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Antioxidant and Chemical Activity of South American Chocolate

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Abstract

Cocoa (Theobroma cacao L.), and its derived product chocolate, are foods with recognized beneficial health properties, mainly associated with their high content of polyphenols and other bioactive compounds. In Perú there are several companies engaged in the manufacture of products based on Peruvian cacao. There is for example La Ibérica, a Peruvian company located in Arequipa, with over 100 years of recognized experience. The aim of this study was to analyze the chocolate (52% cocoa solids) of La Ibérica, in order to assess its nutritional potential and content of health beneficial compounds. Proximate analysis of chocolate showed high carbohydrate (53.9%), fat (32.7%) and protein (6.5%) contents. The fatty acid profile, determined by gas chromatography, showed mainly the presence of palmitic (26.5%), stearic (32.0%) and oleic (38.3%) acids. The concentration of theobromine (0.4 g/100g chocolate), assessed by HPLC chromatography, was 2.4 times greater than caffeine (0.17 g/100g chocolate). The content of total phenolic compounds, quantified by spectrophotometric method, was 1.5%. The epicatechin concentration, measured by HPLC chromatography, was 1.9 times greater than catechin (63.7 and 34.2 mg/100 g chocolate, respectively). The antioxidant activity in the DPPH test, expressed as median effective concentration, was 0.14 mg/mL. Antioxidant activity in the ORAC test was 489.1 µmol Trolox equivalents/g chocolate. This chocolate contains a good amount of methylxanthines and antioxidant compounds that may be beneficial for the prevention of cardiovascular diseases. However, moderate consumption of this product is recommended, due to its high energy value and fat content.

Keywords: antioxidant; chocolate; fatty acids; methylxanthines; phenolics;

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1. Introduction

Cocoa (Theobroma cacao L.) and chocolate, are promising foods due to their beneficial health properties which can be explained by their high content of polyphenols and other bioactive compounds [1,2]. The main nutritional ingredients of cocoa beans are fat, carbohydrates, proteins and minerals; they also contain other bioactive compounds such as xanthines and polyphenolic compounds [3]. Consumption of cocoa flavonoids can improve aspects of cognitive function, improve cerebral blood flow and reduce the risk of coronary disease [2,4].

Chocolate has polyphenolic compounds known as flavan-3-oles or catechins and procyanidins. Certain studies have shown that the consumption of polyphenols in chocolate lead to significant improvements in long-term cardiovascular health such as blood pressure, vascular tone, endothelial function, insulin resistance and glucose tolerance [2,5].

Cocoa, the main component of chocolate, is rich in methylxanthines such as theobromine and caffeine. These alkaloids are pharmacologically active and have the physiological capacity to modulate the central nervous system [2,6]. They also act as vasodilators and have toning and antineural properties [7]. In Peru there are several companies dedicated to the manufacture of products associated with gastronomy, pastry and confectionery. One of them is "La Ibérica", a company from Arequipa region recognized as one of the most outstanding brands in the chocolate industry nationwide. There are no previous studies on the nutritional composition and antioxidant activity of a Peruvian chocolate. The objective of this study is to analyze this chocolate with 52% cocoa solids in order to know their nutritional potential and antioxidant activity.

2. Materials and Methods

2.1 Proximate analysis

Moisture content: Approximately 2 g of sample was weighed into a pre-weighed dish and moisture analyzer equipment (Sartorius Moisture Analyzer, Model MA35,) was programmed at a temperature of 135 °C for 6 minutes. The final weight and the percentage of humidity provided by the equipment were recorded.

Protein content: The Kjeldahl method was used for protein determination, consisting of 3 stages: digestion, distillation and titration. For the calculation of the percentage of proteins in the sample, the value of 6.25 was used as a protein factor.

Fat content: The percentage of fat in the sample was determined by the Soxhlet method. The amount of fat extracted from 2 g of sample by petroleum ether was calculated and reported as a percentage.

Total Fiber content: A fiber extractor was used in which 0.5 g of defatted sample was subjected to acid hydrolysis with 1.25% sulfuric acid, followed by a basic hydrolysis with 1.25% sodium hydroxide. The obtained residue was weighed and reported as percentage of total fiber in the sample.

Ash content: 0.5 g of simple was weighed into a dry crucible and subjected to total calcination in the muffle furnace (Thermo Scientific™ - Model FB1310M) at 525 °C for 3 hours. The weight of the residue obtained was calculated and reported as a percentage of ash in the sample.

Carbohydrate content: The percentage of carbohydrates in the sample was calculated by difference, starting from a value of 100% and subtracting the other components (moisture, fat, fiber, protein and ash) as percentages [8].

