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Procyanidin and Catechin Contents and Antioxidant Capacity of Cocoa and Chocolate Products

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Cocoa and chocolate products from major brands were analyzed blind for total antioxidant capacity (AOC) (lipophilic and hydrophilic $ORAC_{FL}$), catechins, and procyanidins (monomer through polymers). Accuracy of analyses was ascertained by comparing analyses on a NIST standard reference chocolate with NIST certified values. Procyanidin (PC) content was related to the nonfat cocoa solid (NFCS) content. The natural cocoa powders (average 87% of NFCS) contained the highest levels of AOC (826 ± 103 μmol of TE/g) and PCs (40.8 ± 8.3 mg/g). Alkalized cocoa (Dutched powders, average 80% NFCS) contained lower AOC (402 ± 6 μmol of TE /g) and PCs (8.9 ± 2.7 mg/g). Unsweetened chocolates or chocolate liquor (50% NFCS) contained 496 ± 40 μmol of TE /g of AOC and 22.3 ± 2.9 mg/g of PCs. Milk chocolates, which contain the least amount of NFCS (7.1%), had the lowest concentrations of AOC (80 ± 10 μmol of TE /g) and PCs (2.7 ± 0.5 mg/g). One serving of cocoa (5 g) or chocolate (15 or 40 g, depending upon the type of chocolate) provides 2000−9100 μmol of TE of AOC and 45−517 mg of PCs, amounts that exceed the amount in a serving of the majority of foods consumed in America. The monomers through trimers, which are thought to be directly bioavailable, contributed 30% of the total PCs in chocolates. Hydrophilic antioxidant capacity contributed >90% of AOC in all products. The correlation coefficient between AOC and PCs in chocolates was 0.92, suggesting that PCs are the dominant antioxidants in cocoa and chocolates. These results indicate that NFCS is correlated with AOC and PC in cocoa and chocolate products. Alkalizing dramatically decreased both the procyanidin content and antioxidant capacity, although not to the same extent.

KEYWORDS: Antioxidant; cocoa; chocolates; procyanidins; catechin; $ORAC_{FL}$

INTRODUCTION

A number of studies have shown that the consumption of cocoa and chocolate products has positive health effects for humans. These health effects have been assumed to be due to the high antioxidant capacity (AOC) of flavonoids in cocoa, which is higher than those in tea and red wine per serving (*1*). Procyanidins are the major flavonoids in cocoa. Procyanidins in cocoa consist of oligomers and polymers of (+)-catechin and (−)-epicatechin. The size of procyanidins is described as the degree of polymerization (DP). Procyanidins in cocoa have suppressive effects on low-density lipoprotein (LDL) oxidation and the development of atherosclerosis (*2*). Procyanidin-rich cocoa induces nitric-oxide-dependent vasodilation and may improve hypertension in men (*3*). Short-term administration of dark chocolate led to a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons (*4*). Pentameric procyanidins from cocoa selectively inhibited growth of human breast cancer cells (*5*). The procyanidin content in cocoa is higher than those in blueberry and cranberry on a dry weight basis (*6*). We have calculated that the American population consumes 58 mg of procyanidins per day on average; procyanidins from chocolate and cocoa products account for a major source (*7*).

A large number of raw and processed chocolate and cocoa products originate from the tropical cacao tree. When the dehulled cocoa beans are roasted and ground into chocolate liquor, about half of the resulting product consists of nonfat cocoa solids and half as cocoa butter (the fat component). Pure chocolate liquor is known as unsweetened, bitter, or baking chocolate. Natural cocoa powder is chocolate liquor from which variable amounts of cocoa butter have been extracted by pressing. Dark chocolate is a sweetened chocolate with no or a small amount of added milk. Milk chocolate is a sweetened chocolate with added milk powder or condensed milk. Dutched cocoa powder is made by pulverizing defatted chocolate liquor,

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followed by alkali treatment to neutralize its natural acidity. Dutched powders are milder in taste and have a deeper and warmer color than natural cocoa powders (*8*).

Little is known about the variation in antioxidant, (+)-catechin, (−)-epicatechin, and procyanidin contents in commercially available products. In this study, cocoa and chocolate products from major commercial manufacturers and a NIST standard reference chocolate (SRM 2384) were analyzed blind for total antioxidant capacity, (+)-catechin, (−)-epicatechin, and procyanidins.

