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Obtention and Characterization of Phenolic Extracts from Different Cocoa Sources

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The aim of this study was to evaluate several cocoa sources to obtain a rich phenol extract for use as an ingredient in the food industry. Two types of phenolic extracts, complete and purified, from different cocoa sources (beans, nibs, liquor, and cocoa powder) were investigated. UPLC-MS/MS was used to identify and quantify the phenolic composition of the extracts, and the Folin−Ciocalteu and vanillin assays were used to determine the total phenolic and flavan-3-ol contents, respectively. The DPPH and ORAC assays were used to measure their antioxidant activity. The results of the analysis of the composition of the extracts revealed that the major fraction was procyanidins, followed by flavones and phenolic acids. From the obtained results, the nib could be considered the most interesting source for obtaining a rich phenolic cocoa extract because of its rich phenolic profile content and high antioxidant activity in comparison with the other cocoa sources.

KEYWORDS: Antioxidant activity; cocoa sources; phenolic compound; DPPH; ORAC; UPLC-MS/MS

INTRODUCTION

In recent years, there has been a growing interest in polyphenols because of their physiological health benefits, including antioxidant (*1−3*), anti-inflammatory (*4*), and antiatherogenic effects (*5*), among others. Cocoa beans and derivative are an important food source rich in polyphenolic compounds. Different studies have shown that the consumption of cocoa products has positive and various health effects (*6, 7*). These benefits have been mainly attributed to the high antioxidant activity of the cocoa samples. Currently, the protective health potential of cocoa polyphenols has been also related to the protection of the oxidative modification of low-density lipoproteins (LDL), a consequence of oxidative stress (*8*).

The most abundant polyphenol group in cocoa is the procyanidins (flavan-3-ol), which can range in molecular weight from monomers (catechin and epicatechin) to long-chain polymers (with degree of polymerization higher than decamers). Moreover, in smaller amounts, gallocatechin and epicagallocatechin, have also been quantified (*9*). The procyanidins have been ascribed as the main group responsible for the antioxidant activity of the cocoa beans, and this activity could also be related with their degree of polymerization (*1, 10*). However, the phenolic fraction of the cocoa is complex and contains other phenolic groups that could also contribute to the health benefits of cocoa.

Despite the high polyphenol content in the beans and their derivate products, this can vary greatly between different varieties and origins of cocoa beans and also through the manufacturing procedures (*2, 11*). The processing of raw cocoa includes a number of stages, in which cocoa beans are transformed into nibs, liquor, and cocoa powder that are used as ingredients in the formulation of the different cocoa-based foods. The first step, bean fermentation, is crucial for bringing out the desirable flavor for the cocoa derivative. The next step involves roasting and grinding the beans to obtain the nibs. The time and temperature conditions during the roasting process affect the polyphenol stability as well as the taste of the cocoa products (*11*). The result of nib grinding is a liquid paste known as cocoa liquor. Finally, the last step involves pressing the cocoa liquor to remove a high percentage of the cocoa butter, leaving a solid cake, which is milled into a fine powder cocoa.

Numerous analytical techniques have focused on the identification and quantification of the procyanidins in cocoa samples using different chromatography techniques (*3, 10, 12*). Among all of the methods used, the coupling of liquid chromatography and mass spectrometry has been shown to be a powerful tool for identifying procyanidins in cocoa products. For instance, Sanchez-Rabaneda et al. (*13*) identified some other phenolic groups in powder cocoa, such as flavonol and flavone glycosides. Nevertheless, there is not much information available about the determination of phenolic groups, such as flavonoids and phenolic acids, in cocoa beans and derivative.

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Several assays have been used to measure the antioxidant activity available in plant sources; however, all of the methods differ in their principle. The TRAP and ORAC methods are based on the measurement of the scavenging of free radicals. In contrast, other methods, such as DPPH or APPH, measure the antioxidant scavenging ability toward stable radical species. Several methods, such as APPH (*1*), DPPH (*14*), and ORAC (*4, 15*), have been mainly used to determine the antioxidant capacity of cocoa samples.

The interest of this work was focused on the evaluation of the cocoa bean and its derivative as potential sources of bioactive polyphenols, which can be used as ingredients in the food and chocolate industry. Therefore, bearing in mind that the polyphenol content can be modified during the processing of the cocoa, the first aim of this study was to characterize the composition of two different phenolic cocoa extracts obtained from several cocoa sources (beans, nibs, liquor, and powder) to evaluate the most interesting source and the effect of the technological process on the phenolic composition. Furthermore, this study evaluates the antioxidant activity of the different phenolic extracts using the DPPH and ORAC assays with the aim of establishing which phenolic group would best describe the antioxidant activity from the different cocoa sources.

MATERIALS AND METHODS

Chemicals and Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), (+)-catechin, (−)-epicatechin, caffeine, theobromine, caffeic acid, syringic acid, protocatechuic acid, epigallocatechin, hydroxybenzoic acid, naringenin, and myricetin were obtained from Sigma Aldrich (St. Louis, MO). Fluorescein (FL), Folin−Ciocalteu reagent, kaempferol, dimer B_2, vanillic acid, and quercetin-rhamnoside were from Fluka Co. (Buchs, Switzerland). Ferulic acid, coumaric acid, cinnamic acid, chlorogenic acid, quercetin, kaempferol-rutinoside, apigenin, apigenin-glucoside, luteolin, kaempferol-glucoside, and luteolin-glucoside were purchased from Extrasynthese (Genay, France); and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Acros Organics (Fair Lawn, NJ).