2.2 Fattyacids profile

About 100 mg of previously extracted fat sample was weighed into a 14 mL Falcon tube, 10 mL of n-pentane was added to dissolve the sample and then 100 µL of 2N potassium hydroxide in methanol. The Falcon tube was vortexed for 1 min and centrifuged for 6 min at 5000 rpm at 10 °C. A volume of 1.5 mL of the supernatant was transferred to a vial through a Phenomenex 0.45 µm filter.

Subsequently, the vial was injected into the GC-MS (Agilent Technologies 7890 A-5975C) with helium as a carrier gas and a DB-5MS column (60m x 250µm x 0.25µm), injection temperature 250 °C, detector temperature MS of 230 °C, Split ratio 200: 1 and a total running time of 36 min. The column was maintained at 100 °C for 1 min, then the temperature was increased 20 °C/min to 190 °C, maintained for 1 min, the temperature was further increased 3 °C/min to 210 °C, kept for 1 min and then increased to 1 °C/min to the final temperature of 230 °C. The injection volume of the sample was 5 µL. The identification of the compounds was carried out by comparing the mass spectra of the fatty acids with the mass spectra provided by the NIST 08 library and with standards of fatty acid methyl esters [9].

2.3 Theobromine and Caffeine

0.2 g of defatted sample was weighed and placed in a round-bottom flask, and then 40 mL of ultrapure type I water was added and refluxed for 30 min. The extract was centrifuged for 5 minutes at 5000 rpm and brought to a final volume of 50 mL in a volumetric flask. Next, 2 mL of the aqueous solution was placed into the previously conditioned Sep-pak C18 filter and the sample was eluted with 10 mL of chloroform. The solvent was evaporated and the residue obtained was dissolved with 5 mL of ultrapure water type I and transferred to a vial for injection in the HPLC-DAD. The elution system was acetonitrile-water (20:80), isocratic, with a running time of 8 min, injection volume of 20 µL and a flow rate of 1.2 mL/min.

For the preparation of the calibration curve, stock solutions of theobromine (0.15 mg/mL) and caffeine (0.1 mg/mL) were prepared, 5 dilutions from these stock solutions were evaluated in the same way as the samples [10].

2.4 Total Phenolics

0.5 g of defatted sample was weighed into a 15 mL Falcon tube, 5 mL of 80% ethanol was added, the mixture was stirred for 5 minutes on ultrasound equipment and centrifuged at 10 °C, 5000 rpm, for 10 min. The supernatant was transferred to a 25 mL volumetric flask (the previous procedure was repeated 3 times) and brought to volume with 80% ethanol. Then 50 µL of this solution was taken and mixed with 1000 µL of 10% Folin Ciocalteu plus 1000 µL of 7.5% Na_2CO_3 and 970 µL of ultrapure type I water.

The mixture was allowed to stand for 15 min at room temperature and in a dark place. Finally, the absorbance of the solution was read at 750 nm in a spectrophotometer (Spectroquant® Pharo 300). For the preparation of the calibration curve of gallic acid, solutions of this standard were prepared at different concentrations, which were analyzed in the same way as the sample. The total phenol content was expressed as milligram-equivalents of gallic acid per gram of sample (mg AG/g) [11].

2.5 Catechin and Epicatechin

Approximately 0.5 g of defatted sample was weighed in a centrifuge tube, 5 mL of 80% ethanol was added and the mixture was sonicated for 15 min and then centrifuged for 10 min at 5000 rpm, 10 °C; the supernatant was transferred to a 10 mL volumetric flask. The above procedure was repeated and the solution was brought to volume with 80% ethanol. A volume of 1.8 mL of the solution was then transferred to a vial through a Phenomenex 0.45 μm filter and 10 μL of the filtrate was injected into the ultra high performance liquid chromatograph (UHPLC Ultimate 3000) with a RP column 18e (LiChroCART® 250-4) at a temperature of 35 °C. The mobile phase used was acetonitrile - 2.5% acetic acid at a flow of 1.5 mL/min. The wavelength for detection of compounds was 280 nm. The total time of the chromatographic run was 30 min.

For the preparation of the calibration curves for catechin and epicatechin, stock solutions of both substances were prepared at a concentration of 0.2 mg/mL. Six dilutions were made from these solutions, which were analyzed as previously described for the sample [12].

2.6 Antioxidantactivity (DPPH test)

0.5 g of the defatted sample was weighed into a Falcon tube and 5 mL of 80% ethanol was added, sonicated for 5 min and centrifuged for 10 min at 5000 rpm, 10 °C. The supernatant obtained was placed in a 25 mL volumetric flask (this procedure was repeated 3 times). All supernatants were pooled and brought to volume with 80% ethanol. From this solution dilutions were prepared with concentrations of 0.063 to 0.625 mg/mL, each of them were mixed with 3950 μL of DPPH (2,2-diphenyl-1-picryl hydrazyl) and enough 80% ethanol to complete a final volume of 4000 μL. The test tubes were then placed in the dark for 30 min. To prepare a DPPH control solution, 1.97 mg of DPPH was dissolved in 5 mL and then brought to volume with 80% ethanol in a 50 mL volumetric flask. The reduction of DPPH was determined at a wavelength of 517 nm in a spectrophotometer. The percentage of antioxidant activity was calculated with the following formula: % AA = 100 x (1 - (Absorbance sample / Absorbance DPPH control)). The median effective concentration (EC_{50}) of the antioxidant activity was obtained from the curve of the percentage of antioxidant activity versus concentration of the sample (mg/ mL) [13].

