. *Journal of Functional Foods*, **50**, 164-171. <https://doi.org/10.1016/j.jff.2018.10.003>
- [18] Tafurt, G., Suarez, O., Lares, M.C., Álvarez, C. and Liconte, N. (2021) Antioxidant Capacity of a Dark Chocolate from Organic and Unfermented Cocoa Beans. *Revista Digital de Postgrado*, **10**, 1-8. <https://doi.org/10.37910/RDP.2021.10.1.e280>
- [19] Torres-Moreno, M., Tarrega, A., Costell, E. and Blanch, C. (2012) Dark Chocolate Acceptability: Influence of Cocoa Origin and Processing Conditions. *Journal of Science Food and Agriculture*, **92**, 404-411. <https://doi.org/10.1002/jsfa.4592>
- [20] Gu, L., Kelm, M.A. and Hammerstone, J.F. (2004) Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption. *Journal of Nutrition*, **134**, 613-617. <https://doi.org/10.1093/jn/134.3.613>
- [21] Gu, L., House, S.E., Wu, S., Ou, B. and Prior, R.L. (2006) Procyanidin and Catechin Contents and Antioxidant Capacity of Cocoa and Chocolate Products. *Journal of*

- Agricultural and Food Chemistry*, **53**, 4057-4061. <https://doi.org/10.1021/jf060360r>
- [22] Vinson, J., Proch, J., Bose, P., Muchler, S., Tafera, P., Shuta, D., et al. (2006) Chocolate Is a Powerful *ex Vivo* and *in Vivo* Antioxidant, an Antiatherosclerotic Agent in an Animal Model and a Significant Contributor to Antioxidants in the European and American Diets. *Journal of Agricultural Food Chemistry*, **54**, 8071-8076. <https://doi.org/10.1021/jf062175j>
- [23] De Oliveira, T.B. and Genovese, M.I. (2013) Chemical Composition of Cupuaçu (*Theobroma grandiflorum*) and Cocoa (*Theobroma cacao*) Liquors and Their Effects on Streptozotocin-Induced Diabetic Rats. *Food Research International*, **51**, 929-935. <https://doi.org/10.1016/j.foodres.2013.02.019>
- [24] Cambrai, A., Marchioni, E., Julien-David, D. and Marcic, C. (2017) Discrimination of Cocoa Bean Origin by Chocolate Polyphenol Chromatographic Analysis and Chemometrics. *Food Analytical Methods*, **10**, 1991-2000. <https://doi.org/10.1007/s12161-016-0744-7>
- [25] Martini, S., Conte, A. and Tagliazucchi, D. (2018) Comprehensive Evaluation of Phenolic Profile in Dark Chocolate and Dark Chocolate Enriched with Sakura Green Tea Leaves or Turmeric Powder. *Food Research International*, **112**, 1-16. <https://doi.org/10.1016/j.foodres.2018.06.020>
- [26] Fayeulle, N., Meudec, E., Boulet, J.C., Vallverdu-Queralt, A., Hue, C., Boulanger, R., Cheynier, V. and Sommerer, N. (2019) Fast Discrimination of Chocolate Quality Based on Average-Mass Spectra Fingerprints of Cocoa Polyphenols. *Journal of Agricultural and Food Chemistry*, **67**, 2723-2731. <https://doi.org/10.1021/acs.jafc.8b06456>
- [27] Gopalakrishnan, B., Sahib, K.A., Selvam, R. and Lakshmanan, G. (2021) Antioxidant Activity in Toffees and Selected Medicinal Plants. *Annals of the Romanian Society for Cell Biology*, **25**, 1294-1300.
- [28] Torres-Moreno, M., Torres-Casana, E., Salas-Salvadó, J. and Blanch, C. (2015) Nutritional Composition and Fatty Acids Profile in Cocoa Beans and Chocolates with Different Geographical Origin and Processing Conditions, *Food Chemistry*, **166**, 125-132. <https://doi.org/10.1016/j.foodchem.2014.05.141>
- [29] Oliveira, L.B., De Melo, J.C., Da Boa Morte, E.C., De Jesus, R.M., Teixeira, L.S.G. and Korn, M.G.A. (2021) Multi-Element Determination in Chocolate Bars by Microwave-Induced Plasma Optical Emission Spectrometry. *Food Chemistry*, **351**, Article ID: 129285. <https://doi.org/10.1016/j.foodchem.2021.129285>
- [30] DEl Rei, J. and Medeiros, F. (2011) Chocolate e os benefícios cardiovasculares. *Revista do Hospital Universitário Pedro Ernesto, UERJ*, **10**, 54-59.
