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Re-examination of Chromogenic Quantitative Assays for Determining Flavonoid Content

Yu-Chi Ho,[†] Hui-Tzu Yu,[§] and Nan-Wei Su*[,†]

[†]Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan

[§]Department of Food Science, Fu Jen Catholic University, New Taipei 24205, Taiwan

ABSTRACT: Flavonoids in plants have gained worldwide attention because of their benefits for human health. This study compared three analytical procedures commonly used for determining flavonoid content in plant samples in terms of chromogenic relationships and the reaction products of different flavonoid structures by means of using flavonoid standards with flavone, flavonol, flavanone, flavanol, and isoflavone and analytes such as phenolic acids commonly found in plant extracts. Procedure A produced a stable color reaction between 3-hydroxy-4-keto-flavonoids (flavonols) and 5-hydroxyflavones and was highly sensitive. Procedure B produced color reactions among most of the flavonoids, but the reaction products had different colors and faded over time. Procedure B also produced a color reaction with caffeic and chlorogenic acid. Procedure C was the most sensitive. It produced a color reaction and, like procedure A, could be used to quantify flavonols and 5-hydroxyflavones, but also showed color reaction toward caffeic and chlorogenic acid. On the basis of the results, the current three procedures are not satisfactory for determining all of the types of flavonoid. Two issues needed to be clarified before a promising determination of flavonoid content could be performed with chromogenic assays. The first is a survey of the literature to screen the possible predominant component of flavonoid in analytes. The other is guided by the predominant flavonoid; a promising calibration curve for flavonoid detection can be established on the basis of the selection of an appropriate method and a chemical standard with an equivalent dose response to the predominant flavonoid.

KEYWORDS: *flavonoid, aluminum chloride, catechin, flavonol, flavone*

INTRODUCTION

In plants, flavonoids are a class of polyphenolic compounds synthesized by the phenylpropanoid pathway. In recent years, flavonoids were found to have particular biological activities and therefore have gained increasing attention. Previous studies showed that flavonoids have antibacterial,[1] antioxidizing,[2−4] and anti-inflammatory[5] activities, as well as functions for prophylaxis of cancer and cardiovascular diseases.[6,7]

A consistent analytical method for the quantification of flavonoids is lacking because the types and contents of flavonoids vary by plant species. The Folin−Ciocalteu assay based on determining the reducing power of analyte is commonly employed to deduce the total polyphenolic content of analyte, and results are often expressed as gallic acid equivalent, whereas the data cannot represent the flavonoid content of samples. Furthermore, several published papers described chromogenic approaches to quantify total flavonoid content in samples. Three procedures for chromogenic analysis have been used to determine flavonoid content in plant extracts: two involve aluminum ions but different reaction conditions for color reactions;[8−11] another allows 2-aminoethyl diphenylborate to form a colored complex with flavonoid.[12] In terms of classifying chemical structures of natural products, flavonoid compounds are types of polyphenolic compounds. However, in the papers, flavonoid content was higher than total phenolic content in plant samples, which is unusual.[13,14] Although the two quantifying approaches both use an appropriate standard as a calibration curve for detection, whether the standards selected have equivalent dose responses may adversely affect the results.

In this study we performed Folin−Ciocalteu colorimetry and compared the three procedures in quantifying the content of flavonoids, specifically flavone, flavonol, flavanone, flavanol, and isoflavone and associated glycosides with different structures as analytes, in plant samples. Moreover, because most previous studies involved the use of plant extracts as experimental samples, we investigated whether the common phenolic acids, such as gallic, caffeic, ferulic, and chlorogenic acid, in plant extracts interfere with the quantification of flavonoids; all of the analytes were also subjected to Folin−Ciocalteu assay for comparison. Additionally, we investigated the possible chromogenic mechanism of the three procedures.

MATERIALS AND METHODS

Materials. The chemical standards of flavonoid including flavone (6-hydroxyflavone, apigenin), flavonols (fisetin, quercetin), flavanone (hesperetin), flavanols (catechin, epicatechin), and flavonol glycoside (rutin) were from Sigma-Aldrich (St. Louis, MO, USA). Soy isoflavone (with genistein 11.9%, daidzein 8.0%, genistin 0.5%, and daidzin 0.3%) was from Glory Biotech (Taipei, Taiwan). The phenolic acids gallic, caffeic, ferulic, and chlorogenic acid were from Sigma-Aldrich. Sodium nitrite, potassium acetate, aluminum chloride, and sodium hydroxide were from Wako Pure Chemical Industries (Osaka, Japan). Folin−Ciocalteu's reagent and methanol were from Merck KGaA (Darmstadt, Germany). 2-Aminoethyl diphenylborate was from Sigma-Aldrich. All other chemicals were of analytical grade and

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obtained from local suppliers in Taiwan. The chemical structures of the flavonoids and phenolic acids used in this study are in Figure 1.

