

Original Article

Comparative study of different cocoa (*Theobroma cacao* L.) clones in terms of their phenolics and anthocyanins contents

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Abstract

Polyphenols were analysed at 280 nm by HPLC device using a Photodiode Array Detector (PDA). Anthocyanins were separated with the SEP-PAK Vac 6cc 1000 mg (Waters) column and measured at 520 nm with a PDA. Nineteen cacao clones from Cameroon genebank were analysed. Fresh and fermented-like seeds were used. Two main polyphenols were present in our samples: catechin and epicatechin. Epicatechin represents 2–4% DM of defatted cocoa seed powder. Undefined substances called A, B and C were also found in cocoa seeds. Substance A is discussed as a derivative of caffeic acid and an ester-bound compound. Substances B and C are oligomeres of proanthocyanidins. Protocatechiuc acid and quercetin were not detected. Two anthocyanins were found in cocoa seeds: cyanidin-3-galactoside and cyanidin-3-arabinoside. They represent 0.02–0.4% DM of defatted cocoa seed powder. Total phenols, catechin, epicatechin and anthocyanin in fresh and fermented-like beans were genotype-dependent. Polyphenols from seeds of two different pods from the same clone showed a quantitative significant difference. Spearman's correlation test showed that there is no correlation between the number of seeds per pod, weight of pod and content of polyphenolic compounds. Nevertheless, a negative correlation was found between the number of seeds per pod and the catechin content ($r = -0.463$, $P < 0.01$). Groupings of samples were observed using PCA and hierarchical cluster analysis. The separation between groups is related to their polyphenol and anthocyanin contents.

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1. Introduction

In higher plants, many phenomena and roles are attributable to the secondary metabolites. The nature of polyphenol compounds in plants is complex. Phenols have been associated with plant and tissue maturation processes, defence mechanisms (Kubo and Matsumoto,

1984), and sensory characterization of plant-derived food products (Cimato et al., 1990).

Cocoa beans are rich in polyphenols, contributing some 12–18% of the dry weight of the whole bean (Bravo, 1998). Cocoa bean polyphenols have long been associated with the flavour and colour of chocolate. In aerobic fermentation, brown pigments are formed from polyphenols (Forsyth, 1952). The polyphenols epicatechin and catechin are oxidized to quinones, and condensation of proteins and polyphenols results in a reduction of astringency and bitter flavour. The quinones can also complex with amino acids, peptides and proteins and polymerize with other flavonoids.

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High-molecular weight tannins complex with proteins through hydrogen bonding, and the result of these reactions is a brown, water insoluble pigment. Fresh cocoa beans contain purple anthocyanidin pigments, 3- β -galactosyl- and 3- α -L-arabinosyl-cyanidins. During fermentation, these pigments are hydrolysed by glycosidases, resulting in a bleaching of the cotyledons (Forsyth and Quesnel, 1957).

Cocoa flavonoids have been reported to have a wide range of biological properties, including modulating eicosanoid synthesis, increasing nitric oxide synthesis, lowering the rate of low-density lipoprotein (LDL) oxidation, inhibiting platelet activation, stimulating the production of anti-inflammatory cytokines, and inhibiting the production of certain proinflammatory cytokines (Waterhouse et al., 1996; Kondo et al., 1996; Mao et al., 1999; Karim et al., 2000; Mao et al., 2000; Rein et al., 2000; Schramm et al., 2001; Wan et al., 2001). These diverse biological activities are thought to be attributable to a group of polyphenol compounds present in cocoa, including the flavan-3-ol monomers (–)-epicatechin and (+)-catechin and several procyanidin oligomers built upon these monomeric units.

Recently, Lee et al. (2003) showed that cocoa has more phenolic phytochemical and a higher antioxidant capacity than teas and red wine. In *T. cacao* L., studies of phenolic compounds have been focused on roasting beans and cocoa liquor due to their importance in the chocolate industry. Little attention has been paid to raw cocoa and very rarely the spectrum of polyphenols in fresh beans has been investigated concurrently as the basis of phenolic phytochemicals present in chocolate. Cocoa beans used in the confectionery industry come from a wide range of geographical areas, and may have different chemical and organoleptic properties. The chocolate producer must therefore select and combine these beans in various proportions in order to meet certain quality standards and economic specification. Manufacturers have to produce chocolates of constant flavour using raw material that is variable. For this reason, it is important for them to have analytical criteria for the rapid estimation of the quality of the raw material.

