JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 8139–8144 8139
DOI:10.1021/jf1005935

Thorough Study of Reactivity of Various Compound Classes toward the Folin−Ciocalteu Reagent

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A thorough study was done to test the reactivity of the Folin−Ciocalteu (F-C) reagent toward various compound classes. Over 80 compounds were tested. Compound classes included phenols, thiols, vitamins, amino acids, proteins, nucleotide bases, unsaturated fatty acids, carbohydrates, organic acids, inorganic ions, metal complexes, aldehydes, and ketones. All phenols, proteins, and thiols tested were reactive toward the reagent. Many vitamin derivatives were also reactive, as were the inorganic ions Fe^{+2}, Mn^{2+}, I^{-}, and SO_3^{2-}. Other compounds showing reactivity included the nucleotide base guanine and the trioses glyceraldehyde and dihydroxyacetone. Copper complexation enhanced the reactivity of salicylate derivatives toward the reagent, whereas zinc complexation did not. Several amino acids and sugars that were reported to be reactive toward the F-C reagent in earlier studies were found not to be reactive in this study, at least in the concentrations used. Reaction kinetics of each compound with the F-C reagent were also measured. Most compounds tested showed a biphasic kinetic pattern with half-lives under 1 min. Trolox and ascorbic acid displayed a rapid monophasic pattern in which the reaction reached end point within 1 min. In summary, this study has shown that the F-C reagent is significantly reactive toward other compounds besides phenols. As other investigators have suggested, the F-C assay should be seen as a measure of total antioxidant capacity rather than phenolic content. Because phenolics are the most abundant antioxidants in most plants, it gives a rough approximation of total phenolic content in most cases.

KEYWORDS: Folin−Ciocalteu assay; phenolics; thiols; antioxidants; antioxidant assays

INTRODUCTION

The Folin−Ciocalteu (F-C) assay was developed in 1927 for the measurement of tyrosine (*1*). The reagent consists of a mixture of sodium molybdate, sodium tungstate, and other reagents. Upon reaction with phenols, it produces a blue color, which absorbs at 765 nm. It is believed that the blue color is due to a complexed Mo(V) species (*2*). The assay has been used for many years by the food and agricultural industries to determine the phenolic content of plant products (*3*). A modification of this assay, called the Folin−Lowry assay, is used for the quantitation of proteins (*4*).

Folin and Ciocalteu noted during the development of the assay that tryptophan, which is nonphenolic, is reactive toward the reagent and contributes toward protein absorbance in the Folin−Lowry assay (*1*). Over the years, there have been numerous reports that the F-C reagent reacts with other antioxidants besides phenols (*3*). Possible contributors mentioned include proteins, carbohydrates, amino acids, nucleotides, thiols, unsaturated fatty acids, vitamins, amines, aldehydes, and ketones.

Ikawa and co-workers (*5*) performed an exhaustive study to determine the scope of reactivity of the F-C reagent toward nitrogenous compounds. They tested over 60 nitrogen-containing compounds representing several chemical classes. Their findings indicated that several of these classes showed considerable reactivity toward the F-C reagent. Classes showing reactivity included hydrazines, hydroxylamines, guanidines, tertiary amines, aromatic amines, pyrroles, and indoles. Because some compounds in these classes are present in plants, one might think that measurement of phenols by the F-C method could possibly give too high an estimate of phenolic content. One study showed only a moderate correlation ($r = 0.64$) between phenolic content of olive oils determined by F-C and HPLC methods (*6*).

An analogous study to determine the reactivity of various classes of non-nitrogenous compounds toward the F-C reagent has not yet been reported. Nonphenolic, non-nitrogenous antioxidants are common in plants. These include vitamins and thiols. In all probability, these compounds are also reactive toward the F-C reagent. Therefore, we believe that a study of this type would be of interest to researchers.

