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Evolution of cocoa flavanol analytics: impact on reporting and cross-study comparison†

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Cocoa flavanols (CF) are a group of dietary bioactives that have been studied for their potential health benefits for over two decades. In this time, multiple methods for CF testing have evolved, introducing the potential for differences in reported CF content. The reliable characterization of CF content in food and test materials used in clinical studies is critical to comparisons of research studies over time, as well as critical to enabling the systematic reviews and meta-analyses required to support dietary recommendations of bioactives. In this work, we compared two analytical methods that have been widely applied to characterize materials used in clinical research and a method newly recognized by AOAC as the official method for CF analysis. Differences in accuracy of −36% to +20% were observed when comparing CF contents determined with these methods, supporting the notion that CF values determined across methods are not directly comparable. To address differences, a linear regression model was developed to predict CF values. This approach was cross-validated and directly applied to the conversion of CF values published in key scientific papers on the benefits of CF. This work provides a valid tool to compare CF values reported across these different methods and enables comparisons and interpretation of studies investigating the bioactivity of CF.

Introduction

Flavanols, including (−)-epicatechin and (+)-catechin, and their related oligomers, the procyanidins, are dietary bioactives present in foods and beverages like tea,[1,2] apple,[3] grapes,[4] cocoa,[5,6] berries[7,8] and nuts.[9] Among the most researched flavanols and procyanidins are those found in cocoa, collectively referred as cocoa flavanols (CF). CF consist of a mixture of flavanols, mainly (−)-epicatechin, and a mixture of different procyanidins with varying degrees of polymerization (DP), up to ten or more.[6,10] CF are often reported as the sum of all DP and this approach has been widely adopted by clinical researchers and regulatory bodies. Accumulating evidences suggest that the intake of CF mediates beneficial cardiovascular effects and improvements in cognitive performance in humans, supporting a role of these bioactives in primary disease prevention and healthy aging.[11–13] Due to these advancements, industry and regulatory agencies are also showing interest in CF. In this context, the standardization of analytical tools to quantify CF becomes essential to harmonize the reporting of CF values across laboratories and enable wide-ranging comparisons.[14]

Standardization of testing for dietary bioactives is often challenging but essential for developing scientific understanding of a bioactive, as well as delivering consistent quality to ensure the efficacy and safety of consumer products. Although analytical data on the mineral and vitamin composition of commercial products have been well documented,[15,16] the reliable and accurate characterization of botanical bioactives suffers from the lack of accurate testing, reference materials and well defined targeted molecules.[17] In the case of CF, it is essential to provide the tools to characterize the different materials used in clinical research and commercial products with consistency. This would power critical comparisons of scientific research, empower scientists and regulators to determine safe and efficacious levels of intake, and provide means to the development and regulation of CF-containing products available in the market.

Over the last few decades, various methods have been developed for the quantification of CF, using different approaches to address the lack of commercially available reference materials.[6,18–20] The intrinsic complexity of flavanol chemical structures in cocoa-based materials posed challenges to obtain a detailed compositional analysis. One of the first available methods that quantified flavanols/procyanidins up to decamers relied on a composite standard based on flavanols specifically isolated from cocoa;[6,10] the standard was prepared in-house and distributed upon request. Of note is that this method developed by Adamson *et al.* (hereafter referred as Pre-AOAC method; Table 1) was largely intended as a research

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Table 1 Summary of method characteristics including, calibration approach, analytical performances validation, accreditation status and references

