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Predictive Relationship between Polyphenol and Nonfat Cocoa Solids Content of Chocolate

KAREN A. COOPER, ESTHER CAMPOS-GIMÉNEZ, DIEGO JIMÉNEZ ALVAREZ, ANDREAS RYTZ, KORNÉL NAGY, AND GARY WILLIAMSON*

Nestlé Research Centre, Lausanne, Switzerland

Chocolate is often labeled with percent cocoa solids content. It is assumed that higher cocoa solids contents are indicative of higher polyphenol concentrations, which have potential health benefits. However, cocoa solids include polyphenol-free cocoa butter and polyphenol-rich nonfat cocoa solids (NFCS). In this study the strength of the relationship between NFCS content (estimated by theobromine as a proxy) and polyphenol content was tested in chocolate samples with labeled cocoa solids contents in the range of 20–100%, grouped as dark ($n = 46$), milk ($n = 8$), and those chocolates containing inclusions such as wafers or nuts ($n = 15$). The relationship was calculated with regard to both total polyphenol content and individual polyphenols. In dark chocolates, NFCS is linearly related to total polyphenols ($r^2 = 0.73$). Total polyphenol content appears to be systematically slightly higher for milk chocolates than estimated by the dark chocolate model, whereas for chocolates containing other ingredients, the estimates fall close to or slightly below the model results. This shows that extra components such as milk, wafers, or nuts might influence the measurements of both theobromine and polyphenol contents. For each of the six main polyphenols (as well as their sum), the relationship with the estimated NFCS was much lower than for total polyphenols ($r^2 < 0.40$), but these relationships were independent of the nature of the chocolate type, indicating that they might still have some predictive capabilities.

KEYWORDS: Chocolate; procyanidins; epicatechin; antioxidant; cocoa; polyphenols; theobromine

INTRODUCTION

Chocolate and cocoa deliver potential health benefits as measured by improvements in key risk factors for heart disease and related illnesses (*1–5*). The health benefits are related to the fact that they are rich sources of flavanols (monomeric units of catechin or epicatechin) and procyanidins (polymeric chains of these monomers) (*6*). In cocoa beans, the monomer of epicatechin can comprise up to 35% of the polyphenol content (*7*), with recent research indicating that this compound could be the primary active component of cocoa responsible for its associated vascular health benefits (*8*).

In general, plain dark chocolate contains cocoa liquor (and/or cocoa powder), cocoa butter, sugar, lecithin, and flavorings. Nonfat cocoa solids (NFCS) from cocoa liquor and powder are sources of polyphenols and so, theoretically, the higher the NFCS content, the higher the polyphenol content of the resultant chocolate. However, percent cocoa solids declared on the chocolate wrapping comprises liquor, powder, and butter (polyphenol-free) combined, which, depending on the ratio, will have an impact on the final polyphenol content. In cocoa liquor, the ratio of intrinsic cocoa butter to NFCS may further vary by cocoa bean type: an average of 54% cocoa butter with a range of 48–57% has been reported (*9*). The final polyphenol content and profile of the cocoa and chocolate are largely related to the processing, especially the steps of cocoa bean fermentation (*7*) and the alkalinization of cocoa powder (*10*). For example, during bean fermentation the losses of epicatechin are considerable by the third day, and these losses will continue for as long as fermentation is permitted. However, it is not known whether epicatechin is truly lost. It could be used to form larger tannins, in which case the profile, rather than the total polyphenol content, alters.

The use of nonfat cocoa solids as a marker for polyphenol content has been strengthened by several recent papers. Miller et al. (*11*) found a strong relationship between NFCS and oxygen radical absorbance capacity, total polyphenols, and total procyanidins in a broad range of cocoa-containing products from the United States. Gu et al. (*10*) also found a strong correlation between the content of NFCS and total procyanidin content (up to decamer level), but by averaging within categories such as cocoa powder or dark chocolate and eliminating individual variation. In a previous study we showed that chocolate could have a large variation in content of individual polyphenols but that there are predictive concentration relationships between them (*12*). On this basis, it was considered that a large variety of individual chocolates might give an indication as to the

* Author to whom correspondence should be addressed (e-mail gary.williamson@rdls.nestle.com; telephone 00 41 21 785 8546; fax 00 41 21 785 8544).

