

Chemical Study and Antioxidant Activity of Piura's White Cocoa

J. Blancas^a, O. Acha^a, I. Collantes^b, N. Rodriguez^a, A. Castro^c, N. Ramos^c

^a LABICER - Facultad de Ciencias - Universidad Nacional de Ingeniería, Lima, Peru

^b Facultad de Ingeniería Química - Universidad Nacional de Ingeniería, Lima, Peru

^c RENATU - Facultad de Farmacia y Bioquímica - Universidad Nacional Mayor de San Marcos, Lima, Peru

Abstract

Piura's white cocoa, called this way because it possesses whitish beans, is recognized as a high quality cocoa, for this reason it is highly appreciated by national and international chocolate producers. However, its chemical composition has not been studied thoroughly as well as other properties like antioxidant activity (AA) and total phenolic content (TPC). Therefore, this research project was aimed to: I) assess antioxidant activity and total phenolic content and II) characterize molecular structures of the diverse compounds by liquid chromatography - mass spectrometry.

Different beans of Piura's white cocoa were analyzed: I) violate beans, II) whitish beans, III) brown beans, IV) a mixture of different beans, and V) fermented beans (different beans). In order to assess the antioxidant activity and identify secondary metabolites being present, organic extraction was carried out by maceration with ethanol after cocoa defeating with petroleum ether. TPC was quantified by Folin-Ciocalteu method; results expressed in Gallic acid equivalents ranged from 7.15 to 44.37 mg GAE/g. Antioxidant activity determination was performed using two different colorimetric methods: di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) free radical inhibition and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS^{•+}) free radical inhibition. Results expressed in Trolox equivalents ranged from 40.18 to 403.75 $\mu\text{mol TE/g}$ for DPPH and from 64.02 to 501.87 $\mu\text{mol TE/g}$ for ABTS. Brown and violet beans exhibited the highest values. A high correlation between TPC and antioxidant activity was found, $R^2= 0.9984$ for TPC and DPPH assay; and $R^2= 0.9968$ for TPC and ABTS assay.

Samples consisting of a mixture of different beans were analyzed by liquid chromatography coupled with electrospray mass spectrometry (HPLC-ESI-MS) in positive mode. Organic extract was fractioned before analysis, thus ethyl acetate and butanol fractions were analyzed. Several polyphenols were detected: Hydroxynamic acids, anthocyanins, flavan-3-ols, and procyanidin dimers.

Keywords: Piura's white cocoa, total phenolic content, antioxidant activity, LC-MS.

1. Introduction

Cocoa is recognized as a very rich source of antioxidant compounds, which possess many beneficial properties for human health such as cardiovascular benefits, cancer prevention, anti-aging properties, and strengthening of the immune system [1-3]. Antioxidant compounds found in cocoa are mainly procyanidins which are oligomers of catechins and epicatechins, in cocoa they are basically from monomers to pentamers, but molecules having even 18 cyanidin units have been found [4,5].

Most common compounds found are procyanidins A, procyanidins B, and diverse polyphenols like quercetin, kaempferol, cyanidin, and others, along with their respective glycosides [4-6]. Other common compounds are Nitrogen compounds like theobromine, the characteristic alkaloid of cocoa, and clovamide which is a naturally occurring caffeoyl conjugate representative of cocoa [4, 7]. Different techniques are used for characterizing and identifying compounds, like mass spectrometry, IR spectroscopy, nuclear magnetic resonance spectroscopy and others, but different techniques are necessary in most cases. However, in recent years liquid chromatography - mass spectrometry (LC-MS) has increased its capacity to identify structures with high accuracy in different matrix thanks to the development of technologies like tandem mass spectrometry [8, 9].

Antioxidant activity of feeds and isolated compounds can be quantified using in vitro techniques like DPPH and ABTS which are among the most popular and simple assays. DPPH assay consists of measuring the reduction of the radical cation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) as it reacts with antioxidants from the analyzed sample. ABTS assay it is very similar, but in this assay free radicals are produced by reaction between 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and potassium persulfate [10]. Folin-Ciocalteu assay it is used for assessing total phenolic content in-vitro. Folin-Ciocalteu reagent is a phosphorus-tungsten-molybdenum solution that is reduced by phenol compounds to produce molybdenum (+5) whose blue color is measured [11].