	Pre-AOAC	AOAC2012.24	AOAC2020.05
HPLC requirements	Normal phase silica column (Phenomenex-Lichrosphere): fluorescence detection	Normal phase diol column (Phenomenex-Develosil): fluorescence detection	Normal phase diol column (Waters-Torus diol): fluorescence detection
Mode of separation	Normal phase liquid chromatography	Hydrophilic interaction liquid chromatography	Hydrophilic interaction liquid chromatography
Solvents and run time	Dichloromethane, methanol, acetic acid and water; 50 minutes	Acetic acid, acetonitrile, methanol and water; 90 min	Acetic acid, acetonitrile, methanol and water; 16 min
Calibration approach	Commercially available (−)-epicatechin (for DP1) and a mixture of partially purified procyanidins (DP2–10; DP purity ranged from 99% for DP2 to <60% for DP10)	Commercially available (−)-epicatechin (for DP1) and relative response factors (for DP2–10)[a]	Cocoa flavanol extract reference material NIST RM8403 for (DP1–DP7)
Total CF definition	DP1–10	DP1–10	DP1–7
Precision	Not determined	Intermediate precision %RSD = 12% for extract	Intermediate precision %RSD = 2% for extract
Accuracy	Not determined	Not determined	Accuracy 82–105% for total CF from 0.1 to 500 mg g^{-1} (milk chocolate to cocoa extract)
Accredited method	No	Yes – AOAC first action status (2012.24) recommended for repeal	Yes – AOAC first action status (2020.05)
Relevant publications	Ref. 10 and 26	Ref. 18, 22 and 27	Ref. 24, 25 and 28
Weaknesses/ comments	Limited transferability; analytical standard not reproducible, making the method not available today	>90% column failure rate; experimental development limited by use of RRFs; RRF not determined for new matrices	Robustness performances and method versatility enhanced by the use of NIST RM 8403[25]

[a] NIST baking chocolate RM 2384 (not used for quantification; now archived by NIST). Flavanol monomers (DP1); flavanol monomers and procyanidins with a degree of polymerization up to 10 (DP1–10); flavanol monomers and procyanidins with a degree of polymerization up to 7 (DP1–7).

method.[6,10] As such, Pre-AOAC was used for over a decade for the reporting of CF content of materials used in clinical research, as well as for database development.[21] While a useful tool for research, the limitations of this method (Table 1) prevented its wide adoption and application in commercial laboratories, resulting in the need for analytical improvements that could enable reliable transferability and the opportunity for wider adoption.[18,22]

The first step in standardizing CF analysis only occurred in 2012 with the multi-lab validation of the method by Robbins *et al.* and the subsequent recognition as a First Action Official Method of Analysis by AOAC.[18] A key advancement in this method that enable implementation more broadly was the use of relative response factors based on the simple, commercially available monomeric flavanol, (−)-epicatechin. The AOAC2012.24 method offered for the first time a consistent method for a broad range of foods and CF concentrations, opening the door for broader method utilization and the potential for standardization in CF reporting. While a significant advancement at the time, the AOAC2012.24 method had shortcomings that limited its ease-of-use, accuracy and robustness (Table 1), leading to an official recommendation to repeal the method accreditation by an AOAC expert review panel.[23]

More recently, and supported by improvements in analytical technology, a new method was developed by Bussy *et al.* and recently accredited first action status by AOAC (AOAC2020.05).[24] This new method presented significant improvements in analysis time and reliability compared to AOAC2012.24, and more importantly, included the use of a reference material (RM 8403) for CF quantification developed by the National Institute of Standard and Technology (NIST, U. S. Department of Commerce).[25] The availability of such a reference material became critical as, in contradiction with monomeric flavanols for which proper analytical standards have always been available, procyanidins lacked the commercial availability of the chemically diverse reference materials needed to support their quantification.

The Pre-AOAC and AOAC2012.24 methods have been widely used (primarily in research settings) since their development; however, little is known about the differences in reported CF values between these methods. Now with the accreditation of AOAC 2020.05, there is further need to examine and compare reported values across the range of analytical methods that have commonly been applied to CF. Understanding these differences is of primary importance in the evaluation and comparison of the CF reported in the existing literature, and even more important to the integration of our knowledge on the efficacy and safety of CF derived from past and future studies. Thus, the purpose of this work is to examine how the methodological changes have impacted CF reporting, and as needed, develop models that enable comparisons in reported values. We hypothesized that the continuous improvements of CF analytics have led to shifts in method accuracy that would impact reported values of CF. To study this, we compared the differences in CF values reported with Pre-AOAC, AOAC2012.24 and newly approved AOAC2020.05 methods and where possible, investigated whether differences could be assigned to flavanol (DP1) and/or (DP2+) procyanidin quantification. Using these results, we then developed and implemented a model that could permit the direct comparison of CF values when assessed with the different methods that could enable cross-study comparisons.

