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Quantitation of the Hydroxycinnamic Acid Derivatives and the Glycosides of Flavonols and Flavones by UV Absorbance after Identification by LC-MS

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ABSTRACT: A general approach was developed to quantify hydroxycinnamic acid derivatives and the glycosides of flavonols and flavones using UV molar relative response factors (MRRFs). More than 90 standards were analyzed by LC-MS and divided into five groups based on the λ_{max} of their band I absorbance profiles. For each group, a commercially available standard was chosen as the group reference standard. Response factors were determined for each standard in each group as purchased (MRRF) and, when possible, after vacuum drying ($MRRF_D$). The $MRRF_D$ values for 17 compounds whose λ_{max} values fell within ±2 nm of the group reference standard were 1.01 ± 0.03. MRRF values for compounds whose λ_{max} values fell within ±10 nm of the group reference standard were 0.96 ± 0.13. Group reference standards were used to quantify 44 compounds in Chinese lettuce, red onion, and white tea. This approach allows quantitation of numerous compounds for which there are no standards.

KEYWORDS: *quantitation, UV absorbance, flavonol glycosides, flavone glycosides, hydroxycinnamic acid derivatives, molar relative response factors, foods*

INTRODUCTION

Flavonoids and phenolic acids are the most common and widely distributed of the plant secondary metabolites.[1−4] Recent advances in high performance liquid chromatography (HPLC) and mass and tandem mass spectrometry (MS and MS^n) have made identification of these compounds routine practice. The only shortcoming of the MS methods is their inability to differentiate isomeric structures.[5−8] Quantitation of these compounds, however, remains a challenge. This is primarily due to the tremendous variety of phenolic compounds (some estimates are as high as 8000 different structures for flavonoids) and the lack of standards (less than 3% are available commercially). Even with the availability of standards, maintaining an appropriate collection in any laboratory would be a logistical and financial impossibility.

UV absorbance following LC separation is the most common and convenient way for the quantitation of phenolic compounds.[9−12] Phenolic compounds may have one or two absorption bands in the UV; band I (305−390 nm) comes from the B-ring cinnamoyl structure (Figure 1), and band II (230−300 nm) comes from the A-ring benzoyl or benzene structure. Substitution at different positions affects the molar absorptivity and the wavelength maxima of each band. On the basis of these two absorption bands, phenolic compounds can be divided into two groups: hydroxycinnamic acid derivatives and glycosides of flavonols and flavones have a strong UV band I absorption and weaker band II absorption (Figure 2), and hydroxybenzoic acid derivatives, catechins, isoflavones, and flavanones have UV band II absorption. Anthocyanins also have a strong visible absorption band.

This study focuses on the quantitation of phenolics with strong band I absorption, i.e. the hydroxycinnamic acid

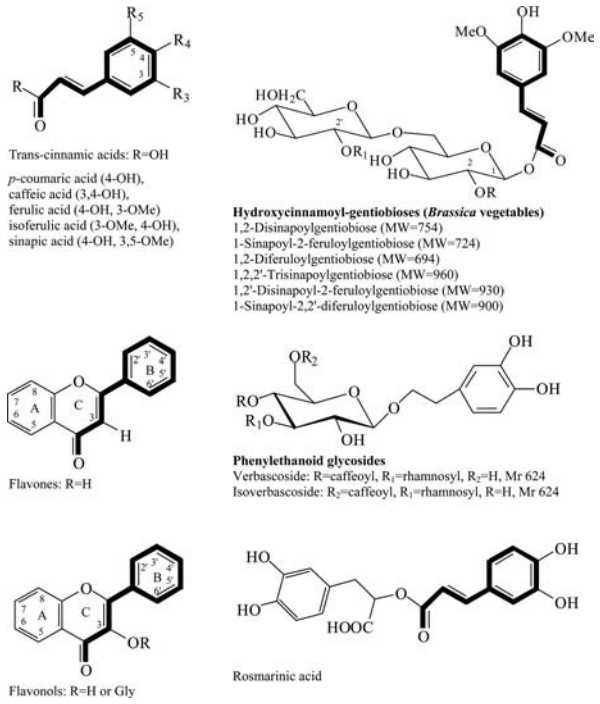

Figure 1. Structures for hydroxycinnamates, flavones, and flavonols.

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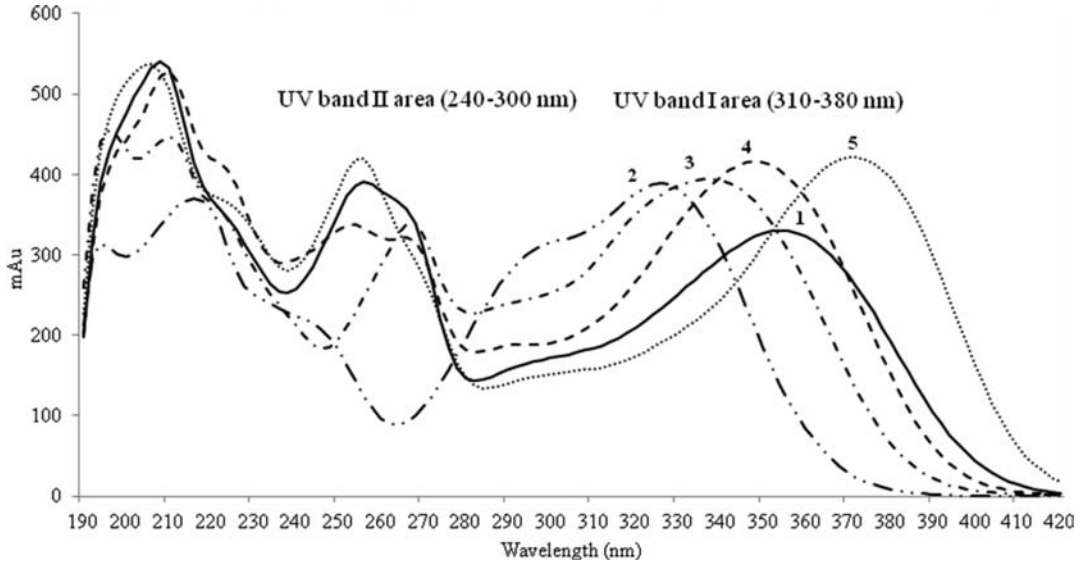

Figure 2. UV spectra of five dried standards at the same molar concentration: 1 = rutin; 2 = chlorogenic acid; 3 = apigenin; 4 = luteolin; and 5 = quercetin.

Table 1. Hydroxycinnamic Acids and Derivatives at 326 nm[a]

	position									
compd	R	R3	R4	R5	MW	λ_{max} (nm)	MRRF 326	$MRRF_D$ 326	$MRRF_P$ 326	$MRRF_R$ 336
rutin									**0.61**	**1.00**
Group Ia										
chlorogenic acid	Q	1	1		354	326	**1.00**	**1.00**	**1.00**	**1.24**
caffeic acid		1	1		180	324	0.95	0.96	1.00	1.24
caffeic acid methyl ester	OMe	1	1		194	324	0.94	0.94	1.00	1.24
ferulic acid		2	1		194	324	0.96	1.00	1.00	1.24
isoferulic acid		2	1		194	324	0.95	0.99	1.00	1.24
sinapinic acid		2	1	2	224	324	0.96	1.02	1.00	1.24
1,3-dicaffeoylquinic acid	Q-C	1	1		516	328	1.65		2.00	2.48
1,5-dicaffeoylquinic acid	T-C,-C	1	1		516	328	1.75		2.00	2.48
chicoric acid	Q-Q_t-C	1	1		474	328	1.63		2.00	2.48
verbascoside	Q-Q_t-PE	1	1		624	328	0.97		1.00	1.24
isoverbascoside	Q-Q_t-PE	1	1		624	328	0.95		1.00	1.24
rosmarinic acid	Q-Q_t-PE PP	1	1		360	328	0.98		1.00	1.24
Group Ib										
p-coumaric acid			1		164	310	0.72	0.73	0.73	0.90
kaempferol 3-*O*-glucoside					448	348	0.83	0.83	0.83	1.02
kaempferol 3-*O*-*p*-coumaroyl-glucoside					594	310	1.63	1.56	1.56	1.93

