

Chemical and phytochemical characteristics as biochemical descriptors of diversity in cocoa seeds from a collection from southern Ecuador

Características químicas y fitoquímicas como descriptores bioquímicos de la diversidad en semillas de cacao de una colección del sur de Ecuador

Características químicas e fitoquímicas como descriptores bioquímicos da diversidade em sementes de cacau de uma coleção do sul do Equador

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Abstract

The objective of this work was to determine the content of some chemical and phytochemical characteristics, in seeds of 60 cocoa trees from a collection in southern Ecuador; to identify their potential as biochemical descriptors. Brix degrees ($^{\circ}$ Brix), moisture (MO), lipids (LI), total phenols (TP), and antioxidant activity (AA) were determined. Statistical analysis indicated that there was low variability in $^{\circ}$ Brix, MO and LI; and high variability in TP and AA. $^{\circ}$ Brix was distributed in five classes; MO, LI, and AA in four and TP in three; several trees presented high contents and close to the standards: $^{\circ}$ Brix (16-21.34 $^{\circ}$ Brix), MO (7-7.90%), LI (50.03-60.71%), TP (5.05-14.46 mg GAE.g⁻¹) and AA (92.48-275.16 mg TE.g⁻¹). A significant correlation ($p < 0.01$) was found between LI and TP ($r = -0.334$), and between TP and AA ($r = 0.802$). The TP and AA variables showed a high positive correlation, while LI and TP a low and negative. The accumulated variance was 64.54%, represented by TP and AA. It is concluded that the variability was influenced by the genotype and was high in TP, and AA. TP and AA constituted excellent biochemical descriptors of diversity in cocoa seeds. The trees FCA58, FCA59, FCA48, FCA45, and FCA46 presented the highest values of TP and AA, so they were promising as cultivars, for plant breeding and industry, among others.

Resumen

El objetivo del presente trabajo fue determinar el contenido de algunas características químicas y fitoquímicas en semillas de 60 árboles de cacao de una colección del sur de Ecuador, para identificar su potencial como descriptores bioquímicos. Se determinaron contenidos de grados Brix ($^{\circ}$ Brix), humedad (HU), lípidos (LI), fenoles totales (FT) y actividad antioxidante (AA). El análisis estadístico indicó que hubo baja variabilidad en $^{\circ}$ Brix, HU y LI; y alta variabilidad en FT y AA. $^{\circ}$ Brix se distribuyó en cinco clases; HU, LI y AA en cuatro; y FT en tres; varios árboles presentaron contenidos altos y cercanos a los estándares: en $^{\circ}$ Brix (16-21,34 $^{\circ}$ Brix), HU (7-7,90 %), LI (50,03-60,71 %), FT (5,05-14,46 mg GAE.g⁻¹) y AA (92,48-275,16 mg TE.g⁻¹). Se encontró correlación significativa ($p < 0,01$) entre LI y FT ($r = -0,334$), y entre FT y AA ($r = 0,802$). Las variables FT y AA mostraron alta correlación positiva, mientras que LI y FT baja y negativa. La varianza acumulada fue de 64,54%, representada por FT y AA. Se concluye que la variabilidad estuvo influenciada por el genotipo y fue alta en FT y AA. Los FT y la AA constituyeron excelentes descriptores bioquímicos de la diversidad en semillas de cacao. Los árboles FCA58, FCA59, FCA48, FCA45 y FCA46 presentaron los mayores valores de FT y AA, por lo que resultaron promisorios como cultivares, para el fitomejoramiento y la industria, entre otros.

Palabras clave: grados Brix, humedad, lípidos, fenoles totales, actividad antioxidante.

