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Quantitation of Flavanols, Proanthocyanidins, Isoflavones, Flavanones, Dihydrochalcones, Stilbenes, Benzoic Acid Derivatives Using Ultraviolet Absorbance after Identification by Liquid Chromatography–Mass Spectrometry

Long-Ze Lin* and James M. Harnly

Food Composition and Methods Development Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, 10300 Baltimore Avenue, Beltsville, Maryland 20705-3000, United States

ABSTRACT: A general method was developed for the systematic quantitation of flavanols, proanthocyanidins, isoflavones, flavanones, dihydrochalcones, stilbenes, and hydroxybenzoic acid derivatives (mainly hydrolyzable tannins) based on UV band II absorbance arising from the benzoyl structure. The compound structures and the wavelength maximum were well correlated and were divided into four groups: the flavanols and proanthocyanidins at 278 nm, hydrolyzable tannins at 274 nm, flavanones at 288 nm, and isoflavones at 260 nm. Within each group, molar relative response factors (MRRFs) were computed for each compound based on the absorbance ratio of the compound and the group reference standard. Response factors were computed for the compounds as purchased (MRRF), after drying ($MRRF_D$), and as the best predicted value ($MRRF_P$). Concentrations for each compound were computed based on calibration with the group reference standard and the $MRRF_P$. The quantitation of catechins, proanthocyanidins, and gallic acid derivatives in white tea was used as an example.

KEYWORDS: *quantitation, hydroxybenzoic acids, flavanols, proanthocyanidins, flavanones, isoflavones, hydrolyzable tannins, UV, response factors, green tea*

INTRODUCTION

Polyphenols are widely distributed in foods and have potential health effects in the prevention of human chronic diseases.[1−4] Biological, epidemiological, and clinical studies aimed at establishing the relationship between dietary polyphenols and health outcomes require accurate analytical methods to determine the concentration of individual polyphenols in foods. While new liquid chromatography (LC) and mass spectrometry (MS) technology has made identification of polyphenols relatively routine (with the exception of isomer identification), quantitation of the many phenolic compounds is still problematic. This difficulty arises from the large variety of compounds and the lack of standards.

Previous research presented a general approach for quantifying hydroxycinnamic acid derivatives and glycosides of flavonols and flavones based on the strong UV band I absorbance (310−380 nm) of their cinnamoyl structure. Using an LC with diode array and MS detection (LC-DAD-MS), the phenolic compounds were first identified and then quantified. Five general groups were established based on the wavelength maximum (λ_{max}) of the band I absorbance peak. Inexpensive, commercially available compounds were selected as reference standards for each group. Molar relative response factors (MRRFs) were computed for each compound based on the ratio of the peak areas of authentic standards and the group reference standards. Accuracy based on calibration with the group reference standards was found, in the worst case, to be ±13% for more than 80 tested compounds.[5]

This study presents a similar approach for quantitating flavanols, proanthocyanidins, isoflavones, flavanones, dihydrochalcones, stilbenes, and hydroxybenzoic acids and their derivatives (mainly hydrolyzable tannins) (Figure 1) based on the UV band II absorbance of their benzoyl structure after identification by LC-MS. The band II maxima (λ_{max}) ranged from 250 to 300 nm. Because these compounds do not contain a cinnamoyl structure, they lack a band I absorbance peak.[6]

The compounds discussed in this study are frequently found in foods. Flavanols, mainly catechins, exist in their free forms at high concentrations in a variety of green teas, green tea products, chocolates, and others foods. In addition, oligomers of the flavanols (proanthocyanidins) are distributed widely in many plant-derived foods.[2,4,6−8] Hydroxybenzoic acids, especially gallic acid, are found in their free form, as simple esters, and as more complex forms called hydrolyzable tannins in many plants.[2,9−12] Isoflavones are less well distributed but no less important.[6,14] They are found mainly in plants from the Leguminosae family, especially soybean and soybean products.[4,13] Flavanones exist primarily in citrus fruits, juices, and peels.[4,15] Dihydrochalcones, such as phloretin and phloridzin from apples, have similar UV absorbance profiles to the flavanones.[2,4] *trans*- and *cis*-Resveratrol and their glycosides, the important grape polyphenols, have also been studied.[2,9]

In this study, we examined the structure, λ_{max}, and MRRF values of authentic standards for more than 55 compounds. On the basis of the band II λ_{max}, which strongly correlated with structure, the compounds were divided into four major groups, and a group reference standard was selected for each group.

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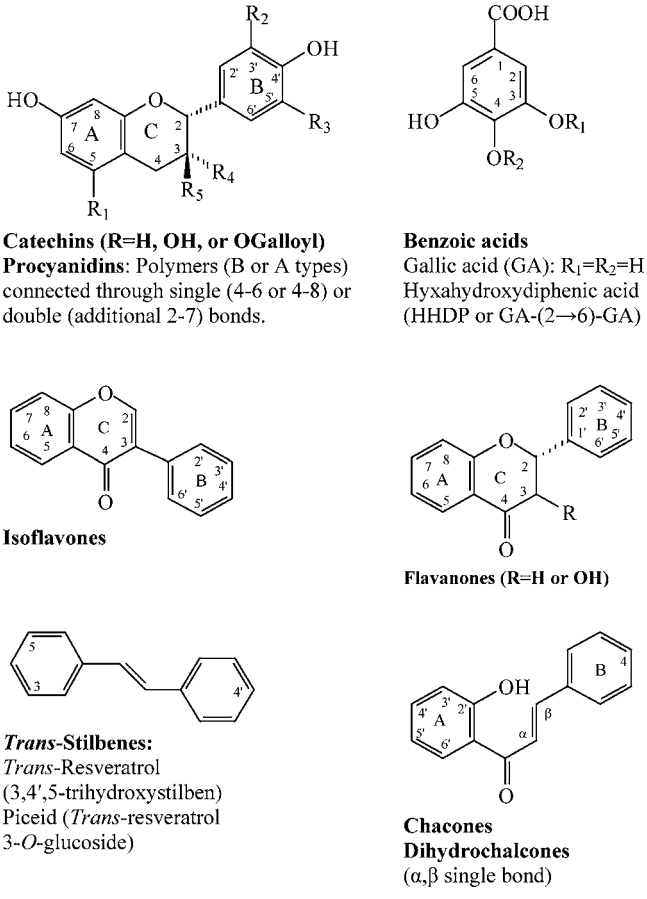

Figure 1. Structures of some food polyphenol standards.

