

## Polyphenolic Content and Composition and Antioxidative Activity of Different Cocoa Liquors

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### Abstract

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Cocoa liquor used in the confectionery industry comes from a wide range of geographical areas and may have different chemical compositions, sensory properties, and nutritional values. We found it interesting to study the polyphenolic content and composition of cocoa liquors for their potential use in industrial production. Six defatted samples originating from different countries were extracted with aqueous methanol (70%, v/v), and the polyphenolic profiles were determined using RP-HPLC method. According to our results, all samples of cocoa liquors have similar polyphenolic profiles, however, quantitatively varied. In the samples, about 13 compounds were identified by comparison of their retention times and UV spectra, and the quantified peaks were (+)-catechin, (–)-epicatechin, (–)-gallocatechin, (–)-epigallocatechin, caffeic acid derivative, caffeine, and theobromine. Also, several peaks were identified as oligomeric procyanidins. The free-radical scavenging activity was determined by the DPPH<sup>•</sup> (1,1'-diphenyl-2-picrylhydrazyl) and Oxygen Radical Antioxidant Capacity (ORAC) assays. The order of antioxidant activity of the cocoa liquors studied was the same with both methods (Madagascar > Mexico > Ecuador > Venezuela > Sao Tome > Ghana samples). In addition, correlation between the antioxidant capacity and polyphenolic content was also determined, a high correlation coefficient having been obtained by both methods ( $R^2 = 0.9868$  for DPPH, and 0.9375 for ORAC).

**Keywords:** antioxidative activity; cocoa liquor; DPPH; flavan-3-ols; HPLC; methylxanthine

Cocoa liquor (CL) is the main raw material for the production of chocolate and chocolate-based products and is a key factor in determining the sensory and nutritional values of chocolate. The composition of cocoa liquors depends on the cocoa bean variety, post harvest processes (fermentation and drying), and roasting conditions (WOLLGAST & ANKLAM 2000). Three varieties of cocoa beans are produced worldwide: Forastero (bulk grade), Criollo (fine grade), their hybrid Trinitario (fine grade),

and National, the third fine variety of cocoa from Ecuador (COUNET *et al.* 2004). Each cocoa exhibits its own composition and sensory properties. It is well known that cocoa bean is a rich source of many structurally different biologically active compounds, among them polyphenolics and xanthine alkaloids being predominant. Polyphenolics and xanthine alkaloids, which comprise approximately 14–20% of the whole bean weight, are very important in affecting the quality of cocoa beans (KIM & KEENEY

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1984; BRAVO 1998; WOLLGAST & ANKLAM 2000). The reactions of polyphenols along with sugars and amino acids are responsible for the production of flavour and colour of the roasted cocoa beans, while alkaloids contribute bitterness to the fermented beans (NAZARUDDIN *et al.* 2006a).

Recently, cocoa bean polyphenols have attracted a lot of attention due to their possible beneficial effects on human health. The health promoting effects of polyphenols are believed to be due to the relatively high antioxidant activity of these compounds protecting against chronic diseases by reducing oxidative damage. Cocoa polyphenols 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 (LAMUELA-RAVENTOS *et al.* 2005; DING *et al.* 2006; VINSON *et al.* 2006).

The polyphenolic content of raw cocoa beans differs substantially from those in cocoa powder, cocoa liquor or chocolate, which are produced by processes involving fermentation, drying, and roasting. Each stage in the processing of cocoa alters its chemistry. During cocoa fermentation, polyphenols are subjected to biochemical modifications through oxidation and polymerisation and binding with proteins, hence decreasing their solubility and astringency effects (NAZARUDDIN *et al.* 2006a). Subsequently, during drying, the amount of polyphenols is substantially reduced mainly by enzymatic browning (KIM & KEENEY 1984; KYI *et al.* 2005). On the contrary, the roasting process, which is responsible for reducing bitter and acidic tastes, caused small changes in polyphenolic concentration (MISNAWI *et al.* 2002; NAZARUDDIN *et al.* 2006b). The manufacturers must select and combine raw materials in various proportions in order to meet certain economic specifications and quality standards, including constant flavour and functional properties of the product. Thus, it is important for them to have analytical data for the determination of the quality of the raw material. The aim of this study was to determine the polyphenolic patterns in different cocoa liquors (Ecuador, Ghana, Madagascar, Mexico, Sao Tome, and Venezuela samples) using HPLC with UV-PDA detection, and their antioxidant activity by DPPH and ORAC methods. In addition, the correlation

