BFJ
113,9

1094

Received September 2009
Revised April 2010
Accepted April 2010

Phenolic compounds, flavonoids and antioxidant activity in different cocoa samples from organic and conventional cultivation

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Abstract

Purpose – Due to the importance attributed to the phenolic compounds present in cocoa samples for their beneficial effects on health, the purpose of this paper was to analyze four samples of organically and conventionally cultivated cocoa from the south area of Bahia for their composition of phenolics, flavonoids and their antioxidant activity.

Design/methodology/approach – Non-fermented beans, fermented beans, roasted nibs and cocoa liquor were analyzed using spectrophotometry.

Findings – In general, the samples that contained a higher level of phenolics and flavonoids were the roasted nibs and the non-fermented samples in both cultivation systems. The fermented beans and the liquor contained a lower level.

Practical implications – The relationship between the concentration of total phenols and the capacity to "mop up" free radicals from the cocoa extracts appears to be highly significant. The extracts with a higher concentration of phenols also show higher antioxidant activity (non-fermented beans extracts and organically and conventionally cultivated nibs).

Originality/value – This work brings an important contribution in the field of agriculture at a time when organic systems of cultivation are an alternative to the conventional system and that pollutes the environment and produces food that contains quantities of chemical contaminants that can damage the health of the consumer. The comparison in phenolic compounds content, flavonoids and antioxidant activity in organic and conventional systems is original and of great importance, showing that the ecological cropping systems are less harmful to the environment and promote improvements to the chemical composition of foods.

Keywords Cocoa, Organic cocoa, Phenolics, Antioxidant activity, Flavonoids, Health foods

Paper type Research paper

1. Introduction

In recent years, there has been an increase in interest in polyphenols and their derivatives because they are important sources of phenolic compounds. Studies have shown that the consumption of cocoa products has a positive effect on health. These benefits are mainly attributed to the high antioxidant activity present in cocoa. Currently, the potential protective health benefit from cocoa's polyphenols is linked to the protection from oxidation caused by low-density lipoprotein (LDL), a consequence

British Food Journal
Vol. 113 No. 9, 2011
pp. 1094-1102

0007-070X
DOI 10.1108/00070701111174550

of oxidative stress (Ortega *et al.*, 2008). Therefore, the interest in natural compounds with antioxidant properties, such as polyphenols, is increasing.

Polyphenols are micronutrients present in plants and in our diet. These substances are secondary metabolites of plants and are involved in protecting the plant against UV radiation and pathogen attack (Manach, 2004). It is known that polyphenols play a role in the prevention of many diseases associated with oxidative stress, such as diseases associated with the cardiovascular system and neurodegenerative diseases. These molecules modulate the activity of a wide range of enzymes and cell receptors (Middleton *et al.*, 2000), "mop up" free radicals (Bors *et al.*, 1994) and chelate transition metal ions (Korkina and Afanas'ev, 1997).

Flavonoids are a class of polyphenols that have a mechanism for protecting molecules (lipids, proteins and nucleic acids) from oxidative damage by suppressing the inflammatory response and modulating vascular homeostasis (Demrow, 1995). Flavonoids absorbed by the diet work together with the endogenous antioxidant defense system, enzymes, small antioxidant molecules and vitamins (Rein, 2000). The consumption of foods and beverages rich in flavonoids may be associated with an increase in the antioxidant capacity of plasma, the reduction of platelet aggregation and improved endothelial function (Zhu *et al.*, 2002).

Oxidation is a metabolic process that leads to energy production needed for essential cell activities. Oxidants are compounds produced by normal metabolism in the body, and, if not controlled, can cause extensive damage. Oxidative stress has been associated with the development of many chronic and degenerative diseases, such as cancer, heart disease and Alzheimer's, and it is also involved in the aging process (Shahidi, 1996; Lang and Lozano, 1998; Roesler *et al.*, 2007). Clinical and epidemiological studies have shown evidence that phenolic antioxidants from cereals, fruits and vegetables are the main factors that contribute to significantly reducing the incidence of chronic and degenerative diseases (Shahidi, 1996). Thus, the importance of finding natural antioxidants has increased in recent years. Typical compounds that possess antioxidant activity include the class of phenols, phenolic acids and their derivatives, flavonoids, tocopherols, phospholipids, amino acids, phytic acid, ascorbic acid, sterols and pigments. Phenolic antioxidants are compounds that act as terminators for free radicals (Roesler *et al.*, 2007).

