JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Spectrophotometric Determination of Phenolic Compounds by Enzymatic and Chemical Methods—A Comparison of Structure–Activity Relationship

YAM-TAK MA AND PETER C. K. CHEUNG*

Department of Biology, Food and Nutritional Sciences Program, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

A spectrophotometric determination of phenolic compounds by a peroxidase-catalyzed enzymatic (PE) method and the Folin−Ciocalteu (FC) chemical method was compared for their structure−activity relationship. In the PE method, the reaction time of 19 phenolic compounds with different chemical structures was found to be within 15 min, with those having bulky substituents showing slower reactivity. The responses of the phenolic compounds toward the PE method in terms of molar absorbance were positively correlated with the nucleophilicity of their corresponding phenoxyl radicals. An increase in the nucleophilicity by substitution of methoxyl and hydroxyl (electron-donating) groups enhanced the responses; while a decrease in nucleophilicity by substitution of the allyl carboxylic (electron-withdrawing) groups lowered the responses of phenolic compounds toward the enzymatic assay. The responses of phenolic compounds to the enzymatic method were found to be independent of the degree of hydroxylation while those to the FC assay were affected by both the position and degree of hydroxylation. Interfering chemicals found in the FC assay, including vitamin C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (a vitamin E analogue), and *tert*-butylhydroquinone (a commercial antioxidant) were insensitive to the enzymatic assay. The PE method appears to have a higher specificity toward phenolic compounds and subject to less interferences from other antioxidants than the FC method.

KEYWORDS: Phenolics; enzymatic analysis; Folin−Ciocalteu; structure−activity relationship

INTRODUCTION

Research interests in polyphenols have considerably increased in the past decades, owing to their antioxidant activities and their possible roles in the prevention of several oxidative stress mediated diseases (*1*). Polyphenols, which are abundantly found in a wide range of plants and fruits, are more effective than other traditional dietary antioxidants such as vitamin C in vitro (*2*−*4*). Despite being highly precise and accurate, chromatographic quantification of phenolic compounds in food matrix is hindered by a limited number of commercial standards, laborious sample preparation and isolation procedures, as well as the widely diversified chemical structures of unknown polyphenols (*5*). Thus, the total phenolic content, instead of individual phenolic compounds that have antioxidant activity, of a plant or fruit extracts is usually determined for comparison or screening purpose, particularly in which the phenolic profiles are largely unknown.

Among all the methods in determining of total phenolic content, the Folin−Ciocalteu (FC) assay (*6*) is the most widely used one in the past decades owing to its simplicity and high reproducibility (*7*). The FC method is based on the reduction (electron-donating) of a phosphotungstates−phosphomolybdates complex by reductants (or antioxidants) to a blue chromogen (*8*). Therefore, it is not surprising to find excellent linear correlations between the total phenolic contents obtained by the FC method and the antioxidant activity determined by assay based on electron-transfer mechanism (e.g., ferric reduction antioxidant assay) that has been reported widely (*7*). However, the FC reagent is nonspecific to phenolic compounds, and interferences caused by a wide range of organic and inorganic substances have been reported (*8*, *9*). Recently, modifications of the FC method have been made to eliminate those particular types of interferences which are specific to the sample involved. However, laborious pretreatments such as solid-phase extraction and thermal elimination of vitamin C are required (*2*), and various shortcomings including destruction of natural antioxidants were observed (*10*). Therefore, a simpler method with higher specificity in the determination of polyphenols is necessary.

A new spectrophotometric-enzymatic method has recently been developed for determination of total phenolic content in tea and wine (*11*). A plausible mechanism of the above enzymatic reaction is shown in **Figure 1**. It involves (a) an oxidation of the amine group of 4-aminophenazone (4-AP) by

* Corresponding author. Tel.: 852-26096144. Fax: 852-26035646. E-mail: petercheung@cuhk.edu.hk.

