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ESR determination of the reactions between selected phenolic acids and free radicals or transition metals

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Abstract

Five phenolic acids commonly present in wheat grain and fractions were examined and compared for their radical-scavenging properties and chelating capacities. The free radical-scavenging properties were evaluated against 2,2-diphenyl-1-picryhydrazyl radical (DPPH·), radical cation ($ABTS^{\cdot+}$), peroxide radical anion ($O_2^{\cdot-}$) and hydroxyl radical (HO·), whereas the chelating properties were evaluated against Fe (II) and Cu (II) using spectrophotometric and electron spin resonance measurements. These phenolic acids differed in their abilities to react with and quench HO·, $O_2^{\cdot-}$, $ABTS^{\cdot+}$ and DPPH·, as well as their capacities to form chelating complexes with transition metals. 4-Hydroxybenzoic acid had neither free radical-scavenging nor chelating activity under the experimental conditions. Strong structure–activity relationships were observed in the present study. Both substituents on the phenyl ring and the conjugated carbon skeleton may influence the antioxidant properties of phenolic acids. The presence of an additional methoxyl group in the ortho position of the hydroxyl group showed a strong influence on the chelating property of phenolic acids and their radical-scavenging capacity against $O_2^{\cdot-}$, $ABTS^{\cdot+}$ and DPPH·, but not on their HO·-scavenging activity.
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1. Introduction

Growing evidence suggests that a high consumption of whole-grain foods, vegetables, and fruits may reduce the risk of several aging-related diseases, including cancer and cardiovascular diseases (De Ancos, González, & Cano, 2000; Jacobs, Meyer, & Solvoll, 2001; Jacobs, Meyer, Kushi, & Folsom, 1998; Liu et al., 1999; Nicodemus, Jacobs, & Folsom, 2001; Slavin, Jacobs, & Marquart, 1997; Slavin, Marquart, & Jacobs, 2000). The beneficial effects are mainly ascribed to phytochemicals, such as insoluble and soluble fibres, vitamins, carotenoids, and natural antioxidants (Ames, Shigen, & Hagen, 1993; Giugliano, 2000; Katsube et al., 2004). Dietary antioxidative components may modulate cellular oxidative status and protect important biological molecules such as DNA, protein, and membrane lipid from oxidative damage, and consequently reduce the risk of several chronic diseases, including cancer and cardiovascular disease (Adom, Sorrells, & Liu, 2003; Halliwell, Gutteridge, & Cross, 1992; Wang & Zheng, 2001; Yu et al., 2002a).

Significant levels of antioxidant activities, total phenolic contents, individual phenolic acids, carotenoids and tocopherols were detected in wheat grain, grain fractions, and wheat-based food products, suggesting the potential utilization of wheat and wheat-based food products in human health promotion and disease prevention (Abdel-Aal & Hucl, 2003; Adom & Liu, 2002;

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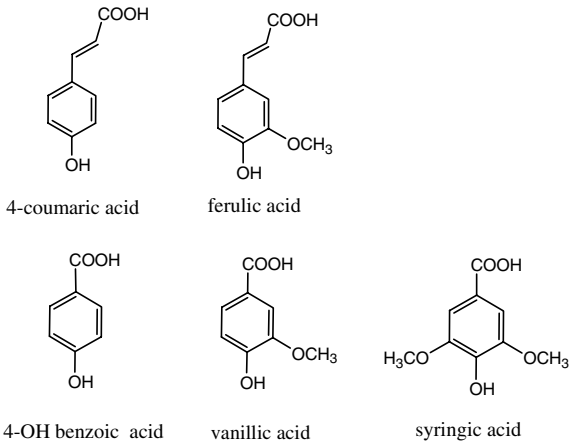

Fig. 1. Structures of phenolic acids present in wheat grain and fractions. 4-coumaric and ferulic acids are cinnamic acid derivatives, while 4-OH benzoic, vanillic, and syringic acids are benzoic acid derivatives.

Adom et al., 2003; Onyeneho & Hettiarachchy, 1992; Yu, Haley, Perret, & Harris, 2002b; Yu et al., 2002a; Yu, Perret, Harris, Wilson, & Haley, 2003; Zhou, Laux, & Yu, 2004; Zielinski & Kozlowska, 2000). Several phenolic acids, including ferulic, vanillic, 4-coumaric, caffeic, chlorogenic, gentisic, syringic, and 4-hydroxybenzoic acids, have been detected in wheat and wheat fractions (Adom et al., 2003; Onyeneho & Hettiarachchy, 1992; Zhou et al., 2004). These phenolic acids are derivatives of either benzoic or cinnamic acids (Fig. 1). Phenolic acids are believed to contribute to the overall antioxidant activities of wheat, as well as fruits, vegetables and other grains (Adom et al., 2003; Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001; Clifford, 1999; Kern et al., 2003a; Kern et al., 2003b; Zhou et al., 2004).