Cocoa (*Theobroma cacao* L., Sterculiaceae family), from South America, contains high amounts of bioactive compounds, in particular polyphenols. It has been known for decades that beans from the cacao tree contain very high levels of polyphenols (12 to 20 per cent of their defatted dry weight) compared to other plants (Brito *et al.*, 2002; Sanchez-Rabaneda *et al.* 2003). According to Brito (2000), 60 per cent of these compounds are procyanidins, as flavan-3-ol condensates, containing between 2 and 18 molecules of (+)-catechin or (−)-epicatechin (Hammerstone *et al.*, 1999). However, the content of polyphenols and their derivatives in the beans may vary due to the variety of the cocoa, the origin of the beans or by the industrial methods used to process the cocoa (Summa *et al.*, 2006; Othman *et al.*, 2007).

The link between the phenolic compounds and the bitter taste and astringency of fermented beans has been known since 1930 (Brito, 2000; Soares, 2001). Indeed, recent studies have attempted to improve the taste of chocolate by using the enzyme polyphenol oxidase (PPO) or by heating (autoclaving) to decrease the phenolic compounds present in the beans (Soares, 2001; Brito *et al.*, 2001, 2002). Soares (2001)

found a decrease of 15 per cent and 24 per cent of the total phenol content in cocoa nibs (cocoa beans that are fermented and dried prior to test and germ removal) through an enzymatic treatment with PPO.

Studies done *in vitro* and *in vivo* show that flavonoids from cocoa are able to modulate or decrease the activation of platelets and thereby assist in the maintenance of cardiovascular health. However, with the discovery of the functional properties of the phenolic compounds in cocoa in the late 1990s, research has also been directed towards maintaining these compounds in chocolate and cocoa without changing the taste (Kealey *et al.*, 2004; Efraim, 2004).

The proven beneficial implications for human health confirm the need for the determination and quantification of total phenolic compounds and simple flavonoids (epicatechin and catechin) found in cocoa products and other foods (Wollgast and Anklan, 2000). The objective of the present work was to evaluate the antioxidant activity of cocoa (non-fermented beans, fermented beans, *nibs* and liquor) coming from two different cultivation systems and to esteem the content the total phenolics compounds and flavonoids.

2. Materials and methods

2.1 Material

2.1.1 Non-fermented beans. A total of 30 fruits were collected randomly from each type of cocoa cultivation (organic and conventional) at three different times. The fruits were opened 48 hours after harvesting. The organically cultivated fruits were collected from farms that were certified as organic by IBD (Biodynamic Institute) and were monitored by a cocoa-processing factory located in South Bahia. The conventionally cultivated fruits were collected from farms in the same region. The fruits were hulled without allowing any contact with water in order to prevent the loss of the compounds of interest. The beans were frozen at -18°C and lyophilized. Next, the episterms were removed, and the germ and cotyledons were crushed in a mill under nitrogen gas. This procedure was performed without light to avoid the oxidation of the compounds of interest.

2.1.2 Fermented beans, roasted nibs *and cocoa liquor*. Organic materials and conventional processes were obtained from a South Bahia company with an IBD certification for processing organic cacao in three different periods (March, June and December), according to the production company's processor. The samples were refrigerated until the time of analysis.

To analyze the fermented beans, dry beans and roasted nibs, Testa was removed and then the sample was crushed in the dark and stored under nitrogen gas to prevent oxidation of compounds of interest. The liquor was melted at 30 °C for homogenization improved.

2.2 Methods

2.2.1 Determination of total phenolics. The shredded samples were defatted with petroleum ether at a ratio of 1:2 (sample: solvent) five times. The quantification of phenolic compounds was performed using the Folin-Ciocalteu method. In this method, the phenolic compounds in the sample reduce a reagent and form a blue complex whose intensity increases linearly to 765 nm. This method is described by Swain and Hillis (1959) and cited by Roesler *et al.* (2007). For extraction, 100 mg of defatted sample

and 10 ml of distilled water were placed in centrifuge tubes. The sealed tubes were agitated in a shaker type vortex for 5 minutes and then centrifuged for 20 minutes. The total amount of phenols from each extract was quantified using a standard curve prepared with gallic acid. For the color reaction, an aliquot from the 0.5 mL of aqueous extract (concentration 10 mg.mL^{-1}) was added to 2.5 mL of a 10 per cent aqueous solution of Folin-Ciocalteu reagent and to 2.0 mL of 7.5 per cent sodium carbonate. The mixture was incubated for 5 minutes in a water bath at 50°C, and the absorbance was measured at 765 nm (using the blank as reference). The quantification of the phenolic compounds in the samples extracts was performed in triplicate.