10.1021/jf070084w CCC: $37.00
Published on Web 04/19/2007

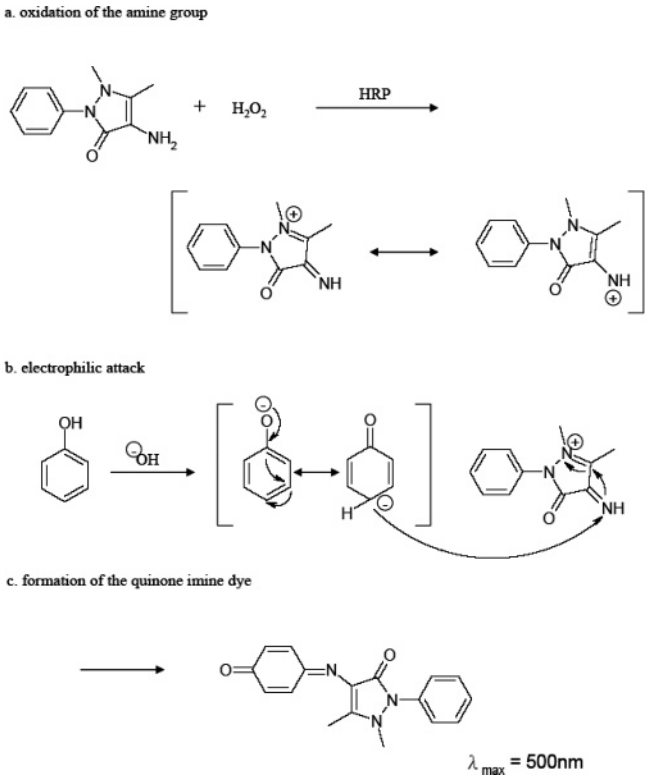


Figure 1. Proposed mechanism of the reaction of 4-aminophenazone (4-AP) with phenol in the enzymatic method.

hydrogen peroxide in the presence of horseradish peroxidase; (b) an electrophilic attack of the oxidized 4-AP by a phenoxyl radical; and (c) the formation of a quinone-imine colored adduct (*12*). The absorbance of the reaction product is proportional to the amount of phenolic compounds in the solution. A high dose-dependent correlation was found between the absorbance and the concentrations of polyphenol contents in 12 teas and 39 wine samples including catechin (0−200 μM) by this enzymatic method (*11*). Three common interfering compounds for the FC method, namely sulfite, citrate, and ascorbate, were found to be insensitive to this peroxidase-catalyzed enzymatic method (PE) (*11*). The total phenolic content in those samples determined by the FC method was generally higher than that determined by the PE method, probably owing to the presence of organic and inorganic interfering compounds (*11*).

Despite the high specificity toward polyphenols and the near absence of common interfering substances, the mechanisms involved in the PE method are still uncertain (*13*). Due to the large diversity in the structure of different phenolic compounds, the time taken to attain equilibrium of the enzymatic reactions and the response for each phenolic standard are expected to be different. In the present study, the reaction kinetics of 19 phenolic compounds of different structural subclasses with various degrees of hydroxylation toward the PE method were investigated. In addition, the responses of these phenolic compounds as well as some common antioxidants and interfering compounds toward the PE method and the FC method were compared. These results would facilitate the establishment of a more accurate method in determining the phenolic content in other phenolic-rich foods.

MATERIALS AND METHODS

Chemicals. All phenolic compounds (**Table 1**) were from Sigma-Aldrich Chemical Co. (St. Louis, MO), except apigenin and sinapic acid which were from Fluka Chemika (Buchs, Switzerland). Folin−Ciocalteu reagent (FC reagent), horseradish peroxidase (P2088) (HRP), and 4-aminophenazone (4-AP) were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Hydrogen peroxide (30% w/v) and HPLC grade methanol were from BDH Chemical Co. (London, U.K.). Sodium carbonate and phenol were from Riedel-de Haën Fine Chemicals (Germany). Ascorbic acid, citric acid, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, a hydrophilic derivative of tocopherol) and *tert*-butylhydroquinone (TBHQ, a commercial standard) were from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Sample Preparation. Stock solutions (10 mM) of the 19 phenolic compounds were prepared in methanol and kept at 4 °C in the dark. Possible interfering compounds including citric acid, trolox (a vitamin E analogue), TBHQ (a commercial antioxidant), and phenol were also prepared similarly, except that vitamin C was prepared in distilled water. All the stock solutions were being evaluated at four different concentrations (10 mM, 1 mM, 0.1 mM, and 0.01 mM) prepared by dilution with corresponding solvents prior to analysis.

Enzymatic Reaction Kinetics of Phenolic Compounds. The enzymatic determination of total phenolic content was based on the method described by Stevanato (*11*) with some modifications. Three milliliters of 30 mM 4-aminophenazone (4-AP), 3 mL of 20 mM hydrogen peroxide (H_2O_2), and 1.5 mL of 6.6 μM HRP, which were all freshly prepared with 0.1 M potassium phosphate buffer solution (pH 8.0), were mixed thoroughly and made up to a total volume of 30 mL with the same buffer solution to form the enzyme−reagent working solution. They were only mixed just before the experiments as the peroxidase was very sensitive and could lose its function after a couple of hours. The experiments were carried out in a 96-well microplate (Sarstedt, U.S.). To each well, 225 μL of the enzyme−reagent working solution and 25 μL of phenolic compounds (1 mM) or blank (methanol or water) were added. The plate was then shaken for 30 s, and the absorbance was taken using a UV−visible spectrophotometric microplate kinetics reader (SPECTRA Max 250, Gene, U.S.) at 500 nm every minute for a 20 min time period. All samples were evaluated in triplicate. It was assumed that the time taken for the enzymatic reaction to complete was indicated by a leveling-off of the absorbance from a plot of absorbance versus time.

