DECEMBER 1999
VOLUME 47, NUMBER 12

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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RAPID COMMUNICATIONS

Phenol Antioxidant Quantity and Quality in Foods: Cocoa, Dark Chocolate, and Milk Chocolate

Keywords: *Antioxidant; chocolate; cocoa; polyphenols; lipid peroxidation; low-density lipoproteins*

INTRODUCTION

Foods and beverages derived from cocoa beans have been consumed by humans since 460 A.D. Cocoa pods from the cocoa tree (*Theobroma cacao*) are harvested and the beans removed and fermented. Dried and roasted beans contain about 300 chemicals including caffeine, theobromine, and phenethylamine. Chocolate liquor is prepared by finely grinding the nib of the cocoa bean and is the basis for all chocolate products. Cocoa powder is made by removing part of the cocoa butter from the liquor. Bittersweet chocolate, sometimes called dark chocolate, contains at least 15% chocolate liquor but may contain as much as 60% with the remainder being cocoa butter, sugar, and other additives. Milk chocolate is the predominant form of chocolate consumed in the United States and typically contains 10−12% chocolate liquor (Apgar and Tarka, 1998). In this paper the term chocolate will refer to cocoa, milk, and dark chocolate.

The appeal of chocolate is universal, but the pleasures of eating chocolate products may perhaps be tempered by their fat and sugar content. However, in a series of human feeding studies it has been shown that the high proportion of stearic acid in the cocoa butter of chocolate does not adversely affect plasma lipids (Kris-Etherton and Mustad, 1994). Two recent reports of antioxidant activity have increased interest in the health aspects of chocolate: an in vitro LDL oxidation study (Waterhouse et al., 1996) and a short-term in vivo study (Kondo et al., 1996). Epicatechin, the major monomeric polyphenol antioxidant in chocolate (Sanbongi et al., 1998) and an extract of chocolate liquor were both found to stimulate cellular immune response in vitro (Sanbongi et al., 1997).

Polyphenol consumption as flavonoids has been shown to decrease the risk of heart disease in a cross-cultural epidemiological study (Hertog et al., 1995). Most recently, an epidemiology study found that Harvard male graduates who ate a "moderate" amount of chocolate and other candy had a 36% lower risk of death compared with noncandy eaters (Lee and Paffenbarger, 1998). The authors speculate that it is the antioxidants present in the chocolate that provide a health benefit. These reports have prompted us to investigate in detail the antioxidant properties of polyphenols in cocoa and two types of chocolate using an in vitro model of heart disease.

MATERIALS AND METHODS

Sample Preparation. Coded market product samples were received in sealed containers from the American Cocoa Research Institute. Each product was provided as triplicate samples, and all samples were analyzed in a blinded fashion. Representative samples were removed and defatted by multiple extraction with hexane. The freeze-dried samples were then accurately weighed (~50 mg) in duplicate. They were extracted with methanolic HCl with vortexing every 30 min for 2 h (Vinson et al., 1998). Hot cocoa mixes were purchased from local supermarkets and extracted with hot water.

Analysis. Total polyphenols were measured in duplicate for each sample by the Folin−Ciocalteu oxidation−reduction colorimetric method using catechin as the standard. Using a published procedure, we measured the quality of the phenolic antioxidants by dose−response inhibition of human low-density lipoprotein (LDL + VLDL) oxidized for 6 h with 25 μM cupric ions (Vinson et al., 1995a). Quality is the concentration of polyphenols to inhibit the oxidation 50% (IC_{50}). The oxidation mimics the first step in atherogenesis (Steinberg et al., 1989). The phenol antioxidant index (PAOXI) (Vinson and Hontz, 1995) was calculated by dividing the quantity of total phenols in μmol/kg or μmol/L by the quality (IC_{50}, the polyphenol concentration to inhibit the oxidation of LDL + VLDL 50%). Statistical comparisons were made using a Students' t test with $p < 0.05$ being considered significant.

10.1021/jf990312p CCC: $18.00 © 1999 American Chemical Society
Published on Web 11/20/1999

Table 1. Polyphenol Antioxidant Parameters of Cocoa, Chocolate, and Other Products