Polyphenols were analysed in mature plant, seedling and tissue cultures of cocoa (Jalal and Collin, 1977). Leucocyanidin were fractioned in cocoa seed by Quesnel (1968). The extent to which genotype, geographical origin and fermentation method and pod size affect cocoa polyphenol content has not been unequivocally established and studies addressing these questions are rather scarce.

The aim of this study was to use HPLC to determine polyphenolic contents in raw cocoa beans and fermented-like seeds from Cameroon and then to assess the potential relationships between phenolic compounds and geographical origin. The correlation between pod

size, number of beans per pod and polyphenolic content are discussed.

2. Materials and methods

2.1. Samples

Cocoa seeds were from ripe, genetically undefined pods harvested at the “Institut de Recherche Agricole pour le Développement (IRAD)” Nkoemvone, Ebolowa (Cameroon). Seeds were analysed at the Institute of Applied Botany, University of Hamburg, Germany. Upon arrival, morphological parameters (weight of pod, number and weight of seeds per pod) were measured for each clone. Unfermented seeds were taken from the pods (7 days after harvesting), shock-frozen in liquid nitrogen after removal of testae and freeze-dried. For fermentation-like studies, seeds with testae were placed in Petri dishes (≈ 10 seeds/dish) and incubated at 25 °C for 1 and 2 days before processing.

2.2. Reagents and standards

Epicatechin and quercetin were obtained from Sigma. Protocatechiuc acid and catechin were from Aldrich and Fluka, respectively. 3- α -L-arabinosyl cyanidin and 3- β -D-galactosyl cyanidin were purchased from Polyphenols AS. All solvents used were of analytical grade purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Extraction of polyphenols

Prior to extraction, preliminary assays (Waterman and Mole, 1994) showed that the solvent acetone/water 60:40 (v/v) and the different procedures described herein for the fractionation of the different phenol groups maximized the percentage of recovery.

Two grams of lyophilized cotyledon were milled to a fine powder in a Retsch Kroll or Resch MM 200 (Germany) laboratory mill with 10 mL of *n*-hexane for fat removal. Most of the residual seed fat was extracted by flushing the powder with 25 mL *n*-hexane in a Buchner funnel.

The phenolic compounds were extracted by agitating 0.5 g of the fat-free sample on ice three times with 50 mL 60% aqueous acetone with constant shaking. After centrifugation at room temperature at 5000 rpm for 15 min, the three supernatants were combined in a flask containing 2 mL of glacial acetic acid. The acetone was removed by rotary evaporation under partial vacuum at 40 °C. The aqueous phase obtained was adjusted to 100 mL with Milli-Q Plus water in a volumetric flask.

Total contents of polyphenolic compounds were analysed from this aqueous phase.

To clean the sample, 30 mL of the previous aqueous phase were mixed five times with 30 mL ethyl acetate. After 1 min shaking, the aqueous phases were discarded and the organic phases combined, dried by addition of 20 g anhydrous Na_2SO_4 and filtered after 5 min in the dark with Whatman paper. The salt residue was discarded and the clear organic phase was dried at 40 °C under vacuum. The dried extract of polyphenolic compounds was dissolved in 5 mL of pure methanol (Lichrosolv, Merck) and filtered with Millipore paper (0.45 μm). The pure polyphenol extracts were stored at –20 °C until HPLC analysed.

2.4. Purification of anthocyanins

Purification of anthocyanins was conducted from the 100 mL aqueous phase using a Sep-Pak[®] Vac C18 6cc column (Waters). The column was first eluted with a mixture of pure methanol (10 mL): 2% acetic acid (10 mL). A 20 mL aliquot of the aqueous phase sample was loaded onto the column and washed with 2 × 5 mL 2% acetic acid. Anthocyanins were then eluted twice from the column with 5 mL pure methanol analytical grade (Lichrosolv, Merck). The elute fractions were combined and dried by rotary evaporation. The residues were re-suspended in 2 mL of a mixture of pure methanol and acetic acid 2%.

2.5. Analysis of polyphenolic compounds by reverse-phase HPLC

Total contents of polyphenolic compounds in the cocoa bean extracts were determined according to the Folin–Ciocalteu procedure (Singleton and Rossi, 1965).