Recently, a nutritional study was conducted to evaluate the absorption of hydroxycinnamates in humans after the administration of a testing high-bran wheat cereal (Kern et al., 2003b). The results from this study suggested that ferulic and sinapic acids, both cinnamic acid derivatives, could be absorbed by humans from wheat-based cereal foods. In addition, ferulic, vannilic, coumaric and cinnamic acids might prevent peroxidation of lipid and protein induced by hydroxyl and peroxyl radicals in synaptosomal and neuronal cell cultures (Kanski, Aksenova, Stoyanova, & Butterfield, 2002). These studies suggested that these phenolic acids may be bioavailable and contribute to the overall in vivo beneficial effects of wheat and wheat-based food products.

A few studies have been conducted to investigate the antioxidant properties of the phenolic acids including those present in wheat and wheat bran. Pulido, Bravo, and Saura-Calixto (2000) reported that ferulic acid had significant reducing power, as measured by a modified ferric reducing/antioxidant power (FRAP) assay. Earlier, in 1999, a study compared the activity of benzoic acid derivatives in prevention of human LDL oxidation to that of corresponding cinnamic acid derivatives (Natella, Nardini, Felice, & Scaccini, 1999). Ferulic, vanillic, syringic, 4-coumaric and 4-OH benzoic acids all showed significant activity in prevention of human LDL oxidation (Natella et al., 1999), quenching cation radical $ABTS^{\bullet+}$ (Yeh & Yen, 2003), and protecting protein molecules from radical attacks, as determined by the oxygen radical absorbing capacity (ORAC) assay (Yeh & Yen, 2003). In addition, ferulic and 4-coumaric acids, at a concentration of 20 μM, quenched 27.3% and 7.0% of DPPH radicals in ethanol (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). To our knowledge, no investigation was conducted to evaluate these phenolic acids for their chelating capacities, radical-scavenging activities against hydroxyl ($HO^{\bullet}$) and peroxide anion ($O_2^{\bullet-}$) radicals, or to test their potential synergistic effects in antioxidant properties. These data are critical for better understanding and utilization of wheat and wheat-based food products in improving human nutrition and health.

Wheat is a major agricultural commodity and dietary component across the world. The average annual farm gate value of hard winter wheat (*Triticum aestivum*) is over $300 million in Colorado (USA) alone (Yu et al., 2002a). Recently, the wheat producers have had to search for new value-added marketing opportunities for wheat because the farmers have suffered from the record low price of the wheat market. The new consumer preference of low-Carb foods may further decrease the global consumption of wheat. Identification of the health promoting factors and novel value-added utilizations of wheat is needed for enhancing its marketing potential, and to benefit both the agricultural economy and human health.

The present study was conducted to determine the radical-scavenging activities of the phenolic acids detected in wheat, and their chelating capacities. The chelating capacities against Fe^{2+} and Cu^{2+} were examined by spectrophotometric and electron spin resonance (ESR) spectroscopic methods, respectively. Peroxide anion ($O_2^{\bullet-}$), cation ($ABTS^{\bullet+}$), hydroxyl ($OH^{\bullet}$), and neutral DPPH radicals were employed in this study. Antioxidant–radical reactions were measured using both spectrophotometric and ESR methods. The tested phenolic acids included ferulic, 4-coumaric, vanillic, syringic, and 4-OH benzoic acids. Also determined were the potential synergistic effects among the selected phenolic acids in their antioxidant activities. In addition, the chemical structure–antioxidant activity relationships were considered. This research is part of our continuous efforts to promote the improved production and utilization of value-added wheat in health promotion and disease prevention.

2. Materials and methods

2.1. Materials

5-tert-Butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) was a gift from Dr. B. Kalyanaraman in the Biophysics Research Institute and Free Radical Research Center, at the Medical College of Wisconsin (Milwaukee, WI). High purity ferulic, 4-coumaric, syringic, vanillic, and 4-hydroxybenzoic acids were purchased from Sigma-Aldrich (St. Louis, MO). Disodium ethylenediaminetetraacetate (EDTA), hydroxylamine hydrochloride, 2,2′-bipyridyl, 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^{\bullet}$), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, hypoxanthine (HPX), xanthine oxidase (XOD), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 5,5-dimethyl *N*-oxide pyrroline (DMPO), diethylenetriaminepentaacetic acid (DTPA), and superoxide dismutase (SOD) were also obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of the highest commercial grade and used without further purification.