Determination of Molar Absorbance of Phenolic Compounds. On the basis of the above results, a 15 min incubation time was found to be required for the peroxidase-catalyzed enzymatic reactions to complete. The experimental procedures used in this section were similar to those in the reaction kinetics study except the incubation time was set at 15 min, and four concentrations (10 mM, 1 mM, 0.1 mM, and 0.01 mM) of the phenolic compounds were evaluated in triplicate. The dose-dependent response for each phenolic compound was also evaluated.

The procedures of the FC method were adapted from the method described by Singleton and Rossi (*6*) with some modifications. First, 0.5 mL of phenolic compounds was mixed with 0.5 mL of the Folin−Ciocalteu reagent, and the mixture was allowed to stand at room temperature for 3 min. Then, 0.5 mL of saturated sodium carbonate (35% aqueous solution) was added to the mixture followed by the addition of 3.5 mL of distilled water. The mixture was kept in the dark with occasional shaking for 90 min, and the absorbance at 725 nm was measured using a UV−vis spectrophotometer (Genesys5, Spectronic Instruments, U.S.).

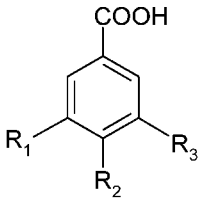
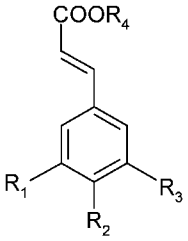
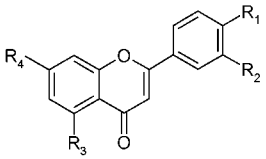
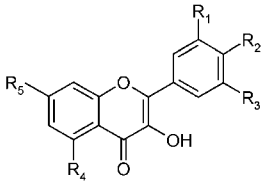
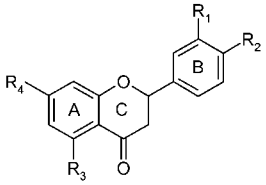
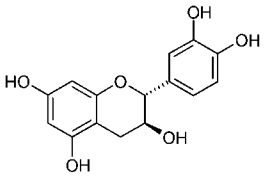
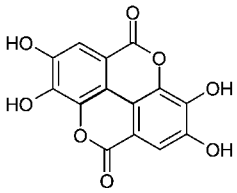
The molar absorbance (ϵ) of the phenolic compounds was determined by plotting their respective standard curves of different concentrations (10 mM, 1 mM, 0.1 mM, and 0.01 mM) as defined below:

$$\text{molar absorbance } (\epsilon) = A/cl$$

where A = absorbance, c = sample concentration in mol/L, and l = length of light path through the cuvette in cm.

Statistical Analysis. Pearson's correlation coefficient (two-tailed) ($p < 0.01$) in bivariate correlations was used to determine dose-dependent responses between the concentrations of phenolic compounds and their corresponding absorbance as well as the correlations between the degree of hydroxylation of individual phenolic compounds and their molar absorbance determined by the two methods. All statistical analyses were done by the Statistical Package for Social Sciences (*14*).

Table 1. Chemical Structures of 19 Phenolic Compounds in Different Subclasses

Subclass/compound	Chemical structure				
Benzoic acids		R_1	R_2	R_3	
Gallic acid		OH	OH	OH	
m-Hydroxybenzoic acid		OH	H	H	
Protocatechuic acid		H	OH	OH	
Vanillic acid		OCH_3	OH	H	
Syringic acid		OCH_3	OH	OCH_3	
Cinnamic acids		R_1	R_2	R_3	R_4
Trans-cinnamic acid		H	H	H	H
p-coumaric acid		H	OH	H	H
Caffeic acid		H	OH	OH	H
Ferulic acid		OCH_3	OH	H	H
Sinapic acid		OCH_3	OH	OCH_3	H
Chlorogenic acid		H	OH	OH	5-quinic acid
Flavones		R_1	R_2	R_3	R_4
Apigenin		OH	H	OH	OH
Luteolin		OH	OH	OH	OH
Flavonols		R_1	R_2	R_3	R_4 R_5
Quercetin		OH	OH	H	OH OH
Myricetin		OH	OH	OH	OH OH
Flavanones		R_1	R_2	R_3	R_4
Naringenin		H	OH	OH	OH
Hesperetin		H	OCH_3	OH	OH
Flavanols					
Catechin					
Others					
Ellagic acid					

RESULTS AND DISCUSSION

Enzymatic Reaction Kinetics of Phenolic Compounds. The different enzymatic reaction kinetics of the 19 phenolic compounds shown in **Figure 2** indicated that the enzymatic reactions were completed on or before 15 min for all phenolic compounds. On the basis of the time taken for the PE reaction to complete, the 19 phenolic compounds can be divided into three groups. Gallic acid, *trans*-cinnamic acid, and protocatechuic acid belonged to the fast-reacting group (0−5 min); while vanillic acid, syringic acid, quercetin, myricetin, and catechin belonged to the medium fast-reacting group (6−10 min); and the remaining ones belonged to the slow-reacting group (11−15 min) (**Table 2**).