MATERIALS AND METHODS

Reference Compounds. (+)-Catechin and (−)-epicatechin were purchased from Sigma Chemical Co. (St. Louis, MO). A composite procyanidin oligomer standard containing monomers through decamers was purified from cocoa and has been described previously (*9*). A polymeric procyanidin fraction with an average DP of 36.1 was used as a polymer standard. This polymer fraction does not contain procyanidins with DP ≤ 10. It was fractionated from blueberries on a Sephadex LH-20 column. Characterization of this polymer fraction has been described previously (*6*).

Cocoa and Chocolate Samples. Commercially available chocolate and cocoa products ($n = 19$), including a NIST standard reference chocolate (SRM 2384), were collected and coded by the American Cocoa Research Institute. Identity-blind samples were sent to the Arkansas Children's Nutrition Center and Brunswick Laboratories for analyses. Final data were sent to the American Cocoa Research Institute and decoded.

Procyanidin Extraction and Normal Phase HPLC-ESI/MS. Samples (1 g) were extracted in a 15-mL screw-cap tube with 10 mL of hexane to remove the lipids. The tube was centrifuged at 3500 rpm for 10 min, and the hexane was decanted. Hexane was evaporated in the hood overnight. The defatted sample was extracted with mixed solvent (acetone/water/acetic acid, 70:29.5:0.5 v/v/v). After the addition of solvent, the tube was vortexed for 30 s followed by sonication at 37 °C for 10 min. The tube was kept at room temperature for 50 min. At the end of extraction, the tube was centrifuged at 3500 rpm for 15 min. Part of the supernatant (7.5 mL) was pipetted out, and the acetone was evaporated at 25 °C in a SpeedVac (SC210A, Thermo, Marietta, OH) under vacuum (1.5 Torr). The residue after evaporation of acetone was dissolved in ≈6 mL of 30% (v/v) aqueous methanol and loaded onto a Sephadex LH-20 column. The column (6 × 1.5 cm) was packed with 3 g of Sephadex LH-20, which was equilibrated with 30% (v/v) aqueous methanol for over 4 h before use. After the sample had been loaded, the column was washed with 40 mL of 30% methanol/water to remove sugars and other phenols. Proanthocyanidins were recovered from the column by elution with 70 mL of 70% (v/v) aqueous acetone. Effluents were evaporated to dryness under vacuum in a SpeedVac at 25 °C. The dried substance was dissolved in the extraction solvent and transferred to a volumetric flask. The final volume was brought up to 5 mL. The solution was centrifuged at 14000 rpm before 10 μL was injected for normal phase HPLC-ESI/MS analyses.

Chromatographic separation was performed on an Agilent 1100 HPLC system consisting of a binary pump, a quaternary pump, a solvent degasser, an autosampler, a thermostated column compartment, and a fluorescence detector (Agilent Technologies, Wilmington, DE). Separation was carried out on a 250 × 4.6 mm Phenomenex Luna Silica (2) column (Phenomenex, Torrance, CA) with a particle size of 5 μm at a column temperature of 37 °C. The tertiary mobile phase consisted of (A) methylene chloride, (B) methanol, and (C) acetic acid and water (1:1 v/v). The 70 min gradient was as follows: 0−20 min, 14.0−23.6% B linear; 20−50 min, 23.6−35.0% B linear; 50−55 min, 35.0−86.0% B linear; 55−65 min, 86.0% B isocratic; 65−70 min, 86.0−14.0% B linear; followed by 10 min of re-equilibration of the column before the next run. A constant 4.0% C was kept throughout the gradient. The excitation and emission wavelengths were 276 and 316 nm, respectively, for fluorescence detection. Effluent was then introduced into an Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) with electrospray interface. Mass spectra in negative mode were acquired using a published method (*10*).

Table 1. Fat and Nonfat Cocoa Solid (NFCS) Contents in Cocoa and Chocolate Products

product type	% fat (av)	av % NFCS supplied by manufacturers	min % NFCS (from SOI, where applicable[a])
milk chocolate	32	7.1	4
dark chocolate	36	22.9	15
baking chips	29	18.6	14
unsweetened chocolate	50	49.5	40
natural powders	13.6	87.4	80
Dutched powders	15.5	80.2	78

[a] Standards of identity (SOI) requirements for chocolate and cocoa products established by the U.S. Food and Drug Administration, which specifies the required percentage of chocolate liquor or cocoa butter. Baking chips do not have a corresponding SOI requirement.

