Electronic Supporting Information for

Flavanol Concentrations do not Predict Dipeptidyl Peptidase-IV Inhibitory Activities of Four Cocoas with Different Processing Histories

Caroline M. Ryan[a], Weslie Khoo[b], Amanda C. Stewart[a], Sean F. O'Keefe[a], Joshua D. Lambert[b], and Andrew P. Neilson[a]*

[a]Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA
[b]Department of Food Science, Pennsylvania State University, University Park, PA

***Corresponding Author:** Andrew P. Neilson, 1981 Kraft Dr. Rm 1013, Blacksburg, VA 24060; Email: andrewn@vt.edu; Phone: 1-540-231-8391; Fax: 1-540-231-9293

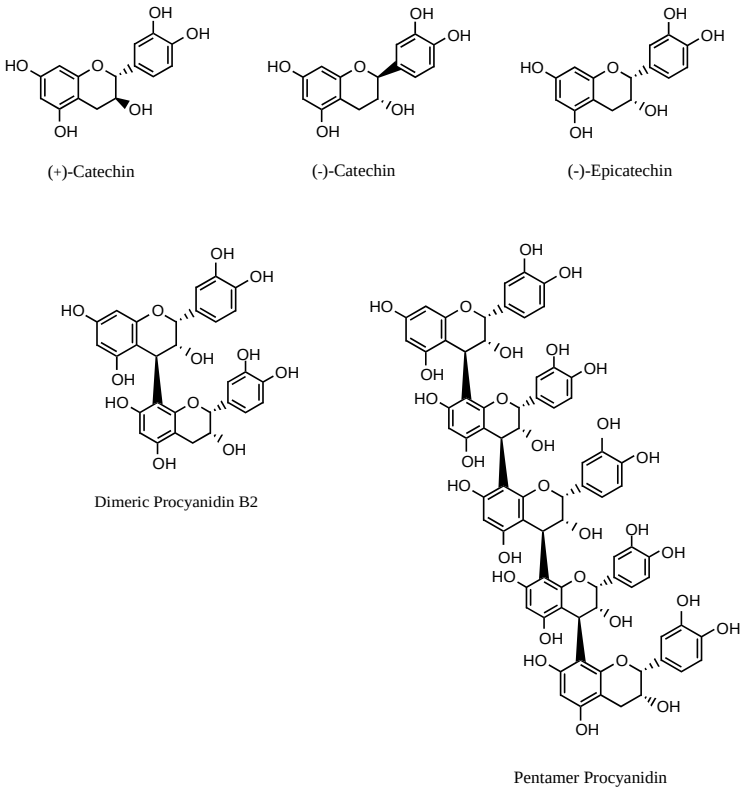

Figure S1. *Representative structures of flavanols commonly found in cocoa*

EXPERIMENTAL

DPP4 Inhibition. Inhibition of DPP4 was measured according to the method utilized by Lin *et al.*[38] with modifications. DPP4 (human, recombinant) was diluted with 100 mM Tris buffer (pH 8) to a final concentration of 2 mU/mL. Cocoa extracts were dissolved in dimethylsulfoxide (DMSO) and further diluted (0-8000 µg CE/mL) so that all dilutions contained 10% DMSO. In a 96-well plate, 50 µL CE solution (n=6 per product and dose; 0-2000 µg CE/mL final concentration in the reaction with 2.5% DMSO final concentration, accounting for all reagent additions prior to reaction quenching) and 50 µL of the DPP4 solution were combined. A second plate was prepared with the same cocoa extract solutions, replacing the enzyme solution with 50 µL of 100 mM Tris buffer (pH 8) without the enzyme in order to account for the absorbance of the pigments naturally present