2.2.2 Flavonoid determination. The quantification of phenolic compounds from the group of flavonoids was made following a previously published method (Lee *et al.*, 2003). The total concentration of flavonoids was determined using a colorimetric reaction. To perform the reaction, 1 mL of sample was transferred using a 10 mL measuring flask containing 4 mL of water. Then, 0.3 mL of 5 per cent sodium nitrite solution was added. After five minutes, 0.3 mL of 10 per cent aluminum chloride solution was added. After one minute, 2 mL of a 1M sodium hydroxide was added. Complete the volume with distilled water and measuring flask. The amount of flavonoids in each extract was quantified using a standard curve prepared with epicatechin. Subsequently, the absorbance was measured at 510 nm using the blank as reference.

2.2.3 Determination of the capacity to "mop up" free radicals. The stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH) has been frequently used to assess the ability of natural antioxidants to "mop up" free radicals (Roesler *et al.*, 2007). To determine the antioxidant capacity, an ethanol solution of DPPH 0.004% m.v^{-1} was prepared. From the aqueous extracts with different concentrations, solutions were prepared by adding 1 mL of the extract and 3 mL of the DPPH solution. The initial concentration of the sample was 10 mg.mL^{-1}, and the final concentration of extracts in the cuvette was 3 mg.mL^{-1} to 25 mg.mL^{-1}. Each sample was incubated for 30 minutes at ambient temperature in the dark. The same procedure was performed for the gallic acid.

The control was prepared using the above procedure without the addition of extract, and ethanol was used for the baseline correction. The DPPH solution was prepared, stored in bottles covered with aluminum foil and kept in the dark at 4°C until use for analysis. The per cent decrease in absorbance was measured for each concentration, and the ability to "mop up" free radicals was quantified by the observed decrease in absorbance. Changes in the absorbance of the sample were measured at 517 nm. The ability to "mop up" free radicals was expressed as a percentage of the inhibition of the radical oxidation and calculated using the following formula (Vinson *et al.*, 2001):

$$\%\text{Inhibition} = ((A_{DPPH} - A_{Extr})/A_{DPPH})^{*}100$$

In this formula, ADPPH is the absorbance in the DPPH solution, and AExtr is the absorbance of the sample in solution. AExtr was calculated as the difference in absorbance of solution aqueous sample to test against the blank. The value of IC50 is defined as the final concentration in μg.mL^{-1} of dry extract in the cuvette required to decrease the initial concentration of DPPH by 50 per cent.

2.3 Statistical analysis

The results were subjected to a randomization test to determine if patterns in the data appeared by chance. To verify differences between cultures, between the variables, and

within the organic and conventional cultivations, the Kruskal-Wallis test used. This is a nonparametric test to determine if there is a difference between the medians of groups. To test for the existence of differences between groups, a non-parametric multiple comparisons test was conducted (Siegel and Castellan, 2006). This test checks which means are different from others using a significance level of 5 per cent. The tests were done using the program MINITAB ® 14.

3 Results and discussion

3.1 Total phenolic compounds and flavonoids

According Tables I and II, all samples coming from organic cultivation presented higher values for samples coming from conventional cultivation. But there was no statistical difference at 5 per cent significance level. The observed concentrations of total phenolic compounds ranged from 195.95 $mg.g^{-1}$ until 301.43 $mg.g^{-1}$ in organic cultivation and 155.25 $mg.g^{-1}$ until 289.43 $mg.g^{-1}$ in conventional cultivation (Table I). The concentrations of flavonoids ranged from 128.79 $mg.g^{-1}$ until 214.03 $mg.g^{-1}$ in organic cultivation and 100.70 $mg.g^{-1}$ until 211.89 $mg.g^{-1}$ in conventional cultivation (Table II).