Calibration using the group reference standards was extended to the other compounds using the MRRFs. This general approach permits quantitation of flavanols, proanthocyanidins, isoflavones, flavanones, dihydrochalcones, and hydrolyzable tannins in plant materials using a few inexpensive and commercially available standards. The quantitation of the catechins, proanthocyanidins, and gallic acid derivatives of white green tea was used as the example.

MATERIALS AND METHODS

Standards. (+)-Catechin, (−)-epicatechin, (−)-catechin-3-*O*-gallate, (−)-epicatechin-3-*O*-gallate, (−)-gallocatechin, (−)-epigallocatechin, (−)-gallocatechin-3-*O*-gallate, (−)-epigallocatechin-3-*O*-gallate, phloretin, phloridzin dihydrate, *trans*-resveratrol, daidzein, daidzin, puerarin, formononetin, genistein, genistin, glycetein, glycetin, biochanin A, prunetin, gallic acid, salicylic acid, gentisic acid, protocatechuic acid, vanillic acid, 2,4,6-trihydroxybenzoic acid, 2,3,4-trihydroxybenzoic acid, and syringic acid were obtained from Sigma-Aldrich Chemical Co. (Saint Louis, MO). 2′,6-Dihydroxyflavanone and procyanidin A_2 were obtained from Indofine Chemical Co. (Somerville, NJ). Theaflavin, theaflavin 3- and 3′-*O*-gallate mixture, procyanidin B_1, procyanidin B_2, procyanidin C_1, (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, (−)-gallocatechin, (−)-epigallocatechin, (−)-epigallocatechin-3-*O*-gallate, (+)-taxifolin, daidzin, genistein, genistin, and glycetin were obtained from Chromadex, Inc. (Irvine, CA). Pinocembrin, liguiritigenin, 5-hydroxy-7-methoxyflavanone, naringenin, naringin, sukuranetin, isosukuranetin, didymin, poncitrin, eriodictyol, eriodictyol-7-*O*-glucoside, neoeriocitrin eriocitrin, hesperetin, hesperidin, neohesperidin, (−)-homoeriodictyol, flavanomarein, and the remaining compounds in the tables were purchased from Extrasynthese (Genay, Cedex, France).

The purities of commercial standards are generally categorized as reagent or high-performance liquid chromatography (HPLC) grade. In some cases, the moisture content was listed or the molecular weight was presented with water of hydration. Few had both purity and moisture content. As in our previous study, the moisture (or solvent) content was determined when sufficient quantities of the compound were available.[5] To remove the water of crystallization or absorbed compounds were dried under vacuum at 110 °C until a constant weight was reached (around 24 h).

Other Chemicals. HPLC grade solvents (methanol, acetonitrile), formic acid, and dimethyl sulfoxide (DMSO) were purchased from VWR International, Inc. (Clarksburg, MD). HPLC water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA).

Standard Solutions. Standards were prepared by weighing 3.00−6.00 mg standards into 10 mL volumetric flasks. The individual standards were first dissolved in 2 mL of DMSO and then brought to volume with aqueous methanol (60/40, v/v). These standards were analyzed by HPLC-MS to check for impurities.

Mixtures of 3−5 standards and the group reference standard were then prepared with the same molar concentration and injected in HPLC three times. Each mixed solution was prepared at three concentration levels (as prepared and diluted 1:4 and 1:16) to provide a range of signals suitable for determining the MRRF values. The standard deviations (RSDs) for the peak areas were all ≤5%. Peak areas for peaks whose height fell in the linear range were used to compute the MRRF and $MRRF_D$ values. [5] This was done for all compound standards as purchased and, when sufficient material was available, after drying.

Plant Material and Quantitative Extract. One dried white tea (WT-1) was powdered and passed through 60 mesh sieves prior to extraction. [5] The ground powders (100.0 mg for tea) were extracted with methanol−water (10.000 mL, 60:40, v/v) using an FS30 Ultrasonic sonicator (Fisher Scientific, Pittsburgh, PA) at 40 kHz and 100 W for 60 min at room temperature, respectively. The slurry mixture was centrifuged at 2500 rpm for 15 min, the supernatant was filtered through a 17 mm (0.45 μm) PVDF syringe filter (VWR Scientific, Seattle, WA), 50 μL of the extract was injected into the HPLC, and contents for the polyphenols expressed as mg/100 g dried food were then calculated. [5]

HPLC and MS. The HPLC-DAD−electrospray ionization/mass spectrometer (ESI/MS) was previously described as were the conditions for identifying the phenolic compounds in food samples.[16] The same conditions were used to check for the UV and mass detectable impurities of the standards. Entire UV spectra were archived from the DAD for the entire chromatographic run. The wavelengths at 260, 274, 278, 288, and 354 nm were monitored in real time to obtain chromatograms for each group of compounds. Absorbances around each λ_{max} were acquired at 2 nm intervals in both directions (±6 nm).[5]

Concentration Calculation. Simplistically, the molar concentration for each phenolic compound can be computed as:

$$C = C_{\text{reference standard}}/\text{MRRF}$$

More specifically, the concentration in units of mg/100 g dry plant materials was calculated as:

$$C\ (\text{mg}/100\ \text{g}) = [1000 \times A_x \times \text{MW}_x \times W_s \times V_s] / [\text{A}_s \times \text{MW}_s \times W_x \times V_x \times \text{MRRF}]$$

or the weight percent as:

$$C\ (\%, \text{w/w}) = [100 \times A_x \times \text{MW}_x \times W_s \times V_s] / [\text{A}_s \times \text{MW}_s \times W_x \times V_x \times \text{MRRF}]$$

where A_x, MW_x, W_x, V_x and A_s, MW_s, W_s, V_s are the peak area (μAu or mAu), molecular weight, and weight (mg) of the testing sample (subscripted x) and the standard (subscripted s) in same volume of the extract or solution, respectively. Depending on the calculation, $MRRF_D$ and $MRRF_P$ were substituted for MRRF.