Catechin and Epicatechin Analyses Using Reversed Phase HPLC. One gram of cocoa or chocolate products was extracted with 10 mL of methanol in a 50 mL polypropylene tube by sonication for 15 min. The tube was centrifuged at 3000 rpm for 10 min and the supernatant decanted to a clean 50 mL tube. The sample was extracted with another 10 mL of methanol. The methanol extracts were combined. The extract (0.5 mL) was diluted with 1.0 mL of distilled water and filtered through a 45 μm film for HPLC injection. Chromatographic separation was performed on an Agilent 1100 HPLC system using a Phenomenex Luna Phenyl Hexyl column (250 × 4.6 mm, 5 μm) and a phenyl hexyl guard column. The mobile phase consisted of (A) water/acetonitrile/acetic acid (89:9:2, v/v/v) and (B) acetonitrile/water (80:20, v/v). The gradient was as follows: 0−10 min, 0% B; 10−25 min, 0−40% B; 25−32 min, 40−100% B; 32−35 min, 100% B, at a flow rate of 1.0 mL/min. The column was equilibrated for 10 min before the next injection. Column temperature was set at 37 °C. (+)-Catechin and (−)-epicatechin eluted at 10.2 and 16.0 min, respectively, and were monitored using a UV detector at 280 nm.

Antioxidant Capacity Analysis. Sample preparation followed the method published previously (*11*). Both hydrophilic and lipophilic oxygen radical absorbance capciety ($ORAC_{FL}$) assays were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnologies, Durham, NC), which was equipped with a temperature-controlled incubation chamber and two injection pumps. The temperature of the incubator was set at 37 °C. The procedures were based on the modified $ORAC_{FL}$ method (*12*) and on that reported by Ou and co-workers (*13*, *14*). The data are expressed as micromoles of Trolox equivalents per gram (μmol of TE/g).

RESULTS AND DISCUSSION

Characteristics of Cocoa and Chocolate Products. There is a wide variation of fat and nonfat cocoa solid contents in different cocoa and chocolate products (**Table 1**). Natural powders and the Dutched cocoa powders had the highest content of nonfat cocoa solids (NFCS; >80%) and the lowest fat (15%). The unsweetened chocolates had equal amounts of both. Milk chocolates had the least amount of NFCS (7.1%) and a significant amount of fat (32%). All products analyzed met or exceeded the minimum percentage of NFCS as calculated from the requirements for specific standardized cacao products established by the U.S. Food and Drug Administration.

Catechin, Epicatechin, and Procyanidin Contents. Procyanidins in cocoa and chocolate products were separated using normal phase HPLC in accordance with their degree of polymerization (**Figure 1**). Monomers through heptamers were identified on ESI/MS as $[M - H]^-$. Octamers through undecamers were identified as $[M - 2H]^{2-}$. Polymeric procyanidins with DP > 10 eluted as a single peak at ≈55 min. Polymers were unstable in negative ESI/MS and cleaved extensively into fragments (**Table 2**). The structures of these

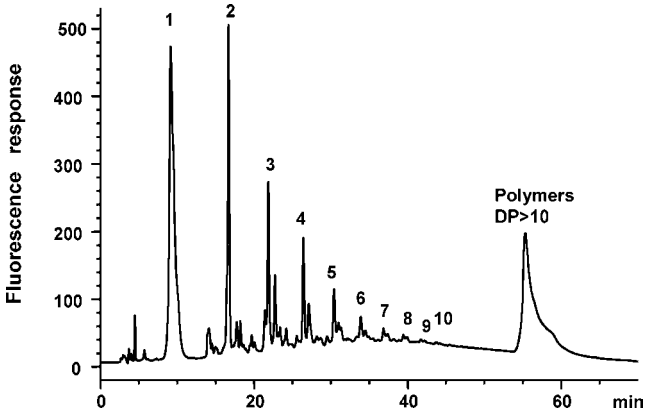

Figure 1. Chromatogram of procyanidins from natural cocoa powder with fluorescent detection; numbers above the peaks denote degree of polymerization of procyanidins.