Phenol Extract Preparation. *Samples.* A total of four different cocoa sources were considered in the study: cocoa beans, cocoa nibs, cocoa liquor, and cocoa powder. All cocoa samples corresponded to Forastero variety from Ghana (West Africa). The Forastero variety is easy to cultivate and has a pungent aroma.

The four cocoa sources were obtained by the typical manufacturing process. The "cocoa bean" is obtained by fermentation and roasting of the fresh cocoa bean. During roasting, the bean color changes to a rich brown, and the aroma of chocolate comes through. After roasting, the cocoa bean shells are cracked and removed, leaving the essence of pure chocolate called the "nib". For Dutch roasts, an alkaline solution is added to produce nibs that are darker and less acidic in flavor. The roasted nibs are milled through a process that liquefies the cocoa butter in the nibs and forms "cocoa liquor". The cocoa liquor is pressed, and the remaining cakes of cocoa solids are pulverized into "cocoa powder".

Two kinds of phenolic extracts were obtained from the four cocoa sources, the phenol cocoa extract (CE), by solid/liquid extraction, and the purified extract (PCE), by solid phase extraction (SPE).

Phenol Cocoa Extract (CE). The phenol CE extraction was based on the work by Hammerstone et al. (*10*), slightly modified. Cocoa beans and cocoa nibs were ground in a laboratory mill for 30 s before the phenol extraction to obtain a homogeneous material. To achieve maximum extraction efficiency during the sample preparation, 15 g of raw material was first defatted four times with 125 mL of hexane for 20 min in an orbital shaker (Selecta, Barcelona, Spain) at 200 rpm and subsequently centrifuged for 15 min at 15000*g*. The defatted cocoa sample was then extracted four times with solvent extraction at a ratio of 1:5. After the addition of this solution, the tubes were vortexed for 3 min and then were centrifuged for 15 min at 15000*g*. The supernatants were combined from the centrifuged tubes and filtered with glass wool. The organic solvent was removed by rotary evaporation (Büchi, Labortechnick AG, Switzerland) under partial vacuum at 30 °C. Finally, the water extract was freeze-dried in Lyobeta 15 equipment (Ima-Telstar, Spain) to obtain the CE.

Purified Phenol Cocoa Extract (PCE). CE (100 mg suspended in 1 mL of Milli-Q water) was purified by off-line SPE using a SPE manifold (Visiprep, Supelco). The cartridge was C_{18}, 1 g (Waters). The cartridge was preconditioned with methanol and then with Milli-Q water. After the freeze-dried CE had been loaded, the cartridge was cleaned by passage of Milli-Q water. Finally, the polyphenols were eluted with solvent extraction solution. The eluted fraction was concentrated by rotary evaporation under a partial vacuum at 30 °C and was finally freeze-dried to obtain the PCE.

Chromatographic Analysis of the Phenolic Extracts. *Ultraperformance liquid chromatography.* (UPLC) analyses were performed using a Waters Acquity ultraperformance liquid chromatography system (Waters, Milford, MA), equipped with a binary pump system (Waters).

The UPLC analyses were performed using an Acquity High Strength Silica (HSS) T3 column (100 mm × 2.1 mm i.d., 1.8 μm particle size) (Waters) with a binary mobile phase. Solvent A was water/acetic acid (0.2%) and B was acetonitrile. Two chromatography methods were developed to characterize the phenolic profile in the cocoa samples: the first one, to analyze procyandins and alkaloids, and a second one to analyze the flavonoids and phenolic acids. The analysis of the procyanidins in the cocoa samples was as reported in our previous work (*16*). For the analysis of the phenolic acids and flavonoids, the gradient elution at 30 °C was as follows: 0−2 min, 5% B isocratic; 2−6 min, 5−18% B; 6−7 min, 18% B isocratic; 7−10 min, 18−21% B; 10−12 min, 21−23% B; 12−16 min, 23−40% B; 16−16.10 min, 40−100% B; 16.10−18 min, 100% B isocratic; 18−18.10 min, 100−5% B; 18.10−20 min, 5% B isocratic. In both analytical methods, the flow rate was 0.4 mL min^{-1} and the sample volume injected was 2.5 μL.

The lyophilized CE and PCE were dissolved in solvent extraction solution and filtered through 0.22 μm nylon filters prior to chromatographic analysis. The stock standard solutions of (+)-catechin, (−)-epicatechin, caffeine, theobromine, and dimer B_2 were dissolved in solvent extraction solution. Meanwhile, the rest of the standards were dissolved in methanol. All stock standard solutions were prepared weekly and stored in a dark glass flask at −80 °C. The results of the quantification of the procyanidins were expressed as milligrams of catechin per gram of lyophilized phenolic extract; the phenolic acids and the aglycon flavonoids were expressed as micrograms of standard per gram of lyophilized phenolic extract. All of the flavonoid glycosides were quantified as micrograms of the corresponding aglycon per gram of lyophilized phenolic extract.

MS/MS Conditions. The UPLC system was coupled to a TQD mass spectrometer (Waters) using a Z-spray electrospray ionization (ESI) source. The MS was operated in negative mode to analyze the phenolic compounds. In contrast to the analysis of the alkaloids (caffeine and theobromine), the MS was operated in the positive mode. However, both analyses were done in a single run. The data were acquired in selected reaction monitoring (SRM). The ionization source working conditions were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L h^{-1}; desolvation gas flow rate, 800 L h^{-1}; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. The dwell time established for each transition was 30 ms. Data acquisition was carried out with MassLynx v 4.1 software.