[a]The average moisture content of chlorogenic acid was 6.4% (5.9−7.0%, n = 4). Functional groups: 1 = OH; 2 = *O*-methyl; 3 = *O*-glycoside; 4 = glucose; 5 = *O*-apigenin. Abbreviations: C, caffeoyl; Gl, glucoside; Me, methyl; PE, phenylethanoid; PP, phenylpropanoid; Q, quinic acid; T, tartaric acid. MRRF values: MRRF, molar relative response function for standards as purchased,; $MRRF_D$, molar relative response function for vacuum dried standards; $MRRF_P$, proposed molar relative response function based on λ_{max}; $MRRF_R$, proposed molar relative response function based on rutin.

derivatives and the glycosides of flavonols and flavones. The common conjugated bond system of these compounds, arising from the hydroxycinnamoyl structure, produces a strong band I absorption and makes these compounds good candidates for quantitation using a common standard. The effects of substitution at different positions on the absorption spectra and on the wavelength of maximum absorption (λ_{max}) for band I were carefully examined for a variety of purified phenolic standards.

In this study, more that 90 phenolic compounds were divided into five groups on the basis of their λ_{max} values. A readily available commercial standard was selected for calibration of each group, and molar relative response factors (MRRFs) were computed for the rest of the compounds.[13−17] In addition, calibration for all the the groups based on a single standard, was considered. Ideally, the database constituted by Tables 1−5 will expand as more samples are analyzed. The phenolic compounds of Chinese lettuce, white tea, and red onion were used as examples of the quantitation method.

MATERIALS AND METHODS

Standards. Apigenin, apigenin 6-*C*-glucoside, luteolin, diosmin (diosmetin 7-*O*-rutinoside), quercetin dihydrate, rutin trihydrate (quercetin 3-*O*-rutinoside), myricetin, kaempferol, quercetin 3-*O*-rhamnoside, rhamnetin, isorhamnetin, nobiletin, morin, chlorogenic acid, caffeic acid, ferulic acid, isoferulic acid, sinapic acid, *p*-coumaric acid, and ellagic acid were obtained from Sigma-Aldrich Chemical Co.

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Table 2. Apigenin and Related Flavones at 336 nm[a]

compd	position 3′	4′	5′	5	6	7	8	MW	λ_{max} (nm)	MRRF 336	$MRRF_D$ 336	$MRRF_P$ 336	$MRRF_R$ 354
rutin												**0.71**	**1.00**
apigenin		1		1		3		270	336	**1.00**	**1.00**	**1.00**	**0.97**
apigenin 7-*O*-glucoside		1		1		3		432	336	0.99	1.04	1.00	0.97
rhoifolin		1		1		3		578	336	1.00	1.03	1.00	0.97
vitexin		1		1		1	4	432	336	0.95	1.02	1.00	0.97
genkwanin		1		1		2		284	336	0.99	0.98	1.00	0.97
acacetin		2		1		1		284	334	1.08	1.07	1.00	0.97
isorhoifolin		1		1		5		578	336	0.78		1.00	0.97
vitexin		1		1		1	4	578	336	0.84		1.00	0.97
isovitexin		1		1	4	1		432	336	0.87		1.00	0.97
isovitexin-7-*O*-glucoside		1		1	4	3		594	336	0.90		1.00	0.97
scutellarein		1		1	1	1		286	336	0.75		1.00	0.97
scutellarin		1		1	1	3		462	336	0.75		1.00	0.97
linarin		2		1		3		592	334	0.96		1.00	0.97
fortunellin		2		1		3		592	334	1.06		1.00	0.97
hinokiflavone (3′,6-biapigenin)	5	1		1	5	1		538	338	1.92		2.00	1.94
cupressuflavone (8,8′-biapigenin)		1		1		1	5	538	330	1.58		2.00	1.94
gardenin A	2	2	2	1	2	2	2	418	338	0.89			
eupatorin	2	2		1	2	1		344	342	1.11			
sinensetin	2	2		2	2	2		372	330	1.15			
5,7,4′-trimethylapigenin		2		2		2		312	328	1.04			
tangeritin		2		2	2	2	2	372	324	1.08			
bailcalein				1	1	1		270	316	0.41			
chrysin				1		1		254	314	0.53			
wogonin				1		1	2	284	274	0.31			

[a]The average moisture content of apigenin was 5.4% (4.8−5.8%, n = 3). Functional groups, abbreviations, and MRRF definitions are the same as those in Table 1.

Table 3. Luteolin and Related Flavones at 348 nm[a]

compd	position 3′	4′	5′	5	6	7	8	MW	λ_{max} (nm)	MRRF 348	$MRRF_D$ 348	$MRRF_P$ 348	$MRRF_R$ 354
rutin												**0.83**	**1.00**
luteolin (1)	1	1		1		1		286	348	**1.00**	**1.00**	**1.00**	**1.20**
luteolin (2)	1	1		1		1		286	348	1.00	1.04	1.00	1.20
luteolin 7-*O*-glucoside (1)	1	1		1		1		448	348	1.11	1.02	1.00	1.20
diosmin	1	2		1		3		608	348	1.09	1.02	1.00	1.20
luteolin 7-*O*-glucoside (2)	1	1		1		3		448	348	0.95		1.00	1.20
luteolin 7,3′-*O*-diglucoside (2)	3	1		1		3		610	342	1.01		1.00	1.20
diosmetin	1	2		1		1		300	348	0.71		1.00	1.20
neodiosmin	1	2		1		3		608	348	1.06		1.00	1.20
orientin	1	1		1		1	4	448	348	1.05		1.00	1.20
homoorientin	1	1		1	4	1		448	348	1.08		1.00	1.20
chrysoeriol	2	1		1		1		300	348	1.11		1.00	1.20
luteolin 6-methoxy	1	1		1	2	1		316	348	1.10		1.00	1.20
luteolin 6,7-dimethoxy	1	1		1	2	2		330	346	1.14		1.00	1.20
tricetin	2	1	2	1		1		302	352	0.91			
luteolin 4′-*O*-glucoside	1	3		1		1		448	338	0.94			

[a]The average moisture content of luteolin was 8.6% (8.2−8.8%, n = 3). Functional groups, abbreviations, and MRRF definitions are the same as those in Table 1.

(Saint Louis, MO). Apigenin, luteolin, rutin, quercetin, chlorogenic acid, luteolin 7-*O*-glucoside, diosmetin, quercetin 3-*O*-glucoside, kaempferol, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-6″-*p*-coumaroylglucoside, rhamnetin, isorhamnetin, wogonin, diosmetin, 1,3-*O*,*O*-dicaffeoylquinic acid, 1,5-*O*,*O*-dicaffeoylquinic acid, vebascoside, isoverbascoside, and chicoric acid were obtained from Chromadex, Inc. (Irvine, CA). Apigenin, luteolin, rutin, isorhamnetin, rosmarinic acid, kaemperol 3-*O*-glucoside, kaempferol 3-*O*-6″-*p*-coumaroylglucoside, and methyl caffeate were obtained from Indofine Chemical Co. (Somerville, NJ). All the flavonoid standards in the tables were also purchased from Extrasynthese (Genay, Cedex, France).

The slightly yellow powders of caffeic acid from the supplier were dissolved in methanol−water (60:40) and recrystallized to produce white crystals for the experiment.