2.2. Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity

$O_2^{\bullet-}$ scavenging activity was determined by ESR method and the xanthine/xanthine oxidase system was used to generate the $O_2^{\bullet-}$ (Noda, Kohno, Mori, & Packer, 1999). The antioxidant–radical reaction was initiated by addition of xanthine oxidase solution (XOD), whereas 5-tert-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) was used as the trapping agent (Zhao, Joseph, Zhang, Karoui, & Kalyanaraman, 2001). The total volume of the reaction mixture was 100 μl and the final concentrations were 2 mM for xanthine, 100 mM for BMPO, 0.2 mM for diethylenetriaminepentaacetic acid (DTPA), 2 mM for all phenolic acids, and 0.08 units/ml for XOD. All other reagents were prepared with 5 mM phosphate buffer (pH 7.4), except that phenolic acids were dissolved in 50% acetone. The ESR spectra were recorded at 2 min of reaction at ambient temperature with 10 mW incident microwave power and 100 kHz field modulation of 1 G. Superoxide dismutase (SOD) was used as the antioxidant standard for quantification of the $O_2^{\bullet-}$ scavenging activity.

2.3. Hydroxyl radical (HO$^{\bullet}$) scavenging activity

Hydroxyl radical (HO$^{\bullet}$) scavenging capacities of the phenolic acids were examined by the ESR method. ESR assay was based on the competition between the trapping agent and the antioxidative phenolic acids (Madsen, Nielsen, Bertelsen, & Skibsted, 1996). HO$^{\bullet}$ was generated by Fenton reaction, while 5,5-dimethyl *N*-oxide pyrroline (DMPO) was used as the trapping agent. Fifty percent of acetone was used as the solvent to dissolve individual phenolic acids. The reaction mixture contained 10 μl of 3 mM freshly prepared $FeSO_4$, 80 μl of 0.75 mM EDTA, 15 μl of 1 M DMPO, 15 μl of 0.5 mM H_2O_2, and 30 μl of phenolic acid solution or solvent for the blank. The final concentration was 2 mM for all phenolic acids. The ESR measurements were conducted at 1 and 20 min of each reaction at ambient temperature, using a Varian E-109X-Band ESR spectrometer (Varian, Inc., Palo Alto, CA) in the Center for Food Safety and Applied Nutrition at the Food and Drug Administration (College Park, MD), with the following spectrometer settings: microwave power of 10 mW, field modulation frequency of 100 kHz, and a modulation amplitude of 1 G.

2.4. Radical cation ($ABTS^{\bullet+}$) scavenging activity

Radical scavenging capacity of the phenolic acids was evaluated against $ABTS^{\bullet+}$, generated by the chemical method according to a previously reported spectrophotometric procedure (Zhou et al., 2004; Miller & Rice-Evans, 1997). $ABTS^{\bullet+}$ was prepared by oxidizing a 5 mM aqueous solution of ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, with manganese dioxide at ambient temperature for 30 min. The $ABTS^{\bullet+}$–antioxidant reaction mixture contained 1.0 ml of $ABTS^{\bullet+}$ with an absorbance of 0.8 at 734 nm, and 100 μl of 100 μM phenolic acid or 100 μl of 50% acetone solution for the control. The absorbance at 734 nm was measured at 1 min of the reaction, and the trolox equivalent was calculated using a standard curve prepared using trolox. The $ABTS^{\bullet+}$ scavenging capacity of phenolic acids was expressed as mmoles of trolox equivalents per mmole of phenolic acid.

To test the potential synergistic effect among the phenolic acids against $ABTS^{\bullet+}$, total radical scavenging capacities of selected combinations of two phenolic acids were determined according to the protocol described above using $ABTS^{\bullet+}$. For instance, to determine the potential synergistic effect between ferulic and syringic acids, the selected phenolic acid combinations include (a) 100% ferulic acid, (b) 50% ferulic and 50% syringic acids, and (c) 100% syringic acids. The trolox equivalents were calculated and used to compare the total $ABTS^{\bullet+}$ scavenging capacities of the phenolic acid combinations with that of the two individual phenolic acids to determine the synergistic effect between them.

