

Original article

The antioxidant capacity of cocoa products: contribution to the Spanish diet

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Summary Chocolate and soluble cocoa powders are dietary sources of antioxidants. The literature reports antioxidant capacity (AC) derived from aqueous-organic extracts of cocoa polyphenols; however, the residue of these extracts contains appreciable amounts of nonextractable polyphenols (condensed tannins or proanthocyanidins), which may become bioactive antioxidants after colonic fermentation. The objective of this work was to determine the total AC of cocoa products including both extractable and nonextractable polyphenols. Three methods were used: ferric reducing/antioxidant power, 2,2,-diphenyl-1-picrylhydrazyl and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cations' free-radical scavenging capacity. With this last method, AC ranged from 42 to 79 and from 22 to 60 μmol Trolox per gram of sample for extractable and nonextractable polyphenols, respectively. Addition of milk reduces the AC of cocoa products by about 30%. On the other hand, it was estimated that cocoa products account for about 10% of the total AC of the Spanish diet.

Keywords Antioxidant capacity, chocolate, condensed tannins, polyphenols.

Introduction

Natural plant antioxidants are common dietary compounds that have potential health benefits. One of the aims of the ongoing research is to elucidate the role of specific food antioxidants, because of the hypothesis that they afford protection against chronic diseases by reducing oxidative damage (Willcox *et al.*, 2004). Cocoa contains a wide range of antioxidants, soluble phenolic compounds, such as phenolic acids, epicatechin, proanthocyanidins and insoluble polymeric phenolics (Dreosti, 2000; Scalbert & Williamson, 2000). The antioxidant capacity (AC) of cocoa has been the focus of numerous *in vitro* and *in vivo* studies (Steinberg *et al.*, 2003). In these studies, the AC is usually determined in aqueous organic extracts, but the AC derived from nonextractable polyphenolic compounds, which is retained in the extraction residues, is ignored. The main goal of this work was to determine the total AC of cocoa products, including that derived from nonextractable condensed tannins (CT).

It is also the case that the relative contribution of the individual foods to the AC of the whole diet, which is usually unknown, is a key factor for evaluating its

nutritional significance. However, studies addressing AC of the whole diet are scarce (Wu *et al.*, 2004; Saura-Calixto & Goñi, 2006). The second objective of this work was to estimate the contribution of the most widely consumed cocoa products (chocolate and cocoa beverages) to the total AC of the Spanish diet.

Materials and methods**Samples**

Commercial cocoa soluble powders (CSP) and chocolate bars, widely consumed in Spain, were selected. Dark and milk chocolates (52% and 34% of cocoa content, respectively) belong to Valor S.A., which provided for this study the cocoa paste used in both chocolates. The CSP were Cola Cao "original" (CSP1), from Nutrexpa S.A. and Nestquik "instant" (CSP2) was from Nestlé España S.A. The cocoa content of these products was not included in the label information. CSP were dissolved in water and milk (the standard consumption procedure) at 70 °C. Centrifugation at 2500 *g* for 10 min was performed to remove some insoluble particles. To avoid protein interference in AC measurements, CSP dissolved in milk were diluted with 0.3 mol L⁻¹ of acetate buffer (pH 3.6) and precipitated proteins were removed by centrifugation.

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Chemicals

Stable free radical 2,2,-diphenyl-1-picrylhydrazyl (DPPH[•]) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of α -tocopherol were supplied by Aldrich Co. (St Louis, MO, USA) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) by Fluka Chemicals (Steinheim, Germany). Potassium persulfate was purchased from Sigma-Aldrich (Steinheim, Germany) and FeCl₃·6H₂O, acetone, 1-butanol, ethanol and methanol from Panreac Química S.A. (Madrid, Spain). All reagents used were of analytical grade.