between the antioxidant activity and polyphenolic content was also determined.

## MATERIALS AND METHODS

**Materials.** Methanol, hexane, and Folin-Ciocalteu reagent (FC) were obtained from Merck (Darmstadt, Germany). (+)-Catechin (C), (–)-epicatechin (EC), caffeic acid (CA), (–)-gallic acid (GC), (–)-epigallocatechin (EGC), theobromine (T), caffeine (CAF), 1,1'-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH), and fluorescein (FL) were purchased from Sigma (St. Louis, USA).

Cocoa liquors originating from Ecuador, Ghana, Madagascar, Mexico, Sao Tome, and Venezuela were purchased from Kraš, Food Industry, d.d., Zagreb, Croatia.

**Preparation of extract.** Cocoa liquors were defatted three times with hexane. After air-drying, to remove residual hexane, 1 g of the defatted sample was extracted twice with 10 ml of 70% aqueous methanol (v/v) in an ultrasonic bath. The combined supernatants, after centrifugation for 10 min (5000 rpm,  $r = 13.8$  cm), were concentrated in a rotary evaporator till dry and then dissolved in 5 ml of 70% (v/v) methanol ( $\gamma = 0.2$  g defatted cocoa liquors – DCL/ml). The prepared extracts were subjected to HPLC analysis and total polyphenolic content and antioxidant capacity determinations. The extracts were prepared and analysed in triplicates.

**HPLC analyses.** HPLC analyses were performed using Varian LC Star System with Star Solvent Delivery System 9010, Injector Rheodine 7125, Polychrom 9065 (UV-Diode Array Detector),  $\lambda = 280$  nm (Palo Alto, USA). The extracts obtained were filtered through 0.45  $\mu\text{m}$  filter and separated on RP-LC column (Eurospher, 250 mm  $\times$  4 mm, 5  $\mu\text{m}$  I.D., Knauer, Berlin, Germany) using the mobile phase: (A) water:acetic acid (97.5:2.5, v/v); (B) acetonitrile, in gradient elution: 0 min 97% A, 0–13 min 91% A, 13–18 min 89% A, 18–25 min 82% A, 25–45 min 70% A and 45–50 min 97% A. The detection was performed with UV-PDA detector at 278 nm (GUYOT *et al.* 1998).

The identification of the compounds was achieved by comparing their UV spectra and retention times of the separated peaks with the retention times of the standards. The identified phenolic com-

pounds were quantified using the external standard method, quantification being based on the peak area. The calibration curves of the standards were made by diluting the stock standards with methanol to yield 0.1–0.8 mg/l (–)-epicatechin, 0.01–0.6 mg/l catechin, 0.25–1.50 mg/l caffeic acid, 0.05–0.6 mg/l gallic acid, epigallocatechin, and caffeine, 1.0–2.1 mg/l theobromine. Caffeic acid derivative, identified by polarity and spectral data in comparison with caffeic acid, was quantified based on caffeic acid as standard.

**Total polyphenolic content.** Total polyphenolic content was determined by Folin-Ciocalteu method (SINGLETON *et al.* 1999). Methanol extracts of cocoa liquors were diluted to 0.60 mg of DCL/ml for the measurement, and 0.5 ml of the diluted extract was added to 2.5 ml FC solution (1:10 diluted FC reagent with water). After 5 min, 2 ml of Na<sub>2</sub>CO<sub>3</sub> (75 g/l) was added followed by incubation at 50° C for 5 minutes. The absorbance was measured at 760 nm and the results were expressed as mg of (–)-epicatechin equivalent per g DCL using the appropriate standard curve (six points from 0.01 mg/l to 0.1 mg/l (–)-epicatechin in 70% methanol).