Chromatographic analyses were carried out on Waters HPLC system equipped with an A2-200 automatic injector, Knauer HPLC pump 64, Knauer HPLC-programme 50 solvent controller, Waters 996 Photodiode Array Detector (PDA) and analysed with MillenniumTM 3.2 software (Millipore Corporation, Milford, MA, USA). Separation of polyphenols was performed on a LicroCart 250-4 octadecylsilyl (ODS) C18, 5 μm particle [RP-18 (5 μm)] column (Merck) at 26 °C. The guard column consisted of a LicroCart 4-4 Lichrospher 100 RP-18 (5 μm) (Merck). The binary mobile phase (Table 1) consisted of 2% acetic acid in water (A) and acetonitrile–water–concentrated acetic acid mixture (4:9:1 v/v/v) (B). Twenty microlitres of sample was injected into the column. The separation of polyphenols was monitored using a PDA detector at 280 nm and anthocyanins were recorded at 520 nm. Identification of each peak was confirmed by comparison of retention time and coelution with authentic standards of protocatechiuc acid, catechinhydrate,

Table 1
Binary gradient used for the separation of polyphenol compounds and anthocyanins present in cocoa beans

Time (min)	Fuel flow (ml min^{-1})	Solvent A (%)	Solvent B (%)
0	1.2	90	10
8	1.2	90	10
38	1.1	77	23
50	1.0	60	40
70	1.0	10	90
73	1.0	10	90
78	1.2	90	10
93	1.2	90	10

epicatechin, quercetin, cyanidin-3-galactoside and cyanidin-3-arabinoside.

2.6. Statistical analysis

Data were subjected to analysis of variance and correlations between size of fruit, number of seeds per pod and content of polyphenolic compounds using the software SPSS 10.0 for windows. Mean values were compared using the least significant difference (LSD) test. Principal component analysis (PCA) was realized to describe the variability of chemical data. This analysis was performed with SPAD 4.01 (Système portable d'analyse des données) software.

3. Results and discussion

The total contents of polyphenolic compounds of cocoa beans were determined and expressed as epicatechin equivalent. The average total content ranged from 67 to 149.2 mg g^{-1} DM in freshly harvested bean, 102.3 to 139.6 mg g^{-1} DM in 1-day-old fermented-like bean and from 101.3 to 143.6 mg g^{-1} DM in 2-day-old fermented beans (Table 2). Different trends and range of concentrations were found in studies that reported the levels of polyphenols in cocoa beans from an Ecuadorian selfed and heterozygous population of clone EET 95 (Luna et al., 2002).

Total content of polyphenolic compounds in freshly harvested beans compared to fermented-like beans (Table 3) showed that from the same pod there is no regular evolution of this determinant during the fermentation-like process. In some clones, total phenolic compounds increased about 25% after 2 days (SNK10, T79/467, UPA143), decreased in others between 14% and 25% (ICS84, ICS1) while remaining constant in the clones SNK413, IMC60, ICS95 and UPA134. According to Forsyth (1952), losses of total phenol are due to diffusion out of the cotyledons and can be calculated as 24% after 60 h of fermentation, reaching 58% after 8

Table 2
Total polyphenol, catechin, epicatechin and anthocyanin contents in freshly harvested seeds of different cocoa pods from different clones