Table 2. Identification of Procyanidin Oligomers on ESI/MS in Negative Mode

procyanidin	*m/z*	procyanidin	*m/z*
monomers	289.0	heptamers	2018.3
dimers	577.0	octamers	1153.2[a]
trimers	865.1	nenomer	1296.9[a]
tetramers	1153.3	decamers	1441.2[a]
pentamers	1441.3	polymers	1439.4, 1151.4, 863.4[b]
hexamers	1729.4		

[a] Doubly charged ions $[M - 2H]^{2-}$. [b] Singly charged ion or fragments.

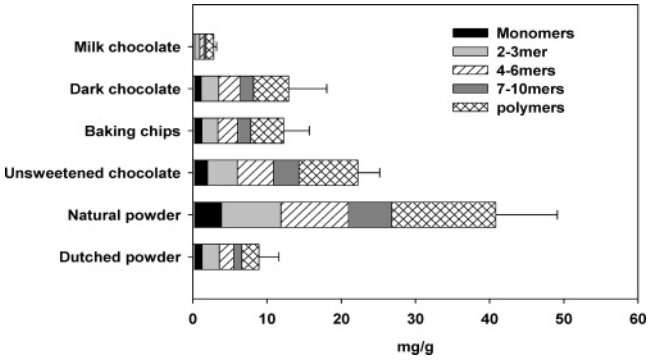

Figure 2. Procyanidin content of cocoa products of different types.

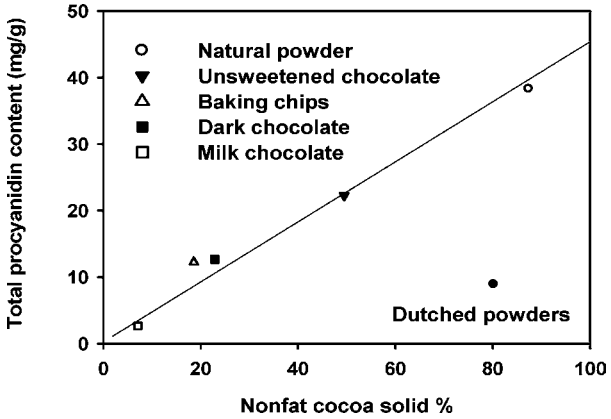

Figure 3. Correlation between nonfat cocoa solid and total procyanidin content in different types of cocoa and chocolate products.

fragments have been reported (*10*). Procyanidins were grouped as monomers, 2−3-mers, 4−6-mers, 7−10-mers, and polymers, and their concentrations in various cocoa and chocolate products are presented in **Table 3**. Total procyanidin content varied markedly in cocoa and chocolate products of the same type. Total procyanidin content in dark chocolate ranged from 8.5 to 19.8 mg/g. Procyanidin contents in different product types are compared in **Figure 2**. Total procyanidin contents in cocoa and chocolate products were related to their NFCS contents (r = 0.99), with the Dutched cocoa as an outlier (**Figure 3**). Natural cocoa powder had the highest NFCS contents and also the highest total procyanidin content. Milk chocolate had the lowest NFCS contents and also the lowest total procyanidin content. The Dutched powders contained similar amounts of NFCS to natural powder, yet the procyanidin content was much lower, indicating degradation of procyanidins as a result of alkali

Table 3. Catechin and Procyanidin Contents of Cocoa and Chocolate Products[a]