Resumo

O objetivo deste trabalho foi determinar o conteúdo de algumas características químicas e fitoquímicas em sementes de 60 cacauzeiros de uma coleção no sul do Equador, para identificar seu potencial como descritores bioquímicos. Graus Brix ($^{\circ}$ Brix), umidade (UM), lipídios (LI), fenóis totais (FT) e atividade antioxidante (AA) foram determinados. A análise estatística indicou baixa variabilidade em $^{\circ}$ Brix, HU e LI; e alta variabilidade em FT e AA. $^{\circ}$ Brix foi distribuído em cinco classes; UM, LI e AA em quatro; e FT em três; várias árvores apresentaram teores elevados e próximos aos padrões: em $^{\circ}$ Brix (16-21,34 $^{\circ}$ Bx), UM (7-7,90%), LI (50,03-60,71%), FT (5,05-14,46 mg GAE.g⁻¹) e AA (92,48-275,16 mg TE.g⁻¹). Foi encontrada correlação significativa ($p < 0,01$) entre LI e FT ($r = -0,334$), e entre FT e AA ($r = 0,802$). As variáveis FT e AA apresentaram alta correlação positiva, enquanto LI e FT baixa e negativa. A variância acumulada foi de 64,54%, representada por FT e AA. Conclui-se que a variabilidade foi influenciada pelo genótipo e foi alta em FT e AA. FT e AA foram excelentes descritores bioquímicos de diversidade em sementes de cacau. As árvores FCA58, FCA59, FCA48, FCA45 e FCA46 apresentaram os maiores valores de FT e AA, por isso se mostraram promissoras como cultivares, para melhoramento de plantas e indústria, entre outras.

Palavras-chave: graus Brix, umidade, lipídios, fenóis totais, atividade antioxidante.

Introduction

Cocoa (*Theobroma cacao* L., Malvaceae family) is an important item in the economy of many countries dedicated to the production of its seeds, called “almonds” or “beans” because it generates foreign exchange, and jobs in its production and marketing chain. Among the

main exporters of America is Ecuador, which has the fourth position worldwide (Alcívar *et al.*, 2021). Knowledge of the diversity of chemical substances contained in cocoa seeds is a fundamental key to the selection of materials to be used in the genetic breeding of the crop (Quevedo *et al.*, 2020). In addition to nutritional and sensory quality, markets have evolved to offer consumers products with biomolecules that benefit their health, among them phenolic compounds especially catechins, flavonoids, anthocyanins, and proanthocyanins, secondary metabolites responsible for the antioxidant activity (AA) of cocoa (Castro *et al.*, 2016). In this regard, Vázquez *et al.* (2016) have pointed out that endogenous enzymes in cocoa seeds, when activated, are fundamental in the development of biomolecules associated with flavor and aroma.

Lipids, also called “fats”, allow the cocoa to have an adequate consistency when processed to obtain chocolate, with saturated fatty acids being the most present (Lares *et al.*, 2012). However, in some cases the “defatting” of cocoa liquor increases the content of phenols, and antioxidants, improving its quality (Castro *et al.*, 2016). In this regard, some works point out that cocoa seeds are characterized by their high phenol content (Quiñones *et al.*, 2013; Ordoñez *et al.*, 2020) and the amount varies according to the clone. Variations in phenol content and antioxidant activity have been reported (Zapata *et al.*, 2013; Bustamante *et al.*, 2015) due to the post-harvest processing and morphoagronomic characteristics of the cultivar. Based on these premises and the morphoagronomic diversity found in the UTMACH collection of *T. cacao* trees (Quevedo *et al.*, 2020), the objective of this research was to determine the content of some chemical ($^{\circ}$ Brix, MO, LI) and phytochemical (TP and AA) characteristics in seeds of 60 cocoa trees from the UTMACH collection, in southern Ecuador, with the purpose to identify their potential as biochemical descriptors.

Materials and methods

The research was carried out at the Granja Experimental Santa Inés de la Universidad Técnica de Machala (UTMACH), “El Oro” province, Ecuador; coordinates 3°17'30" S, 79° 54'51" W; with clay loam soil, order Entisols; located between dry and semi-humid forest with annual average temperatures of 28 °C (minimum 24 °C, maximum 30 °C), relative humidity of 80% and rainfall between 500 and 1,000 mm, distributed in two periods: one of higher rainfall from December to May (rainy period), and another of lower rainfall from June to November (dry period).

Plant material

Sixty trees of approximately 42 years of age were selected, representatives of the morphoagronomic diversity of the UTMACH cocoa collection, obtained by Quevedo *et al.* (2020). Trees with continuous numbering from 01 to 60 with the prefix FCA (Facultad de Ciencias Agropecuarias) were identified.