In the PE reaction, since other substrates such as 4-AP and H_2O_2 as shown in **Figure 1** were in excess and the incubation temperature and pH of the reagents were kept constant, the

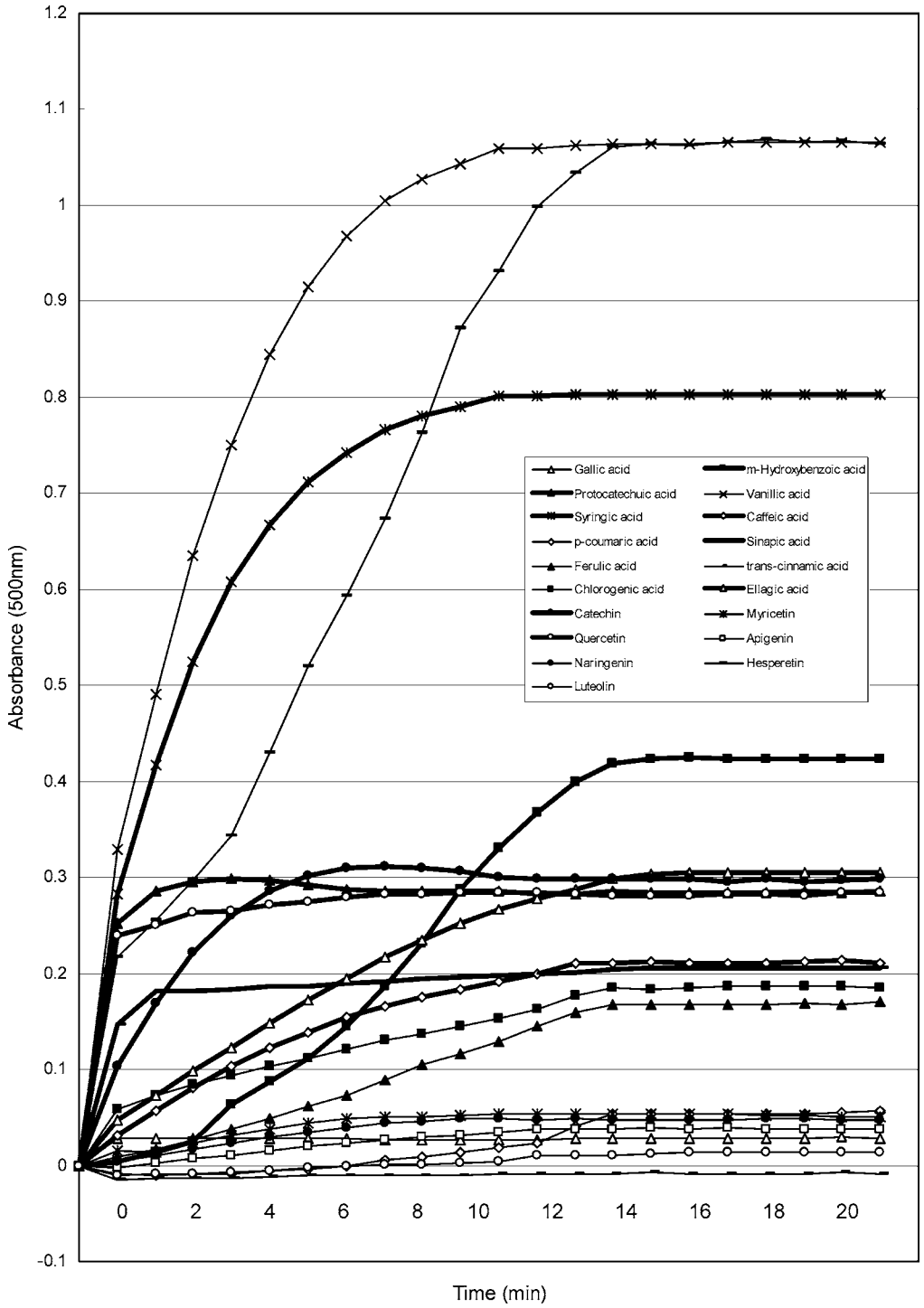


Figure 2. Reaction kinetics of the 19 phenolic standards (1 mM) in the enzymatic assay.

reaction rates were dependent mainly on the structural difference of the phenolic compounds. Phenolic compounds belonging to the subclasses with the same functional groups exhibited similar reaction kinetics by using a similar time to attain the maximum absorbance. All cinnamic acids except *trans*-cinnamic acid, all flavones, and all flavanones took about 11−15 min to complete the reactions; while all flavonols and flavanols took about 7−8 min for complete reactions. However, benzoic acids demonstrated a wide range of reaction times, in which at least one subclass member was found in each of the three reacting groups.