in the substrate and extracts. The plates were covered and incubated at 37°C for 10 min. Then, 50 µL 1 mM GP-pNA chromogenic substrate was added to each well, followed by 50 µL 100 mM Tris buffer (pH 8). The plates were then incubated at 37°C for 60 min. After incubation, 50 µL 3% acetic acid was added to each well to end the reaction and absorbance was read at 405 nm. The 0 µg/mL sample was used as the negative control to measure full (uninhibited) DPP4 activity (negative control). The DPP4 inhibitor Ile-Pro-Ile (Diprotin A) was diluted to the same concentrations and % DMSO as the cocoa extracts and used as the positive control. Inhibition values were expressed as % DPP4 activity (negative control: 100%). Activity in each well was calculated as follows:

$$\%DPP4 = \frac{(A_{I,S,E} - A_{I,S})}{(\bar{A}_{S,E} - \bar{A}_{S})} x\ 100$$

Where:

$A_{I,S,E}$ = individual absorbance value of reaction containing the inhibitor (positive control, negative control or CE), substrate, and enzyme at each inhibitor dose

$\bar{A}_{I,S}$ = average absorbance value of the inhibitor (positive control, negative control or CE) and substrate (no enzyme) reaction at the same inhibitor dose

$\bar{A}_{S,E}$ = average absorbance value of the substrate and enzyme reaction without inhibitor (0 µg/mL)

$\bar{A}_{S}$ = average absorbance value of the substrate without enzyme and inhibitor

Complex Polymeric Pigments. Measurement of polymeric pigments as an indication of complex oxidative/roasting products in cocoa was performed per Wang *et al.*[39] and Zou *et al.*[40] These compounds were crudely separated into arbitrary classifications based on their affinities for polar versus non-polar extraction solvents and spectrophotometric properties. Ethyl acetate, butanol, 95% ethanol, and distilled deionized water were utilized as extraction solvents for extraction of pigments from CEs. In the method of Zou *et al.*[40], theaflavins corresponded to those compounds soluble in ethyl acetate, thearubigins were soluble in butanol, and theabrownins in 95% ethanol, corresponding to extraction of compounds with increased size and complexity with increase solvent polarity. Wang *et al* states that theabrownins are soluble in water and not polar solvents[39], which is why we added a water extraction to separate out even larger, water-soluble compounds. While the specific structures of complex polymeric pigment compounds in cocoa are likely different than those found in tea, the principle of compound separation based on size and polarity is similar. To fractionate based on polarity, 50 mg of each CE (n=4) was combined with 200 µL ethyl acetate. The solutions were vortexed and sonicated to mix, then centrifuged (17,000 x *g*, 5 min) and the supernatant retained. Extraction was repeated two more times, pooling the supernatants of each individual CE sample. The entire extraction procedure was then repeated using butanol, 95% ethanol, then distilled deionized water sequentially, utilizing the same CE pellet throughout all extractions once previous supernatants had been removed. Water and ethanol extracts were then diluted 10x and 5x, respectively, in order to be within the measureable absorbance range. 250 µL of each pooled supernatant (n=4 for each CE sample and solvent) was transferred to a 96-well plate and absorbance read at 380 nm. Flavanols absorb strongly at ~280 nm due to the aromatic rings[41], but typically not at wavelengths >300 nm[42]. Theaflavins have maximal absorbance at 380 nm,[43, 44] which is why we used this area of the spectrum to measure oxidative polymers. At this wavelength, native cocoa flavanols (including PCs) do not exhibit appreciable absorbance, whereas chromogens in complex oxidation/condensation products similar to theaflavins and more complex compounds can be measured and interpreted as complex polymers formed due to cocoa fermentation and roasting. The entire extraction procedure was then repeated using the original cocoa samples rather than CEs, in order to determine the impact of the original extraction (performed with 70:28:2 acetone/water/acetic acid) on pigment extraction. Briefly, 250 mg cocoa sample (n=4) was snap frozen in liquid N_2 and ground to powder. The extraction procedure remained the same as that used for the cocoa extracts except that solvent volume utilized was 1 mL instead of 200 µL. Water and ethanol extracts were both diluted 2x in order to be within the measureable absorbance range. 250 µL of each pooled supernatant was transferred to a 96-well plate and absorbance was read at 380 nm.

