Supplementary Information

Reliable, accessible and transferable method for the quantification of flavanols and procyanidins in foodstuffs and dietary supplements.

Table S1. Summary of commercial samples investigated for cocoa flavanol content.

Cocoa Powder	Universal Product code	Production Code/lot code
Ghiradelli 100% Cocoa Unsweetened	47599 61703	P4708H2
Ghiradelli 100% Cocoa Unsweetened	47599 61703	P4718H2
Ghiradelli 100% Cocoa Unsweetened	47599 61703	P3708H3
Navitas Organic Cacao Powder	58847 00087	1901812101 E 7:52
Navitas Organic Cacao Powder	58847 00087	1301735212C 18:50
Navitas Organic Cacao Powder	58847 00087	1901820601D 06:20:32
Nestle Toll House Cocoa	28000 21495	817950954B 03:07
Nestle Toll House Cocoa	28000 21495	818650954A 13.42
Nestle Toll House Cocoa	28000 21495	809250954B 00:40
Dark Chocolate		
Dove Dark	40000 45985	822DEELZ03
Dove Dark	40000 45985	822CGELZ03
Dove Dark	40000 40189	819AFELZ03
Hershey Special Dark	34000 17414	45A13911
Hershey Special Dark	34000 19528	44R13912
Hershey Special Dark	34000 19528	44R13921
Flavabar Dark Chocolate	5374000736	7361-02
Dietary supplements		
Flavamix	53740 00702	30827617
CocoaVia Stick Pack Sweetened	5048700200	647CAIFP12
Reserveage True Energy Capsules	94922 01565	201806970
Reserveage True Energy Capsules	94922 01565	201807087
CocoaVia Capsules	50487 00243	R12230
CocoaVia Capsules	50487 00243	R12332

Supplementatry information: Purity assessment of primary standards

Purity of primary standards was determined using a modified version of AOAC2012.24.

Primary standards were dissolved in acetone:water:acetic acid (70:29.5:0.5) to reach the concentration of 0.2 mg/mL. After an injection of 30 µL, a binary gradient between A (acetonitrile:acetic acid; 98:2, v/v) and B (methanol:water:acetic acid; 95:3:2, v/v) at a flow rate of 1.0 mL/min. The gradient was applied as follows: (0 min,0.0% B), (35 min, 40% B), (40 min, 40% B), (63 min, 100% B), (70 min, 100% B) and (76 min, 7.0% B) with a post-run time set to 10 min. 10 µL were injected on the column (Develosil 100 Diol-5, 250 x 4.6 mm, 5 µm). The column temperature was kept at 35 °C while the auto-sampler was maintained at 5 °C. A diod array detector (Merch Hitachi LaChrom DAD) was operated at the absorption wavelength of 278 nm. Purity was determined for each of the procyanidin primary standard as the ratio of the target signal area to the sum of all signal area integrated on the chromatogram. In addition, identity of the primary standards was determined via HPLC-MS, with parent ions and fragments showing coincidence from expected values for procyandins (Lin et al. JAFC 2014, 62:9387). Additional identity of primary standards was done with HPLC coupled to high resolution MS, confirming the results.

Supplementatry information: Characterization of secondary standard

Analysis of the CF content in the CEC was carried out by three different analysts, using different hardware (HPLC and column) on different days in order to minimize bias introduced by single analysis. The chromatographic conditions used were those from an AOAC-accepted method (AOAC2012.24), but using flavanol and procyanidin primary standards for the quantification of the CEC. Individual calibration curves were built for the individual DP1-7 flavanols and procyanidins. DP 8-10 were not included because they were not properly resolved, which is further discussed in the main manuscript. HPLC-FLD signals acquired with low gain were used for the quantification of DP1-4, while signals obtained with high gain were used for the quantification of DP5-7. Calibration curves were built by plotting the signal area versus the experimental concentration (determined with experimental weights and dilution factors). After linear regression, the calibration curve was used to determine the concentration of DP1-7 in the CEC. Five data points were used (50, 80, 100, 160 and 200% of target concentration; zero was not included).

1.1 Primary standard preparation

Solution of primary standards isolated from cocoa were prepared fresh daily by dissolving solid in volumetric flasks with acetone:water:acetic acid (AWAc; 70:29.5:0.5; v/v), filtered through a 0.45 µm PTFE syringe filter, transferred to an amber glass LC-autosampler vial and stored at 5 °C. To support quantitative analysis, CF primary standards were divided between three calibration solutions. Solution one comporised (–)-epicatechin and procyanidins with a DP of 4 and 7. Solution two included procyanidins with a DP of 2 and 5, and solution three included procyanidins with a DP of 3 and 6. Stock solutions were prepared to reach 200% of the target concentration in the CEC. The 200% solution was diluted to 50, 80, 100 and 160%, giving five calibration data points for each of the ten oligomers.