One factor that affects antioxidant activity is the cocoa processing. To turn cocoa into chocolate, cocoa bean has to be fermented and dried. Such processes are very important because they produce cocoa flavor and aroma, but antioxidant compounds are destroyed or modified during these processes [12].

Several studies concerning antioxidant compounds from cocoa have been developed in different countries, some of them are Colombia, Brazil, Ivory Coast, Ghana, among others, but studies regarding Peruvian cocoa are still few [13-15]. Among the great variety of cocoa that Peru possesses there is a special cocoa known as Piura's white cocoa. This cocoa has a particular characteristic, the color of some of their beans which are whitish (Figure 1). Piura's white cocoa is also recognized for its quality, fragrance, and flavor, reason why this cocoa has received national and international awards [16].



Figure 1 – Piura's white cocoa beans exhibiting different colors including whitish. Image taken from "Manual del Cultivo de Cacao Blanco de Piura" [16].

The objective of this study was to evaluate the antioxidant activity of different beans of Piura's white cocoa using DPPH and ABTS assays; quantify total phenol compounds by Folin-Ciocalteu assay and characterize main compounds by LC-MS analysis. This study is the first step of a more complex study about composition and characteristics of cocoa beans from Piura.

2. Experimental

2.1. Chemicals

Solvents used for obtaining and fractionation of organic extract were analytical grade: petroleum ether (40-60 °C), ethanol, chloroform, ethyl acetate, and butanol; all of them were purchased from PanReac. Water was purified by a Purelab CLASSIC UV system. The reagents used to measure antioxidant capacity and phenol total content, Folin & Ciocalteu's reagent 2N, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich. Gallic acid was purchased from Chromadex. Sodium carbonate and Potassium persulfate were obtained from PanReac. Methanol HPLC grade was obtained from Merck. Acetonitrile LC-MS grade and trifluoroacetic acid were purchased from Sigma-Aldrich.

2.2. Instrumentation

Cocoa beans were grinded by a Willey Mill Standars N ° 03 (Thomas scientific). Antioxidant and total phenol assays were carried out using a UV-1800 spectrophotometer (Shimadzu).

Analytic characterization was performed by using a Shimadzu HPLC System consisting of a SCL 10A VP binary pump, a DAD detector, a SPD MI10A VP, and a SIL 10AF auto sampler. The HPLC system was coupled to a Bruker Daltonics Esquire 3000 ion trap mass spectrometer, with electrospray ionization (ESI) source.

2.3. Obtaining of cocoa extracts

Cocoa fruits and beans (fermented in boxes and sun-dried) were collected from farms located in Piura (Peru). Fruits were opened in the laboratory; beans from fruits and fermented beans were dried in stove at less than 40 °C until constant weight. Cocoa husk was manually separated from beans, and beans were turned into cocoa powder with a blade grinder.

Beans of cocoa had different colors, beans were classified into violate, whitish and brown. Thus, 5 samples were used for analysis: I) violate beans, II) whitish beans, III) brown beans, IV) a mixture of different beans, and V) fermented beans (different beans).

Obtaining of cocoa extract for each sample was carried out following the next method: 10 grams of cocoa powder were defatted with 300 ml by Soxhlet. Defatted samples were extracted four times with 300 ml of ethanol shaking for 4 hours each time. Ethanol of extracts was evaporated at vacuum.

2.4. Total phenolic content by Folin-Ciocalteu assay

Sample stock solutions and Gallic acid, used as standard, were prepared by dissolving each sample in water. Working solutions used to build the standard regression curve were prepared by dissolving different volumes of stock solutions in water.

Folin-Ciocalteu assay was performed by using a method previously described by Amzad & Shah [17], with some modifications. Working solutions or water (blank) (250 μ L) were added to Folin-Ciocalteu reagent 1N (125 μ L), and then after mixing 20% sodium carbonate (625 μ L) was added. After incubation for 90 minutes at room temperature in darkness measurements were recorded at 760 nm.

TPC is reported as Gallic acid Equivalents (GAE) obtained by comparing observances of samples with the standard regression curve. Data was processed by Minitab 17.