Protein Precipitation. Protein precipitation activities of the CEs were assessed by haze formation per Watrelot *et al.*[45]. Citrate/phosphate buffer (pH 3.8) was prepared by combining 0.1 M citric acid and 0.2 M sodium phosphate solutions (13:7). 2 mg/mL BSA in citrate/phosphate buffer was prepared as the working protein solution. CEs (n=4) were dissolved in DMSO and further diluted (0-4000 µg CE/mL) with DMSO and citrate/phosphate buffer so that each dilution contained 10% DMSO. In the top four rows of a 96-well plate, 100 µL of 2 mg/mL BSA in buffer was combined with 100 µL diluted CE solution (n=4; 0-2000 µg CE/mL final in reaction with 5% final DMSO concentration). It was confirmed that a 5% DMSO would not induce protein precipitation, to minimize background precipitation in the assay. 100 µL citrate/phosphate buffer without BSA was combined with 100 µL diluted CE solution (0-2000 µg CE/mL final in reaction, n=4) as a negative control. Plates were gently shaken and loss of light (absorbance and/or scattering) was read at 650 nm to quantify turbidity due to precipitated protein. Values were corrected by subtracting average negative control values for each concentration.

RESULTS AND DISCUSSION

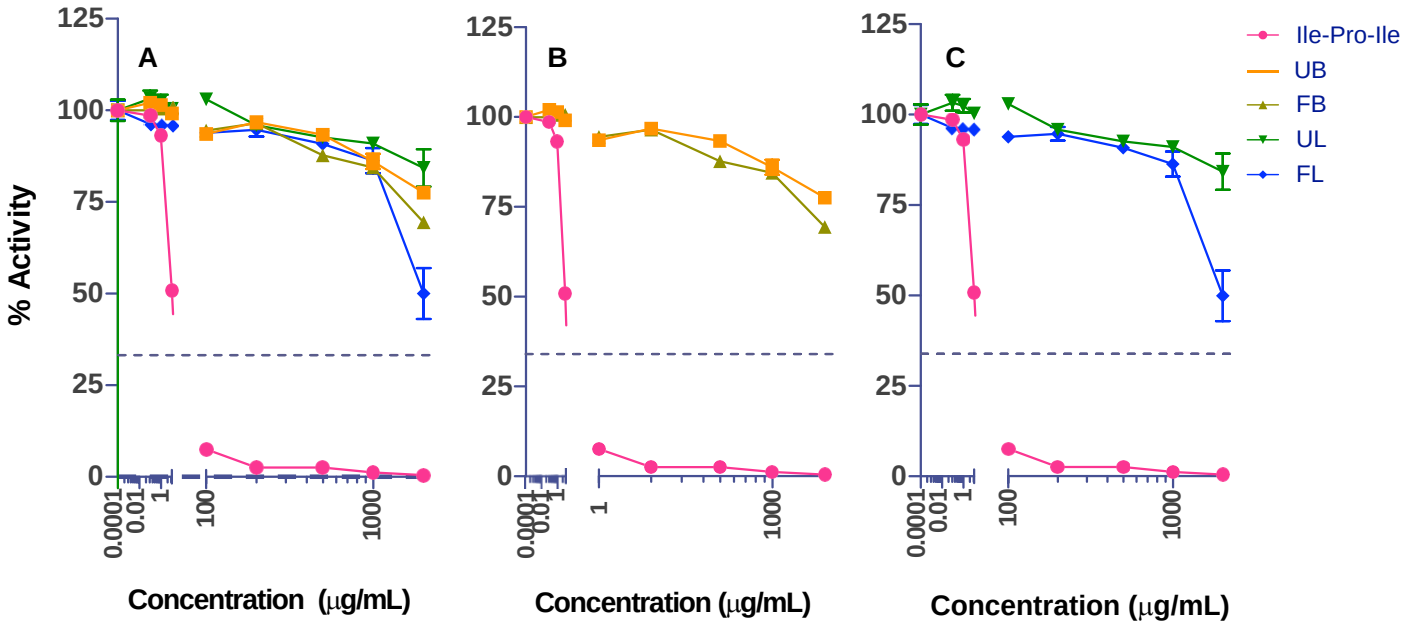

Figure S2. *Dipeptidyl peptidase-IV (DPP4) activity (relative to negative control, 100% activity) for (A) all cocoa extracts versus the Ile-Pro-Ile control; (B) unfermented and fermented bean CEs; and (C) unfermented and fermented liquor CEs. Dotted line represents 75% DPP4 activity (i.e 25% inhibition). Activity at 0 µg CE/mL was plotted as 0.0001 µg/mL in order to facilitate graphing on a logarithmic scale. Concentration at which DPP4 activity was inhibited 25% (25% inhibitory concentration, IC_{25}) by Ile-Pro-Ile was calculated using four parameters sigmoidal analysis.*