2.5. Radical DPPH scavenging activity

Radical DPPH scavenging capacities of individual selected phenolic acids were determined by an electron spin resonance (ESR) spectrometry method, using the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^{\bullet}$) (Yu et al., 2002a). ESR analysis was conducted using

a Varian E-109X-Band ESR spectrometer (Varian, Inc. Palo Alto, CA) at ambient temperature in the Center for Food Safety and Applied Nutrition at the Food and Drug Administration (College Park, MD). Each phenolic acid solution was mixed with DPPH· stock solution to initiate the antioxidant–radical reaction. The final concentration was 250 μM for DPPH· in all reaction mixtures. The final concentration was 50 μM in the reaction mixture for all phenolic acids, and the control reaction contained no antioxidant. Both DPPH· and phenolic acid solutions were prepared with 50% acetone in water (v/v). ESR signals were recorded at 1, 25, and 75 min following the start of the reaction, with 20 mW incident microwave power, 100 kHz field modulation of 2 G (Santiago, Hiramatsu, & Mori, 1992; Yu et al., 2002a). The scavenging activity of each phenolic acid was estimated by comparing the DPPH· in the antioxidant–radical reaction mixture and that in the control reaction for the same period of reaction time, and expressed as % DPPH· remaining.

The potential synergistic effects among the selected phenolic acids in quenching DPPH· were examined using a spectrophotometric method. Total free radical-scavenging capacities of selected combinations of the wheat phenolic acids were estimated and compared using DPPH· (Yu et al., 2002a). For instance, to determine the potential synergistic effect between ferulic and syringic acids, the selected phenolic acid combinations included (a) 100% ferulic acid, (b) 75% ferulic and 25% syringic acids, (c) 50% ferulic and 50% syringic acids, (d) 25% ferulic and 75% syringic acids and (e) 100% syringic acids. The initial concentrations were 100 M for DPPH· and 100 μM for total phenolic acids in all antioxidant–radical reactions. Both DPPH· and individual phenolic acid solutions were prepared with 50% acetone in water (v/v). The absorbance at 517 nm was measured against a blank of the solvent at 0.5, 1, 1.5, 2, 2.5, and 3 min of the radical–antioxidant reaction and used to estimate the remaining radical levels according to a standard curve. A_{517nm}, at 3 min of reaction, was used to compare the DPPH· scavenging capacity of each combination of phenolic acids under the experimental conditions.

2.6. Chelating activity against Fe^{2+} and Cu^{2+}

A 2,2′-bipyridyl competition assay was conducted to measure the Fe^{2+} chelating activity of individual phenolic acids and their potential synergistic effects (Yu et al., 2003). The reaction mixture contained 0.1 ml of 1 mM $FeSO_4$ solution, 50 μl of 100 μM phenolic acid in 50% acetone, 0.3 ml of 10% hydroxylamine–HCl, 0.4 ml of 2,2′-bipyridyl solution (0.1% in 0.2 M HCl), and 0.8 ml of Tris–HCl buffer (pH 7.4). The absorbance at 522 nm was measured and used to determine Fe^{2+}-chelating activity with EDTA as a standard.

ESR measurements were carried out to determine the potential chelating capacity of the phenolic acids against Cu^{2+}, based on a previously described condition with slight modification (Antholine, Basosi, Hyde, Lyman, & Petering, 1984). Briefly, 150 μl of 10 mM individual phenolic acid solution was mixed with 150 μl of 2 mM copper chloride ($CuCl_2$) solution. ESR spectra were recorded with 40 MW incident microwave power and 100 kHz field modulation of 5 G at 77 K.

2.7. Statistic analysis

Data were collected as means ± SD for triplicate determinations. Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were conducted to identify differences among means. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Superoxide anion radical ($O_2^{\cdot -}$) scavenging activity

$O_2^{\cdot -}$-scavenging activity of the phenolic acids were evaluated by ESR measurements. These phenolic acids included 4-coumaric, ferulic, syringic, vanillic and 4-OH benzoic acids. Ferulic and 4-coumaric acids are cinnamic acid derivatives, whereas syringic, vanillic, and 4-OH benzoic acids are benzoic acid derivatives (Fig. 1). ESR measurements showed that syringic acid, the 3,5-dimethoxyl derivative of 4-OH benzoic acid, had strongest $O_2^{\cdot -}$ scavenging activity among all tested phenolic acids, followed by ferulic and 4-coumaric acids (Fig. 2), indicating that an additional *o*-methoxyl (OCH_3) group on the phenyl ring may enhance the $O_2^{\cdot -}$ scavenging activity of either benzoic or cinnamic acid derivatives. Also noted was that 4-OH benzoic acid was not able to scavenge $O_2^{\cdot -}$ in the reaction mixture under the experimental conditions. The $O_2^{\cdot -}$ scavenging activities of individual phenolic acids were also compared by ESR quantification (Fig. 3). Ferulic, syringic, 4-coumaric, and vanillic acids had $O_2^{\cdot -}$ scavenging activity, but 4-OH benzoic acid did not quench free radicals at all (Fig. 3). Furthermore, syringic, ferulic, 4-coumaric, and vanillic acids significantly differed in their $O_2^{\cdot -}$ scavenging activities (Fig. 3).