2.7 ORAC (Oxygen Radical Absorbance Capacity)test

250 mg of deffated sample was weighed in a 14 mL Falcon tube. An extraction was carried out in 5 mL of the mixture acetone: distilled water: acetic acid (35: 14.9: 0.1) with sonication for 30 min. The extract was centrifugated for 10 min at 3000 rpm, 4 °C; then the supernatant was diluted 1/625withbuffer phosphate.

For the reactions buffer phosphate 75 mmol/L was used (final reaction mixture: 200 μL). The reaction mixture consisted of 20 μL sample and 120 μL fluorescein (70 nmol/L final concentration). The solutions were placed in the wells of black 96-well plates. The mixture was preincubated for 15 min at 37 °C before adding 60 μL 2,2'-Azobis (2methylpropionamidine) dihydrochloride (AAPH) solution (12 mmol/L final concentration). The fluorescence was measured in a microplate reader (Hidex Chameleon) every minute for 80 min. Excitation and emission filters were 485 nm and 520 nm, respectively. A blank using phosphate buffer instead of the sample and eight calibration solutions using Trolox (1-8 μmol/L final concentration) were also carried out in the same run. Antioxidant curves (fluorescence versus time) were normalized to the curve of the blank by multiplying original data by the factor (fluorescence of blank at t=0/fluorescence sample at t=0). From the normalized curves, the area under the fluorescence decay curve (AUC) and the net AUC were calculated as follows:

$$AUC = 1 + \sum_{i=2}^{i=n} f_1/f_0$$

net AUC = AUCsample - AUCblank where f_0 was the initial fluorescence reading at 0 min and f_i was the fluorescence reading at time i. Linear regression equations between net AUC and antioxidant concentration were calculated for all the samples. Antioxidant activity (ORAC value) was calculated by using a Trolox calibration curve [14].

3. Results and Discussion

Analysis of bioactive compounds and antioxidant activity of cocoa and chocolate are important in order to assess the nutritional potential and health benefits of these products.

Cocoa solids (cocoa paste), cocoa butter, sugar and lecithin as an emulsifier are the main ingredients for the elaboration of chocolate. Therefore, the main nutritional components in chocolate are carbohydrates, fat and proteins. Salinas & Bolívar investigated various types of Venezuelan chocolates; they contain carbohydrates in a range of 48-52%, fat 30-37% and proteins 5-13% [15]. Torres-Moreno et al. investigated chocolates from Ghana and Ecuador, despite being from different locations, the percentages of carbohydrates, fat and protein were very similar (60.0, 30.5 and 6.4%, respectively) [16]. Chocolate 52% from La Ibérica showed a nutritional composition similar to that of chocolates from Ghana and Ecuador, with carbohydrate, fat and protein contents of 54, 33 and 6%, respectively (Table 1). Its energy value calculated for 100g of chocolate is 539.4 kcal; this value must be taken into account when calculating consumers' diets.

Table 1: Proximate analysis of chocolate 52%

Component	Content (%)
Moisture	1.82 ± 0.03
Ash	1.81 ± 0.03
Protein	6.47 ± 0.04
Fat	32.72 ± 0.21
Fiber	3.24 ± 0.09
Carbohydrates	53.95 ± 0.07

Cocoa fat contains mainly triglycerides of oleic, stearic and palmitic acids, these fatty acids are also the main ones in chocolate. For example, chocolates from Ghana and Ecuador showed concentrations of palmitic, stearic, oleic and linoleic acid equal to 28.0, 35.2, 32.8 and 2.2% [16].
La Ibérica´s chocolate contained similar percentages of said fatty acids (Table 2). The highest percentage consisted of saturated (58.8%), followed by mono and polyunsaturated fatty acids (38.0 and 2.4%). Although the content of stearic acid in chocolates is high, it is a saturated fatty acid considered non-atherogenic, since the excess is converted to oleic acid in the liver by a desaturase enzyme [15].

Table 2: Fatty acid profile of couverture chocolate.