This phenomenon may be explained by the complexity and bulkiness of the phenolic compounds. Since the PE reaction involves an electrophilic attack by a phenolic compound toward 4-AP that is previously oxidized by H_2O_2 in the presence of HRP, the reaction rate could be hindered by any bulky substituents (e.g., methoxyl and allyl carboxylic acid group) in the aromatic rings of the phenolic compounds. Benzoic acids possess the simplest structures and generally completed the reaction in a shorter reaction time than the phenolic compounds in other subclasses. Vanillic acid and syringic acid, which are benzoic acids with one and two methoxyl substituents, respectively, had a longer enzymatic reaction time than other benzoic acids such as gallic acid, probably due to the stronger steric hindrance in the former.

Spectrophotometric Responses of Phenolic Compounds Determined by the PE Method and the FC Methods. Table 3 summarizes the molar absorbance and Pearson's correlation of various phenolic compounds determined by the PE method

Table 2. Grouping of the Phenolic Compounds According to the Time Taken To Complete the Enzymatic Reaction

phenolic compd	subclass	no. of OH	time taken (min)
fast-reacting group (0–5 min)			
gallic acid	benzoic acids	3	<1
trans-cinnamic acid	cinnamic acids	0	<1
protocatechuic acid	benzoic acids	2	2
medium-fast reacting group (6–10 min)			
vanillic acid	benzoic acids	1	10
syringic acid	benzoic acids	1	10
quercetin	flavonols	5	8
myricetin	flavonols	6	8
catechin	flavanols	5	7
slow-reacting group (11–15 min)			
m-hydroxybenzoic acid	benzoic acids	1	13
p-courmaric acid	cinnamic acids	1	14
caffeic acid	cinnamic acids	2	13
ferulic acid	cinnamic acids	1	14
sinapic acid	cinnamic acids	1	15
chlorogenic acid	cinnamic acids	2	15
apigenin	flavones	3	12
luteolin	flavones	4	13
naringenin	flavanones	3	11
hesperetin	flavanones	2	15
ellagic acid	others	4	15

Table 3. Molar Absorbance and Pearson's Correlation of Phenolic Compounds Determined by the PE Method and FC Method

		PE method		FC method	
subclass/ compounds	no. of OH	molar absorbance ($M^{-1}\,cm^{-1}$)	R^2 [a]	molar absorbance ($M^{-1}\,cm^{-1}$)	R^2
benzoic acids					
gallic acid	3	28.0	0.903	1310	0.994
m-hydroxybenzoic acid	1	337	0.999	397	0.999
protocatechuic acid	2	250	0.995	1150	0.991
vanillic acid	1	1061	0.998	584	0.991
syringic acid	1	805.2	0.999	907	0.995
cinnamic acids					
trans-cinnamic acid	0	n.d.[b]	n.d.	n.d.	n.d.
p-coumaric acid	1	41.2	0.969	612	1.000
caffeic acid	2	215	0.998	1610	0.998
ferulic acid	1	149	0.997	708.6	0.993
sinapic acid	1	205	0.999	965	0.990
chlorogenic acid	2	182	0.995	1094	0.996
flavones					
apigenin	3	309.1	0.999	668	0.998
luteolin	4	997	0.988	495	0.988
flavonols					
quercetin	5	278	0.998	1920	0.998
myricetin	6	400	0.998	2950	1.000
flavanones					
naringenin	3	45.4	0.959	1009	0.999
hesperetin	2	940	1.000	602.6	0.998
flavanols					
catechin	5	279	0.998	1480	0.996
others					
ellagic acid	4	304.6	0.996	2310	0.997

[a] R^2: Pearson's correlation coefficient. [b] n.d.: not detected.

and the FC methods. The correlations between the concentrations of all samples and their corresponding absorbance were very high for both methods, with nearly all samples having a Pearson's correlation coefficient over 0.99, indicating both methods were very sensitive and dose-dependent. However, as represented by the molar absorbance, which is proportional to the sample molar concentration, the response of individual phenolic compounds toward the two methods was very different. For example, gallic acid (1310 $M^{-1}\,cm^{-1}$) and naringenin (1009 $M^{-1}\,cm^{-1}$) had a high molar absorbance in the FC method, while both of them had a low molar absorbance (28.0 $M^{-1}\,cm^{-1}$ and 45.4 $M^{-1}\,cm^{-1}$) in the PE method. On the other hand, vanillic acid, syringic acid, luteolin, and hesperetin possessed a high molar absorbance (805.2−1061 $M^{-1}\,cm^{-1}$) in the PE method, while all of them only responded moderately (495−907 $M^{-1}\,cm^{-1}$) in the FC method.