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Table 1. Characteristics of Chocolates Used ($n = 69$)[a]

	plain dark chocolate ($n = 46$, labeled = 32)		milk chocolate ($n = 8$, labeled = 2)		chocolate + inclusions ($n = 15$, labeled = 5)	
	mean	range	mean	range	mean	range
labeled cocoa content[b] (%)	66	34–100	23	20–26[c]	57	28–64[c]
nonfat cocoa solids						
theobromine (%)	0.60	0.26–1.16	0.17	0.09–0.28	0.48	0.25–0.75
calcd nonfat cocoa solids	22.9	9.7–43.9	6.6	3.6–10.5	18.3	9.3–28.7
calcd cocoa liquor content (%)	49.8	21.1–95.5	14.3	7.8–22.7	39.7	20.2–62.3
calcd addition of cocoa butter (%)	11.8	0.0–24.2	14.4	12.2–16.5[c]	6.3	0.0–13.4[c]
polyphenols						
total polyphenols (mg of ECE/g)	10.9	3.4–23.4	5.9	5.2–8.5	7.9	4.1–13.9
sum of six polyphenols (mg/g)	2.00	0.18–4.99	0.67	0.46–1.22	1.64	0.64–3.04
epicatechin (mg/g)	0.78	0.07–1.94	0.27	0.19–0.50	0.64	0.26–1.20
catechin (mg/g)	0.22	0.07–0.52	0.08	0.04–0.12	0.18	0.06–0.40
dimer B2 (mg/g)	0.46	0.04–1.17	0.15	0.11–0.26	0.38	0.15–0.72
dimer B5 (mg/g)	0.09	0–0.24	0.03	0.01–0.05	0.07	0.02–0.15
trimer C1 (mg/g)	0.32	0–0.91	0.11	0.04–0.20	0.26	0.09–0.46
tetramer D (mg/g)	0.13	0–0.39	0.10	0.02–0.44	0.11	0.03–0.23

[a] Total polyphenols were measured according to the Folin−Ciocalteu method reported in epicatechin equivalents (ECE) per gram of chocolate. All other polyphenols are given as milligrams per gram of product. The calculation for cocoa content is described under Methodology. [b] $n = 39$, as only 39 of the 69 chocolates stated a cocoa solids content on the label. [c] Only a limited number in this group had labeled percent cocoa content.

strength of the relationship between high nonfat cocoa content and polyphenol content (both total and individual) and whether it can be used to identify chocolates high in polyphenols, especially those individual cocoa polyphenols considered to be most beneficial for health such as epicatechin (*8*). In the current study, different relationships were examined by means of linear regression analysis for their potential predictability in chocolate, with emphasis on the predictive relationship between NFCS and polyphenol content.

METHODOLOGY

Reference Compounds. A reference sample (SRM 2384 baking chocolate) was obtained from the National Institute of Standards and Technology (http://www.nist.gov/srm) with certified catechin and epicatechin values. (+)-Catechin and (−)-epicatechin were sourced from Sigma. Procyanidin B2 was purchased from Extrasynthèse (Genay, France).

Chocolate Samples. Sixty-nine chocolate samples (46 plain dark, 8 milk, and 15 dark with other added ingredients) were sourced from different countries (Australia, Belgium, Brazil, Canada, France, Italy, Japan, The Philippines, Russia, South Africa, Switzerland, and the United States), covering a wide range of brands. The labeled cocoa contents were in the range of 20–100%, although only 39 chocolates declared a cocoa solids content (**Table 1**).

Polyphenol Extraction and Ultra Performance Liquid Chromatography (UPLC) Analysis. The method used for the chromatographic quantification of individual polyphenols was the same as that recently published (*12*). Briefly, ground chocolate samples were defatted and triple extracted with a mixture of acetone/water/acetic acid (70:28:2). The solvent was evaporated under vacuum at 35 °C and the final extract diluted with water. Cocoa procyanidins were analyzed by UPLC with UV photodiode array detection. Catechin and epicatechin were quantified by comparison with pure external standards. Procyanidins B2, B5, C1, and D were quantified using the epicatechin standard. The repeatability was excellent, leading to very small 95% confidence intervals (i.e., mean ± 0.01 mg/g for catechin, B2, C, and D, epicatechin ± 0.2 mg/g, and B5 ± 0.003 mg/g). All analyses were performed in duplicate.

