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Flavanols and Methylxanthines in Commercially Available Dark Chocolate: A Study of the Correlation with Nonfat Cocoa Solids

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ABSTRACT: Intake of flavanols, a subgroup of dietary polyphenols present in many fruits and vegetables, may be associated with health benefits, particularly with reducing the risk of coronary diseases. Cocoa and chocolate products are rich in flavanol monomers, oligomers, and polymers (procyanidins). This study used normal phase HPLC to detect, identify, and quantify epicatechin, catechin, total monomers, procyanidin oligomers and polymers in 14 commercially available chocolate bars. In addition, methylxanthines (theobromine and caffeine) were also quantified. Nonfat cocoa solids (NFCS) were determined both gravimetrically and by calculation from theobromine contents. The flavanol levels of 12 commonly consumed brands of dark chocolate have been quantified and correlated with % theobromine and % NFCS. Epicatechin comprised the largest fraction of total chocolate flavonoids, with the remainder being catechin and procyanidins. Calculated NFCS did not reflect epicatechin (R^2 = 0.41) or total flavanol contents (R^2 = 0.49). Epicatechin (R^2 = 0.96) was a reliable marker of total flavanols, catechin (R^2 = 0.67) to a lesser extent. All dark chocolate tested contained higher levels of total flavanols (93.5−651.1 mg of epicatechin equiv/100 g of product) than a milk or a white "chocolate" (40.6 and 0.0 mg of epicatechin equiv/100 g, respectively). The amount and integrity of procyanidins often suffer in the manufacturing of chocolate, chiefly due to oxidation and alkalinization. In this study, the labeled cocoa content of the chocolate did not always reflect analyzed levels of flavonoids. Increasingly, high % NFCS is being used commercially to reflect chocolate quality. If the flavanol content of chocolate is accepted to be a key determinant of health benefits, then continued monitoring of flavanol levels in commercially available chocolate products may be essential for consumer assurance.

KEYWORDS: chocolate, epicatechin, flavanols, procyanidins, nonfat cocoa solids (NFCS), theobromine

INTRODUCTION

Cocoa-containing foods are a rich source of a subclass of polyphenolic flavonoids called the flavanols (also known as flavan-3-ols or catechins). Two monomers found within this class, epicatechin and catechin, serve as building blocks for oligomers and polymers known as procyanidins or condensed tannins. Procyanidins with a chain length of over 10 units have been reported in cocoa.[1,2] During the production of chocolate, the initial levels of flavonoids in the cocoa bean will change during fermentation, drying, roasting, alkalinization, and storage conditions due to oxidation and thermal effects.[3] Additionally, cocoa is rich in the methylxanthines theobromine and caffeine. These alkaloids are pharmacologically active, and the physiological effects they induce comprise modulation of the central nervous system (including mood changes) and the gastrointestinal tract and increased diuresis.[4]

The interest in flavonoids as health-protective agents increased following publication of the Dutch Zutphen Elderly Study.[5] A subset of this study linked epidemiological observation of reduced blood pressure and 15-year cardiovascular and all-cause mortality to increased intake of cocoa-containing foods.[6] Further human studies[7−11] also suggested that cocoa or dark chocolate may be able to reduce the risk of cardiovascular disease (CVD) and stroke.

It has been proposed that flavonoids improve endothelial function, a biomarker for cardiovascular risk, by modulating antioxidant, vasorelaxant, antithrombotic, antiproliferative, and anti-inflammatory processes (reviewed in ref 12). The key mechanism underlying these (transient) effects seems to be stimulation of nitric oxide release, giving rise to vasorelaxation.[8,9]

Cocoa flavonoids consistently and significantly increased nitric oxide-dependent endothelial function in a number of studies.[8−11] Furthermore, insulin sensitivity and glucose tolerance was improved, and plasma concentrations of total and LDL cholesterol were reduced.[13] LDL peroxidation, which in vivo promotes atherogenesis, could be significantly delayed ex vivo by cocoa flavonoids,[14,15] as could platelet aggregation and adhesion.[16,17] There is evidence that this effect may be relevant in the human organism, as F(2)-isoprostane levels, a marker of in vivo LDL peroxidation, were reduced after cocoa ingestion.[18] Longer term effects of cocoa flavonoids seem to involve modulation of inflammatory mediators and signal transduction pathways.[19]

Epicatechin, the major monomeric cocoa flavanol, seems likely to be the main agent of the protective effects associated with cocoa.[11] Flavanols, particularly (−)-epicatechin, are readily absorbed, and plasma concentrations peak after around 2 h.[20] Evidence suggests that procyanidin dimers, which are present in considerable amounts in cocoa products, are absorbed and metabolized to some extent.[21] Procyanidins of higher degrees of polymerization (DP) do not seem to be bioavailable in the small intestine; they are, however, subject to fermentation by the colonic microflora,[20] giving rise to relatively well absorbed low molecular weight phenolics.[22,23]

The publicity generated by research into the putative beneficial effects of cocoa flavonoids has led to a significant interest in

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chocolate products and, in particular, dark chocolate. Levels of chocolate flavanols reported in the literature range from 2500 mg/100 g in baking chocolate (which consists of pure cocoa liquor of 100% cocoa solids)[24] to 43 mg/100 g in milk chocolate.[25] There are no specific regulations governing labeling of dark chocolate or of its polyphenol or flavonoid contents.