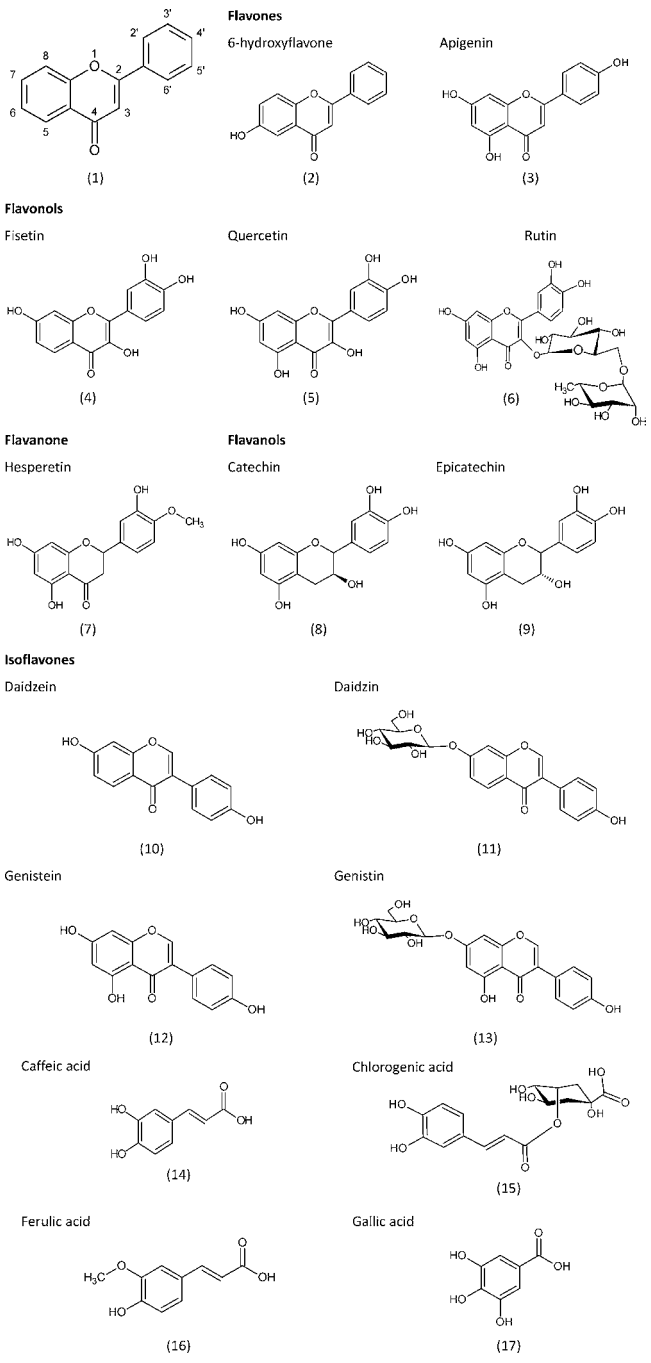

Figure 1. Chemical structures of flavonoids (flavones, flavonol, flavanone, flavanol, and isoflavone) and phenolic acids used in this study.

Sample Preparation. Each chemical standard was dissolved in an 80% (v/v) methanol aqueous solution, starting with 2000 μg/mL, and then serially diluted to the final concentrations of 1000, 500, 250, 100, 50, 25, 10, and 5 μg/mL for determining the difference in absorbance of the reaction mixtures between the samples with and without the test compounds. The reaction mixture with 80% methanol instead of the test compound was the blank control.

Chromogenic Analyses with Folin−Ciocalteu Assay and the Three Procedures. Folin−Ciocalteu colorimetry was adapted from the method of Singelton et al.[15] Briefly, 0.1 mL of sample was mixed with 7.9 mL of distilled water, then 0.5 mL of Folin−Ciocalteu reagent was added. The mixture was mixed well and incubated for 5 min, and then 1.5 mL of 20% (w/v) aqueous sodium carbonate solution was added to a volume of 10 mL. The mixture was allowed to stand at 30 °C for 30 min, and then absorbance was determined at 765 nm.

Procedure A was adapted from Woisky and Salatino[8] and Chang et al.[9] Briefly, 0.5 mL of sample was mixed with 1.5 mL of 95% ethanol. The mixture was added to 0.1 mL of 10% (w/v) of aqueous aluminum chloride solution and 0.1 mL of 1 M aqueous potassium acetate solution. Distilled water was added to a volume of 5 mL, and the mixture was allowed to stand at room temperature for 30 min. Absorbance was determined at 415 nm.

Procedure B was adapted from Jia et al.[10] and Yoo et al.[11] Briefly, 1 mL of sample was mixed with 4 mL of distilled water and then added to 0.3 mL of 5% (w/v) aqueous sodium nitrite solution. After 5 min of reaction, 0.6 mL of 10% aqueous aluminum chloride solution was added. After 6 min, 2 mL of 1 M sodium hydroxide was added, and distilled water was added to a volume of 10 mL. The mixture underwent centrifugation at 3000 rpm for 3 min, and absorbance in the supernatant was determined at 510 nm.

Procedure C was adapted from Hariri et al.[12] In total, 2 mL of sample was mixed with 100 μL of 2-aminoethyl diphenylborate (1% w/v, in methanol) and allowed to stand for 20 min. Absorbance was determined at 404 nm.

Statistical Analysis. Statistical analysis involved the use of SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Comparisons involved ANOVA under the general linear model. Significant differences between means were determined by Duncan's multiple-range tests at $P < 0.05$. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Color Reactions of Different Flavonoids with Folin-Ciocalteu Assay and the Three Procedures. All flavonoids used in this study showed to be reactive toward Folin−Ciocalteu's reagent (Table 1), and the corresponding intensity of absorption at 765 nm was proportional to the concentration of each sample. Because Folin−Ciocalteu colorimetry is based on the chemical reduction of the reagent, the results indicated the B ring of the flavonoid structure with two hydroxyl groups, such as catechin, epicatechin, fisetin, and quercetin, exhibited a stronger reducing capacity.

Procedure A had significant chromogenic effects for flavonols such as fisetin, quercetin, and rutin, and flavones with the 5-hydroxyl structure, such as apigenin. For test compounds such as 6-hydroxyflavone, hesperetin, catechins, and isoflavones, even with concentrations as high as 250 μg/mL, the absorbances at 415 nm were almost the same as that of the blank control. Therefore, procedure A was considered to be not suitable for determining the flavonoid content in a sample unless samples contained flavonols or flavones with 5-hydroxyl structure as their predominant flavonoid.