Sample preparation

Five fruits per tree were harvested according to homogeneity criteria (maturity, size, color, shape) and health (no pests, diseases, or mechanical damage). Immediately, the seeds were extracted and placed in a wood fermenter made of white laurel (*Cordia alliodora* (Ruiz & Pav.) Oken) for three days. The samples were placed separately in mesh bags, duly labeled, removing the seeds every 24 hours for 10 min, then they were dried in a marquee for 10 days. The fermented and dry seeds from each tree were placed in identified Zip® plastic bags, to be transferred to the laboratory.

Obtaining the aqueous extract

From each tree, in triplicate, 0.1 g of fermented and dry seed sample without seed coat, ground (Daewoo DCG362 electric mill), and sieved (100 mm) were weighed on an analytical balance, placed

in Eppendorf tubes; then three (3) mL of boiling double-distilled water was added and centrifuged at 1,000 rpm for 15 min (MiniSpin plus-Eppendorf AG, Hamburg). The aqueous solution was transferred to centrifuge tubes rooted to five (5) mL with double-distilled water and then to test tubes (Vacutainer TM) at -4 °C, for which the Electrolux freezer (EFCC20A6HQW) was used.

Study variables

Brix degrees. Five ripe and healthy fruits were taken from each tree; they were opened with the help of a wooden mallet to extract the fresh seeds with juicy mucilage from the central part of the fruit, to which three Brix degree readings were made with a refractometer (BOECO-103).

Moisture. The percentage of moisture was obtained with a digital moisture meter (SAMAP-O-TEST 40 model, program 29), according to Popa & Popescu (2017), calibrated for cocoa. Three readings per tree were done when the temperature was maintained at 20 °C, and 100 g.tree⁻¹ of fermented and dry seeds with seed coat were used.

Lipids. The Soxhlet extraction method (NTE INEN 535:2013) was applied; for the extraction and quantification 3 g.tree⁻¹ of fermented and dry seeds without seed coat were used, and three repetitions per sample were made (Luque & Priego, 2010).

Total phenols. They were determined by the Folin-Ciocalteu method, modified by Kraujalyte *et al.* (2015).

For quantification, a calibration curve was prepared with gallic acid (GA) ratio 1:10 (V:V), a method modified by Zhapan *et al.* (2021).

The results were expressed in equivalent milligrams of GA (GAE) per gram of dry sample (mg GAE.g⁻¹).

Antioxidant activity. It was quantified according to the method described by Ordoñez *et al.* (2020) and expressed in equivalents milligrams of Trolox (TE) per gram of dry sample (mg TE.g⁻¹).

Statistical analysis

Descriptive statistics of summary and frequency distribution were performed in the study variables, PCA analysis, and hierarchical cluster analysis, in order to know the physicochemical relationships between the study trees.

Results and discussion

The descriptive statistical analysis showed that there was variability in the contents of Brix degrees, moisture, lipids, total phenols (TP), and antioxidant activity (AA) in the seeds of the 60 cocoa trees (table 1). In the first three variables, low values of standard deviation and coefficient of variation (about 20 %) were observed. Whereas, for TP and AA, the variability was higher.

In the frequency distribution, it was found that °Brix presented five classes or categories; moisture, lipids, and AA four classes, and TP three (table 2).

Table 1. Descriptive statistics of Brix degrees, moisture, lipids, total phenols (TP), and antioxidant activity (AA) in seeds of 60 cocoa trees.

Descriptive statistics	Brix degrees (°Brix)	Moisture (%)	Lipids (%)	TP (mg GAE.g ⁻¹)	AA (mg TE.g ⁻¹)
Mean	17.72	6.45	48.70	4.79	82.16
Standard deviation	1.88	1.36	7.73	2.72	61.03
Range	7.67	5.10	30.52	13.67	271.59
Minimum value	13.67	5.00	30.19	0.79	3.57
Maximum value	21.34	10.10	60.71	14.46	275.16
Mode	20.00	500			
Median	1716	6.00	49.71	4.36	69.61
Variance	3.55	1.85	59.77	7.38	3724.20
Coefficient of variation (%)	10.63	21.12	15.87	56.78	74.28
Standard error	0.24	0.18	1.00	0.35	7.88

Table 2. Frequency distribution of variables: Brix degrees, moisture, lipids, total phenols, and antioxidant activity in seeds of 60 cocoa trees.