To assess potential health benefits, it is important to be aware of the composition of biologically active compounds in commercially available chocolate. Many of the studies that have looked at the in vivo bioactivity of cocoa flavanols have used levels much higher than that present in most products. At present, it is impossible for consumers seeking to increase their consumption of cocoa polyphenols to obtain information about levels in particular products. There have been few papers describing the determination of levels of nonfat cocoa solids (NFCS) in chocolate.[1,24−27] NFCS is a gauge of the quantity of the flavanol-rich fat-free component of chocolate.[27] Additionally, to our knowledge, quantitative data for bioactive components in individual brands of dark chocolate are lacking. It would seem important to understand more clearly the link between NFCS and flavanol contents of commercially available chocolate. We have determined levels of flavanols (monomeric catechins and procyanidins) and methylxanthines in a range of popular chocolate brands, using a modified NP-HPLC method.

MATERIALS AND METHODS

Materials. Solvents used, *n*-hexane, methanol (MeOH), acetonitrile (ACN), and acetic acid (HOAc), were of HPLC grade and were obtained from Fisher Scientific (U.K.). Acetone was obtained from Alfa Aesar (U.K.). Water was prepared using a Millipore Milli-Q purification system. (−)-Epicatechin, theobromine, caffeine, catechol, and taxifolin standards were of analytical grade and purchased from Extrasynthèse (France).

The following chocolate brands were bought in Leeds (U.K.) between October 2009 and November 2010: 12 dark chocolate brands (listed alphabetically), Cadbury Bournville Plain; Co-op Fairtrade Dark; Green & Black's Organic Dark 70%; Harcourt-Cooze Willie's Cacao, Venezuelan Black, Rio Caribe Superior; Hôtel Chocolat Single Estate, Hacienda Iara Plantation, Ecuador; Lindt Excellence Intense 70%; Marks & Spencer Swiss Extra Fine; Nestlé Heaven Swiss Dark; Sainsbury Belgian Dark; Tesco Finest Swiss Plain; Thorntons Antioxi Choc, Berry Boost; and Thorntons Dark French; one milk chocolate, Cadbury Dairy Milk; and one white chocolate, Cadbury Dream. All dark chocolate samples contained between 39 and 72% cocoa solids (as labeled), except Harcourt-Cooze and Hôtel Chocolat, which contained 100% cocoa solids. Thorntons Antioxi Choc Berry Boost contained inclusion pieces (blueberries and raspberry extract). For each brand, two bars from two different batches (typically 6 months apart) were purchased, except for the chocolate bars with sample codes 8 and 14, of which only one bar was purchased due to lack of availability. Samples were coded 1−14 (coding bears no relation to the order in above list) and prepared as described by Robbins et al.,[28] with minor modifications as outlined below. Samples from all chocolate types were prepared using the same method. A small part of each chocolate bar (approximately 2 g) was shredded by knife into small pieces, which were milled with liquid nitrogen using a cryogenic laboratory mill. Samples were stored at −20 °C until analyzed.

Fat Extraction. To eliminate lipids from samples, ground chocolate (0.5 g) was weighed accurately in a Falcon tube (50 mL). Fat was extracted three times with *n*-hexane (15 mL) by vortexing (1 min) and sonication (10 min, 38 ± 2 °C). Tubes were centrifuged (5 min, 38 °C, 3000*g*, using a tabletop centrifuge) and carefully decanted. The defatted samples were dried under a gentle stream of nitrogen.

Flavonoid Extraction. Phenolic compounds were extracted three times from the defatted, dried samples in Falcon tubes (50 mL) with 2.5 mL of acetone/water/acetic acid (70:28:2, v/v/v) by vortexing (1 min) and subsequent sonication (10 min, 38 ± 2 °C). Samples were then centrifuged (5 min, 38 °C, 3000*g*) and carefully decanted, and supernatants were pooled. This procedure was repeated three times. The pooled supernatants (approximately 7.5 mL each) were diluted 1:3 in extraction solvent and filtered with 0.45 μm PTFE filters (Chromacol, U.K.). Catechol (at a final concentration of 100 μM) was added as an external standard, and the samples were used directly for HPLC analysis.

NFCS Determination by Weight. The remains of the initial chocolate samples, after fat and flavonoid extraction, were allowed to dry in a fume hood for 5 days; the remaining mass, which largely represents NFCS, was determined gravimetrically. NFCS was not able to be determined with this method for white and milk chocolate due to the high levels of protein they contained.

NFCS by Calculation. According to Cooper et al.,[1] theobromine widely serves as a marker of NFCS in the chocolate industry. Thus, NFCS levels can be calculated as 38.0 × % theobromine (w/w) (90% confidence interval: 34.4 and 42.4 × % theobromine).