Materials and methods

CF analysis

CF analysis was done following the procedures and conditions described by Adamson *et al.* (Pre-AOAC),[10] Robbins *et al.* (AOAC2012.24)[18] and Bussy *et al.* (AOAC2020.05).[24] In all methods, CF were resolved based on their degree of polymerization, using fluorescence detection, and then the individual components sum to quantify total CF. The main differences among these methods included: (i) mode of separation, (ii) calibration approach and (iii) total CF definition (Table 1).

Sample sets

Data were collected on a variety of cocoa-based samples (dark chocolate, cocoa powder, cocoa extract and cocoa extract-based materials like dietary supplement capsules and powder drink mixes); the manner these data were collected and compared varied. Because the mixture of partially purified procyanidins used as analytical standards in the Pre-AOAC method is no longer available and it is impossible to reproduce, a direct side-by-side comparison of Pre-AOAC and AOAC2020.05 could not be conducted. Instead, the comparison of these methodologies was based on the re-analysis (using RM 8403 and AOAC2020.05) of samples that were previously analyzed with the Pre-AOAC method (n = 15; 15–470 mg of CF per g per AOAC2020). Importantly, these samples (all solids with very low water activity) were properly stored until being re-analyzed with AOAC2020.05 method. Based on information reported in the literature,[29] the decay of CF would be expected to be minimal under these conditions (<2% per year), thus reducing the risk of significant changes in total CF content over time. A different set of samples was used to establish the comparison between AOAC2012.24 and AOAC2020.05 (n = 45; 4–480 mg of CF per g per AOAC2020) and for the comparison between Pre-AOAC and AOAC2012.24 (n = 26; 17–537 mg g^{-1} per Pre-AOAC). Samples were mostly distributed either below 200 mg of CF per g or above 350 mg of CF per g. This distribution of CF in the samples reflects CF content in different cocoa-based products such as chocolate and cocoa powder (with a CF levels < 200 mg g^{-1}) and materials like cocoa extract and cocoa extract-based materials (with CF levels > 350 mg g^{-1}).

Statistical analysis

Total CF was expressed in mg g^{-1} and used to analyze the differences between methods as well as evaluate and model the bias introduced by each method. Differences between two methods were expressed relative to the average value between the two methods studied for each of the three comparisons established. Data analysis started by creating a Bland–Altman plot for each of the three comparisons to identify possible trends in method differences and evaluate the magnitude and significance of the bias. A 95% confidence interval was estimated on the mean and on a single measurement. If the 95% confidence interval on the mean didn't include 0% difference, the difference between methods was deemed significant. The mean difference was calculated as the average of the relative differences observed across all data points. The 95% confidence interval on the mean was determined as mean difference ± confidence. The confidence was determined as the product of the t-value and the standard error on the relative differences. When significant bias was observed when comparing two methods, the data set was fitted with the appropriate model.

Cross validation

For the model established between AOAC2012.24 and AOAC2020.05, it was possible to run samples side-by-side with the two methods and thus to verify model accuracy. For this purpose, a new set of samples was analyzed with these two methods. This set of samples included total CF contents from 7 to 520 mg g^{-1}, and included dark chocolate, cocoa liquor, cocoa powder, dietary supplement drink mixes, dietary supplement capsules and cocoa extract samples. The predictive model developed in this study was applied to total CF values determined per AOAC2012.24 to predict values that would be determined by AOAC2020.05. The ratio of predicted total CF content to experimental total CF content (AOAC2020.05) estimated the accuracy of the model. The cross-validations of the two other models were not possible due to retirement of Pre-AOAC method (as described earlier).

Results and discussion

Comparison of total CF contents

Differences among the Pre-AOAC, AOAC2012.24 and AOAC2020.05 methods for the determination of CF were investigated using Bland–Altman plots (Fig. 1). No outliers were identified using Grubbs test on relative differences between methods for each of the three models. The results obtained showed significant differences in the CF values reported with the three methods tested, with biases ranging from −36.5% to +19.8% (Table 2). It should be noticed that no significant trend was observed for relative differences between method as function of total CF content, which suggests that differences observed between methods were proportional to the CF content measured.