Procedure B could detect flavonols, flavanones, and especially flavanols. However, procedure B did not detect 6-hydroxyflavone, apigenin, and isoflavones. According to Jia et al.[10] and Yoo et al.,[11] the absorbance wavelength used for detection in procedure B was 510 nm, and the colors of reaction products should be brownish-red. Nevertheless, we obtained different colors for the reaction products of flavones, flavonols, and flavanols with procedure B. Figure 2 shows the appearance and colors of reaction products for apigenin, quercetin, and catechin at the same concentration (i.e., 500 μg/mL) and absorbance spectra at 380−600 nm with procedure B. The colors of reaction products for quercetin and apigenin were bright orange-yellow and pale yellow, respectively, and that of catechin was bright red. The absorbance spectra for these three flavonoids differed greatly. The chromogenic products for

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Table 1. Color Reactions of Selected Flavonoid Standards Used To Determine Flavonoid Content with Procedures A, B, and C and the Folin−Ciocalteu Assay

	Folin−Ciocalteu assay		procedure A		procedure B		procedure C	
flavonoid[a]	μg/mL	absorbance (765 nm)	μg/mL	absorbance (415 nm)	μg/mL	absorbance (510 nm)	μg/mL	absorbance (404 nm)
flavones								
6-hydroxyflavone (**2**)	50	0.02 ± 0.00	50	0.01 ± 0.00	100	0.00 ± 0.00	5	0.00 ± 0.00
	100	0.04 ± 0.01	100	0.03 ± 0.01	250	0.00 ± 0.00	10	0.00 ± 0.00
	250	0.08 ± 0.01	250	0.07 ± 0.01	500	0.00 ± 0.01	25	0.01 ± 0.00
							50	0.02 ± 0.00
apigenin (**3**)	50	0.06 ± 0.00	50	0.08 ± 0.00	100	0.01 ± 0.00	5	0.12 ± 0.00
	100	0.11 ± 0.00	100	0.17 ± 0.01	250	0.01 ± 0.00	10	0.22 ± 0.00
	250	0.23 ± 0.00	250	0.39 ± 0.01	500	0.02 ± 0.01	25	0.49 ± 0.01
			500	0.80 ± 0.02			50	0.92 ± 0.00
flavonols								
fisetin (**4**)	50	0.09 ± 0.00	50	0.38 ± 0.01	100	0.08 ± 0.00	5	0.31 ± 0.00
	100	0.17 ± 0.00	100	0.76 ± 0.02	250	0.20 ± 0.01	10	0.62 ± 0.00
	250	0.40 ± 0.01	250	1.52 ± 0.10	500	0.39 ± 0.05	25	1.23 ± 0.01
							50	1.40 ± 0.00
quercetin (**5**)	50	0.07 ± 0.00	50	0.31 ± 0.00	100	0.07 ± 0.00	5	0.37 ± 0.00
	100	0.14 ± 0.00	100	0.62 ± 0.01	250	0.16 ± 0.01	10	0.70 ± 0.02
	250	0.34 ± 0.01	250	1.37 ± 0.05	500	0.29 ± 0.02	25	1.30 ± 0.00
							50	1.46 ± 0.00
rutin (**6**)	50	0.04 ± 0.00	50	0.13 ± 0.00	100	0.09 ± 0.00	5	0.12 ± 0.00
	100	0.09 ± 0.01	100	0.26 ± 0.00	250	0.23 ± 0.01	10	0.23 ± 0.00
	250	0.20 ± 0.00	250	0.61 ± 0.03	500	0.44 ± 0.03	25	0.67 ± 0.01
							50	1.15 ± 0.00
flavanone								
hesperetin (**7**)	50	0.05 ± 0.00	50	0.00 ± 0.00	100	0.03 ± 0.00	5	0.00 ± 0.00
	100	0.08 ± 0.00	100	0.01 ± 0.00	250	0.07 ± 0.00	10	0.01 ± 0.00
	250	0.19 ± 0.00	250	0.03 ± 0.00	500	0.15 ± 0.00	25	0.02 ± 0.00
							50	0.04 ± 0.00
flavanols								
catechin (**8**)	50	0.22 ± 0.02	50	0.00 ± 0.00	100	0.25 ± 0.02	5	0.00 ± 0.00
	100	0.44 ± 0.02	100	0.00 ± 0.00	250	0.56 ± 0.03	10	0.00 ± 0.00
	250	1.05 ± 0.04	250	0.00 ± 0.00	500	1.09 ± 0.02	25	0.00 ± 0.00
							50	0.00 ± 0.00
epicatechin (**9**)	50	0.19 ± 0.00	50	0.00 ± 0.00	100	0.18 ± 0.01	5	0.00 ± 0.00
	100	0.36 ± 0.00	100	0.00 ± 0.00	250	0.40 ± 0.02	10	0.00 ± 0.00
	250	0.75 ± 0.00	250	0.00 ± 0.00	500	0.94 ± 0.02	25	0.00 ± 0.00
							50	0.00 ± 0.00
isoflavones (**10**−**13**)								
daidzin 0.25%	50	0.02 ± 0.00	50	0.02 ± 0.02	100	0.01 ± 0.00	5	0.00 ± 0.00
genistin 0.46%	100	0.05 ± 0.00	100	0.02 ± 0.00	250	0.02 ± 0.00	10	0.01 ± 0.00
daidzein 7.94%	250	0.10 ± 0.01	250	0.05 ± 0.00	500	0.05 ± 0.00	25	0.02 ± 0.00
genistein 11.85%							50	0.05 ± 0.00

[a]The number in parentheses refers to the chemical structure of the compound in Figure 1.

quercetin and apigenin had low absorbance intensity only at 510 nm, so procedure B was not suitable for quantifying the flavonoid content of the samples containing quercetin or apigenin as the predominant component of flavonoid.