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Table 1. Catechins and Proanthocyanidins at 278 nm[a]

	position										278 nm		
compd	3′	4′	5′	2	3	4	5	7	MW	λ_{max} (nm)	$MRRF_F$	$MRRF_D$	$MRRF_P$
catechin (1)	OH	OH			OH		OH	OH	290	280	1.00	1.00	1.00
catechin (2)	OH	OH			OH		OH	OH	290	280	1.02	1.02	1.00
epicatechin (1)	OH	OH			OH		OH	OH	290	280	0.97	0.98	1.00
epicatechin (2)	OH	OH			OH		OH	OH	290	280	1.08	0.96	1.00
catechin-3-*O*-gallate	OH	OH			Ga		OH	OH	442	278	3.91		3.80
epicatechin-3-*O*-gallate (1)	OH	OH			Ga		OH	OH	442	278	3.79		3.80
epicatechin-3-*O*-gallate (2)	OH	OH			Ga		OH	OH	442	278	3.91	3.74	3.80
gallocatechin	OH	OH	OH		OH		OH	OH	306	270	0.31		0.30
epigallocatechin (1)	OH	OH	OH		OH		OH	OH	306	270	0.29		0.30
epigallocatechin (2)	OH	OH	OH		OH		OH	OH	306	270	0.30	0.29	0.30
gallocatechin-3-*O*-gallate	OH	OH	OH		Ga		OH	OH	458	274	3.42		3.10
epigallocatechin-3-*O*-gallate (1)		OH	OH	OH		Ga		OH	458	274	3.00	3.14	3.10
epigallocatechin-3-*O*-gallate (2)		OH	OH	OH		Ga	OH	OH	458	274	3.10		3.10
procyanidin B1	OH	OH			OH	C8′	OH	OH	578	280	1.67		2.00
procyanidin B2	OH	OH			OH	C8′	OH	OH	578	280	1.85		2.00
procyanidin C1	OH	OH			OH	C8′	OH	OH	866	280	2.24	3.01	3.00
procyanidin A2	OH	OH		OC7′	OH	C8′	OH	OH	576	278	1.95		2.00
gallic acid	see Table 5								170	274	2.60	2.80	2.80
theaflavin									564	272	4.65	5.34	5.34
theaflavin 3- or 3′-*O*-gallate									716	274	6.07	7.15	8.14

[a]The average moisture content for (+)-catechin (1) was 4.22% (3.91−4.63% for three determinations). Functional groups: OH, hydroxyl; C, catechin; Ga, gallate; C8′, the C8 of the second monomer; and OC7′, the O−C7 of the second monomer.

RESULTS AND DISCUSSION

MRRF. The MRRF of a compound is the ratio of the molar absorptivity of the compound to that of a reference compound. In this study, the response factors were computed based on the peak areas from chromatograms of authentic standards of comparable molar concentration. Any standard that had an impurity observable in the chromatogram was discarded. Tables 1 and 3−5 show response factors for compounds as purchased (MRRF) and, when sufficient compound was available, after vacuum drying ($MRRF_D$). The predicted response factors ($MRRF_P$) represent our best estimate of the "true" value of response factor.

Differences between the MRRF and the $MRRF_D$ values arose from differences in the moisture (or solvent) content of the standards before drying. In general, increased water of hydration was observed with increased polarity of the compound. A strong indicator that the difference in the MRRF values arises from differences in the solvent content, and not a difference in molar absorptivity, is the agreement of the wavelength maximum (λ_{max}) of the band II absorbance. Changes in molar absorptivity are usually accompanied by a shift of λ_{max}. A previous study showed that, for the flavonoid band I absorbance, shifts of less than 10 nm were not accompanied by significant changes in molar absorptivity.[5]

Flavanols, Catechins, and Proanthocyanidins (Group 1). As shown in Figure 1, the flavanols have two chiral centers (C-2 and C-3), which can form four stereoisomers. Thus, eight common catechins, (+)-catechin (C) and (−)-epicatechin (EC), (−)-gallocatechin (GC or 5′-hydroxycatechin) and (−)-epigallocatechin (EGC), (−)-catechin 3-*O*-gallate (CG) and (−)-epicatechin 3-*O*-gallate (ECG), (−)-gallocatechin 3-*O*-gallate (GCG) and (−)-epigallocatechin 3-*O*-gallate (EGCG), exist as four stereoisomer pairs. All have the same 2*R* configuration but different configurations at their 3-positions. In each pair, the first compound (no prefix) has an *S* configuration at C3 (i.e., 3*S*), while the second compound, with the "epi" prefix, has a 3*R* configuration. Each compound also has a 2*S* configuration isomer denoted by adding an "*ent-*" prefix to the name of their 2*R* isomers. Besides the eight common catechins, afzelechin (i.e., 3′-deoxycatechin), epiafzelechin, fisetinidol (i.e., 5-deoxycatechin), epifisetinidol, robinetinidol (i.e., 5-deoxygallocatechin), epirobinetinidol, and their gallates and glycosides have also been reported in some plants. Afzelechin has been detected in green tea and strawberries in trace amounts.[2,4,6−8,17]

Standards were purchased for each of the eight common catechins; some were quite expensive, resulting in limited quantities. Five of the standards were also purchased from a second supplier. Table 1 presents MRRF and $MRRF_D$ values using catechin as a reference standard. These values were measured at 278 nm, although their peak maxima are at the wavelengths noted in Table 1. In general, we observed no difference in response for the isomers. An extra hydroxyl in the B ring induced reduced response for GC and EGC, and substitution of gallate at the 3-position appears to add equally to the absorbance of CG, ECG, GCG, and EGCG. The $MRRF_D$ value for procyanidin C1 also suggests that the response factors are additive.