Total Polyphenols Analysis. One gram of chocolate was treated with 10 mg of protease, and the polyphenols were extracted in hot water for 1 h under reflux and determined by spectrophotometry after reaction with Folin−Ciocalteu reagent. All analyses were performed in duplicate. The results were expressed in milligrams of epicatechin equivalents per gram of chocolate. This method corresponds to the method described in the *Official Journal of the European Communities* (*13*). The 95% confidence intervals are proportional to the mean (±6%).

Theobromine Analysis. Extraction was performed with water/ammonia 4:1 (v/v); the extract was acidified with diluted hydrochloric acid and clarified with Carrez reagents (*14*). The determination of theobromine was achieved by reversed-phase HPLC with UV detection at 274 nm. The 95% confidence intervals are very small (theobromine ± 0.03 g/100 g).

Estimation of NFCS, Cocoa Liquor, and Added Cocoa Butter. The use of theobromine as a marker of NFCS is well established within the confectionery industry (*14, 15*). An average theobromine content of 2.63% has been reported for NFCS, with a 90% confidence interval of 2.36−2.91% (*16*). As a consequence, NFCS can be estimated on average as 38.0 × theobromine, with a 90% confidence interval ranging between 34.4 and 42.4 × theobromine (which indicates that there is a 90% confidence that the true NFCS content will be between 80 and 120% of the estimated average value). Cocoa liquor content is then estimated using NFCS content as a proxy: on average, cocoa liquor = NFCS × 100/46 (*9*). Finally, the addition of cocoa butter is estimated by subtraction [added cocoa butter = labeled cocoa content − cocoa liquor (calculated to contain approximately 54% cocoa butter)].

Data Analysis. Simple linear regression is used to predict polyphenol contents as a function of the estimated NFCS content. The models are built using the dark chocolate data only. The coefficient of determination between two variables (r^2) is used to express how much of the variability of polyphenol content is predicted by the estimated NFCS content. This index is completed by the root-mean-square error (RMSE) that gives the average difference between the observed and predicted values (*17*). The models are then used to predict polyphenol contents of samples other than dark chocolate (i.e., milk and inclusions). The RMSE values of these groups of products are compared to the RMSE of dark chocolate to test whether the precision is comparable. The mean prediction error (MPE), that is, the average bias of the model for a set of samples, was also calculated. By construction of the regression, the MPE index is null for the dark chocolate group. For the two other groups, this index allows estimation of whether the dark chocolate model gives any systemic bias in either direction for polyphenol content.

RESULTS AND DISCUSSION

Chocolate Characteristics. Table 1 gives the means and ranges of labeled cocoa content, theobromine, total polyphenols, and individual polyphenols for the 69 chocolates used in this study. Using theobromine as a proxy, the contents of NFCS, cocoa liquor, and added cocoa butter are estimated. NFCS