**DPPH radical scavenging assay.** The scavenging activity was estimated according to the method of BRAND-WILLIAMS *et al.* (1995). Briefly, methanol extracts of cocoa liquor were diluted to 15, 12.5, 10, 7.5 and 5 mg of DCL per ml and 50 µl of each dilution was added to 3.992 ml of 100 µM methanol DPPH' solution. The decrease in absorbance was determined at 515 nm using UV spectrophotometer, Cary 3, Varian (Palo Alto, USA), at 0 min and every 2 min until the reaction reached the plateau.

The remaining DPPH' concentration in the reaction medium was calculated from the calibration curve. For each concentration tested, the reaction kinetics was plotted. From these graphs the percentage of the residual DPPH' was determined, and the values were transferred into another graph showing the percentage of the residual DPPH' as a function of the cocoa liquor concentration in the diluted methanol extract. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH' concentration by 50% (EC<sub>50</sub> – mg/ml). Antiradical efficiency (AE) was also calculated (AE = 1/EC<sub>50</sub>).

**Oxygen Radical Absorbance Capacity Assay (ORAC).** The oxygen radical absorbance capacity was determined according to the method of NINFALI *et al.* (2005). The final reaction mixture for

the assay (3 ml) was prepared as follows: 2.25 ml of 0.04 µM fluorescein sodium salt in 0.075M sodium phosphate buffer, pH 7.0, and 0.375 ml of diluted samples or 25 µM Trolox as standard. The control was 0.075M sodium phosphate buffer. The reaction mixtures were incubated for 30 min at 37° C followed by the reaction initiation with 0.375 ml 152mM AAPH, and fluorescence was read every minute up to value zero at 485 nm excitation, 520 nm emission. Fluorescence was measured by Varian Cary Eclipse Spectrofluorimeter (Palo Alto, USA). The results were calculated as ORAC values using the differences of the areas under fluorescein decay curve between the blank and the sample according to the formula:

$$\text{ORAC } (\mu\text{mol Trolox equivalent/g}) = \frac{[(A_s - A_b)/(A_t - A_b)] k \times a \times h}{1}$$

where:

A<sub>s</sub> – area under the curve (AUC) of fluorescein in the sample

A<sub>t</sub> – AUC of the Trolox

A<sub>b</sub> – AUC of the control

k – dilution factor

a – concentration of Trolox in µmol/l

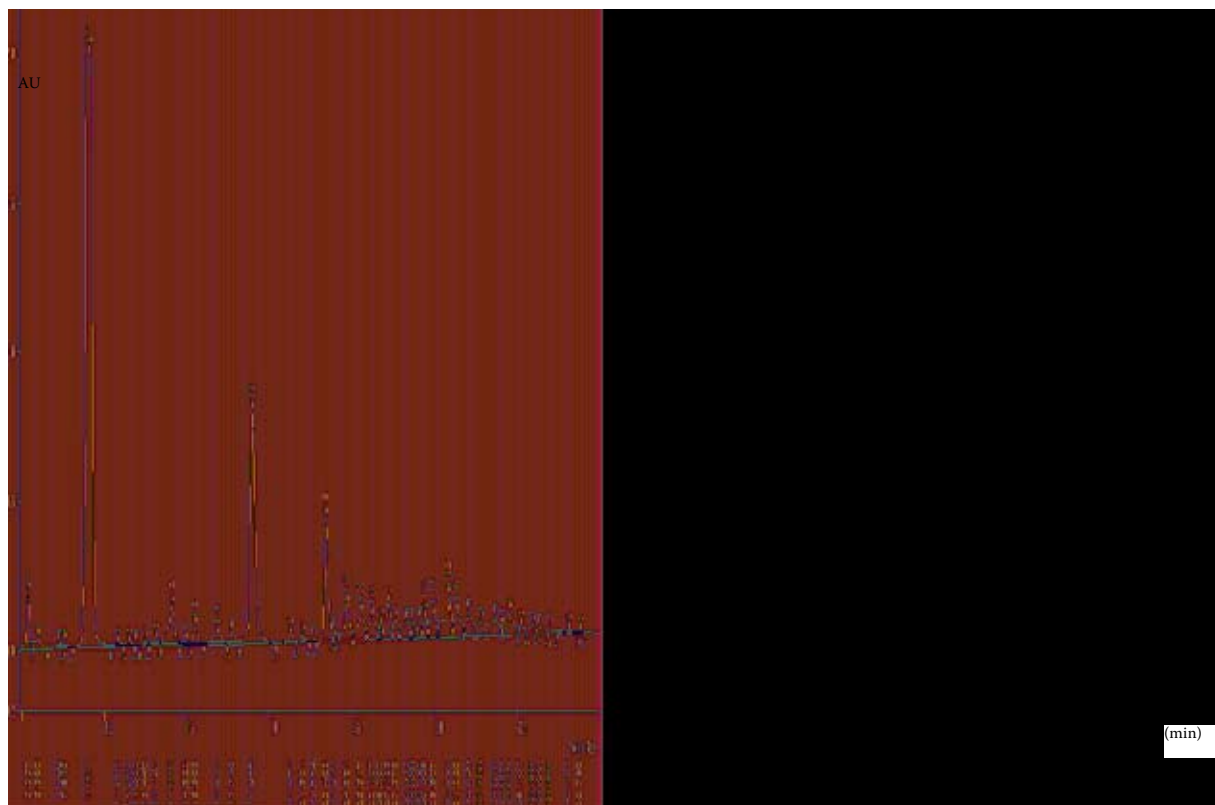
h – ratio between the litres of extract and the grams of DCL

The results were expressed as µmol Trolox equivalent per gram of DCL.

**Statistical analysis.** All measurements were performed in triplicates (n = 3) and the values were averaged and reported along with the standard deviation (± S.D). Statistical analysis was performed using the program Statistica 7.1. The differences between the means were analysed by ANOVA test followed by the post hoc test Tukey's HSD. A significant difference was considered at the level of P < 0.05.

## RESULTS AND DISCUSSION

Polyphenolic profiles of cocoa liquor samples were analysed by reverse-phase HPLC coupled to the photo diode-array detector. Various elution phases (aqueous acetonitril and/or methanol with trifluoroacetic acid, phosphate buffer, or acetic acid) and corresponding chromatographic gradients were applied to achieve an appropriate resolution, as well as a better signal in the UV-PDA detector (ARTS *et al.* 2000; NATSUME *et*



Peak assignment: 1. theobromine, 2. caffeic acid derivate, 3. (+)-catechin, 4. (–)-gallocatechin, 5. caffeine, 6. (–)-epicatechin, 7. (–)-epigallocatechin, 8–13. procyanidins

Figure 1. Typical HPLC chromatogram of a cocoa liquor sample, monitored at 278 nm

*al.* 2000; WOLLGAST *et al.* 2001). Finally, the following chromatographic conditions were used (binary mobile phase with water:acetic acid (97.5:2.5, v/v) and acetonitrile, gradient elution) which allowed the separation of different polyphenols classes including phenolic acids, monomeric catechins, procyanidins, and methylxantines. A typical RP-HPLC chromatogram of a cocoa liquor sample is shown in Figure 1.