product	total phenols dry weight (μmol/g)[a]	IC_{50} (μM)	total phenols PAOXI × 10^3	% increase in lag time vs control
		Milk Chocolate		
sample 1	84.1 ± 1.2	0.45	187	26
sample 2	60.0 ± 1.0	0.44	136	
sample 3	44.5 ± 1.3	0.41	109	
sample 4	37.8 ± 1.6	0.34	111	
sample 5	34.5 ± 0.3	0.41	84.1	
average	52.2 ± 20.4	0.41 ± 0.04	136 ± 36.3	
		Dark Chocolate		
sample 1	104 ± 1.8	0.20	520	
sample 2	153 ± 5.8	0.29	528	
sample 3	136 ± 2.4	0.28	486	55
sample 4	114 ± 4.6	0.24	475	
sample 5	118 ± 4.4	0.24	492	
sample 6	129 ± 2.7	0.26	496	
average	126 ± 17.4	0.25 ± 0.03	500 ± 20.4	
		Cocoa		
sample 1	277 ± 2.0	0.40	693	
sample 2	128 ± 1.0	0.27	474	42
sample 3	258 ± 3.0	0.26	992	
sample 4	232 ± 0.6	0.34	682	
average	224 ± 66.4	0.32 ± 0.07	710 ± 213	
		Hot Cocoa Mixes		
sample 1	9.8			
sample 2	11.2			
sample 3	9.5			
sample 4	4.4			
sample 5	5.9			
average	8.2 ± 2.9			
epicatechin		0.19		63
quercetin		0.22		146
beet	53.4	1.54	34.6	
asparagus	40.2	0.28	144	
spinach	27.6	0.86	32.1	17
sweet potato	13.7	0.75	18.3	42
apple	6.4	0.31	20.6	
orange	18.9	0.34	55.6	
black tea	12.0	0.38	31.5	57
red wine	9.6	0.45	21.3	127
chocolate	133.9[b]	0.33[b]	378[b]	41[c]

[a] μmol/mL for beverages. [b] Average of cocoa and chocolate samples. [c] Average for three representative cocoa and chocolate samples.

Epicatechin and catechin, two monomeric polyphenols in chocolate, were analyzed in six samples (two each of milk, dark, and cocoa powder) by HPLC using a 25 cm × 4.6 mm, 5 μm LC-DP C_8 column (Supelco, Bellefonte, PA) with a 280 nm detector and conditions as follows: solvent A, 4% acetic acid in H_2O; solvent B, 1 acetic acid/12.5 methanol/12.5 water; 0−15 min, 100% A, 2 mL/min; 1.5−12 min, 100% A to 100% B, 2 mL/min. Epicatechin had a retention time of 9.80 min and catechin 7.10 min, and their identity was confirmed by spectral scan and spiking.

Ex Vivo Oxidative Susceptibility. Extracts from a representative of each of the three types of chocolate were spiked into plasma at 100 μM polyphenols as catechin equivalents and allowed to equilibrate. Lipoprotein-bound antioxidant activity was then measured by means of the lag times of the cupric ion oxidation of the LDL + VLDL isolated from human plasma and compared with a control with no antioxidants added. This oxidation was done under standard conditions as previously described (Vinson et al., 1995b)

RESULTS AND DISCUSSION

Results of the phenol analysis of the samples are shown in Table 1. We used our extraction method for polyphenols in vegetables (Vinson et al., 1998). The same combination of 50% methanol/water was recently found to quantitatively remove catechins from fruits and vegetables (Arts and Hollman, 1998). No interference was found with the sugars in the chocolates as determined by spiking experiments. It was also found that extraction with 100% methanol, compared with 50% methanol/water as used in the analysis procedure, produced the same phenol concentration of the extracts. This result shows there is no interference from proteins and sugars that would be insoluble in pure methanol. The average deviation of duplicate analyses of the same sample was 1.9%. Variation within sample was 4.1%. We found a wide variation in total phenol content within each type of chocolate. Despite the wide variations, there were significant differences among the types of chocolate with the order of polyphenol content being cocoa > dark chocolate > milk chocolate > hot cocoa, $p < 0.01$ using a two-sample t test. The order followed the decreased amount of chocolate liquor in the products. The instant hot cocoa products were very low in polyphenols, as expected from the low amount of cocoa solids in the retail samples.

Epicatechin and catechin were found in all of the six samples analyzed by HPLC. These two monomeric polyphenols were minor components of the chocolates, ranging from 1.97 to 2.76 mol % of the phenols in the milk chocolate, from 2.98 to 5.48 mol % in the dark chocolate, and from 5.45 to 6.11 mol % in the cocoa powder. Assuming a 35% content of fat and moisture, the concentrations of the catechins are 15−16 mg/100 g in milk chocolate, 48−137 mg/100 g in dark chocolate, and 296−327 mg/100 g in cocoa powder. A recent HPLC analysis of single samples of European chocolate found 16 mg/100 g in milk chocolate and 54 mg/100 g in dark chocolate (Arts et al., 1999). Another report found 300 mg/100 g in cocoa powder, which also agrees well with our results (Bonvehi and Coll, 1997). The two catechins' total concentration in chocolate correlates well with the total polyphenols as measured by the Folin method, Pearson correlation coefficient 0.9326, $p < 0.01$.

The quality of the phenol antioxidants was assessed using the IC_{50} for LDL + VLDL oxidation, with smaller values indicating a higher quality. Quality of the antioxidants was due to free-radical scavenging activity and not chelation as the concentration of polyphenols for 50% inhibition was <1 μM and cupric ion 25 μM in the oxidation medium. There was less percent variation within the groups for this parameter than for the total polyphenol content. The quality order was dark chocolate > cocoa > milk chocolate. The dark chocolate and cocoa were significantly different from the milk chocolate ($p < 0.05$) but not from each other. There was no correlation between the quantity of phenolic antioxidants in the chocolate and the quality as measured by IC_{50}, $p > 0.05$. There was also no correlation between the amount of epicatechin and catechin in the chocolates and the IC_{50}. Theobromine and caffeine, two major ingredients in chocolate, were neither prooxidants nor antioxidants in our model of oxidation. We believe that the quality of antioxidants in chocolate is a complex function of all the antioxidants in the mixture, which may change during processing. This includes the monomeric antioxidants such as catechin (IC_{50} of 0.19 μM; Vinson et al., 1995a) and the oligomeric and polymeric catechins or tannins that are also excellent antioxidants in other in vitro models (Hagerman et al., 1998). PAOXI, the quantity/quality index, was significantly different among the products ($p < 0.01$) with the order cocoa > dark chocolate > milk chocolate.