Variables	Class	Class denomination	Absolute frequency (trees)	Relative frequency (%)
Brix degrees (°Brix)	13 – 14.99	Very low	4	6.67
	15 – 16.99	Low	21	35.00
	17 – 18.99	Medium	18	30.00
	19 – 20.99	High	13	21.67
	21 – 22.99	Very high	4	6.67
Moisture (%)	5 – 6.49	Very low	31	51.67
	6.50 – 7.99	Low	20	33.33
	8 – 9.49	High	8	13.33
	9.50 – 10.99	Very high	1	1.67
Lipids (%)	30 – 39.99	Very low	8	13.33
	40 – 49.99	Low	23	38.33
	50 – 59.99	High	28	46.67
	60 – 69.99	Very high	1	1.67

Table 2. Frequency distribution of variables: Brix degrees, moisture, lipids, total phenols, and antioxidant activity in seeds of 60 cocoa trees (continuación).

Variables	Class	Class denomination	Absolute frequency (trees)	Relative frequency (%)
Total phenols (mg GAE.g ⁻¹)	0 – 4.99	Low	37	61.67
	5 – 9.99	Medium	21	35.00
	10 – 14.99	High	2	3.33
Antioxidant activity (mg TE.g ⁻¹)	0 – 74.99	Very low	32	53.33
	75 – 149.99	Low	21	35.00
	150 – 224.99	High	6	10.00
	225 – 299.99	Very high	1	1.67

In °Brix degrees, classes two, three and four were made up of 35, 30 and 21.67 % of the 60 trees evaluated, representing 86.67 %; while the first and last class obtained 6.67 %, respectively, thus demonstrating a high diversity. When comparing the °Brix with those obtained in other works, it was observed that the values of 26 trees located between classes two and three (table 2; figure 1) corresponded to the range indicated by Guzmán & Gómez (2014), between 16±0.4 and 17±0.4 °Bx, for cocoa trees from six municipalities in southern Bolívar, Colombia.

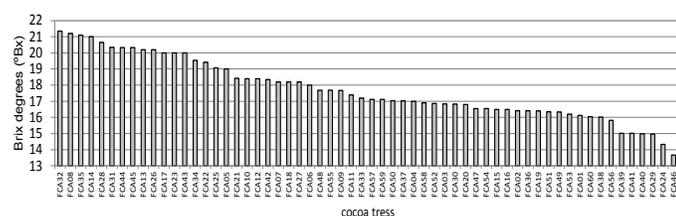
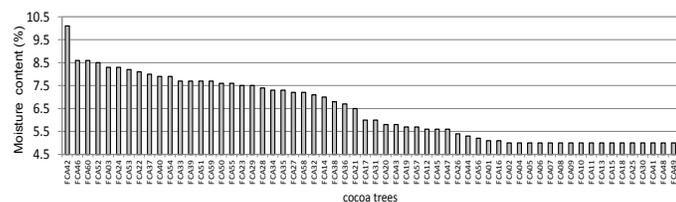
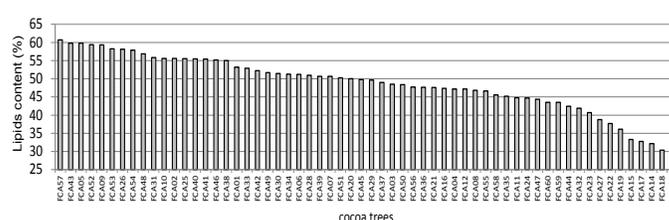
Of the 60 cocoa trees, 25 recorded values greater than and equal to 18 °Brix; of these, four presented between 18, and 18.2 °Brix, and 21 between 18.34, and 21.34 °Brix (figure 1). This result, together with the classes described (table 2), showed that 25 trees presented medium to very high °Brix contents. Values similar to those reported by Loureiro *et al.* (2017) who classified PH-16 cocoa pulp as high, with a value of around 18 °Brix. In Ecuador, Vallejo *et al.* (2016) reported 16 °Brix for national cocoa, and 15 °Brix for Trinitario CCN-51, which differs from the results obtained in this work, 53 of 60 trees exceeded 16 °Brix.