3.2. Hydroxyl radical (HO·) scavenging activity

4-Coumaric, ferulic, syringic, and vanillic acids exhibited significant HO· scavenging capacities under the experimental conditions (Fig. 4), but 4-hydroxybenzoic acid had no ability to react with and quench HO·. 4-Coumaric acid, the 4-OH derivative of cinnamic acid, showed strongest activity in reacting with and quenching

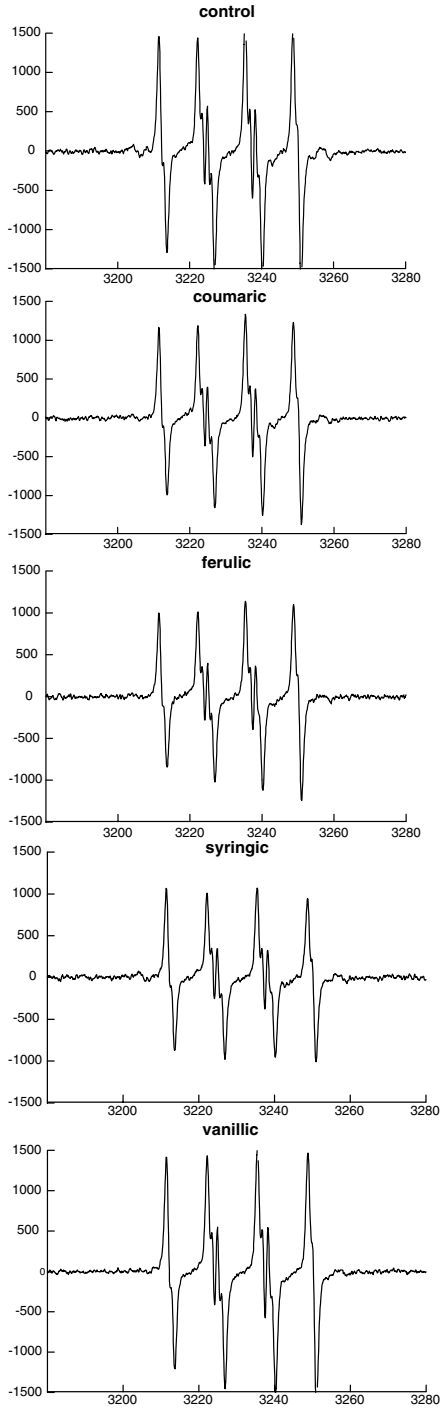

Fig. 2. $O_2^{\bullet-}$ scavenging activities of phenolic acids determined by ESR. Coumaric, ferulic, syringic, and vanillic stand for 4-coumaric, ferulic, syringic, and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid had no $O_2^{\bullet-}$ scavenging activity under the experimental conditions. The reaction mixture contained 2 mM xanthine, 100 mM BMPO, 0.2 mM diethylenetriaminepentaacetic acid (DTPA), 2 mM phenolic acid, and 0.08 units/ml XOD in a total volume of 100 μl. The ESR spectra were recorded at 2 min of the reaction at ambient temperature.

HO$^{\bullet}$ (Fig. 4). Also notable was that the HO$^{\bullet}$ scavenging activities of the phenolic acids were time-dependent, with a greater HO$^{\bullet}$ scavenging activity associated with a