Total Polyphenol Content. *Total polyphenol content (TP).* was determinated according to the Folin−Ciocalteu colorimetric method. An aliquot of lyophilized phenolic extract dissolved in solvent extraction was diluted to 5 mL of Milli-Q water, and 0.5 mL of Folin−Ciocalteu reagent was added. After 3 min, 1 mL of sodium carbonate was added and the content was mixed to obtain a final volume of 10 mL with Milli-Q water. After 60 min of standing in darkness, the color was measured in the spectrophotometer at 725 nm.

Total Flavan-3-ol (TF). The content of flavan-3-ols in the phenolic extracts was measured with the vanillin assay described by Sarker et al. (*17*). The total volume fixed was 6 mL, the lyophilized cocoa extract, CE or PCE, was dissolved in 1 mL of methanol, 2.5 mL of 1% vanillin in methanol, and 2.5 mL of HCl. The reaction mixture was incubated

Table 1. Optimized SRM Conditions for the Analysis of Procyanidins, Phenolic Acids, Flavones and Alkaloids by UPLC-MS/MS

standard	[M − H]	SRM_1 (quantification)	cone coltage (V)	collision energy (eV)	SRM_2 (confirmation)	cone voltage (V)	collision energy (eV)
				ESI (−)			
procyanidins							
catechin	$[M - H]^•$	289 > 245	45	10	289 > 205	45	15
epicatechin	$[M - H]^•$	289 > 245	45	10	289 > 179	45	15
procyanidin B2	$[M - H]^•$	577 > 289	45	20	577 > 425	45	15
epigallocatechin	$[M - H]^•$	305 > 125	40	15	305 > 165	40	15
phenolic acids							
hydroxybenzoic acid	$[M - H]^•$	137 > 93	30	15			
vanillic acid	$[M - H]^•$	167 > 123	30	10	167 > 152	30	15
caffeic acid	$[M - H]^•$	179 > 135	35	15	179 > 117	35	20
syringic acid	$[M - H]^•$	197 > 153	30	10	197 > 182	30	15
ferulic acid	$[M - H]^•$	193 > 134	30	15	193 > 178	30	10
vanillin	$[M - H]^•$	151 > 136	20	10	151 > 92	20	15
coumaric acid	$[M - H]^•$	163 > 119	25	10	163 > 117	25	25
cinnamic acid	$[M - H]^•$	147 > 103	20	10			
chlorogenic acid	$[M - H]^•$	353 > 191	30	10	353 > 127	30	30
protocatechuic acid		153 > 109	45	15	153 > 91	45	25
flavones							
quercetin	$[M - H]^•$	301 > 151	40	15	301 > 125	40	20
quercetin-glucoside	$[M - H]^•$	463 > 300	50	25	463 > 151	45	30
quercetin-rutinoside	$[M - H]^•$	609 > 300	45	25	609 > 151	45	35
quercetin-arabinoside	$[M - H]^•$	433 > 300	45	20	433 > 151	45	25
dihydroquercetin	$[M - H]^•$	303 > 285	45	10	303 > 177	45	10
dihydroxykaempferol	$[M - H]^•$	287 > 259	45	10	287 > 269	45	10
kaempferol-rutinoside	$[M - H]^•$	593 > 285	50	25	593 > 255	50	40
naringenin	$[M - H]^•$	271 > 151	40	15	271 > 119	40	20
naringenin-glucoside	$[M - H]^•$	433 > 271	45	10	433 > 164	45	25
myricetin-glucoside	$[M - H]^•$	479 > 316	45	20	479 > 179	45	30
luteolin	$[M - H]^•$	285 > 133	55	25	285 > 151	55	25
luteolin-glucoside	$[M - H]^•$	447 > 285	50	25	447 > 256	50	40
apigenin	$[M - H]^•$	269 > 117	60	25	269 > 151	60	25
apigenin-glucoside	$[M - H]^•$	431 > 268	45	25	431 > 311	45	30
				ESI (+)			
alkaloids							
caffein	$[M - H]^+$	195 > 138	45	15	195 > 110	45	20
theobromine	$[M - H]^+$	181 > 163	45	15	181 > 140	45	10

for 15 min in the dark, and the absorbance at 500 nm was measured. The TP and TF contents were calculated from the calibration curve using catechin as a standard and expressed as milligrams of catechin per gram of lyophilized phenolic extract.

Antioxidant Activity. *DPPH Assay.* The scavenging activity of the phenolic extracts toward the stable free radical DPPH was measured. An aliquot of 50 μL, at concentrations varied from 4−6 to 2.5−4 mg mL^{-1} for CE and PCE in the solvent extraction solution respectively, was mixed with 2.95 mL of 0.1 mM methanolic DPPH solution. The mixture was shaken and left to read for 5 min in the spectrophotometer at 517 nm. The scavenging activity of the DPPH radical was calculated using the equation

$$\text{scavenging activity (\%)} = \left(\frac{A - B}{A}\right) \times 100$$

where A is the initial absorbance of the mixture and B is the absorbance after 5 min. Triplicate measurements were carried out for each sample. The EC_{50} was determined from the plotted graph of the scavenging activity against the concentration of the cocoa extracts.