HPLC. The filtered, diluted chocolate extracts were analyzed by NP-HPLC, using an Agilent 1200 series HPLC-DAD system. The method used in this study was as described in Robbins et al.,[28] with some modifications. Sample injection volume was 5 μL. The column was a Develosil Diol (250 mm × 4.6 mm, 5 μm particle size and 100 Å packing density; Phenomenex, U.K.). The mobile phase was a binary gradient with a flow rate of 0.6 mL min^{-1} and consisted of (A) acidic acetonitrile (ACN/HOAc, 98:2; v/v) and (B) acidic aqueous methanol (CH_3OH/H_2O/HOAc, 95:3:2; v/v/v). The starting mobile phase condition was 7% B, held isocratically for 3 min. Subsequently, solvent B was increased to 37.6% (3−57 min) and then to 100% B (57−60 min). The conditions were held at 100% B for 7 min prior to returning to 7% B (67−73 min) with a final isocratic run of 7% B from 73 to 83 min. A cyano Security Guard cartridge (4 × 3.0 mm; Phenomenex, U.K.) acted as a precolumn to protect the main column. The column oven was set to 35 °C. All flavonoids (mono-, oligo-, and polymers) were determined using fluorescence detection with an excitation wavelength of 230 nm and emission at 321 nm. Theobromine and caffeine were detected using a photodiode array detector at 280 nm.

Recovery from samples spiked with various concentrations of taxifolin (internal standard), epicatechin, and theobromine were 101% (n = 4; CV = 2.7%), 120% (n = 3; CV = 3.9%), and 111% (n = 3; CV = 2.7%), respectively. The external standard catechol was added to all samples; the mean CV over the course of the analysis was 9.8%. Individual compounds were confirmed by comparison with literature values of their relative retention times,[28] order of elution, and nature of UV spectra (compared to pure (−)-epicatechin and theobromine standards). All determinations were performed in duplicate. Six separate extractions from two chocolate bar samples from two different batches (n = 6) were analyzed, except for chocolate bars 8 and 14 (n = 3). The mean precisions (of analyses of extracts from the same sample) were 5.9% for monomers, 7.3% for procyanidin oligomers, 5.8% for total flavanols (range from 0.9 to 17.5%; median 5.0%), and 3.9% for theobromine. The mean accuracies (determined values of known concentrations of epicatechin samples) at LOQ were 137.4% (n = 12; SD = ±6.5%) and 98.6% (n = 12; SD = ±4.7%) at midrange concentration (100 μM). Levels of flavanols and procyanidins measured in the chocolate samples were expressed in terms of epicatechin equivalents (ECE).

The limit of detection (LOD) was, in accordance with Risner,[29] calculated as 3 times the standard deviation (SD) of the lowest standard analyzed as a sample, whereas the limit of quantification (LOQ) was 10 times the SD of the lowest standard analyzed as a sample.

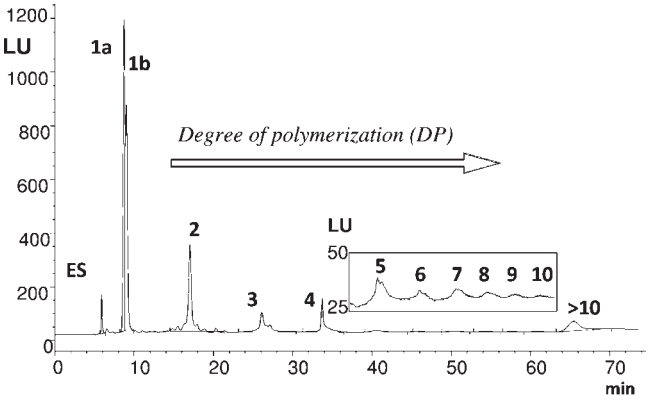

Figure 1. Typical chromatogram of an extract (from chocolate 14). ES, external standard (catechol). Numbers refer to degree of polymerization, that is, 1a, epicatechin; 1b, catechin; 2, procyanidin dimer; 3, procyanidin trimer, and so on; >10, unresolved procyanidins with degree of polymerization greater than 10. Although epicatechin and catechin run very close, the values calculated using the procedure described did not differ from values derived from studies using RP-HPLC (data not shown).

For (−)-epicatechin, the LOD was 0.55 mg/100 g (0.14 μg/mL) and the LOQ, 0.76 mg/100 g (0.19 μg/mL); for theobromine, the LOD was 1.1 mg/100 g (0.28 μg/mL) and the LOQ, 3.7 mg/100 g (0.93 μg/mL); and for caffeine, the LOD was 3.12 mg/100 g (0.78 μg/mL) and the LOQ, 10.4 mg/100 g (2.60 μg/mL). The mean CV of the epicatechin standard curves, ranging from 0.05 to 500 μM (during which the curve was linear), was 6.5% (n = 12). Theobromine standard curves (ranging from 5 μM to 4 mM, during which the curve was linear) had a mean CV of 8.1% (n = 3), caffeine (range = 4.6 μM−4.6 mM, during which the curve was linear) of 4.3% (n = 3).