Of the four moisture classes, the first stood out for grouping close to half of the trees, the second 33.33%, and the last two few individuals (table 2, figure 2).

The mean moisture content of the seeds of the trees studied (table 1) was higher than those reported by Guzmán & Gómez (2014), in cocoas from six municipalities in southern Bolívar (Colombia), between 4.22±1.3, and 6.62±1.7 %; although, some of the trees (figure 2) were placed in that range. The mean also contrasted with the research of Lares *et al.* (2012), who obtained 4.31±0.06 % of moisture in fermented and dry cocoa from the Barlovento region, Venezuela.

The values of some trees were similar to those indicated by Steinau *et al.* (2017), and Lares *et al.* (2013), between 6.8, and 7.1 % moisture in seeds dried with seed coat, in trinitario cocoa. According to the standards -classification and quality requirements- the maximum moisture value established is 7 % (INEN, 2018; ICONTEC, 2012). For their part, CAOBISCO *et al.* (2015) indicated a moisture content of 7 %, and an absolute maximum of 8 %, for well-fermented and dry seeds. Based on this, it was found that, out of the 20 trees, in the second class, 17 recorded between 7, and 7.9 % moisture; there was little variation in moisture content (table 1), adjusted according to standards or technical norms (INEN, 2018).

Regarding lipids, 46.67 % of the trees were in the third class and 38.33 % in the second class, equivalent to 85 % (29 trees), with contents from medium to high; FCA57 qualified as very high (table 2; figure 3).

**Figure 1. Brix degrees in seeds of 60 cocoa trees from the Universidad Tecnica de Machala (UTMACH) collection.****Figure 2. Moisture content in seeds of 60 cocoa trees from the Universidad Tecnica de Machala (UTMACH) collection.****Figure 3. Lipid content in the seeds of 60 cocoa trees from the Universidad Tecnica de Machala (UTMACH) collection.**

The values obtained in several trees are similar to those published by Graziani *et al.* (2003), for three types of cocoa from Cumboto locality, Venezuela; 50.99% in Criollo, 49.52% in forastero amazónico, and 52.24% in trinitario. The percentage of FCA33 has similarity with that indicated by Lares *et al.* (2013), of 52.85±0.21 % in fermented and dried cocoa in Chuao region, Venezuela; while that of the rest of the trees was different.

Although, the values obtained diverge from other works (figure 3); only two trees FCA13 and FCA18 were close to that reported by Castro *et al.* (2016), 30.71 % in fermented and dry cocoa seeds;

and two other trees were close to that reported by Lares *et al.* (2012), 46.27±0.30 %. CAOBISCO *et al.* (2015) pointed out contents for intact seeds of 43.6 and 44.2 % in Ecuador, and 44.6 % in Brazil, and for dry “dehulled” seeds of 55-58 % in West Africa; the results obtained in some of the trees (figure 3) are related to the mentioned amounts; as well as, with the work of Steinau *et al.* (2017), who reported 38.95 and 40.73 % in pre-dried seeds of category A.

The variability found in lipid content may be due primarily to the genetic condition of each tree (tables 1 and 2), representatives of the morphoagronomic diversity of the UTMACH collection (Quevedo *et al.*, 2020); because they had the same environmental and management conditions. In this regard, Lares *et al.* (2012) explained that genetic and environmental factors have a decisive influence on the lipid content in seeds, mainly in the cotyledons; which varies according to the type of cocoa, being lower in the forastero amazónico and higher in the trinitario.

For TP content, 61.67% of the trees were in the first class, 35% in the second class and 3.33% in the third class (table 2); in the last two classes, classified as medium and high, respectively, there were 29 trees (figure 4).

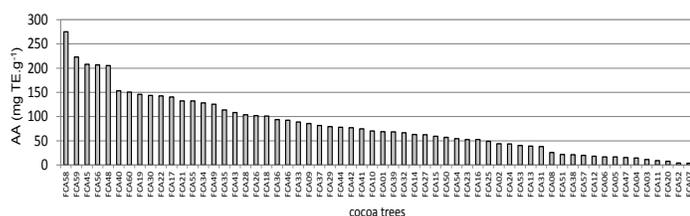


Figure 4. Total phenol content (TP) in seeds of 60 cocoa trees from the Universidad Tecnica de Machala (UTMACH) collection.