Clones	Weight of pods (g)	Number of seeds/pod	Total polyphenols (mg g ⁻¹)	Catechin (mg kg ⁻¹)	Epicatechin (mg kg ⁻¹)	Cyanidin-3-galactoside (mg kg ⁻¹)	Cyanidin-3-arabinoside (mg kg ⁻¹)
SNK10 (A)	304.97	32	119.7	836	28 186	853	978
SNK10 (B)	271.73	42	122.9	725	29 028	820	880
SNK413	376.03	31	103.5	1036	29 839	1031	1453
SNK64 (A)	412.00	52	96.8	895	29 046	650	2375
SNK64 (B)	299.79	30	105.7	1389	27 787	1483	2485
SNK60	389.20	26	114.6	890	30 924	997	1692
SNK478	362.06	45	122.2	497	34 750	601	1040
SNK480 (A)	324.14	46	86.6	694	24 499	494	821
SNK480 (B)	354.52	46	117.6	899	33 230	1833	2186
SNK476	352.52	25	149.2	863	41 519	1544	2732
SNK16	322.47	35	134.4	1234	43 903	396	941
SNK505 (A)	357.86	30	136.2	833	42 926	308	2273
SNK505 (B)	277.89	37	109.6	328	31 697	n.d.	673
SNK417 (A)	326.68	44	67.0	125	14 435	94	404
SNK417 (B)	226.37	43	75.8	179	16 989	79	502
ICS84 (A)	405.06	44	129.0	503	36 282	1486	3212
ICS84 (B)	566.15	40	147.8	672	39 098	1288	1693
ICS84 (C)	517.26	40	121.4	968	32 618	1099	1114
ICS84 (D)	464.08	22	94.1	609	29 452	438	659
ICS1 (A)	321.16	34	126.6	332	35 623	455	1508
ICS1 (B)	324.61	46	134.6	525	38 692	321	894
ICS95 (A)	337.70	34	100.7	1192	30 894	294	466
ICS95 (B)	640.52	38	139.4	867	43 154	1781	4552
ICS95 (C)	351.24	40	122.7	545	37 319	561	1581
ICS95 (D)	359.93	39	147.9	431	34 606	1006	1388
UPA134 (A)	148.80	29	118.5	737	29 788	1807	2452
UPA134 (B)	283.64	34	104.0	781	20 833	590	639
UPA143 (A)	417.21	28	88.9	555	18 639	369	491
UPA143 (B)	352.59	36	118.2	974	33 225	699	1327
UPA143 (C)	277.33	28	116.9	1295	29 910	1402	1776
UPA143 (D)	375.73	22	114.4	1059	35 545	792	1096
UPA143 (E)	896.08	44	103.5	782	35 639	379	940
IMC60 (A)	571.04	35	131.2	1239	27 196	1829	2027
IMC60 (B)	383.00	26	139.4	1442	34 363	2817	4443
T79/467 (A)	695.5	57	84.4	170	16 880	448	1661
T79/467 (B)	548.05	34	94.1	688	20 193	1313	2389
T79/501 (A)	368.90	45	101.0	n.d.	26 852	477	1012
T79/501 (B)	380.64	31	112.9	507	28 563	1362	1795

Depending on the availability of plant material, one to five pods were analysed per clone. Results are expressed as unit/g or kg of defatted dried cocoa bean powder. Results are average of duplicate analyses.

days. Phenolic compounds also complex with cocoa proteins and polysaccharides (Forsyth et al., 1958; Zak and Keeney, 1976). On the other hand, increase of polyphenols during fermentation-like process may reflect a formation of polymeric proanthocyanins.

Polyphenolic compounds were identified by their chromatographic behaviour and UV spectra (280 nm), HPLC and chromatographic comparisons with authentic standards. The chromatogram pattern (Fig. 1a) found in cocoa bean was similar to that described by Elwers (2002) from cocoa of other countries. The predominant polyphenols identified in freeze-dried defatted cocoa bean were catechin and epicatechin. Protocatechiuc acid and quercetin were not detected.

Epicatechin represented 2–4% (Table 2) of the dry mass of defatted powder while catechin was about 0.05–0.1% of defatted bean.

It is well established that (–)-epicatechin is the main polyphenol found in cocoa beans (Kim and Keeny, 1984; Nelson and Sharpless, 2003; Zhu et al., 2003). Protocatechiuc acid is discussed to be synthesized in cocoa beans after pod infection and therefore involved in defence mechanisms vis-à-vis pathogens and parasites. Three unidentified compounds were revealed with retention time at ≈ 6.9 min (substance A); ≈ 12.6 min (substance B) and ≈ 20.4 min (substance C). Substance A, due to the PDA results is a derivative of caffeic acid and an ester-bound compound. Substance B and C are

Table 3

Changes in total polyphenol, catechin, epicatechin and anthocyanin contents during fermentation-like incubation at 25 °C of different cocoa beans from different clones (otherwise as in Table 2)