Table 2 compares the MRRF values obtained in the current study with values reported previously.[18,19] None of the standards from the previous studies were reported to be dried. Responses for the vacuum-dried standards ($MRRF_D$ values) for the current study are denoted with an asterisk. If we assume that C and EC have a similar response based on values from the current study and previous work,[18] and that the earlier C value[19] is biased low (so that response factors in Table 2 were computed relative to EC rather than C), then the MRRF values shown in Table 2 are consistent. Wang et al.[18] reported differences in responses between CG and ECG and between GCG and EGCG, whereas this study (Tables 1 and 3−5) and Pelillo et al.[19] did not. On the basis of this data, we

Table 2. Comparison of Catechin MRRFs

	Wang et al.	Pelillo et al.	current study
compd	280 nm	270 nm	278 nm
C	1.00	0.58	1.00[a]
EC	1.03	1.00	0.97[a]
GC	0.29		0.31
EGC	0.24	0.47	0.29[a]
CG	4.70		3.91
ECG	3.94		3.74[a]
GCG	3.56	2.55	3.41
EGCG	2.66	2.77	3.14[a]
gallic acid		2.60	2.80[a]

[a]Values based on vacuum-dried standards.

felt comfortable in assuming that there was no systematic difference between the responses of the isomers.

The assigned $MRRF_P$ values for the stereoisomer pairs were 3.8, 0.3, and 3.1, respectively (Table 1). These values were consistent with the response factors from previous studies and with the $MRRF_D$ values of 2.8 and 3.0 for gallic acid and procyanidin C_1, respectively.[18,19] As compared to the $MRRF_P$ values, the average $MRRF_D$ values were 0.98 ± 0.04, indicating that the assigned values were in close agreement with the experimental data. The 4% standard deviation was consistent with the uncertainty observed for repeat determinations of the moisture content in this study (endnotes for Tables 1 and 3−5) and our previous study on cinnamoyl-based band I absorbance.[5] The average MRRF value was 0.96 ± 0.10, indicating that failure to account for the solvent content biased the results slightly lower and introduced greater uncertainty.

The assumed additive property of the response factors suggests that the experimental $MRRF_D$ value for theaflavin 3-*O*-gallate might be low. There is no absolute assurance that the chemical bond between two compounds, such as theaflavin and gallic acid, does not have a slight influence on the molar absorptivity of the new compound. However, because there was no significant shift in λ_{max}, the assumption that the molar absorptivities are additive seems reasonable. Thus, theaflavin gallate (Table1) was assigned an $MRRF_P$ value of 8.2 and not 7.2. Similarly, we assigned theaflavin 3,3′-digallate an $MRRF_P$ value of 11.0 due to the addition of a second gallate moiety. These $MRRF_P$ values represent a reasonable approximation until a suitable standard becomes available.

Proanthocyanidins (condensed tannins) are the various length polymers (oligomers) of flavanols (catechins and their ent- isomers) and have been well studied.[4,6−8,10,20] They are linked mainly through single (B type) or double (A type) bonds. The number of flavanol units in a compound is described by its degree of polymerization (DP). Only a few proanthocyanidin standards, such as procyanidin B_1 [epicatechin-(4β→8)-catechin], B_2 [epicatechin-(4β→8)-epicatechin], A_2 [epicatechin-(4β→8,2β→*O*→7)-epicatechin], and C_1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin], are available.

Table 1 shows that there was no shift in λ_{max} for the dimer standards, as compared to the monomers, and their MRRF values ranged from 1.7 to 2.0. The $MRRF_D$ value for procyanidin C_1 was 3.0, three times that of catechin. Previous studies reported relative molar absorptivity response ratios for the monomers, dimers, and trimers of 1.0, 1.7, and 2.9 at 280 nm.[8,21] These data also support the assignment of the $MRRF_P$ values of 2.0 and 3.0 to the dimers and trimers, respectively. Thus, it can be assumed that the $MRRF_P$ values of the oligomers consisting only of catechin or epicatechin are equal to their DP values.

Isoflavonoids (Group 2). To date, more than 1400 isoflavonoids (only 10% glycosylated), with a wide variety of structures, have been found in plants. However, quantitative methods and analytical values are primarily limited to those found in soybeans and soybean products. Consequently, there is a need for the development of more comprehensive quantitative methods.[2,4,13] In this study, MRRF values were computed for 11 isoflavonoid standards, both aglycones and glycosides, at 260 nm using genistein as the group reference standard (Table 3). When possible, standards were obtained from two suppliers and, when sufficient standard was available, dried to provide $MRRF_D$ values. As compared to genistein, the MRRF values of genistin (genistein 7-*O*-glucoside), prunetin (genistein 7-methyl), and biochanin A (genistein 4′-methyl) were very close to 1.00, and all had a λ_{max} of 260 nm. This indicated that methylation and glycosylation at the 7- or 4′-