Standard Purity. All the phenolic standards were examined initially for inhomogeneity, for undissolved particles after dissolution,

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Table 4. Flavonol 3-*O*- or 3,7-*O*-Glycosides at 354 nm[a]

	position										
compd	3′	4′	5′	3	5	7	MW	λ_{max} (nm)	MRRF 354	$MRRF_D$ 354	$MRRF_P$ 354	$MRRF_R$ 354
rutin (1)	1	1		3	1	1	610	354	**1.00**	**1.00**	**1.00**	**1.00**
rutin (2)	1	1		3	1	1	610	354	0.98	1.00	1.00	1.00
quercetin 3-*O*-glucoside	1	1		3	1	1	464	354	0.95	0.98	1.00	1.00
quercetin 3-*O*-galactoside	1	1		3	1	1	464	354	1.04		1.00	1.00
quercetin 3-*O*-rhamnoside (1)	1	1		3	1	1	448	354	0.89		1.00	1.00
quercetin 3-*O*-rhamnoside (2)	1	1		3	1	1	448	354	0.79		1.00	1.00
quercetin 3-*O*-arabinosylglucoside	1	1		3	1	1	596	354	0.99		1.00	1.00
isorhamnetin-3-*O*-glucoside	2	1		1	1	1	478	354	0.95		1.00	1.00
isorhamnetin-3-*O*-rutinoside	2	1		1	1	1	624	354	1.00		1.00	1.00
myricetin-3-*O*-rhamnoside	1	1	1	3	1	1	464	352	0.83		1.00	1.00
kaempferol 3-*O*-glucoside		1		3	1	1	448	348	0.95	0.96	1.00	1.00
kaempferol 3-*O*-rutinoside		1		3	1	1	594	348	0.88	0.88	1.00	1.00
kaempferol 3-*O*-robinoside-7-*O*-rhamnoside		1		3	1	3	740	348	0.62		1.00	1.00
syringetin-3-*O*-glucoside	2	2	2	3	1	1	508	358	1.13		1.00	1.00
syringetin-3-*O*-galactoside	2	2	2	3	1	1	508	358	1.05		1.00	1.00

[a]The average moisture content of rutin (i.e., rutin·3hydrate) was 10.0% (9.8−10.2%, $n = 6$). Functional groups, abbreviations, and MRRF definitions are the same as those in Table 1.

Table 5. Flavonol Aglycones and 7-*O*, 4′-*O*-, and 8-*O*-Glycosides at 368 nm[a]

	position												
compd	2′	3′	4′	5′	3	5	7	8	MW	λ_{max} (nm)	MRRF 368	$MRRF_D$ 368	$MRRF_P$ 368	$MRRF_R$ 354
Rutin													**0.69**	**1.00**
quercetin		1	1		1	1	1		302	372	**1.00**	**1.00**	**1.00**	**1.05**
myricetin		1	1	1	1	1	1		318	372	1.05	1.03	1.00	1.05
tamarixetin		1	2		1	1	1		316	370	1.16		1.00	1.05
rhamnetin		1	1		1	1	2		316	370	1.20		1.00	1.05
isorhamnetin (1)		2	1		1	1	2		316	370	1.05		1.00	1.05
isorhamnetin (2)		2	1		1	1	1		316	370	0.97		1.00	1.05
syringetin		2	1	2	1	1	1		346	374	1.27		1.00	1.05
kaempferol			1		1	1	1		286	368	1.03	1.08	1.00	1.05
quercetin-4′-*O*-glucoside		1	3		1	1	1		464	366	0.96	0.90	1.00	1.05
myricetin 3′,4′,5′-trimethoxy		2	2	2	1	1	1		360	366	0.93		1.00	1.05
kaempferol 7-*O*-neohesperidoside			1		1	1	3		594	366	1.07		1.00	1.05
robinetin		1	1	1	1		1		302	362	1.04	1.04	1.00	1.05
fisetin		1	1	1	1		1		286	360	1.12			
geraldol		2	1		1		1		300	360	1.18			
galangin					1	1	1		270	360	0.72			
geraldol 4′-methoxy		2	2		1		1		314	358	1.10			
3,7,4′-trihydroxyflavone			1		1		1		270	354	0.92			
morin	1		1		1	1	1		302	352	0.59			
herbacetin		1			1	1	1	1	302	352	0.53			
datiscetin	1				1	1	1		286	348	0.40			
gossypetin		1	1		1	1	1	1	318	348	0.50			
gossypetin-8-*O*-glucoside		1	1		1	1	1	3	480	378	0.79			
ellagic acid									302	368	0.57	0.59	0.59	0.62

[a]The average moisture content of quercetin·2hydrate was 11.7% (11.6−12.3%, $n = 6$). Functional groups, abbreviations, and MRRF definitions are the same as those in Table 1.

and for UV-sensitive contaminants in the chromatogram. Observation of any undissolved particles was a basis for eliminating the standard.

The molecular weights presented in Tables 1−5 do not include hydration. The purity of the standards listed by the suppliers on the label covered a range of specifications, including reagent grade, purity ≥95%, or purity 95.5% by HPLC. Only 20 of the standards had both HPLC purity and specifications for water/solvent content.

To remove crystalline water/solvent content and/or absorbed moisture, the standards were vacuum-dried (National Appliance Co, Portland, OR) at 110 °C until a constant weight was reached (around 24 h). This was done only if there was sufficient standard with which to make accurate weight measurements.

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. Accurately weighed 3.00−6.00 mg standards were put into 10 mL volumetric flasks (±0.2 mL). The standards were first dissolved in 2 mL of DMSO and then brought to volume with aqueous methanol (60/40, v/v). Each solution was mixed with the

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Table 6. Concentration of the Phenolic Compounds in Chinese Lettuce[a]

compd (peak no.)	group	MW	λ_{max}	value used for calculation			
				$MRRF_D$	$MRRF_P$	MRRF	$MRRF_R$
chlorogenic acid (1)	Ia	354	328	46.8 ± 1.4	46.8 ± 1.4	49.8 ± 1.5	46.7 ± 1.3
caffeoyltaric aicd (2)	Ia	312	328		37.5 ± 1.5	39.9 ± 1.6	37.4 ± 1.3
caffeic acid (3)	Ia	180	328	4.8 ± 0.2	4.8 ± 0.2	5.1 ± 0.2	4.7 ± 0.2
caffeoylmalic aicd (4)	Ia	206	328		6.1 ± 0.2	6.9 ± 0.2	6.0 ± 0.2
3,5-dicaffeoylquinic acid (12)	Ia	516	328		91.2 ± 3.6	97 ± 3.9	91.0 ± 3.5
di-*O* - caffeoyltartaric acid (13)	Ia	474	328		451 ± 14	480 ± 14	448 ± 13
4,5-dicaffeoylquinic aicd (14)	Ia	516	328		10.1 ± 0.5	10.7 ± 0.5	9.9 ± 0.4
meso-di-*O*-caffeoyltartaric acid (15)	Ia	474	328		15.0 ± 0.6	16 ± 0.6	14.8 ± 0.5
caffeoyl-*p*-coumaroyltartaric acid (16)	Ia	458	328		20.2 ± 0.8	21.5 ± 0.9	19.8 ± 0.7
caffoylferuloyltartaric aicd (17)	Ia	488	328		22.2 ± 0.9	23.6 ± 0.9	21.8 ± 0.7
Total Group Ia				**51.6 ± 1.5**	**705 ± 31**	**751 ± 34**	**700 ± 31**
luteolin 7-*O*-glucoside (7)	III	448	348	4.2 ± 0.2	4.2 ± 0.2	4.6 ± 0.1	4.3 ± 0.2
luteolin 3-*O*-glucuronide (9)	III	464	348		50.4 ± 2.0	54.8 ± 2.2	51.4 ± 2.1
Total Group 3				**4.2 ± 0.2**	**54.6 ± 2.2**	**59.4 ± 2.4**	**55.7 ± 2.3**
quercetin 3-glucoside (6)	IV	464	354	8.3 ± 0.3	8.3 ± 0.3	9.2 ± 0.4	8.3 ± 0.3
quercetin 3-*O*-glucuronide (8)	IV	478	354		9.1 ± 0.4	10.0 ± 0.4	9.1 ± 0.4
quercetin 3-*O*-malonylglucoside (10)	IV	550	354		31.9 ± 1.3	35.1 ± 1.4	31.9 ± 1.3
quercetin 3-*O*-malonylglucoside (11)	IV	550	354		5.8 ± 0.2	6.4 ± 0.2	5.8 ± 0.2
Total Group IV				**8.3 ± 0.3**	**55.2 ± 2.2**	**60.7 ± 2.4**	**55.2 ± 2.2**
quercetin 7-glucoside (5)	V	464	368		6.8 ± 0.3	7.6 ± 0.3	7.0 ± 0.3
Total				**64.1 ± 1.6**	**823 ± 37**	**879 ± 40**	**818 ± 37**

[a]Concentration is in mg/100 g ± standard deviation.