Clones		Total polyphenols (mg g ⁻¹)	Catechin (mg kg ⁻¹)	Epicatechin (mg kg ⁻¹)	Cyanidin-3-galactoside (mg kg ⁻¹)	Cyanidin-3-arabinoside (mg kg ⁻¹)
SNK413	Unfermented	103.5	1036	29839	1031	1453
	Fermented-like (d ₁)	112.8	1121	30723	1080	1481
	Fermented-like (d ₂)	105.3	917	28037	1036	1563
SNK480	Unfermented	86.6	694	24499	494	821
	Fermented-like (d ₁)	119.8	1298	33098	2127	4552
	Fermented-like (d ₂)	116.2	1319	32980	1024	3821
T79/467	Unfermented	94.1	688	20193	1313	2389
	Fermented-like (d ₁)	112.1	950	26632	1925	3507
	Fermented-like (d ₂)	126.6	1278	31358	2554	5031
IMC60	Unfermented	139.4	1442	34363	2817	4443
	Fermented-like (d ₁)	139.6	1618	34936	2590	3915
	Fermented-like (d ₂)	141.6	1735	37652	2359	3473
ICS95	Unfermented	139.4	867	43154	1781	4552
	Fermented-like (d ₁)	135.7	742	42419	1497	3543
	Fermented-like (d ₂)	143.6	739	44644	1045	3198
UPA134	Unfermented	118.5	737	29788	1807	2452
	Fermented-like (d ₁)	113.2	735	28626	1596	2120
	Fermented-like (d ₂)	107.9	814	26761	1228	2184
ICS1	Unfermented	126.6	332	35623	455	1508
	Fermented-like (d ₁)	116.0	370	30584	354	2455
	Fermented-like (d ₂)	103.0	272	2401	149	1639
ICS84	Unfermented	129	503	36285	1486	3212
	Fermented-like (d ₁)	1023	337	27691	1220	2752
	Fermented-like (d ₂)	1143	330	32795	1172	2617
UPA143	Unfermented	118.2	974	33225	699	1327
	Fermented-like (d ₁)	138.6	1274	40089	1283	2422
	Fermented-like (d ₂)	130.2	1085	35884	1008	1672

oligomers of proanthocyanidins. Porter et al. (1991) established the presence of procyanidin oligomers through heptamers in cocoa using column and thin-layer chromatography and negative fast atom bombardment mass spectrometry. Evidence of cocoa procyanidin oligomers through octamers was reported by Clapperton et al. (1992) who used a combination of column chromatography, reversed-phase HPLC, and positive liquid secondary ion mass spectrometry. Monomeric and oligomeric procyanidins present in cocoa and chocolate were separated and identified by Hammerstone et al. (1999) using a modified normal-phase high-liquid chromatography method coupled with on-line mass spectrometry analysis using an atmospheric pressure ionization electrospray chamber.

During fermentation-like incubation, change in polyphenol compounds depended on the clones and the metabolites analysed (Table 3). Fermentation of cocoa

beans is critical for the development of precursors for chocolate flavour. Complex interactions among the polyphenols to form high-molecular weight tannins and their association with proteins play a major role in the overall quality of fermented cocoa beans for chocolate production (Forsyth, 1952).

Fig. 1b shows a typical chromatogram of anthocyanin extract. The major anthocyanin compounds in freshly harvested bean included cyanidin-3-arabinoside ranging from 466 to 4552 mg kg⁻¹ of dry and defatted beans, and cyanidin-3-galactoside ranging from 294 to 2817 mg kg⁻¹ (Table 2). Contents of cyanidin-3-arabinoside in all clones were consistently higher in unfermented and fermented-like beans compared to cyanidin-3-galactoside. During fermentation-like incubation, variation in anthocyanin was clone and pod dependent (Table 3). The average value of total anthocyanin in freshly harvested, fermented-like (d₁) and fermented-like (d₂) beans from nine cocoa