RESULTS

One white, one milk, and 12 dark chocolates were tested. The modified HPLC method employed here is an excellent method for the standard analysis and quantification of chocolate procyanidins. The method allowed the simultaneous separation and quantification of epicatechin, catechin, and different procyanidin oligomers up to a degree of polymerization of 10 (using fluorescence detection). At the same time, theobromine and caffeine (using UV absorption) were analyzed in samples extracted from chocolate bars in a quick and simple procedure without further purification.

Flavanol Contents. The flavanol content was determined in extracts from two bars each representing two batches of white, milk, and dark chocolate. Figure 1 shows a typical chromatogram in fluorescence detection mode. The method permitted epicatechin and catechin to be resolved and the levels of both compounds to be quantified. The progressive broadening of the peaks was due to the number of possible isomers, which increased exponentially with DP.[30] Figure 2 and Table 1 illustrate the distribution of all flavanols and procyanidins in the analyzed chocolate. Epicatechin represented the dominant single flavanol in most of the analyzed cocoa products, followed by dimers and trimers (combined values), a finding that was in accordance with Cooper et al.[31] The mean epicatechin-to-catechin ratio determined here was 1:0.39. Mean epicatechin contents were 0.0 ± 0.0 mg/100 g for the white chocolate (sample 1), 10.0 ± 1.3 mg/100 g for the milk chocolate (sample 2), and ranged from 29.8 ± 2.6 to 269.7 ± 20.2 mg/100 g for the dark chocolates (samples 3−14). Procyanidins (DP ≥ 2) were found to be 0.0 ± 0.0 mg/100 g for white chocolate, 27.3 ± 7.4 mg/100 g for milk, and between 48.4 ± 4.3 and 334.0 ± 5.9 mg/100 g for all of the dark chocolates. Total monomers constituted approximately a third to a half of total flavanols and polymers (DP ≥ 11) about 10% of total flavanols.

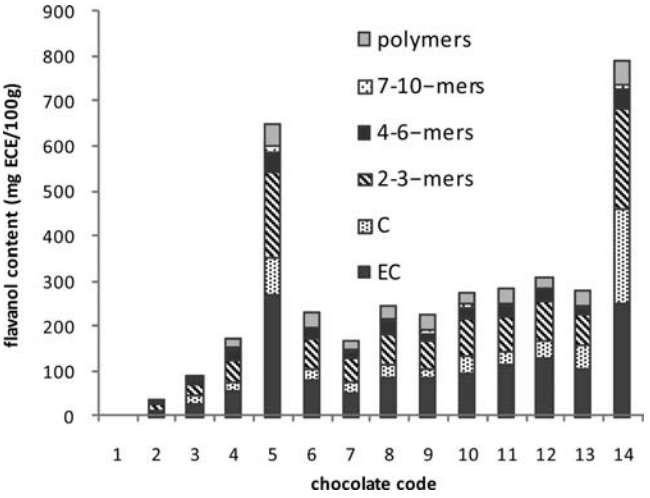

Figure 2. Mean distribution of total flavanols in the chocolate bars analyzed. Results are expressed as milligrams of epicatechin equivalents per 100 g.

Total flavanols were determined as 40.6 ± 9.3 mg/100 g in milk chocolate and ranged from 93.5 ± 8.1 to 792.8 ± 27.0 mg/100 g in dark chocolate. The highest levels of both epicatechin and procyanidins were present in sample 14, followed by sample 5. Chocolate 5 contained unspecified levels of raspberry extract and blueberry inclusion pieces. These additions seemed unlikely to have contributed to measured flavanol levels, as raspberries and blueberries contain low to moderate levels of flavanols and relatively high levels of anthocyanidins and flavonols.[32]

Epicatechin correlated better (R^2 = 0.96) than catechin (R^2 = 0.67) with total flavanol levels in all samples (Figure 3), indicating a greater degree of variation in catechin than epicatechin content of the products.

Methylxanthines, NFCS. The contents of methylxanthines (theobromine and caffeine) are shown in Table 2 as a percent of chocolate weight. Theobromine levels may serve as a proxy for total polyphenols and can be used to calculate NFCS (see Materials and Methods). The correlation of experimentally determined and calculated % NFCS for the 12 dark chocolates (based on theobromine levels; Table 2) was R^2 = 0.68 (Figure 4). In further correlations, the calculated (rather than measured) % NFCS levels were used. The correlation coefficient of calculated % NFCS content and epicatechin was 0.41, and for total flavanol content 0.49 for the 12 dark chocolates (Figure 5). Calculated % NFCS did not closely mirror measured monomer and procyanidin levels (Figure 6). As mentioned previously, samples 5 and 14 were notable exceptions, with both their epicatechin and procyanidin levels being considerably higher than their % theobromine or % NFCS would predict.

