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Fluorescent detection of (-)-epicatechin in microsamples from cacao seeds and cocoa products: Comparison with Folin-Ciocalteu method

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Abstract

Polyphenolic compounds of the flavanoid family are abundantly present in cacao seed and its cocoa products. Results from studies using cocoa products indicate beneficial effects of flavanols on cardiovascular endpoints. Evidence indicates that (-)-epicatechin is the main cacao flavanol associated with cardiovascular effects, so the accurate quantification of its content in cacao seeds or cocoa products is important. Common methods for the quantification of phenolic content in cocoa products are based on the reaction of phenols with colorimetric reagents such as the Folin-Ciocalteu (FC) In this study, we compared the FC method of phenolic determinations using 2 different standards (gallic acid and (-)-epicatechin) to construct calibration curves. We compare these results with those obtained from a simple fluorometric method (Ex₂₈₀/Em₃₂₀ nm) used to determine catechin/(-)-epicatechin content in samples of cacao seeds and cocoa products. Values obtained from the FC method determination of polyphenols yield an overestimation of phenol (flavonoid) content when gallic acid is used as standard. Moreover, the epicatechin is a more reliable standard because of its abundance in cacao seeds and cocoa products. The use of fluorometric spectra yields a simple and highly quantitative means for a more precise and rapid quantification of cacao catechins. Fluorometric values are essentially in agreement with those reported using more cumbersome methods. In conclusion, the use of fluorescence emission spectra is a quick, practical and suitable means to quantifying catechins in cacao seeds and cocoa products.

Keywords

Cacao seed; Cocoa products; *Theobroma cacao*; Phenolics; Rapid method of analysis; Folin-Ciocalteu; fluorescence; flavonoids; flavanols; (-)-epicatechin; catechin; Food analysis; Food composition

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Introduction

Polyphenolic compounds of the flavonoid family are abundantly present in natural products (Natsume, et al., 2000). Plants such *Theobroma cacao* produce a diversity of phenolic compounds where flavonoids, anthocyanins and tannins are the major phenols present (Sanbongi, et al., 1998, Tomas-Barberan, et al., 2007). Cacao seeds contain, by weight, very large amounts of flavonoids (in particular the flavanols catechin and (-)-epicatechin) (Cienfuegos-Jovellanos, et al., 2009). Catechin and (-)-epicatechin are present in cacao in a ratio of $\approx 40/60$. Studies of cocoa products indicate beneficial effects of (-)-epicatechin on cardiovascular parameters such as blood pressure (hypertension) (Corti, et al., 2009). Flavonoids also exert other beneficial health-related effects. These compounds reportedly act as free radical scavengers and inhibitors of eicosanoid biosynthesis; in model systems, they also reduce low-density lipoprotein oxidation, prevent platelet aggregation and protect the heart from ischemia-reperfusion injury (Ainsworth and Gillespie, 2007, Xu, et al., 2007, Yamazaki, et al., 2008). The flavanol content of cocoa products varies with the origin of the cacao seeds and their subsequent processing. Workers in this field need a rapid and reliable method of quantifying and comparing catechin content of various samples so that accurate concentration-dependence data can be obtained.

The most common method for the quantification of phenolic content in plant products is based on the reaction of phenols with a colorimetric reagent such as Prussian blue (Budini et al., 1980) or Folin-Ciocalteu (FC) (Ainsworth and Gillespie, 2007). This method relies on the reaction of phosphomolybdate and phosphotungstate with phenolic and polyphenolic compounds (Ainsworth and Gillespie, 2007; Cicco, et al., 2009). A major limitation of the FC method is its lack of specificity, since other products of oxidation can interfere with the measurements, thereby causing over-estimation of the true polyphenol content (Ainsworth and Gillespie, 2007). Although this colorimetric method, producing an “apparent” polyphenol content, is not very reliable, it is routinely used in various laboratories for phenolic determinations. During a comparison of different cacao products, it can be useful to give us an approximate idea of (-)-epicatechin (EPI) and catechin (CAT) level, since their monomers and polymers are abundant in cocoa samples. On the contrary, a simple and rapid fluorometric method facilitates a quantification of the EPI/CAT content in cocoa-containing products and in biological samples such as human blood.

