DIETARY SUPPLEMENTS

Development of a Potential Reference Material for Evaluating Antioxidant Activity

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Phenolic phytochemical are known to perform several functions ranging from phytoprotectants, to protecting lipids in food products, to antioxidant activity in animals and humans. The need for a common standard mixture containing multiple phenolic phytochemicals is critical for the development of a robust validation assay for accurately quantifying antioxidant activity in various matrixes. Different research groups have used A wide array of single purified reference phenolic compounds in this regard. A 5 compound mixture (caffeic acid, morin hydrate, hesperetin, catechin hydrate, and epigallocatechin gallate) containing phenolic compounds from 4 subgroups (phenolic acid, flavone, flavanone, and flavan-3-ol) was prepared. The mixture was assayed for stability evaluation by high-performance liquid chromatography (HPLC) using a diode array detection procedure for a 3 month time interval. HPLC analysis confirmed that there was no significant interaction between different components of the mixture. The among-sample relative standard deviation (RSD) of all 5 phenolic compounds, as well as the total HPLC area, was <1%. The RSD due to instrument variation was <2% and the total RSD among-days was <5%. These results unambiguously suggest that the sample was stable for a 3 month time interval in an amber vial stored in a refrigerator below 5°C. This mixture is currently being used for the single-laboratory validation study for the assay of total phenolic content by the Folin-Ciocalteu method and the antioxidant capacity by oxygen radical absorbing capacity procedure.

An antioxidant may be defined as an enzyme or other organic molecule that can counteract the damaging effects of oxygen in tissues. Although the term technically applies to molecules reacting with oxygen, it is often applied to molecules that protect from any free radical (molecule with unpaired electron; 1, 2). Antioxidants have been a major focus of researchers in the areas of food, nutrition, and biological sciences over the past decade (3). The number of publications on antioxidants and oxidative stress has nearly quadrupled during the past decade, and large numbers of reviews, books, and international conferences have been organized on this subject around the world (4–9). The literature on antioxidants is growing at a rapid pace, as over 13 000 000 hits were obtained when the term "antioxidant" was searched on Google search engine.

Clinical trials and epidemiological studies suggest an inverse correlation between dietary intake of fruits and vegetables and occurrence of inflammation, cardiovascular diseases, certain forms of cancers, and age-related disorders (10–14). The health beneficial properties of fruits and vegetables have been partially ascribed to dietary antioxidants such as polyphenols, vitamins E and C, phenolic acids, and carotenoids. The health beneficial effects of dietary antioxidants have generated significant interest to health and food science researchers, nutritional and medical professionals, and consumers for assaying antioxidant activity of foods, food products, and their constituents. Conflicting results on the antioxidant activity for individual polyphenolic compounds, herbs, spices, teas, and other foods are common in the literature (2). These variations in published results include the large number of single compound reference standards, the type of assay system, the presence of interfering or interacting compounds, the nature of the substrate for oxidation, the mode of oxidation, specificity of assay, and the mode of preparing sample for the antioxidant assay (2, 15–19).

One objective of the 2 International Congress on the Antioxidant Methods Meetings held in Orlando, Florida, in 2005 and 2006 was to develop standardized chemical methods for measuring antioxidant activity in food and biological systems. A first step in standardizing antioxidant assay procedures was the development of a multicomponent reference material because a wide range of single purified

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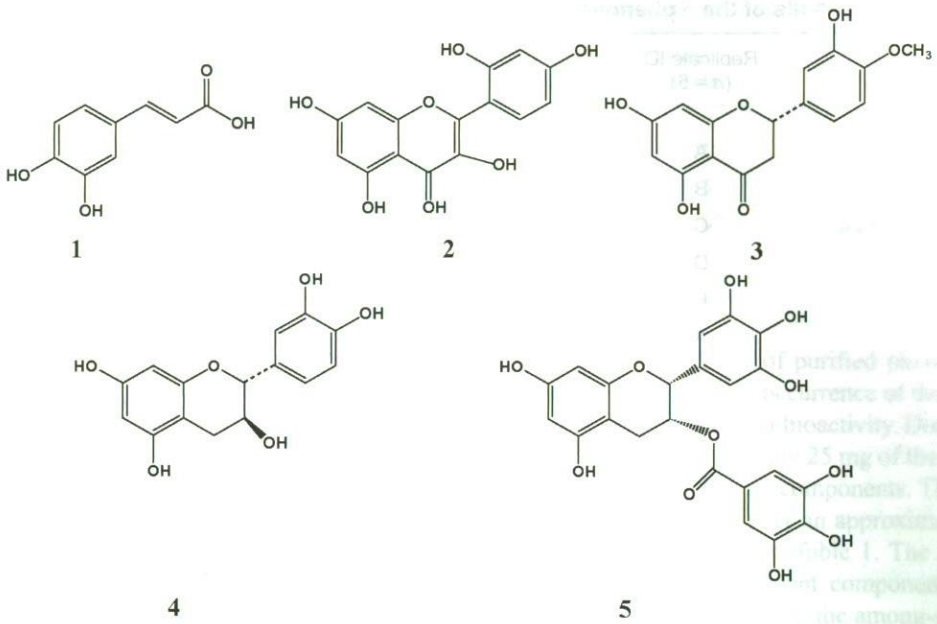