2.5. Antioxidant assays

For both DPPH and ABTS assay, sample stock solutions and Trolox, used as standard, were prepared by dissolving each sample in methanol. Working solutions used to build regression curves were prepared by dissolving different volumes of stock solutions in methanol.

DPPH assay, which measures the reduction of the radical cation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by antioxidants, was performed by using a method previously described by Sharma & Bhat [18] with some modifications. DPPH solution 300 μ M was prepared by dissolving DPPH reactive in methanol. Working solutions or methanol (blank) (600 μ L) were added to DPPH (300 μ L). Absorbance was read at 517 nm after incubating the mixture for 30 min at room temperature in darkness.

ABTS assay was performed by using a method previously described by Celik *et al.* [19], with some modifications. ABTS radicals were generated by chemical reaction with potassium persulfate ($K_2S_2O_8$). For this purpose, a stock solution of ABTS (7 mM) and $K_2S_2O_8$ 2,45 mM was prepared and allowed to stand in darkness at room temperature all the night. The working solution was prepared by taking a volume of the stock solution and diluting it in methanol until its absorbance at 734 nm was 0.70 ± 0.02 . Working solutions or MeOH (blank) (100 μ L) were added to DPPH (900 μ L). Absorbance was read at 734 nm after incubating the mixture for 7 min at room temperature in darkness.

In both assays, antioxidant activity is reported as Trolox Equivalents (TE) obtained by comparing IC50 values of standard Trolox and samples, IC50 is the concentration of standard Trolox or sample necessary to inhibit 50% of radical cation. Data was processed by Minitab 17.

2.6. Liquid-liquid partition

Sample consisting of a mixture of different beans was analyzed by LC-MS. Before analysis extract of the sample was fractionated by liquid-liquid partition. Cocoa extract (0.7 g) was solubilized in water (25 mL). The solubilized extract was transferred to a glass column (1 cm intern diameter, 70 cm length). 25 mL of chloroform were added little by little to the column, and as the chloroform shows a higher density when compared to water, it passes through the polar phase in an intimate contact eluting low polarity substances. The procedure was repeated one more time. The chloroform extract was evaporated at vacuum, resulting in a whitish residue. The aqueous phase was then subjected to an ethyl acetate and butanol partition (with 50 mL of organic solvent each time), resulting in the production of a brown ethyl acetate fraction (mass = 85 mg; yield = 12.14 %) and butanol fraction (mass = 365 mg; yield = 52.20 %). Ethyl acetate and butanol fractions were analyzed by LC-MS.

2.7. Liquid chromatography - mass spectrometry analysis

LC-MS technique used was high liquid chromatography coupled with electrospray mass spectrometry (HPLC-ESI-MS) in positive mode. Ethyl acetate and butanol fractions were dissolved in methanol (1 mg/mL) and membrane-filtered (0.45 μ m). Solutions were analyzed at room temperature using a C18 Luna HPLC column (5 μ m, 4.6 x 250 mm; Phenomenex). Injection volume was 2 μ L and flow rate was set at 1 ml/min. The mobile phase consisted of A = H₂O (0.1% TFA) and B = CH₃CN. Gradient consisted of: 5% B for 5 min; gradient to 100% B in 45 min; 100% B for 5 min; and gradient to 5% B in 10 min.

The ion source temperature was 320°C and capillary voltage was set at -4000V (positive mode) and plat offset -500V. Nitrogen served as nebulizer gas regulated at 27 psi and a flow rate of 7 L/min. The mass spectrometer was operated in full-scan mode monitoring positive ions. Fragmentation of $[M+H]^+$ molecular ions into specific product ions was performed in enhanced product ion (EPI) mode induced by collision with nitrogen. Instrument control and data acquisition were performed using the Esquire 5.2 software.

3. Results and discussion

3.1. Total phenolic content

TPC assessment was performed by using the Folin-Ciocalteu method which is the most common phenolic assay. Although this method is widely used it presents different interferences since this colorimetric method is based on a Red-Ox reaction between the Folin-Ciocalteu solution and a reducing agent (which is not necessarily a phenolic compound), therefore Folin-Ciocalteu assay has to be used only if it is known that most reducing compounds in the sample are phenolic. Main interferences are reducing sugars and ascorbic acid [11]. In this study, the standard used was Gallic acid because it is the most common standard for this assay.

