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ORIGINAL PAPER

Influence of geographical origin in the physical and bioactive parameters of single origin dark chocolate

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Abstract

Dark chocolate presents exclusive characteristics that make it a food product with worldwide consumption and also as an ingredient in several food industries. Although chocolate is an energy-dense food, it is also rich in bioactive compounds and recent studies have demonstrated health benefits from a moderate consumption. Therefore, the quantification of the bioactive compounds of different types of cocoa, from different geographical origins, is of great importance to recognize the importance of single origin dark chocolate from the nutritional point of view. Dark chocolate produced from Amelonado variety presented higher values of hardness (5592 g), plastic viscosity (2.87 Pa.s) and yield value (12.91 Pa). Both dark chocolates from Peru, Piura Blanco and Chuncho, presented higher results in total phenolic content, total antioxidant capacity, caffeine and vitamin E. Additionally, sample Piura Blanco presented a higher content of theobromine (720.7 mg/100 g), lactic acid (1153.2 mg/100 g), succinic acid (679.4 mg/100 g) and oxalic acid (468.5 mg/100 g). On the other hand, chocolate from São Tomé presented a higher content of sucrose (38.22%) and SFA (62.38% of total fat). The results obtained demonstrate the existence of heterogeneity in cocoa varieties, supporting decision-makers in the selection of the most suitable cocoa for specific market needs.

Keywords Chocolate · Single origin · Cocoa · Amelonado · Piura Blanco · Chuncho

Introduction

The unique characteristics of cocoa make it an important food product, with large consumption worldwide, as an ingredient of desserts, pastries, or chocolate-covered products [1], but where the simple chocolate bar is one of the most popular. Since 2016/2017, the global production of cocoa beans has presented steady values around 4.6—4.7 Mtonnes per year, however for the first time a record production of over 5 Mtonnes was reported in season 2020/21. Africa provides 73.4% of the world's production, mostly from the Ivory Coast and Ghana, the two largest cocoa producers and representing around 58% of world production [2]. The market makes a difference between "bulk" and "fine-flavor" cocoa. Bulk cocoa represents around 95% of the cocoa market [3] and is composed mostly of the *Forastero* variety, more productive, with a higher yield than the *Criollo* variety, more vigorous and less susceptible to diseases. Chocolate products using *Forastero* present rich chocolate flavour but miss complexity or fruity notes. The *Criollo* variety is one of the "fine-flavour" cocoa, presenting lower productivity, and higher susceptibility to diseases/external attacks, but presenting a higher aromatic flavour, with a nutty and fruity profile, therefore highly priced. Such differences may be a consequence of a genetic condition, however, climate, rainfall, sunshine hours and location also contribute to the

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formation of the final flavour [4, 5]. Dark chocolate consists of a suspension of non-fat particles, including a blend of cocoa beans with sugar, in a continuous fat phase of cocoa butter, using lecithin as an emulsifier [6]. The high content of carbohydrates and fat, mostly from cocoa butter and sugar, is the main responsible for the high energy density of dark chocolate, above 470 kcal/100 g [7], contributing to obesity and cardiovascular diseases if excessively consumed [8]. Dark chocolate is also known for including a set of bioactive components with antioxidant properties such as phenolic compounds, including catechins, epicatechin, anthocyanins, proanthocyanidins [9] and flavonoids [10]. Phenolic compounds, or polyphenols, are one of the largest groups of substances in plants including more than 8000 phenolic structures known actually, result of the secondary metabolites of plants and stored in the pigment cells of the cotyledons of cocoa beans, coloured from white to deep purple [11] and protecting beans from damage and diseases [12]. Such polyphenols are identified with health benefits such as protective effects against cell damage, increased immune response, beneficial effects on the cardiovascular system and antioxidant protection [7]. The antioxidant properties of theobromine and caffeine, in cocoa and chocolate, are also recorded in the literature, presenting the capacity to stimulate the central nervous system, bronchial relaxation, increased secretion of gastric acids and diuresis [13, 14]. However, the incidence of such components in cocoa beans is related to variety, geographical location [14, 15], post-harvest treatments [16] and industrial processing [17, 18]. The industrial processing of dry cocoa beans into chocolate starts with roasting, crucial for the development of flavour, where the intensity depends on the time–temperature binomial, around 5–120 min and 120–150 ºC. However, roasting temperature contributes to a significant reduction in phenolics and related antioxidant activity [11, 16, 19]. The next stage is grinding, usually including two stages: i) conversion of solid nibs into a dark brown fluid named "liquor", and ii) reduction of particle size [11]. Refining used roll refiners with controlled temperature, for reducing particle size below 30 µm [5] and producing a smooth texture. Conching is carried out above 50 ºC for several hours, for decreasing moisture content and undesirable volatile compounds (e.g. such as acetic acid), deepening the color, aroma and contributing to the proper texture, viscosity and flavour. During this stage, cocoa butter and lecithin can be added to adjust viscosity [5]. Tempering is a process where melting and cooling of chocolate follows defined steps to obtain the form V of cocoa butter, the most suitable crystalline structure of cocoa butter among six different polymorphic forms [20]. Such steps include the pre-crystallization of a small proportion of triglycerides, around 1–3% of the total, through four key steps: i) melting at 50 ºC; ii) cooling to crystallization (32 ºC); iii) crystallization (27 ºC), and iv) conversion of unstable crystals (29–31 ºC). Tempering influences subsequent stages, such as demoulding, but affects mostly the characteristics of the final product [5]. A proper tempered chocolate presents a shiny and even-coloured surface, the typical snap, melts smoothly inside the mouth, while untempered chocolate presents a greyish haze [21] or brown coloured surface [7] and a chewy to grainy texture [20].

Therefore, the present study was set up to evaluate physico-chemical parameters, rheological behaviour, nutritional composition, bioactive compounds, organic acids and methylxanthines, of single-origin commercial dark chocolates from Peru and São Tomé.

