SUPPORTING INFORMATION

for

Oligomeric cocoa procyanidins possess enhanced bioactivity compared to monomeric and polymeric cocoa procyanidins for preventing the development of obesity, insulin resistance, and impaired glucose tolerance during high-fat feeding

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Cocoa Flavanol Extraction. A flavanol-rich cocoa extract (CE) was produced following the methods of Adamson *et al.* [1] and Robbins *et al.* [2] with modifications. For methodological details, see Supporting Information. Commercially-available non-alkalized natural cocoa powder (The Hershey Co., Hersey, PA) was defatted by dispersing 100 g cocoa powder in 400 mL hexane, sonication (10 min), stirring (5 min) and centrifugation (5 min, 20 °C, 5,000 x *g*). The supernatant was discarded and the extraction repeated. Residual hexane was evaporated from defatted cocoa at room temperature. Flavanols were extracted by dispersing defatted cocoa in 400 mL acetone: water: glacial acetic acid (70:28:2 v/v/v) solution, sonication (10 min), stirring (5 min) and centrifugation (5 min, 20 °C, 5,000 x *g*). The supernatant was collected and the extraction was repeated. The supernatants were pooled, acetone was evaporated using a rotary evaporator (40-45 °C), and the remaining extract (predominantly water) was frozen at −80 °C.and freeze-dried for ≥2 d. After freeze-drying, CE was crushed into a powder, weighed, and stored at −80 °C. The final extraction yield from 100 g cocoa powder was ~12.8 g CE.

Cocoa Extract Fractionation. CE was fractionated by solid phase extraction to produce three fractions with different mDP values (monomers, oligomers, and polymers) according to the method used by Sun *et al.* [3] with modifications. For methodological details, see Supporting Information. A tC_{18} Sep-Pak SPE column (20 cc, 5 g sorbent) (Waters, Milford, MA) was attached on top of a C_{18} Sep-Pak SPE column (20 cc, 5 g sorbent). The columns were preconditioned with 10 mL methanol (MeOH) followed by 10 mL distilled, deionized water (ddH_2O, pH 7.0) on a vacuum manifold. CE was dissolved in acetone: water: glacial acetic acid (70:28:2 v/v/v) to 0.1 g/mL and 1.5 mL of the CE solution was loaded onto the column. Highly polar compounds (primarily phenolic acids) were eluted with 10 mL ddH_2O and discarded. Monomers and oligomers were eluted together with 35 mL ethyl acetate, concentrated by rotary evaporation, and set aside. The polymer fraction was then eluted with 40 mL MeOH,

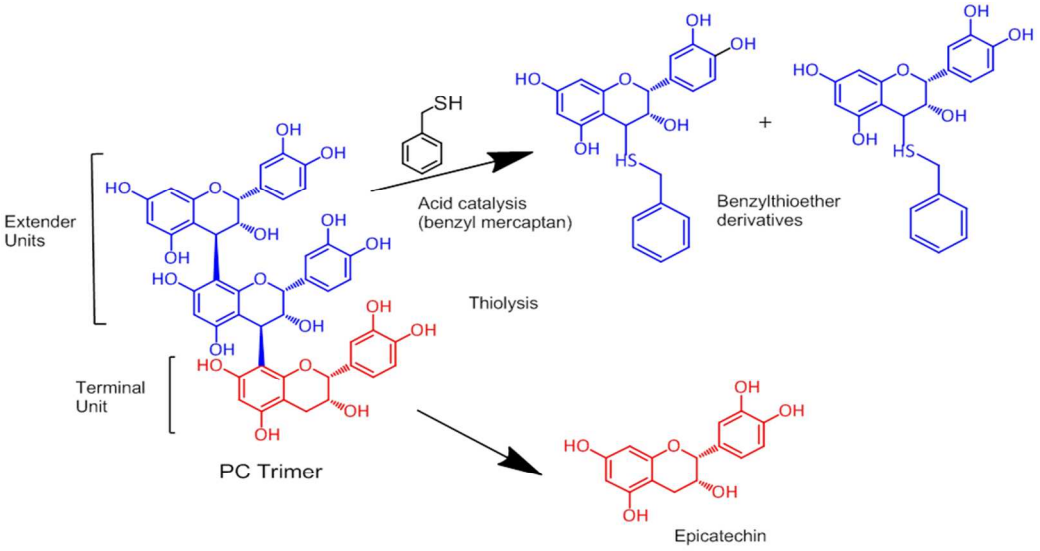
concentrated by rotary evaporation, dispersed in ddH_2O and freeze-dried as described above until a dark brown powder remained. A new set of tC_{18} Sep-Pak + C_{18} Sep-Pak columns was then pre-conditioned, and the combined monomer/oligomer fraction (dissolved in 1.5 mL of the acetone: water: glacial acetic acid solution) was loaded onto the column. Monomers were eluted with 35 mL diethyl ether, concentrated by rotary evaporation, dispersed in ddH_2O and freeze-dried until a light brown powder remained. Oligomers were eluted with 40 mL, concentrated by rotary evaporation, dispersed in ddH_2O and freeze-dried until a cream-colored powder remained. All three fractions were stored at −20 °C for 2 d, weighed, and stored −80 °C. An average of ~155 mg monomer, ~282 mg oligomer, and ~502 mg polymer were produced from each batch of 10 replicate fractionations (a total of 15 mL containing 1.5 g CE, or 1.5 mL containing 0.15 g CE per replicate). Fractions produced from separate batches were combined to produce a single uniform lot of each fraction.