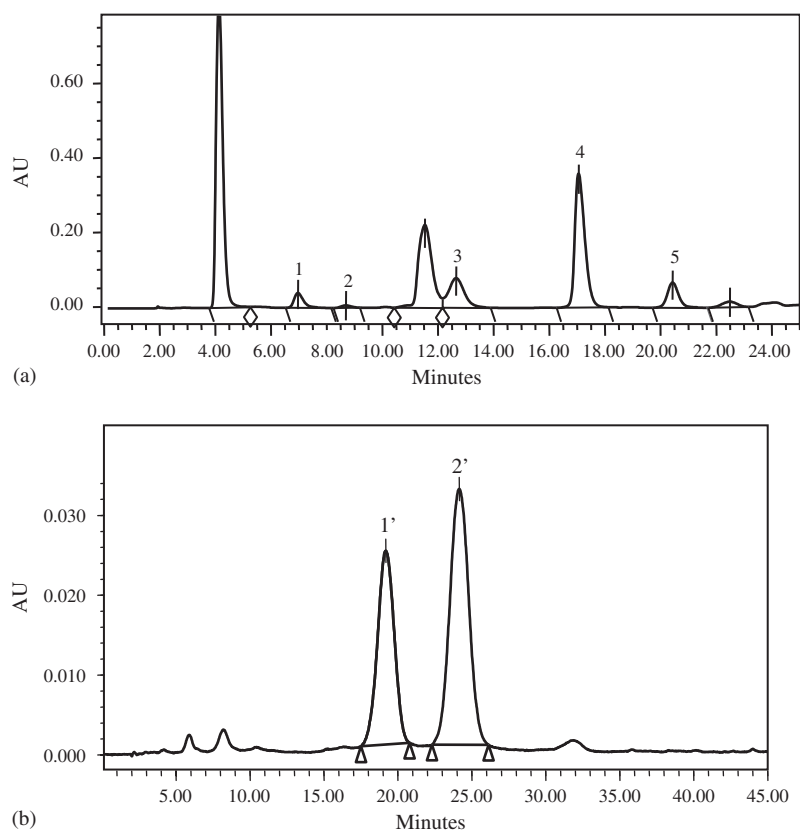


Fig. 1. HPLC chromatograms of (a) polyphenols and (b) anthocyanins in cocoa seeds. Chromatographic conditions are described in the text. Peak identification: 1—Substance A, 2—Catechinhydrate, 3—Substance B, 4—Epicatechin, 5—Substance C, 1'—cyanidin-3-galactoside (retention time, 19.123 min), 2'—cyanidin-3-arabinoside (retention time, 24.463 min). The other peaks in (a) are those of purine alkaloids and their derivatives. Thus peaks at 4.5 and 11.5 min are theobromine and caffeine, respectively.

genotypes was 6587.5, 8239 and 6720 per mg kg⁻¹ DM respectively.

Spearman's correlation test showed that there is no correlation between the number of seeds per pod, weight of pod and content of polyphenolic compounds. The absence of significant correlation between polyphenol contents and morphological parameters may derive from evolutionary differences according to environmental factor than gene linkages. Nevertheless, a negative correlation was found between the number of seeds per pod and the catechin content ($r = -0.463$, $P < 0.01$). A high positive significant correlation ($r = 0.842$, $P < 0.01$) was found between the total content of polyphenolic compounds and the epicatechin accumulation in cocoa beans. In the same way total content of polyphenolic compounds and cyanidin-3-galactoside showed a positive correlation with a coefficient $r = 0.842$ at $P < 0.01$.

A PCA was carried out on the data from all the clones studied. The three first principal components represent 93.76% of the total variability, the total phenols, catechin, epicatechin and anthocyanin contents being the dominating features in the first principal component (58.60% of the total variability) and epicatechin content

the feature with highest weight in the second principal component (21.60% of the total variability). Catechin content showed the highest weight in the third principal component (14.06% of the total variability). Fig. 2 shows a classical representation of the scores on PC1 and PC2 (79.70% of total variance). Bean of clone IMC60 B contains a large amount of catechin, cyanidin-3-galactoside and cyanidin-3-arabinoside, and this is why PC1 clearly separates it from the other samples. Examining a two-dimensional scores plot in the space defined by PC1 and PC2 shows that distribution of samples follows a pattern. The separation between groups is related to the polyphenolic content of beans. This was clarified using the LSD test. Average means of polyphenolic compounds within group were found to be statistically different ($P < 0.05$). The first group comprising 8 cocoa clones contains high amounts of total phenols, catechin, epicatechin and anthocyanin (dominating features in the first principal component). The second group (6 clones) contains average amounts of total phenol and epicatechin compared to the total average amounts. The third group (12 clones) contains average amounts of catechin and cyanidin-3-galactoside compared to the total average amounts. Group 4 (clones