In an effort to improve the quantification of catechin(s) content without the use of complex methodologies such HPLC and mass spectrophotometry, we measured polyphenol content present in cacao seeds and cocoa products with the FC method using gallic acid or EPI as standards and compared the values obtained with those derived from the fluorescent spectra of catechins ($\text{Ex}_{280}/\text{Em}_{320}$ nm) (Cho, et al., 1989).

Material and Methods

Defatting of samples

The samples used in this study are noted in table 1. For defatting 1 gram of sample was added to 3.5 ml of hexane, vortexed, centrifuged (1,350 g for 15 min) and the supernatant discarded. The resulting pellet was re-extracted 2 more times by the same procedure and the final hexane in pellets was evaporated to dryness under a stream of N_2 . For assay, samples were reconstituted in water.

Polyphenol extraction from cacao seeds and cocoa products

Fifty milligrams of the defatted sample was added to 0.5 ml of the extraction solvent (acetone: water: acetic acid: 70:29.5:0.5) and vortexed until the sample was homogeneously distributed. The sample was then incubated (37°C , 30 min) with continuous stirring.

Samples were centrifuged (3,600 g, 15 min). The extraction was repeated once more and all the supernatants mixed. Solvents were evaporated until the volume was ~50 μL . The extract obtained was used to measure total polyphenol content following the Singleton-Rossi modified FC method or the fluorometric approach (Roura, et al., 2006).

Folin-Ciocalteu assay

Assays were performed using FC reagent from Sigma-Aldrich, sodium carbonate buffer and gallic acid, EPI or CAT as standards. Standards were dissolved in distilled H_2O at room temperature and the developed color assessed at 765 nm. The assay was run in a total final volume of 1000 μL . Aqueous samples or standards (100 μL) were added to test tubes containing 700 μL of distilled water and 50 μL of FC reagent. The mixtures were vortexed and allowed to equilibrate (20°C, 10 min) after which, color was stabilized by the addition of sodium carbonate (150 μL of a 0.7 mM solution); then, the mixture was vortexed and incubated at 40 °C for 20 min. Tubes were cooled rapidly in ice and 200 μL of each sample were placed into a 96 well microplate. The developed color was read at 765 nm using a microplate reader (uQuant, Biotek, Instruments Inc.). All samples were assayed at least in triplicate and the phenolic content was expressed as μM (mean $\pm$ SD).

Fluorescence Assay

Here, 1 μL of blanks, standards or de-fatted samples was diluted to 600 μL using distilled water and fluorescence assessed at $\text{Ex}_{280}/\text{Em}_{320}$ nm using a spectrofluorometer (Perkin-Elmer LS55). Samples were assayed at least in triplicate.

Statistical analysis

All standard curves were analyzed using a linear regression of data calculating the coefficient of correlation (r) at a 95% confidence level. Statistical significance = $P \leq 0.05$.

Results

FC standard curves

Gallic acid, EPI and CAT standard curves obtained using the FC methodology were linear over the range of 10-140 μM of the polyphenol (Figure 1). The gallic acid standard curve has a relatively small slope, the slope using EPI was more favorable for a sensitive assay. There was no difference between EPI and CAT curves (data not shown); thus, we make further reference only to EPI standard curves. The slopes of the two curves in Figure 1 are statistically different ($p < 0.001$) and, confirming literature data, therefore the phenolic content in cacao samples can be misinterpreted when gallic acid is used as the standard in the FC assay. Basically, use of gallate as a standard in assessing EPI and CAT will lead to over-estimation of the EPI/CAT content by the ratio of the slopes, or by about 57% (difference between slopes). Concentrations lower than 20 μM with gallate as a standard and 10 μM with EPI as standard resulted in undetectable measurements (Figure 2B insert)

Fluorescence standard curves

The fluorescence emission spectrum of EPI ($\text{Ex}_{280}/\text{Em}_{320}$ nm) is shown in Figure 2A. Emission intensity increases as a linear function of the concentration, with a sensitivity of ~0.5 μM (Figure 2B). Given the lack of difference between EPI vs. CAT slopes, we only make further reference to EPI curves.