Quantification of Total Polyphenols by the Folin-Ciocalteau Assay. CE and the three fractions were diluted to 0.2 mg/mL in 40% EtOH. Samples (100 µL) were combined with 900 µL distilled deionized H_2O and 2.5 mL 0.2 N Folin-Ciocalteau regaent (Sigma, St. Louis, MO) and vortexed. Sodium carbonate (2 mL, 7.5% w/v aqueous solution, Sigma) was added and the samples were vortexed. Samples were allowed to sit at room temperature for 2 h, and the absorbance was measured at 765 nm. Samples were analyzed in triplicate and quantified based on an external standard curve prepared with gallic acid standards (0-0.5 g/L) in 40% EtOH. Total polyphenol content was expressed as g gallic acid equivalents (GAE)/g of material.

Estimation of mDP by Thiolysis. Thiolysis was employed to determine the mean degree of polymerization (mDP) of the flavan-3-ols that comprised each fraction based on the methods described by Guyot *et al.* [4] and Gu *et al.* [5] with modifications. Thiolysis involves an acid-catalyzed reaction in which the interflavan C-C bonds linking the monomeric residues together are broken to release the terminal flavanol unit (see schematic in Supporting Information, **Figure S3**) [6]. The reaction substrates (CE or the monomer, oligomer, or polymer fractions) were first diluted to 0.5 mg/mL in MeOH. Then, 50 µL of the diluted reaction substrate, 50 µL HCl (3.3% w/v) and 100 µL benzyl mercaptan (5% w/v in MeOH) were combined. Controls (to estimate the monomer content in unthiolyzed samples) were prepared by combining 50 µL of diluted reaction substrate with 150 µL MeOH. Samples and controls were heated in a water bath (90 °C for 5 min) and then cooled on ice for 5-10 min to stop the reaction. Samples and controls were prepared for UPLC/MS-MS analysis by combining 100 µL reaction product with 900 µL 0.1% formic acid in water/0.1% formic acid in acetonitrile (ACN) (95:5) in an HPLC vial. It is important to note that benzyl mercaptan is flammable and has significant oral and inhalation toxicity. Benzyl mercaptan should be kept in closed containers under a chemical fume hood.

UPLC-MS/MS Analysis of Thiolysis Products. Thiolysis reaction products were quantified by UPLC-MS/MS. UPLC analysis was performed on Waters (Milford, MA) Acquity H-class separations module. A Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm particle size) was used and maintained at 40°C. Binary gradient elution was carried out with 0.1% formic acid in water (phase A) and 0.1% formic acid in ACN (phase B). Linear gradient elution was performed at a 0.6 mL/min solvent flow rate based on the following program: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7–10.5 min). The sample temperature was kept at 10 °C. UPLC effluent was analyzed by (−)-electrospray ionization (ESI)