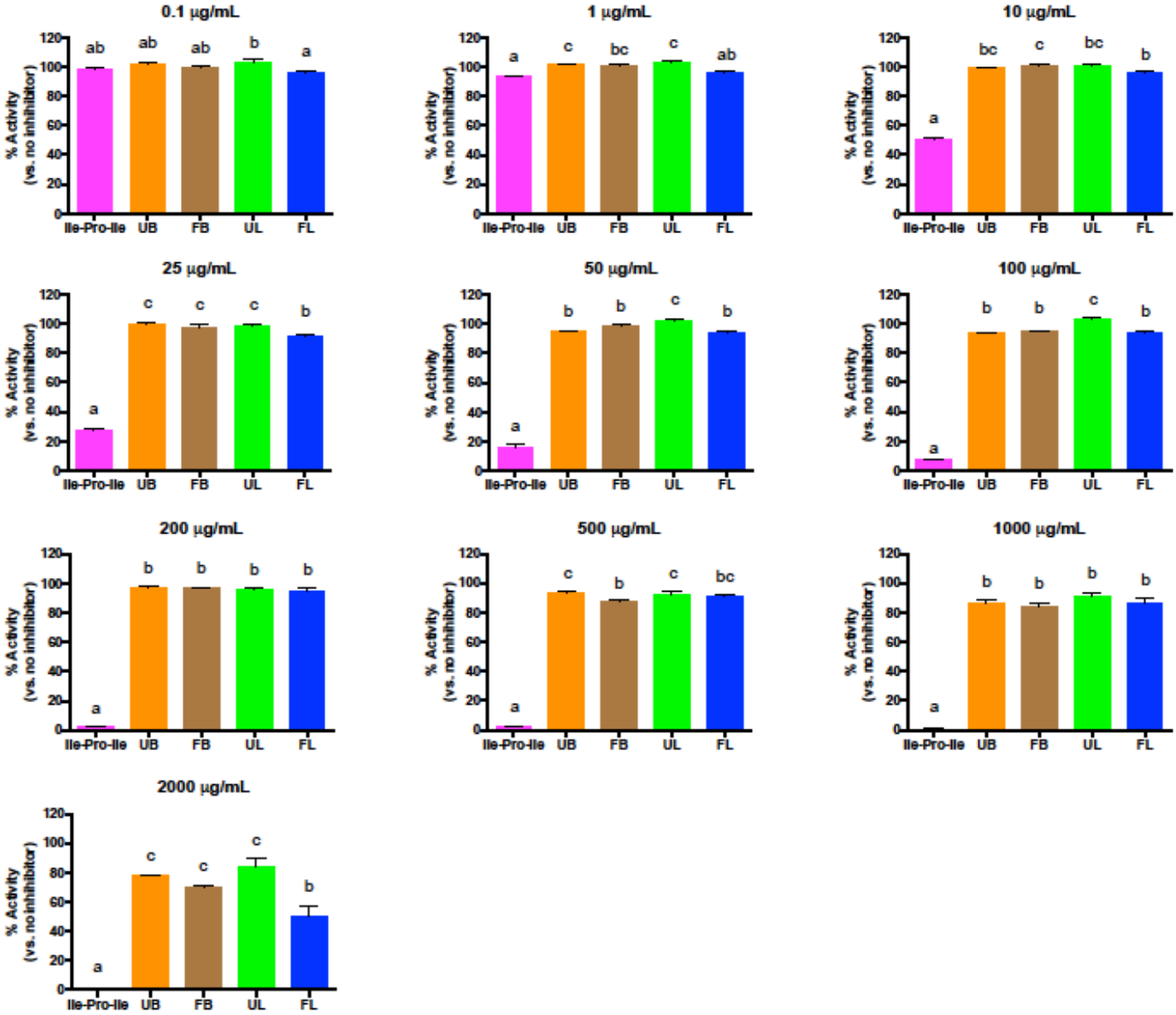

Figure S3. *% DPP4 activity remaining in the presence of each CE at various concentrations (mean ± SEM). Bars not sharing a common superscript are significantly different (1-way ANOVA with Tukey's post hoc test between all means, $P<0.05$).*