ORAC Assay. The ORAC assay was performed as described by Ou et al. (*18*), slightly modified, in a FLUORstar optima spectrofluorometric (BMG GmbH, Offenburg, Germany). The reaction was carried out in phosphate buffer (pH 7.4). AAPH was used as a peroxyl generator (prepared in assay buffer at 37 °C immediately before each assay), Trolox as a standard (concentration range from 12.5 to 100 μM), and assay buffer as a blank. The ORAC values were calculated by a linear regression equation between Trolox or the sample concentration and the net area under the FL decay curve. The results are expressed as micromoles of Trolox equivalent per gram of lyophilized phenolic extract.

Statistical Analysis. All data were expressed as mean ± standard deviation. Data were analyzed by a one-way ANOVA test to assess differences between means. A significant difference was considered at level of $p < 0.05$. All of the statistical analyses were carried out using STATGRAPHICS Plus 5.1.

RESULTS AND DISCUSSION

Analysis of the Phenolic Profile of CE and PCE by UPLC-MS/MS. In this study, two types of phenolic extracts, CE and PCE, from different cocoa sources (bean, nib, liquor, and powder) were analyzed to evaluate differences in their polyphenol content and the relationship with the technological treatment of the cocoa sources. Most of the studies carried out on the phenolic fraction of cocoa and its derivatives have focused on the procyanidin group (*1, 10*). Thus, a UPLC-MS/MS method was applied to quantify the procyanidins in the cocoa extracts, as in our previous work (*16*). Additionally, a chromatographic method was developed to identify and quantify other phenolic compounds, such as flavonoids and phenolic acids, using phenol standards. Accordingly, to obtain the structural information of each phenolic compound, the deprotonated molecule was first studied by the negative mode $[M - H]^-$ in full-scan mode (by scanning from m/z 80 to 800). Then the fragmentation of these precursors ions was investigated under various conditions to study their molecular structure through the use of different collision energies. The optimal collision energy, cone voltage, and transition chosen for the SRM experiment are listed in **Table 1**. Two characteristic SRM transitions were selected for each compound: SRM_1 for quantification and SRM_2 for confirmation.

The concentrations of the phenolic compounds identified in CE and PCE are shown in **Tables 2** and **3**, respectively. The components of the phenolic extracts were classified into four

Table 2. Quantification by UPLC-MS/MS of Phenolic Compounds (Procyanidins, Phenolic Acids and Flavones) and Alkaloids in CE from Different Cocoa Sources[a]

phenol	bean	nib	liquor	powder
	Milligrams of Catechin per Gram of Lyophilized CE			
procyanidins				
catechin	0.335 ± 0.083a	2.374 ± 0.090b	1.223 ± 0.127c	1.803 ± 0.321d
epicatechin	3.011 ± 0.053a	5.638 ± 0.061b	3.873 ± 0.217c	0.922 ± 0.074d
dimer	8.214 ± 0.521a	13.20 ± 0.157b	8.341 ± 0.481a	2.914 ± 0.174c
trimer	4.411 ± 0.283a	6.216 ± 0.410a	3.776 ± 0.182b	0.398 ± 0.022c
tetramer	1.768 ± 0.287a	1.571 ± 0.080b	1.191 ± 0.111c	0.028 ± 0.021d
pentamer	0.046 ± 0.017a	0.157 ± 0.015b	0.021 ± 0.010a	nd
hexamer	0.156 ± 0.064a	0.100 ± 0.040a	0.115 ± 0.082a	nd
heptamer	0.027 ± 0.009a	0.018 ± 0.003a	0.021 ± 0.013a	nd
octamer	0.023 ± 0.010a	0.017 ± 0.001a	0.012 ± 0.017b	nd
nonamer	0.020 ± 0.010a	0.011 ± 0.000b	0.009 ± 0.012b	nd
epigallocatechin	0.090 ± 0.011ab	0.167 ± 0.042c	0.109 ± 0.263a	0.061 ± 0.030b
	Milligrams of Alkaloid per Gram of Lyophilized CE			
alkaloids				
theobromine	43.42 ± 4.541a	39.12 ± 1.212b	44.62 ± 3.612c	38.72 ± 0.133d
caffeine	4.500 ± 0.124a	2.941 ± 0.082b	3.624 ± 0.200a	3.303 ± 0.272bc
	Micrograms of Standard per Gram of Lyophilized CE			
phenolic acids				
hydroxybenzoic acid	40.25 ± 12.34a	31.30 ± 9.112b	40.03 ± 5.034a	51.84 ± 1.183c
vanillic acid	26.40 ± 6.492a	18.76 ± 4.178b	17.44 ± 11.06ab	15.13 ± 3.325b
caffeic acid	74.43 ± 7.607a	74.25 ± 4.462a	72.83 ± 8.116a	71.59 ± 4.968a
syringic acid	8.073 ± 0.314a	9.514 ± 2.597a	8.170 ± 2.393a	12.26 ± 3.093b
ferulic acid	3.316 ± 0.274a	2.472 ± 0.131b	1.495 ± 1.296bc	2.111 ± 0.195b
vanillin	4.822 ± 1.839a	3.080 ± 0.572b	2.752 ± 0.443b	1.720 ± 0.127c
coumaric acid	25.90 ± 5.086a	24.14 ± 2.860a	18.29 ± 1.102b	24.52 ± 1.581a
cinnamic acid	13.36 ± 5.354a	6.442 ± 2.748b	17.06 ± 5.820a	28.81 ± 5.341c
chlorogenic acid	23.63 ± 4.538a	27.68 ± 3.393a	35.18 ± 4.020b	18.14 ± 0.342c
protocatechuic acid	478.2 ± 74.08a	380.6 ± 106.4b	478.2 ± 53.64a	1055 ± 155.1c
	Micrograms of Standard per Gram of Lyophilized CE			
flavones				
quercetin	102.1 ± 26.79a	201.6 ± 56.91b	238.9 ± 35.30b	57.37 ± 3.512c
quercetin-glucoside	2940 ± 192.9a	3094 ± 230.9b	2215 ± 262.3c	590.5 ± 58.65d
quercetin-rutinoside	21.77 ± 1.532a	24.77 ± 1.235b	24.72 ± 2.214b	23.17 ± 1.168b
quercetin-arabinoside	3169 ± 51.15a	3429 ± 103.0a	2706 ± 87.57b	664.9 ± 15.61c
dihydroquercetin	95.64 ± 6.253a	132.2 ± 16.37b	111.9 ± 2.377c	60.27 ± 4.672d
dihydroxykaempferol	712.7 ± 51.84a	1012 ± 85.07b	725.5 ± 31.17a	171.4 ± 4.521c
kaempferol-rutinoside	502.2 ± 19.07a	648.8 ± 56.28b	551.7 ± 39.38a	579.0 ± 99.99a
naringenin	23.91 ± 0.342a	26.26 ± 0.693ab	27.05 ± 3.360ab	5.076 ± 0.766b
naringenin-glucoside	70.96 ± 4.701a	92.43 ± 10.59b	64.60 ± 8.234a	20.18 ± 1.580c
myricetin-glucoside	118.6 ± 17.92a	143.2 ± 8.530b	138.2 ± 11.00c	161.3 ± 125.7d
luteolin	90.09 ± 20.80a	82.87 ± 14.02b	106.4 ± 18.53c	29.06 ± 1.713d
luteolin-glucoside	179.9 ± 52.02a	251.8 ± 76.51b	226.5 ± 72.27b	157.1 ± 53.33a
apigenin	2.722 ± 0.172a	2.354 ± 0.208a	3.826 ± 1.195b	0.967 ± 0.129c
apigenin-glucoside	0.890 ± 0.088a	1.268 ± 0.264b	1.051 ± 0.182c	0.735 ± 0.155a
total polyphenols content (TP)	259.9 ± 8.413a	302.5 ± 5.157a	257.5 ± 5.982a	123.9 ± 10.41b
total flavan-3-ol (TF)	163.5 ± 17.87a	175.2 ± 10.70a	168.8 ± 6.047a	49.53 ± 5.143b