coupled to tandem mass spectrometry (MS/MS) on a Waters Acquity triple quadrupole (TQD) MS. Ionization was performed in (−) mode, with a capillary voltage of −4.25 kV, a cone voltage of 30.0 V, an extractor voltage of 3.0 V, and source and desolvation temperatures of 150 and 400 °C respectively. Cone and desolvation gasses were N_2 with flows rates of 75 and 900 L/h respectively. MS/MS was performed using Ar as the collision gas at a flow rate of 0.25 mL/min in the collision cell. Data acquisition was carried out with MassLynx software (version 4.1, Waters). MS data collection was set to 10 points/peak with an average peak width of 6 s. The auto-dwell setting was used to automatically calculate dwell time based on an interscan delay time of 0.02 s. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 µL/min) in combination with a background flow of 50% phase A/50% phase B at 0.6 mL min. Intellistart automatically selected the most abundant daughter ion, optimized the source cone voltage and MS/MS collision energy, and generated a single multi-reaction monitoring (MRM) transition for each compound. MRM was performed on parent ions $[M-H]^-$ and signature daughter ions following collision-induced dissociation (CID). MRM mass span was 0.2 Da, the inter-channel delays and inter-scan times were both 1.0 sec. MRM and CID parameters for each compound of interest are listed in **Table S1**. All compound peaks were processed and quantified using the QuanLynx function of MassLynx software.



Calculated mDP of PC trimer:

$$Calculated\ mDP\ (PCs\ only) = \frac{Net\ monomer + Net\ Derivative}{Net\ Monomer}$$

$$= \frac{1\ monomer + 2\ derivatives}{1\ monomer} = 3\ (trimer)$$

Figure S1. *Schematic representation of the thiolysis reaction; adapted from Meagher, L. P.; Lane, G.; Sivakumaran, S.; Tavendale, M. H.; Fraser, K., Characterization of condensed tannins from Lotus species by thiolytic degradation and electrospray mass spectrometry. Anim. Feed Sci. Technol. 2004, 117, 151-163 (with permission).*

Table S1. MRM Settings for MRM Detection of Favanol Monomers and Thiolyzed Monomer Derivatives by UPLC-MS/MS.

Compound	t_R[a] (min)	MW (g mol^{-1})	[M – H]$^-$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
C/EC	2.5-4.1	290.142	288.98	245.05	40	14
ECG	4.2-5.0	441.952	440.92	169.00	38	16
C/EC benzylthioether derivative	6.6-8.5	412.031	410.94	124.97	30	18
ECG benylthioether derivative	6.8-8.4	563.824	563.05	287.06	38	16

[a]retention time

Standard Curve for Thiolysis. Thiolysis products were quantified using external monomer standards due to the lack of available authentic flavanol benzylthioether standards. The mDP was calculated from the concentration data produced by the UPLC-MS/MS method using the following equations:

$$net\ monomers = \Sigma\ monomers_{thiolyzed\ sample} - \Sigma\ monomers_{unthiolyzed\ control}$$

$$calculated\ mDP\ (DP\ \geq\ 2\ only) = \frac{net\ monomers + \Sigma\ derivatives_{thiolyzed\ sample}}{net\ nonomers}$$

To calibrate the thiolysis values (calculated mDP values) obtained by this calculation, a standard curve relating actual mDP vs. calculated mDP (thiolysis value) was produced by thiolyzing known standards covering DP 2-10 by the method described above (*n*=5 replicates per compound) at concentrations of 0.1 mg/mL in methanol (lower concentrations are used for standards vs. 0.5 mg/mL for extracts, as the standards represent higher concentrations of individual compounds). Least-squares regression analysis was used to determine the relationship between actual mDP and measured mDP (calculated by thiolysis) (**Figure S2**).