1.2 Sample preparation

The CEC extract was prepared as described previously. The extract was weighed in a volumetric flask and dissolved in AWAC. The content of the flask was transferred to a centrifuge tube, vortexed, sonicated for 5 min at 50 °C, and centrifuged for 5 min at 1700 rcf. The supernatant was then purified using a pass-though solid-phase extraction cartridge (SPE, Strata SCX, Phenomenex

500 mg, 3 mL, 55 µm, 70 Å). The cartridge was conditioned with water until 5 mm of water remained on top of the cartridge sorbent and then the cartridge was loaded with the CEC solution and eluted until 5 mm remained on top of the cartridge sorbent. The cartridge was then again loaded with the CEC solution and eluted with the eluate being collected. The eluate was filtered through a 0.45 µm PTFE syringe filter, transferred to an amber glass LC-autosampler vial and stored at 5 °C for no longer than 4 days prior to analysis.

1.3 HPLC conditions

Analysts I and II analyzed samples on an Agilent HPLC Infinity 1260, while Analyst III used an Agilent HPLC Infinity 1200 (Agilent Technologies, Santa Clara, CA, USA). After an injection of 5 µL, a binary gradient between A (acetonitrile:acetic acid; 98:2, v/v) and B (methanol:water:acetic acid; 95:3:2, v/v) at a flow rate of 1.0 mL/min. The gradient was applied as follows: (0 min,7.0% B), (3 min, 7.0% B), (60 min, 37.6% B), (63 min, 100% B), (70 min, 100% B) and (76 min, 7.0% B) with a post-run time set to 10 min. 10 µL were injected on the column (Develosil 100 Diol-5, 250 x 4.6 mm, 5 µm; cyano guard column, 4.0 x 3.2 mm). The column temperature was kept at 35 °C while the auto-sampler was maintained at 5 °C. A fluorimetric detector (FLD; Agilent FLD G1321B) was operated at 230 nm and 321 nm for excitation and emission wavelenths, respectively. Several settings were tested relative to the optimal gain used for QC testing on the instrument in use. Optimal gain was evaluated on single injection of standard solution containing (−)-epicatechin and procyanidins with a DP of 5. The first gain selected was the highest that allowed the detection of (−)-epicatechin without saturation of the signal. This gain was used for the quantification of DP1-4. The second gain selected was the highest that allowed the detection of DP5 without saturation of the signal. This gain was used for the quantification of DP5-7. Each sample and calibration solution was analyzed using the two gains.

Table S2. Summary of column investigated for the separation of cocoa flavanols by degree of polymerization.

	Name	Chemistry	Length (mm)	Diameter (mm)	Pore size (Å)	Particle size (μm)	Manufacturer
1	Luna	Diol	100	4.6	200	3.0	Phenomenex
2	Kromasil	Diol	100	2.1	100	5.0	Supelco
3	Polar-diol	Diol	100	4.6	120	1.8	Sepax
4	Polar-diol	Diol	250	4.6	100	5.0	Sepax
5	Develosil	Diol	250	4.6	120	5.0	Nomura
6	YMC-triart	Diol	100	3.0	120	1.9	YMC
7	Torus	Diol	100	3.0	130	1.7	Waters
8	GreenSep	Diol	100	3.0	120	1.9	ES Industries
9	Torus	Diol	100	2.1	130	1.7	Waters
10	Cortecs	HILIC (proprietary)	100	2.1	90	1.6	Waters
11	ACQUITY BEH	polyethoxysilane	100	2.1	130	1.6	Waters
12	Kinetex	poly(dihydroxy)siloxane	100	2.1	100	1.7	Phenomenex
13	Polar-100	polyethyleneglycol	100	2.1	120	1.8	Sepax
14	ACQUITY BEH	Amide	100	2.1	130	1.7	Waters
15	TSK gel	Amide	100	2.0	80	2.0	Supelco

Supplementatry information: Method Validation Experimental Design

Method validation included the evaluation of the accuracy, repeatability, intermediate precision, specificity, interferences, linearity, robustness, stability and limit of quantification parameters (see supplementary information Table S3).

Accuracy was calculated in two ways depending on material – via spike-and-recovery and standard addition. Spike-and-recovery was employed for a powdered drink mix that contained no CF (non-cocoa-based powdered drink mix), and standard addition was performed for materials with flavanol and/or procyanidins background levels (cocoa extract, cocoa powder and dark chocolate). In all cases, accuracy was determined using CEC. A single preparation of a sample was diluted and spiked with three increasing levels of extract calibrant. The best fit linear curve for (amount measured)=f(amount spiked) was used to determined accuracy as the ratio of the unspiked sample to x-axis intercept. All accuracy estimates were performed in triplicate.