DISCUSSION

The NP-HPLC method employed here had good reproducibility, with mean CV values between analyses of extracts from the same chocolate sample of 5.9% for monomers, 7.3% for total procyanidins, and 3.9% for theobromine. Analysis of the samples

Table 1. Catechin and Procyanidin Contents (Milligrams per 100 g of Product ± SD, Expressed in Epicatechin Equivalents) of the Different Chocolate Bars

sample[a]	epicatechin	catechin	total monomers	DP 2−3	DP 4−6	DP 7−10	polymers (DP ≥ 11)	sum of procyanidins (DP ≥ 2)	sum of all flavanols
1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	10.0 ± 1.3	3.4 ± 1.4	13.3 ± 2.3	12.7 ± 1.4	6.0 ± 2.5	0.0 ± 0.0	5.9 ± 5.8	27.3 ± 7.4	40.6 ± 0.3
3	29.8 ± 2.6	15.9 ± 3.1	45.7 ± 4.	27.3 ± 1.8	9.9 ± 1.1	2.0 ± 0.5	8.5 ± 2.7	48.4 ± 4.3	93.5 ± 8.1
4	58.3 ± 8.8	19.5 ± 4.9	77.8 ± 6.8	49.0 ± 5.8	21.6 ± 6.2	4.1 ± 1.5	19.1 ± 3.0	93.8 ± 10.3	171.6 ± 16.5
5	269.7 ± 20.2	86.0 ± 23.4	355.7 ± 39.7	186.9 ± 22.9	44.5 ± 12.9	12.1 ± 2.0	51.9 ± 12.3	295.4 ± 42.8	651.1 ± 80.9
6	81.4 ± 15.7	26.3 ± 7.3	107.7 ± 23.0	67.8 ± 11.1	22.2 ± 13.6	2.3 ± 0.6	31.6 ± 10.0	123.9 ± 12.4	231.6 ± 30.4
7	51.4 ± 3.8	27.1 ± 3.5	78.5 ± 3.9	49.3 ± 7.8	18.4 ± 10.7	2.6 ± 0.6	19.0 ± 7.7	89.3 ± 15.7	167.8 ± 18.0
8[b]	87.7 ± 5.3	26.7 ± 3.8	114.3 ± 8.7	69.9 ± 6.2	29.5 ± 2.9	4.7 ± 0.2	29.1 ± 7.3	133.3 ± 10.8	247.6 ± 19.0
9	88.1 ± 5.5	17.4 ± 2.9	105.5 ± 7.0	61.5 ± 4.0	18.7 ± 2.2	5.3 ± 1.1	34.2 ± 7.4	119.7 ± 12.5	225.2 ± 18.2
10	97.6 ± 8.9	39.7 ± 7.6	137.3 ± 13.4	79.7 ± 11.4	25.6 ± 7.5	6.6 ± 3.6	26.3 ± 7.9	138.2 ± 23.2	275.5 ± 35.3
11	115.6 ± 11.0	28.0 ± 3.2	143.6 ± 8.1	80.3 ± 6.6	22.5 ± 4.5	6.2 ± 1.3	33.7 ± 11.0	142.7 ± 21.0	286.3 ± 29.0
12	131.5 ± 13.7	37.1 ± 2.9	168.7 ± 13.9	89.0 ± 9.5	25.9 ± 8.1	4.3 ± 1.6	22.4 ± 13.7	141.5 ± 24.8	310.2 ± 34.7
13	105.0 ± 17.7	52.7 ± 21.9	157.7 ± 39.4	69.4 ± 6.0	13.1 ± 2.0	5.1 ± 2.4	33.8 ± 1.7	121.4 ± 5.3	279.2 ± 38.3
14[b]	253.2 ± 29.4	205.7 ± 33.6	458.9 ± 25.4	225.2 ± 15.3	44.6 ± 11.6	9.1 ± 3.5	55.0 ± 2.3	334.0 ± 5.9	792.8 ± 27.0

[a] $n = 6$ unless otherwise noted. Samples 1 and 2 were white and milk chocolate, respectively. [b] $n = 3$.

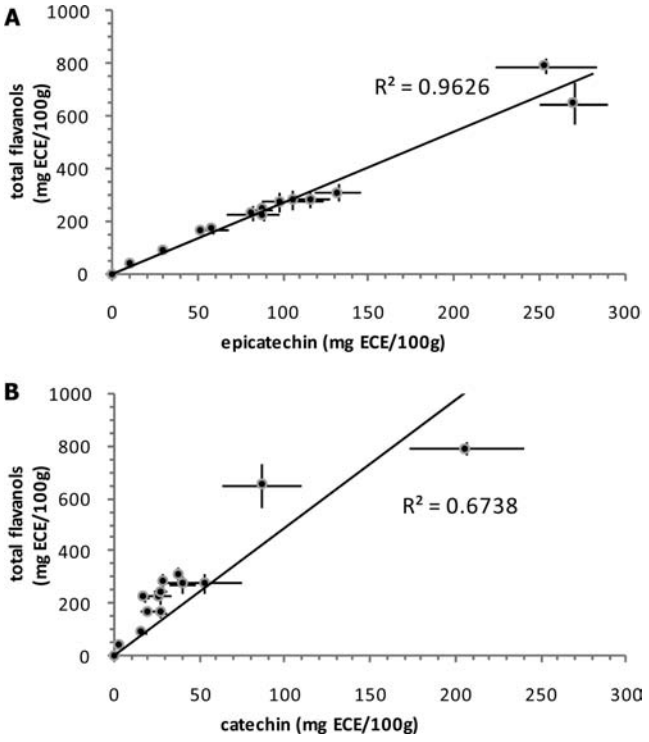

Figure 3. Epicatechin (A) and catechin (B) plotted against total flavanols, expressed as milligrams of ECE per 100 g, for all chocolate bars analyzed. Epicatechin (A) correlates closely with total flavanol content (irrespective of processing conditions), catechin (B) less so. Results are ±SD. Trendline is forced through the origin.