Table 7. Concentration of the Phenolic Compounds in Red Onion[a]

compd (peak no.)	group	MW	λ_{max}	value used for calculation			
				$MRRF_D$	$MRRF_P$	MRRF	$MRRF_R$
quercetin 3,7,4′-*O*-triglucoside (1)	IV	788	344		2.0 ± 0.1	2.2 ± 0.1	2.0 ± 0.1
quercetin 3,4′-*O*-diglucoside (3)	IV	626	344		124 ± 5	140 ± 6	124 ± 5
isorhamnetin 3,4′-*O*-diglucoside (4)	IV	640	344		7.5 ± 0.3	8.3 ± 0.3	7.5 ± 0.3
quercetin 3-*O*-glucoside (5)	IV	464	354	4.5 ± 0.2	4.5 ± 0.2	5.2 ± 0.2	4.5 ± 0.2
Total Group IV				**4.5 ± 0.2**	**138 ± 6**	**156 ± 6**	**138 ± 6**
quercetin 7,4′-*O*-diglucoside (2)	V	626	366		6.9 ± 0.3	7.7 ± 0.3	7.0 ± 0.3
quercetin 4′-*O*-glucoside (6)	V	464	366	286 ± 11	286 ± 11	320 ± 13	292 ± 12
isorhamnetin 4′-*O*-glucoside (7)	V	478	366		22.0 ± 0.9	24.6 ± 1.0	22.4 ± 0.9
quercetin (8)	V	302	372	66.9 ± 2.7	66.9 ± 2.7	74.8 ± 3.0	68.2 ± 2.7
Total Group V				**353 ± 12**	**382 ± 15**	**427 ± 17**	**390 ± 16**
Total				**358 ± 12**	**520 ± 22**	**583 ± 24**	**528 ± 22**

[a]Concentration is in mg/100 g ± standard deviation.

solutions of its reference and 3−5 other standards in same molar concentration, and injected twice. Each mixed solution was prepared at three concentration levels (three different dilution factors) to provide a range of signals suitable for the quantitation of the major and minor polyphenols in food samples. The standard deviation (RSD) for each peak was < ±5.0, and the average integrated peak absorbance fell in the linear range. Peak areas were used to calculate MRRF values.[9]

Sample Preparation. Chinese lettuce (*Lactuca sativa* L. var. *asparagine*) (Compositae) and red onion (*Allium cepa* L.) (Amaryllidaceae) were bought in local food stores in Maryland and freeze-dried. White tea (*Camellia sinensis* L. var. *white tea*) (Theaceae) was obtained from a local store in Kunming, China. The dry materials were powdered and passed through 60 mesh sieves, and the ground powders (100.0 mg) were put into a test tube and extracted with 10.000 mL of aqueous methanol (60/40 v/v) by sonication at room temperature for 60 min. The slurry mixture was centrifuged at 2500 rpm for 15 min, 8.000 mL of the supernatant was filtered through a 17 mm (0.45 μm) PVDF syringe filter (VWR Scientific, Seattle, WA, USA), and 10 μL of the extract (or extract-1; 20 μL for red onion) was injected into the HPLC. Each sample extract was prepared in triplicate, and each preparation was analyzed in duplicate.[9]

The extract for quantitation was prepared using a series of three extractions. The first extraction was made as described above, and 8.000 mL (accurately determined by the weight, error ≤1 mg) of the supernatant was drawn off and named extract 1. Then 8.000 mL of fresh solvent was added to the sample tube. After centrifuging, 8.000 mL of the supernatant was again drawn off and named extract 2. Extract 2 contained one-fifth of the mass of extract 1 and 100% of the mass from the second extraction. This was followed by a third extraction. In this study, the result showed that the new mass from the second extraction for each of the three samples contributed less than 5% of the total mass of the first extractions, so second and third extractions were not needed. Thus, a single extraction was suitable for a quantitative determination.

LC and MS. The liquid chromatographer−diode array detector−electrospray ionization/mass spectrometer (LC-DAD-ESI/MS) was previously described, as were the conditions for identifying the phenolic compounds in the three samples.[7] 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 326, 336, 348, 354, and 368 nm were monitored in real time and for the peak intensity of

Table 8. Concentration of Phenolic Compounds in White Tea[a]

compd (peak no.)	group	MW	λmax	value used for calculation: $MRRF_D$	$MRRF_P$	MRRF	$MRRF_R$
kaempferol							
3-*O*-6²-*E*-*p*-coumaroylglucoside (10)	Ib	594	310	9.7 ± 0.4	9.7 ± 0.4	10.3 ± 0.4	9.5 ± 0.4
3-*O*-6″-*Z*-*p*-coumaroylglucoside (11)	Ib	594	310		1.03 ± 0.04	1.10 ± 0.04	1.01 ± 0.04
3-*O*-*p*-coumaroylhexoside (12)	Ib	594	310		2.4 ± 0.1	2.6 ± 0.1	2.35 ± 0.1
3-*O*-di-*p*-coumaroylhexoside (14)	Ib	740	310		0.80 ± 3.6	0.82 ± 0.04	0.81 ± 0.04
3-*O*-2″,6″-di-*E*-*p*-coumaroylgalactoside (15)	Ib	740	310		25.0 ± 1.0	26.6 ± 1.1	24.5 ± 1.0
3-*O*-2″,6″-di-*Z*-*p*-coumaroylglucoside (16A)	Ib	740	310		3.0 ± 0.1	3.1 ± 0.1	2.94 ± 0.1
3-*O*-2″*Z*,6″*E*-di-*p*-coumaroylglucoside (16B)	Ib	740	310		2.4 ± 0.1	2.6 ± 0.1	2.35 ± 0.1
3-*O*-di-*p*-coumaroylhexoside (17)	Ib	740	310		0.83 ± 0.04	0.81 ± 0.04	0.82 ± 0.04
Total Group Ib				**9.7 ± 0.4**	**45.1 ± 1.9**	**48.0 ± 2.0**	**42.1 ± 1.8**
myricetin 3-*O*-galactoside (1)	IV	480	354		2.5 ± 0.1	2.7 ± 0.1	2.5 ± 0.1
myricetin 3-*O*-galucoside (2)	IV	480	348		1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.1
quercetin 3-*O*-galactosylrutinoside (3)	IV	772	354		1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
quercetin 3-*O*-glucosylrutinoside (4)	IV	772	354		4.8 ± 0.2	5.3 ± 0.2	4.8 ± 0.2
kaempferol 3-*O*-glucosylrutinoside (5A)	IV	756	354		9.7 ± 0.5	10.7 ± 0.5	9.7 ± 0.5
rutin (5B)	IV	610	354	2.1 ± 0.1	2.1 ± 0.1	2.4 ± 0.1	2.1 ± 0.1
quercetin 3-*O*-glucoside (6)	IV	464	354	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
kaempferol 3-*O*-glucosylrutinoside (7)	IV	756	354		22.1 ± 1.0	24.3 ± 0.1	22.1 ± 1.0
kaempferol 3-*O*-rutinoside (8)	IV	594	354		8.4 ± 0.5	9.3 ± 0.1	8.4 ± 0.5
kaempferol 3-*O*-glucoside (9)	IV	448	354	2.3 ± 0.1	2.3 ± 0.1	2.6 ± 0.1	2.3 ± 0.1
Total Group IV				**5.7 ± 0.2**	**56.6 ± 2.4**	**62.2 ± 2.6**	**56.6 ± 2.4**
kaempferol (13, V, 286, 368 nm)				0.80 ± 0.04	0.80 ± 0.04	0.80 ± 0.04	0.80 ± 0.04
Total Group V				**0.8 ± 0.04**	**0.8 ± 0.04**	**0.8 ± 0.04**	**0.8 ± 0.04**
Total				**16.2 ± 0.5**	**102.5 ± 4.6**	**110.0 ± 5.0**	**99.5 ± 4.5**