Given the results obtained, we investigated whether the differences in CF values reported with the tested methods were also observed when assessing the levels of specific CF constituents, particularly monomeric flavanols (DP1). To accomplish this, a linear regression analysis of DP1 contents measured against the average DP1 content across the three methods was performed (Fig. 2). Two outliers were identified when examining DP1 relative differences between methods and were thus removed. Coefficients of determination (R^2) above 0.96 confirmed the linear relation between the DP1 contents measured with each method, and slopes close to 1 reflected the similarity

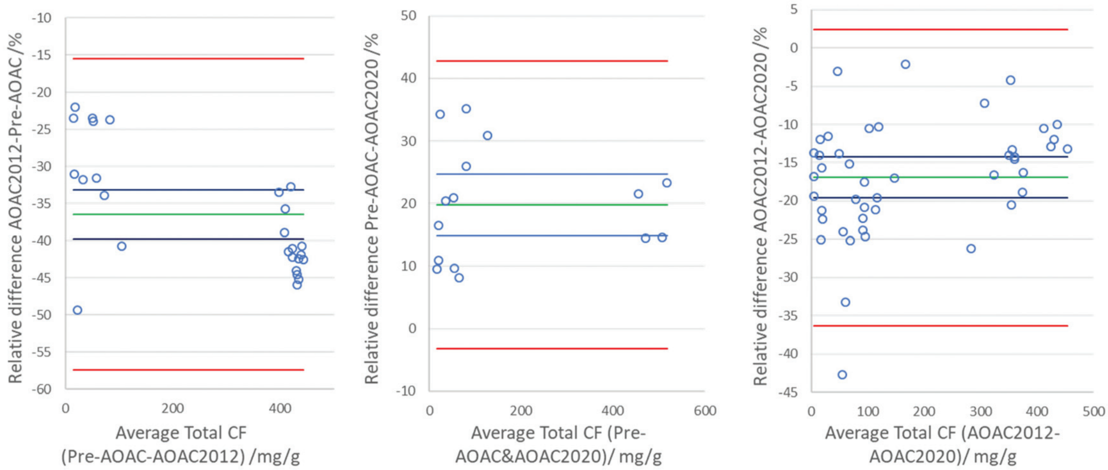

Fig. 1 Bland–Altman plots of differences between total CF determined by two methods (Pre-AOAC, AOAC2012.24 and AOAC2020.05) relative to the average total CF measured by the two methods plotted. For each plot, the green line represents the mean relative difference, the blue line and red lines show 95% confidence interval on the mean and on a single measure, respectively.

Table 2 Summary of comparison between methods with mean relative difference, 95% CI on measure and mean for each of three models. For each model, bias is expressed using the first method as a reference

	Pre-AOAC–AOAC2012.24	AOAC2020.05–Pre-AOAC	AOAC2020.05–AOAC2012.24
Bias (%)	−36.5	19.8	−16.9
95% CI on measure (%)	−(57.4;15.5)	(−3.2;42.8)	(2.4;-36.3)
95% CI on mean (%)	−(33.2;39.8)	(14.9;24.7)	(−14.3;-19.3)

in DP1 levels determined with the different methods. Furthermore, DP1 levels were compared with the values determined using an independent method that was validated for the quantification of DP1 *via* a different analytical approach (AOAC2013.04, data not shown). Thus, the results obtained showed a significant level of agreement in DP1 levels among the methods (ESI†), supporting the notion that Pre-AOAC, AOAC2012.24 and AOAC2020.05 have an acceptable and similar accuracy to quantify DP1 levels. This finding is not entirely unexpected given the long-standing commercial availability of (−)-epicatechin as a reference material in all these methods. Instead, it is possible to argue that the discrepancies in CF values reported among the three methods could correspond to discrepancies in procyanidin quantification. In this context, the reliability improvement associated with the observation and modelling of differences between total CF values determined will mostly be associated with better estimation of procyanidin content (DP2–7). This argument seems to be consistent with the different calibration approaches to quantify procyanidins used by the tested methods (Table 1), and the challenges associated with the assessment of this complex set of compounds.