Fatty acid	t_R (min)	Relative Concentration (%)
Miristic (14:0)	15.95	0.05
Palmitoleic (16:1)	20.97	0.21
Palmitic(16:0)	21.84	26.55
Margaric (17:2)	25.38	0.18
Linoleic (18:2)	28.45	2.42
Oleic (18:1)	29.08	38.35
Stearic (18:0)	30.25	31.99

According to Todorovic et al. [3], 50 g of dark chocolate or 30 g of cocoa powder have enough theobromine to produce a neurophysiological effect. Although caffeine stimulates the CNS five times more than theobromine, the latter has a longer life and is found in greater quantities in chocolate [17, 18].
Various types of chocolate made in Serbia had theobromine contents between 0.5 to 2.2 g/100g and caffeine between 0.03-0.10 g/100g. Chocolate 52% from La Ibérica, presents lower values of theobromine (0.4 g/100g), and higher caffeine (0.17 g/100g). The content of methylxanthines in the sample of La Ibérica is much higher than for example Milka milk chocolate (theobromine: 0.1 g/100g, caffeine: 0.01 g/100g) [19].
Numerous studies state the beneficial effects of cocoa polyphenols on cardiovascular health. In a randomized controlled trial conducted by Pereira et al. in 60 healthy young volunteers, it was established that daily consumption of 10 g of chocolate 70% per day induces several beneficial effects on blood pressure and vascular function; therefore, chocolate with a high content of phenolic compounds may be a promising functional food for the primary prevention of cardiovascular diseases [20].
Pimentel et al. demonstrated that 49 g of a dark chocolate has the same amount of flavonoids as 196 ml of Tannat wine, which is the recommended daily amount to produce beneficial health effects in an adult of 70 kg of weight [21].
Chocolate 52% of La Ibérica showed a high content of phenolic compounds (1.5 g/100g), higher than that reported for various chocolates made in Serbia (0.7 - 1.3 g/100g) [3]. The difference is mainly due to the cocoa variety and the process of making chocolate. It is known that the amount of polyphenols depends on the cocoa genetics and the time of fermentation chosen for the post-harvest process. The concentration of polyphenols in cocoa decreases as the fermentation time increases [22]. Also, the concentration of polyphenols in chocolate depends on the percentage of cocoa solids used [23]. The flavanolsare a subgroup of the flavonoid family, whose main representatives are catechin and epicatechin. Some studies report epicatechin as the most active compound, responsible for the effects on cardiovascular health associated with cocoa and chocolate [24]. Alañón et al. evaluated 41 samples of various chocolate types obtained in the markets of Reading, UK. The contents of epicatechin and catechin in these chocolates were 8.1-89.5 and 1.840.8 mg/100g, respectively. The content of these flavanols was always higher in dark chocolates than in milk chocolates [2]. A study on dark chocolates from Nestlé-Turkey showed lower values of epicatechin and catechin (4.7-7.9 and 2.4-6.9 mg/100 g, respectively) [24]. The contents of catechin and epicatechin in La Ibérica´s chocolate

(34.2 ± 0.0 and 63.7 ± 0.1 mg/100g, respectively) were similar to that of the chocolates of Reading.

The antioxidant activity was measured by the DPPH and ORAC test. Both methods are frequently used to measure the ability to neutralize radicals in an *in vitro* system and give a preliminary idea of what might happen in an *in vivo* system.

In the DPPH test, the activity is expressed as mean effective concentration (EC_{50}), meaning that at a lower EC_{50}, better antioxidant activity. Vertuani et al. evaluated the antioxidant activity (DPPH test) of various milk and dark chocolates. The antioxidant activity of dark chocolates (EC_{50} = 0.28-0.81 mg/mL) was better than those of milk chocolates (EC_{50} = 3.82-7.72 mg/mL) [25]. La Ibérica´s chocolate (EC_{50} = 0.14 ± 0.00 mg/mL) showed better antioxidant activity than chocolates evaluated by Vertuani et al.; however, its potency is much lower than ascorbic acid (EC_{50} = 0.005 mg/mL).

The antioxidant activity in the ORAC test is reported through the comparison with the antioxidant Trolox, a vitamin E analogue. The ORAC test is used for various samples (food, beverages, plasma, etc.) and lately, it has also been applied to cosmetics and nutraceuticals [26]. The value obtained for thechocolate 52% of La Ibérica (489.1 ± 8.84 µmol TE/g) was higher compared to milk chocolates (71.3 µmol TE/g), but lower than dark chocolates (1031.9 µmol TE/g) evaluated by Wu et al [27].

4. Conclusions

The chocolate 52% of La Ibéricashows a high content of carbohydrates (54%) and fat (33%). The fatty acid profile is characterized mainly by high concentrations of palmitic (27%), stearic (32%) and oleic (38%) acids. The chocolate contains a good amount of methylxanthines (theobromine and caffeine) and antioxidant phenolic compounds that may be beneficial for the prevention of cardiovascular diseases. However, moderate consumption of this product is recommended, due to its high energy value and fat content.

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