Materials and methods

Sample preparation and reagents

Chocolate samples with different percentages of cocoa and geographical origin were used, as presented in Table 1, and obtained from the company "Melgão Cacau e Chocolate" (Montemor-o-Novo, Portugal). Chocolate samples with peruvian cocoa were identified as "Chocolate Piura Blanco" (variety *Nacional*) and "Chocolate Chuncho" (variety *Criollo*), while the sample with cocoa from S. Tomé was identified as "Chocolate Amelonado" (variety *Forastero*). Samples were processed and analysed in triplicate.

All reagents, standards, and solvents were obtained from Merck, Sigma or Panreac (Lisbon, Portugal) and were of analytical or chromatographic grade. The mobile phases were prepared according to Panda et al. [7].

Determination

The viscosity and yield value of the molten chocolates were determined at 40 ºC using a viscosimeter (VT 550, Thermo Scientific HAAKE, Waltham, MA, USA) equipped with a MV-DIN concentric cylinder geometry (bob diameter: 19.36 mm, bob height: 58.08 mm; cup diameter: 21.00 mm), according to the International Office of Cocoa [22].

The determination of hardness (in grams) was performed at 20 ºC, using a texture analyser (TA.XT Plus100, Stable

Table 1 List of chocolate samples

Sample	Code	Origin	Declared cocoa content (%)
Chocolate Piura Blanco	Ch-PB	Perú	74
Chocolate Chuncho	Ch-C	Perú	70
Chocolate Amelonado	Ch-A	S. Tomé	62

Micro Systems, Godalming, UK) equipped with a 3 mm Ø aluminium probe, according to Monteiro et al. [23].

Color determination

The evaluation of colour was determined with a portable colorimeter (CR400, Minolta, Osaka, Japan), with reference to CIELAB system. The calculation of the parameter whiteness index (WI) was performed according to Monteiro et al. [23].

Determination of titrable acidity and pH

Titrable acidity was measured according to method 970.21 [25]. For pH measurement, a 1 g portion of the chocolate sample was dispersed in 10 mL of distilled tap water, and the pH value was registered using a digital pH meter (HI11312, Hanna Instruments, Woonsocket, RI, USA).

Determination of fat content and fatty acids composition

Chocolate samples were grinded and homogeneized, and then, 5 g of the homogeneized samples were placed in a Soxhlet apparatus and extracted with petroleum ether (40—-60 ºC), under reflux, for 4 h. The extract was evaporated in a rotavapor at a temperature of 40 ºC and placed in an oven at 80 ºC for a period of 45 min. After cooling the extracted fat was weighed for fat content calculation.

Fatty acid methyl esters (FAME), for gas chromatographic analysis, were prepared by transmethylation with KOH in methanol solution (2 mol/L). Separation and quantification of FAME were performed in a gas chromatograph with a flame ionization detector (GC-FID), Trace GC 2000, from Thermo Quest (CE Instruments, Rodano, Milan, Italy). The chromatographic column was a DB 23 (J & W Scientific, Agilent Technologies, Santa Clara, CA, USA) with 60 m length, with 0.25 mm I.D. and 0.25 µm phase thickness. The oven temperature was raised from 70 ºC up to 195 ºC (isotherm of 30 min) at a rate of 5 ºC/min and then raised to 225 ºC (isotherm of 60 min), at the same rate. The injector temperature was set to 220 ºC and the detector temperature to 280 °C. Helium was used as carrier gas, at a constant pressure of 70 kPa. The fatty acids were identified by comparison of the relative retention times (RRT), the relation between the retention time (RT) of each fatty acid to the RT of C16 (methyl hexadecanoate), obtained in the samples, with those obtained in a standard mixture of 52 FAME (Nu-Chek-Prep Inc., Elysian, MN, USA). Quantification was made after converting the relative areas percentages (% area) into weight percentages of total fatty acids (g/100 g), by multiplying % area with the correction factors, calculated from the analysis, of a standard mixture of known composition, in the same conditions (52 FAME -Nu-Chek-Prep Inc., Elysian, MN, USA).

Determination of moisture content

Moisture content was performed by the gravimetric method 931.04 [25], weighing accurately 2 g of sample (AG245, Mettler Toledo, Schweiz, Switzerland).

Determination of total phenolic content, total antioxidant capacity and HPLC phenolic compounds profile

The extraction was performed in triplicate with Polytron homogenization (Ika, Ultra-Turrax T25, Staufen, Germany), for 1 min, using a 1.8 g chocolate sample in 15 mL methanol (100%), followed by 5 min ultrasonic bath (Bransonic, Branson 5200, Branson, MO, USA), and leaving overnight on a rotary shaker (Robbins Scientific, model 16,021, Sunnyvale, CA, USA) inside the refrigerator at 4 ºC (Radiber, Sa, UKS-5000, Barcelona, Spain), followed by centrifugation during 20 min at 4500 × *g* (Sigma, model 2K15 with rotor 12139H, Osterode am Harz, Germany) and the clear supernatant was collected and maintained at -20 ºC (Bosch, KGS3722, Stuttgart, Germany) till methanolic extracts further analysis [7].

Total phenolic content (TPC) was analysed spectrophotometrically at 725 nm according to Panda et al. and Swain and Hillis [7, 26], with 50 µL methanolic extract dilution with 100 µL methanol, 2400 µL ultrapure water, and 150 µL Folin-Ciocalteu reactant 0.25 molmol/L, followed by vortex shaking (Heidolph, Reax top, Staufen, Germany) and 3 min incubation at room temperature, followed by the addition of 300 µL sodium carbonate 0.5 molmol/L, vortex and let incubate for 2 h, in dark at room temperature. Calibration was done with gallic acid from 3 to 300 µg/mL. Results were expressed as mg of gallic acid equivalent (GAE) per 100 g of sample.