[a] Determination of total polyphenol content by Folin−Ciocalteu and flavan-3-ol by vanillin assay in CE. Values in the same row followed by different letters are significantly different ($p < 0.05$). nd, not detected.

groups: procyanidins, alkaloids, phenolic acids, and flavones. The procyanidin content varied significantly in relation to the cocoa source, mainly in the CE extracts. The results indicated that the nib could be the most interesting cocoa source for phenolic extracts. This could be related to the grinding and roasting operations during the transformation of the cocoa beans into nibs. Grinding and roasting could increase the damage to the cell that leads to a breakdown of the cell wall. These changes in the cytoplasmatic material, especially the loss of cell disruption, lead to major solubility and diffusion of the phenols compounds. As a consequence, these modifications in the cell wall structure during the roasting and grinding process could explain the increased phenolic compound content found in the cocoa nib extracts in comparison with the cocoa bean extracts.

On the other hand, lower concentration in the cocoa liquor has been observed. Also, it could be due to the pressing and milling processes that could produce a loss of the phenolic compounds. During these manufacturing steps oxidation and condensation reactions are present, which could contribute as well to a loss in the phenol content. However, it depends on the pressing and milling conditions to influence the loss of the phenolic compounds. It has been seen that mild condition in these processes did not really influence the variation on the phenolic content (*9*). In this work, the procyanidin content for liquor CE and PCE decreased in comparison with nib CE and PCE; however, these contents are nearly the same as those of the cocoa bean CE (**Figure 1**). Therefore, in this case, it can be concluded that mild conditions were applied to the nib to obtain the liquor.

The lowest phenolic content observed in the powder CE and PCE (**Tables 2** and **3**) in comparison with the phenolic extracts of the other cocoa sources could be attributed to the degradation as consequence of the temperature during the pressing process of the cocoa liquor, to obtain the cocoa butter and the "cocoa cakes" that are finally pulverized into cocoa power. In addition

Table 3. Quantification by UPLC-MS/MS of Phenolic Compounds (Procyanidins, Phenolic Acids and Flavones) and Alkaloids in PCE from Different Cocoa Sources[a]