MATERIALS AND METHODS

The thiol derivative 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (PTCA) was synthesized in our laboratory according to a method formerly reported in the literature (*7*). The metal complexes $Cu(II)(DIPS)_4$ (*8*), $Zn(II)(DTBS)_2$ (*8*), and $Cu(II)_2(DTBS)_4$ (*9*) were synthesized according to

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Published on Web 06/29/2010

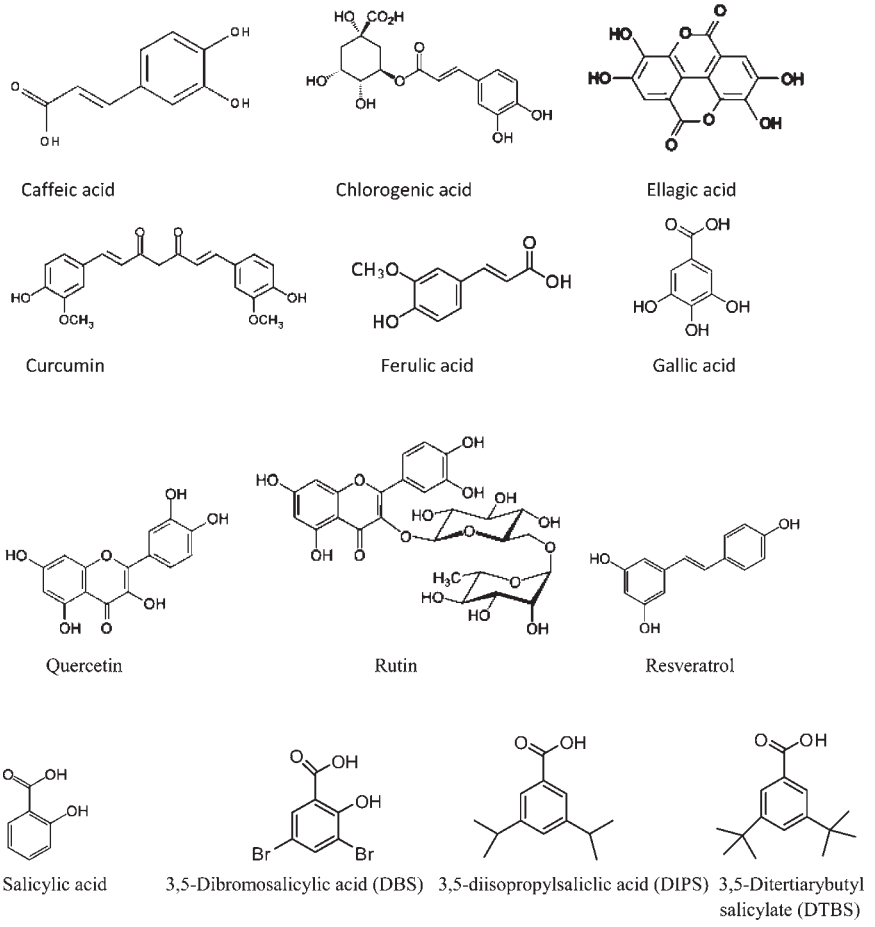

Figure 1. Chemical structures of phenolic compounds used in this study.

methods reported in the literature. $Cu(II)_2(DBS)_4$ and $Zn(II)(DTBS)_2$ were synthesized in our laboratory according to a procedure listed below. All other materials were purchased either from Fisher Scientific Co., Houston, TX, or Sigma-Aldrich Co., St. Louis, MO.