Total antioxidant capacity (TAC) was assessed by the colorimetric methods DPPH (2,2-Diphenyl-1-picrylhydrazyl), ORAC (Oxygen Radical Absorbance Capacity) and FRAP (Ferric Reducing Antioxidant Power) assays, according to slightly modified procedures of Brand-Williams et al., Ou et al. and Benzie and Strain [27–29]. Calibration curves were done with Trolox (from 0 to 600 µmol/L) for each different antioxidant capacity assay and results were expressed in µmol of Trolox Equivalents (TE) per 100 g sample.

The DPPH method, in whose the purple compound colour turns yellow after reduction in the presence of antioxidant compounds, was performed according to Monteiro et al. [24], after incubation of 110 µL methanolic extract with 40 µL water and 2850 µL diluted DPPH for 2 h in dark and at room temperature, followed by absorbance reading at

580 nm wavelength in a spectrophotometer (Thermo Fisher Scientific, Unicam RS232C, Waltham, MA, USA).

For oxygen radical absorbance capacity (ORAC), chocolate samples were prepared in a grinder (Moulinex A980, Écully, France), and a 1.0 g sample was first extracted three times with 10 mL of hexane, and the obtained residue was in turn extracted three times with 10 mL of methanol and the three resulting volumes combined. The methanolic extract from each sample was properly diluted to fit the calibration curve, as described by Ou et al. and Al-Duais et al. [28, 30]. The analysis were performed in 96 well microplates (Greiner) for fluorescence analysis, and a microplate reader (FluoStar Optima, BMG Labtech, Offenburg, Germany) was used with 485 nm and 520 nm filters, excitation, and emission, respectively.

Ferric Reducing Antioxidant Power (FRAP) assay, was developed by Benzie and Strain [29], and is based on the rapid reduction of ferric-tripyridyltriazine (FeIII-TPTZ) by the antioxidants in sample, forming a blue-coloured product, the ferrous-tripyridyltriazine (FeII-TPTZ). Briefly, 20 µL of methanolic extract were diluted in 80 µL MeOH, and 3 mL of FRAP solution (heated to 37 °C), and after incubation at 37 °C, for 20 min, the absorbance at 593 nm wavelength was read.

HPLC phenolic compounds profile was performed according to Pereira et al. [31]. The HPLC system (Waters, Milford, MA, USA) was equipped with a refrigerated automatic injector (Alliance 2690) with a quaternary pump, column oven (Jetstream 2 plus) and UV–Vis diode array detector (Waters 996), with acquisition and control system software for chromatography (Empower Pro 2002 *v.*5.0, Waters, Milford, MA, USA). The phenolic compounds quantification was based on an external standard curve using mixed standard solutions, ranging from 5 to 150 mg/L. The hydroxycinnamic acids (chlorogenic, caffeic, coumaric, and ferulic) and resveratrol, were integrated at 325 nm wavelength; the hydroxybenzoic acids (gallic, *p*-hydroxybenzoic, vanillic, and syringic), catechin, procyanidin B_1, naringenin, naringin and ellagic acid at 280 nm; and rutin, quercetin, and kaempferol were integrated at 340 nm.

HPLC methylxanthines profile determination

Methylxanthines extraction and HPLC profile were done in the same Waters HPLC as for phenolic compounds and organic acids, with procedures adapted from Panda et al. [7]. Separation was performed in a C18 (4.6 × 250 mm, 5 µm) column (Waters, Spherisorb ODS2, Milford, MA, USA) kept at 30 °C. Identification was done based on compound retention time and characteristic absorption spectrum. Integration was done at 205 nm, and quantification was based on the external standard technique, using mixed standards solutions in the concentration range of 5 to 200 mg/L, 5 to 150 mg/L, and 0.2 to 100 mg/L for theobromine, caffeine, and theophylline, respectively.

HPLC organic acids profile determination

Organic acids extraction was performed as described by Panda et al. [7]. The analysis was done using the same HPLC system reported before. Separation was made in a Rezex™ ROA column (Phenomenex, Torrance, CA, USA) as described by Pereira et al. [31]. Quantification was based on the external standard technique, from a standard curve of peak area versus concentration of oxalic, citric, tartaric, malic, lactic, succinic, and acetic acids mix standard solutions ranging from 5 to 160 g/L (for oxalic acid) and 20 to 2000 g/L (for the others organic acids).

HPLC free sugars and sugar alcohols determination

Free sugars extraction was done according to Panda et al. [7], weighing 5 g of chocolate sample dissolved with hot ultrapure water followed by protein precipitation with Carrez I and II solutions (2 mL each), made up the volume to 100 mL with ultrapure water and filtrated 0.45 µm nylon syringe filter (Alwsci, Labfil, Lisbon, Portugal). The analysis of individual sugars and sugar alcohols was performed in an HPLC equipped with a solvent module 126, a column oven (Beckman System Gold, Brea, CA, USA), an autosampler (Spark, Mi, Emmen, Netherlands), a refractive index detector (RID) (Waters, RID 2414, Milford, MA, USA), connected to the acquisition and control system software, 32Karat software v.8 (Beckman Coulter, Brea, CA, USA). Separation was made in a cation (calcium) exchange column (Waters Sugar-Pak I, 6.5 × 300 mm, 10 µm, Milford, MA, USA) held at 90 ºC. EDTA calcium 0.1 mmolmol/L aqueous mobile phase, with 0.5 mL/min flow rate, 20 µL injection volume, and 20 min run time, were used. Identification was done by comparison with standards-specific retention times. Quantification was based on an external standard calibration technique, standard curves of peak area versus concentration, and a range of 0 to 5 g/L mix of standard solutions of sucrose, glucose, fructose, and mannitol were used. Total sugar content was considered the sum of the free sugars and sugar alcohols determined individually and was expressed in % (w/w).