phenol	bean	nib	liquor	powder
		Milligrams of Catechin per Gram of Lyophilized PCE		
procyanidins				
catechin	0.434 ± 0.055a	3.119 ± 0.319b*	1.532 ± 0.261a	6.974 ± 0.288c*
epicatechin	4.314 ± 0.371a*	6.147 ± 0.727b	4.721 ± 0.300c*	2.344 ± 0.241d*
dimer	10.43 ± 1.804a	14.81 ± 3.356a	10.38 ± 1.067b*	10.67 ± 0.429c*
trimer	6.369 ± 0.890a*	7.600 ± 0.466b	5.232 ± 0.652c*	1.027 ± 0.004d*
tetramer	2.879 ± 0.129a*	2.512 ± 0.069b*	1.889 ± 0.054a*	0.120 ± 0.027c*
pentamer	0.079 ± 0.013a*	0.077 ± 0.010a*	0.040 ± 0.007b*	0.009 ± 0.001c
hexamer	0.359 ± 0.014a*	0.286 ± 0.069ab*	0.221 ± 0.031b*	nd
heptamer	0.042 ± 0.001a	0.033 ± 0.003b	0.023 ± 0.002b	nd
octamer	0.033 ± 0.004a	0.029 ± 0.003a	0.020 ± 0.002b	nd
nonamer	0.032 ± 0.004a	0.025 ± 0.003a*	0.018 ± 0.002b	nd
epigallocatechin	0.117 ± 0.039a	0.176 ± 0.069b	0.113 ± 0.391a	0.096 ± 0.0382c
		Milligrams of Alkaloid per Gram of Lyophilized PCE		
alkaloids				
theobromine	70.27 ± 4.016a*	64.87 ± 1.532a*	65.74 ± 3.266a*	89.66 ± 8.914b*
caffeine	7.195 ± 0.918a*	4.991 ± 0.156b*	5.169 ± 0.270a*	9.030 ± 0.257c*
		Micrograms of Standard per Gram of Lyophilized PCE		
phenolic acids				
hydroxybenzoic acid	21.15 ± 7.526ab*	17.07 ± 7.105b*	17.91 ± 5.606b*	42.77 ± 9.904a
vanillic acid	20.95 ± 7.620a	21.50 ± 16.13a	20.70 ± 11.66a	41.74 ± 19.78a*
caffeic acid	88.88 ± 18.42a	76.72 ± 9.509a	78.18 ± 11.34a	99.76 ± 46.12a*
syringic acid	13.14 ± 3.526a*	11.49 ± 3.003a	15.22 ± 1.610a	26.10 ± 5.295b*
ferulic acid	4.515 ± 0.805a	3.667 ± 1.740a	3.367 ± 0.741ab*	3.445 ± 2.054b
vanillin	3.573 ± 0.954ab	3.208 ± 1.119b	2.242 ± 0.660b	9.027 ± 3.584b*
coumaric acid	41.97 ± 9.513ab*	34.89 ± 5.534ab*	51.87 ± 5.006b*	30.47 ± 6.386a
cinnamic acid	24.98 ± 4.980a*	25.02 ± 4.506a*	31.43 ± 1.821a*	91.28 ± 23.15b*
chlorogenic acid	31.38 ± 10.69a*	32.33 ± 1.883a	54.21 ± 12.01b*	21.91 ± 6.720c
protocatechuic acid	134.6 ± 55.93a*	69.07 ± 36.76a*	64.14 ± 31.61a*	226.0 ± 86.33b*
		Micrograms of Standard per Gram of Lyophilized PCE		
flavones				
quercetin	140.4 ± 41.80a	259.9 ± 76.13b	263.2 ± 80.40b	93.17 ± 27.00c
quercetin-glucoside	3649 ± 1005a	3464 ± 914.5a	3066 ± 748.5b	1284 ± 300.8c*
quercetin-rhamnoside	29.12 ± 5.189a	19.39 ± 3.433ab	14.67 ± 1.285b	18.15 ± 6.310c
quercetin-rutinoside	99.64 ± 23.66a*	18.53 ± 1.632b*	17.87 ± 1.561b*	21.52 ± 8.301c
quercetin-arabinoside	4501 ± 749.8a*	4215 ± 499.5a	3629 ± 783.7b	1622 ± 285.4c*
dihydroquercetin	96.68 ± 35.17a	95.44 ± 32.87b	86.09 ± 27.59b	76.09 ± 58.13b
dihydroxykaempferol	781.0 ± 134.6a	878.9 ± 103.7b	753.5 ± 96.58a	378.7 ± 115.4c*
kaempferol-rutinoside	834.9 ± 25.94a*	741.4 ± 70.67a	701.3 ± 68.59a*	1144 ± 395.9b*
kaempferol-glucoside	1198 ± 233.1a	14.07 ± 1.070b	14.46 ± 1.267b	17.31 ± 6.809b
naringenin	23.38 ± 7.664a	25.92 ± 6.980a	30.91 ± 8.047a	8.775 ± 2.721b
naringenin-glucoside	65.59 ± 20.28a	79.28 ± 27.65b	69.38 ± 17.84a	42.43 ± 18.58c
myricetin-glucoside	91.10 ± 10.10a	107.7 ± 13.42a*	108.3 ± 16.33a*	71.91 ± 60.57a
luteolin	182.0 ± 3.250a*	111.7 ± 30.29b	159.8 ± 64.57a	75.01 ± 15.38c*
luteolin-glucoside	486.9 ± 273.2a*	255.7 ± 94.27b	247.6 ± 110.0b	362.0 ± 170.9b*
apigenin	2.736 ± 1.182a	3.034 ± 1.249a	3.883 ± 1.710b	1.753 ± 0.837c
apigenin-glucoside	1.714 ± 0.368a*	1.048 ± 0.195a	1.187 ± 0.310a	1.310 ± 0.490a
total polyphenols content (TP)	399.9 ± 28.14a*	437.2 ± 25.12a*	378.8 ± 11.39a*	296.5 ± 22.28b*
total flavan-3-ol (TF)	292.2 ± 7.256a*	306.6 ± 3.873a*	275.9 ± 3.894a*	117.2 ± 7.450b*

[a] Determination of total polyphenol content by Folin−Ciocalteu and flavan-3-ol by vanillin assay in PCE. Values in the same row followed by different letters are significantly different ($p < 0.05$). An asterisk indicates significant difference from the CE ($p < 0.05$). nd, not detected.

with this last step, it is noteworthy that the content of catechin is higher for the powder CE and PCE than for the other cocoa source extracts. This could be due to an isomerization of the epicatechin to catechin or to the monomerization of procyanidins during the manufacturing process of the cocoa power.