		catechins (mg/g)			procyanidins (mg/g)					
product type	no.	catechin[b]	epicatechin[b]	sum[b]	monomers[c]	2–3-mers	4–6-mers	7–10-mers	polymers	total
milk chocolate	1	0.12	0.18	0.30	0.25	0.49	0.38	0.17	0.88	2.16
	2	0.05	0.18	0.23	0.27	0.57	0.58	0.31	1.11	2.84
	3	0.08	0.24	0.32	0.30	0.61	0.68	0.41	1.14	3.14
dark chocolate	1	0.25	0.52	0.77	0.99	1.84	1.80	1.02	2.87	8.52
	2	0.4	0.64	1.04	1.05	1.68	1.92	1.20	2.86	8.72
	3	0.12	0.75	0.87	0.89	1.78	2.29	1.41	4.47	10.84
	4	0.11	1.06	1.17	1.09	2.73	3.83	2.47	6.64	16.76
	5	0.33	1.25	1.58	1.66	3.74	4.54	2.95	6.97	19.85
baking chips	1	0.35	0.66	1.01	0.95	1.63	1.91	1.21	3.01	8.71
	2	0.50	1.01	1.51	1.51	2.23	2.55	1.52	4.67	12.49
	3	0.26	1.07	1.33	1.24	2.59	3.60	2.47	5.68	15.57
unsweetened chocolate	1	0.52	2.01	2.53	1.83	3.46	4.17	3.18	6.12	18.76
	2	1.17	2.00	3.17	2.52	4.22	4.16	2.85	6.22	19.97
	3	1.06	1.76	2.82	2.82	4.78	5.63	3.66	8.30	25.20
	NIST[d]	0.23	1.24	1.47	1.51	4.05	5.53	3.95	10.01	25.05
natural powder	1	0.61	2.29	2.9	3.54	7.09	7.36	4.40	9.80	32.19
	2	0.78	1.58	2.36	3.63	7.87	9.06	5.59	15.49	41.64
	3	0.90	2.58	3.48	4.49	8.98	10.94	7.45	16.85	48.70
Dutched powder	1	0.23	0.18	0.41	1.08	1.96	1.47	0.85	1.65	7.02
	2	0.35	0.38	0.73	1.44	2.69	2.39	1.34	2.96	10.82

[a] Data are average of duplicate tests. [b] Data obtained using reverse phase HPLC. [c] Data obtained using normal phase HPLC. [d] NIST standard reference chocolate (SRM 2384).

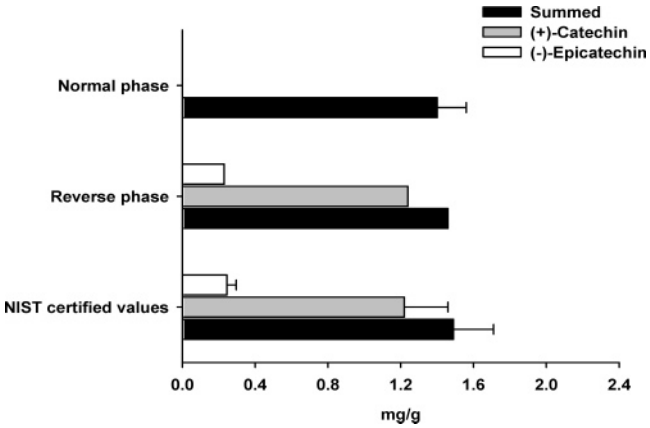

Figure 4. Comparison of catechin, epicatechin, and summed values with NIST certified contents on NIST standard reference chocolate (SRM 2384).

treatment. The unstable nature of flavanols in a basic environment has been well-known (*15*). Procyanidin monomers, dimers, and trimers were considered to be bioavailable as they can be absorbed and are present in blood (*16*, *17*). On average, ≈30% of procyanidins in cocoa were monomers through trimers. This implies a higher bioavailability of procyanidins from cocoa than in other foods, such as grapes, where polymers are dominant (*7*).

(+)-Catechin and (−)-epicatechin coeluted and thus could not be distinguished using normal phase HPLC. Both compounds are biologically active; however, their absorption appears to be different with higher absorption of epicatechin than catechin (*18*). To overcome the shortcoming of normal phase HPLC, (+)-catechin and (−)-epicatechin were quantified using reverse phase HPLC (**Table 3**). (−)-Epicatechin was higher than (+)-catechin in most cocoa products. The (+)-catechin and (−)-epicatechin contents in the standard reference chocolate were consistent with the NIST certified values (**Figure 4**). The monomer content (catechin and epicatechin combined) determined on normal phase HPLC also agreed with the NIST values, indicating that the analytical methods used in this study were accurate and reliable (*19*).

Antioxidant Capacity. Unsweetened chocolate has been found to possess an extremely high antioxidant capacity (*20*). In this study, lipophilic and hydrophilic antioxidant capacities of several types of cocoa and chocolate products were determined (**Table 4**). Hydrophilic fractions contributed >90% of the total antioxidant capacity in all cocoa products. Remarkably, dramatic variation of antioxidant capacity was observed across these samples. Factors such as percentage of NFCS content, other ingredients, and processing method may eventually affect their antioxidant capacity. Procyanidin content was highly correlated with the antioxidant capacity ($r = 0.92$; **Figure 5**), suggesting that procyanidins account for a major portion of the antioxidants in cocoa products.