The value of total polyphenols found in non-fermented beans (284.63 $mg.g^{-1}$) that were organically cultivated was higher than the total polyphenols found in non-fermented beans that were conventionally cultivated (274.41 $mg.g^{-1}$). This value is higher than that found by Ortega *et al.* (2008) (259.9 $mg.g^{-1}$ in non-fermented beans from organic cultivate). In a study with non-fermented cocoa beans, Efraim *et al.* (2006) reported values of 238.45 mg/g. Another study, Niemenak *et al.* (2006), comparing the phenolic compounds from different clones of cocoa found values that

Table I.
Content of total phenolic compounds with standard deviations in different samples from organic and conventional cocoa cultivation

	Total Phenolics ($mg.g^{-1}$)	
	Organic cultivation	Conventional cultivation
Non-fermented beans	$284.63^{a} \pm 3{,}22$	$274.41^{a} \pm 5{,}22$
Fermented beans	$233.32^{a} \pm 0{,}33$	$221.82^{a} \pm 0{,}53$
Roasted *nibs*	$301.43^{a} \pm 0{,}55$	$289.43^{a} \pm 0{,}75$
Cocoa *liquor*	$195.95^{a} \pm 0{,}55$	$155.25^{a} \pm 0{,}54$

Values marked with the same letter in the same line between partial averages do not differ significantly ($p > 0.05$)

Table II.
Content of flavonoids with standard deviations in different samples from organic and conventional cocoa cultivation

	Flavonoids ($mg.g^{-1}$)	
	Organic cultivation	Conventional cultivation
Non-fermented beans	$197.85^{a} \pm 0{,}33$	$183.61^{a} \pm 2{,}31$
Fermented beans	$173.63^{a} \pm 0{,}43$	$168.80^{a} \pm 3{,}23$
Roasted *nibs*	$214.03^{a} \pm 0{,}65$	$211.89^{a} \pm 1{,}25$
Cocoa *liquor*	$128.79^{a} \pm 0{,}95$	$100.70^{a} \pm 2{,}35$

Notes: Values marked with the same letter in the same line between partial averages do not differ significantly ($p > 0.05$)

ranged from 84.4 $mg.g^{-1}$ to 149.2 $mg.g^{-1}$ of total phenols. In a study working with beans of lyophilized cocoa from different genotypes, Soares (2001) found values of 330 $mg.g^{-1}$ total phenols; this is considerably higher than values found in this work for non-fermented beans.

The contents of phenolic compounds found in fermented beans from organic cultivation showed a higher value (233.32 $mg.g^{-1}$) than those found in fermented beans (221.82 $mg.g^{-1}$) from conventional cultivation. The statistical analysis found that there is a non-significant difference ($P > 0.05$) between the content of total phenols of samples from fermented cocoa that is organically or conventionally cultivated. Another study, Efraim *et al.* (2006), with fresh and fermented cocoa beans found values that ranged from 86.75 $mg.g^{-1}$ to 149.49 $mg.g^{-1}$ for fermented beans. From the non-fermented beans to fermented beans, the decrease was 14.51 per cent for the organic cultivation and 19.2 per cent for the conventional cultivation. Among other reactions during the fermentation, the oxidation of anthocyanins and the complexation of amino acids with phenolic compounds (and the subsequent formation of quinones) may explain the decrease in total phenols during the fermentation stage.

The content of phenolic compounds found in *nibs* (301.43 $mg.g^{-1}$) from the organic cultivation was higher than the content found for *nibs* (289.43 $mg.g^{-1}$) from the conventional cultivation, but there is no significant difference at the 5 per cent level. The values found for the organic cultivation of *nibs* (301.43 $mg.g^{-1}$) were close to those reported by Ortega *et al.* (2008) (302.5 $mg.g^{-1}$), who worked with the characterization of phenolic compounds in different samples of cocoa. Furthermore, there was a lower concentration of polyphenols in cocoa liquor coming from conventional cultivation.

The flavonoids varied significantly between the *nibs* and liquor samples in conventional and organic cultivation, ranging between 214.03 $mg.g^{-1}$ and 128.79 $mg.g^{-1}$ in the organic system and between 211.89 $mg.g^{-1}$ and 100.71 $mg.g^{-1}$ in the conventional system. The other samples showed no significant difference at a 5 per cent level of significance. When comparing the samples from organic and conventional cultivation, higher values were observed for samples that were organically cultivated; however, no significant difference was seen at the 5 per cent level of significance (Table II).