Total phenolic content

Extractable polyphenols (EPP)

Chocolate and cocoa paste were milled and sieved into particles size ≤ 2 mm before being defatted using a Soxhlet apparatus (Soxhlet System HT Tecator, Höganäs, Sweden), which performs a continuous light petroleum circulation over the sample during 30 min. CSP were used for analysis directly. The extraction of antioxidants was carried out following the procedure commonly used in our lab for vegetable foods. A gram of the sample was treated with 40 mL of acidic methanol/water (50:50, pH 2.0), and was thoroughly shaken at room temperature for 1 h. After centrifugation at 2500 g for 10 min, the supernatant was recovered. Forty millilitres of acetone/water (70:30) was added to the residue, and shaking and centrifugation were repeated. Both extracts were combined. Extracts were used to estimate both EPP content and AC. EPP of the extracts were determined according to the Folin-Ciocalteu procedure (Singleton *et al.*, 1999) using gallic acid as standard and expressing the results as milligram of gallic acid equivalents (GAE) per gram of the sample.

Nonextractable polyphenols

The residue obtained from the aqueous-organic extractions was treated with 5% HCl in *n*-butanol (100 °C, 3 h) as was described by Reed *et al.* (1982). CT were determined by reading the absorbance at 555 nm. Carob pod (*Ceratonia siliqua*) CT (Nestlé, Ltd. Vers-Chez-les-Blanes, Switzerland) were used as standard. Previously, it was checked that the UV/VIS spectra from cocoa and cocoa products CT and the standard (carob pod) were similar. Results were expressed as mg CT g⁻¹ sample.

AC assays

Ferric reducing/antioxidant power assay

The reduction power was estimated according to the procedure described by Benzie & Strain (1999), with

some modifications introduced in our laboratory (Pulido *et al.*, 2000). The ferric reducing/antioxidant power (FRAP) reagent contained 2.5 mL of a 10 mmol L⁻¹ TPTZ solution in 40 mmol L⁻¹ HCl, plus 2.5 mL of 20 mmol L⁻¹ FeCl₃·6H₂O, plus 25 mL of 0.3 mol L⁻¹ acetate buffer at pH 3.6. Then, 900 μ L of the FRAP reagent was mixed with 90 μ L of distilled water and 30 μ L of sample. Readings at 595 nm were taken every 15 s using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a thermostatted auto cell-holder. Temperature was maintained at 37 °C. The readings at 30 min were selected for calculation of FRAP values. Solutions of known Trolox concentration were used for calibration and results were expressed as micromoles of Trolox equivalents (TE) per gram.

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay

The AC was estimated in terms of radical scavenging activity following the procedure described elsewhere (Re *et al.*, 1999) with some modifications (Pulido *et al.*, 2003). Briefly, ABTS radical cation (ABTS^{•+}) was produced by reacting ABTS with 10 mL of 2.45 mmol L⁻¹ of potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS^{•+} solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 730 nm. After addition of 0.1 mL of the sample to 3.9 mL of diluted ABTS^{•+} solution, absorbance readings were taken every 20 s using a Beckman DU-640 spectrophotometer. The reaction was monitored over 6 min. In the presence of antioxidant, inhibition of absorbance vs. time was plotted and the area below the curve (0–6 min) was calculated. Solutions of known Trolox concentration were used for AC equivalents.

2,2,-diphenyl-1-picrylhydrazyl radical cation assay

The AC was measured in terms of free radical-scavenging ability according to the DPPH[•] method (Brand-Williams *et al.*, 1995) with some modifications (Sanchez-Moreno *et al.*, 1998). Briefly, 0.1 mL of the methanolic extractions at various concentrations were added to 3.9 mL of DPPH[•] solution, and the absorbance at 515 nm was measured until the reaction reached the plateau. A calibration curve at 515 nm was made to calculate the remaining DPPH[•] concentration in the reaction medium. The parameter EC₅₀, which reflects 50% depletion of DPPH[•] free radical, was expressed in terms of grams of cocoa or chocolate equivalent per gram of DPPH[•] in the reaction medium. The time taken to reach the steady state at EC₅₀ (T_{EC50}) was calculated. This method is not suitable with butanol; therefore, AC from CT was not measured by this assay.