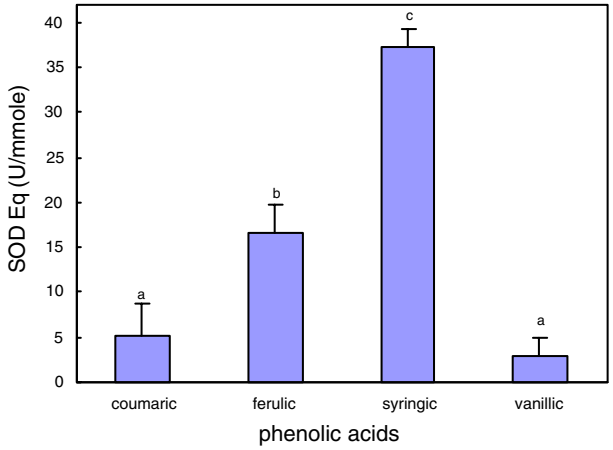

Fig. 3. Comparison of $O_2^{\bullet-}$ scavenging capacities of the phenolic acids. $O_2^{\bullet-}$ scavenging capacities of phenolic acids were expressed as superoxide dismutase (SOD) equivalent (U) per mmole phenolic acid. Coumaric, ferulic, syringic and vanillic stand for 4-coumaric, ferulic, syringic and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid had no $O_2^{\bullet-}$ scavenging activity under the experimental conditions. All tests were conducted in triplicate and the means are used. The vertical bars represent the standard deviation of each data point ($n = 3$). Values marked by the same latter are not significantly different ($P < 0.05$).

longer reaction time between phenolic acid and HO$^{\bullet}$ (Fig. 4).

3.3. Radical cation $ABTS^{\bullet+}$ scavenging activity

The $ABTS^{\bullet+}$ scavenging capacities of each selected phenolic acid and combinations of the phenolic acids were examined. The trolox equivalent against the $ABTS^{\bullet+}$ radical ranged from 1.12 to 1.66 mmoles per mmole of phenolic acid for ferulic, 4-coumaric, syringic and vanillic acids (Fig. 5), but no $ABTS^{\bullet+}$ scavenging capacity was detected for 4-OH benzoic acid under the experimental conditions. The four phenolic acids differed in their $ABTS^{\bullet+}$ scavenging capacities. Of the two cinnamic acid derivatives, ferulic acid had a greater $ABTS^{\bullet+}$ scavenging capacity than did 4-coumaric; and for the benzoic acid derivatives, syringic acid had the strongest $ABTS^{\bullet+}$ scavenging capacity, followed by vanillic acid (Fig. 5), suggesting that the presence of OCH_3 in ortho to the OH group may significantly enhance the $ABTS^{\bullet+}$ scavenging capacity of phenolic acids. None of the phenolic acid combinations exhibited an $ABTS^{\bullet+}$ scavenging capacity greater than that of either constituent phenolic acids alone (Fig. 5), indicating no synergistic effects between these phenolic acids in their reactions with $ABTS^{\bullet+}$.

3.4. Radical DPPH scavenging activity

ESR measurements were conducted in 50% acetone. ESR spectra showed that derivatives of both cinnamic and benzoic acids might directly react with and quench