The flavonoids found in organically cultivated fermented beans showed a higher value (173.63 $mg.g^{-1}$) compared to conventionally cultivated fermented beans (168.80 $mg.g^{-1}$). The statistical analysis demonstrated that there was no significant difference ($P > 0.05$) in the flavonoid content of fermented samples between the organic and conventional cultivations. In a study characterizing the phenolic compounds in different samples of cocoa, Ortega *et al.* (2008) found a value of 163.5 $mg.g^{-1}$, which is similar to the values found in this study.

The values for *nibs* grown by organic and conventional cultivation were 214.03 $mg.g^{-1}$ and 211.03 $mg.g^{-1}$, respectively. The observed values were higher than those reported by Ortega *et al.* (2008) (175.2 $mg.g^{-1}$).

Although the method of Folin-Ciocalteau is mainly used for the quantification of phenolic compounds, the Folin-Ciocalteau reagent may interact with other non-phenolic compounds and thereby lead to an overestimation of total phenols (Stephane *et al.*, 2005). Thus, some authors suggest an additional step in the analysis of total phenols, the quantification of ascorbic acid, or its destruction by heat or acidic conditions (Vinson *et al.*, 2001).

3.2 Antioxidant activity

The ability to "mop up" free radicals using the stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH) was initially chosen because of its simple methodology. The potential of different extracts of cocoa samples to "mop up" free radicals was expressed as the final concentration of extract required to inhibit the oxidation of the DPPH radical by 50 per cent. The results are described in Table III.

The antioxidant substances present in cocoa extracts react with DPPH, a stable radical. The degree of discoloration indicates the antioxidant potential of the extract. An extract that shows high potential to "mop up" free radicals has a low IC_{50} value. Thus, a small amount of extract is capable of decreasing the initial concentration of DPPH radical by 50 per cent. The lower IC_{50} values were obtained by gallic acid ($0.96\ \mu g.mL^{-1}$) and the organically and conventionally cultivated *nibs* ($4.22\ \mu g.mL^{-1}$ and $5.29\ \mu g.mL^{-1}$, respectively)

The IC_{50} for the organic and conventional nibs was $4.22\ \mu g.mL^{-1}$ and $5.29\ \mu g.mL^{-1}$, respectively. As reported in the determination of phenolic compounds, the *nibs* had a high content of phenols and therefore a high potential to "mop up" free radicals.

The relationship between the concentration of total phenols and the ability to "mop up" free radicals from the cocoa extract appears to be quite significant. The extracts with the higher concentrations of total phenols are the extracts with higher antioxidant activity (the extract from the non-fermented beans and the organically and conventionally cultivated *nibs*).

Studies indicate that the correlation between total phenols and antioxidant capacity may depend on the method, the hydrophobic or hydrophilic characteristics of the test system and the antioxidants tested. Several studies in the determination of phenolic compounds have shown that the addition of fermented or non-fermented cocoa liquor will improve the content of phenolic compounds in chocolate and therefore meet the calls for better health.

The results of this study indicate the presence of compounds with high antioxidant capacity from different cocoa samples (non-fermented beans, fermented beans, *nibs* and liquor).

4 Conclusions

The results presented in this study prove that there is variability in the content of phenolic compounds in the samples coming from different cultivate systems. The organic nibs had a higher content of phenolic compounds, flavonoids and antioxidant

	IC_{50} ($\mu g.mL^{-1}.m.v^{-1}$)	
	Organic cultivation	Conventional cultivation
Non-fermented beans	$6.43^{a} \pm 1{,}33$	$7.76^{b} \pm 1{,}44$
Fermented beans	$7.06^{a} \pm 0{,}85$	$8.66^{b} \pm 2{,}85$
Roasted *nibs*	$4.22^{a} \pm 0{,}64$	$5.29^{b} \pm 1{,}14$
Cocoa *liquor*	$7.67^{a} \pm 1{,}05$	$9.87^{b} \pm 1{,}15$

The IC_{50} value was obtained using three replicates from five different concentrations of extracts. This covered the range from low to high inhibition of oxidation inhibition of the DPPH radical

Table III.
Determination of the ability to "mop up" free radicals (DPPH)

activity in comparison with non-fermented beans, fermented beans and liquor in conventional cultivate system. The lowest levels were observed in conventional liquor, the main raw material for chocolate. All results were higher in samples from organic cultivate. Other studies can contribute by performing the monitoring process of conventional and organic cultivate.

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