- [31] Grassia, M., Salvatori, G., Roberti, M., Planeta, D. and Cinquanta, L. (2019) Polyphenols, Methylxanthines, Fatty Acids and Minerals in Cocoa Beans and Cocoa Products. *Journal of Food Measurement and Characterization*, **13**, 1721-1728. <https://doi.org/10.1007/s11694-019-00089-5>
- [32] Ceplac (Comissão Executiva da Lavoura Cacauera). <https://www.gov.br/agricultura/pt-br/assuntos/ceplac>
- [33] Lima, D.G., Padovani, R.M., Rodriguez-Amaya, D.B., Farfán, J.A., Nonato, C.T., De Lima, M.T., et al. (2011) Tabela Brasileira de Composição de Alimentos-TACO 4ª Edição Revisada e Ampliada. Núcleo de Estudos e Pesquisas em Alimentação-NEPA Universidade Estadual de Campinas-UNICAMP. [http://www.cfn.org.br/wp-content/uploads/2017/03/taco\\_4\\_edicao\\_ampliada\\_e\\_revogada.pdf](http://www.cfn.org.br/wp-content/uploads/2017/03/taco_4_edicao_ampliada_e_revogada.pdf)
- [34] Seem, S.A., Yuan, Y.V. and Tou, J.C. (2019) Chocolate and Chocolate Constituents



- Influence Bone Health and Osteoporosis Risk. *Nutrition*, **65**, 74-84. <https://doi.org/10.1016/j.nut.2019.02.011>
- [35] European Union (2000) Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 Relating to Cocoa and Chocolate Products Intended for Human Consumption. <https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32000L0036>
- [36] Rodriguez-Negrette, A.C., Huck-Iriart, C. and Herrera, M.I. (2019) Physical Chemical Properties of Shea/Cocoa Butter Blends and Their Potential for Chocolate Manufacture. *Journal of American Oil Chemical Society*, **96**, 239-248. <https://doi.org/10.1002/aocs.12189>
- [37] Matsume, M., Osakabe, N., Yamagishi, M., Takizawa, T., Nakamura, T., Miyatake, H., et al. (2000) Analysis of Polyphenols in Cacao Liquor, Cocoa and Chocolate by Normal-Phase and Reversed-Phase HPLC. *Bioscience, Biotechnology and Biochemistry*, **64**, 2581-2587. <https://doi.org/10.1271/bbb.64.2581>
- [38] De Maria, C.A.B. and Moreira, R.F.A. (2007) Cafeína: Revisão sobre métodos de análise. *Química Nova*, **30**, 99-105. <https://doi.org/10.1590/S0100-40422007000100021>
- [39] Ribeiro, F.A.L., Ferreira, M.M.C., Morano, S.C., Da Silva, L.R. and Schneider, R.P. (2008) Planilha de validação: Uma nova ferramenta para estimar figuras de mérito na validação de métodos analíticos univariados. *Química Nova*, **31**, 200-228. <https://doi.org/10.1590/S0100-40422008000100029>
- [40] Veiga, R.S., Mendonça, S., Mendes, P.B., Paulino, N., Mimica, M.J., Lagareiro Netto, A.A., et al. (2017) Artepillin C and Phenolic Compounds Responsible for Antimicrobial and Antioxidant Activity of Green Propolis and *Baccharis dracunculifolia* DC. *Journal of Applied Microbiology*, **122**, 911-920. <https://doi.org/10.1111/jam.13400>
- [41] Hammer, Ø., Harper, D.A.T. and Ryan, P.D. (2001) Past: Paleontological Statistics software Package for Education and Data Analysis. *Palaeontologia Electronica*, **4**, 9 p. [http://palaeo-electronica.org/2001\\_1/past/issue1\\_01.htm](http://palaeo-electronica.org/2001_1/past/issue1_01.htm)
- [42] De Melo, C.W.B., Bandeira, M.J., Maciel, L.F., Bispo, E.S., De Souza, E.O. and Soares, S.E. (2020) Chemical Composition and Fatty Acids Profile of Chocolates Produced with Different Cocoa (*Theobroma cacao* L.) Cultivars. *Food Science and Technology*, **40**, 326-333. <https://doi.org/10.1590/fst.43018>
- [43] Alves, A.B. and Bragagnolo, N. (2002) Determinação simultânea de teobromina e cafeína em chás por cromatografia líquida de alta eficiência. *Revista Brasileira de Ciências Farmacêuticas*, **38**, 237-243. <https://doi.org/10.1590/S1516-93322002000200013>
- [44] Ramli, N., Yatim, A.M., Said, M. and Hok, H.C. (2001) HPLC Determination of Methylxanthines and Polyphenols Levels in Cocoa and Chocolate Products. *Malaysian Journal of Analytical Sciences*, **7**, 377-386.