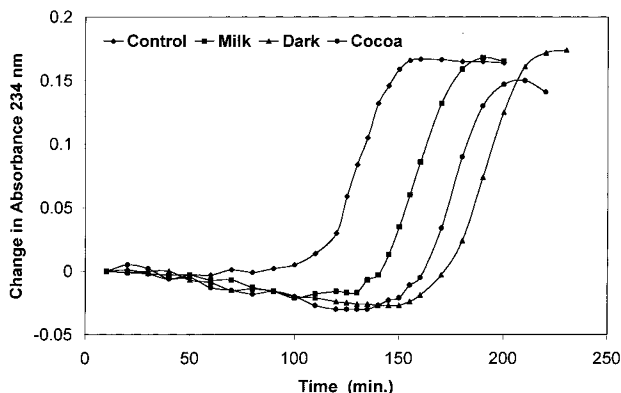

Figure 1. Ex vivo spiking of plasma with cocoa and chocolate extracts containing 100 μM catechin equivalents and the cupric ion oxidation of isolated LDL + VLDL as measured by conjugated dienes formation at 234 nm.

Results of the plasma spiking study are shown in Figure 1, and the lag times are listed in Table 1. Chocolate extracts substantially increased the lag time (time when the rate of oxidation rises sharply) vs the control. This increase is a measure of the ability of the polyphenols from chocolate to enrich the lipoproteins and prevent oxidation. There is a highly significant negative correlation between the lag time and the IC_{50}, correlation coefficient −0.996, $p = 0.01$. Thus, the quality of the antioxidants determines the lag time.

Some interesting comparisons can be made between the antioxidant properties of chocolate and other foods and beverages as shown in Table 1. The average total polyphenol content of dry, defatted chocolate was 133.9 μmol/g. The vegetable with the highest dry weight total phenols was beet, 53.4 μmol/g (Vinson et al., 1998), apple 6.4 μmol/g (Vinson, 1998), and black tea 12.0 μmol/mL (Vinson, 1998). Chocolate has a higher polyphenol content than the 23 vegetables and the several fruits and beverages studied. The IC_{50} average for chocolate products is 0.33 μM and is superior to the average for vegetables, 0.69 μM (Vinson et al., 1998). PAOXI values for chocolate averaged 378 $\times$ 10^3, while the highest vegetable, asparagus, was 144 $\times$ 10^3 and the highest commonly consumed beverage was black tea, 31.5 $\times$ 10^3 (Vinson and Dabbagh, 1998).

An important antioxidant parameter with respect to heart disease is the lipoprotein-bound antioxidant activity. The value for epicatechin, a polyphenol component of chocolate, was 63% greater than the control. The average increase in lag time for the three representative chocolate products was 41%, as shown in Table 1. All beverages, such as wine and tea, that had this antioxidant activity were also found to produce an increase in lag time after ingestion (Vinson et al., 1999).

Thus, chocolates contain both a high quantity and quality of phenol antioxidants. When expressed as catechin equivalents on a fresh weight basis, the average chocolate contains 28.7 mg/g, assuming a 35% content of fat and moisture. Forty grams of chocolate is the reference amount commonly consumed per eating occasion. This serving of milk chocolate, the most popular type of chocolate, provides 394 mg of polyphenol antioxidants and a serving of dark chocolate 951 mg. For comparison, the average black tea contains 943 mg/240 mL serving and a red wine 431 mg/240 mL (Vinson, 1998). The average hot cocoa mix made according to the instructions provides 45 mg of polyphenols in a 240 mL serving. Using 5 g of the average cocoa powder (35% fat and moisture), a homemade serving of hot cocoa has 211 mg of polyphenols as catechin.

A recent published report confirmed our ex vivo results. Defatted cocoa caused a temporal increase in LDL oxidation lag time 2 h after human consumption, indicating in vivo enrichment (Kondo, 1996). Also, recently epicatechin was absorbed following human consumption of 40 g of dark chocolate (Richelle et al., 1999). It remains to be shown whether polyphenols from market chocolates can act as antioxidants following ingestion since fat is a known in vivo prooxidant (Lechleitner et al., 1994). Polyphenols from chocolate may provide additional antioxidant protection for lower density lipoproteins and thus be beneficial for preventing heart disease.

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Received for review April 1, 1999. Accepted October 22, 1999. This work was presented at the 217th American Chemical Society National Meeting in the symposium “Chemistry and Health Benefits of Caffeinated Beverages”. The American Cocoa Research Institute funded the research.

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JF990312P

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