Precision (repeatability, intermediate precision and reproducibility) was determined by relative standard deviation on concentrations (%RSD) between triplicates. Repeatability was determined as three individual preparations of a sample determined the same day by a single analyst. Intermediate precision was determined as a sample prepared and determined by three analysts on three different HPLC systems, columns and days within a single lab. Reproducibility was estimated as a sample prepared and measured by three analysts in three different labs. Samples were analyzed at their nominal concentrations ranging from approximatively 3 mg/g (dark chocolate) to approximatively 500 mg/g (cocoa extract).

Limit of quantification was determined for individual oligomers by the ratio of ten times the standard deviation of the signal area (at a concentration between WS1 and WS2) to the slope the calibration curve (ICH, 1994). Linearity was measured through the coefficient of determination of the ten individual calibration curves.

Specificity was calculated for DP1-7 as the difference between retention times in sample and CEC relative to the the retention time in the CEC. Eventual interferences were estimated by measuring signals in blank matrix (only available for drinks) and comparing the signal area to that of the signal eluting at the same retention time.

Stability was assessed daily at freezer (-18 °C) and autosampler (5 °C) temperatures. Standard solutions were prepared the day of analysis and the stability experiment carried out for nine consecutive days.

Robustness of the method was evaluated by making small but deliberate changes to experimental conditions, including column temperature (± 1 °C), flow rate (± 0.01 mL/min), diluent composition (70:30:0.5 to 1 acetic acid in AWAC), mobile phase A composition (98 ± 1:2 acetonitrile:acetic acid) and mobile phase B (95 ± 1:3 ± 1:2 methanol:water:acetic acid). The impact of chromatography results was evaluated by retention time shift compared to standard conditions.

Supplementary Information: Additional Method Validation Results

Specificity and interferences

Specificity is the quality of the method to identify and determine cocoa flavonols (CFs) without bias from the sample matrix. Interferences were evaluated by analyzing blank placebo of the drink mix. No peaks were observed in the range of retention times covered by CFs with a degree of polymerization (DP) 1-7. This confirms the absence of interferences of the drink mix formulation with the CF oligomer assay described in this article. Specificity is defined by the ability of the method to identify CF oligomers regardless of the sample matrices. For the five sample matrices studied, the HPLC-FLD method showed high specificity with retention drift of <1% determined by comparing retention times in samples against those in a cocoa extract calibrant (CEC). The results confirmed the method's ability to identify CF oligomers in cocoa extracts, cocoa powder, drink mix, capsules and dark chocholate with specificity.

Linearity and sensitivity

Linearity was verified by determining coefficient of determination (r^2) for each DP. Coefficient of determination were systematically ≥0.99. These values demonstrate the linear response of the fluorescence detection to concentration variation for the range described in Section 2.4. Limit of quantification (LOQ) was determined for each degree of polymerization using the standard deviation method described in Supplementatry information: Method Validation Experimental Design. LOQ was defined as ten times the ratio of ten times the standard deviation of the signal area to the slope the calibration curve defined minimum operating concentration. Method variability usually increases as concentration decreases. This approach computes sensitivity (slope) and precision (standard deviation on signal area) to determine the lowest working concentration (higher the sensitivity and precision, lower the LOQ). Across matrices and DP, LOQ was systematically below the lowest concentration of the calibration curve. Together, the coefficient of determination ($r^2 \geq 0.99$) and the LOQ<WS1 demonstrate that the range of concentrations covered by the calibration curve injections (WS1-5) corresponds to liner response of the method to CF concentration.

Table S3. Summary of limit of quantification for studied cocoa matrices.

LOQ (ng/µL)*	DP1	DP2	DP3	DP4	DP5	DP6	DP7
Extract	8.1	4.0	4.4	3.1	2.9	2.6	3.9
Powder	2.7	1.8	1.3	0.6	1.5	1.0	0.8
Drink Mix	0.7	0.4	0.7	1.7	2.4	1.0	2.1
Supplement Capsules	3.3	5.0	0.8	1.2	1.2	2.7	2.1
Dark Chocolate	4.5	5.1	5.7	4.6	3.2	2.8	4.5
Estimated lower calibration point	58.7	42.7	45.1	38.5	32.5	25.0	19.0

*injection volume 2 µL

Stability

Stability of the samples was assessed at storage (−18 °C) and autosampler (5 °C) conditions. Stability was determined by comparing total CF concentration determined at a defined time point against initial measurement of the sample. Loss of total CF was limited to ≤7% for up to 4 days in analysis conditions.