- [45] Vertuani, S., Scalambra, E., Trotta, V., Bino, A., Malisardi, G., Baldisserotto, A. and Manfredini, S. (2014) Evaluation of Antiradical Activity of Different Cocoa and Chocolate Products: Relation with Lipid and Protein Composition. *Journal of Medicinal Food*, **17**, 1-5. <https://doi.org/10.1089/jmf.2013.0110>
- [46] Waterhouse, A.L., Sirley, J.R. and Donovan, J.L. (1996) Antioxidants in Chocolate. *The Lancet*, **348**, 834. [https://doi.org/10.1016/S0140-6736\(05\)65262-2](https://doi.org/10.1016/S0140-6736(05)65262-2)
- [47] Serra-Bonvehí, J. and Ventura Coll, F. (1997) Evaluation of Bitterness and Astringency of Polyphenolic Compounds in Cocoa Powder. *Food Chemistry*, **60**, 365-370. [https://doi.org/10.1016/S0308-8146\(96\)00353-6](https://doi.org/10.1016/S0308-8146(96)00353-6)

- [48] Kealey, K.S., Snyder, R.M., Romaczyk, L.J., Geyer, H.M., Hammerstone, J.F., Buck, M.M., *et al.* (2000) Method for Producing Fat and/or Solids from Cocoa Beans. <http://www.google.com/patents/US6015913>
- [49] Natsume, M., Osakabe, N., Yamagishi, M., Takizawa, T., Nakamura, T., Miyatake, H., *et al.* (2000) Analyses of Polyphenols in Cacao Liquor, Cocoa and Chocolate by Normal-Phase and Reverse-Phase HPLC. *Bioscience Biotechnology and Biochemistry*, **64**, 2581-2657. <https://doi.org/10.1271/bbb.64.2581>
- [50] Adamson, G.E., Lazarus, S.A., Mitchell, A.E., Prior, R.L., Cao, G., Jacobs, P.H., *et al.* (1999) HPLC Method for the Quantification of Procyanidins in Cocoa and Chocolate Samples and Correlation to Total Antioxidant Capacity. *Journal of Agricultural and Food Chemistry*, **47**, 4184-4188. <https://doi.org/10.1021/jf990317m>
- [51] Luna, F., Crouzillat, D., Cirou, L. and Bucheli, P. (2002) Chemical Composition and Flavor of Ecuadorian Cocoa Liquor. *Journal of Agricultural and Food Chemistry*, **50**, 3527-3532. <https://doi.org/10.1021/jf0116597>
- [52] Ramirez-Sanchez, I., Maya, L., Ceballos, G. and Villarreal, F. (2010) Fluorescent Detection of (-)-epicatechin in Microsamples from Cacao Seeds and Cocoa Products: Comparison with Folin Ciocalteu Method. *Journal of Food Composition and Analysis*, **23**, 790-793. <https://doi.org/10.1016/j.jfca.2010.03.014>