Procedure C was sensitive for flavonols. With concentrations of 5 μg/mL for analytes such as fisetin and quercetin, the products were bright yellow and gave absorbance intensity at 404 nm higher than 0.30. Furthermore, with increasing concentration, the absorbance intensity at 404 nm matched the linear relationship of quantification. Procedure C could also be used to quantify apigenin, chemically classified as a flavone with 5-hydroxyl structure, and the reaction product was bright yellow, but could not be used to quantify 6-hydroxyflavone, because the reaction product did not have absorbance at 404 nm. In addition, the reaction products for other analytes, such as hesperetin, catechins, and isoflavones, did not show absorbance at 404 nm with procedure C either. Thus, procedure C was not suitable for quantifying 6-hydroxyflavone, hesperetin, catechins, and isoflavones. Table 2 summarizes the flavonoids with reaction products that could be chromogenically quantified in the study.

Color Reactions of Selected Phenolic Acids with Folin−Ciocalteu Assay and the Three Procedures. Table 3 shows the color reactions of four common phenolic acids of plant extracts with Folin−Ciocalteu assay and the three procedures. All four selected phenolic acids showed to be reactive toward Folin−Ciocalteu reagent, and the products exhibited a broad absorption with a maximum at 765 nm.

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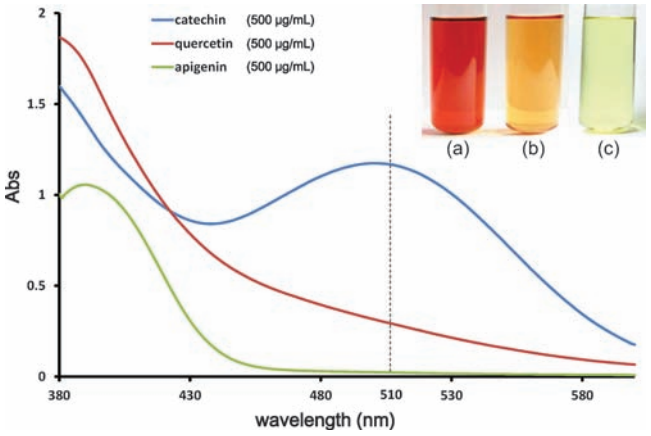

Figure 2. Absorption spectra (380−600 nm) of reaction products for apigenin, quercetin, and catechin at 500 μg/mL with procedure B. (Inset) Outward appearance and color of reaction products for (a) catechin, (b) quercetin, and (c) apigenin.

Among them, caffeic acid showed the most reactivity toward the reagent, implying that it possesses the most capability to inhibit the oxidation of the reagent. Although various phenolic acids responded differently to the reagent, the intensity of absorption was proportional to the concentration of individual sample.

In terms of quantitative interference toward the three procedures for flavonoid determination, with procedure A, the reaction products of the four phenolics had no absorbance at 415 nm. Even at concentrations of 250 μg/mL, caffeic and chlorogenic acid showed absorbances of 0.1 and 0.12, respectively. Ferulic and gallic acid showed no bright yellow color.

With procedure B, the reaction products for the four phenolic acids all had absorbances at 510 nm. Caffeic and chlorogenic acid showed sensitive color reactions. Even at 100 μg/mL, the reaction products were bright red.

With procedure C, the chromogenic products for caffeic and chlorogenic acid were bright yellow. This reaction was very sensitive. At 50 μg/mL, the reaction products with chlorogenic and caffeic acid showed absorbances of 1.14 and 0.48 at 404 nm, respectively, whereas the reaction products for ferulic and gallic acid showed little absorbance.

Thus, the common phenolic acids in plant extracts may interfere with the quantification of flavonoids. In particular, with procedures B and C used to determine flavonoid content, the chromogenic results overestimate the flavonoid content if phenolic acids such as caffeic and chlorogenic acid are also present in the analytes.

Stability of Reaction Products with the Three Procedures. The reaction products obtained with procedures A, B, and C were determined at 415, 510, and 404 nm, respectively, with colors of bright yellow, brownish-red, and bright yellow, respectively. We performed a stability test by incubating the reaction products at room temperature and measuring the absorption spectra and intensity of the reaction products at different times. Figure 3a shows the absorption spectra with the range of visible wavelength for the reaction products of quercetin (at 500 μg/mL) with procedure A. The spectra and intensities of the reaction product almost completely overlapped within 60 min, so the stability of the reaction products for quercetin was good and did not change over time. Figure 3b shows the absorption spectra for the reaction products of catechin (500 μg/mL) with procedure B. The stability of this reaction product was poor, and the absorbance at 510 nm decreased gradually over time. After 20 min, the absorbance of the reaction product at 510 nm was only 80% of the initial intensity, which indicated severe decolorization. As well, a larger error would occur in quantifying the flavonoid content with procedure B. Figure 3c shows the absorption spectra for the reaction products of quercetin (50

Table 2. Calibration Curve Equations, Correction Coefficients, and Detection Ranges of the Three Procedures for Determining the Flavonoid Content Used in This Study[a]

				procedure		
flavonoid	calibration curve equation (n = 3)	correlation coefficient (n = 3)	linear range (μg/mL) (n = 3)	A	B	C
flavone						
apigenin	$y = 0.0016x + 0.0015$	0.9995	50−500	△		
	$y = 0.0178x + 0.0386$	0.9993	5−50			○
flavonols						
fisetin	$y = 0.0570x + 0.0969$	0.9910	25−250	○		
	$y = 0.1662x + 0.1065$	0.9922	100−1000		○	
	$y = 0.0232x + 0.0649$	0.9980	5−50			○
quercetin	$y = 0.0530x + 0.0476$	0.9975	25−250	○		
	$y = 0.1070x - 0.0413$	0.9926	100−500		○	
	$y = 0.0232x + 0.1342$	0.9995	5−25			○
rutin	$y = 0.0025x + 0.0022$	0.9983	25−250	△		
	$y = 0.2494x - 0.2170$	0.9415	100−1000		○	
	$y = 0.0231x + 0.0227$	0.9897	5−50			○
flavanone						
hesperetin	$y = 0.0003x - 0.018$	0.9999	100−1000		△	
flavanols						
catechin	$y = 0.4657x - 0.2805$	0.9865	100−1000		○	
epicatechin	$y = 0.0150x + 0.0674$	0.9798	100−1000		○	

[a] ○ indicates that the compound could be quantified with the procedure. △ indicates that the compound is not recommended to be quantified by the procedure despite a good correlation coefficient because the unsuitable slope of the calibration curve equation would enlarge the deviation.