The values of 59 trees, out of 60, coincide with the range indicated by Tello *et al.* (2020), in eight clones of Mexican cocoa (criollos and trinitarios); from 1.01 to 31.12 mg GAE.g⁻¹ in dry seeds, and from 0.75 to 7.92 mg GAE.g⁻¹ in fermented seeds. In addition, the contents of 35 trees were in the range indicated by Avendaño *et al.* (2021), from 7.50 to 85.20 mg GAE.g⁻¹ in dry seeds.

The results obtained differ from other studies such as those conducted by Castro *et al.* (2016), on fermented and dry seeds (53.90 mg GAE.g⁻¹) and Zapata *et al.* (2013), on fermented seeds (22.58 to 50.23 mg GAE.g⁻¹). The variation in TP content, of the 60 cocoa trees (tables 1 and 2), was mainly attributed to the effect of the genotype. In this regard, some authors have indicated that cocoa clone or genotype affects TP content (Quiñones *et al.*, 2013; Avendaño *et al.*, 2021). According to Zapata *et al.* (2013); Castro *et al.* (2016), and Vázquez *et al.* (2016), the quantity and quality of phenolic compounds can vary by internal factors, such as genetic diversity, lipid content; and external factors such as temperature, crop management, seed processing (fermentation, drying, others). The low TP content in the 60 cocoa trees (tables 1 and 2), can be observed in the light tonality of the seeds, which is indicative of lower phenol content and higher lipid content, allowing them to be very aromatic (Vázquez *et al.*, 2016).

The results of AA showed that the largest number of trees was in the first class (53.33 %), followed by the second (35 %), with contents classified as low and medium, respectively (table 2), in the other two classes there were few trees that stood out for having high levels (FCA59 to FCA60) and very high (FCA58) (figure 5).

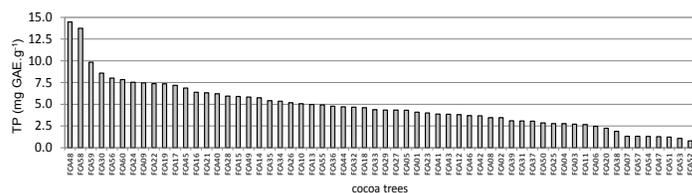


Figure 5. Antioxidant activity (AA) in seeds of 60 cocoa trees from Universidad Tecnica de Machala (UTMACH) collection.

The AA of 18 cocoa trees was located in the range obtained by Zapata *et al.* (2013), from 63.51 to 116.29 mg TE.g⁻¹ in fermented seeds of five cocoa clones. In contrast, the values of 22 trees -from FCA46 to FCA36- (figure 5), exceeded that indicated by Zapata *et al.* (2013), for dry and fermented seeds (90.36 mg TE.g⁻¹); while in the rest of the trees (38) they were lower and ranged from 3.57 to 88.80 mg TE.g⁻¹. Based on the results, it was established that AA varied in the 60 trees with levels ranging from very low to very high (table 2), being a discriminating variable in this case.

Six trees in the second class, designated as low (table 2), had high AA; and trees in classes three and four had very high AA, for a total of thirteen; individuals in the last two classes were promising for the food, pharmaceutical, and cosmetic industries. The high variability observed in the AA, in the 60 trees (tables 1 and 2), confirms the biochemical diversity in the seeds. This result agrees with the report of Avendaño *et al.* (2021), who found differences among cocoa phenotypes for AA.

Regarding the correlation between variables, it was observed that it was highly significant ($p < 0.01$) only between lipids, and FT, and between FT, and AA (table 3); which showed the tendencies: the lower the lipid content, the higher the TFA, and the higher the TFA, the higher the AA.

Table 3. Spearman's correlation coefficients between the variables.