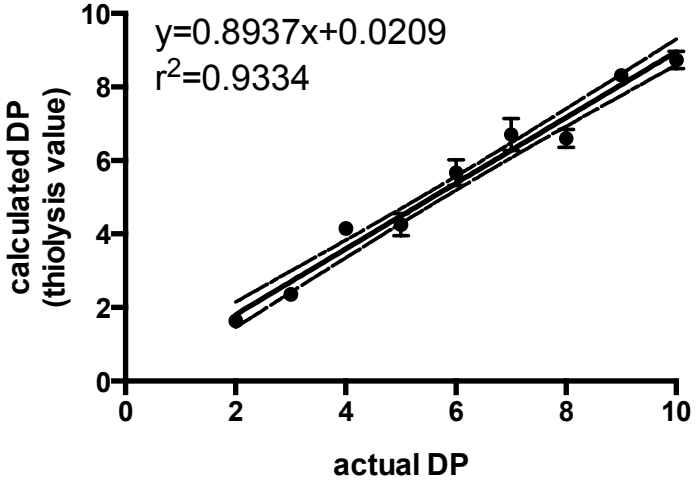


Figure S2. *Thiolysis standard curve relating the calculated mDP (thiolysis value) vs. actual mDP of authentic procyanidin standards (plotted values are means ± SEM of n=5 replicates per compound). The least-squares regression line was plotted using each replicate value as an individual point. Dotted lines represent the 95% confidence interval of the regression line.*

Using this curve, actual mDP values were determined from the calculated mDP (thiolysis) values using the following equation:

$$actual\ mDP\ (DP \geq 2\ only) = \frac{calculated\ mDP - y\ intercept}{slope}$$

As monomer concentrations in the unthiolyzed sample are subtracted during the mDP calculation, the original mDP calculation only accounts for flavanol species with DP ≥ 2. To account for the monomers as well, the mDP was corrected by including the monomer concentrations in the unthiolyzed sample (with a defined DP of 1) in the mDP calculation as follows:

$$mDP\ (all\ flavanols) = \frac{\left[1 \times \Sigma\ \text{monomers}_{unthiolyzed\ control}\right] + \left[actual\ mDP\ (DP \geq 2\ only) \times\ net\ monomers\right]}{\Sigma\ \text{monomers}_{unthiolyzed\ control} + net\ monomers}$$

Normal-Phase HPLC of Cocoa Procyanidins. Cocoa fractionation was further evaluated by normal-phase HPLC profiling [7]. Analyses were performed on an Agilent Technologies (Santa Clara, CA) 1260 Infinity HPLC equipped with a solvent degasser, quaternary pump, an autosampler with temperature control, a thermostat column compartment, and a fluorescence detector (FLD). Separations were carried out using a Develosil Diol column (100 Å, 250 × 4.6 mm, 5 μm particle size) equipped with a Luna HILIC guard column (4 × 3.0 mm ID SecurityGuard cartridge and cartridge holder) (both from Phenomenex, Torrance, CA). The column temperature was 35°C. Binary gradient elution employing 2% acetic acid (v/v) in ACN (phase A) and 2% acetic acid (v/v) and 3% ddH_2O (v/v) in MeOH (phase B) was performed at a flow rate of 1 mL/min. The gradient was as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min, 0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min, and 7.0% B at 76 min. FLD excitation and emission wavelengths were 230 nm and 321 nm, respectively. CE and cocoa fractions were prepared at 10 mg/mL in acetone: water: acetic acid (70:28:2, v/v/v) immediately prior to analysis. All the samples and standards were held at 5 °C in the autosampler before injection. Injection volume was 5 μL. Mixtures of authentic standards consisting of monomers (DP 1: C, EC, ECG), PC oligomers (dimers-hexamers), and PC polymers (heptamers-decamers) were prepared and used as a reference for comparison of elution profiles.

UPLC-MS/MS Analysis of Cocoa Procyanidins. CE and cocoa fractions were analyzed by UPLC-MS/MS (*n*=3) to quantify individual monomer and procyanidin species up to decamers. For methodological details, see Supporting Information. CE and cocoa fractions were prepared by diluting to 0.05 mg/mL in 0.1% formic acid in water/0.1% formic acid in ACN (95:5). UPLC separations and MS/MS analyses were performed on the Acquity UPLC-TQD and UPLC HSS T3 column described above (2.1 mm × 100 mm, 1.8 μm particle size). Column temperature,

sample temperature, mobile phases, flow rate, gradient, ESI parameters, MS/MS CID settings, data collection parameters, and Intellistart tuning of authentic standards to generate MRM functions were the same as described above. MRM parameters for monomers and PCs are listed in **Table S2**.

Table S2. MS/MS Settings for MRM Detection of Monomers and Procyanidins.