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Table 3. Color Reactions of Four Phenolic Acids with Procedures A, B, and C and the Folin−Ciocalteu Assay

	Folin−Ciocalteu assay		procedure A		procedure B		procedure C	
phenolic acid[a]	μg/mL	absorbance (765 nm)	μg/mL	absorbance (415 nm)	μg/mL	absorbance (510 nm)	μg/mL	absorbance (404 nm)
caffeic acid (**14**)	50	0.14 ± 0.01	50	0.02 ± 0.02	100	0.29 ± 0.00	5	0.03 ± 0.01
	100	0.26 ± 0.01	100	0.05 ± 0.00	250	0.69 ± 0.01	10	0.07 ± 0.00
	250	0.71 ± 0.05	250	0.10 ± 0.01	500	1.19 ± 0.02	25	0.18 ± 0.00
							50	0.48 ± 0.00
chlorogenic acid (**15**)	50	0.08 ± 0.00	50	0.02 ± 0.00	100	0.16 ± 0.02	5	0.14 ± 0.00
	100	0.16 ± 0.01	100	0.05 ± 0.00	250	0.38 ± 0.05	10	0.28 ± 0.00
	250	0.28 ± 0.01	250	0.12 ± 0.00	500	0.65 ± 0.08	25	0.68 ± 0.00
							50	1.14 ± 0.00
ferulic acid (**16**)	50	0.11 ± 0.00	50	0.00 ± 0.00	100	0.05 ± 0.01	5	0.00 ± 0.00
	100	0.20 ± 0.00	100	0.00 ± 0.00	250	0.11 ± 0.00	10	0.00 ± 0.00
	250	0.44 ± 0.01	250	0.00 ± 0.00	500	0.17 ± 0.01	25	0.00 ± 0.00
							50	0.00 ± 0.00
gallic acid (**17**)	50	0.07 ± 0.00	50	0.00 ± 0.00	100	0.01 ± 0.00	5	0.00 ± 0.00
	100	0.12 ± 0.01	100	0.00 ± 0.00	250	0.03 ± 0.00	10	0.00 ± 0.00
	250	0.31 ± 0.02	250	0.00 ± 0.00	500	0.07 ± 0.00	25	0.00 ± 0.00
							50	0.00 ± 0.00

[a]The number in parentheses refers to the chemical structure of the compound in Figure 1.

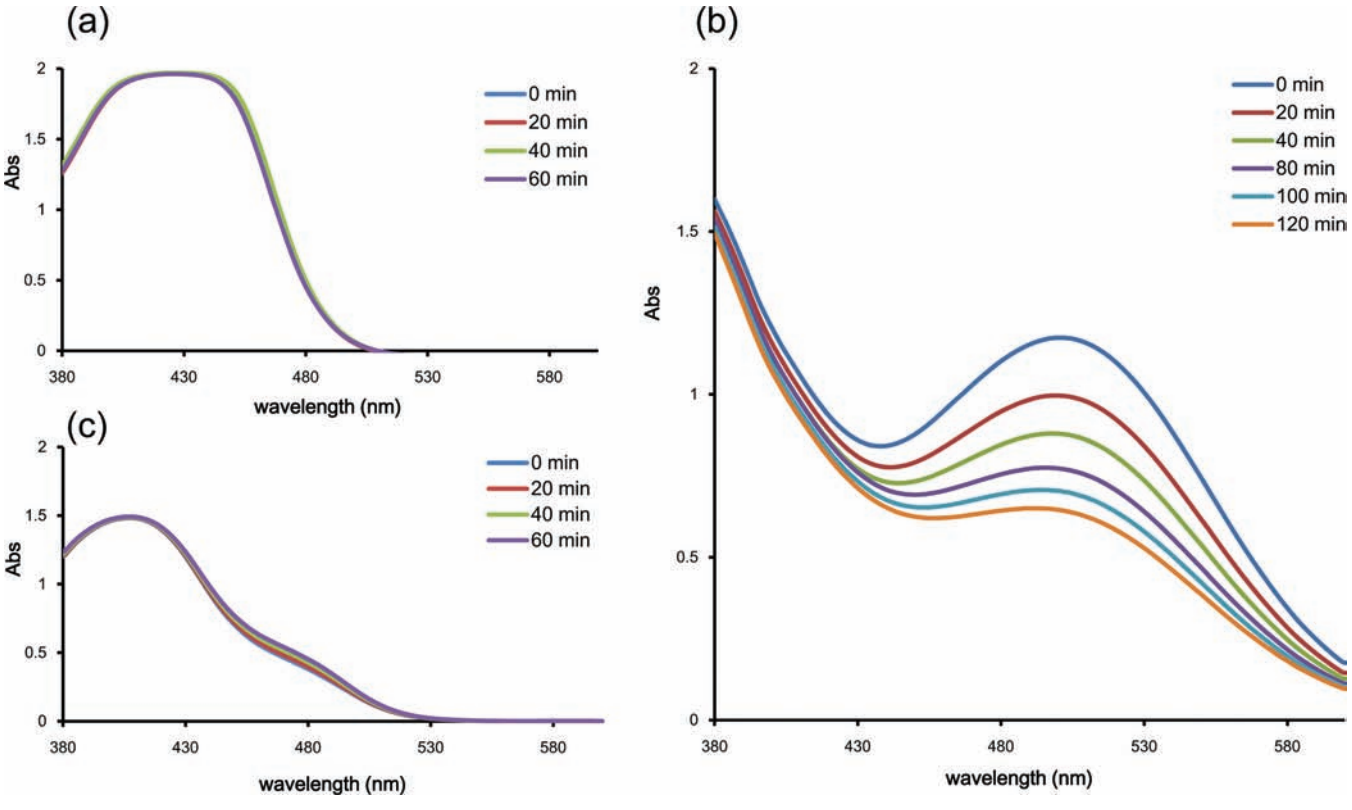

Figure 3. Absorption spectra and intensities of the reaction products for various times: (a) quercetin (500 μg/mL) with procedure A; (b) catechin (500 μg/mL) with procedure B; (c) quercetin (50 μg/mL) with procedure C.