[a]Concentration is in mg/100 g ± standard deviation.

each compound. Repeated runs were just required to have LC data only. To check the absorption changes at wavelengths around the band I λ_{max}, for each of the five group reference standards, data were also acquired at 2 nm intervals in both directions (λ_{max} ± 10 nm).

RESULTS AND DISCUSSION

Groups Based on UV Band I Absorption Maxima. LC-DAD-ESI/MS was used to organize more than 90 standards into five major groups as determined by their structure and the peak maxima (λ_{max}) of their band I absorbance profile (Tables 1−5). In general, the degree of structural similarity is accurately reflected by the similarity of the band I λ_{max} values. The total spectra were less informative, since the band II λ_{max} values were not well correlated with the structures of the compounds in these five groups. Common names have been used in Tables 1−8 to reduce the column width and table size.

Group I is composed of hydroxycinnamic acid derivatives (λ_{max} = 310 and 324−336 nm), and groups II−V (λ_{max} = 330−380 nm) are composed of the glycosylated flavonols and flavones. The shift between group I and the others is understandable with the inclusion of hydroxycinnamic acid into the flavonoid structure of the latter compounds. As shown in Tables 2−5, we see shifts to higher wavelengths for groups II−V as the structures change from hydroxyls at the 4′, 3, and 5 positions (group II), to hydroxyls at the 3′, 4′, 3, and 5 positions (group III), to hydroxyls at the 3′, 4′, 3, 5, and 7 positions (group V), and a slight decrease in λ_{max} with glycosylation at the 3 position (group IV).

Molar Relative Response Factors (MRRF). For each group, an inexpensive, commercially available standard was chosen as the group reference standard (chlorogenic acid, apigenin, luteolin, rutin, and quercetin in Tables 1−5) and used as the basis for the MRRF calculations. Then, molar response factors were computed for each compound on the basis of the integrated peak absorbance of standards as purchased (MRRF) and, when possible, after vacuum drying ($MRRF_D$). The water and solvent content of the group reference standards ranged from 4.85% to 12.26%, as noted at the bottom of each table. Repeat drying determinations gave a precision of ±3%.

Two additional MRRF values were computed. The proposed response factor ($MRRF_P$) represents our best estimates of the true response factors for the compounds within each group. The rutin response factor ($MRRF_R$) was computed using rutin as the master reference standard for standards in all groups. These response factors are discussed in more detail in later sections.

Hydroxycinnamic Acid Derivatives (Group I). The majority of hydroxycinnamic acid derivatives are formed from hydroxycinnamic acid and nonchromophores (i.e., non-UV absorbing groups) such as mono-, di-, and trisaccharides and aliphatic acids, such as quinic, malic, tartaric, shikimic, and tetrahydroxyhexanedioic acids (Figure 1). These compounds are found as the main phenolic compounds in many common plant derived foods such as coffee beans, common beans, brassica vegetables, and many Compositae plants. [3,7,8,18] However, most of these compounds are not commercially available.

Table 1 shows the structure, molecular weight, λ_{max}, and MRRF values of the hydroxycinnamates. In general, they have hydroxyl groups at the R_3 and R_4 positions and a variety of structures at the R position. The lack of a hydroxyl at the R_3 position for *p*-coumaric acid produces a shift of λ_{max} to a lower wavelength. Consequently, group I has been subdivided into group Ia with all the non-*p*-coumaric hydroxycinnamates and group Ib containing *p*-coumaric acid and its derivatives. With chlorogenic acid as the group reference standard, the MRRF

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and $MRRF_D$ values for caffeic, ferulic, sinapic, and isoferulic acid and caffeic methyl ester were all close to 1.00. Substitution at the R position or methoxylation at the R_3 position had no effect on the λ_{max} or the molar absorptivity, as reflected by the MRRF and $MRRF_D$ values.

Provided λ_{max} does not shift, the molar absorptivities of these compounds should, in theory, be additive. Thus, although the MRRF values for the dicaffeoylquinic and dicaffeoyltartaric acids are 1.65, 1.75, and 1.63, it is assumed that the $MRRF_D$ would be 2.00 and that the deviation of the MRRF values from 2.00 is the result of the degree of hydration. Similarly, compounds with three caffeoyl units would be expected to have $MRRF_D$ values of 3.0. It can be shown that the molar absorptivities for tiliroside (kaempferol 3-*O*-*p*-coumaroylglucoside) and related compounds are additive. The $MRRF_D$ values for kaempferol 3-glucoside and the *p*-coumaroyl group are 0.83 and 0.73, respectively, at 326 nm. The $MRRF_D$ for tiliroside was 1.56, as predicted by the sum of the components.

Flavones: Apigenin Glycosides and Their Derivatives (Group II). The flavone UV spectra contain two maxima: band II from the A-ring benzoyl system (250−300 nm) and band I from the B-ring cinnamoyl system (305−350 nm).[19] The λ_{max} for band I falls in two clusters: 334−338 nm for the apigenins (group II) (Figure 2 and Table 2) and 342−350 nm for the luteolins (group III) (Figure 2 and Table 3). The compounds are listed by their deviation from λ_{max} of the group reference standard.

Table 2 shows that the dominant structure for the apigenin derived compounds consists of a hydroxyl at the 4′ and 5 positions and a hydroxyl, methoxy, or *O*-glycosyl function at the 7 position. None of the variations at the 7 position caused a deviation from the λ_{max} at 336 nm, supporting the observation that nonchromophore substitutions at this position do not have a significant effect on the molar absorptivity.[17] Varied substitution at the 3′, 6, and 8 positions caused slight shifts of λ_{max} to lower wavelengths, but the loss of the hydroxyl at the 4′ position (such as chrysin, baicalein, wogonin) caused the loss of the typical band I λ_{max}.

Two reagent grade dimeric apigenins, hinokiflavone and cupressuflavone (not dried), had MRRF values of 1.92 and 1.58, respectively. The former had a λ_{max} of 338 nm, but the latter had a maximum shifted to 330 nm. Sufficient standard was lacking to determine $MRRF_D$ values for either one. The results for hinokiflavone support the additive nature of the molar absorptivities when there is no shift in λ_{max}. Thus, calibration with apigenin offers the possibility of quantifying dimeric apigenins found in more than 200 biflavonyls in such plants as *Ginkgo biloba*. [1,20]

Flavones: Luteolin Glycosides and Their Derivatives (Group III). The basic structure of luteolin (Table 3) is similar to that of apigenin (Table 2) with an additional hydroxyl group at the 3′ position. Like apigenin, substitution at the 4′, 5, and 7 positions with a nonchromophore does not alter the molar absorptivity or λ_{max} significantly. For luteolin 7-*O*-glucoside and diosmin (4′-methylluteolin), the $MRRF_D$ values were close to 1.00 and the values for λ_{max} did not shift. $MRRF_D$ values were not available for the rest of the compounds.