TPC of samples are presented in Table 1. Brown and violet beans showed the major content, their TPC are not significantly different ($P < 0.05$). Although violet color in cocoa beans is very intense, anthocyanins (compounds responsible of violet color) are not the main phenolic compounds in cocoa but flavan-3-ol and procyanidins which are colorless or slightly red [20]. Fermented and dried sample showed the lowest phenolic content; it has previously reported that fermentation reduces concentration of phenolic compounds by phenomena like enzymatic oxidation, oxygen diffusion throughout beans and the diffusion of polyphenols into fermentation sweating [21].

Table 1 – Piura’s white cocoa beans total phenolic content and antioxidant activity.

Sample	TPC (mg GAE/g)	DPPH ($\mu\text{mol TE/g}$)	ABTS ($\mu\text{mol TE/g}$)
Violet	43.22 \pm 0.57 ^a	380.19 \pm 12.69 ^{ab}	473.19 \pm 19.88 ^{ab}
White	38.23 \pm 0.55 ^b	331.39 \pm 14.28 ^c	405.18 \pm 14.82 ^c
Brown	44.37 \pm 0.57 ^a	403.75 \pm 14.55 ^a	501.87 \pm 16.66 ^a
Mix	41.84 \pm 0.56 ^c	365.12 \pm 14.01 ^b	450.55 \pm 16.88 ^b
Fermented and dried	7.15 \pm 0.11 ^d	40.18 \pm 2.24 ^d	64.02 \pm 3.21 ^d

Data are presented as mean \pm SD (n=3). Values within a column followed by the same superscript letters are not significantly different at the level ($P < 0.05$).

Several studies concerning TPC of cocoa and derivatives has been performed, however it is difficult to compare all results because of differences in: samples and methods for extracting phenols. Our results can be compared with those of some previous studies up to a point, results reported by some studies [13, 15, 22, and 23] are shown in Table 2. Among these studies, the one carried out by Thomas Barberan *et al.* stands out, authors analyzed beans from Ivory Coast, Colombia, Equatorial Guinea, Ecuador, Venezuela, Peru and Dominican Republic; samples from Peru and Dominican Republic exhibited the lowest values (50 and 40 mg GAE/g respectively). Our results are very similar to those reported by Thomas Barberan. In addition, Piura’s white cocoa belongs to Criollo variety which generally possesses lower concentrations of phenolic compounds than Forastero and Trinitario varieties [24].

Table 2 – Total phenolic content and antioxidant activity reported by previous works.

Author	Sample	TPC (mg GAE/g)	AA ($\mu\text{mol TE/g}$)
Niemenak <i>et al.</i> , 2006	Beans from Cameroon	86.6 - 139.4	-
Gu <i>et al.</i> , 2006	Natural cocoa powder available in USA	-	709 - 899 ^a
Thomas Barberan <i>et al.</i> , 2007	Beans from Ivory Coast, Colombia, Equatorial Guinea, Ecuador, Venezuela, Peru and Dominican Republic	40.0 - 84.2	-
Miller <i>et al.</i> , 2008	Natural cocoa powder available in USA	-	615 - 846 ^a
Di mattia <i>et al.</i> , 2012	Beans from different regions of Costa Rica	-	600 - 875 ^b
Carrillo, Londoño & Gil, 2014	Beans from different regions of Colombia	44.94 - 70.09	387.29 - 639.51 ^a
Oracz & Nebesny, 2014	Beans from Brazil, Ecuador, Venezuela, Papua New Guinea, Ghana, Indonesia and Cameroon	76.14 - 173.58	482 - 1406 ^b 323 - 1370 ^c

Data are presented as ranges of means as reported by respective authors.

^a values reported using the ORAC method.

^b values reported using the ABTS method.

^c values reported using the DPPH method.

3.2. Antioxidant activity

Antioxidant evaluation was performed using two simple and very common methods: DPPH and ABTS assays. Both methods are similar since DPPH and ABTS molecular structures are alike; and reaction mechanisms are similar, however both methods can exhibit different yields depending on the nature of the tested antioxidant [10]. In this study, the standard used was Trolox because structure and size of this artificial antioxidant are adequate to get high yields of reaction. Procyanidins, main antioxidants found in cocoa, have reported high antioxidant activities and comparable to that of Trolox [25].