Although the detailed mechanism for the PE method is still uncertain, the difference in the responses between phenolic compounds was probably dependent on the nucleophilicity of the corresponding phenoxyl radicals, which are formed under a slightly alkaline medium. On the other hand, the FC method measures the ability of a substance to reduce the phosphotungstates−phosphomolybdates complex to blue reaction products (*7*). Hence, the response of phenolic compounds toward the FC method is expected to be closely related to their antioxidant activities, which are also affected by their chemical structures. The different responses of individual phenolic compounds toward the PE and FC methods may be explained in the scope of the following structure−activity relationships.

Substitution with Electron-Donating Groups. When the hydrogen atoms of the aromatic ring are substituted by electron-donating groups (e.g., hydroxyl and methoxyl groups), the nucleophilicity of the phenoxyl radical would increase, facilitating its electrophilic attack to the oxidized 4-AP. Methoxyl-substituted phenolics generally had greater responses than the hydroxyl-substituted ones in the PE assay. For instance, methoxyl-substituted benzoic acids, including vanillic acid and syringic acid, possessed much higher molar absorbance (805.2−1061 $M^{-1}\,cm^{-1}$) than other hydroxyl-substituted benzoic acids (28.0−337 $M^{-1}\,cm^{-1}$) (**Table 3**). In the PE assay, the molar absorbance of hesperetin (940 $M^{-1}\,cm^{-1}$), which consists of a methoxyl group at the 4′ position of the B ring (**Figure 1**), was nearly 20 times that of naringenin (45.4 $M^{-1}\,cm^{-1}$) which has the same ring structure except a hydroxyl group is substituted at the same position (**Table 3**).

Substituted methoxyl groups could improve the response of phenolic compound toward the FC reaction when compared with those having hydrogen atoms at the corresponding positions. For instance, syringic acid (907 $M^{-1}\,cm^{-1}$) and vanillic acid (584 $M^{-1}\,cm^{-1}$) had higher response than *m*-hydroxybenzoic acid (397 $M^{-1}\,cm^{-1}$) toward the FC reaction, which was consistent with the trend in their antioxidant activities (*15*). However, the substitution of a methoxyl group instead of a hydroxyl group reduced the antioxidant activity and the molar absorbance in the FC method because methoxyl-substituted phenoxy radicals are unable to be stabilized by intramolecular hydrogen bonding (*16*). For example, the molar absorbance of hesperetin, which has a methoxyl group at the 4′ position of the B ring, was only 60% of that of naringenin which possesses the same structure except a hydroxyl group is substituted at that position (**Table 3**). Substitution of methoxyl groups (sinapic acid) instead of hydroxyl group (caffeic acid) at the 3- and 5-positions in the aromatic ring reduced its response toward the FC method (**Table 3**), which was consistent with the trend in their antioxidant activities (*17*).

Substitution with Electron-Withdrawing Groups. Allyl carboxylic acid and carboxylic acid groups are the examples of electron-withdrawing groups, which stabilize the phenoxyl radical by reducing the electron density in the aromatic ring, reducing its nucleophilicity. Having one more double bond, the allyl carboxylic acid (−CH═CH−COOH) group is a stronger electron-withdrawing group than carboxylic acid. Hence, in the

PE assay, the molar absorbance of cinnamic acids (−CH═CH−COOH group) was generally lower than that of the corresponding benzoic acids (−COOH group). For example, the molar absorbance of ferulic acid and sinapic acid was only $^1/_5$ and $^1/_4$ of that of their benzoic counterparts, vanillic acid and syringic acid, respectively (**Table 3**).

Generally, having the same number of hydroxyl and methoxyl groups at the same position, hydroxycinnamic acids tended to be more sensitive to the FC reaction than hydroxybenzoic acids. For instance, in the FC assay, the molar absorbance of caffeic acid (1610 M^{-1} cm^{-1}) was greater than protocatechuic acid (1150 M^{-1} cm^{-1}), which is its hydroxybenzoic acid counterpart. The −CH═CH−COOH group linked to the phenyl ring of the hydroxycinnamic acids might improve the hydrogen-donating ability by stabilizing the subsequent radical by resonance (*15*, *18*).

Position of Hydroxylation. The position of hydroxyl group in the aromatic ring also contributed to the response of specific phenolic compound toward the PE assay. Phenolic compounds, such as *p*-coumaric acid and naringenin, with their *para*-position being occupied by −CH═CH−COOH in their aromatic rings were found to have low molar absorbance (41.2 and 45.4 M^{-1} cm^{-1}, respectively) in the PE assay. It has been reported that when the *para*-position of the phenol was occupied, the reaction would take place in the *ortho*-position, but in a less effective way (*12*).