Vitamins E, A, D_2 and β-carotene determination

The quantification of vitamins E, A, D_2 and β-carotene was determined by HPLC and based in the method described by Roseiro et al. al [32]. with some changes. The saponification of 5 g of each sample, added of 0.2 g of L-ascorbic acid, was carried out in a water bath at 80 ºC for 15 min with 20 mL of 11% KOH solution in a mixture of ethanol

and water (55:45, v/v). After saponification, samples were cooled in tap water for 1 min and 6 mL of water and 12 mL of 25 µg/mL BHT solution in n-hexane were added. The samples were vigorously mixed for 2 min and centrifuged at 1500 × g for 5 min, in order to accelerate the separation of phases. The upper layer (n-hexane) was then dried over anhydrous Na_2SO_4 and filtered through a 0.45 µm hydrophobic membrane (Acrodisc, Waters, Milford, MA, USA). The chromatographic separation was performed using a normal-phase silica column (Waters Silic 125 mm × 4.6 mm i.d., 5 µm, Waters, Milford, MA, USA), with fluorescence detection for tocopherols (excitation wavelength of 295 nm and emission wavelength of 325 nm) and UV–Vis detection for beta-carotene (450 nm), retinol (325 nm) and vitamin D_2 (265 nm) in series, using 1% v/v isopropanol in n-hexane as solvent at a flow rate of 1 mL/min. The tocopherols (vitamin E), retinol, vitamin D_2 and beta-carotene contents were calculated based on the external standard technique.

Vitamins B determination

The quantification of vitamins B (thiamine, riboflavin, niacin, pantothenic acid and pyridoxine was determined according to Panda et al. [7].

Statistical analysis

The average, standard deviation, and 0.95 confidence interval values were determined. Experimental data were subjected to one-way ANOVA (pairwise comparison of means with Scheffé test) in order to compare the average values of different samples. A Principal Component Analysis (PCA) was used to study the inter-sample and inter-variable (nutritional attributes) relationships. All statistical analysis were carried out with IBM SPSS Statistics (Version 22).

Results and discussion

The behaviour of chocolate samples was studied under a controlled shear rate, at a temperature of 40 °C, and all chocolate samples exhibited a shear-thinning with a yield stress, related to the energy required to start the flow of chocolate [33]. The highest value for plastic viscosity was observed in chocolate Amelonado (2.87 Pa.s), followed by Piura Blanco (2.13 Pa.s) and Chuncho (1.36 Pa.s). A similar trend was observed in yield stress: higher in Amelonado (12.91 Pa), followed by Piura Blanco and Chuncho. The obtained values of plastic viscosity and yield stress are similar to those reported in the literature [33–35]. The relation between Casson parameters and the structure of chocolate has been reported by Glicerina et al. [36]. The increase in Casson parameters is related to the increase of solid particles in the matrix, such as sugar, leading to a higher number of contact points between particles, thus requiring more energy to initiate flow [36]. At the same time, the effect of fat is proportionately much higher for the plastic viscosity, where most of the fat is partially tied to the particle surface such as cocoa solids and sugar, with a large effect of lubricating the flow when it takes place, decreasing viscosity [37, 38].

The highest hardness value was observed on Amelonado (5592 g), followed by Chuncho (4224 g) and Piura Blanco (3797 g), similar to the reported in previous works with dark chocolate [39], using samples with comparable dimensions. Hardness is an attribute of texture that results from a predetermined microstructure and, therefore, depends not only on the composition but also on processing conditions, especially the hardening and crystallization of the fatty phase of chocolate. Thus, proper hardness determines the durability against physical damage and temperature changes [40].

The appearance is a major quality parameter for the chocolate industry and is traditionally evaluated by a sensory panel [41]. Nevertheless, several methodologies have been reported successfully these last years for instrumental measurement of chocolate, such as computer vision [42], colorimeter [43] and glossmeter [44]. The WI adimensional parameter is the most used colour parameter in chocolate storage, calculated from components L*, a* and b* from the CIELAB colour system. In the present work, Piura Blanco and Chuncho presented the highest WI values (27.06 to 27.10), followed by Amelonado (26.30), comparable to similar reports on dark chocolate [23, 43, 45, 46]. Under certain conditions, the visual and structural properties of chocolate are affected in a phenomenon known as “fat bloom”, where fat crystals, at a certain polymorphic form, affect incident light forming a whitish/greyish film covering the entire surface and making the appearance unacceptable for consumers [42]. The most accepted theory is a polymorphic transition from the V form to the more stable VI form or a re-crystallization of other forms after the fusion of the less stable polymorphic forms [47]. The obtained WI results are below reported values for dark chocolate with fat bloom [41], an indicator of proper tempering and temperature stability during storage.

Sugars and fat were the main components, representing 70 – 80% of the total weight, in agreement with previous studies using similar chocolates [7, 48]. Fat content presented higher results in Chuncho (43.70%) and Piura Blanco (43.23%), due to the higher cocoa liquor used in the formulation and, consequently, higher cocoa butter content [5]. As expected, total sugars presented the highest results in Amelonado (42.26%), a consequence of the lowest cocoa content (Table 1). Moisture presented values between 1.25% (Amelonado) and 2.32% (Chuncho), slightly above the recommended value of 1% which may impact shelf-life and flow behaviour [5, 38].

According to the literature, the acidity in cocoa liquor is a consequence of the production of acetic and lactic acids under anaerobic conditions during the early stages of fermentation, affecting the development of proper flavours from precursors [5]. During the transformation of cocoa beans into cocoa liquor, roasting [49] and conching [50] are responsible for the remotion of some volatile acids formed during the fermentation, removing certain undesirable components and promoting adequate flavour development [5]. The observed values of total acidity were proportional to the cocoa liquor content in each chocolate, from 10.62 mEq NaOH/100 g (Amelonado) to 15.98 mEq NaOH/100 g (Piura Blanco). As expected, the highest pH value was observed in the Amelonado chocolate while the lowest pH was observed in Piura Blanco, a consequence of total acidity.