Figure 1. Structures of 5 phenolic compounds: (1) caffeic acid, (2) hesperetin, (3) morin, (4) catechin, and (5) epigallocatechin gallate.

compounds have been used by different researchers. The multicomponent phenolic mixture described in this study is currently being used as a reference material for the single-laboratory validation (SLV) study for assay of total phenolics by the Folin-Ciocalteu assay and antioxidant capacity by the oxygen radical absorbing capacity assay procedure.

This article describes the preparation and stability studies of a 5 phenolic compound mixture, a potential reference standard material for assaying antioxidant activity of phenolic antioxidants present in different food matrixes.

Experimental

Reagents

(**a**) *Caffeic acid, (-)-epigallocatechin gallate, and hesperetin.*—Sigma Chemical Co. (St. Louis, MO).

(**b**) *(+)-Catechin hydrate and morin hydrate.*—Aldrich Chemical Co. (Milwaukee, WI).

(**c**) *Genistein.*—Indofine Chemical Co. (Somerville, NJ).

(**d**) *HPLC grade methanol and formic acid.*—Fisher Chemical Co. (Fair Lawn, NJ).

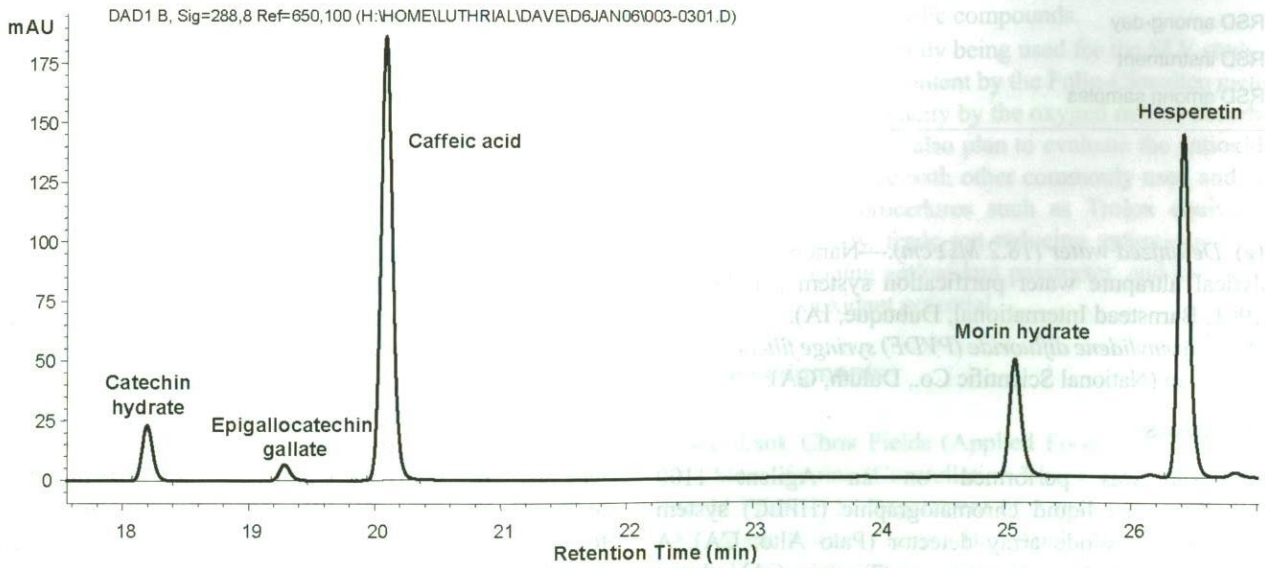

Figure 2. Typical HPLC analysis chromatogram of a mixture of 5 compounds used for the stability study.