The position of hydroxylation also accounts for the difference in the responses of flavonoids in the FC assay. Hydroxylation of the B ring is the major contributor for antioxidant activity of flavonoids. Quercetin and catechin were found to possess high molar absorbance (1480−1920 M^{-1} cm^{-1}) in the FC method (**Figure 1**). This was consistent with the previous findings that flavonoids with 3′,4′-dihydroxy configuration possessed strong antioxidant activity (*19*). Myricetin had the highest molar absorbance (2950 M^{-1} cm^{-1}) because it has an additional hydroxyl group in its 5′ position, leading to a higher response to the FC method and enhanced antioxidant activities. Apigenin (668 M^{-1} cm^{-1}), luteolin (495 M^{-1} cm^{-1}), naringenin (1009 M^{-1} cm^{-1}), and hesperitin (602.6 M^{-1} cm^{-1}), which all lack the 3-hydroxyl group in the A ring, possessed lower antioxidant activities and weaker responses toward the FC method.

Degree of Hydroxylation. The response of phenolic compounds to the PE reaction was found to be independent of the degree of hydroxylation. Phenolic compounds with the same degree of hydroxylation within the same subclass, for example, vanillic acid and *m*-hydroxybenzoic, had a large difference in their molar absorbance (**Table 3**). These data agreed with previous findings that each phenolic compound can form only one phenoxyl radical, which is the fundamental step of antioxidant properties of polyphenols, because the oxidized compounds are more difficult to donate electrons further (*11*). *trans*-Cinnamic acid, which is a cinnamic acid without any hydroxyl group on its ring, did not demonstrate an increase of absorbance in the PE method. Since the phenoxyl radical became less nucleophilic due to the absence of electron-donating group (−OH) and the presence of an electron-withdrawing group (−CH═CH−COOH), it could not react with 4-AP efficiently.

On the contrary, the degree of hydroxylation correlated well with the molar absorbance determined by the FC method (R^2 = 0.766, $p < 0.01$), which agreed with previous findings that antioxidant activity of a phenolic compound increased with the degree of hydroxylation (*16*), particularly on the *ortho* or *para* position (*20*). The molar absorbance of protocatechuic acid (1150 M^{-1} cm^{-1}) and caffeic acid (1610 M^{-1} cm^{-1}), which have a second hydroxyl group presented at the *ortho*-position of their aromatic ring, was higher than that of phenolics having only one hydroxyl group [e.g., *m*-hydroxybenzoic acid (397 M^{-1} cm^{-1}) and vanillic acid (584 M^{-1} cm^{-1})]. The reason was probably due to the increase in their antioxidant activity by the stabilization of the phenoxyl radical through an intramolecular hydrogen bond (*16*). *trans*-Cinnamic acid, which contains no hydroxyl group on its ring, did not demonstrate an increase of absorbance in the FC assay. *trans*-Cinnamic acid did not possess hydrogen-donating ability as its phenoxyl radical could not be well stabilized without the substituted hydroxyl groups (*15*).

Table 4. Molar Absorbance and Pearson's Correlation of Common Interfering Compounds Determined by the PE Method and FC Method

	enzymatic method		FC method	
interfering compds	molar absorbance (M^{-1} cm^{-1})	R^2 [a]	molar absorbance (M^{-1} cm^{-1})	R^2
vitamin C	n.d.[b]	–	11.7	0.999
citric acid	n.d.	–	n.d.	–
trolox	0.002	0.994	25.5	0.999
TBHQ	1.26	0.429	27.8	0.978
phenol	8.20	0.321	25.4	0.819

[a] R^2: Pearson's correlation coefficient. [b] n.d.: not detected.

Interferences to the Enzymatic Method and FC Method. **Table 4** shows the molar absorbance and Pearson's correlation of some common interfering compounds determined by the PE method and the FC method. Vitamin C and citric acid could not be detected by the PE method, owing to their inability to form a nucleophilic phenoxyl radical. Trolox and TBHQ had demonstrated very weak (0.00220 and 1.26 M^{-1} cm^{-1}) responses toward the PE method. Vitamin C (11.7 M^{-1} cm^{-1}), trolox (25.5 M^{-1} cm^{-1}), and TBHQ (27.8 M^{-1} cm^{-1}), which were previously identified as interfering compounds in the FC method (*9*), had moderate molar absorbance toward the FC assay, while citric acid was insensitive to both the PE and FC methods. Being a common interfering substance that has no substitution of other functional groups, phenol has been found to be an ineffective antioxidant (*16*). The unsubstituted phenoxyl radical was not nucleophilic enough to attack the oxidized 4-AP, so poor dose-dependent response (R^2 = 0.321) was found in the enzymatic method.