The content of sugars is shown in Table 2. Sucrose is the most common sugar in unfermented cocoa beans, representing about 90% of the total sugars of the cotyledons [5] and plays a fundamental role in the definition of the quality of chocolate, namely particle size distribution, texture and sensorial perception [51]. The results of sucrose ranged from 27.65% (Piura Blanco) to 38.22% (Amelonado) and, as expected, presented an inverse correlation with the declared cocoa content of chocolate (Table 1). Glucose and fructose are the main fermentable sugars in cocoa pulp, converted by yeasts and lactic acid bacteria into ethanol and lactic acid and residual traces are usually observed after 72 h [52]. Therefore, glucose values in tested chocolates ranged from 0.34% to 0.54%, higher in Piura Blanco, while fructose values ranged from 0.51% to 1.53%, higher in Piura Blanco (Table 2), consequence of the method and time of harvesting and the type and origin of cocoa beans [5]. Mannitol is part of the polyols naturally present in cocoa shells at low concentrations, but concentration increases during fermentation as a result of the action of heterofermentative species, such as *L. fermentum* [53]. The concentration of mannitol ranged from 0.40% to 2.21%, higher in Amelonado.

Fermentation is a key step for the development of the complexity of flavours starting from the precursors. After pod harvest, cocoa beans and pulp are transferred to heaps or boxes for fermentations lasting from 1 to 6 days, depending on the variety [5]. The fermentation of the mucilaginous pulp of cocoa beans occurs naturally, triggered by anaerobic yeasts and lactic acid bacteria converting sugars and citric acid into lactic acid, ethanol, and carbon dioxide [12]. The highest concentrations of organic acids were reported on Piura Blanco, namely lactic acid (1153.20 mg/100 g), succinic acid (679.43 mg/100 g) and oxalic acid (468.46 mg/100 g). Tartaric acid presented the lowest results, between 21.50 mg/100 g (Amelonado) and 57.79 mg/100 g (Chuncho). According to literature, the production of organic acid during fermentation and retained by the cotyledons of cocoa depends on the duration of fermentation, diffusion rate and drying method [5].

The quantity and composition of fat are factors affecting the mechanical behaviour of chocolate [54–56], being

Table 2 Physical and chemical properties of studied chocolates

Sample	Piura Blanco	Chuncho	Amelonado
Hardness (g)	3797b ± 585	4224b ± 381	5592a ± 1182
Plastic viscosity (Pa.s)	2.13b ± 0.02	1.36c ± 0.04	2.87a ± 0.06
Yield value (Pa)	8.38b ± 0.36	7.07b ± 1.05	12.91a ± 1.00
WI frontside	27.06a ± 0.23	27.10a ± 0.29	26.30b ± 0.40
Fat (%)	43.23a ± 0.04	43.70a ± 0.75	37.87b ± 0.36
Sugars (%)	31.20 ± 1.66	31.10 ± 0.92	42.26 ± 1.32
Moisture (%)	2.05b ± 0.02	2.32a ± 0.02	1.25c ± 0.02
Acidity (mEq NaOH/100 g)	15.98a ± 0.04	15.69a ± 0.46	10.62b ± 0.47
pH	5.05b ± 0.01	5.15a ± 0.02	5.18a ± 0.01
Sucrose (%)	27.65b ± 1.77	29.84b ± 1.10	38.22a ± 1.40
Glucose (%)	0.54a ± 0.02	0.34b ± 0.01	0.37b ± 0.03
Fructose (%)	1.53a ± 0.10	0.51b ± 0.02	1.47a ± 0.04
Mannitol (%)	1.48b ± 0.19	0.40c ± 0.03	2.21a ± 0.17
Lactic acid (mg/100 g)	1153.20a ± 29.86	672.66b ± 59.77	835.92b ± 85.58
Acetic acid (mg/100 g)	823.83a ± 282.07	520.08a ± 52.69	354.74a ± 188.27
Malic acid (mg/100 g)	706.12a ± 123.86	540.69a ± 37.57	578.93a ± 65.35
Succinic acid (mg/100 g)	679.43a ± 21.09	555.83b ± 25.09	512.76b ± 19.55
Citric acid (mg/100 g)	563.40a ± 77.79	563.78a ± 174.07	608.92a ± 119.32
Oxalic acid (mg/100 g)	468.46a ± 10.52	319.61b ± 34.29	286.99b ± 25.09
Tartaric acid (mg/100 g)	55.59a ± 43.74	57.79a ± 37.15	21.50a ± 16.79

Means followed by a common letter within the same row are not significantly different

related to climate, harvest time, agricultural method [57], geographical origin [6], and industrial processing [58]. The composition of fatty acid was analysed, and the results are listed in Table 3. The distribution of the main fatty acids varied as follows: palmitic acid (between 24.93 and 26.09%), stearic acid (between 31.56 and 34.92%), oleic acid (between 32.91 and 36.43%), linoleic acid (between 2.64 and 3.70%), and α-linolenic acid (between 0.07 and 0.29%). Similar distributions have been reported previously in dark chocolates produced from *Criollo* and *Forastero* varieties [49] and dark chocolate from Africa and America [6, 42]. More than 33% of total fat was composed of monounsaturated fatty acids (MUFA), while only 2.74—3.93% were polyunsaturated fatty acids. Overall, the studied chocolates presented similar profiles, however, Chuncho presented lower SFA (57.78%) and higher MUFA (36.81%), a consequence of the higher content on C18:1 (*cis* 9). On the other hand, Amelonado presented the highest SFA (62.38%) and lower content on MUFA together with Piura Blanco.