Statistical analysis

Extractions were performed in three different samples for each cocoa product. Determinations were performed by triplicate in each extract. Results are expressed as means values with the corresponding standard deviation (SD). Comparison of the means of three measurements using a significant level of $P < 0.05$ was performed by one-way analysis of variance (ANOVA) using the Statgraphics Computer System, version 5.1.

Results and discussion

Total antioxidant capacity of cocoa products

Most natural antioxidants are multifunctional and their activity in complex heterogeneous foods cannot be evaluated by a single method (Frankel & Meyer, 2000). In the present work, three different methods were used to measure AC: FRAP, ABTS and DPPH.

Total phenolic content and AC of defatted cocoa products are shown in Table 1. Dark and milk chocolates and cocoa paste presented high fat contents (352.3 ± 13.8 , 325.5 ± 4.2 and 535.0 ± 9.6 mg g⁻¹, respectively), which had to be removed in order to avoid interference in AC assays. The results were not affected as the AC associated with the fat was negligible. This was verified using the DPPH method, a procedure suitable for fats, and the AC of the cocoa fat accounted for less than 0.2% of AC in defatted cocoa.

Cocoa products presented appreciable concentrations of total EPP (10–18 mg g⁻¹), providing an AC that ranged from 62 to 150 µmol of TE per gram (FRAP) and 42–80 µmol of TE per gram (ABTS). These values are in the same range as reported by other authors (Vinson *et al.*, 1999; Scalbert & Williamson, 2000). Significant correlation was found between total phenolic content and AC of cocoa products. Moreover, antiox-

idant kinetics of cocoa products was compared using the parameter T_{EC50} , measured by the DPPH method. This reflects the time taken by the antioxidant to deplete the initial concentration of DPPH° free radical by 50%. The results, which ranged from 21 to 33 min, classified cocoa products as 'intermediate' antioxidants. For reference, ascorbic acid (T_{EC50} : 1.15 ± 0.08 min) is classified as rapid while quercetin is considered to be slow (T_{EC50} : 63.28 ± 3.15 min) (Sanchez-Moreno *et al.*, 1998).

The cocoa component of a cocoa product is the main ingredient determining its AC, although the milk content may influence the AC. The presence of milk in milk chocolate partially accounts for the fact that its AC was about 50% lower than that of dark chocolate although the phenolic content was only 30% lower. Similarly, the AC of CSP solubilised in water was 35% higher than that of CSP solubilised in milk (Fig. 1). Statistical

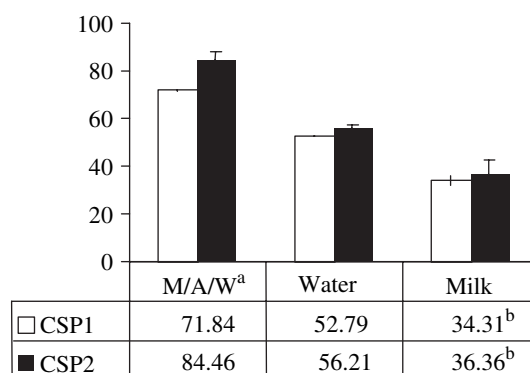


Figure 1 Antioxidant capacity, measured by ferric reducing/antioxidant power (FRAP) method in cocoa soluble products (CSP) using different solvents. Results expressed as Trolox equivalents (µmol g dry weight). Each value represents means of three replicates. ^aM/A/W: methanol, acetone water (25:35:40) extraction; ^bMeans with the same letter are not significantly different at the 5% level.