μg/mL) with procedure C. The spectra of the reaction product within the incubation of 60 min were almost the same, which indicated that the reaction products with procedure C were very stable.

Evaluation of Structural Requirements for Complexation with the Three Procedures. Previous study showed that with procedure A, the chromogenic mechanism might be the formation of a bright yellow, stable complex by complexation of aluminum ion and the keto group at C-4 and the hydroxyl group at C-3 or C-5 of the flavonoid structure.[9,17] According to Table 1, the flavonoids that can be chromogenically quantified with procedure A were mainly flavonols such as fisetin, quercetin, and rutin. Besides flavonols, flavones with the 5-hydroxy-4-keto structure, such as apigenin, can also form a

bright-yellow complex with procedure A, but the chromogenic capability was weaker than that of flavonol. The flavones without the 5-hydroxy structure, such as 6-hydroxyflavone, cannot be quantified with procedure A because the corresponding reaction product had no absorbance at 415 nm. In addition, hesperetin and genistein, chemically classified as a flavanone and an isoflavone, respectively, both with 5-hydroxy-4-keto in the chemical structure, cannot be quantified with procedure A either. Therefore, we considered that among all of the subclasses of flavonoids, only flavonols (with 3-hydroxy-4-keto structure) and partial flavones (with the 5-hydroxy-4-keto structure) can form bright-yellow reaction products with procedure A, which indicated procedure A was just suitable for the samples containing flavonols or 5-hydroxyflavones as the predominant component of flavonoid. Samples containing the predominant flavonoid with other subclasses of flavonoid, such as flavanone, flavanol, and isoflavone, even with 5-hydroxy-4-keto structure in the formula, should not be recommended for use with procedure A for quantitative analysis. Figure 4a shows the possible chromogenic complexes generated by chelating aluminum ion with the functional substituents of flavonol and flavone with procedure A.

Procedures A and B both use an aluminum ion to form a colored complex with the reactant flavonoid. Mainly different in procedure B, the color reaction occurs in a strong alkaline environment. Procedure B had the lowest sensitivity among the three procedures and therefore required a higher sample concentration for the reactions (Table 1). Furthermore, the colors of the reaction products of different flavonoids differ greatly (Figure 2). Thus, under alkaline conditions, aluminum ions and different flavonoids form different aluminum-chelated complexes. Mabry et al.[17] proposed that aluminum ion can chelate with the *o*-dihydroxyl flavonoid group and produce bathochromic shifts of absorption bands in the spectrum. We showed that with procedure B, the absorbance was 510 nm for the reaction products of all tested *o*-dihydroxyl group flavonoids. However, only the reaction product for catechin with *o*-dihydroxyl groups such as the 3′- and 4′-hydroxyls with catechin in the structure had the highest absorbance at 510 nm. For flavonoids with 3-hydroxy-4-keto or 5-hydroxy-4-keto structure, together with *o*-dihydroxyl groups such as fisetin, quercetin, and rutin, the absorbance of the reaction product was greatly reduced at 510 nm. Furthermore, for flavonoids with 3-hydroxy-4-keto structure but without *o*-dihydroxyl groups in the structure of the B ring, the absorbance of the reaction products was lowest at 510 nm. Therefore, for flavonoids with 3-hydroxy-4-keto or 5-hydroxy-4-keto structure, aluminum ion may prefer binding to the 3-hydroxyl and 4-keto oxygen or to the 5-hydroxyl and 4-keto oxygen of the flavonoid molecule, rather than the *o*-dihydroxyl groups that can lead to reduced absorbance at 510 nm. Moreover, a double complex may occur, which would result in the final products having different colors. Thus, in terms of chemical structures, the binding intensities of the aluminum-chelated complexes formed by flavonoids and aluminum ions are in the following descending order: flavonoid with 3-hydroxy-4-keto > 5-hydroxy-4-keto > *o*-dihydroxyl group. In addition, because plant phenolic acids usually contain two adjacent hydroxyls, which are highly similar to the 3′- and 4′-dihydroxyls in catechins, phenolic acids such as caffeic and chlorogenic acid may chelate with aluminum ion to form the chromogenic complex. On the basis of the above, procedure B was recommended only for samples containing flavanols with *o*-dihydroxyl structure as the predominant component of flavonoid and lacking *o*-dihydroxyl phenolic acids because the presence of any 3- or 5-hydroxy-4-keto structure in analytes would interfere in the reaction and cause inconsistent coloring of the reaction product. Figure 4b shows the possible chromgenic complexes generated by chelating an aluminum ion with the *o*-dihydroxyl group of catechin with procedure B.