Table 3. Isoflavones and Their 7-*O*-Glucosides at 260 nm[a]

	position							260 nm		
compd	4′	5	6	7	8	MW	λ_{max} (nm)	MRRF	$MRRF_D$	$MRRF_P$
genistein (1)	OH	OH		OH		270	260	1.00	1.00	1.00
genistein (2)	OH	OH		OH		270	260	1.03	1.04	1.00
genistin (1)	OH	OH		OGl		432	260	0.93		1.00
genistin (2)	OH	OH		OGl		432	260	1.06		1.00
prunetin	OH	OH		OCH_3		284	260	0.98		1.00
biochanin A	OCH_3	OH		OH		284	260	0.99	1.00	1.00
daidzein (1)	OH			OH		254	248	0.66		0.66
daidzein (2)	OH			OH		254	248	0.66	0.66	0.66
daidzin	OH			OGl		416	248	0.66		0.66
puerarin	OH			OH	CGl	416	248	0.76		0.66
formononetin	OCH_3			OH		268	248	0.70		0.66
glycetein	OH		OCH_3	OH		284	256	0.81		0.80
glycetin (1)	OH		OCH_3	OGl		446	256	0.80		0.80
glycetin (2)	OH		OCH_3	OGl		446	256	0.79	0.80	0.80

[a]The average moisture content for genistein (1) was 1.25% (1.15−1.35 for four determinations). Functional groups: OH, hydroxyl; OCH_3, methoxy; OGl, *O*-glucoside; and CGl, *C*-glucoside.

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Table 4. Flavanones, Chalcones, and Resveratrols at 288 nm[a]

	position												288 nm		
compd	2′	3′	4′	5′	2	3	4	5	6	7	8	MW	λ_{max} (nm)	MRRF	$MRRF_D$	$MRRF_P$
hesperitin		OH	OCH_3					OH		OH		302	288	1.00	1.00	1.00
hesperidin		OH	OCH_3					OH		OGl		610	284	0.98	0.97	1.00
neohesperidin		OH	OCH_3					OH		OGl		610	284	1.04	1.00	1.00
pinocembrin								OH		OH		256	290	0.98	0.98	1.00
5-hydroxy-7-methoxyflavanone									OH	OCH_3			270	286	0.98	1.00
naringenin			OH					OH	OH			272	290	0.94	0.99	1.00
naringin			OH					OH		OGl		580	284	0.97	0.95	1.00
sukuranetin			OH					OH		OCH3		286	288	1.06	1.06	1.00
isosukaranetin			OCH_3					OH		OH		286	290	1.05	1.05	1.00
didymin			OCH_3					OH		OGl		584	284	1.04		1.00
poncitrin		OH	OH	OH				OH		OGl		584	284	0.96	0.97	1.00
eriodictyol		OH	OH					OH		OH		288	288	1.03	1.05	1.00
eriodictyol-7-*O*-glucoside		OH	OH					OH		OGl		450	284	1.08	1.03	1.00
eriocitrin		OH	OH					OH		OGl		596	284	1.07	---	1.00
neoeriucutrin		OH	OH					OH		OGl		596	284	1.09	1.02	1.00
(+)-taxifolin		OH	OH			OH		OH		OH		304	288	1.04		1.00
(−)-homoeriodictyol		OH	OCH_3					OH		OH		302	288	1.10		1.00
flavanomarein		OH	OH							OH	OGl	450	284	0.82		
isoxanthohumol			OH					OCH_3		OH	P	354	290	0.80	0.87	1.00
liguiritigenin							OH			OH		256	276	0.63	0.60	0.60
2′,6-dihydroxyflavanone	OH								OH			256	258	0.13		
phloretin			OH		OH		OH		OH			274	286	1.17	1.19	1.19
phloridzin			OH		OH		OH		CGl			436	288	0.99		1.19
xanthohumol			OH		OH	P	OH		OCH3			354	224,382	0.20	0.22	
trans-resveratrol			OH			OH		OH				228	306,318		1.12	1.12
piceid			OH			OGl		OH				390	306,318		1.00	1.00

[a]The average moisture content for hesperetin was 1.58% (1.40−1.81% for three determinations). Functional groups: OH, hydroxyl; OCH_3, methoxy; OGl, *O*-glycoside; CGL, C-glycoside; and P, prenylated.

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Table 5. Hydroxybenzoic Acids and Hydrolysable Tannins at 274 nm[a]

	position								274 nm		
compd	1	2	3	4	5	6	MW	λ_{max} (nm)	MRRF	$MRRF_D$	$MRRF_P$
gallic acid (1)	COOH		OH	OH	OH		170	274	1.00	1.00	1.00
gallic acid (2)	COOH		OH	OH	OH		170	274	0.98		1.00
salicylic acid	COOH	OH					138	302	0.08		0.08
gentisic acid	COOH	OH			OH		154	328	0.04		0.04
protocatechuic acid	COOH		OH	OH			154	260, 294	0.60		0.60
vanillic acid	COOH		OCH_3	OH			168	260, 294	0.79		0.79
2,4,6-trihydroxybenzoic acid	COOH	OH		OH		OH	170	256, 294sh	0.32		0.32
2,3,4-trihydroxybenzoic acid	COOH	OH	OH	OH			170	266	0.95		0.95
syringic acid	COOH		OCH_3	OH	OCH_3		198	276	1.30		1.30

[a]The average moisture content for gallic acid was 2.65% (2.58−2.75% for three determinations). Functional groups: OH, hydroxyl; OCH_3, methoxy; COOH, carboxyl; and no standard for HHDP.

Table 6. Concentration of Catechins, Proanthocyanidins, and Gallic Acid Derivatives in White Tea