Antioxidant activities of samples are presented in table 2. Brown and violet beans showed the highest antioxidant activities, their activities are not significantly different ($P < 0.05$). Fermented and dried sample showed the lowest antioxidant activity; this effect of fermentation over antioxidant activity has been previously reported [12]. In order to understand the real relation between color of beans and antioxidant activity, it is necessary to characterize compounds in these beans in the future. Antioxidant activity is higher for ABTS assay which demonstrates that reaction between ABTS and antioxidants in the sample is more efficient.

Several studies concerning antioxidant activity of cocoa and derivatives has been performed, however it is difficult to compare results because of differences in: samples, methods for extracting antioxidant compounds, antioxidant activity assay methodologies and used standards. Our results can be compared with those of some previous studies up to a point, results reported by some studies [13, 15, 21, 26, and 27] are shown in Table 2. Among these studies, the one carried out by Oracz & Nebesny stands out, authors analyzed cocoa beans from Brazil, Ecuador, Venezuela, Papua New Guinea, Ghana, Indonesia and Cameroon, they found values between 323 and 1370 $\mu\text{mol TE/g}$ for DPPH assay, and values between 482 and 1406 $\mu\text{mol TE/g}$ for ABTS. Brazilian beans showed the highest antioxidant activities, and Papua New Guinea beans the lowest ones. Our results are only a little higher than those for Papua New Guinea beans. Thomas Barberan *et al.* has reported low TPC values for Peruvian beans, as antioxidant activity is related to total phenolic content our results are compatible with those previous results.

3.3. Correlation between TPC and antioxidant activity

Our results exhibit a correlation very close to linearity between TPC and antioxidant activities for both methods (Figure 2). Genovese & Lannes [28] have founded a significant correlation ($R = 0.977$) between the phenolic content and antioxidant activity of cocoa and derivatives. Zhou *et al.* [29] studied TPC and the antioxidant activity of mangosteen, a procyanidins-rich fruit and reported very good correlations between TPC and DPPH assay ($R^2 = 0.9736$); and between TPC and ABTS assay ($R^2 = 0.9728$).

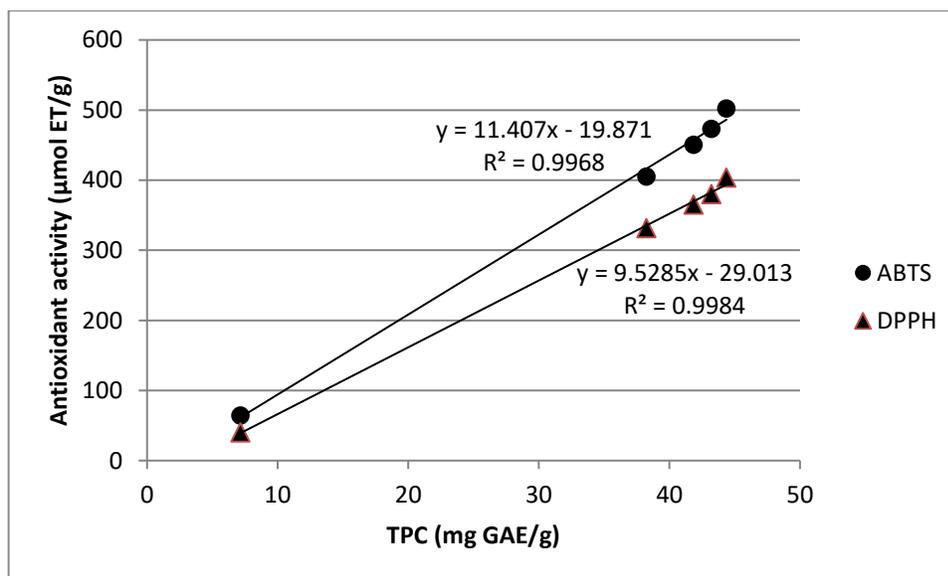


Figure 2 – Relationship between antioxidant activity and total phenolic content.

3.4. Liquid chromatography - mass spectrometry analysis

Compounds in ethyl acetate and butanol fractions were identified by analyzing the respective total ion chromatograms (TIC). TICs were interpreted and compared with bibliography. As all compound present in ethyl acetate fraction were also present in butanol fraction, TIC of butanol fraction was used for discussions. TIC of butanol fraction is shown in Figure 3.