The U.S. Food and Drug Administration has established the reference amounts customarily consumed for cocoa and chocolate products: ≈5 g for natural/Dutched powders, 15 g for baking chips and unsweetened chocolate, and 40 g for milk/dark chocolate, respectively. A serving, based on the reference amount, of milk chocolate, dark chocolate, or unsweetened chocolate would supply on average 108, 517, and 312 mg of procyanidins; and 3200, 9100, and 6950 Trolox equivalents (TE) of antioxidant capacity (AOC), respectively (**Table 4**). These amounts exceed those in most foods on a per-serving basis. The consumption of one serving of these chocolates would provide more procyanidins and antioxidant capacity than the average daily amount consumed in the United States (*7*, *20*).

Table 4. Total Antioxidant Capacities of Cocoa and Chocolate Products

product type	no.	ORAC (μmol of TE/g) hydrophilic	lipophilic	total	serving size (g)	μmol of TE/serving
milk chocolate	1	64	4	68		
	2	76	10	86		
	3	80	6	86		
mean ± SD		73 ± 8	6.7 ± 3.1	80 ± 10	40	3200
dark chocolate	1	195	16	211		
	2	150	11	161		
	3	172	6	178		
	4	231	6	237		
	5	344	5	349		
mean ± SD		218 ± 76	8.8 ± 4.7	227 ± 74	40	9100
baking chips	1	134	14	148		
	2	204	12	216		
	3	234	8	242		
mean ± SD		191 ± 51	11 ± 3.1	202 ± 48	15	3030
unsweetened chocolate	1	509	7	516		
	2	516	7	523		
	3	444	6	450		
mean ± SD		490 ± 40	6.7 ± 0.6	496 ± 40	14	6950
	NIST[a]	375	6	381		
natural powder	1	865	6	871		
	2	703	6	709		
	3	893	6	899		
mean ± SD		820 ± 103	6 ± 0	826 ± 103	5	4100
Dutched powder	1	395	2	397		
	2	402	4	406		
mean ± SD		399 ± 5	3.0 ± 1.4	402 ± 6	5	2000

[a] NIST standard reference chocolate (SRM 2384).

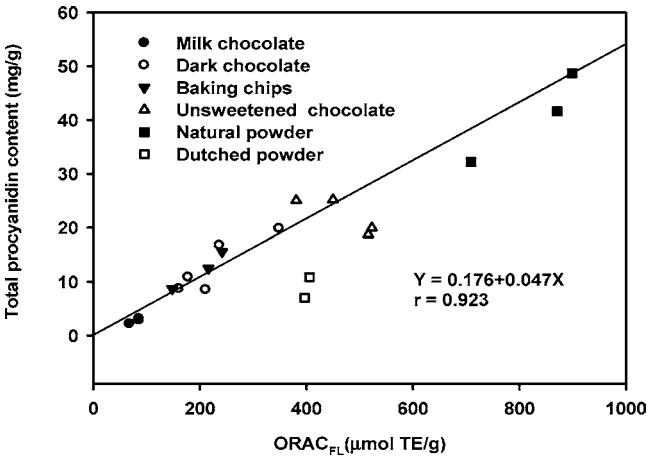

Figure 5. Correlation between total antioxidant capacity and total procyanidin contents.

In summary, this study demonstrated that cocoa and chocolate products contain a substantial amount of procyanidins and are excellent sources of antioxidants. Antioxidant capacity in cocoa and chocolate products reflect the procyanidin content, and both antioxidant capacity and procyanidin content are related to the content of nonfat cocoa solids. Certain food-processing methods, such as the Dutching process, markedly decreased procyanidin content and AOC.

ABBREVIATIONS USED

AOC, antioxidant capacity; DP, degree of polymerization; NFCS, nonfat cocoa solid; NIST, National Institute of Standards

and Technology; $ORAC_{FL}$, oxygen radical absorbance capacity; PC, procyanidins; TE, Trolox equivalent.

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