As expected, the stability of CEC was better at low temperatures (−18 °C), where total CF DP1-7 loss did not exceed 11% after 9 days (Table S4). This will support the experimental work involving large number of injections that may exceed autosampler capacity.

The lowest stability was observed with the dark chocolate matrix and in solution oxidoreductive degradation was hypothesized to be the responsible of fluctuating CF concentration. To evaluate this hypothesis, experiments were repeated at autosampler temperature in presence of ascorbic acid. The sample showed better stability over 5 days, confirming the role of oxidoreductive degradation in CF stability (data not shown).

The data presented in Table 5 suggests that appropriate sample stability for the routine implementation of this method was attained. Given the current analysis time, incorporating calibration curves, blanks, system suitability and bracketing injections, a single HPLC instrument can analysze of 90-100 samples per day.

Table S4. Summary of Cocoa Extract Calibrant (CEC) stability results at analysis (autosampler, 5 °C) and storage (freezer, −18 °C) temperatures. Results are expressed in % relatively to the content CF DP1-7 on day one.

Matrix	Autosampler		Freezer	
	4 days	9 days	4 days	9 days
Extract	96	89	97	94
Powder	103	106	101	106
Drink mix	97	94	99	98
Supplement capsules	96	91	97	96
Dark chocolate	93	89	99	92

Robustness

Robustness evaluates the ability of the method to perform normally under deliberate changes of experiemental conditions. The impact of fluctuating experimental conditions was evaluated through specificity. Deliberate changes in dilutent composition (0.5 or 1 % of acetic acid) led to only minors shift in retention times (0.00 to 0.05%). In contrast, higher shift of retention times were observed when modifying the mobile phase composition. However, these results ranged from 0.04 to 1.44 % highlighting the method robustness to fluctuation in mobile phase composition. Finally, the study of method robustness to column temperature (50 ± 1 °C) and flow rate (1.00 ± 0.01 mL/min) did not lead to significant retention time shift (<1 %).

Table S5

Concentration of cocoa flavanols were determined in singlicate for selected cocoa powders, dark chocolate and cocoa-based dietary supplements. Data are expressed in mg/g of flavanols and procyanidins with a degree of polymerization (DP) of 1-7.

Sample	DP1	DP2	DP3	DP4	DP5	DP6	DP7	Total DP1-7
Powder A -1	1.3	1.8	1.6	0.8	0.6	0.5	0.4	6.9
Powder A -2	1.1	1.6	1.5	0.7	0.5	0.4	0.3	6.1
Powder A -3	1.2	1.6	1.5	0.7	0.5	0.4	0.3	6.2
Powder B -1	3.3	3.6	3.2	1.6	1.1	0.8	0.7	14.3
Powder B -2	2.5	2.8	2.7	1.1	0.8	0.5	0.5	10.9
Powder B -3	3.8	4.2	4.0	2.2	1.8	1.3	1.0	18.2
Powder C -1	3.6	3.9	3.6	2.4	2.0	1.6	1.3	18.5
Powder C -2	3.2	3.3	2.8	1.8	1.5	1.2	0.9	14.5
Powder C -3	3.0	3.2	2.8	1.8	1.4	1.1	0.9	14.1
Chocolate A -1	0.9	0.7	0.6	0.5	0.4	0.3	0.2	3.6
Chocolate A -2	1.0	0.7	0.6	0.5	0.4	0.3	0.2	3.6
Chocolate A -3	0.8	0.6	0.6	0.5	0.4	0.3	0.2	3.4
Chocolate B -1	0.5	0.4	0.4	0.3	0.2	0.2	0.2	2.2
Chocolate B -2	0.4	0.4	0.4	0.3	0.2	0.2	0.1	2.0
Chocolate B -3	0.4	0.4	0.3	0.3	0.2	0.2	0.1	1.8
Chocolate C	3.1	2.3	2.4	2.1	1.9	1.6	1.3	14.7
Supplement A	18.5	12.7	13.9	12.6	11.7	10.2	8.6	88.1
Supplement B -1	49.5	8.9	0.0	0.0	0.0	0.0	0.0	58.4
Supplement B -2	46.9	9.0	0.0	0.0	0.0	0.0	0.0	55.9
Supplement C -1	78.1	56.6	60.1	52.0	45.2	35.7	27.5	355
Supplement C -2	78.3	58.4	63.2	55.7	49.3	39.0	30.4	374
Supplement D	28.1	20.6	21.3	18.6	15.9	12.5	9.7	127