Development of a model for total CF comparison across methods

In order to model and eventually predict the differences in CF reporting across methods, a linear regression of the total CF content determined with each method was investigated. The graphical representation of these linear models is represented in Fig. 3 and shows that the relationship between total CF measured between each pair of methods is indeed linear. In this context, no significant trend was observed on relative residual (data not shown). The model thus suggests that Pre-AOAC overestimated total CF content on average by 34.0% when compared to AOAC2012.24 (Fig. 3). In addition, it was shown that Pre-AOAC testing overestimated total CF content on average by 20.7% and AOAC2012.24 underestimated total CF on average by 13.0% when these were compared to AOAC2020.05. Importantly, these figures did not change drastically when the samples with a high content of CF were excluded from the linear regression (Fig. 3). The slopes of the Pre-AOAC to AOAC2012.24 and Pre-AOAC (0.72 and 0.87 respectively) to AOAC2020.05 models changed by approximately 0.06 while the slope of the AOAC2012.24 to AOAC2020.05 (1.207) model changed by 0.03. Thus, using these linear models and the confidence interval determined on each slope (Table 3), it would be possible to predict the total CF content comparable to AOAC2020.05 from total CF values determined from Pre-AOAC and AOAC2012.24.

Before application of the models proposed above, the analytical and statistical approach described here was validated. For conversion between AOAC2012.24 and AOAC2020.05 methods, additional samples to the one used to build the model were available. Thus, the cross validation of the AOAC2012.24–AOAC2020.05 model was used to evaluate the appropriateness

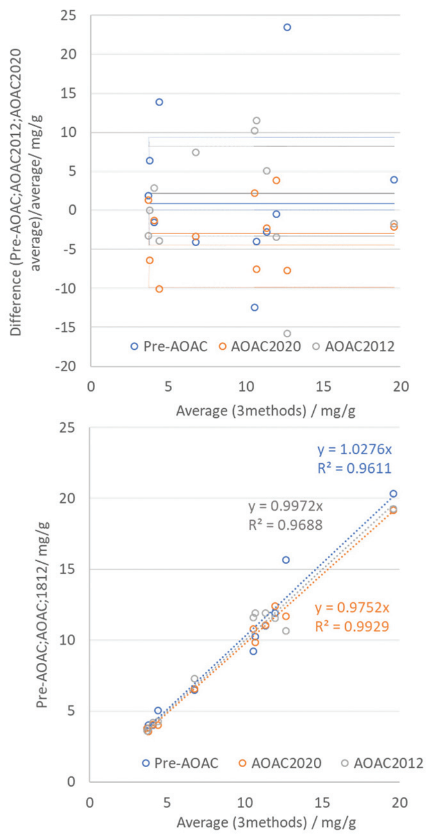

Fig. 2 Comparison of DP1 content across methods; Pre-AOAC (blue), AOAC2012 (orange) and AOAC2020 (grey). Top: Bland–Altman plot of relative difference of DP1 content as a function of the average DP1 content determined with three method. Bottom: Linear regression of DP1 content measured by each method as a function of the average DP1 measured by the three methods.

of the analytical and statistical approach described in this study to build predictive models. Twenty cocoa-based samples covering the range of CF concentrations were analyzed with the two methods side-by-side and using the linear regression model (Table 4). The average model accuracy across 20 samples was 101.9% and single-point model accuracy ranged from 89 to 113% (one Grubbs outlier excluded). This cross validation demonstrated that the model between AOAC2012.24 and AOAC2020.05 can be reliably implemented to estimate CF contents per AOAC2020.05 using data generated using AOAC2012.24. Additionally, the data collected for this cross-validation were used to gain insights on the contribution of each DP to the method bias. The correlation established for each DP (Fig. S2†) confirmed that DP1 was consistent across the two methods and that biases in CF values can be mainly explained by differences in procyanidin quantification. The results showed that the larger the DP, the larger the difference between methods; however it is important to consider that these larger DP contribute less to the total CF. Thus, while the differences are larger with larger DPs, the overall quantitative impact is muted by their smaller contributions to total CF reporting. While linear models could potentially be built for each DP, currently the extremely limited reporting of CF as a breakdown by DP in clinical research limits the applicability of such models. The two models involving Pre-AOAC were built using the same approach than AOAC2012.24–AOAC2020.05 and were thus expected to be reliable and applicable to total CF estimation in historical data.