3-*O*-Mono- and 3,7- and 3,4′-*O*-Diglycosides of Flavonols (Group IV). Nearly 80% of the flavonols, primarily quercetins, kaempferols, isorhamnetins, and myricetins, are glycosylated at the 3 position[1] and can be quantified using rutin as a group reference standard. Table 4 shows that glycosylation at the 3 position has little effect on λ_{max} for quercetin. Glycosylation at the 3 position had a slight effect (−2 nm) on the λ_{max} of myricetin and a larger effect (−6 nm) on kaempferol. In the latter case, it is not possible to separate the effect of glycosylation at the 3 position from lack of hydroxylation at the 3′ position. The $MRRF_D$ values suggested a slight change in the molar absorptivity for kaempferol 3-*O*-rutinoside.

Previous research characterized the effect of methylation and glycosylation on the band I shifts.[19] Methylation and glycosylation at the 3 and 4′ positions produced shifts of λ_{max} to shorter wavelengths by 12−17 nm and 3−10 nm, respectively. The shift with glycosylation at the 3 position may be consistent with the kaempferol data (Table 4).

In Table 7, glucosylation of quercetin and isorhamnetin in red onion at the 4′ position resulted in a shift of 10 nm. The previous report also found that methylation at the 5 position produced a 5−15 nm shift to shorter wavelengths for both band I and II and no shifts were seen for substitution at the 3′, 5′, 6, and 7 positions.[19] We did not see any shift for isorhamnetin with hydroxylation at the 3′ position. The previous report provided no data for changes in the molar absorptivity.

More than 20% of the glycosides of flavonols in plants are found in an acylated form.[1] Thus, quantitation of these compounds is important. Green teas and *Ginkgo biloba* leaf contain several dozen flavonol hydroxycinnamoyl glycosides.[20,21] All the green leaf brassica vegetables contain hydroxycinnamoyl glycosides as their main flavonoids; more than 100 have been reported.[8,22−27] In two studies, the isolated kaempferol 3-*O*-hydroxyferuloyl diglucoside-7-*O*-glucoside was used to quantify its analogues in the extracts.[25,26] In most cases, however, quantitation was achieved using nonacylated glycosides, such as rutin,[22] and led to errors of approximately 40% (for one hydroxycinnamoyl) by failing to account for the shift of λ_{max} and the change in the molar absorptivities. More accurate results can be obtained using chlorogenic acid as a group reference standard and appropriate $MRRF_p$ values.

In theory, compounds with acylglycosides formed with nonchromophores, such as aliphatic acyls (e.g., acetyl or malonyl), and with UV absorbers with no band I absorbance, such as hydroxybenzoyl compounds, will have the same λ_{max} and $MRRF_D$ values as their parent flavonoids in group IV. Unfortunately, no standards were available to support this hypothesis. However, acylglycosides were observed in Chinese lettuce (Table 6). Two isomers of quercetin 3-*O*-malonylglucoside showed no shift in their values for λ_{max}. From Table 1, quercetin and kaempferol 3-acylglycosides, with caffeoyl, feruloyl, or sinapoyl groups, would be predicted to have a λ_{max} of 326 nm and $MRRF_D$ values close to 1.83 (1.00 from the hydroxycinnamates and 0.83 from kaempferol). These values are suitable for the quantitation of the 3-hydroxycinnamoylacylglycosides of kaempferol, quercetin, isorhamnetin, and myricetin.

Acylglycosides formed from the UV band I active hydroxycinnamoyls will have λ_{max} closer to that of the group I compounds and MRRF values that reflect the sum of the hydroxycinnamoyl and 3-glycosylflavonol units. Thus, using chlorogenic acid as the group reference standard, the $MRRF_D$ for tiliroside at 326 nm was 1.56, the sum of $MRRF_D$ values of both units) (Table 1). Similar shifts in λ_{max} were observed for *p*-coumaroylglycosides in white tea (Table 8). The $MRRF_D$ values for 3-*O*-*p*-coumaroyglucoside and kaempferol 3-di-*p*-coumaroylglucoside would be predicted to be 1.56 and 2.29

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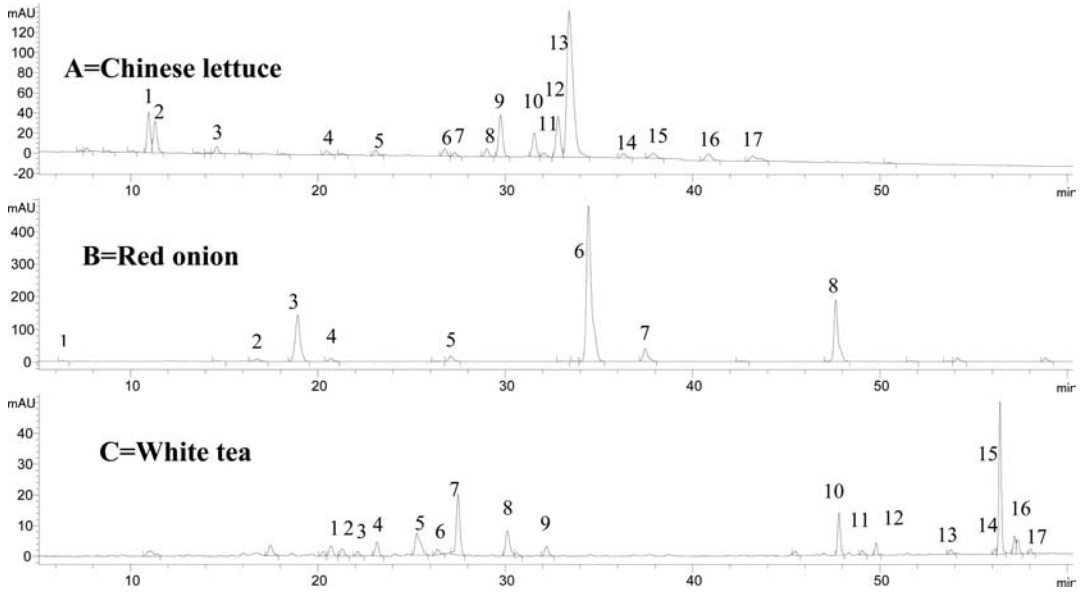

Figure 3. Chromatograms (354 nm) of the flavonoids of Chinese lettuce (A), red onions (B), and white tea (C).

(1.46 from di-*p*-coumaryl and 0.83 from kaempferol), respectively, at 326 nm.

Flavonols: Aglycones and other Glycosides (Group V). Flavonols usually exist in plants as glycosides. Free aglycones are only detected in a limited number of foods as minor components and contribute little to the total flavonoid content.[1,2] However, for completeness, it is important to consider these compounds. Eighteen flavonol aglycones, together with kaempferol 7-*O*-neohesperidoside, quercetin 4′-*O*-glucoside, gossypetin-8-*O*-glucoside, and ellagic acid, are listed in Table 5. Most have a UV band I λ_{max} between 352 and 374 nm. $MRRF_D$ values fell between 0.90 and 1.08, despite the λ_{max} varying by 10 nm.

Ellagic acid, a complex polyphenol, is found with its glycosides and ellagitannins as the beneficial components in numerous fruits, such as pomegranates, strawberries, muscadine grapes, cranberries, and walnuts.[28] It has a λ_{max} at 368 nm, identical to that of kaempferol, and has a $MRRF_D$ value of 0.59 using quercetin as a group reference standard (Table 5). Thus, this value can be used to quantify such compounds.

Accuracy of Response Factors. $MRRF_D$ values are listed in Tables 1−5 for 17 compounds that have a λ_{max} within 2 nm of that of the group reference standard. These compounds had an average $MRRF_D$ value of 1.01 ± 0.03. The precision agrees well with the ±3% relative standard deviation reported earlier for repeat drying measurements of the group reference standards. The average MRRF value for the same compounds was 1.00 ± 0.06. The larger standard deviation indicates that the differences in the degree of hydration of the individual compounds and the group reference standard introduced an inaccuracy of ±0.05 (the quadratic difference). These data suggest that if the λ_{max} are in good agreement (±2 nm), then the $MRRF_D$ values should have a value of 1.00 (or a multiple of 1.00).