In the samples, 10–15 compounds were identified by comparison of their retention times and UV spectra. The identified peaks of (+)-catechin, (–)-epicatechin, (–)-gallocatechin, (–)-epigallocatechin, caffeine, and theobromine were confirmed by spiking the samples with standard mixtures. Furthermore, some compounds were tentatively identified via their spectral features and a review of the literature data. In all extracts, a distinctive peak at  $R_t = 14\text{--}15$  min (peak number 2) exhibited spectral characteristics similar to those of caffeic acid ( $\lambda_{\max} = 235$  nm and 318 nm). Comparing its spectral data with those of caffeic acid (Figure 2), this peak was identified as a caffeic acid derivative and quantified using caffeic acid standard. In

many other similar studies on cocoa and cocoa based products such compound was not identified except in the work by NIEMENAK *et al.* (2006). These authors analysed aqueous acetone (60%, v/v) extracts of cocoa clones from Cameron gene bank using RP-HPLC with a binary mobile phase gradient consisting of acetonitrile/water/2% acetic acid. The predominant polyphenols identified were (–)-epicatechin and (+)-catechin, however, three unidentified compounds were also revealed. Substance A, which was eluted before (+)-catechin, was assigned as an ester bound caffeic acid derivative. In addition, SUÁREZ *et al.* (2005) used similar chromatographic conditions for the separation and quantification of different classes of polyphenolic compounds in cedar and they identified hydrocaffeic acid, which was eluted before (+)-catechin, and far before caffeic acid.

As can be seen in a typical chromatogram (Figure 1), several peaks that appeared after 25 min were also detected, which are presumed to correspond to other polyphenolic compounds, among them procyanidins with a higher polymerisation degree being predominant. The peaks denoted in the chromato-

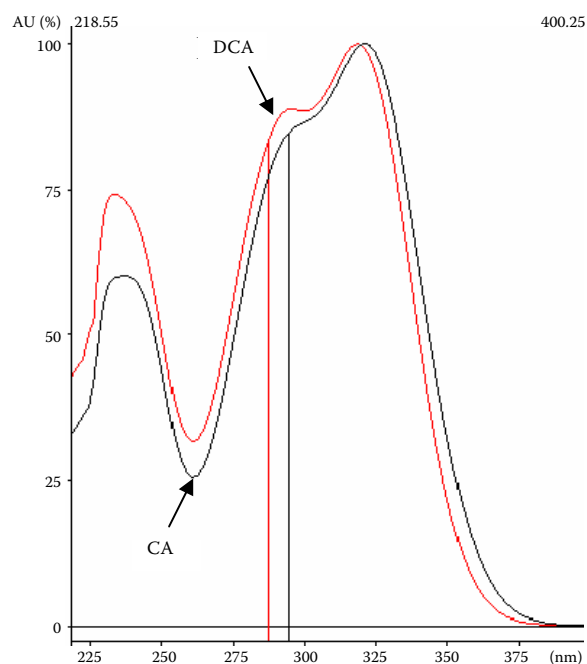


Figure 2. Comparison of UV spectra of the peak at retention time of 14 min in cocoa liquor extract (DCA) with UV spectra of caffeic acid (CA)

gram with numbers between 8–13 showed spectra similar to those of (+)-catechin or (–)-epicatechin (absorption maximums at 231 and 278 nm). In comparing the spectrum of (–)-epicatechin with these peaks, good matches (> 95% similarity) were obtained, the latter having been identified as oligomeric procyanidins. Although monomers and small oligomers may be separated from polymeric procyanidins by RP-HPLC, the latter ones appear as a broad unresolved peak towards the end of the chromatogram. Therefore, methods based on NP-HPLC have been developed to separate oligo-

meric and polymeric procyanidins on a molecular mass basis (ADAMSON *et al.* 1999; HAMMERSTONE *et al.* 1999; NATSUME *et al.* 2000; WOLLGAST *et al.* 2001; COUNET *et al.* 2004; GU *et al.* 2006).

The analytically determined polyphenolics and methylxanthines compositions of cocoa liquor samples with an appropriate statistic analysis are presented in Table 1.