Compound	t_R[a] (min)	MW (g mol^{-1})	[M – H]$^{-}$[b] (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
procyanidin dimer B_1	2.68	578.136	577.136	289.105	38	24
(−)-epigallocatechin	2.76	306.038	305.038	124.977	40	22
unknown dimer 1[c]	2.92	578.136	577.136	425.102	36	16
(±)-catechin	2.99	290.028	289.028	245.057	36	14
procyanidin trimer T_2	3.09	866.218	865.218	289.102	36	48
unknown dimer 2[c]	3.29	578.136	577.136	425.102	36	16
procyanidin dimer B_2	3.34	578.136	577.136	425.102	36	16
(−)-epicatechin	3.63	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	3.67	458.038	457.038	168.982	34	16
procyanidin trimer C_1	3.82	866.218	865.218	287.085	46	32
cinnamtannin tetramer A_2	3.97	1154.808	576.404	125.020	26	34
procyanidin dimer B_2 gallate	3.99	730.164	729.164	407.129	42	32
procyanidin octamer	4.04	2307.17	1152.58	125.17	48	68
unknown dimer 3[c]	4.07	578.136	577.136	425.102	36	16
procyanidin pentamer	4.10	1442.820	720.410	125.022	26	44
procyanidin hexamer	4.23	1731.038	864.519	125.020	32	56
procyanidin nonamers	4.33	2586.36	864.12	125.17	28	46
procyanidin heptamer	4.41	2018.80	1008.40	125.17	36	56
(−)-epicatechin gallate	4.60	442.076	441.076	168.968	38	18
procyanidin decamers	4.60	2883.55	960.18	125.17	30	52
procyanidin dimer B_5	4.64	578.136	577.136	289.107	30	26

[a]retention time

[b]All MRMs used singly-charged parent ions except for cinnamtannin tetramer A_2, pentamers, hexamers, heptamers, octamers, which were doubly-charged ($[M – 2H]^{2-}$), and nonamers and decamers, which were triply-charged ($[M – 3H]^{3-}$)

[c]likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

Table S3. Total Amounts of Cocoa Extract (CE) and Cocoa Fractions Produced for Mice Feed.

Fraction	Total Obtained (g)	Yield (mg fraction/g extract)
CE	87.96	----
monomers	1.42	78.23
oligomers	2.68	181.21
polymers	4.77	316.32

Table S4. Review of current literature exploring the anti-obesity and anti-diabetic effects of cocoa flavanols in animal models.

Paper	Species	Model	Product Tested	Dose	Major Findings
Tomaru *et al.* 2007 [8]	C57BL/KsJ Mice	db/db[a]	CLPr[b]	0.5% & 1% (w/w diet)	Both doses reduced resting blood glucose levels.
Yamashita *et al.* 2012 [9]	C57BL/6 Mice	HF[c] diet (30% fat)	CLPr	0.5% & 2% (w/w diet)	Both doses reduced: • blood glucose levels after OGTT[d] • body weight • WAT[e] weight • total cholesterol
Matsui *et al.* 2005 [10]	Wistar Rats	HF diet	Cocoa powder	12.5% (w/w diet)	Dose reduced: • body weight • mesenteric-WAT
Ruzaidi *et al.* 2005 [11]	Wistar Rats	STZ[f]-induced diabetes	CE[g]	1, 2, & 3% (w/w diet)	Doses of 1 & 3% reduced: • glucose levels • total cholesterol All doses reduced TAG[h]
Jalil *et al.* 2008 [12]	Sprague-Dawley Rats	STZ induced diabetes + HF diet (49% fat)	CE	600 mg/kg BW*d	Dose reduced: • blood glucose levels after OGTT • plasma FFA[i]
Jalil *et al.* 2009 [13]	Sprague-Dawley Rats	STZ induced diabetes + HF diet (49% fat)	CE	600 mg/kg BW*d	Dose reduced: • blood glucose levels after OGTT • total cholesterol • LDL[j] cholesterol • TAG
Sanchez *et al.* 2010 [14]	Zucker Fatty Rats	Standard diet (4% fat)	SCF[k]	5% (w/w diet)	Dose reduced: • body weight • plasma glucose levels • plasma insulin levels • plasma TAG

[a] Mice were induced with diabetes and obesity prior to study
[b] Cocoa liquor procyanidins
[c] High-fat diet
[d] Oral glucose tolerance test
[e] White adipose tissue
[f] Streptozotocin
[g] Cocoa extract
[h] Triglyceride
[i] Free fatty acid
[j] Low-density lipoprotein
[k] Soluble cocoa fiber

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