Table 3 Summary of comparison between methods with slope, coefficient of determination (R^2) and 95% confidence interval on slopes for each of three models

	AOAC2012–Pre-AOAC	Pre-AOAC–AOAC2020	AOAC2012–AOAC2020
Slope	0.660	1.207	0.870
95% CI on slope	0.648–0.671	1.174–1.239	0.857–0.883
R^2	0.9947	0.9962	0.9946

Estimation of total CF content per AOAC2020.05 in published data

Over the last two decades, the Pre-AOAC method, and to a lesser extent AOAC2012.24 method were used to determine total CF content in test materials that were used in numerous clinical trials. The AOAC2012.24 method was mostly adopted for total

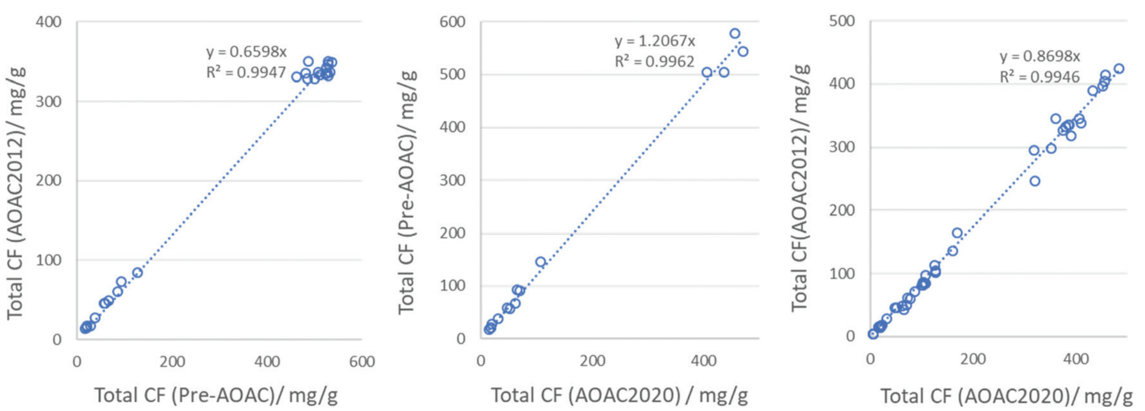

Fig. 3 Linear regression between total CF content determined by the three method Pre-AOAC, AOAC2012 and AOAC2020.

Table 4 Cross validation of predicted total CF content (AOAC2020.05) from total CF determined by AOAC2012.24

Material	AOAC2020.05 (mg g^{-1})	AOAC2012.24 (mg g^{-1})	Predicted AOAC2020 (mg g^{-1})	Relative difference (%)	Model accuracy (%)
Chocolate	7.2	6.8	7.8	9	109
Chocolate	7.4	7.1	8.2	10	110
Liquor	22	21	24	5	105
Liquor	23	21	24	5	105
Powder	69	61	70	1	101
Powder	70	58	67	−5	95
Powder	71	59	68	−4	96
Powder	72	60	69	−3	97
Powder	74	57	66	−11	89
Mix[a]	78	90	104	33	133
Powder	85	76	87	3	103
Mix	94	92	106	13	113
Mix	96	89	102	6	106
Mix	118	111	128	8	108
Capsule	269	228	262	−3	97
Extract	388	337	387	0	100
Extract	390	341	392	1	101
Extract	494	445	511	3	103
Extract	503	439	505	0	100
Extract	518	450	518	0	100

[a] Denotes Grubbs outlier based on relative differences between predicted and measured value.