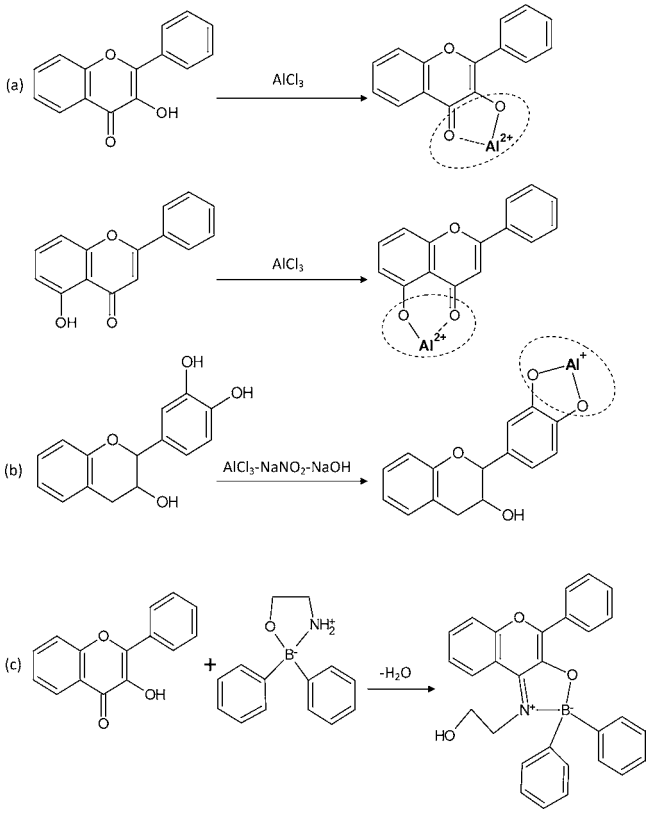

Figure 4. Possible chemical reactions resulting in chromgenic complexes with the three procedures: (a) complexation by chelating aluminum ion with the 3- or 5-hydroxy-4-keto structure of flavonols and flavones with procedure A; (b) complexation by chelating aluminum ion with the *o*-dihydroxyl group of catechin with procedure B; (c) chromogenic complex generated by reacting 2-aminoethyl diphenylborate with the 3-hydroxy-4-keto-flavonoid structure with procedure C.

The principle of procedure C is that 2-aminoethyl diphenylborate reacts with flavonoid molecules to form complexes with a bathochromic shift of the absorption maximum. Previously, 2-aminoethyl diphenylborate was employed as the spray reagent for detecting some flavonoids by the formation of a yellow spot on thin layer chromatography. Thus, 2-aminoethyl diphenylborate is also called a flavone reagent or Neu's reagent. Previous study revealed that the complexation occurs only at the 3-hydroxy-4-keto oxygen site.[16] According to Table 1, flavonoids with the 3-hydroxy-4-keto structure were able to react with the reagent to generate the chromogenic complex with absorbance at 404 nm. However, a flavone with a 5-hydroxyl group, such as apigenin, also reacted with 2-aminoethyl diphenylborate to form a complex, but the complex was less capable of chromogenicity at 404 nm. Flavonoids without the 4-keto oxygen, such as catechins, would escape this chromogenic complexation. Furthermore, a flavanone with 5-hydroxy-4-keto structure

such as hesperetin also escaped this chromogenic complexation. Therefore, like procedure A, the 4-keto site and a free 3- or 5-hydroxyl group in the structure of flavone are essential for the complexation with procedure C. In addition, caffeic and chlorogenic acid can react with 2-aminoethyl diphenylborate to form the chromogenic complex. The spatial arrangement of the structures of caffeic and chlorogenic acid may be similar to the 3(or 5)-hydroxy-4-keto structure of flavonol, which leads to color reactions. The phenomenon could be observed with the mild color reaction of caffeic and chlorogenic acid with procedure A (Table 3). However, on the basis of detection sensitivity, procedure C may have the potential to be used in routine assay for samples containing flavonols or 5-hydroxy-flavones. Figure 4c shows the chromogenic complexes generated by reacting 2-aminoethyl diphenylborate with the 3-hydroxy-4-keto flavonoid structure with procedure C, as described by Jork et al.[16]

Proximate analysis is used extensively to determine the composition of a food or material such as total protein, fat, carbohydrate, and ash. The protocol for proximate analysis of food components should be applicable to any food sample on the basis of a specific scientific criterion. An example is the Folin−Ciocalteu method, which is widely used for determining total phenolic content in wine.[18] This colorimetry method is based on a chemical reduction of Folin−Ciocalteu reagent, a mixture of tungsten and molybdenum oxides, and has a fairly equivalent response to the content of phenolics. Although the method was developed for use in the analysis of wines and grapes, it was used extensively for total phenolic levels in other samples.

Recently, many studies have determined total flavonoid contents. The quantitative method involves one of the three procedures we tested. According to this work, these three procedures are not currently satisfactory for quantitative analysis toward all types of flavonoids. With procedures A and C, the color reaction was mainly due to the 3-hydroxy-4-keto structure of flavonols and the 5-hydroxy-4-keto structure of flavones. However, procedure B is recommended to quantify catechin-rich samples only, because the presence of a 3- or 5-hydroxy-4-keto structure in the analyte can cause inconsistent coloring of the reaction product and difficulty in accurate quantification. For a sample containing an unknown component of flavonoid for determining flavonoid content, a previous survey of the literature to screen a possible predominant flavonoid in this analyte was absolutely required because it could cause the data with merit based on the selection of an appropriate chromogenic assay and a chemical standard with an equivalent dose response to this predominant flavonoid. Otherwise, any chromogenic analysis for measuring the flavonoid content of anonymous samples with each of the three procedures could not be considered a promising determination. A supposed case selecting an appropriate assay and chemical standard for determining the flavonoid content was provided as follows. As an analyte with its predominant flavonoid component being flavanol, procedure B could be chosen as the appropriate assay with a quantitative calibration curve prepared by catechin standard to carry on the detection. In the case above, procedures A and C were not suitable for the quantitation because flavanol gave poor absorbance with both procedures. Furthermore, if quercetin was used as the standard for preparing the quantitative curve with procedure B, the outcome of quantitation would be seriously overestimated because quercetin was not a promising standard with procedure B. We searched Google Scholar using the term "total flavonoid content" and found more than 60 academic reports containing the term, but we caution against considering these results of chromogenic quantitative determinations as total flavonoid content because no information can be used to guide the selection of appropriate colorimetric procedures, including the selection of a promising standard for preparing the quantitative curve in these results.