Synthesis of Cu or Zn Chelates of 3,5-Dibromosalicylate (DBS) and 3,5-Di-*tert*-butylsalicylate (DTBS). The metal chelates were synthesized from commercially available substituted salicylic acids (Aldrich) and copper(II) chlorides or zinc(II) chlorides (Aldrich) using methods that have been developed (*8*, *9*). Solutions of the sodium salt of each substituted salicylic acid were prepared by dissolving respective substituted salicylic acid in deionized H_2O by adding equivalent moles of NaOH. This solution was filtered to remove any insoluble impurity and vigorously stirred with a magnetic stirrer, and the pH was adjusted to the range of 9−11 using 10% sodium hydroxide and/or 10% HCl. For copper complexes the molar ratio of the respective salicylate salt to copper(II) chlorides was 2:1, and for zinc chelates the molar ratio of the respective salicylate salt to zinc(II) chlorides was 1:1. A filtered deionized water solution of 1:1 or 2:1 equivalents of the metal chloride was added dropwise with a separatory funnel to a vigorously stirred solution of respective sodium salicylate. Vigorous stirring was employed to provide shearing and avoid entrapment of any of the starting materials in the precipitating product of this reaction. Following the completion of this addition, the precipitate were collected by filtration, washed with deionized water, and dried overnight at room temperature in a sintered glass filter funnel attached to a laboratory vacuum (15 mmHg). The metal complexes were characterized by melting point or decomposition range, elemental analysis, and Fourier transform infrared spectroscopy (Nicolet FT-IR spectrometer). The elemental analyses for the two new compounds, $Cu(II)_2(DBS)_4$ and $Zn(II)(DTBS)_2$, were within acceptable ranges.

Measurement of Reactivity of Compounds toward the Folin−Ciocalteu Reagent (End Point Assay). A slightly modified version of the method of Singleton et al. was used (*10*). A 50 mg quantity of each test compound was dissolved in either water or ethanol, dependent upon

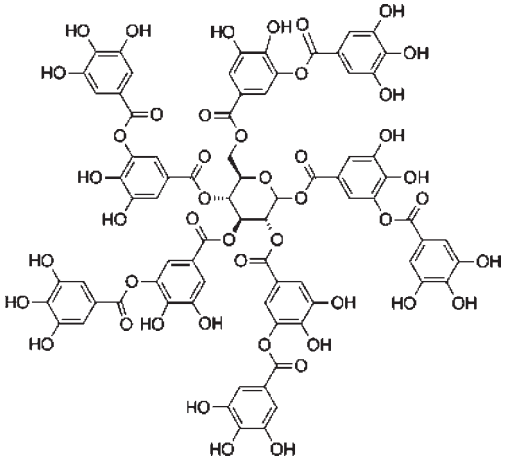

Figure 2. Chemical structure of tannic acid.

solubility. For compounds suspected of having high reactivity, the following dilutions were made: 1:10, 1:20, 1:30, 1:50, 1:100. For compounds suspected of having low reactivity the dilutions made were 1:5, 2:5, 3:5, and 4:5. Cuvettes were prepared such that there were three replicates for each of the dilutions mentioned above. To each cuvette was added 1.58 mL of water, 0.1 mL of F-C reagent, and 20 μL of the proper dilution of test compound. The contents of the cuvettes were stirred and allowed to stand 5 min. After this, 0.3 mL of a 20% aqueous sodium carbonate solution was added to each cuvette. The contents of the cuvettes were again stirred and incubated at 45 °C for 30 min in a dry bath. Absorbances were read at 765 nm using a Bio-Rad Smart Spec 3000 spectrophotometer. Graphs of absorbance versus concentration were prepared using Sigma Plot software. The activity of the compounds is expressed in terms of gallic