						concn. (mg/100 g)	
peak no.	compd	$[M - H]^-(m/z)$	λ_{max} (nm)	group	$MRRF_P$	group ref. standard	authentic standard
1	1-galloylglucose*	331	274	4	1.0	30.9 ± 1.2	
2	6-galloylglucose*	331	274	4	1.0	247 ± 10	
3A	3-galloylquinic acid	343	274	4	1.0	107 ± 4	
3B	4-galloylquinic acid	343	274	4	1.0	150 ± 4	
3C	5-galloylquinic acid (theogallin)	343	274	4	1.0	1890 ± 76	
4	gallic acid	169	274	4	1.0	219 ± 9	219 ± 9
6	stricitinin isomer	633	274	4	3.0	30.0 ± 1.2	
7	digalloylglucose	483	274	4	2.0	46.7 ± 1.9	
8	digalloylglucose	483	274	4	2.0	35.4 ± 1.4	
10	stricitinin isomer	633	274	4	3.0	25.1 ± 1.0	
11	digalloylglucose	483	274	4	2.0	133 ± 5	
13	stricitinin	633	274	4	3.0	999 ± 37	
14	trigalloylglucoses	635	274	4	3.0	97.8 ± 3.9	
17	trigalloylglucoses	635	274	4	3.0	48.8 ± 2.0	
18	trigalloylglucoses	635	274	4	3.0	172 ± 7	
20	trigalloylglucose	635	274	4	3.0	740 ± 30	
22	1,2-digalloyl-4,6-HHDP-glucose	785	274	4	4.0	73.2 ± 2.9	
total tannins and qalloylquinic acids						**5045 ± 202**	
5	gallocatechin (GC)	305	270	1	0.3	136 ± 5	132 ± 5
9	epigallocatechin (EGC)	305	270	1	0.3	820 ± 33	806 ± 32
12	catechin (C)	289	278	1	1.0	20.5 ± 0.8	20.5 ± 0.8
15	epicatechin (EC)	289	278	1	1.0	67.6 ± 2.7	67.6 ± 2.7
19	epigallocatechin 3-*O*-gallate (EGCG)	457	274	1	3.1	9971 ± 399	9950 ± 398
23	gallocatechin 3-*O*-gallate (GCG)	457	274	1	3.1	683 ± 27	675 ± 27
25	epicatechin 3-*O*-gallate (ECG)*	441	274	1	3.8	4346 ± 174	4315 ± 173
26	gallocatechin 3,5-di-*O*-galltate*	609	274	1	5.9	217 ± 9	
27	epigallocatechin 3,5-di-*O*-galltate	609	274	1	5.9	363 ± 16	
28	catechin 3-*O*-gallate (CG)	441	274	1	3.8	173 ± 7	170 ± 7
29	epiafzelechin 3-*O*-gallate (EAG)	425	274	1	3.8	638 ± 26	
total catechins						**17436 ± 697**	
16	procyanidin B2 (19.95/12.96)	577	278	1	2.0	11.6 ± 0.5	11.6 ± 0.5
21	theasinensin A or D [EGCG-(2′→2′)-EGCG]	913	278	1	6.2	212 ± 8	
24	catechin-(4α→8)-epicatechin 3-*O*-gallate	729	278	1	4.8	130 ± 5	
total proanthocyanidins						**354 ± 14**	

position did not affect the molar absorptivities and the MRRF values significantly. The $MRRF_D$ values were also close to 1.0. The agreement of the MRRF and $MRRF_D$ values is not surprising since the moisture content for genistein, and many of the other isoflavonoids were only about 1%.

Daidzein, another common isoflavonoid, differs structurally from those described above, lacking a hydroxyl group at the 5-position. The MRRF and $MRRF_D$ values were 0.66, and there was a shift of λ_{max} to 248 nm. Glycosylation at the 7- and 8-positions (daidzin and puerarin) and methylation at the 4′-position (formononetin) had no effect on the λ_{max} and little effect on the MRRF values. Consequently, all were assigned $MRRF_P$ values of 0.66.

The addition of a methoxy group at the 6-position shifted the λ_{max} to 256 nm and the MRRF values to 0.80 for glycetein and glycetin, back toward the values for genistein. Although the

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shift of λ_{max} from the group reference standard was only 4 nm, the MRRF and $MRRF_D$ values were consistent at 0.80. Visual inspection showed that the absorbance profile of band II was relatively narrow as compared to that from band I. Consequently, the assigned $MRRF_P$ values were 0.80. Because MS provides little information regarding isomeric structure, differentiation between glycetein and bochanin A, both with a mass of 284 Da, will have to be based on retention time to assign the correct value for $MRRF_P$.

Flavanones, Dihydrochalcones, Chalcones, and Stilbenes (Group 3). Table 4 lists structures and response factors for flavanone, dihydrochalcone, chalcone, and stilbene standards recorded at 288 nm using hesperetin as the group reference standard. The flavanone standards included common aglycones and glycosides found in citrus fruits and juices.[9,16,22] In general, all of the flavanones had a λ_{max} at 288 ± 4 nm and MRRF and $MRRF_D$ values close to 1.0. These data indicated that methylation and glycosylation at the 4′- and 7-positions did not affect the molar absorptivity.

The flavanones with MRRF values significantly different from 1.0 were glycosylated (flavanomarein) or prenylated (isoxanthohumol) at the 8-position or had significantly different hydroxylation sites (liquiritigenin and 2′,6-dihydroxyflavanone). However, only flavanomarein had a λ_{max} within 4 nm of hesperitin. Isoxanthohumol is the extracted flavanone form of xanthohumol, a 3-prenylated chalcone with a dramatically different λ_{max} and an $MRRF_D$ value of 0.22. Both compounds were from *Humulus lupulus* L. and exist in beer produced using this plant. [23]

Two dihydrochalcones (phloretin and phloretin 6-*O*-glucoside) had a λ_{max} close to hesperitin. The glycosylated form had an MRRF value of 0.99, and the aglycone had an $MRRF_D$ value of 1.17. These compounds were assigned $MRRF_P$ values of 1.00 and 1.19, respectively.

The two stilbene standards (resveratrol and resveratrol 3-glucoside) had λ_{max} at 306 nm and 318 but $MRRF_D$ values close to 1.00. These compounds are found in grapes, wines, and some herbs.[2,9] The λ_{max} would not immediately suggest calibration using hesperitin. However, upon identification, such a calibration strategy would provide accurate results.