Tables 1−5 list MRRF values for 36 compounds with a λ_{max} within 2 nm of that of the group reference standard, but no $MRRF_D$ values. For these compounds, the average MRRF value was 0.96 ± 0.13. This standard deviation is considerably larger than the values reported in the previous paragraph and most likely arises from increased variation in the hydration of the standards. The more complex structure of these harder to obtain standards leads to greater solvent interaction. In addition, the less frequent occurrence of many of these compounds in plants leads to lower yields (through purification or synthesis) and makes sufficient material for drying difficult to obtain. Despite the large standard deviation, the agreement of λ_{max} for the compounds and the group reference standards suggests that the $MRRF_D$ values for these compounds should be close to 1.0.

Band I is relatively broad, and λ_{max} is usually not well-defined. Consequently, a wider wavelength range around the λ_{max} of the group reference standards was considered. On the average, the absorbance 10 nm from the λ_{max} decreased by only 7.4%. The average MRRF for compounds in Tables 1−5 within ±10 nm of the λ_{max} of the group reference standard was 0.96 ± 0.13. The mean and standard deviation are the same as those for compounds within ±2 nm. This suggests that these compounds should have $MRRF_D$ values close to 1.00. If, however, the larger standard deviation arises not from differences in hydration but from differences in molar absorptivities, then the inaccuracy introduced by assuming an $MRRF_D$ of 1.00 would be ±26% at the 95% confidence limit, a confidence interval of 0.70−1.22. This would be a worst case scenario.

Compounds with a λ_{max} further than 10 nm from that of the group reference standard have an increasing probability of having a different molar absorptivity. For these compounds, the average MRRF value was 0.70 ± 0.30. The $MRRF_D$ values for these compounds would undoubtedly have a smaller standard deviation but would be expected to differ consistently from 1.0, since there has been a significant shift in λ_{max}. If the large standard deviation was caused only by differences between the molar absorptivities of the compound and the group reference standard, then quantitation based on an MRRF value of 1.00 could produce inaccuracies as great as ±59% (95% confidence limit) in the worst case.

It is necessary to consider the accuracy of the MRRF values in the context of the expected biological variation. For 59 fresh fruits, vegetables, and nuts, Harnly et al.[29] observed an average relative standard deviation of ±330% (95% confidence limit) for 22 flavonoids (reported as aglycones) collected nationwide

at 6 month intervals. Since phenolic compounds are strongly influenced by environmental conditions, this high standard deviation is not surprising. In light of this data, quantitation of phenolic compounds based on an assumed $MRRF_D$ of 1.00 that provides an accuracy of ±26% (95% confidence limit) is reasonable. Even a standard deviation of ±59% (95% confidence limit) is not unreasonable. In many cases, simply knowing the presence or absence of a rare phenolic compound is significant. However, in this study, it was never necessary to base calibration on a standard or compound whose λ_{max} was further than 10 nm from that of the group reference standard.

Proposed Molar Relative Response Factors ($MRRF_P$). On the basis of the data in the previous section, proposed molar relative response factors ($MRRF_P$) were established for the quantitation of the compounds in Tables 1−5. If the λ_{max} of the compound was within 10 nm (most were within 6 nm) of that of the group reference standard, then an $MRRF_P$ value of 1.00 was assigned. If the λ_{max} differed by more than 10 nm, no $MRRF_P$ value was assigned. For these compounds, the MRRF values, regardless of the inaccuracy introduced by differences in degree of hydration, were the best (only) estimates of the ratio of molar absorptivities.

Molar Relative Response Factors Based on Rutin ($MRRF_R$). The usefulness of the group reference standards suggested that quantitation could be accomplished using only a single calibration standard, a master reference standard. Tables 1−5 show the relative responses of the five group reference standards as compared to rutin at the five group wavelengths. It can be seen that if calibration curves are constructed at two wavelengths for reliable results, then the rutin molar relative response factor ($MRRF_R$) is 1.24 at 336 nm for chlorogenic acid and 0.97, 1.20, and 1.05 at 354 nm for apigenin, luteolin, and quercetin, respectively.

Quantitation of Phenolic Compounds in Foods. Hydroxycinnamic acid derivatives and flavonol and flavone glycosides were quantified in three foods (Chinese lettuce, red onion, and white tea) (Tables 6−8) by direct comparison to authentic standards, if available, and by comparison to group and master reference standards using the three response factors (MRRF, $MRRF_P$, and $MRRF_R$) found in Tables 1−5. LC-DAD-ESI/MS data were first used to identify the structure of the compounds and to place them in the correct group (Tables 1−5). It was determined that 41 of the 44 compounds had λ_{max} values very close (0−2 nm) to those of the group reference standards.

Figure 3 shows chromatograms for the three foods, and Tables 6−8 list the identified compounds, λ_{max} values, and computed concentrations. Chinese lettuce contained caffeic acid and its derivatives, and the glycosides of luteolin and quercetin (Figure 3A and Table 6). Red onion contained 3,7,4′-, 7,4′-, 3,4′-, 3-, and 4′-glucosides of flavonols.[30] The 3,7,4′-triglucoside and two 3,4′-diglucosides have their λ_{max} values at 344 nm (Figure 3B and Table 7), which is 10 nm from λ_{max} of the group reference standard. The listed concentrations should be 6% lower than the actual contents, since the MRRF value at 344 nm was 94.1% of the value at 354 nm (Figure 2). White green tea contained kaempferol, and glycosides and *p*-coumaroylglycosides of flavonols (Figure 3C and Table 8).[21] Four of the *p*-coumaroylglycosides (peaks 11, 15, 16A, and 16B) were positively identified using the compounds found in holly oak.[31]

The molar concentration (C) of each of the phenolic compounds was calculated as follows:

$$C = C_R/\mathrm{MRRF} \quad (1)$$

where C_R is the molar concentration of the sample as calculated from the calibration curve of the reference standard. The same equation can be expressed in more detail as follows:

$$C\ (\mathrm{mg}/100\ \mathrm{g}) = 1000A_S\mathrm{MW}_X W_S V_S/(A_X\mathrm{MW}_S V_X W_X \mathrm{MRRF}) \quad (2)$$

where A_X, MW_X, W_X, and A_S, MW_S, W_S, are the peak area, molecular weight, and weight of the sample and the standard, respectively. In each case, the preparation volume of the sample (V_X) and standard (V_S) were 10.0 mL and the injection volumes were 10 μL. Depending on the calculation, $MRRF_P$ and $MRRF_R$ were substituted for MRRF.

The compounds found in the three foods (Tables 6−8) have little overlap with those found in Tables 1−5. However, all the compounds in the foods were found to have λ_{max} values that were in agreement with that of the reference standard of the group in which they were placed. Therefore, $MRRF_P$ values were used to provide quantitation based on calibration for the group reference standards. Similarly, $MRRF_R$ values were available for all the compounds in Tables 6−8. Authentic standards were available for direct calibration and quantitation for only 12 of the compounds in the three foods. Similarly, the lack of standards meant that MRRF values were not available for many of the compounds in Tables 6−8. In these cases, the MRRF values of the group reference standards were assumed.

Tables 6−8 show that, in every case, quantitations based on direct calibration and $MRRF_P$ and $MRRF_R$ values were almost identical. Concentrations based on MRRF values were consistently higher than the other computed concentrations. The hydration of the undried standards leads to lower MRRF values, compared to $MRRF_D$ values. As a result, the absorbance of the sample will give erroneously high concentrations.