Among flavan-3-ols, (–)-epicatechin (0.16 to 0.59 mg/g DCL) and (+)-catechin (0.02–0.42 mg/g DCL) were present in all samples. (–)-Gallocatechin was not detected in Ghana and Venezuela samples, while (–)-epigallocatechin was not detected in cocoa liquor samples originated from Mexico and Venezuela. The contents of (–)-epigallocatechin in some cocoa liquor (Ghana and Ecuador samples) were similar to the levels of (–)-epicatechin, while in Madagascar and Sao Tome samples the levels were even higher. In all samples, caffeic acid-derivative was determined in the range from 0.41 to 1.23 mg/g DCL. Overall, total polyphenolic contents of cocoa liquors obtained by HPLC analysis were in the following order: Madagascar > Mexico > Ecuador > Venezuela > Sao Tome > Ghana samples. Comparing six cocoa liquor samples investigated, the compositions of polyphenols in all samples were similar, but the quantity of the individual polyphenols differed remarkably. Except polyphenols, two methylxanthines were quantified, theobromine and caffeine. The predominant compound in the extracts analysed was theobromine (1.11–1.98 mg/g DCL), while the levels of caffeine were substantially lower, in the range from 0.13–0.43 mg/g DCL.

The levels of total polyphenolic content were determined by Folin-Cicalteu method and the

Table 1. Polyphenolics and methyxanthines contents (mg/g DCL) in different cocoa liquor samples

Cocoa liquor sample	EC	C	DCA	GC	EGC	CAF	T
Ecuador	0.59 ± 0.03 <sup>ab</sup>	0.02 ± 0.00 <sup>d</sup>	0.60 ± 0.04 <sup>b</sup>	0.40 ± 0.03 <sup>a</sup>	0.40 ± 0.03 <sup>ab</sup>	0.43 ± 0.01 <sup>a</sup>	1.21 ± 0.03 <sup>b</sup>
Ghana	0.16 ± 0.02 <sup>c</sup>	0.18 ± 0.12 <sup>b</sup>	0.53 ± 0.05 <sup>b</sup>	n.d.	0.15 ± 0.01 <sup>c</sup>	0.12 ± 0.01 <sup>d</sup>	1.97 ± 0.06 <sup>a</sup>
Madagascar	0.40 ± 0.03 <sup>bc</sup>	0.09 ± 0.00 <sup>c</sup>	1.11 ± 0.11 <sup>a</sup>	0.19 ± 0.01 <sup>b</sup>	0.42 ± 0.01 <sup>a</sup>	0.25 ± 0.01 <sup>c</sup>	1.83 ± 0.05 <sup>a</sup>
Mexico	0.55 ± 0.05 <sup>a</sup>	0.11 ± 0.01 <sup>c</sup>	1.23 ± 0.04 <sup>a</sup>	0.09 ± 0.01 <sup>c</sup>	n.d.	0.33 ± 0.01 <sup>b</sup>	1.14 ± 0.08 <sup>b</sup>
Sao Tome	0.26 ± 0.04 <sup>c</sup>	0.34 ± 0.40 <sup>b</sup>	0.41 ± 0.09 <sup>b</sup>	0.06 ± 0.01 <sup>c</sup>	0.34 ± 0.03 <sup>b</sup>	0.16 ± 0.01 <sup>d</sup>	1.98 ± 0.08 <sup>a</sup>
Venezuela	0.52 ± 0.05 <sup>ab</sup>	0.42 ± 0.48 <sup>a</sup>	0.70 ± 0.03 <sup>b</sup>	n.d.	n.d.	0.38 ± 0.01 <sup>a</sup>	1.11 ± 0.02 <sup>b</sup>

The results are the means ± S.D. ( $n = 3$ ),  $P < 0.05$ ; the values in the same column, followed by the same letter (a–d), are not statistically different ( $P < 0.05$ ) as measured by Tukey's HSD test

(–)-epicatechin (EC), (+)-catechin (C), caffeic acid derivate (DCA), (–)-gallocatechin (GC), (–)-epigallocatechin (EGC), caffeine (CAF), and theobromine (T)