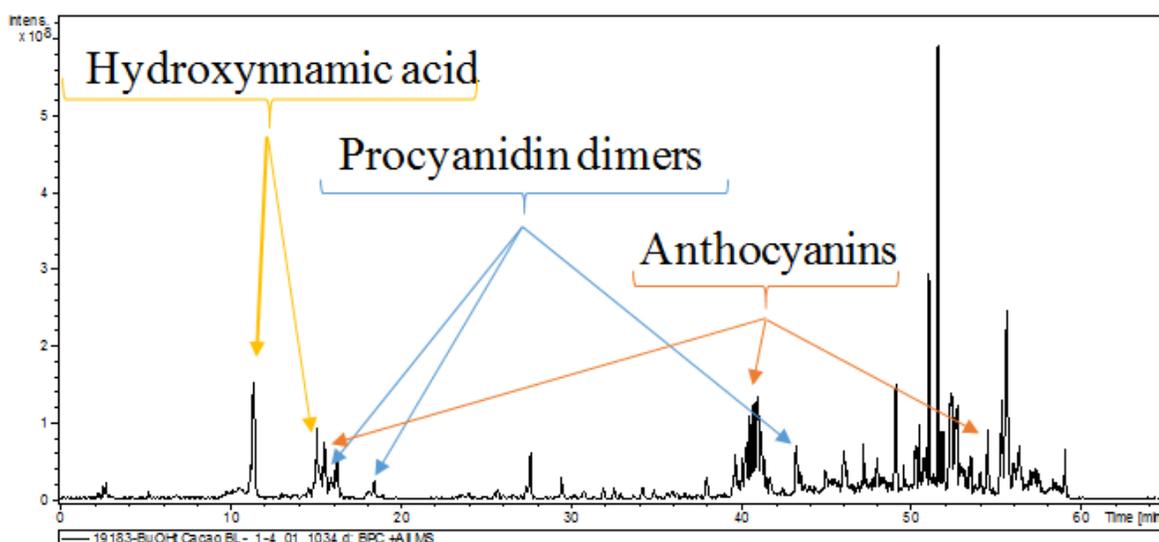


Figure 3 – Total ion chromatogram of butanol fraction.

All detected compounds are polyphenols. Two Hydroxynamic acids have been identified: caffeic acid and ferulic acid, which are compounds synthesized from phenylalanine [4]. The other compounds are anthocyanins, flavan-3-ols and procyanidins. Detected Compounds and previous works that have used like main references [9, 30-33] are shown in Table 3. Structures of main compounds reported in our study are drawn in Figure 4.

Table 3 – compounds detected in butanol fraction.

Peak N°	Identified compound	t _r (min)	m/z [M+H] ⁺ or M ⁺	References
1	Caffeic acid	10.6-11.4	180.98	Kurkin, 2003
2	Ferulic acid	15.1	194.96	
3	Cyanidin-3-O-glucoside	15.1-15.4	449.04	Flamini, De Rosso & Bavaresco, 2015
4	Procyanidin dimer (isomer 1)	15.5-15.6	579	Li & Deinzer, 2007
5	Cyanidin-3-O-pentoside	16.0-16.2	419	Flamini, 2013
6	(epi) catechin	16.0-16.4	291.01	Cádiz-Gurrea <i>et al.</i> , 2014
7	Procyanidin dimer (isomer 2)	18.5	579.08	Li & Deinzer, 2007
8	Acetone derivative of Peonidin pentoside		471.07	Flamini, 2013
9	Malvidin 4-vinylphenol derivative	27.4-27.7	505	
10	Acetone derivative of Peonidin	39.7	581.25	
11	Malvidin-8-ethyl-(epi)catechin derivative	40.3	664.42	
12	Malvidin	40.7	354.28	
13	Malvidin 4-vinylphenol derivative		576.38	
14	Cyanidin-3-O-monoglucoside derivative	41.2	471.34	
15	Procyanidin dimer (isomer 3)	43.3	579	
16	4-vinylphenol-Delphinidin	46.0	419.25	
17	Malvidin pyruvate derivative	52.3	518.36	
18	Malvidin 4-vinylguaicol adduct	54.4	447	
19	Malvidin pyruvate derivate		551.32	
20	Peonidin derivative	55.3	413.24	