The strong band I absorbance arising from the hydroxycinnamoyl structure present in hydroxycinnamates and flavonol and flavone glycosides provides a basis for simplifying the quantitation of these compounds. Inexpensive compounds can be chosen for each group that permits accurate quantitation of all the compounds. Thus, inexpensive quantitation can be achieved for most compounds. More importantly, this systematic approach will permit quantitation of numerous compounds for which there are no standards. In addition, a compilation of λ_{max} for every compound identified in a new food will expand the database and provide for more accurate quantitation.

We have presented a logical and comprehensive approach to the quantitation of hydroxycinnamic acid derivatives and glycosides of flavonols and flavones. The accuracy of the quantitation is dependent on shifts of the wavelength of the peak maximum for band I, changes in the molar absorptivity, and the purity of the standards. We have shown that either five group reference standards or a single master reference standard can be used, and we have characterized the range of accuracy of the method.

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■ REFERENCES

(1) Flavone *O*-glycosides, flavonol *O*-glycosides, biflavonyls. In *The handbook of natural flavonoids*; Harborne, J. B., Baxter, H., Eds.; John Wiley & Sons, Inc.: New York, 1999, *Vol. 1*.

(2) Veitch, N. C.; Grayer, R. J. Flavonoids and their glycosides, including anthocyanins. *Nat. Prod. Rep.* **2008**, *25*, 555−611.

(3) Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*, 1001−1043.

(4) Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287−306.

(5) Cuyckens, F.; Cleays, M. Mass spectrometry in the structural analysis of flavonoids. *J. Mass Spectrom.* **2004**, *39*, 1−15.

(6) Abad-García, B.; Berrueta, L. A.; Garmón-Lobato, S.; Gallo, B.; Vicente, F. A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 5398−5415.

(7) Lin, L.-Z.; Harnly, J. M. A screening method for the identification of glycosylated flavonoids and other phenolic compounds using a standard analytical approach for all plant materials. *J. Agric. Food Chem.* **2007**, *55*, 1084−1096.

(8) Lin, L.-Z.; Sun, J.; Chen, P.; Harnly, J. M. U-HPLC-PDA-EIS/HRMS/MS^n analysis of anthocyanins, flavonol glycosides and hydroxycinnamic acid derivatives in red mustard green (*Brassica juncea* Coss variety). *J. Agric. Food Chem.* **2011**, *59*, 12059−12072.

(9) Lin, L.-Z; Lu, S; Harnly, J. M. Detection and quantitation of glycosylated flavonoid malonates in celery, Chinese celery, and celery seed by LC-DAD-ESI/MS. *J. Agric. Food Chem.* **2007**, *55*, 1321−1326.

(10) Merken, H. M.; Beecher, G. R. Measurement of food flavonoids by high-performance liquid chromatography: A review. *J. Agric. Food Chem.* **2000**, *48*, 577−599.

(11) Kartsova, L. A.; Alekseeva, A. V. Chromatographic and electrophoretic methods for determining polyphenol compounds. *J. Anal. Chem.* **2008**, *63*, 1024−1033.

(12) Spáèil, Z.; Nováková, L.; Solich, P. Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta* **2008**, *76*, 189−199.

(13) Tsuchiya, Y. Photon path distribution and optical responses of turbid media: theoretical analysis based on the microscopic Beer-Lambert law. *Phys. Med. Biol.* **2001**, *46*, 2067−2084.

(14) Brolis, M.; Gabetta, B.; Fuzzati, N.; Pace, R.; Panzeri, F.; Peterlongo, F. Identification by high-performance liquid chromatography−diode array detection−mass spectrometry and quantitation by high-performance liquid chromatography−UV absorbance detection of active constituents of *Hypericum perforatum*. *J. Chromatogr., A* **1998**, *825*, 9−16.

(15) Määttä, K. R.; Kamal-Eldin, A.; Törrönen, A. R. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes* species. *J. Agric. Food Chem.* **2003**, *51*, 6736−6744.

(16) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Mattila, P. H.; González-Paramás, A. M.; Törrönen, A. R. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **2004**, *52*, 4477−4486.

(17) Papagiannopoulos, P.; Wollseifen, H. R.; Mellenthin, A.; Haber, B.; Galensa, R. Identification and quantitation of polyphenolis in carob fruit (*Ceratonia siliqua* L.) and derived products by HPLC-UV-ESI/MSn. *J. Agric. Food Chem.* **2004**, *52*, 3784−3791.

(18) Lin, L.-Z.; Harnly, J. M. Identification of hydroxycinnamoyl-quinic acids of arnica flowers and burdock roots using a standardized LC-DAD-ESI/MS profiling method. *J. Agric. Food Chem.* **2008**, *56*, 10105−10114.

(19) The ultraviolet spectra of flavones and flavonols. In *The systematic identification of flavonoids*; Mabry, T. J., Markham, K. R., Thomas, M. B., Eds.; Springer-Verlag: New York, NY, 1970; Chapters V and VI, pp 41−45 and 165−166.

(20) Lin, L.-Z.; Chen, P.; Ozcan, M.; Harnly, J. M. Chromatographic profiles and identification of new phenolic components of *Ginkgo biloba* leaves and selected products. *J. Agric. Food Chem.* **2008**, *56*, 6671−6679.

(21) Lin, L.-Z.; Chen, P.; Harnly, J. M. New phenolic components and chromatographic profiles of green and fermented teas. *J. Agric. Food Chem.* **2008**, *56*, 8130−8140.

(22) Olsen, H.; Aaby, K.; Borge, G. I. Characterization, quantitation, and yearly variation of the naturally occurring polyphenols in a common red variety of curly kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica* cv. 'Redbor'). *J. Agric. Food Chem.* **2010**, *58*, 11346−11354.

(23) Lin, L.-Z.; Harnly, J. M. Identification of the phenolic components of collard green, kale and Chinese broccoli. *J. Agric. Food Chem.* **2009**, *57*, 7401−7408.

(24) Lin, L. Z.; Harnly, J. M. Phenolic component profiles of mustard greens, yu choy and 15 other Brassica vegetables. *J. Agric. Food Chem.* **2010**, *58*, 6850−6857.

(25) Martínez-Sánchez, A.; Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F. A comparative study of flavonoid compounds, vitamin C, and antioxidant properties of baby leaf Brassicaceae species. *J. Agric. Food Chem.* **2008**, *56*, 2330−2340.

(26) Harbaum, B.; Hubbermann, E. M.; Wolff, C.; Herges, R.; Zhu, Z.; Schwarz, K. Identification of flavonoids and hydroxycinnamic acids in Pak Choi varieties (*Brassica campestris* L. ssp. *chinensis* var. *communis*) by HPLC-ESI-MS(n) and NMR and their quantitation by HPLC-DAD. *J. Agric. Food Chem.* **2007**, *55*, 8251−8260.

(27) Harbaum, B.; Hubbermann, E. M.; Zhu, Z.; Schwarz, K. Impact of fermentation on phenolic compounds in leaves of Pak Choi (*Brassica campestris* L. ssp. *chinensis* var. *communis*) and Chinese leaf mustard (*Brassica juncea* Coss). *J. Agric. Food Chem.* **2008**, *56*, 148−157.

(28) Bakkalbas, E.; Mentes, I. Ö. A.; Artik, N. Food ellagitannins—occurrence, effects of processing and storage. *Food Sci. Nutr.* **2009**, *49*, 283−298.

(29) Harnly, J. M.; Doherty, R. F.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Bhagwat, S.; Gebhardt, S. Flavonoid content of U.S. fruits, vegetables, and nuts. *J. Agric. Food Chem.* **2006**, *54*, 9966−9977.

(30) Slimestad, R.; Fossen, T.; Vågen, I. M. Onions: a source of unique dietary flavonoids. *J. Agric. Food Chem.* **2007**, *55*, 10067−10080.

(31) Karioti, A.; Bilia, A. R.; Skaltsa, H. *Quercus ilex* L.: a rich source of polyacylated flavonoid glucosides. *Food Chem.* **2010**, *123*, 131−142.