Table 1. Reactivity of Various Substances with Folin−Ciocalteu Phenol Reagent

compound	molar mass (g/mol)	GAE (mass)	GAE (molar)
phenolic compounds			
caffeic acid	180.16	1.00	0.958
chlorogenic acid	354.31	0.722	1.36
curcumin	368.40	0.722	1.41
ellagic acid	302.19	1.32	2.12
ferulic acid	194.18	1.05	1.08
gallic acid	188.14	1.00	1.00
quercetin	338.00	1.16	2.08
resveratrol	228.25	1.01	1.23
rutin	610.52	0.568	1.53
salicylic acid	138.12	0.357	0.262
tannic acid	1701.00	0.878	9.04
thiol derivatives			
amifostine	214.22	0.378	0.430
captopril	217.29	0.323	0.373
cysteamine HCl	113.61	0.304	0.184
glutathione	307.30	0.161	0.263
MPG	163.20	0.342	0.297
N-acetylcysteine	163.20	0.395	0.378
penicillamine	149.21	0.333	0.264
PTCA	175.25	0.180	0.141
RibCys	253.23	0.202	0.271
WR-1065	134.24	0.375	0.268
vitamin derivatives			
ascorbic acid	176.12	0.662	0.620
biotin	244.31	0.000	0.000
folic acid	441.40	0.071	0.167
folinic acid	473.44	0.069	0.174
menadione	172.18	0.000	0.000
NADH	709.40	0.054	0.204
nicotinic acid	123.11	0.000	0.000
pyridoxine	205.64	0.211	0.231
retinoic acid	300.42	0.404	0.645
riboflavin	376.36	0.000	0.000
thiamin	337.28	0.183	0.328
Trolox	250.29	0.395	0.525
amino acids			
alanine	89.09	0.000	0.000
arginine	174.20	0.000	0.000
cysteine	121.16	0.281	0.181
glycine	75.07	0.000	0.000
histidine	155.16	0.000	0.000
hydroxyproline	131.13	0.000	0.000
lysine	146.19	0.000	0.000
methionine	149.21	0.000	0.000
tryptophan	204.22	0.413	0.448
tyrosine	181.19	0.397	0.382
nucleotide bases			
adenine	135.13	0.0223	0.0160
guanine	151.13	0.340	0.273
cytosine	111.10	0.000	0.000
thymine	126.11	0.000	0.000
unsaturated fatty acids			
arachidonic acid	304.50	0.0045	0.0160
linoleic acid	280.45	0.000	0.000
linolenic acid	278.42	0.000	0.000
carbohydrates and proteins			
dihydroxyacetone	90.08	0.0444	0.0212
D-deoxyribose	164.16	0.000	0.000
D-fructose	180.16	0.000	0.000
D-glucose	180.16	0.000	0.000
D-glyceraldehyde	90.08	0.0243	0.0120
D-lactose	342.30	0.000	0.000
D-maltose	342.30	0.000	0.000
methylcellulose	~440 kDa	0.000	0.000
potato starch	~1000 kDa	0.000	0.000
D-sucrose	342.30	0.000	0.000
egg albumin	~65 kDa	0.0163	5.63
bovine serum albumin	69.3 kDa	0.0282	10.39
aldehydes, ketones, and carboxylic acids			
cinnamic acid	148.17	0.000	0.000
citric acid	192.12	0.000	0.000
oxalic acid	126.07	0.000	0.000
quinic acid	197.17	0.000	0.000
sodium tartrate	196.07	0.000	0.000
α-ionone	192.30	0.0043	0.0044
2,3-butanedione	86.09	0.180	0.00824
cinnamaldehyde	132.16	0.000	0.000
citronellal	154.25	0.000	0.000
inorganic salts			
iron(II) chloride	126.73	0.149	0.100
manganese(II) chloride	125.84	0.0432	0.0289
sodium nitrite	85.01	0.000	0.000
sodium sulfite	126.04	0.0506	0.0339
potassium iodide	166.00	0.0224	0.0198
miscellaneous compounds			
caffeine	194.19	0.000	0.000
cystamine[a]	225.20	0.000	0.000
glutathione disulfide	612.60	0.000	0.000
menthol	156.27	0.000	0.000
substituted salicylic acids			
3,5-diisopropylsalicylic acid (DIPS)	222.28	0.241	0.285
3,5-dibromosalicylic acid (DBS)	295.93	0.135	0.213
3,5-di-*tert*-butylsalicylic acid (DTBS)	250.34	0.0552	0.0735
metal complexes			
$Zn(II)(DIPS)_2$	543.92	0.0875	0.253
$Zn(II)(DTBS)_2$	600.08	0.0333	0.106
$Cu(II)_2(DIPS)_4$	1084.25	0.0713	0.411
$Cu(II)_2(D\ BS)_4$	1378.78	0.0883	0.647
$Cu(II)_2(DTBS)_4$	1196.47	0.0198	0.126

[a] Cystamine is the disulfide of cysteamine.

acid equivalents (GAE). GAE is defined as the slope of the test compound standard curve divided by the slope of the gallic acid standard curve.