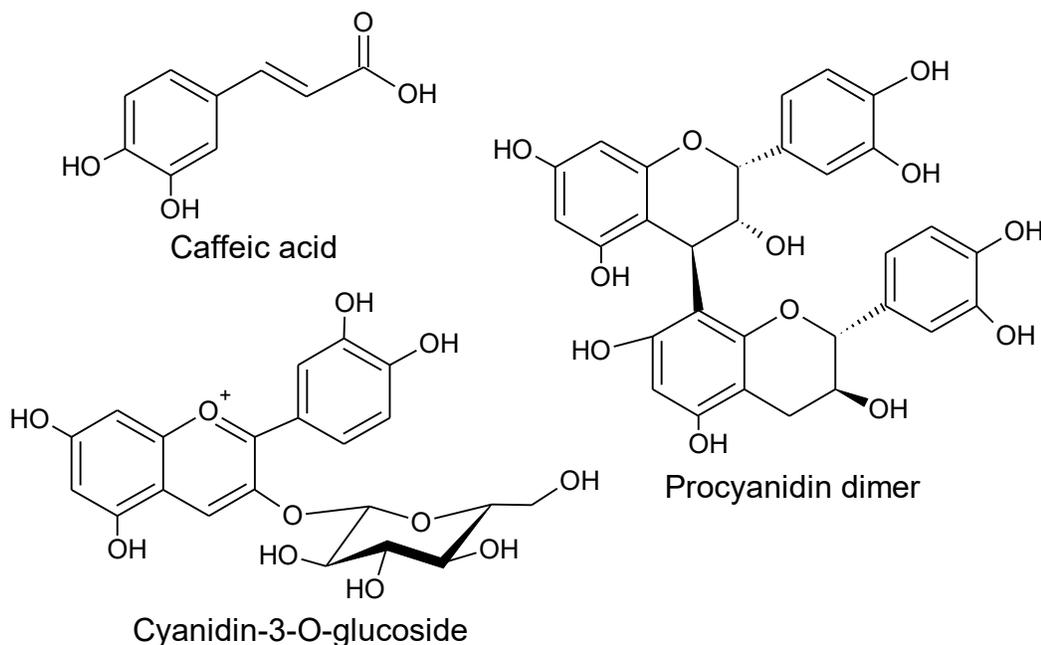


Figure 4 – Some structures found in Piura's white cocoa.

Detected procyanidins have been procyanidin dimers; main procyanidin dimers are B [5]. Heavier procyanidins haven't been found. This lack of heavy procyanidins could explain the relatively low antioxidant activity as antioxidant activity of procyanidins increases with the degree of polymerization [25]. Different anthocyanins have been found, these compounds were derivatives (mainly glycosides) of cyanidin, peonidin, malvidin, delphinidin, and petunidin. Neither theobromine nor clovamide were detected, this was expected because our extraction method was aimed to polyphenols. Apart from type, origin, and processing of cocoa, compounds profile depends on the extraction method. Our results show that our extraction method is mainly useful for anthocyanins.

4. Conclusions

TPC and antioxidant activity (according to DPPH and ABTS methods) of different types of beans of Piura's White cocoa were assessed. TPC ranged from 7.5 to 44.37 mg GAE/g; and antioxidant activity ranged from 40.18 to 403.75 $\mu\text{mol TE/g}$ for DPPH, and from 64.02 to 501.87 $\mu\text{mol TE/g}$ for ABTS. Violate and brown beans possessed the highest values, besides their values were not significantly different ($P < 0.05$), on the other hand fermented beans exhibited the lowest values. Our results were compared with previous works and exhibited relatively low TPC and antioxidant activity. TPC and antioxidant activity exhibited a very good correlation.

Several polyphenols were detected in Piura's White cocoa by HPLC-ESI-MS in positive mode. Detected compounds were different Hydroxynannamic acids, anthocyanins, flavan-3-ols and procyanidin dimers. This work is a first step of a more complex study concerning Piura's white cocoa and their content. This work is also a contribution to the research of Peruvian plants and natural resources.

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