Conclusion. In the enzymatic reaction kinetics study, all phenolic compounds completed the reaction within 15 min with those having bulky substituents having a longer reaction time. The responses of various phenolic compounds to the two assays were found to be closely related to their chemical structures. However, the response of individual phenolic compounds toward the two assays was different, probably due to the difference in the mechanism involved. The PE method is more specific to phenolic compounds, while the FC method reflects better the antioxidant activities of the phenolic compounds. The responses of phenolic compounds toward the PE method were closely related to the nucleophilicity of their corresponding phenoxyl radicals. Substitution by methoxyl and hydroxyl groups (electron-donating group) and with the allyl carboxylic acid group (electron-withdrawing group) in the aromatic ring enhanced the responses of phenolic compounds toward the PE assay. Some common antioxidants, such as vitamin C and trolox (a vitamin E analogue), were found to interfere in the FC method. Therefore, the FC method is not suitable to measure the total phenolic content of food samples which contain high level of non-phenolic antioxidants and other interfering compounds. Although the PE method is more expensive due to the cost of

enzymes, it is more accurate in the determination of the total phenolic content because of its higher specificity toward phenolic compounds and lesser interferences from other antioxidants. Further investigation on the responses of conjugated phenolic compounds (e.g., phenolic glycosides) toward the PE method and the evaluation of its application on polyphenol-rich food samples are underway.

ABBREVIATIONS USED

ϵ, molar absorbance; PE, peroxidase-catalyzed enzymatic; FC, Folin−Ciocalteu; HRP, horseradish peroxidase; TBHQ, *tert*-butylhydroquinone; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

ACKNOWLEDGMENT

We thank K. M. Lo of the Department of Biology, The Chinese University of Hong Kong, for his technical assistance.

LITERATURE CITED

(1) Willcox, J. K.; Ash, S. L.; Catignani, G. L. Antioxidants and prevention of chronic disease. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 275−295.
(2) George, S.; Brat, P.; Alter, P.; Amiot, M. J. Rapid determination of polyphenols and vitamin C in plant-derived products. *J. Agric. Food Chem.***2005**, *53*, 1370−1373.
(3) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.***1997**, *2*, 152−158.
(4) Shi, J.; Nawaz, H.; Pohorly, J.; Mittal, G.; Kakuda, Y.; Jiang, Y. M. Extraction of polyphenolics from plant materials for functional foods − engineering and technology. *Food Rev. Int.* **2005**, *21*, 139−166.
(5) Tsao, R.; Deng, Z. Y. Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B* **2004**, *812*, 85−99.
(6) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.
(7) Huang, D. J.; Ou, B. X.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.
(8) Stratil, P.; Klejdus, B.; Kuban, V. Determination of total content of phenolic compounds and their antioxidant activity in vegetables − evaluation of spectrophotometric methods. *J. Agric. Food Chem.* **2006**, *54*, 607−616.
(9) Prior, R. L.; Wu, X. L.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.
(10) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.
(11) Stevanato, R.; Fabris, S.; Momo, F. New enzymatic method for the determination of total phenolic content in tea and wine. *J. Agric. Food Chem.* **2004**, *52*, 6287−6293.
(12) Fiamegos, Y. C.; Stalikas, C. D.; Pilidis, G. A.; Karayannis, M. I. Synthesis and analytical applications of 4-aminopyrazolone derivatives as chromogenic agents for the spectrophotometric determination of phenols. *Anal. Chim. Acta.* **2000**, *403*, 315−323.
(13) Martinello, F.; Luiz da Silva, E. Mechanism of ascorbic acid interference in biochemical tests that use peroxide and peroxidase to generate chromophore. *Clin. Chim. Acta.* **2006**, *373*, 108−116.
(14) SPSS Inc. *SPSS Base 13.0 Application Guide*; SPSS Inc.: Chicago, 2004.
(15) Cai, Y. Z.; Sun, M.; Xing, J.; Luo, Q.; Corke, H. Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sci.* **2006**, *78*, 2872−2888.
(16) Shahidi, F.; Janitha, P. K.; Wanasundara, P. D. Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 67−103.
(17) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(18) Cheng, Z. Y.; Li, Y. Z.; Chang, W. B. Kinetic deoxyribose degradation assay and its application in assessing the antioxidant activities of phenolic compounds in a Fenton-type reaction system. *Anal. Chim. Acta.* **2003**, *478*, 129−137.
(19) Dziedzic, S. Z.; Hudson, B. J. F. Polyhydroxychalcones and flavanones as antioxidant for edible oils. *Food Chem.* **1983**, *12*, 205−212.
(20) Arts, M. J. T. J.; Dallinga, J. S.; Voss, H. P.; Haenen, G. R. M. M.; Bast, A. A critical appraisal of the use of the antioxidant capacity (TEAC) assay in defining optimal antioxidant structures. *Food Chem.* **2003**, *80*, 409−414.

Received for review January 11, 2007. Revised manuscript received March 12, 2007. Accepted March 13, 2007.

JF070084W