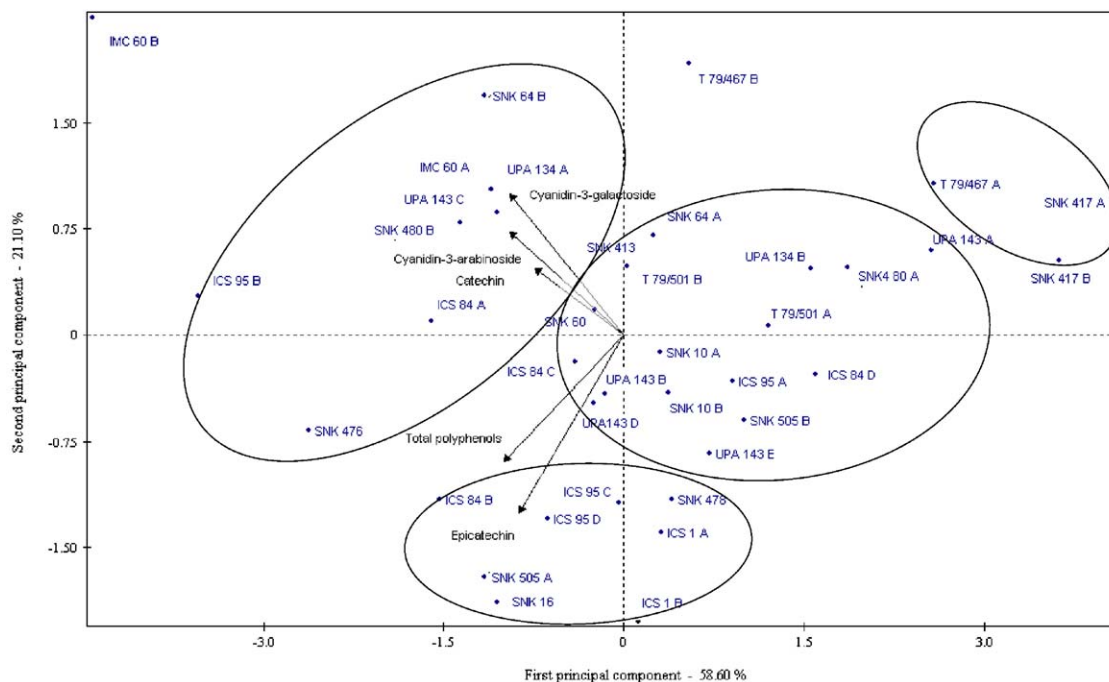


Fig. 2. Loading plot of the first two principal components for total polyphenols, epicatechin, catechin, cyaniding-3-arabinoside and cyaniding-3-galactoside from cocoa of different genotypes.

SNK417A and B, T79/467A) differs from the others by their low content in all the compounds analysed. Cluster analysis also classified samples into four groups according to the distance 0.5 (dendrogram not shown).

There are several internal and external factors affecting the quality and/or quantity of polyphenolic compounds in plants. These include the genetic (varietal and regional) diversity as well as many environmental variables, i.e. growing conditions such as light intensity, humidity, temperature, the use of fertilizers, wounding, infections or other stress factors (Macheix et al., 1990; Chalker-Scott, 1999; Cabrita et al., 2000; Vallejo et al., 2003; Stintzing and Carle, 2004). The quality of polyphenolic compounds found in raw cocoa from Cameroon is in agreement with those from Ghana and Malaysia in spite of their genetic differences (Elwers, 2002). The quantitative differences registered within clones could be explained, at least in part, by the interaction of several genetic, physiological, agronomic (i.e. position of pods on the tree), and environmental factors (microclimate) modifying the final concentration in each pod. For example, it was shown that many environmental factors control the accumulation of anthocyanins in plants (Roubelakis-Angelakis and Kliever, 1986). Besides light and temperature (Kliever, 1977, Cobbina and Miller, 1987; Wang and Zheng, 2001), the availability of plant nutrients also has a great influence on the accumulation of polyphenols (Francis and Atwood, 1961; Doak and Miller, 1968; Piccaglia et al., 2002).

4. Conclusion

Our results suggest that there were no qualitative difference in polyphenol compounds in cocoa beans in spite of their genetic origin and fermentation-like process. As dominating phenolic components epicatechin, catechin, cyanidin-3-galactoside, and cyanidin-3-arabinoside as well as three undefined substances A, B, C were found. Quantitative difference found could be attributed to the growing conditions (microclimate, position of pods on the tree). Negative correlations were found between the number of seeds per pod and catechin content. PCA and hierarchical cluster analysis classified sample according to their polyphenol and anthocyanin contents.

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