Total phenolics from FC method using gallic acid or (-)-epicatechin standard curves

The comparison of values is presented in Figure 3. As demonstrated above (Figure 1), use of the FC assay with gallate as a standard gives consistently higher values for all samples,

falsely high. The total polyphenol content measured by FC using gallic acid as the standard was 151 μM for the sample with 85% cacao content. Milk-40% had the lowest content of polyphenols (46 μM). Natural cocoa powder had 194 μM . The untreated dry cacao seed had the highest concentration (206 μM). When using EPI to generate a standard curve the optical densities recorded resulted in ~36% decrease in polyphenol concentration vs. gallic acid standard. With this approach we found that the chocolate sample with 85% cacao content had 97 μM of polyphenols, Milk-40% = 30 μM , Natural cocoa = 124 μM and cacao seeds = 132 μM .

FC vs. fluorescence method

Using the fluorometric method, cacao seeds and natural cocoa powder were the products with the highest content (47 and 44 μM , respectively). On average (Table 2), the relative content of EPI/CAT-detected by fluorescence was approximately 35% of FC detected total polyphenols using EPI as the standard. Figure 3 reports the average value of 3 polyphenol extractions and contrast the FC gallic acid, FC EPI and fluorometric methods. As can be observed, the relationship between values obtained from FC total polyphenols vs. fluorescence-detected catechins is maintained in any of the samples assayed, independently of the standard curve used. These results suggest that the fluorometric method is a reliable and simple technique to detect EPI and CAT in samples.

Discussion

Results of the study indicate the following that, first, catechin fluorescence spectra can be reliably used to obtain accurate measurements of cocoa flavanol content. This methodology is precise and rapid and does not use special reagents or incubations, making it adequate to improve measurements in cocoa products. Second, our results also suggest that other flavonoids present in cocoa (e.g. quercetin) may also be adequately quantified using its specific fluorescence emission spectra.

HPLC and mass spectrophotometry methodologies are the gold standard for the quantitative analysis of flavonoids in samples. These methods require complex instrumentation not readily available in many laboratories. In addition, they rely on the use non-environmentally friendly solvents. For these reasons, more traditional colorimetric methodologies may be preferred. The FC colorimetric assay is a useful technique to determine total phenolics. Nevertheless, during a comparison of different cacao products, it can be useful to give us an approximate idea of (-)-epicatechin (EPI) and catechin (CAT) level, since their monomers and polymers are abundant in cocoa samples. Usually, the content of polyphenols in plant samples is calculated using gallic acid as a standard, expressed as gallic acid equivalent. However, if the main objective is to estimate the concentration of catechins, gallic acid is not the ideal standard because it has a monophenolic structure and catechins have more than 2 phenolic groups. With this issue in mind, we implemented an alternate standard curve using EPI and compared the observed results with those obtained using gallic acid. Results demonstrate that the gallic acid FC and EPI FC standard curves slopes are statistically different. Using gallic acid results in a ~36% overestimation of polyphenol (flavonoid) content in the analyzed samples.

The FC method is also not specific; substances such as sugars, aromatic amines, sulphur dioxide, ascorbic acid, and organic acids and bases (including some commonly used buffers as Tris) can react with the FC reagent (Roura, et al., 2006). The presence of these substances can thus also contribute to the overestimation of flavonoid content. In an attempt to develop a fast, economical and simplified approach to quantify cocoa catechins, we implemented a fluorometric approach. For this purpose, we took advantage of the fluorescent properties of EPI/CAT. The resulting assay is an order of magnitude more sensitive than the absorbance-

based assay. The fluorescence assay data also agree well with determination made by advanced techniques.