Table 1. HPLC analysis results of the 5 phenolic compound mixtures over approximately 3 months

Day	Replicate ID (n = 5)	Catechin hydrate	Epigallocatechin gallate	Caffeic acid	Morin hydrate	Hesperetin	Total
1 (1/3/06)	A	134.2	38.9	1163.1	322.3	880.1	2538.6
	B	135.1	38.9	1172.1	320.1	883.4	2549.6
	C	136.7	39.5	1184.0	327.0	896.0	2583.2
	D	136.7	39.5	1184.0	327.0	896.0	2583.2
	E	137.1	39.8	1188.5	327.9	897.7	2591.0
4 (1/6/06)	A	138.6	39.9	1198.6	328.4	907.1	2612.6
	B	138.8	40.1	1199.5	328.2	908.7	2615.3
	C	137.4	39.6	1184.9	324.0	897.4	2583.3
	D	136.3	39.2	1175.7	322.1	891.9	2565.2
	E	139.1	40.0	1199.4	329.1	909.7	2617.3
11 (1/13/06)	A	126.7	36.1	1123.8	289.8	819.5	2395.9
	B	130.8	37.2	1159.0	300.4	844.7	2472.1
	C	132.2	37.8	1174.4	305.5	854.5	2504.4
	D	127.2	36.1	1127.8	294.3	820.8	2406.2
	E	126.7	35.9	1124.7	293.8	817.4	2398.5
74 (3/17/06)	A	129.9	37.6	1153.3	305.9	839.0	2465.7
	B	133.4	38.6	1183.4	319.2	865.0	2539.6
	C	133.3	38.5	1182.8	316.4	862.1	2533.1
	D	135.8	39.1	1202.6	321.5	875.4	2574.4
	E	134.3	38.6	1193.0	322.2	871.1	2559.2
85 (3/28/06)	A	126.7	36.1	1123.8	289.8	819.5	2395.9
	B	130.8	37.2	1159.0	300.4	844.7	2472.1
	C	132.2	37.8	1174.4	305.5	854.5	2504.4
	D	127.2	36.1	1127.8	294.3	820.8	2406.2
	E	126.7	35.9	1124.7	293.8	817.4	2398.5
Mean HPLC peak area		132.96	38.16	1167.37	312.36	863.78	2514.62
Among-day standard deviation		4.05	1.39	25.84	13.50	30.84	73.90
Instrument standard deviation		2.01	0.69	12.79	6.68	15.26	36.57
Among-sample standard deviation		0.77	0.26	4.88	2.55	5.83	13.96
RSD among-day		0.03	0.04	0.02	0.04	0.04	0.03
RSD instrument		0.02	0.02	0.01	0.02	0.02	0.01
RSD among samples		0.01	0.01	0.00	0.01	0.01	0.01

(e) *Deionized water (18.2 MΩ·cm).*—Nanopure diamond analytical ultrapure water purification system (Model No. D11901; Barnstead International, Dubuque, IA).

(f) *Polyvinylidene difluoride (PVDF) syringe filters.*—Pore size 0.45 μm (National Scientific Co., Duluth, GA).

Apparatus

Analysis was performed on an Agilent 1100 high-performance liquid chromatographic (HPLC) system coupled with a diode array detector (Palo Alto, CA). A reversed-phase C_{18} Luna Phenomenex (Torance, CA) column (150 × 4.6 mm; particle size 5 μm), preceded by a guard column (Phenomenex; 4 × 3.0 mm) of the same stationary phase was used for HPLC analyses. The column and guard column were thermostatically controlled at 30°C and the flow rate was set to 0.7 mL/min for the initial 25 min and changed to 1 mL/min for the last 35 min. The mobile phase consisted of 2 solvents: 0.1% formic acid in water (A), and methanol (B). The solvent gradient in volumetric ratios of solvents A and B was as follows: 0–10 min, 6% B to 10% B; 10–25 min, 10% B to 70% B; 50–53 min, 70% B to 100% B; 53–55 min, 100% B to 6% B; 55–60 min 6% B. A wavelength of 288 nm was used to detect the eluent composition. High-performance liquid chromatograms were detected using a photo diode array UV

Table 2. Slope estimates from linear regressions models of observed means onto day

Phenolic compound	Catechin hydrate	Epigallocatechin gallate	Caffeic acid	Morin hydrate	Hesperetin	Total
Slope	–0.053	–0.017	–0.175	–0.161	–0.450	–0.856
Standard error of slope	0.051	0.018	0.318	0.185	0.386	0.947
P-value	0.3752	0.4120	0.6206	0.4469	0.3281	0.4326

detector. The structure of the phenolic compound was determined by comparison of retention time and UV spectrum with the commercially purchased standards.