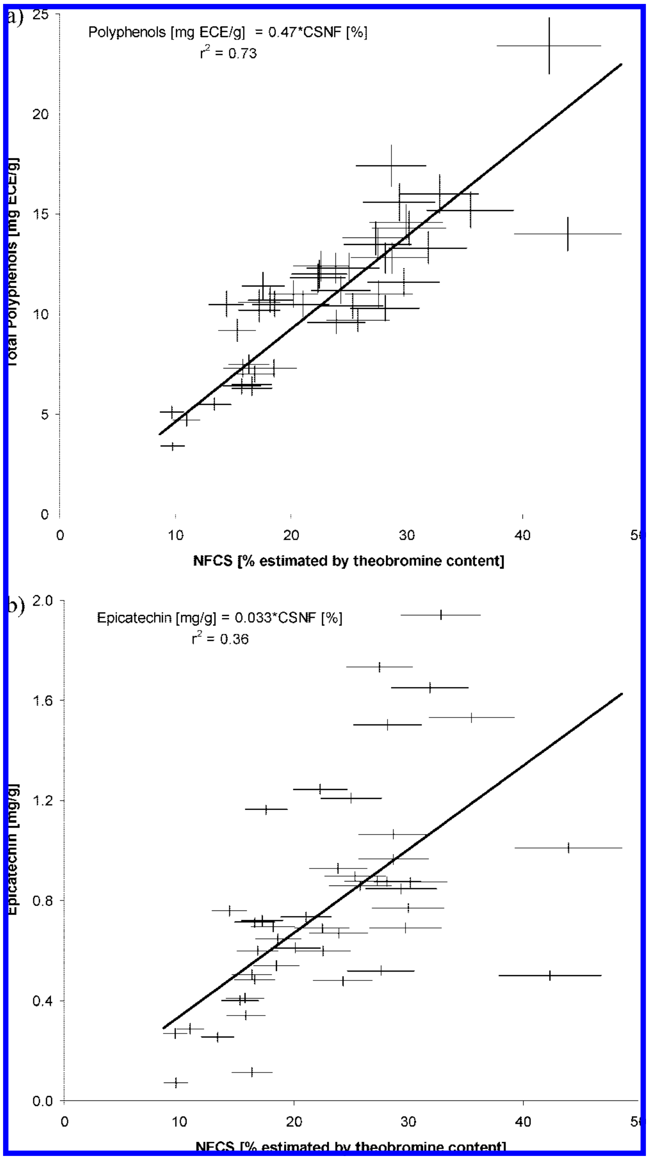

Figure 1. (**a**) Model built using data from 47 plain dark chocolates shows a highly significant relationship between NFCS estimated by theobromine content and total polyphenol content ($p < 0.0001$). Each sample is a cross to visualize the uncertainty of the NFCS estimation as well as the measurement error of total polyphenol content. (**b**) Same model but built for epicatechin concentrations rather than total polyphenols.

content varies between 9.7 and 43.9% in dark chocolate, with a mean of 22.9% (6.6% for milk chocolate and 18.3% for chocolate with inclusions). As a consequence, the cocoa liquor content of dark chocolate, by calculation, varies between 21.1 and 95.5%, with a mean of 49.8% (14.3% for milk chocolate and 39.7% for chocolate with inclusions). Finally, the addition of cocoa butter in dark chocolate varies between 0 and 24.2%, with a mean of 11.8%. Of the 69 chocolates examined, 8 were dark chocolates labeled as containing 70% cocoa solids. On average, these 70% cocoa chocolates contained 0.79 mg/g epicatechin, which accounts for a mean percentage of 40% of the total of six polyphenols (average = 1.99 mg/g, range = 1.26–2.62 mg/g) analyzed by UPLC. However, there is a range of 46–65% of cocoa liquor content as estimated by theobromine content and therefore, by calculation, cocoa butter addition of between 5 and 24%. These data indicate there is a wide variation within a subset of chocolates, which without analysis could be assumed to contain similar concentrations of polyphenols.

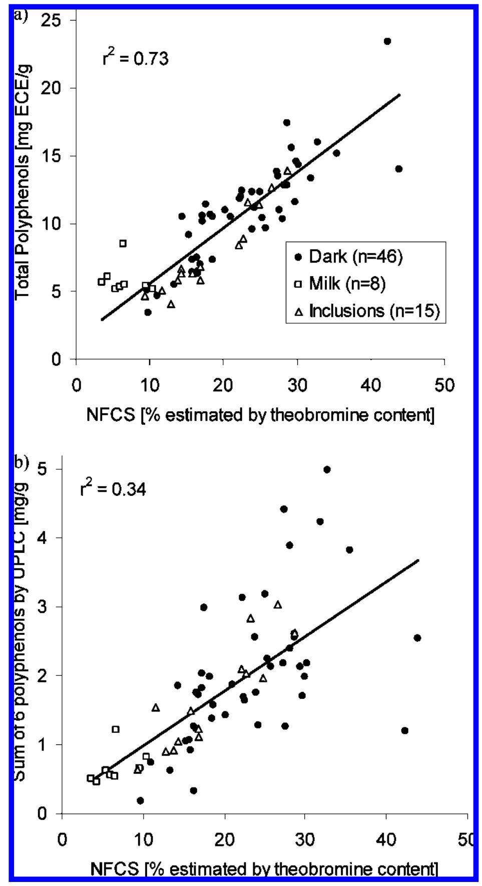

Figure 2. (**a**) Model featuring the same regression line as in **Figure 1a** on the 47 dark samples, but with all other samples plotted on the graph. (**b**) Model with the regression line for dark chocolate only built for the sum of the six individual polyphenols as measured by UPLC, with all other samples plotted on the graph (●, dark chocolate; □, milk chocolate; △, with inclusions).