Table 1 Antioxidant capacity of milk and dark chocolates, cocoa soluble products (CSP) and cocoa paste

	Dark chocolate ^a		Milk chocolate ^a		CSP1		CPS2		Cocoa paste ^a	
	Means	SD	Means	SD	Means	SD	Means	SD	Means	SD
Extractable polyphenols (mg g ⁻¹)	18.16	0.17	13.10 ^b	0.48	10.05	0.33	12.04 ^b	0.97	77.59	1.82
FRAP (µmol TE g ⁻¹)	149.87	8.01	61.50	1.70	71.83	0.34	84.46	3.345	606.14	42.91
ABTS (µmol TE g ⁻¹)	78.80	2.13	42.72 ^b	1.28	42.37 ^b	2.04	54.74	3.55	290.29	2.84
DPPH (EC ₅₀ %)	7.04	0.29	17.31	1.06	13.23	1.70	7.11	0.39	1.68	0.02
Condensed tannins (mg g ⁻¹)	29.40	0.61	11.61 ^b	0.54	11.20 ^b	0.84	13.00 ^b	2.23	111.44	1.25
FRAP (µmol TE g ⁻¹)	144.05	1.82	84.31	0.58	51.83	3.33	60.15	10.10	695.64	29.59
ABTS (µmol TE g ⁻¹)	60.02	1.10	27.12 ^b	0.85	22.26 ^c	1.60	25.88 ^{b,c}	4.41	246.14	5.47

^aResults belongs to samples previously deffated.

^{b,c}Means with the same letter between rows are not significantly different at the 5% level.

Abbreviations: FRAP, ferric reducing/antioxidant power; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; TE, Trolox equivalents.

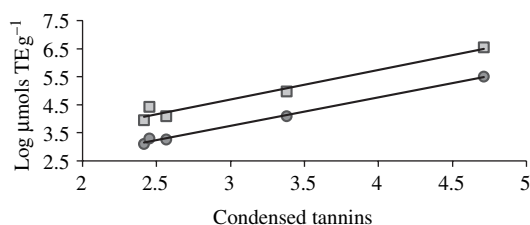


Figure 2 Correlation between antioxidant capacity, measured by ferric reducing/antioxidant power (FRAP; squares) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; circles) assays (results expressed as log mM Trolox equivalents per gram) and condensed tannins (results expressed as log mg g⁻¹ of tannic acid equivalents) in cocoa products. Correlation coefficients: FRAP: $r^2 = 0.9648$ and ABTS: $r^2 = 0.9952$.

differences were found in the AC between CSP1 and CSP2 extracted with organic solvents and water. However, no differences were found with milk extraction ($P < 0.05$). The ability of polyphenols to associate proteins is well known, and the formation of milk protein–polyphenol complexes may explain why the AC of cocoa products is reduced by the addition of milk and milk polyphenols. Serafini *et al.* (2003) reported increases of AC in plasma of volunteers after dark chocolate consumption. This effect was not observed when milk chocolate or dark chocolate plus milk were consumed. The AC decrement has been described in the literature for other polyphenol-rich foods habitually consumed with milk, such as coffee (Sanchez-Gonzalez *et al.*, 2005) and tea (Serafini *et al.*, 1996). Nevertheless, the intestinal availability of milk-complexed EPP after enzymatic digestion could be the object of further research.

Most of the literature cited assumes that the AC of chocolate is derived exclusively from the antioxidants extracted in the corresponding aqueous-organic extracts. However, appreciable amounts of nonextractable polyphenols or CT were found in the residues of these extracts, which ranged from 11 to 29 mg g⁻¹ (Table 1). Most cocoa CT are stable during gastric transit in humans (Rios *et al.*, 2002); nevertheless, CT may be partially metabolized in the colon by the action of intestinal microflora (Deprez *et al.*, 2000). The partial metabolism may also produce an antioxidant environment in the colon. To evaluate the potential contribution of CT to the AC in the gut, the AC of hydrolysed standard of CT was measured, and the corresponding AC was correlated with the CT content of the samples (Fig. 2). The potential AC derived from proanthocyanidins may range from 144 to 52 μmol of Trolox per gram measured by FRAP assay.