using reversed phase (RP)-HPLC (which resolves individual peaks up to a DP of 4, with the remaining procyanidins eluting as one unresolved peak) yielded values for procyanidin monomers on average 4.2 ± 9.8% greater (data not shown) than the ones determined with NP-HPLC, which were used in this study. Epicatechin-to-catechin ratios were similar whether using NP- or RP-HPLC (data not shown).

Gu et al.[24] used a similar extraction method and measured levels of procyanidin polymers (DP > 10) in dark chocolate of 286−697 mg/100 g, 12−33 times higher than the levels detected in our samples. Similarly, the values of Miller et al.[25] for oligomers and polymers, but not for monomers, were higher than the ones detected here. The same authors further described certain individual chocolate samples having dimer levels that were higher than those of the monomers.[25] In this study, monomer levels always were considerably greater than those for dimers.

It is conceivable that the levels of highly oligomeric and polymeric flavanols in cocoa-containing products are routinely underreported. During fermentation of the cacao beans, lower molecular weight polyphenols can condense to form tannins of higher DP, which may complex with proteins and carbohydrates[2] and thus make it difficult to achieve complete extraction from the matrix.[30] Moreover, when using HPLC in fluorescence detection mode for analyzing procyanidin polymers, fluorescence quenching[33] can occur, leading to further underestimation of oligomers and polymers. More aggressive extraction methods (acid-catalyzed hydrolysis, higher temperatures) were shown to increase the amounts of measurable high molecular weight procyanidins.[34] However, such methods would invariably lead to degradation of the more labile monomers. In the present study, an increase in extraction time to 90 min made no difference to extracted amounts of flavonoids, nor did an increase in extraction temperature up to 50 °C (data not shown). As methods of analysis for flavanol polymers become more widely used and may have more significance for consumers, collaborative trials become essential. Further study of quantification of procyanidins is warranted.

The level of theobromine determined for the one milk chocolate under investigation (153 mg/100 g) compares well with the values for nine analyzed milk chocolates (117−196 mg/100 g).[35] Another survey of commercially available chocolate[36] gave values for Cadbury Dairy Milk of 149 mg/100 g, Cadbury Bournville of 474 mg/100 g, and Green & Black's Dark Chocolate of 1037 mg/100 g, which were comparable to the values reported here (153, 525, and 1123 mg/100 g), for the same brands of chocolate, respectively. This suggests, furthermore, that the typical levels of theobromine in these brands seem

Table 2. Gravimetrical and Calculated NFCS and Methylxanthine Contents (Percent by Weight ± SD) in Commercially Available Chocolate Bars

sample[av]	labeled cocoa solids (%)	labeled fat content (%)	measured NFCS (%)	calculated NFCS (%)	measured theobromine (%)	measured caffeine (%)
1	0	33	nd[b]	0.1 ± 0.0	0.00 ± 0.00	0.00 ± 0.00
2	20	30	nd	5.8 ± 0.8	0.15 ± 0.02	0.01 ± 0.01
3	39	27	21.7 ± 2.1	20.0 ± 3.0	0.53 ± 0.08	0.03 ± 0.01
4	51	36	29.0 ± 0.7	32.2 ± 4.9	0.85 ± 0.13	0.06 ± 0.01
5	63	35	31.3 ± 0.7	41.9 ± 1.8	1.10 ± 0.05	0.08 ± 0.01
6	70	41	32.4 ± 1.8	42.7 ± 3.5	1.12 ± 0.09	0.14 ± 0.02
7	70	40	33.4 ± 1.1	39.6 ± 3.4	1.04 ± 0.09	0.09 ± 0.02
8[c]	70	45	32.1 ± 1.0	34.1 ± 0.7	0.90 ± 0.02	0.08 ± 0.00
9	70	40	34.4 ± 3.0	34.6 ± 3.2	0.91 ± 0.08	0.07 ± 0.01
10	72	45	30.5 ± 2.4	36.7 ± 3.5	0.97 ± 0.09	0.09 ± 0.01
11	72	47	32.3 ± 1.8	34.6 ± 3.9	0.91 ± 0.10	0.08 ± 0.01
12	72	46	32.2 ± 0.8	37.0 ± 5.6	0.97 ± 0.15	0.09 ± 0.02
13	100	—[d]	45.2 ± 1.3	43.9 ± 2.0	1.16 ± 0.05	0.15 ± 0.00
14[c]	100	–	43.4 ± 0.7	62.4 ± 1.8	1.64 ± 0.05	0.24 ± 0.04

[av] Samples 1 and 2 were white and milk chocolate bars, respectively. $n = 6$, except where noted otherwise. [b] nd, not determined. [c] $n = 3$. [d] —, not labeled.