By comparison of the complete and purified extracts, CE and PCE, respectively (**Tables 2** and **3**), it is interesting to analyze the procyanidin content, especially the monomer and dimer contents. Particularly, the purification step of the phenolic extract of powder supposed a significant increase in the procyanidins, mainly monomers to trimers. In contrast, the concentration of the oligomers (higher than hexamers) was not significantly increased by the purification of the phenolic extract.

In relation to the alkaloid content, it is worth noting the main content of theobromine in both cocoa extracts, CE and PCE, for all cocoa sources. In addition, all PCEs showed a significant difference in the alkaloid content in comparison with the CE. However, the higher theobromine and caffeine contents were quantified in the PCE from the cocoa powder, despite the lower procyanidin content.

With regard to the flavone content, which was almost 2 times lower than the procyanidin concentration in all of the CEs and PCEs, it was mainly characterized by flavonol type, quercetin-glycoside forms, dihydroxykaempferol, and kaempferol. The total flavone content was significantly different for all CEs, which ranged from 2.5 to 9.1 mg per lyophilized phenolic extract. In contrast, only the cocoa powder PCE showed the lowest and significantly different content in comparison with the other PCEs. Conversely, in powder PCE the concentration of the main flavonol type, catechin, as well as other flavones

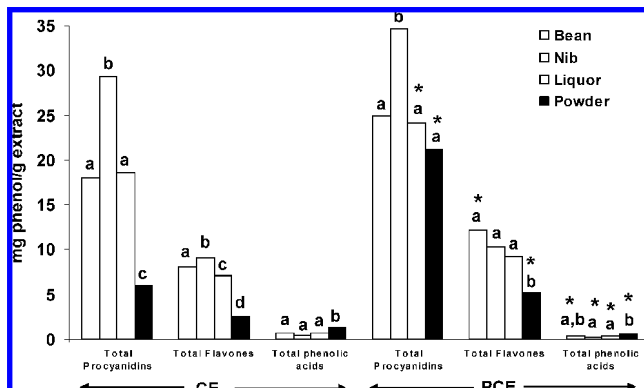

Figure 1. Content of total procyanidins, flavones, and phenolic acids in CE and PCE from different cocoa sources, expressed as milligrams of phenol per gram of cocoa extract. For each phenolic group (procyanidins, flavones, and phenolic acids) for CE and PCE, respectively, means with different letters indicate significant differences between cocoa sources (bean, nib, and power) at the level of $p < 0.05$. Values with an asterisk indicate significant difference for each of the phenolic group (procyanidins, flavones, and phenolic acids) content between CE and PCE ($p < 0.05$).

such as luteolin and luteolin-glucoside were doubled in comparison with the other PCEs. As can be observed in **Figure 1**, the total flavone content also increased in the nib CE in comparison with the other cocoa CEs. However, this trend cannot be seen for the PCE. Only a lower and significant difference in the flavone content is shown in the powder PCE in comparison with the other PCEs.

Protocatechuic acid was found to be the main phenolic acid in all of the CEs and PCEs. However, the total content of the phenolic acid group in all of the cocoa extracts was found as the minor fraction in comparison with the procyanidin and flavone contents (**Figure 1**). Nevertheless, it is interesting to note that the poorest phenolic extracts in terms of procyanidins and flavones, CE and PCE powders, were the richest in phenolic acids (**Tables 2** and **3**). This could be due to the process used to obtain the cocoa powder. Curiously, it was also observed that the phenolic acid profile in all of the cocoa sources decreased in all of the PCEs. These slightly lower values could be attributed to the fact that phenolic acids are the most hydrophilic molecules, so they could be removed with the clean water during the cleanup step in the purification procedure (SPE).

Parallel to the chromatographic analysis of the phenolic extracts, the total phenols were quantified according to the Folin−Ciocalteu and vanillin methods, which are faster and easier methods. The total contents of polyphenolic compounds in all of the extracts were expressed by milligrams of catechin per gram of lyophilized cocoa extract. The results are shown in **Tables 2** and **3**, and these values ranged from 123.9 to 302.5 and from 296.5 to 437.2 for CE and PCE, respectively. The total polyphenol content followed the order nib ≈ bean ≈ liquor > powder for CE and PCE (significant difference, $p < 0.05$). The TP content expressed by the Folin−Ciocalteu was higher than the total sum of polyphenol compounds determined by UPLC-MS/MS. It is generally known that the Folin−Ciocalteu is not a specific test for phenol quantification and that it detects other hydrophilic compounds, such as residual sugars. Therefore, the vanillin assay was considered to be a more specific method for quantifying procyanidins due to the presence of procyanidins as the main phenolic group in cocoa extracts. This test shows more sensitivity and specificity to a narrow range of procyanidins because it is limited to flavonoids with meta-oriented

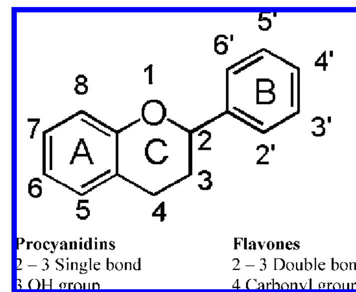

Figure 2. Basic structure and numbering system of procyanidins and flavones.