Prediction Modeling with Dark Chocolate. The data shown in **Table 1** suggest that the percentage cocoa content that appears on the label of chocolate cannot be used accurately by itself to make a prediction of the concentration of polyphenols, because it includes polyphenol-free cocoa butter. However, by eliminating the error from any additional cocoa butter added to the chocolate and from varying intrinsic fat contents from the cocoa bean, it is possible to make a strong predictive model for total polyphenol content from NFCS. **Figure 1a** shows the regression model based on the relationship between total polyphenol content for 46 plain dark chocolate samples and percent NFCS calculated from theobromine. Each point is represented by a cross to indicate the confidence intervals on both axes. The relationship appears to be strong ($r^2 = 0.73$). Approximately one-third of the samples had more, and one-third had less, polyphenols than expected (calculated by the number of crosses that fell to either side of the model without contact with the regression line) from this model, indicating that linear regression is a suitable model for these data. From these data, it could be interpreted that to be 95% confident of having >8 mg of ECE/g of total polyphenols in the chocolate, at least 20% NFCS would be necessary.

Figure 1b is the regression model based on the relationship between epicatechin content for the same set of dark chocolates and percent NFCS. Epicatechin was selected as it is considered to be a major cocoa polyphenol both in concentration and in potential health benefits. The relationship is less strong ($r^2 = 0.36$) than for total polyphenols, making it less predictable from

Table 2. Predictive Model for Total Polyphenol and Individual Polyphenols Based on Plain Dark Chocolate, with Application of the Model to Milk Chocolate and Chocolate with Inclusions[a]

	total polyphenols (mg of ECE/g)	sum of six polyphenols by UPLC (mg/g)	epicatechin (mg/g)	catechin (mg/g)	procyanidin B2 (mg/g)	procyanidin B5 (mg/g)	trimer (mg/g)	tetramer (mg/g)
r^2 (dark)	0.73	0.34	0.36	0.25	0.33	0.36	0.27	0.26
RMSE (dark)	1.89	0.84	0.33	0.09	0.20	0.04	0.15	0.07
RMSE (milk)	2.40	0.22	0.10	0.03	0.05	0.02	0.05	0.02
RMSE (inclusions)	1.49	0.38	0.14	0.08	0.09	0.02	0.05	0.03
MPE (milk)	−1.92	0.00	−0.02	0.03	0.00	−0.01	0.01	0.00
MPE (inclusions)	1.07	0.00	0.00	0.01	−0.01	0.00	0.00	0.00

[a] RMSE, root mean square error, the average distance of the observed value to the model; MPE, mean prediction error, the average bias of the model for these samples (i.e., 0 for dark chocolate samples, because the regression model was obtained on dark chocolate samples only and so by nature of the regression, it is by definition unbiased).