Benzoic Acids and Hydrolyzable Tannins (Group 4). Various forms of benzoic acids are commonly found in fruits. Hydrolyzable tannins are polyesters of a sugar moiety (or other nonaromatic polyhydroxy compounds) and a benzoic acid, usually gallic acid or hexahydroxydiphenic (HHDP) acid (in *S*- or *R*-form). In most cases, the sugar component is glucose, but fructose, xylose, and saccharose are also found. If the acid component is gallic acid, these tannins are called gallotannins, while the esters with hexahydroxydiphenic acid are called ellagitannins because ellagic acid is formed by lactonization of hexahydroxydiphenic acid when the tannins are hydrolyzed. However, most ellagitannins are mixed esters with both hexahydroxydiphenic and gallic acids. Gallo- and ellagitannins can be further linked by C−C and C−O−C bonds to form extensive polymers. In general, the hydrolyzable tannins have many isomeric forms and are found in many plant-derived foods.[10−12]

Table 5 shows a list of common hydroxybenzoic acids with gallic acid as the group reference standard. In almost every case, the addition or removal of a hydroxyl or methoxy group has a pronounced effect on the λ_{max} and the molar absorptivity. Thus, gallic acid will have limited use as a group reference standard. The λ_{max} and the mass of the hydroxybenzoic acids are distinctive, and standards are available for those listed in Table 5. Thus, quantitation of these acids and their derivatives will be more reasonably accomplished by using authentic standards that are very common and very inexpensive.

The fact that hexahydroxydiphenic acid had the same λ_{max} as gallic acid and an MRRF value of 2.00, double that of gallic acid, suggests that the MRRF values of the gallotannins and simple ellagitannins are additive and can be predicted for smaller molecules. For example, monogalloylglucose has one galloyl, and its MRRF should be 1.00; trigalloylglucose has three galloyls, and its MRRF should be 3.00. Similarly, 1-galloyl-4,6-HHDP-glucose has one galloyl and one HHDP and should have an MRRF value of 3.00 (Table 6). This approach, however, cannot predict MRRF values for tannins containing different conjugated systems (other than those for galloyl or HHDP) that would have different λ_{max} and molar absorptivities.

Accuracy of $MRRF_P$ Values. The ideal approach using response factors is to identify the compound using MS and/or MS^n data, determine to which group the compound belongs, and then use the correct $MRRF_P$ value with calibration for the appropriate group reference standard for quantitation. Difficulties arise if a compound has not been previously analyzed and no $MRRF_P$ value exists. There are two possible solutions to this problem. The first is to identify the group that the compound belongs to based on λ_{max} and assume an $MRRF_P$ value of 1.00. The second approach is to match the compound as closely as possible with a compound in the database based on structure and to use its group reference standard and value for $MRRF_P$.

In our previous study of cinnamoyl-containing compounds, we considered the first possibility. We divided the hydroxycinnamic derivatives and the glycosides of flavonols and flavones into five groups. When the λ_{max} of a compound fell within ±2 nm of that of a group reference standard, the $MRRF_D$ values for 17 compounds were 1.01 ± 0.03. These data suggested that there were no shifts in the molar absorptivities since the uncertainty associated with repeat moisture determinations was ±3%. Alternatively, if the λ_{max} of a compound fell within 10 nm of that of a group reference standard, the undried MRRF values for 51 compounds were 0.96 ± 0.13. This result indicated that the assumption of an $MRRF_P$ value of 1.00 for any compound not previously analyzed but, with a λ_{max} within 10 nm of that of a group reference standard, could be analyzed with an accuracy of ±13%.

The approach just described, which assumes an $MRRF_P$ value of 1.0, is not applicable to all the benzoyl-containing compounds examined in the current study. Band II was found to be narrower that band I and the λ_{max} for the 4 group reference standards were much closer together, a range of 260−288 nm for band II as compared to 326−368 nm for band I. Shifts of as little as 2 nm were found to indicate significant changes in the molar absorptivities of the flavanols and isoflavones (Tables 1 and 3). For example, $MRRF_P$ values for EGC, EGCG, and ECG were 0.3, 3.1, and 3.8 with shifts of λ_{max} of 10, 4, and 2 nm, respectively. For the isoflavones, $MRRF_P$ values of 0.66 and 0.8 were observed for daidzein and glycetin with shifts of 12 and 4 nm, respectively, for λ_{max}. In both cases, accurate quantitation can only be achieved through the second approach, that is, match the new compound as closely as possible with a compound in the database based on MS data and use its group reference standard and value for $MRRF_P$.

The need for specific $MRRF_P$ values is also obvious for the hydroxybenzoic acids (Table 5). Significant changes in the