The composition of vitamins is shown in Table 4. Tocopherol is the fat-soluble vitamin E that exists in four different forms [7], namely α–, β–, γ– and δ–tocopherol. Piura Blanco presented the highest values, mainly in γ (2.30 mg/100 g) and α- (0.55 mg/100 g) forms, while β- and δ-tocopherol forms presented negligible values. Considering the vitamin B complex, vitamin B_1 (thiamin) presented higher values in Amelonado (0.14 mg/100 g), while B_2 (riboflavin) presented values around 0.03—0.04 mg/100 g, not influenced by cocoa type. Vitamin B_3 (niacin) presented values around 0.22—0.31 mg/100 g, also not influenced by cocoa type. Vitamin B_5 (pantothenic acid) presented higher values on Piura Blanco, around 0.50 mg/100 g. Vitamin A (retinol), B_6 (pyridoxine) and D_2 (ergocalciferol) presented residual values, below 0.1 mg/100 g. Some carotenoids, like β-carotene, are precursors to vitamin A, presenting also a significant antioxidant activity [59]. The results of β-carotene ranged from 0.11 to 0.17 mg/100 g, higher in Amelonado, and above previously reported values on chocolates from America and Africa [7].

Table 4 Composition of vitamins in chocolate samples (mg/100 g)

	Piura Blanco	Chuncho	Amelonado
Vitamin A (retinol)	0.04a ± 0.00	0.02b ± 0.00	0.04a ± 0.00
Vitamin B1 (thiamin)	0.09b ± 0.01	0.08b ± 0.02	0.14a ± 0.03
Vitamin B2 (riboflavin)	0.03a ± 0.01	0.04a ± 0.01	0.04a ± 0.01
Vitamin B3 (niacin)	0.22a ± 0.06	0.22a ± 0.07	0.31a ± 0.06
Vitamin B5 (pantothenic acid)	0.50a ± 0.03	0.34b ± 0.02	0.27c ± 0.01
Vitamin B6 (pyridoxine)	ND	ND	ND
Vitamin D2 (ergocalciferol)	0.04b ± 0.00	0.08a ± 0.00	0.05b ± 0.00
α-tocopherol	0.55a ± 0.07	0.33b ± 0.04	0.33b ± 0.05
β-tocopherol	0.01a ± 0.00	0.01b ± 0.00	0.01c ± 0.00
γ-tocopherol	2.30a ± 0.12	1.88b ± 0.11	1.54b ± 0.04
δ-tocopherol	0.01a ± 0.00	0.01b ± 0.00	0.00b ± 0.00
β-carotene	0.12b ± 0.01	0.11b ± 0.01	0.17a ± 0.01

Means followed by a common letter within the same row are not significantly different, ND-not detected

TPC, phenolic acids, flavonoids and stilbenes were determined and results are summarized in Table 5. TPC presented higher values in Chuncho (957.8 mg GAE/100 g), followed by Piura Blanco (923.5 mg GAE/100 g) and Amelonado (813.4 mg GAE/100 g). As expected, the lowest TPC values were observed in the chocolate with the lowest cocoa content, in line with previous reports [60]. However, although the lower content, Chuncho presented higher values than Piura Blanco, a consequence of the higher TPC content on roasted cocoa beans (data not shown). In the present work, phenolic acids were the group with the largest contribution

Table 3 Composition of fatty acids in chocolate samples (%, w/w)

	Piura Blanco	Chuncho	Amelonado
C14 Myristic	0.26a ± 0.15	0.15a ± 0.12	0.12a ± 0.06
C16 Palmitic	24.93a ± 0.74	24.96a ± 0.31	26.09a ± 1.42
C16:1 (*cis* 9) Palmitoleic	0.44a ± 0.25	0.38a ± 0.23	0.28a ± 0.05
C17 Margaric	0.20a ± 0.08	0.40a ± 0.11	0.24a ± 0.02
C18 Stearic	33.68b ± 1.24	31.56c ± 1.31	34.92a ± 0.65
C18:1 (*cis* 9) Oleic	33.46b ± 0.69	36.43a ± 0.32	32.91b ± 0.13
C18:2 (*cis* 9,12) Linoleic	3.70a ± 0.59	3.54a ± 0.72	2.64a ± 0.37
C18:3 (*cis* 9,12,15) Linolenic	0.29a ± 0.24	0.07a ± 0.02	0.11a ± 0.13
C20 Arachidic	1.37a ± 0.18	0.71a ± 0.64	1.01a ± 0.11
SFA	60.43ab ± 0.45	57.78b ± 1.40	62.38a ± 0.74
MUFA	33.90b ± 0.43	36.81a ± 0.55	33.18b ± 0.17
PUFA	3.93a ± 0.91	3.57a ± 0.68	2.74a ± 0.51
Total	98.26 ± 0.02	98.15 ± 0.12	98.30 ± 0.03

Means followed by a common letter within the same row are not significantly different

Table 5 Phenolic compounds, methylxanthines and TAC (mg/100 g)