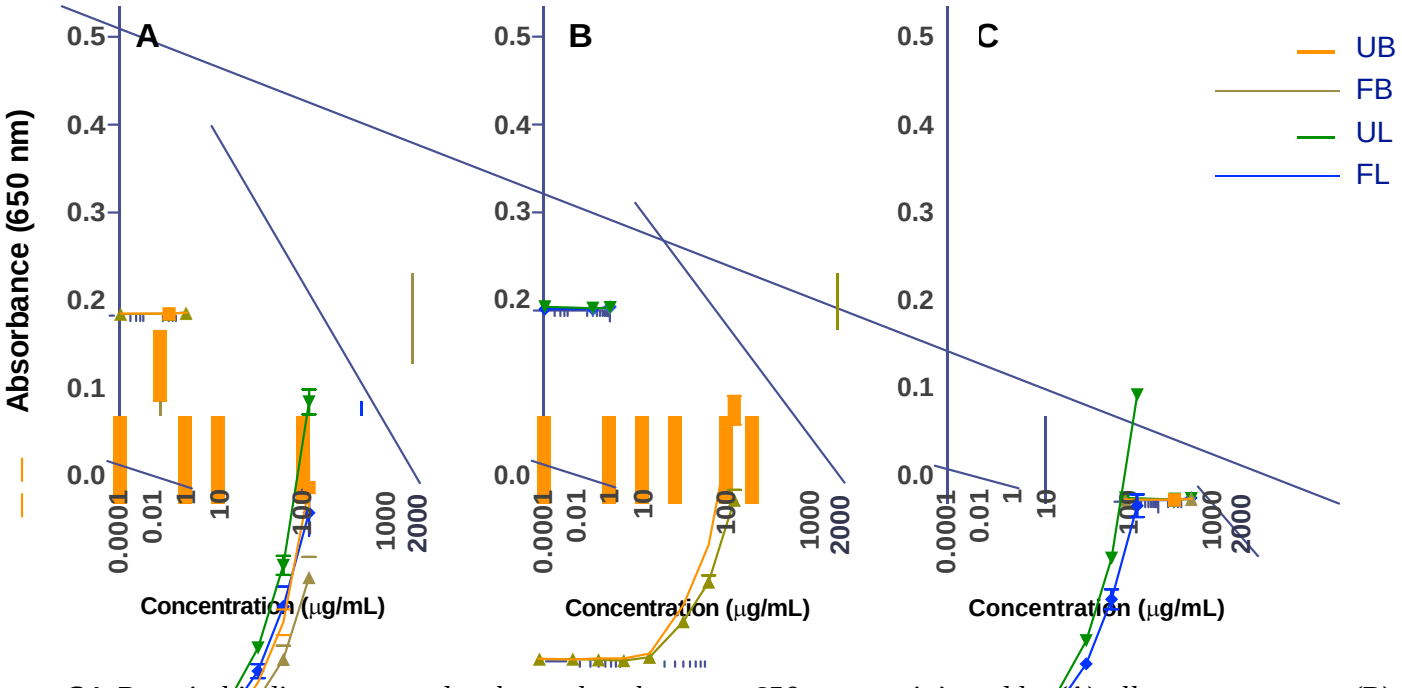

Figure S4. *Protein binding measured as haze absorbance at 650 nm precipitated by (A) all cocoa extracts; (B) unfermented and fermented bean CEs; and (C) unfermented and fermented liquor CEs. Precipitation at 0 µg/mL (− control) was plotted as 0.0001 µg/mL in order to facilitate graphing on a logarithmic scale.*

Table S1. Chemical compositions of cocoa extracts (expressed as mean value)[a]

Measure		Cocoa Extract[b] UB	FB	UL	FL
Total Polyphenols (mg GAE eq./g)		424	232	432	321
Total Flavanols (mg PCB2 eq./g)		327	67.7	170	64.7
Flavanol mDP[c] (monomer residues/molecule)	(excl. monomers)	2.85	4.16	2.51	2.39
	(incl. monomers)	2.04	3.16	1.92	2.00
Individual flavanols (mg/g)	(±)-catechin	0.349	0.0901	0.796	0.409
	(−)-epicatechin	60.6	22.4	48.3	12.7
	DP2	16.8	8.7	21.9	9.6
	DP3	0.314	0.191	4.39	2.4
	DP4	18.3	3.91	8.83	2.13
	DP5	10.5	3.05	7.13	1.59
	DP6	78.4	24.4	47.9	2.19
	DP7	0.35	0.0784	0.151	0.312
Anthocyanins (mg cyanidin-3-glucoside eq./g)		4.85	0.13	1.58	0

[a]Note that we previously published these values (Ryan et al. 2016). They are presented here as known composition values for these extracts, not new data
[b]UB; unfermented beans, FB: fermented beans, UL: unfermented liquor, FL: fermented liquor
[c]mDP: mean degree of polymerization/thiolysis value (oligomers + polymers does not incorporate monomers present prior to thiolysis into the mDP calculation)

Table S2. Enzyme inhibition parameters for cocoa extract samples and positive controls

Parameter[a]	Inhibitor[c] + Control[b]	UB	FB	UL	FL
IC_{25} (µg/mL)	4.82	2135	1585	2871	1076
R^2	0.993	0.902	0.966	0.859	0.899