Table 2. The total polyphenolic content and antioxidant activity determined by DPPH and ORAC methods in different cocoa liquor samples

Cocoa liquor sample	Total polyphenols	EC <sub>50</sub>	1/EC <sub>50</sub>	ORAC
Ecuador	8.14 ± 0.13 <sup>b</sup>	6.595 ± 0.40 <sup>b</sup>	0.15 ± 0.01 <sup>b</sup>	1016.24 ± 131.80 <sup>b</sup>
Ghana	4.01 ± 0.04 <sup>d</sup>	11.01 ± 0.77 <sup>d</sup>	0.09 ± 0.00 <sup>d</sup>	347.90 ± 16.03 <sup>d</sup>
Madagascar	12.65 ± 0.31 <sup>a</sup>	4.82 ± 0.23 <sup>a</sup>	0.21 ± 0.01 <sup>a</sup>	1425.82 ± 21.36 <sup>a</sup>
Mexico	8.37 ± 0.15 <sup>b</sup>	6.19 ± 0.25 <sup>ab</sup>	0.16 ± 0.01 <sup>ab</sup>	1139.34 ± 52.77 <sup>b</sup>
Sao Tome	4.92 ± 0.13 <sup>c</sup>	9.16 ± 0.10 <sup>cd</sup>	0.11 ± 0.00 <sup>cd</sup>	545.70 ± 31.72 <sup>cd</sup>
Venezuela	5.19 ± 0.19 <sup>c</sup>	8.54 ± 0.16 <sup>c</sup>	0.12 ± 0.00 <sup>c</sup>	680.02 ± 35.41 <sup>c</sup>

The results are the means ± S.D. ( $n = 3$ ),  $P < 0.05$ ; values in the same column, followed by the same letter (a–d) are not statistically different ( $P < 0.05$ ) as measured by Tukey's HSD test

Total polyphenols – mg epicatechin equivalent/g DCL; EC<sub>50</sub> – mg DCL/ml; ORAC – μmol Trolox equivalent/g of DCL

data are shown in Table 2. The total polyphenolic content was the highest in the Madagascar sample, followed by the Mexico and Ecuador samples. The levels found in the Venezuela and Sao Tome samples were lower and were followed by the Ghana sample in which the total polyphenolic content was the lowest. The order of the total polyphenolic content in cocoa liquor samples analysed with Folin-Ciocalteu method was the same as the order obtained as a result of the summation of the concentrations of polyphenolic compounds quantified by HPLC.

Most natural antioxidants are multifunctional and their activity in complex heterogeneous foods cannot be evaluated with a single method. Our of different methods, we applied two radical scavenging assays, DPPH' and ORAC. The combination of DPPH and ORAC methods gave valuable information on the significant antioxidant capacity of cocoa liquors. Besides phenolic compounds, the presence of methylxanthine (theobromine and caffeine) and anthocyanins in cocoa beans

may influence the antioxidant capacity as well. The results obtained for total antioxidant activity determined by DPPH' and ORAC assays are shown in Table 2.

DPPH' assay determines the scavenging of stable radical DPPH by antioxidants. EC<sub>50</sub> values, the amount of antioxidant necessary to decrease the initial DPPH' radical concentration by 50%, were determined from the graph of scavenging activity plotted against the concentration of DCL. The lowest EC<sub>50</sub> indicates the highest ability of the extract to act as DPPH' scavenger. Radical scavenging activities of cocoa liquors were in the following order: the Madagascar > Mexico > Ecuador > Venezuela > Sao Tome > Ghana samples.