	Piura Blanco	Chuncho	Amelonado
TPC (mg GAE/100 g)	923.5b ± 2.3	957.8a ± 2.2	813.4c ± 2.9
Phenolic acids (mg/100 g)			
Gallic acid	6.53a ± 0.07	7.33a ± 0.73	6.43a ± 0.65
Ellagic acid	77.99a ± 0.26	78.40a ± 0.38	76.88b ± 0.31
Vanillic acid	8.32a ± 0.26	8.28a ± 0.03	8.39a ± 0.15
p-hydroxybenzoic acid	20.83a ± 0.28	20.73a ± 0.43	17.23b ± 0.24
Ferulic acid	7.50b ± 0.01	7.71a ± 0.01	7.52b ± 0.02
Caffeic acid	13.40b ± 0.02	14.24a ± 0.05	13.46b ± 0.03
Coumaric acid	12.79b ± 0.03	13.18a ± 0.05	12.82b ± 0.04
Chlorogenic acid	9.85a ± 0.11	9.62a ± 0.11	6.51b ± 0.07
Flavonoids (mg/100 g)			
Quercetin	6.42a ± 0.05	6.46a ± 0.04	6.36a ± 0.10
Kaempferol	2.97a ± 0.01	2.98a ± 0.01	2.94a ± 0.02
Catechin	33.37b ± 0.67	36.72a ± 0.65	27.10c ± 0.83
Procyanidin B1	23.18a ± 1.31	24.14a ± 0.67	20.29a ± 1.89
Naringenin	8.53b ± 0.09	9.20a ± 0.09	8.43b ± 0.03
Stilbenes (mg/100 g)			
Resveratrol	8.40a ± 0.07	8.28a ± 0.03	8.29a ± 0.01
Methylxanthines (mg/100 g)			
Theobromine	720.74a ± 54.34	516.24b ± 3.27	557.24b ± 32.94
Caffeine	103.08b ± 10.96	145.96a ± 2.37	46.79c ± 9.85
Theophylline	4.03a ± 0.59	3.12a ± 0.04	4.35a ± 0.47
TAC (μmol TE/100 g)			
DPPH	4048.6a ± 376.5	4650.8a ± 502.0	2970.0b ± 128.5
ORAC	29,583.4a ± 6773.6	34,676.5a ± 2095.6	17,724.3b ± 3154.2
FRAP	2453.4a ± 50.0	2470.6a ± 24.1	1936.5b ± 108.8

Means followed by a common letter within the same row are not significantly different

to the phenolic compounds, followed by flavonoids and, finally, stilbenes. The phenolic acids in higher concentration were ellagic acid (76.88 to 78.40 mg/100 g) and p-hydroxybenzoic acid (17.23 to 20.83 mg/100 g), followed by caffeic acid (13.40 to 14.24 mg/100 g), chlorogenic acid (6.51 to 9.85 mg/100 g), vanillic acid (8.28 to 8.39 mg/100 g), ferulic acid (7.50 to 7.71 mg/100 g) and gallic acid (6.43 to 7.33 mg/100 g). Overall, Chuncho and Piura Blanco presented the highest values on phenolic acids, a consequence of the higher content of cocoa solids. Catechin presented values from 27.10 to 36.72 mg/100 g, highest in Chuncho, followed by procyanidin B1 with values from 20.29 to 24.14 mg/100 g. In fact, previous studies on different types of cocoa have also reported the lowest catechin content on Amelonado, when compared with varieties *Criollo*, *Forastero* and cocoa hybrids [61], probably resulting from a genetic condition of this variety. Other detected flavonoids include quercetin, kaempferol and naringenin, presenting reduced values. Stilbenes presented also reduced values, below 8.40 mg/100 g, not affected by cocoa type.

The results of methylxanthines are presented in Table 5. Theobromine presented values between 516.24 mg/100 g and 720.74 mg/100 g, higher in Piura Blanco ($p < 0.01$), and comparable to previous studies in dark chocolates [13, 62, 63]. As expected, caffeine presented lower values than

Table 6 Principal component analysis summary table

	PC1	PC2
Theobromine	–0.04	0.98*
Lactic acid	–0.19	0.95*
Vitamin E	0.46	0.86*
FRAP	0.91*	0.35
Caffeine	0.98*	-0.03
MUFA	0.89*	-0.43
SFA	–0.94*	0.24
TPC	0.98*	0.14
Eigenvalue	4.69	2.99
% Variance	58.66	37.44
% Cumulative variance	58.66	96.09

* Marked values were considered correlated with the PC ($r \geq 0.7$) following the classification used previously [66, 67]