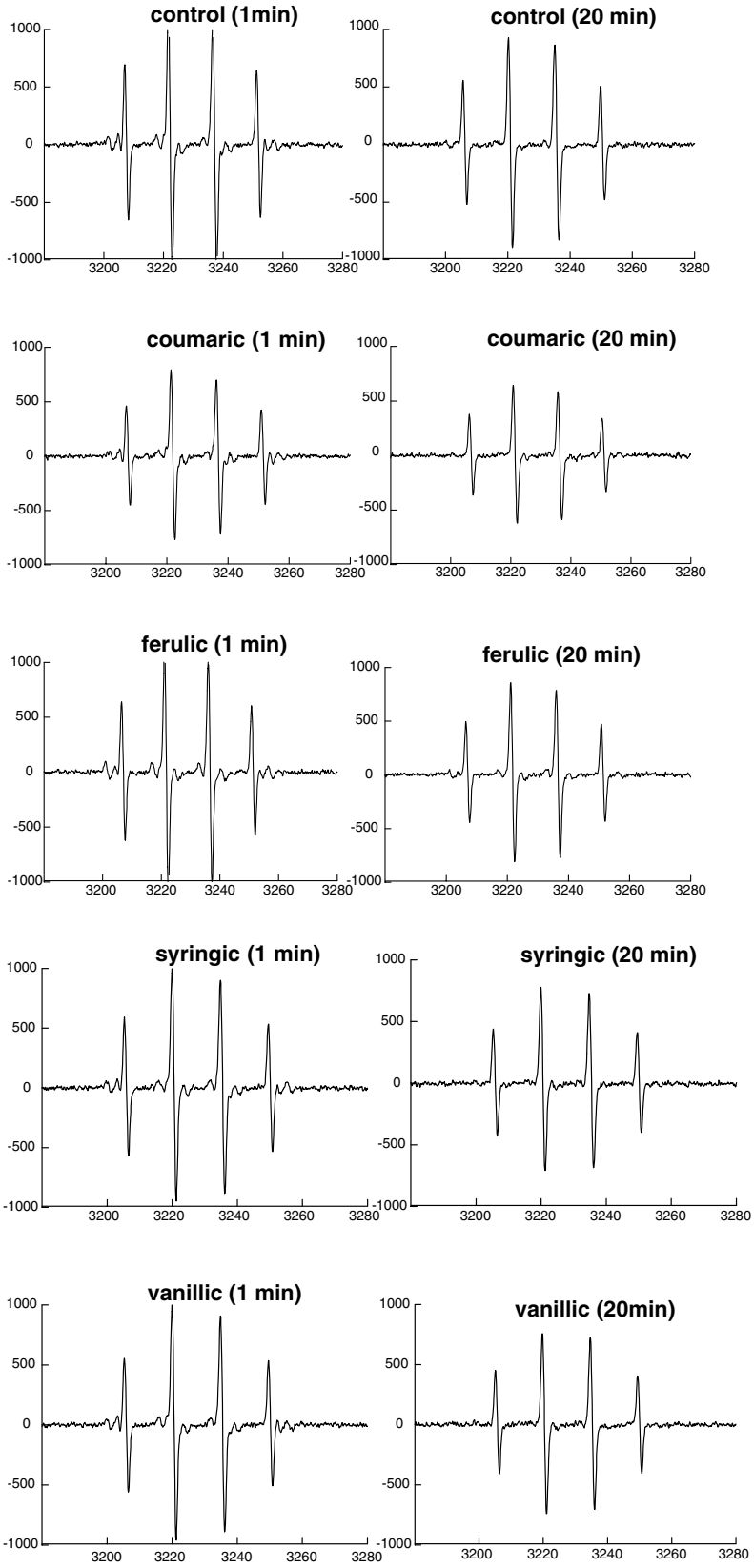

Fig. 4. Hydroxyl radical scavenging activities of phenolic acids determined by ESR. Coumaric, ferulic, syringic, and vanillic stand for 4-coumaric, ferulic, syringic, and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid had no $HO^{\cdot}$ scavenging activity under the experimental conditions. Each reaction mixture contained 10 μl of freshly prepared 3 mM $FeSO_4$, 80 μl of 0.75 mM EDTA, 15 μl of 1 M DMPO, 15 μl of 0.5 mM H_2O_2, and 30 μl of phenolic acid solution. The final concentration of phenolic acid was 2 mM in all reaction mixtures. ESR signals were recorded at 1 and 20 min of the reaction at ambient temperature.

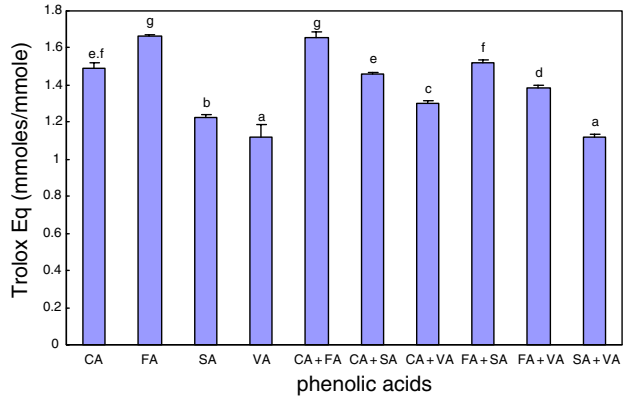

Fig. 5. $ABTS^{\cdot+}$ scavenging properties of phenolic acids. The $ABTS^{\cdot+}$ scavenging activities of phenolic acids were expressed as mmoles of trolox equivalent (Trolox Eq) per mmole of total phenolic acids. CA, FA, SA, VA, represent 4-coumaric, ferulic, syringic, and vanillic acid, respectively. 4-OH benzoic acid showed no $ABTS^{\cdot+}$ scavenging activity under the experimental conditions. The final concentration was 9.1 μM for all phenolic acids. The combined phenolic acids contained 50% of each on a per mole basis. 50% acetone was used in all reactions as the solvent. The vertical bars represent the standard deviation ($n = 3$), and values marked by the same latter are not significantly different ($P < 0.05$).