The ORAC assay is thought to be more relevant because it utilises a biologically relevant radical source (PRIOR *et al.* 2005). That assay provides a controllable source of peroxy radicals that models the reactions of antioxidants with lipids. The peroxy radical reacts with a fluorescent probe to form non-fluorescent product, which can be

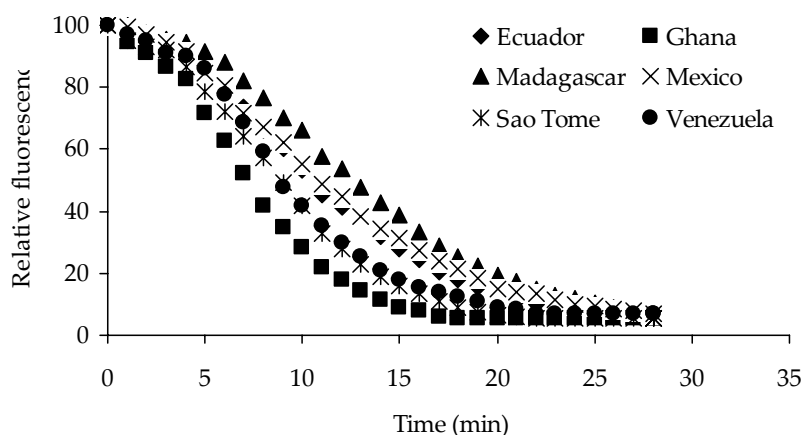


Figure 3. Kinetics of fluorescein quenching with different cocoa liquor samples

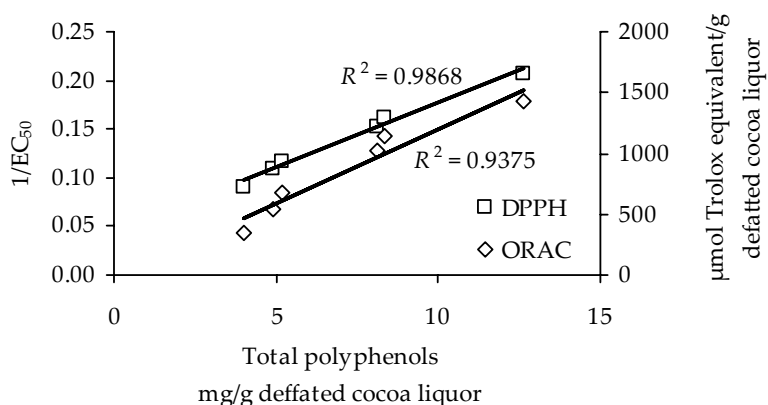


Figure 4. Correlation between the total polyphenolic content and antioxidant activity determined by DPPH and ORAC methods

easily quantified by the fluorescence changes. The decrease of the relative fluorescence of fluorescein with time in the presence of AAPH and cocoa liquor samples is shown in Figure 3. The data obtained with this method were similar and the order of the antioxidant activities was the same as with DPPH method.

In addition, the linear regression analysis showed a high correlation coefficient between the antioxidant activity determined by both methods and total polyphenolic content ( $R^2 = 0.9868$  for DPPH, and  $0.9375$  for ORAC) (Figure 4). A very high correlation coefficient was also found in many other studies using similar analyses (ADAMSON *et al.* 1999; COUNET *et al.* 2004; NINFALI *et al.* 2005; SUN & HO 2005; MILLER *et al.* 2006). The high correlation between the total polyphenol content and antioxidant capacity allows to suggest that the dominant compounds which contribute to the antioxidant capacity are of polyphenolic structure.

## CONCLUSIONS

The varieties of cocoa bean, growing conditions, and fermentation seem to influence the level of polyphenols in cocoa beans and therefore lead to a variety of values we have found in cocoa liquor samples. In all cocoa liquor samples a similar phenolic profile was found, however, with varying proportions of polyphenols. The total polyphenolic contents of cocoa liquors were in the order: the Madagascar > Mexico > Ecuador > Venezuela > Sao Tome > Ghana samples. The same order of the antioxidant activity was determined with both methods applied.

Nowadays, except for the interest in the quality standards and economic specification of raw material, manufacturers pay a lot of attention to declaring their products as functional food. Thus, our data provide additional knowledge to

be considered in promoting the consumption of cocoa products.

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