Measurement of Reaction Kinetics. A single cuvette was prepared containing 1.58 mL of water, 0.1 mL of F-C reagent, and 20 μL of the highest dilution of test compound used in the end point assay. The contents of the cuvettes were stirred and allowed to stand 5 min. This was used as the blank. After this, 0.3 mL of a 20% aqueous sodium carbonate solution was added to the cuvette, and kinetics measurements were made using a Bio-Rad Smart Spec 3000 spectrophotometer. Readings were taken at 30 s intervals over a 1 h period at room temperature. Absorbance versus time was plotted using Sigma Plot software.

RESULTS AND DISCUSSION

Compounds tested in this study were chosen because of earlier reports that these or similar compounds showed reactivity toward the F-C reagent (*3*). We tested several phenolic compounds to serve as a basis of comparison to the other compounds tested in this study. All phenolics tested were significantly active, and the GAE values measured for compounds formerly reported in the literature were consistent with the earlier reported values (*11*). The chemical structures of the phenolic compounds used in this study are shown in **Figures 1** and **2**. **Table 1** shows the results of the end point assay for all compounds tested in this study. The data show that all thiol derivatives and proteins tested, as well as many of the vitamin derivatives, were also reactive toward the reagent.

The chemical structures of the thiol derivatives used in the study are shown in **Figure 3**. Amifostine, captopril, MPG, *N*-acetylcysteine, and penicillamine are currently being used as