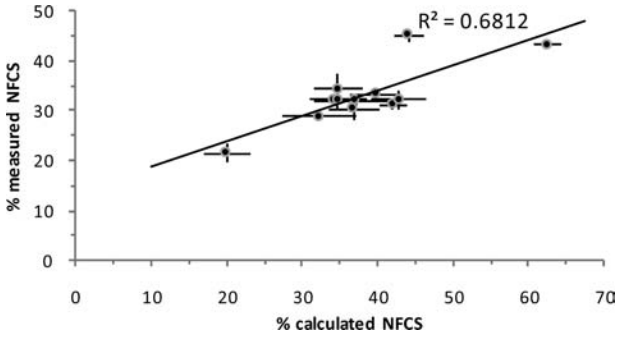

Figure 4. Correlation gravimetrically measured against calculated NFCS (percent by weight) for dark chocolate bars. Results are ±SD.

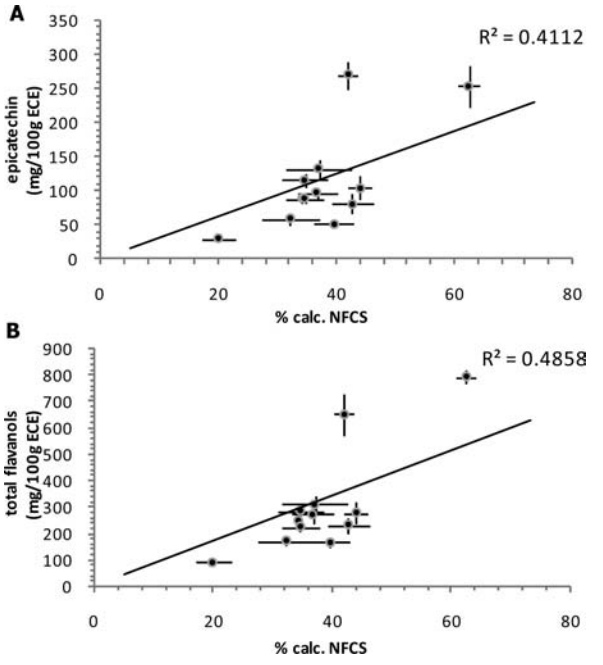

Figure 5. Correlation of epicatechin (A) and total flavanols (B) with calculated percent NFCS (±SD) for dark chocolate bars. Trendline is forced through the origin.

to have been constant over a period of more than a decade. Miller et al.[27] affirmed that chocolate manufacturers typically blend cacao bean types to ensure product uniformity.

Measured theobromine served as a proxy for (measured) NFCS and, in turn, calculated NFCS was assessed to test whether it was a predictor of epicatechin and flavanol levels (Figure 5).

The levels of flavanols determined here varied greatly between different brands. Percent cocoa solids include flavonoid-rich cocoa liquor, cocoa powder, and possibly added flavonoid-free cocoa butter. However, % cocoa solids as stated on the product label, and measured and calculated % NFCS contents do not always correlate with actual flavonoid levels. In contrast, Miller et al.[27] analyzed a range of cocoa-containing products in the U.S. market, including chocolate syrup, chocolate milk, dark and baking chocolate, and cocoa powder, and found a strict linear correlation between experimentally determined NFCS and total procyanidins over all product categories, with a squared linear correlation coefficient, R^2, of 0.95. Across five cocoa product categories, Gu et al.[24] described a linear correlation between NFCS and total procyanidin content with a coefficient of 0.99. Cooper et al.[1] employed calculated % NFCS (estimated from theobromine levels) and found, after analyzing 68 chocolates from various countries, a correlation coefficient R^2 to epicatechin of only 0.36 and to catechin of 0.25. In contrast, another study that employed directly determined NFCS reported a correlation coefficient of 0.83 between % NFCS and epicatechin contents of 19 cocoa-containing products, concluding that directly measured NFCS was more accurate than calculated NFCS.[25]

In the present study, calculated NFCS (which is also the more rapid method) was used as a substitute for directly measured NFCS (correlation $R^2 = 0.68$, Figure 4). Our relationship of R^2, between calculated % NFCS and total flavanols, when all analyzed samples were taken into account, was 0.61. Considering the dark chocolate samples only, R^2 decreased to 0.49 (Figure 5B).

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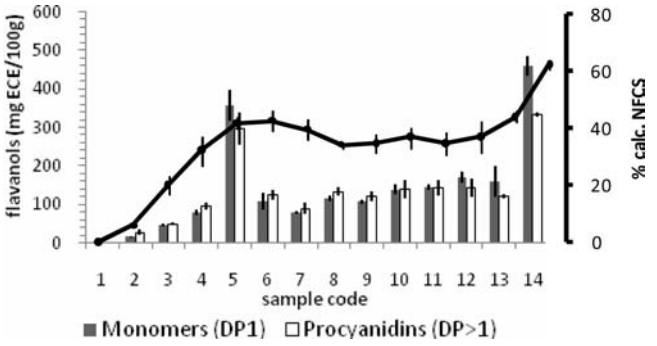

Figure 6. Total flavanols (milligrams of ECE per 100 g ± SD) in monomer (DP1 = epicatechin + catechin) and total procyanidins (DP > 1) compared with calculated NFCS (percent by weight ± SD) for all chocolate bars analyzed.