In conclusion, as flavonoids vary significantly in structure, especially the substituents and the bond type between the C2 and C3 positions of the C ring, different color reactions usually end up with different assays used to determine flavonoid content in samples. The measurements of these different flavonoids and reports of meaningful values in a number represent a great challenge. Unlike Folin−Ciocalteu colorimetry, which can be used to determine total phenolic levels in analyte based on a criterion of chemical reduction of the reagent, procedures A and C are highly specific with flavonoids containing the 3- or 5-hydroxy-4-keto structure and with a double bond at the C2−C3 position of the C ring. Procedure B is not recommended for determining the flavonoid content of an anonymous sample because it is less sensitive. Furthermore, colors of the reaction products are inconsistent using procedure B, and in some cases, severe decolorization can occur. Currently, no satisfactory chromogenic analysis can be used to quantify all types of flavonoids. Therefore, a previous survey of the literature and screening of the predominant flavonoid component in analytes are definitely essential. Afterward, guided by the predominant flavonoid, a promising calibration curve for flavonoid detection can be established on the basis of the selection of an appropriate method and a chemical standard with a dose response equivalent to that of the predominant flavonoid, and then a promising determination can be performed with merit.

AUTHOR INFORMATION

Corresponding Author

*Phone: +886-2-33664819. Fax: +886-2-23632714. E-mail: snw@ntu.edu.tw.

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Notes

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REFERENCES

(1) Celiz, G.; Daz, M.; Audisio, M. C. Antibacterial activity of naringin derivatives against pathogenic strains. *J. Appl. Microbiol.* **2011**, *111*, 731−738.

(2) Dudonne, S.; Poupard, P.; Coutiere, P.; Woillez, M.; Richard, T.; Merillon, J. M.; Vitrac, X. Phenolic composition and antioxidant properties of poplar bud (*Populus nigro*) extract: individual antioxidant contribution of phenolics and transcriptional effect on skin aging. *J. Agric. Food Chem.* **2011**, *59*, 4527−4536.

(3) Liu, M.; Li, X. Q.; Weber, C.; Lee, C. Y.; Brown, J.; Liu, R. H. Antioxidant and antiproliferative activities of raspberries. *J. Agric. Food Chem.* **2002**, *50*, 2926−2930.

(4) Trumbeckaite, S.; Benetis, R.; Bumblauskiene, L.; Burdulis, D.; Janulis, V.; Toleikis, A.; Viskelis, P.; Jakstas, V. *Achillea millefolium* L. s.l. herb extract: antioxidant activity and effect on the rat heart mitochondrial functions. *Food Chem.* **2011**, *127*, 1540−1548.
(5) Kang, S. R.; Il Park, K.; Park, H. S.; Lee, D. H.; Kim, J. A.; Nagappan, A.; Kim, E. H.; Lee, W. S.; Shin, S. C.; Park, M. K.; Han, D. Y.; Kim, G. S. Anti-inflammatory effect of flavonoids isolated from Korea *Citrus aurantium* L. on lipopolysaccharide -induced mouse macrophage RAW 264.7 cells by blocking of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways. *Food Chem.* **2011**, *129*, 1721−1728.
(6) Imai, M.; Kikuchi, H.; Denda, T.; Ohyama, K.; Hirobe, C.; Toyoda, H. Cytotoxic effects of flavonoids against a human colon cancer derived cell line, COLO 201: a potential natural anti-cancer substance. *Cancer Lett.* **2009**, *276*, 74−80.
(7) Hamauzu, Y.; Nosaka, T.; Ito, F.; Suzuki, T.; Torisu, S.; Hashida, M.; Fukuzawa, A.; Ohguchi, M.; Yamanaka, S. Physicochemical characteristics of rapidly dried onion powder and its antiatherogenic effect on rats fed high-fat diet. *Food Chem.* **2011**, *129*, 810−815.
(8) Woisky, R. G.; Salatino, A. Analysis of propolis: some parameters and procedures chemical quality control. *J. Apic. Res.* **1998**, *37*, 99−105.
(9) Chang, C. C.; Yang, M. H.; Wen, H. M.; Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*, 178−182.
(10) Jia, Z. S.; Tang, M. C.; Wu, J. M. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555−559.
(11) Yoo, K. M.; Lee, C. H.; Lee, H. G.; Moon, B. K.; Lee, C. Y. Relative antioxidant and cytoprotective activities of common herbs. *Food Chem.* **2008**, *106*, 929−936.
(12) Hariri, B.; Salle, G.; Andary, C. Involvement of flavonoids in the resistance of two popular cultivars to mistletoe (*Viscum album* L.). *Protoplasma* **1991**, *162*, 20−26.
(13) Hazra, B.; Biswas, S.; Mandal, N. Antioxidant and free radical scavenging activity of *Spondias pinnata*. *BMC Complementary Altern. Med.* **2008**, *8*, 63 (DOI: 10.1186/1472-6882-8-63).
(14) Mandal, S.; Hazra, B.; Sarkar, R.; Biswas, S.; Mandal, N. Assessment of the antioxidant and reactive oxygen species scavenging activity of methanolic extract of *Caesalpinia crista* leaf. *eCAM* **2011**, article ID 173768, 11 pages, DOI: 10.1093/ecam/nep072.
(15) Singelton, V. R.; Orthifer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.
(16) Jork, H.; Funk, W.; Fischer, W.; Wimmer, H. Reagents in alphabetical order. In *Thinlayer Chromatography: Reagents and Detection Methods*; VCH: Weinheim, Germany, 1990; Vol. *1a*, pp 278.
(17) Mabry, T. J.; Markham, K. R.; Thomas, M. B. The UV spectra of flavones and flavonols in the presence of $AlCl_3$ and $AlCl_3$/HCl. In *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, 1970; pp 51.
(18) Singleton, V. L.; Rossi, J. A. J. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.