Table 4. Antioxidant Activity of Lyophilized CE and PCE Based on DPPH and H-ORAC Assays[a]

cocoa source	DPPH[b]		H-ORAC[c]	
	CE	PCE	CE	PCE
bean	0.09 ± 0.01a	0.05 ± 0.00ab	3778.19 ± 296.92a	5534.18 ± 283.36a
nib	0.09 ± 0.00a	0.04 ± 0.00a	3640.44 ± 181.63a	6930.99 ± 568.10b
liquor	0.09 ± 0.01a	0.06 ± 0.00b	3610.27 ± 15.70a	6393.77 ± 265.46b
powder	0.18 ± 0.00b	0.10 ± 0.00c	1683.64 ± 204.20b	5556.91 ± 239.94a

[a] Values are expressed as mean ± standard deviation ($n = 3$). Means in the same column followed by different letters are significantly different ($p < 0.05$). [b] EC_{50} value is defined as the mg of cocoa extract necessary to decrease the initial DPPH radical concentration by 50%. [c] μmol equiv Trolox/g of lyophilized cocoa extract.

unsubstituted hydroxyls and a saturated C_2−C_3 bond and lacking a C_4 carbonyl (see **Figure 2**). As a consequence, the number of procyanidins is proportional to the absorbance of the solution (*17*). Thus, according to the values shown in **Tables 2** and **3**, the cocoa extracts were also classified in the same order as followed in the Folin−Ciocalteau test. Moreover, the values from the vanillin assay in CE powder and PCE were the lowest, which also correlated with the content of procyanidins identified by UPLC-MS/MS, especially with procyanidins with a degree of polymerization higher than the pentamers.

According to the results of the phenolic quantification by UPLC-MS/MS, the total polyphenol content by Folin−Ciocalteu and the total flavanal content by vanillin tests, the purification step of the CE by SPE supposed an important enrichment of the phenolic content, mainly in the extracts from cocoa powder, probably as consequence of the loss of the carbohydrates, which may have been eluted in the cleanup step. As a consequence of the SPE step, the total polyphenolic content increased from CE to PCE around 30−35% for the bean, nib, and liquor extracts, whereas for CE powder the SPE step increases by 58% the phenolic content of the PCE.

Antioxidant Activity Measured of CE and PCE. The cocoa sources have been shown to be subject to change in polyphenol content during the steps of processing. To assess the antioxidant activity in the cocoa products obtained, two different assays were applied. The first one was the DPPH assay, in which the free radical scavenging activities were expressed as EC_{50}, defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. Thus, the lowest EC_{50} indicates the strongest antioxidant capacity. As can be observed in **Table 4**, the EC_{50} values for the phenolic extracts from the cocoa bean, nib, and liquor were not significantly different, ranging from 0.04 to 0.06 and 0.09 for CE and PCE, respectively. In contrast, the CE powder and PCE showed the lowest antioxidant activity, expressed as the highest EC_{50} (being 0.18 and 0.10, respectively).

Total antioxidant activity in most cocoa and chocolate samples assayed by the ORAC method was mainly attributed to the hydrophilic ORAC (H-ORAC) because H-ORAC made up ≥90%

of the total antioxidant capacity (*19*). For that reason, in this study, only the H-ORAC from all of the cocoa extracts was also measured (**Table 4**). The rank order of the antioxidant activity was the same as the DPPH results for the CE. However, the antioxidant activity for the PCE followed the order nib ≈ liquor > bean ≈ powder (significant difference $p < 0.05$). It is noteworthy that the purification step of the PCE powder increased the H-ORAC activity in comparison with the effect of the purification of the PCE of the other cocoa sources. This fact could be explained due to the concentration of some phenolic compounds in this extract such as catechin monomer, which showed statistically the highest content of all the PCEs, and also the levels of some flavones, such as dihydroxykaempferol, kaempferol, luteolin, and quercetin and their glucoside forms increased by at least twice (**Tables 2** and **3**). As **Figure 2** shows, all of these phenolic compounds showed a catechol group in the B ring, capable of attributing the major antioxidant activity to the PCE powder. It could thus appear that the increase in these phenolic compounds was the consequence of the increased antioxidant activity detected by the H-ORAC assay in PCE powder.

Differences in the antioxidant activity of the PCE powder obtained by the DPPH and H-ORAC assays could be due to the fact that the two antioxidant methods are based on different responses to the radical source. The ORAC assay uses a biologically relevant radical source and combines inhibition time and degree of inhibition into a single quantity. It is based on the ability of an antioxidant to quench free radicals by hydrogen donation. In contrast, the DPPH assay is based on the detection of a potential antioxidant to transfer one electron to reduce the DPPH free radical. In addition, by the DPPH method a number of flavonols and procyanidins were shown to be active, although some flavones, such as apigenin, naringin, naringenin, and others, showed no activity (*18*). Moreover, the 2,3-double bond and the 3-OH group, which is also shown in the flavone structures (**Figure 2**) quantified in the cocoa phenolic extracts of this study, appeared to intensify the radical scavenging power. Apparently in our study the flavone content seemed to increase the radical scavenging response of the cocoa phenolic extracts in the DPPH method. Thus, the lowest flavone content in powder PCE, in comparison with the other cocoa sources, could be an explanation for the different antioxidant activity values expressed by DPPH and H-ORAC.

Conclusion. According to the aim of the study, focused on obtaining a rich phenolic cocoa extract for use as an ingredient in the food industry, the nib was shown to be the most interesting source for obtaining a rich phenolic cocoa extract, which showed the highest polyphenol content and also antioxidant activity. These are probably related to the effect of the grinding and roasting operations, which disrupt the cells of the bean tissue, which could explain the greater solubilization of the phenols.

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