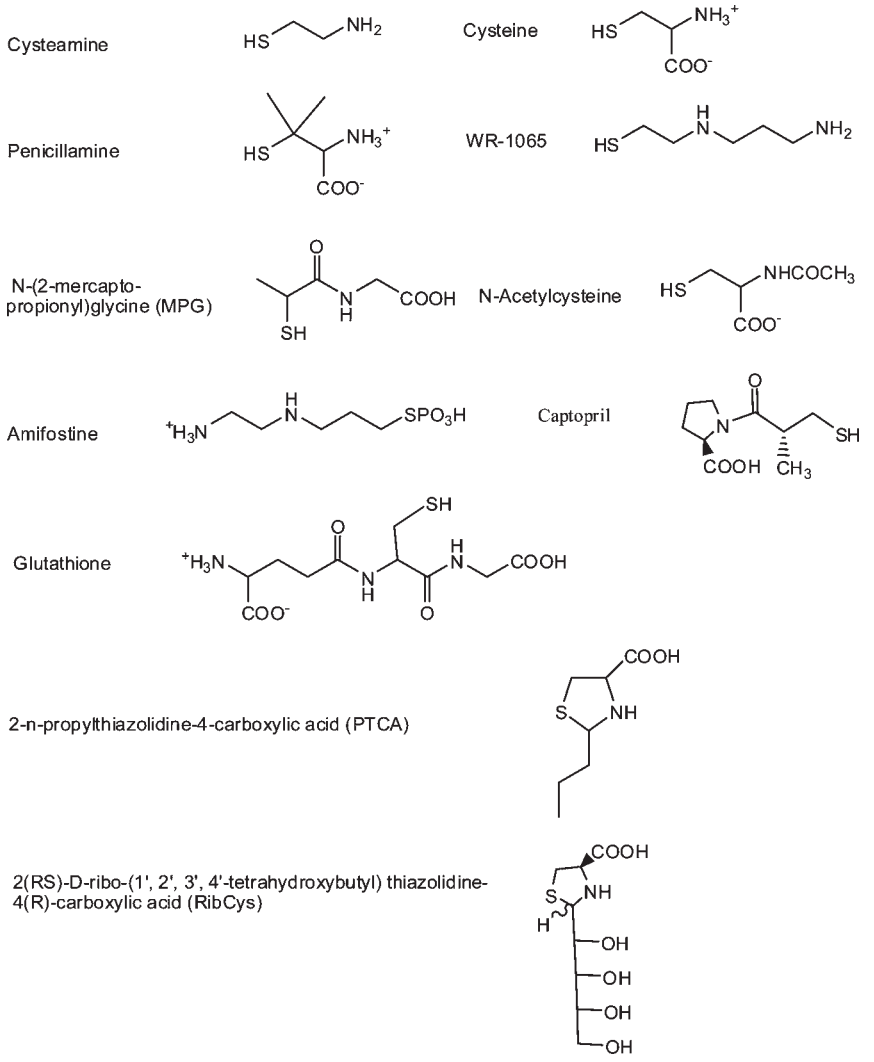

Figure 3. Chemical structures of thiol derivatives used in this study.

drugs. Glutathione, PTCA, and RibCys are compounds of medicinal interest (*7*, *12*−*14*). All thiol derivatives tested had significant activity. In all cases, thiols showed less reactivity than phenolics. Of the thiols tested, amifostine showed the most reactivity, whereas PTCA showed the least. The disulfides tested, glutathione disulfide and cystamine, showed no reactivity under the conditions of the assay. This is in contrast to the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, in which the disulfides show slight reactivity (*15*).

Of the vitamin derivatives tested, ascorbic acid, folic acid, folinic acid, NADH, pyridoxine, retinoic acid (vitamin A derivative), thiamin, and Trolox (vitamin E derivative) had significant activity. Ascorbic and retinoic acids had the greatest reactivity. Because these vitamin derivatives are present in many plants, it would be expected that they would contribute toward the estimation of phenols by the F-C method.

Of the nucleotide bases, adenine showed very slight reactivity, whereas guanine showed considerable reactivity. Thymine and cytosine were unreactive. Of the fatty acids, arachidonic acid had slight reactivity, whereas the other fatty acids tested showed none. Of the carbohydrates tested, only the trioses glyceraldehyde and dihydroxyacetone showed significant reactivity. The inorganic ions Fe^{2+}, Mn^{2+}, I^-, and SO_3^{2-} also showed reactivity toward the reagent. Of the aldehydes, ketones, and carboxylic acids tested, only butanedione and α-ionone had significant activity.

Of the amino acids tested, only tyrosine, tryptophan, and cysteine had significant reactivity toward the F-C reagent. This would imply that cysteine also makes a major contribution toward the absorbance of proteins in the Folin−Lowry assay. It is also interesting to note that bovine serum albumin was almost twice as reactive toward the reagent as egg albumin, on a mole basis. This would imply that proteins may vary considerably in reactivity toward the F-C reagent used in the Folin−Lowry assay, on the basis of their relative contents of tryptophan, tyrosine, and cysteine.

Also, our study found no reactivity, at the concentrations used, for the following compounds, which were earlier reported to be reactive toward the F-C reagent: oleic acid, fructose, glycine, histidine, cytosine, sucrose, potassium nitrite, and organic acids (*3*). We also tested several substituted salicylic acid derivatives and their metal complexes. These compounds are of interest because they have been shown to have desirable cytoprotective properties in cellular and animal models (*8*, *9*, *16*). It is believed that this cytoprotection is due to their ability to scavenge reactive oxygen species (*16*).

We are currently studying these compounds in our laboratory. We felt that they would be of relevance to this paper because they are phenolic in nature. Metal ions are found in plants and may complex with phenolics present. Metal ions have been shown to alter the redox properties of phenols (*17*). Therefore, the effects of metal complexation may be relevant to the determination of phenols by this method.