Furthermore, calculated % NFCS correlated poorly with epicatechin for all chocolate samples (R^2 = 0.56) and dark chocolate samples (R^2 = 0.41) (Figure 5A). Samples 5 and 14 showed epicatechin and flavanol levels that were considerably higher than those predicted by either their theobromine or calculated % NFCS levels. This observation may be due to an exceptionally low degree of degradation of flavonoids during processing in these two brands, whereas theobromine seemed to have been unaffected, leading to a misleading theobromine−flavanol correlation. Commonly, the polyphenol levels in the finished cocoa-containing product are governed chiefly by cocoa bean fermentation[2] and "dutching" (alkalinization) of cocoa powder.[24] According to the producers of chocolate 5, a distinctive, unspecified manufacturing process is proposed to result in a product with particularly high levels of preserved polyphenols.[37] Sample 14, a 100% cocoa solid bar containing relatively high levels of monomeric and oligomeric procyanidins, was produced by an artisan manufacturer employing minimal processing (personal communication). The flavanol levels of sample 13, another 100% cocoa solids product from a different artisan manufacturer, were in agreement with predictions from calculated % NFCS.

Figure 3 shows that epicatechin correlated better with total flavanols (R^2 = 0.96, for all chocolate samples) than catechin (R^2 = 0.67, for all chocolate samples), highlighting the greater level of variation in catechin quantity. Although native cacao beans contain (−)-epicatechin and (+)-catechin (but not (−)-catechin), during the manufacturing process of chocolate, and particularly during alkalinization, some of the (−)-epicatechin may epimerize to (−)-catechin.[3,31] It is not possible, using our method of detection, to distinguish between (+)-catechin and (−)-catechin enantiomers. It has been reported that the manufacturing process of chocolate seems to be most destructive for flavanols, which are more readily absorbed, such as (−)-epicatechin and (+)-catechin.[3,31] This finding has to be taken into account when in the consideration of flavanol values in cocoa products. Epicatechin levels in chocolate may vary greatly (see Table 1), and the simple % cocoa solids stated on the label is no clear indicator of its level.

Polyphenol-rich cocoa and chocolate are likely to have beneficial effects on the cardiovascular system through the active mediator (−)-epicatechin.[11] Transient effects of flavonoids involve modulation of nitric oxide and, among other effects, subsequent improvement of endothelial function. It is conceivable that an increase in the consumption of certain polyphenols (such as cocoa epicatechin) could yield small but significant improvements in certain long-term health outcomes.

Further research is warranted on the overall health effects of other macro- and micronutrients in chocolate. The fat (cocoa butter) in chocolate is rich in stearic acid, which is sometimes referred to as "cholesterol-neutral" due to its suggested propensity to increase high-density (HDL) and decrease low-density lipoprotein (LDL).[15] Sugar consumption evidently is associated with caries, obesity, and diabetes; accordingly, the relatively high sugar content of around 30% w/w in all but the 100% cocoa solid chocolate analyzed here has to be considered in the recommendation of chocolate intake for health reasons. There is contradictory evidence as to whether the composition of chocolate has a significant effect on the absorption of flavanols[38] or methylxanthines.[4] Potential synergistic effects between the bioactive cocoa components, flavanols and methylxanthines, are barely established and need much further scrutiny. Overall, calculated % NFCS was not indicative of the epicatechin and total flavanol profile in the milk and dark chocolates analyzed. Stated levels of % cocoa solids on chocolate bar labels give a fair but not excellent indication of flavonoid content. However, especially in the upmarket dark chocolate sector, products with higher or lower than typical flavanol contents were identified. The two 100% cocoa solids chocolates tested varied 3-fold in their levels of total flavanols, whereas one sample with only 63% of stated cocoa solids was found to have the second highest content of total flavanols in the range of analyzed chocolates, surpassing all other dark chocolates within the 70% cocoa solids bracket by >2-fold.

In many countries it is mandatory to label % cocoa solids in cocoa-containing products. However, there are no regulations concerning labeling of flavonoid levels, although some manufacturers use terms such as "flavonoids", "polyphenols", and "antioxidants". Labeling is an area that can help or confuse consumers, and because dark chocolate is increasingly being marketed as a healthy option, it may be necessary to consider new labeling requirements. As our findings suggest that % cocoa solids may be misleading in certain circumstances, labeling of flavonoid or possibly epicatechin contents may be more pertinent.

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ABBREVIATIONS USED

C, catechin; DP, degree of polymerization; EC, epicatechin; ECE, epicatechin equivalents; FMD, flow-mediated dilation; NFCS, nonfat cocoa solids.

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