CF assessment by industry and regulatory agencies. In order to provide continuity in scientific literature, total CF values determined with the Pre-AOAC and AOAC2012.24 methods must be converted to AOAC2020.05 equivalents. To accomplish this, studies were selected for the significant scientific advancement they represented in the determination of CF efficacy and safety levels of intake. Because CF were measured and reported as the sum of all degree of polymerization, the breakdown of CF content by degree of polymerization was not available for material used in clinical research. Thus, a comparison focused on DP1 such as the one implemented in this manuscript to understand method biases is not possible. For each study, an estimated CF content range was calculated using the 95% confidence interval on the slopes from linear models shown in Table 2. An example of this calculations is as follows, Davison *et al.* reported a CF content of 902 mg in their clinical research material.[30] The slope of the linear modeling of Pre-AOAC into AOAC2020.05 determined in this study was 1.207, with a 95% CI on the slope of 1.174–1.239, which leads to an CF estimate of 747 ± 21 mg per AOAC2020.05 method. The rest of the AOAC2020.05 CF estimates are shown in Table 5 and ranged from 176 to 990 mg. The findings reported by Davison *et al.*, alongside other clinical research studies, have been used to perform systematic reviews and meta-analyses to determine efficacy levels of CF across different health outcomes.[30–36] It is anticipated that the model developed here can be used to strengthen the comparison of clinical studies. In this context, it is insufficient to solely identify the content of CF reported, but it is required to identify how research materials were analytically characterized in different studies to identify the adequate model to estimate CF content per AOAC2020.05. There are many more clinical researches articles discussing the health benefits of CF. Some of these studies detailed the method used for CF characterization[37–39] and the CF content reported could thus be estimated for AOAC2020.05. Unfortunately, CF characterization details in many other studies were not reported which makes the estimation of CF content per AOAC2020.05 impossible. This fact highlights the importance of the reporting of analytical test methods in conjunction with the compositional analysis of the test materials used.

Although not widely used for clinical research material characterization, other methodologies different from the ones tested in this study are available for CF quantification, including but not limited to reverse phase HPLC, thiolysis or LC-MS.[4,19,40] These methods provide wide ranging of flavanol/procyanidin values given the different nature of each analytical approach and the limited availability of flavanol/procyanidin reference materials commercially available. This variety of methods raised the challenge of including new methodologies that may not allow for the direct comparison with AOAC2020.05. In this context, (−)-epicatechin and the total monomeric content (DP1) should be considered as a reference point for comparison across studies. As presented earlier, the monomeric content (DP1) is not significantly impacted by the change in methods over time (Fig. 2), and this phenomenon is expected to be true across a wide range of analytical methods given the long-standing commercial availability of the simple monomeric flavanols.

The relevance of (−)-epicatechin goes beyond its use as a reference standard in analytical testing as it is also considered the main active molecule in CF. While the potential health benefits of procyanidins are still emerging, their direct biological activity is most likely related to actions in the gastro-intestinal tract as these components are not readily absorbed intact into the circulation.[41–43] Procyanidins are catabolized into a range of ring fission products by the microbiota in the colon that are then absorbed, metabolized and present in systemic circulation.[41,42,44] However, the benefits that these ring fission

Table 5 Cocoa flavanol and procyanidin contents in clinical research materials and estimated AOAC2020.05 contents