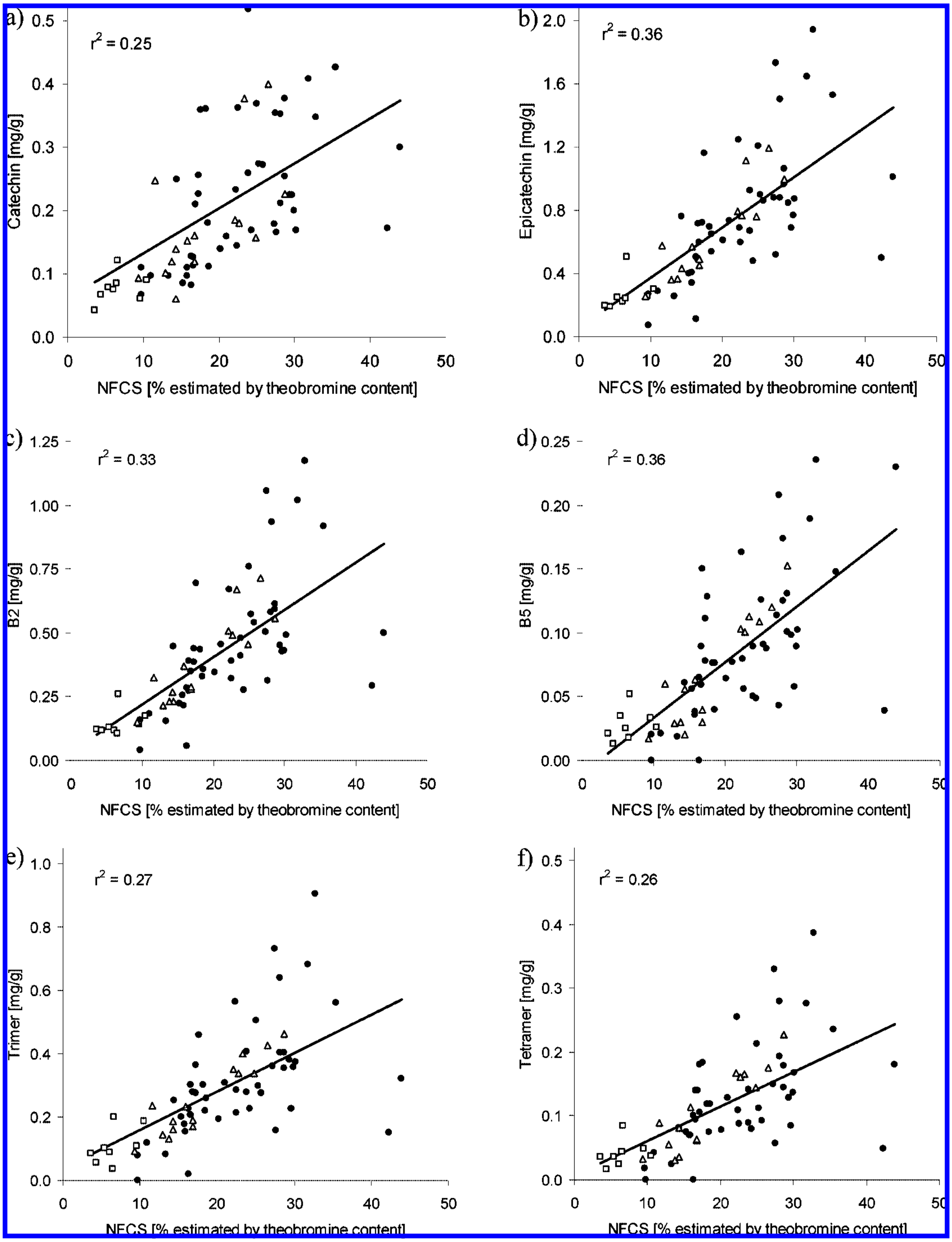

Figure 3. Regression models [based on plain dark chocolate with all other samples (milk, $n = 7$; inclusions, $n = 15$) plotted on the graph] for nonfat cocoa solids and total polyphenols, the six individual polyphenols (●, dark chocolate; □, milk chocolate; △, with inclusions).

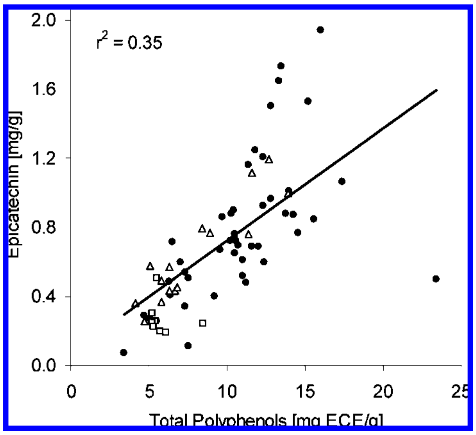

Figure 4. Relationship between total polyphenols and epicatechin content (●, dark chocolate; □, milk chocolate; △, with inclusions).

NFCS content. For example, the range of epicatechin concentrations of the eight dark chocolates labeled "70% cocoa" was ∼2-fold (0.520–1.065 mg/g). The wide range could indicate that the level of epicatechin is influenced by several other factors as it does not correlate well with the cocoa content. Epicatechin levels also do not correlate linearly with the overall polyphenol content, especially in those chocolates considered to be richer in total polyphenols.

Applying the Model to Nondark Chocolate. To see how other ingredients of chocolate influence the relationship between polyphenols and NFCS, the data from milk and other ingredient-added chocolates were evaluated on the same dark chocolate regression plot (**Figure 2a**). An MPE (defined under Data Analysis) of zero would indicate the model estimates correctly the total polyphenol content of these nondark chocolates. However, total polyphenol content in milk chocolate appears to be systematically slightly higher than estimated by this model with an MPE of −1.92 (**Table 2**), whereas chocolate with other added ingredients appears to be estimated closer to the model with an MPE of 1.07, with data points placed below the model or in proximity to it. This can be observed in **Figure 2a**. The RMSE values for both of these groups were very close to that of plain dark chocolate (**Table 2**), indicating that the precision of the model built on dark chocolate should apply similarly to chocolate containing milk and other ingredients. **Figure 2b** is a similar regression for the sum of six major polyphenols measured by UPLC. The correlation with NFCS is much lower than for total polyphenols but without the apparent estimation differences for milk and other ingredients, as shown in **Table 2**. This suggests that either the compounds that could explain the higher result for milk chocolates when using the model in **Figure 2a** are not among the six polyphenols that were determined individually or that there are interferences in the analytical method used for the determination of total polyphenols.