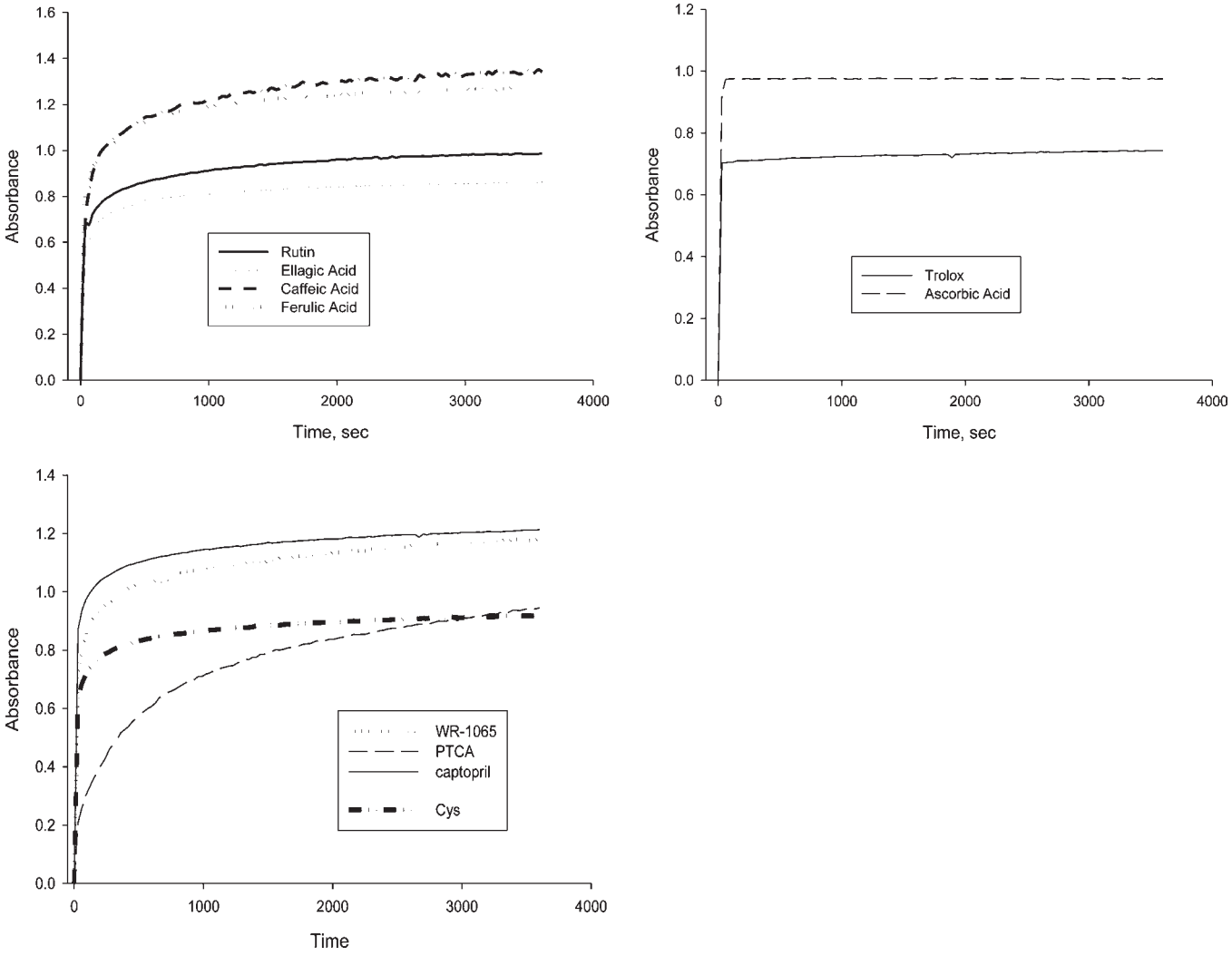

Figure 4. Reaction kinetics of selected compounds toward the F-C reagent.