Authors	Title	Method used	CF intake amount	AOAC2020.05 estimate ± 95% CI	Ref.
Fisher and Hollenberg	Aging and vascular responses to flavanol-rich cocoa	Pre-AOAC	205 mg 821 mg	170 ± 5 mg 680 ± 19 mg	50
Heiss *et al.*	Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers	Pre-AOAC	176–185 mg	146–153 mg	51
Schroeter *et al.*	Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans	Pre-AOAC	917 mg	760 ± 18 mg	48
Davison *et al.*	Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects	Pre-AOAC	451 mg 902 mg	374 ± 11 mg 747 ± 21 mg	30
Desideri *et al.*	Benefits in cognitive function, blood pressure, and insulin resistance through cocoa flavanol consumption in elderly subjects with mild cognitive impairment: the Cocoa, Cognition, and Aging (CoCoA) study	Pre-AOAC	520 mg 990 mg	431 ± 12 mg 820 ± 23 mg	52
Brickman *et al.*	Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults	Pre-AOAC	900 mg	746 ± 21 mg	53
Ottaviani *et al.*	Safety and efficacy of cocoa flavanol intake in healthy adults: a randomized, controlled, double-masked trial	Pre-AOAC	1000 mg 1500 mg 2000 mg	829 ± 23 mg 1243 ± 35 mg 1657 ± 47 mg	54
Sansone *et al.*	Cocoa flavanol intake improves endothelial function and Framingham risk score in healthy men and women: a randomised, controlled, double-masked trial: the Flaviola health study	Pre-AOAC	450 mg 900 mg	373 ± 10 mg 746 ± 21 mg	55
Gratton *et al.*	Dietary flavanols improve cerebral cortical oxygenation and cognition in healthy adults	AOAC2012.24	681 mg	783 ± 12 mg	56
Rodriguez-Mateos *et al.*	Assessing the respective contributions of dietary flavanol monomers and procyanidins in mediating cardiovascular effects in humans: randomized, controlled, double-masked intervention trial	AOAC2012.24	690 mg	793 ± 12	49

products offer are not yet clearly understood. In contrast, the monomeric components, and notably (−)-epicatechin, are absorbed and present as phase II metabolites in the systemic circulation[45–47] and have been proven to modulate vascular function and confer health benefits.[48,49] So while understanding the impact of these methods on the reporting of total CF content is important for the comparison and evaluation of studies, the consistency in monomeric flavanol content across studies may serve as a useful means for comparison, particularly in cases where the biological activity is dependent on systemic absorption.

Conclusion

Over the past two decades, multiple methods for the quantification of total CF content have been developed, employing different chromatographic as well as quantification approaches that introduced potential discrepancies or differences in reported CF content. For the first time, the impact of these methodological differences on CF reporting was evaluated and modelled against several methods, including two methods accredited within the past decade. This study demonstrated through the example of CF that as methods and calibration approaches changed, it should be anticipated for botanical bioactive contents reported to change as well. It was possible to develop models that enabled comparisons and translation of total CF content across methods.

The learnings associated with changes in cocoa flavanol and procyanidins testing methodology can be extended to other botanical bioactives. While the accreditation of method guarantees that method performances have been thoroughly validated and reviewed, it does not address the systematic technical gaps that are inherent to the testing of complex botanical bioactives. The most challenging part of botanical testing remains the access to reliable reference materials that capture the structural diversity and distribution of natural bioactives which can be different from one botanical source to another. One alternative to the comparison of total cocoa flavanol contents is to limit the comparison and standardization of bioactives to the well-defined and characterized entities (*e.g.* (−)-epicatechin or total CF monomeric contents for cocoa). This could offer a practical and achievable resolution and improve the characterization, standardization and research on food bioactives.

With the continued and growing interest in the use of botanicals in dietary supplements, there is a need to develop reliable and transferable methods of analysis to advance research. Advancements in analytical methodologies are expected but can pose distinct challenges for botanical bioactives, particularly if the characterization of botanical bioactives requires the analysis of complex mixtures instead of single components. Through the example of flavanol and procyanidins in cocoa, this study demonstrated that the standardization and harmonization of bioactive content reporting does not need to be compromised by changes in analytical methods, especially if these changes seek to improve analytical tools. The biases introduced by the inevitable but beneficial continuous improvements in analytical characterization methodology, while significant, can be accommodated through method comparisons and development of statistical modelling. The results of this work highlight the importance of considering not only the quantitative reporting of bioactives, but the need to include and evaluate the analytical methods that underlie reporting so as to enable appropriate study comparisons that can ultimately lead to public health recommendations regarding the efficacy and safety of bioactives.

Conflicts of interest

Ugo Bussy, Javier I. Ottaviani and Catherine Kwik-Uribe are currently employed by Mars Incorporated, a company engaged in flavanol research and flavanol-related commercial activities.

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