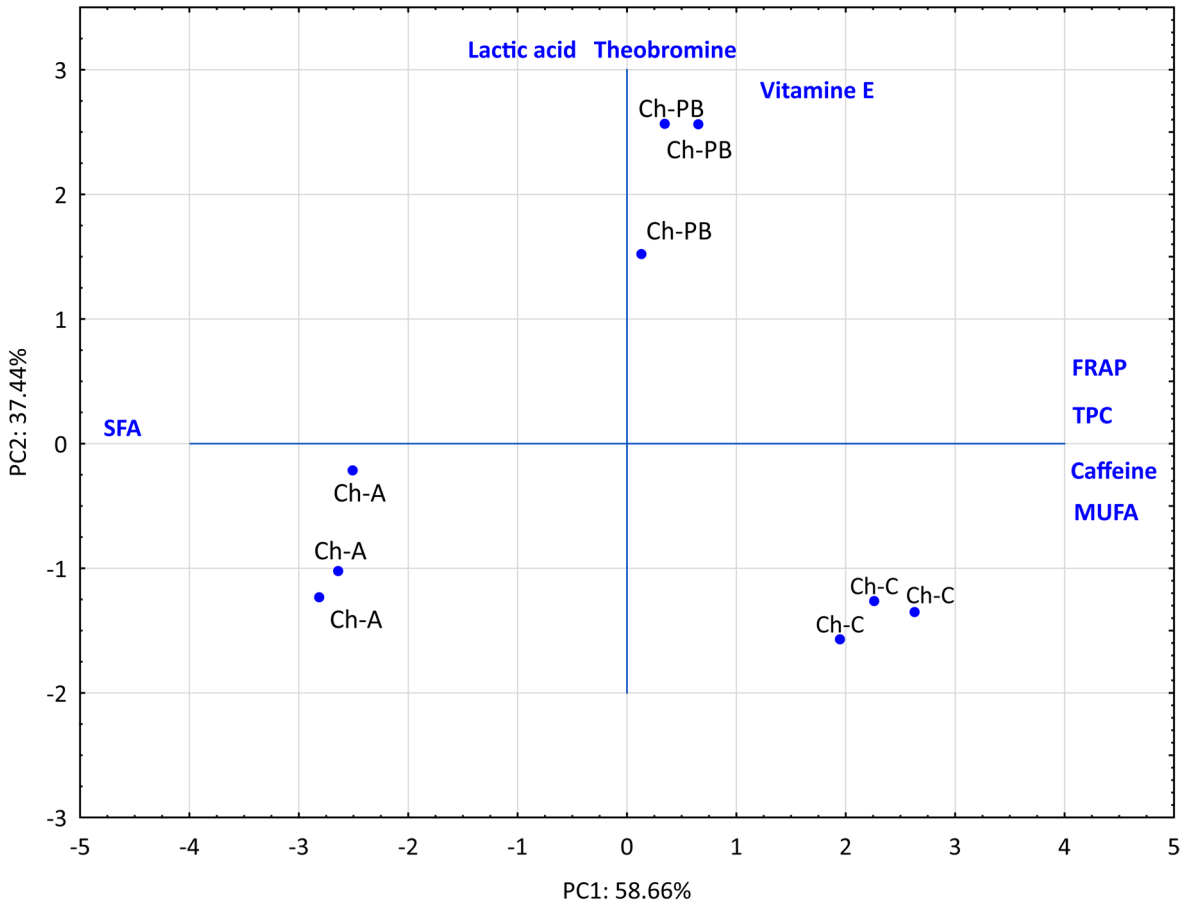

Fig. 1 Samples projection of principal component analysis: PC1(58.66 %) vs PC2(37.44 %) / n = 3. The most important variables for the definition of the two components are shown on the edge of each axis, indicating the direction in which the value of the parameter increases

theobromine, from 46.79 mg/100 g to 145.96 mg/100 g, higher in Chuncho. Theophylline presented results below 4.4 mg/100 g, with no differences between chocolate samples. According to the literature, the concentration of methylxanthines in chocolate depends on the cocoa type [63], and roasting degree of cocoa beans [49], among others.

There is not a standardized method for the evaluation of the antioxidant activity in certain food products, therefore, is recommended the use of more than one method [63]. In the present work, TAC was evaluated using DPPH, ORAC and FRAP; assays and results are presented in Table 5. DPPH measures the scavenging capacity of the free radical of a sample, involving an electron transfer reaction from phenoxide anions to DPPH [63], and results ranged from 2970.0 to 4650.8 µmol TE/100 g (Table 5). Similar results were obtained by Medina-Mendoza et al. [65] on dark chocolates enriched with sauco by-product and sacha inchi oil, where values from 30.6 to 53.1 µmol TE/g were recorded. The results of ORAC ranged from 17,724.3 to 34,676.5 µmol TE/100 g, similar to previous studies on dark chocolates with different cocoa content [63] and different production methods [16]. The results according to the FRAP method ranged from 1936.5 to 2470.6 µmol TE/100 g, higher in Chuncho. Siow et al. [15] presented a positive correlation between antioxidant activity and phenolic content on cocoa nibs from different geographical origins and under different roasting temperatures. Also, Gültekin-Ozgüven et al. [16] observed a strong correlation between total phenolics and total flavonoids with antioxidant capacity in dark chocolate under different processing conditions. Such findings are in agreement with the present study, where correlations among the results for TPC and TAC (ORAC, DPPH and FRAC) were considerably positive ($R^2 > 0.7955$).

Principal component analysis (PCA) was carried out (Table 6) in order to find eventual regional effects on the bioactive / nutritional characteristics of dark chocolate. The main nutritional / functional attributes used in PCA analysis

were FRAP, theobromine, caffeine, SFA, MUFA, TPC, vitamin E and lactic acid.

The similarity map defined by the first two principal components accounted for 96.09% of the total variance. The first component (PC1) condensed 58.66 %, and the second component (PC2) represented 37.44 % of the total variance. The PC1 was heavily loaded with FRAP, caffeine, MUFA, and TPC presenting positive correlations, while SFA presented negative correlations. A positive correlation with PC2 was observed with theobromine, lactic acid and vitamin E. Figure 1 shows the projection of the samples onto the PC1 vs PC2 plane.

With PCA analysis it was possible to highlight the influence of geographical origin in the bioactive parameters of single origin dark chocolate. Thus, both samples from Peru (Chuncho and Piura Blanco) were projected in the right side of the PC1 vs PC2 plane, meaning a higher prevalence of bioactive attributes such as TPC, TAC (evaluated with FRAP), caffeine, and MUFA. On the other hand, chocolate from São Tomé (left side of the PC1 vs PC2 plane) presented a higher content of SFA. In addition, sample Piura Blanco presented a higher content of theobromine, lactic acid and vitamin E (top section of the PC1 vs PC2 plane).

Conclusions

The present study aimed for the evaluation of the geographical origin and type of cocoa in the physical and bioactive parameters of single origin dark chocolate from Peru (Piura Blanco and Chuncho) and São Tomé (Amelonado) acquired locally. Results presented a significant variability in physical–chemical parameters, with higher hardness, plastic viscosity and yield value on Amelonado sample. Both samples from Peru presented higher results on total phenolic content, antioxidant capacity (evaluated with FRAP), caffeine, MUFA and vitamin E. Additionally, sample Piura Blanco presented a higher content of theobromine, lactic acid and vitamin E. On the other hand, sample chocolate from São Tomé presented a higher content of SFA. The obtained results are in agreement with previous studies, on single origin dark chocolates, referring to the impact of the variety, geographical origin, and post processing operations on the nutritional balance. The results presented here are of great importance for consumers, traders, and cocoa producers as will support the selection process of the most suitable type of cocoa for specific markets with specific needs.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval This study does not contain any studies with human or animal participants.

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