Of the three substituted salicylic acids tested, the 3,5-di-isopropylsalicylic acid (DIPS) and 3,5-dibromosalicylic acid had similar reactivity to unsubstituted salicylic acid. However, the 3,5-di-*tert*-butyl (DTBS) derivative was less reactive than salicylic acid. Copper complexation enhanced reactivity of all three substituted salicylic acids, whereas zinc complexation appeared to have little effect. It is believed that this is because copper(II) is able to serve as a redox carrier, whereas zinc(II) is not (*9*, *17*, *18*). Quercetin has been shown to form a complex with Cu(II) that is more redox-active than uncomplexed quercetin. This observation would support the hypothesis that copper(II) is serving as a redox carrier (*17*).

One could propose the following mechanism:

reduced phenol Cu(II) complex → oxidized phenol Cu(I) complex

oxidized phenol Cu(I) complex + oxidized F-C reagent

→ oxidized phenol Cu(II) complex + reduced F-C reagent

Figure 4 shows the kinetic profiles of several compounds used in this study. Kinetic measurements were made for each compound in this study that showed appreciable reactivity toward the F-C reagent. However, because of similar kinetic patterns observed between many compounds, not all are shown in **Figure 4**. Compounds not shown in **Figure 4** displayed kinetic profiles similar to those shown. Most compounds tested displayed what appeared to be a biphasic kinetic pattern with a very fast and a very slow step. Most compounds had half-lives of < 1 min and reached end point within 1 h. Because of the very rapid initial rates, reaction order or rate constants could not be determined by methods we used.

The phenolics showed slight variability, with caffeic acid and curcumin showing the slowest kinetics. The thiols tested showed a pattern similar to that of the phenolics. The only exception was the thiazolidine derivative PTCA, which showed considerably slower kinetics. The low GAE value of PTCA compared to those of the other thiol derivatives may be because the reaction of this compound with the F-C reagent had not reached end point under the conditions used. Of the compounds tested, Trolox and ascorbic acid showed the fastest kinetics, reaching end point in < 1 min. In an earlier study, we noted that Trolox and ascorbic acid displayed a similar kinetic pattern in their reactivity toward the ABTS radical cation (*15*).

This study showed that many nonphenolic compounds do indeed show considerable reactivity toward the F-C reagent. Therefore, our findings confirm the observations of other investigators that the assay should not be viewed as a measure of total phenolic content, but rather a measure of overall antioxidant capacity, similar to the ABTS assay (*3*, *19*). In fact, studies have shown a strong correlation between the F-C and ABTS assays (*19*).

The F-C assay is normally described as an antioxidant capacity (AOX) method that involves a single electron transfer (SET). It is believed that a single electron is transferred from the substrate, presumably a phenol, to the complexed Mo(VI) in the reagent. In this process, Mo(VI) is reduced to Mo(V) (*3*). If this mechanism is correct, one might expect the initial product formed from a phenolic substrate to be a semiquinone. Semiquinones have been identified as the initial product formed upon reaction of phenols with the ABTS radical cation (*18*). The mechanism of the reaction of the F-C reagent with phenols and other compounds needs to be further explored.

The F-C assay is simple to perform and straightforward and yields consistent results. The similarities between the F-C and ABTS assays suggest that the F-C assay, like the ABTS assay, may be useful for assaying compounds of biomedical interest for antioxidant activity. The F-C method has the advantage of not requiring an overnight incubation time for the preparation of reagents.

In most plants, phenolics are the most abundant antioxidants present (*19*). Therefore, the F-C assay gives a good "ballpark" estimation of total phenolic content for most plants. Correction factors for vitamin C have been developed. These involve both the use of specific assays for vitamin C and extraction methods that remove most vitamin C (*20*). More precise assays specific for phenols need to be explored.

NOTE ADDED AFTER ASAP PUBLICATION

There were data errors in the nucleotide section of **Table 1** in the version of this paper published ASAP on June 29, 2010. The correct version published on July 2, 2010.

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Received for review February 17, 2010. Revised manuscript received May 25, 2010. Accepted June 2, 2010. Funding for the project was obtained from Arkansas Space Grant Consortium and NIH Grant P20 RR-16460 from the IDeA Networks of Biomedical Research Excellence (INBRE) Program of the National Center for Research Resources.