The amount of catechins determined by fluorescence, ~35% by sample weight, agrees with the content reported using mass spectrometry (~40%) in similar samples (Joseph, 2008). The 5% difference may be related to the presence of procyanidins (polymers of 4, 6, 8 EPI/CAT) since the fluorescence quantification of these products is not quite linear and in fact, their presence appears to interfere with (i.e. decrease) fluorescence emission (Joseph, 2008, Tomas-Barberan, et al., 2007). It is also possible that the contamination of samples with proteins, which also emit at Ex₂₈₀/Em₃₂₀ spectra, may explain the discrepancy. However, this is likely not the case since the fluorescence in any given sample is ~35% of FC values, including the sample with a high content of milk (which has casein).

Conclusion

Given the abundance of catechins in cacao and the relationship between total phenolics and catechins, we can conclude that the FC method for the measurement of flavanoid content in cocoa samples can be implemented by using EPI as a standard since it allows a better quantitative approximation. The use of fluorescence emission spectra is a quick, practical and highly quantitative approach to measure EPI/CAT in cacao and cocoa samples.

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Figure 1.

Comparison of gallic acid and EPI standard curves with FC method. Concentration-OD standard curves using gallic acid (slope = 7.753×10^{-3} , $r^2 = 0.99754$) (closed circles) or (-)-epicatechin (slope = 1.2226×10^{-2} , $r^2 = 0.99717$) (n=3) (open circles). The slopes are statistically different ($p < 0.001$).

Figure 2.

(A) Representative fluorescence spectra (Ex 280/Em 320 nm) of increasing concentrations of (-)-epicatechin. (B) Concentration-OD curve of (-)-epicatechin (n=3) (slope = 19.5249, $r^2=0.999$). Low concentrations of (-)-epicatechin (graph insert) measured with the FC method were not detected.

Figure 3. Relative comparison of flavonoid concentration in analyzed samples. FC methodology using gallic acid as standard (black bars), FC methodology using (-)-epicatechin as standard (gray bars) and, fluorescence(Ex $_{280}$ /Em $_{320}$ nm) methodology (dashed bars).

Table 1

Listing of cacao products used in the study

ORIGINAL SAMPLE (1 g)	DEFFATED SAMPLE Dry weight (g) mean $\pm$ SD
PULVERIZED RAW CACAO SEED	0.51 $\pm$ 0.07
NATURAL COCOA POWDER	0.82 $\pm$ 0.06
DARK 85%	0.56 $\pm$ 0.05
DARK 72%	0.59 $\pm$ 0.06
DARK 70%	0.56 $\pm$ 0.07
HERSHEY'S 60%	0.63 $\pm$ 0.09
DARK 60%	0.56 $\pm$ 0.07
MILK 40%	0.51 $\pm$ 0.08

Table 2

Comparison of polyphenol concentration with Folin-Ciocalteu (gallic acid or epicatechin standards) and fluorescence methodologies in cocoa samples.

SAMPLE	GA std curve F-C μM (mean $\pm$ SD)	EPI std curve F-C μM (mean $\pm$ SD)	EPI vs. GA F-C std curves % detection	EPI std curve Fluorescence Ex ₂₈₀ /Em ₃₂₀ nm $\pm$ SD	Fluorometric vs. F-C EPI % detection
PULVERIZED CACAO SEED	206 $\pm$ 8	132 $\pm$ 5	64.2	47 $\pm$ 3	35.5
NATURAL COCOA POWDER	194 $\pm$ 6.5	124 $\pm$ 3.5	64	44 $\pm$ 2.5	35.5
DARK 85%	151 $\pm$ 6.5	97 $\pm$ 3.7	64.2	33.5 $\pm$ 3	34.6
DARK 72%	109 $\pm$ 5.5	70 $\pm$ 3	64.5	26.5 $\pm$ 2.5	36
DARK 70%	108 $\pm$ 5.5	70 $\pm$ 3.5	65	21.5 $\pm$ 2.5	31
HERSHEY'S 60%	111 $\pm$ 5	71 $\pm$ 3	64	25 $\pm$ 2	35
DARK 60%	78 $\pm$ 5	50 $\pm$ 3.7	64.5	17.2 $\pm$ 2	34
MILK 40%	46 $\pm$ 5.5	30 $\pm$ 3.5	65	11.8 $\pm$ 1.5	39

GA= Gallic acid; EPI= epicatechin