Preparation of Standard Mixture

Exactly 200 ± 0.1 mg each of caffeic acid, hesperetin, catechin hydrate, and morin hydrate and 25 ± 0.1 mg epigallocatechin gallate standard were weighed on a Mettler Toledo balance (Model AX205, Columbus, OH). The mixture of all 5 phenolic compounds was thoroughly mixed with a mortar and pestle. The solid mixture was transferred to an amber glass vial (8 mL), flushed with nitrogen, and stored at 4°C until analyzed. A small aliquot of 5 ± 0.1 mg of this thoroughly mixed solid was dissolved in a 25 mL volumetric flask in methanol and assayed by HPLC. A fresh solution of this solid mixture was prepared on each day the analysis was carried out over approximately 3 months.

Statistical Analysis

For each of the 5 phenolic compounds (and their total), phenolic stability (relative to time) was examined by fitting a linear regression model to averages of the 5 readings observed each day. Observed levels of the 5 phenolic compounds were compared for consistency of pattern relative to day using a 2-way (compound × day) analysis of variance (ANOVA), after first standardizing the data observed for each compound to zero mean and unit variance. The ANOVA modeled among-day and within-instrument variance components. Among-sample relative standard deviation (RSD) values were calculated, as the ratio of residual standard deviation to mean HPLC peak area, for each phenolic compound. All statistical analyses were accomplished using SAS® Version 9.1.3 Proc MIXED or Proc REG (20).

Results and Discussion

The structures of the 5 phenolic compounds mixture belonging to 4 phenolic subgroups, namely, caffeic acid (phenolic acid), catechin and epigallocatechin-3-gallate (flavan-3-ol), hesperetin (flavanone), and morin (flavone) are shown in Figure 1. A typical high-performance liquid chromatogram of the 5 compounds mixture is presented in Figure 2. The selection of the 5 pure phenolic compounds was based on the factors discussed and suggested at the International Congress on Antioxidant Methods Meetings held in Orlando, Florida, in 2005 and 2006. These factors were solubility, stability of purified phenolic compounds at temperatures below 5°C, occurrence of these phytochemicals in common foods, and their bioactivity. Due to the high cost of epigallocatechin gallate, only 25 mg of the sample was mixed with 200 mg of the other 4 components. The results of HPLC analysis of the mixture over an approximately 3 month time interval are summarized in Table 1. The results indicate no interactions between different components of the phenolic standard mixture. In addition, the among-sample RSD of the HPLC peak area of each of the 5 phenolic compounds, as well as the total HPLC area, was <1%. The RSD due to instrument repeatability was <2%, and the RSD among-days was <5%.

Slope estimates from the linear regression models fitted to data from each of the 5 phenolic compounds were all statistically indistinguishable from zero (Table 2). The area under the curve for each of the 5 phenolic components exhibited a statistically similar pattern relative to day, as illustrated by the raw data of HPLC peak area presented in Table 1. For each of the 5 phenolic components (and the total), phenolic levels were similarly high at days 1, 4, and 74, and phenolic levels were similarly low at days 11 and 85. The nonsignificant (P = 0.9661) compound × day interaction effect in the 2-way (compound × day) ANOVA, by definition, implies that the observed data provided no evidence that the observed pattern from day to day was statistically different for any of the 5 observed phenolic compounds. This confirmed that the observed pattern, relative to day, was statistically consistent for all 5 phenolic compounds.

This mixture is currently being used for the SLV study for assay of total phenolic content by the Folin-Ciocalteu method and the antioxidant capacity by the oxygen radical absorbing capacity procedure. We also plan to evaluate the antioxidant activity of this mixture with other commonly used and cited antioxidant assay procedures such as Trolox equivalence antioxidant capacity, ferric ion reducing antioxidant power, total radical trapping antioxidant parameter, and low density lipoprotein antioxidant potential.

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