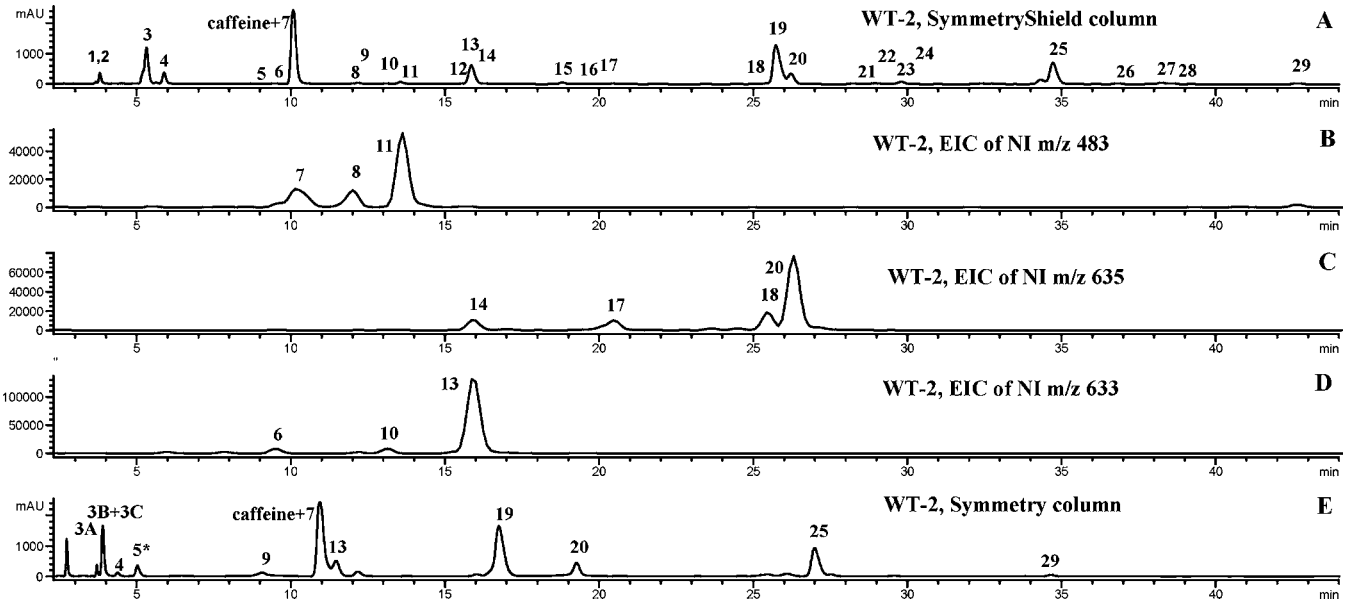

Figure 2. LC (274 nm, A and E) and extracted ion chormatograms (EIC) (B–D).

MRRF values are accompanied by large shifts in the λ_{max}. Conversely, the flavanones, chalcones, and stilbenes (Table 4) were similar to the previously reported cinnamoyl-containing compounds. The average MRRF value for 20 standards with a λ_{max} within 4 nm of that of hesperitin was 1.01 ± 0.09. This suggests that the assumption of an $MRRF_P$ value of 1.0 for these compounds would not be unreasonable.

The need for specific $MRRF_P$ values for the benzoic acids, catechins, flavanones, and isoflavones is not particularly problematic since the numbers of these compounds are much less extensive. As more standards become available, the database (Tables 1 and 3) can be expanded. Thus, quantitation of all but the most exotic compounds will be possible.

Quantitation of Catechins, Proanthocynidins, and Gallic Acid Derivatives in White Tea. The LC-MS study of white tea identified 11 catechins, three proanthocyanidins, gallic acid, three galloylquinic acids, and 13 gallotannins (Figure 2 and Table 6). These phenolic compounds also were found in various green teas.[20,24−27] The catechins have been quantified in two ways: using authentic standards, when available, and using a group reference standard. The catechins were quantified at 278 nm using (+)-catechin as the group reference standard. The $MRRF_P$ values for the stereoisomer pairs were 1.0, 3.8, 0.3, and 3.1, as previously discussed. Concentrations based on authentic and group reference standards were in good agreement (Table 6). Both sets of values fell within the range of concentrations previously published for white teas.[24,25]

Numerous galloylglucoses were identified in the white tea (Figure 2). All have been previously reported.[20,25,26] They were quantified at 274 nm using gallic acid as the group reference standard. 1- and 6-Monogalloylglucoses (peaks 1 and 2, Figure 2A,E) were quantified at 274 nm using MRRFP values of 1.00. 1,3-, 1,2-, 1,6-, and 2,6-Digalloylglucosides have been previously reported in tea.[20,24−27] Three of them were found in white tea: peaks 7, 8, and 11 ($[M - H]^-$ at m/z 483, Figure 2A,B) with peak area ratios of 7:8:11 of 1.6:1.0:43.7 in the extracted ion chromatograms (EIC). They were quantified at 274 nm using an $MRRF_P$ value of 2.00. Peak 7 coeluted with caffeine (Figure 2A) but was resolved using an EIC (Figure 2B). Similarly, trigalloylglucoses (1,2,6- and 1,4,6-trigalloylglucosides) ($[M - H]^-$ at m/z 635) (peaks 14, 17, 18, and 20, Figure 2C) had very small and partially overlapping peaks at 274 nm (Figure 2A). They were cleanly resolved using EIC (Figure 2C) and quantified at 274 nm using $MRRF_P$ values of 3.00 based on the ratio of EIC peaks.

Stricitinin [1β-galloyl-4,6-(*S*)-HHDP-glucose, $[M - H]^-$at m/z 633] (peak 13) and two isomers (peaks 6 and 10, Figure 2D) and 1,2-digalloyl-4,6-HHDP-glucose (peak 22, $[M - H]^-$ at m/z 787) were detected in white tea by both UV and MS (EIC for peak 22 not shown). Stricitinin has been reported as a main tea polyphenol, and 1,2-digalloyl-4,6-HHDP-glucose has been reported in some Chinese green teas.[25−27] Two strictinin isomers, isostrictinin [1β-galloyl-2,3-(*S*)-HHDP-glucose] and gemin D [3-galloyl-4,6-(*S*)-HHDP-glucose],[26,27] have been reported, but positive identification cannot be made at this time using only LC-MS data. These compounds were quantified using $MRRF_P$ values of 5.00 and 6.00, respectively. This may be the first report of some of the trigalloylglucosides and three isomeric stricitinins coexisting in green tea samples.

Theasinensin A contained two EGCG units and was quantified using an $MRRF_P$ value of 6.2. Similarly, the $MRRF_P$ for catechin-(4α→8)-epicatechin 3-*O*-gallate was the sum of values for catechin (1.00) and ECG (3.80) or 4.80. The concentration of 3,5-di-*O*-gallates of epigallocatechin and gallocatechin was calculated using an $MRRF_P$ value of 5.9. Some 3,5-gallates of flavanols have been previously reported in Chinese green teas.[20,26]

As indicated above, after checking the purity of available commercial standards, selecting suitable group reference standards, and determining the $MRRF_P$ values of individual compounds, this approach can provide an easy and convenient method for the quantitation of phenolic compounds in foods.

AUTHOR INFORMATION

Corresponding Author

*Tel: 301-504-9136. Fax: 301-504-8314. E-mail: longze.lin@ars.usda.gov.

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