The reason the model for total polyphenols and NFCS appears to give lower estimations than found for the polyphenol content for milk chocolate is not possible to ascertain from these data. It is possible that the milk or its proteins reacted with the Folin reagent to give a slightly higher result than expected. To clarify this phenomenon, the extraction method was checked with milk present to rule out potential interferences. Standard skimmed milk powder was tested alone and gave an apparent value of 9 mg of ECE/g, with sodium caseinate and whey alone giving 14 and 3 mg of ECE/g, respectively. These data indicate that the milk component of milk chocolate could increase the apparent content of cocoa polyphenols to give a higher value than justified by the cocoa content alone. Conversely, the MPE data for the UPLC-measured individual polyphenols (**Table 2**) suggests that the presence of milk introduces no significant interferences for this assay. On the basis of these observations, the Folin assay gives good prediction only in the absence of milk; however, it is still possible to obtain accurate analysis of polyphenols in milk chocolate with the use of UPLC.

The model also appeared to give higher estimations of total polyphenols for inclusions in dark chocolate with an MPE of +1.07. For this data set, the inclusions were fruits, nuts, wafers, caramel, biscuits, etc. Although some of these ingredients may contain polyphenols themselves and therefore contribute to total polyphenols, it is probable that the amount of polyphenols in the inclusions is lower than that from the chocolate displaced by these ingredients. However, the theobromine calculation should correct for this displacement, and the RMSE and MPE were similar to those of dark chocolate for the individual polyphenols, indicating that the factor that leads to higher results is affecting only total polyphenols, in a similar manner as for milk chocolate but without an obvious pattern.

Predicting Total Polyphenol versus Individual Polyphenol Content. Figure 3 is a series of similar regressions for the six individual polyphenols (epicatechin, catechin, procyanidin B2 and B5, trimer C, and tetramer D) against NFCS. The relationships for the individuals are much lower than for total polyphenols (r^2 < 0.4), making them much less predictable from NFCS content but without any systematic estimation differences for milk chocolate or chocolate containing other ingredients. One hypothesis to explain this could be that processing may affect the smaller oligomeric polyphenols to a greater extent than the polymers. Another explanation might be that the ratio among different isomers/oligomers (including the ones not measured by UPLC) can also vary in individual chocolate types, and this variation is considered only in the measurement of total polyphenols.

Figure 4 indicates the correlation between total polyphenols and epicatechin, this individual polyphenol being selected as it is considered to be the most likely candidate for the health effects of cocoa (*8*). It is interesting to note that high total polyphenol content does not always correlate with high epicatechin content. An outlier that has the highest total amount of polyphenols of 23.4 mg of ECE/g contains only 0.50 mg of epicatechin/g, that is, ∼3-fold lower than expected. Another outlier contains only 16.0 mg of ECE/g total polyphenols but 1.94 mg of epicatechin/g, that is, ∼1.8-fold higher than expected. This again supports the hypothesis that the ratio between polyphenols is likely to be affected by origin and processing. Therefore, epicatechin is not easily or reliably predicted from total polyphenol content. This is important as concentrations of this particular polyphenol may affect the overall potential health benefits of the chocolate.

Conclusion. To conclude, the suggested model can be used to predict total polyphenol content of dark plain chocolate from NFCS content. Individual polyphenols, such as epicatechin, demonstrate less predictive relationships overall but without systematic estimation differences when extrapolated to chocolate containing milk and other ingredients. These conclusions could be explained partly by the different sensitivities of different analytical methods to interferences; that is, the UPLC method is less sensitive to protein interferences than the Folin method. In addition, processing conditions may affect the monomeric and oligomeric polyphenols more than the polymers, and/or the ratio among different isomers/oligomers can also vary in individual chocolate types.

ABBREVIATIONS USED

NIST, National Institute of Standards and Technology; RP-UPLC, reverse phase ultra performance liquid chromatography; HPLC, high-performance liquid chromatography; HDL, high-density lipoprotein; THF, tetrahydrofuran; TFA, trifluoroacetic acid; nd, not detectable; ESI, electrospray ionization; CID, collision-induced dissociation; RMSE, root-mean-square error; MPE, mean prediction error.

SAFETY

Apart from standard caution with all solvents and acids, there are no specific safety criteria for this work.

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