[a]IC_{25}: concentration resulting in 25% inhibition compared to uninhibited activity, R^2: goodness of fit for the chosen model, %I: % inhibition of enzyme activity at the indicated concentration (those near calculated IC_{25} values)
[b]Positive inhibitor control: Ile-Pro-Ile (Diprotin A)

Since our bioactivity studies were performed utilizing cocoa extracts, we were more concerned with their levels of oxidative polymers. However, we also wanted to measure these compounds in the original, non-extracted

cocoa samples as well to see how they were different. When comparing extracts to original products, levels of each compound type varied. The original cocoa products contained higher amounts of the largest, water-soluble compounds than the extracts. Water-soluble compounds were highest in the FL extract, yet UB contained the highest amount (about 0.5-fold higher than FL CE) compared to any of the other samples. UB and UL had higher amounts of ethanol-soluble compounds than FB and FL in both cases of extract and original sample, suggesting that fermentation decreases the concentrations of these compounds. Fermentation and roasting significantly decreased butanol- and water-soluble compounds in the original cocoa samples (UB > FB = UL = FL for both statistical analyses). There were no significant effects of fermentation or roasting on the concentrations of ethyl acetate-soluble compounds. Going forward, it may important to consider the difference in concentrations of these compounds between extracted and non-extracted samples. If one were to explore the area of non-flavanol compound bioactivities, a different extraction procedure may be useful to select for these other compounds.