Contribution to the diet

Recently, several studies have related the intake of cocoa-flavonoids with the prevention of chronic

Table 2 Antioxidant capacity (AC) intake from cocoa products, expressed as Trolox equivalents (TE), in the Spanish diet

	Daily intake (grams per day per person)	FRAP (μmol TE per day per person)	ABTS (μmol TE per day per person)
General population			
Chocolate ^a	6.46 ^c	446.8	257.5
CSP ^b	2.15 ^c	168.2	104.5
Young population			
Chocolate ^a	12.18 ^d	843.8	486.3
CSP ^b	4.23 ^d	330.6	205.4
Adult population			
Chocolate ^a	2.87 ^e	198.8	45.1

^aAC values are means of dark and milk chocolates (including fat content).

^bMean values from CSP1 and CSP2.

^cValues based on Ministerio de Agricultura, Pesca y Alimentación (MAPA, 2000).

^dValues based on Aranceta Bartrina *et al.* (2004).

^eValues based on Wirfält *et al.* (2002).

Abbreviations: FRAP, ferric reducing/antioxidant power; ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); CSP, cocoa soluble products.

diseases, principally cardiovascular diseases (Keen *et al.*, 2005). However, the dose of cocoa flavonoids used in some of these studies is higher than in a normal diet. The average consumption of cocoa products in Spain is 8.6 g per person per day (Ministerio de Agricultura Pesca y Alimentación, 2000), and it has been estimated that about 75% is chocolate and 25% is CSP. On this basis, the contribution of cocoa products to the intake of extractable antioxidants in the Spanish diet is estimated as equivalent to 615 μmol of TE per person per day measured by FRAP assay (Table 2).

These data are representative of the national per capita intake, but the chocolate consumption is not uniform over the population. The daily intake of chocolate and CSP in the school-aged population has been estimated at 12.18 g per day and 4.23 g per day per child of CSP (Aranceta-Bartrina *et al.*, 2004) representing an antioxidant intake of about 1174 μmol of TE per day per child measured by FRAP assay (Table 2). The Spanish adult population interviewed for the European Prospective Investigation into Cancer and Nutrition (EPIC) reported average consumption of 2.87 g of chocolate per day, similar to the values reported by the Italian and Greek populations (Wirfält *et al.*, 2002). The populations of Britain and Norway reported the highest chocolate consumption (10.77 or 10.00 g per day per capita, respectively) (Wirfält *et al.*, 2002). As reference, chocolate represents the major source of catechin monomers in Dutch daily dietary habits (3–20% according to age) (Arts *et al.*, 2001).

Recently in a short-term food intake of a group of Italian adults the consumed chocolate was reported as

negligible (Brighenti *et al.*, 2005). Therefore, the second objective of this work was to estimate if the contribution of cocoa products to the total AC of the Spanish diet is significant. The total AC derived from EPP of the Spanish diet has recently been estimated, without considering cocoa products, at 6014 measured by FRAP assay and 3549 by ABTS^o (both in terms of μmol of TE per person per day) (Saura-Calixto & Goñi, 2006). The intake of EPP by Spaniards was estimated at 1171 mg GAE per person per day. Including cocoa products consumption, it would be 1237 mg GAE per person per day. Thus, cocoa products contribute about 7.3% of the total dietary intake of antioxidants, known as GAE, which provides 9.5% of the AC of the Spanish diet. Nevertheless, further research to elucidate the role of dietary AC in chronic diseases prevention is needed.

Conclusions

The addition of milk to cocoa-based products decreased its AC.

The potential contribution of CT to the AC of cocoa products is in the same range as the AC derived from the EPP.

Although the consumption of cocoa products in Spain is lower than that in other Western diets, it provides a significant AC to the diet.

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