Variables	Moisture	Lipids	Total phenols	Antioxidant activity
Brix degrees	-0.206	-0.179	0.097	0.018
Moisture		-0.047	-0.070	0.159
Lipids			-0.334 **	-0.134
Total phenols				0.802 **

** : Highly significant ($p < 0.01$). * : Significant ($P < 0.05$).

The correlation between TP and AA was positive and high ($r = 0.802$), while that of lipids, and TP was low ($r = -0.334$), although it was negative and highly significant. In other works, the correlation between TP and AA has also been reported (Zapata *et al.*, 2013; Avendaño *et al.*, 2021). For the correlation between lipids, and TP, Castro *et al.* (2016), pointed out that the TP was lower in dry fermented seeds with “fat” and higher in seeds without “fat”. On the other hand, Cienfuegos *et al.* (2016) reported a negative correlation between lipids and TP, which coincides with the results obtained in this research; it should be noted that cocoa from Ecuador (CCN-51), Venezuela (criollo), and Ivory Coast (forastero) were used in this research.

On the other hand, the accumulated variance of the two-dimensional PCA of the variables (figure 6) determined 38.70 % in component 1 and 25.83 % in component 2, for a total of 64.54 % of the total variance explained; of which the most representative and related between them were TP and AA, so that both variables were considered excellent biochemical descriptors of diversity in cocoa seeds.

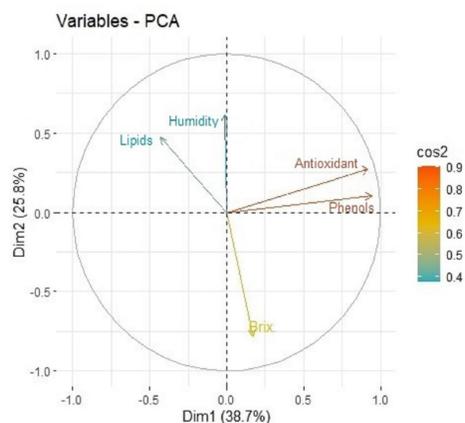


Figure 6. Two-dimensional principal component analysis of the variables studied in seeds of 60 cocoa trees from the Universidad Tecnica de Machala (UTMACH) collection.

Of 60 cocoa trees, FCA58 presented the highest amount of FT and AA; other trees that also stood out were FCA59 and FCA48. These three trees showed greater relation with the phytochemical substances associated with quality (TP and AA), according to previous studies that established that sensory quality is directly proportional to the aromatic phenolic compounds present in cocoa seeds (Delgado *et al.*, 2018).

The hierarchical cluster analysis allowed discriminating the 60 cocoa trees, thus, the most significant were FCA58, FCA59, FCA48, FCA45, and FCA46, which showed the highest values of TP, and AA, compounds that have positive perspectives for human consumption. The dendrogram (figure 7) shows the relationships between the 60 trees studied, which formed 15 groups at a Euclidean distance of 20.

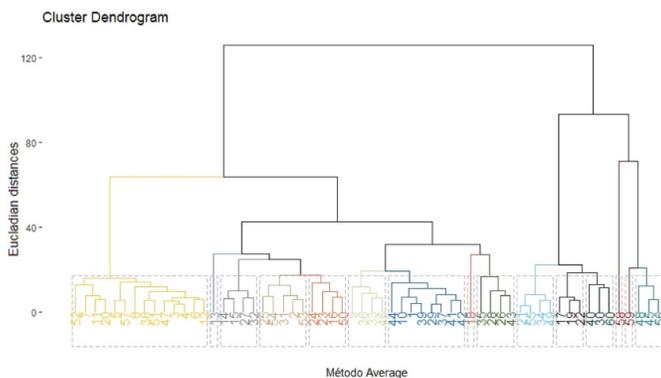


Figure 7. Hierarchical cluster analysis applying Euclidean distances (AVERAGE method) in 60 cocoa trees from the Universidad Tecnica de Machala (UTMACH) collection.

Conclusions

Variability in cocoa seeds was influenced by the genotype; it was low in Brix degrees, moisture, and lipids; and high in total phenols and antioxidant activity. Several trees presented promising contents, towards high or towards the standards, in these parameters. Total phenols and antioxidant activity showed a high positive correlation; and lipids and total phenols, a low negative correlation; both highly significant. Total phenols and antioxidant activity accumulated 64.54% of the variability and constituted excellent biochemical descriptors of diversity in cocoa seeds. The trees FCA58, FCA59, FCA48, FCA45, and FCA46 presented the highest contents of total phenols and antioxidant activity; so they resulted promising as cultivars, for plant breeding, industry, among others.

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