$DPPH^{\cdot}$ in the reaction mixture (Fig. 6). Syringic acid, the 3,5-dimethoxyl derivative of 4-OH benzoic acid, might have the strongest $DPPH^{\cdot}$-scavenging capacity, followed by that of ferulic acid, the 3-methoxyl derivative of 4-OH cinnamic acid (Fig. 6), while 4-coumaric acid exhibited weak activity against $DPPH^{\cdot}$ (Fig. 7). Vanillic and 4-OH benzoic acids showed no activity in quenching $DPPH^{\cdot}$ under the ESR measurement conditions (Figs. 6 and 7). ESR measurements also indicated the time-dependence of phenolic acid–$DPPH^{\cdot}$ reactions for both syringic and ferulic acids (Figs. 6 and 7). In addition, the potential synergistic effects among phenolic acids against $DPPH^{\cdot}$ were examined using a spectrophotometric method with 50% acetone as the solvent for all reaction mixtures. No synergistic effect was observed between any two tested phenolic acids on a same total phenolic acid molarity basis.

3.5. Chelating activity against Fe^{2+} or Cu^{2+}

No chelating activity against Fe^{2+} was detected under the experimental condition using a spectrophotometric method. Interestingly, vanillic and ferulic acids showed Cu^{2+}-chelating capacity according to the ESR measurements (Fig. 8). Syringic and 4-coumaric acids had no interaction with Cu^{2+} under the experimental conditions (Fig. 8).

4. Discussion

Significant levels of ferulic, syringic, 4-coumaric, vanillic and 4-OH benzoic acids were detected in wheat grain, bran, and aleurone fraction of bran (Zhou et al., 2004). These phenolic acids may act as natural antioxidants and play important roles in the health benefits associated with consumption of whole-grain foods (Jacobs et al., 2001; Jacobs et al., 1998; Liu et al., 1999; Nicodemus et al., 2001; Slavin et al., 1997; Slavin et al., 2000). Antioxidants may form chelating complexes with transition metals to reduce their availability as catalysts, and suppress the formation of the first few free radicals in the system, to inhibit the initiation of the free radical-mediated oxidative chain reaction. Antioxidants may also directly react with and quench radicals in the system to terminate oxidative chain reactions. Therefore, this study examined radical-scavenging properties of the five phenolic acids that are present in wheat grain and fractions, and their potential synergistic effects using spectrophotometric and ESR methods. ESR measures the presence of an unpaired electron in the free radicals, and has been successfully used to study radical-scavenging properties of antioxidants (Sripriya, Chandrasekharan, Murty, & Chandra, 1996; Yu et al., 2002a).

Interestingly, 4-OH benzoic acid exhibited no ability to react with and quench $DPPH^{\cdot}$, $ABTS^{\cdot+}$, $O_2^{\cdot-}$ or $HO^{\cdot}$ under the experimental conditions, as measured by either ESR or spectrophotometric methods. This is in contrast to the previous observation that 4-OH benzoic acid, at a concentration of 6.7 μM, had greater $ABTS^{\cdot+}$ scavenging capacity than vanillic and syringic acids on a molarity basis (Yeh & Yen, 2003). To confirm the $ABTS^{\cdot+}$ scavenging property of 4-OH benzoic acid, $ABTS^{\cdot+}$–antioxidant reactions were carried out with 4-OH benzoic acid at concentrations of 0.91, 2.3, 4.6, 9.1, and 91 μM. To further confirm the findings, the experiments were repeated using freshly purchased 4-OH benzoic acid from Sigma-Aldrich. No $ABTS^{\cdot+}$ scavenging activity was detected at any of these concentrations with two purchases of 4-OH benzoic acid. In addition, 4-OH benzoic acid was tested for its oxygen radical absorbing capacity (ORAC) since it showed strong ORAC in the previous study (Yeh et al., 2003). The 4-OH benzoic acid exhibited strong ORAC, although it showed no $ABTS^{\cdot+}$ scavenging capacity under the experimental conditions. These data indicate that 4-OH benzoic acid is not able to scavenge $ABTS^{\cdot+}$ at a greater activity than either vanillic or syringic acids. Also notable was that vanillic acid had greater $ABTS^{\cdot+}$ scavenging capacity than syringic acid, as reported by Yeh and Yen (2003), but syringic acid was a stronger $ABTS^{\cdot+}$ scavenger in the present study (Fig. 5). Furthermore, results from the present study suggest that an additional methoxyl group (OCH_3) in the ortho position to the hydroxyl position on the phenyl ring enhances the $ABTS^{\cdot+}$ scavenging capacity of both 4-OH benzoic and 4-OH cinnamic acid derivatives. This is in contrast to Yeh and Yen's observation (2003) that additional OCH_3 in an ortho position to the hydroxyl group on

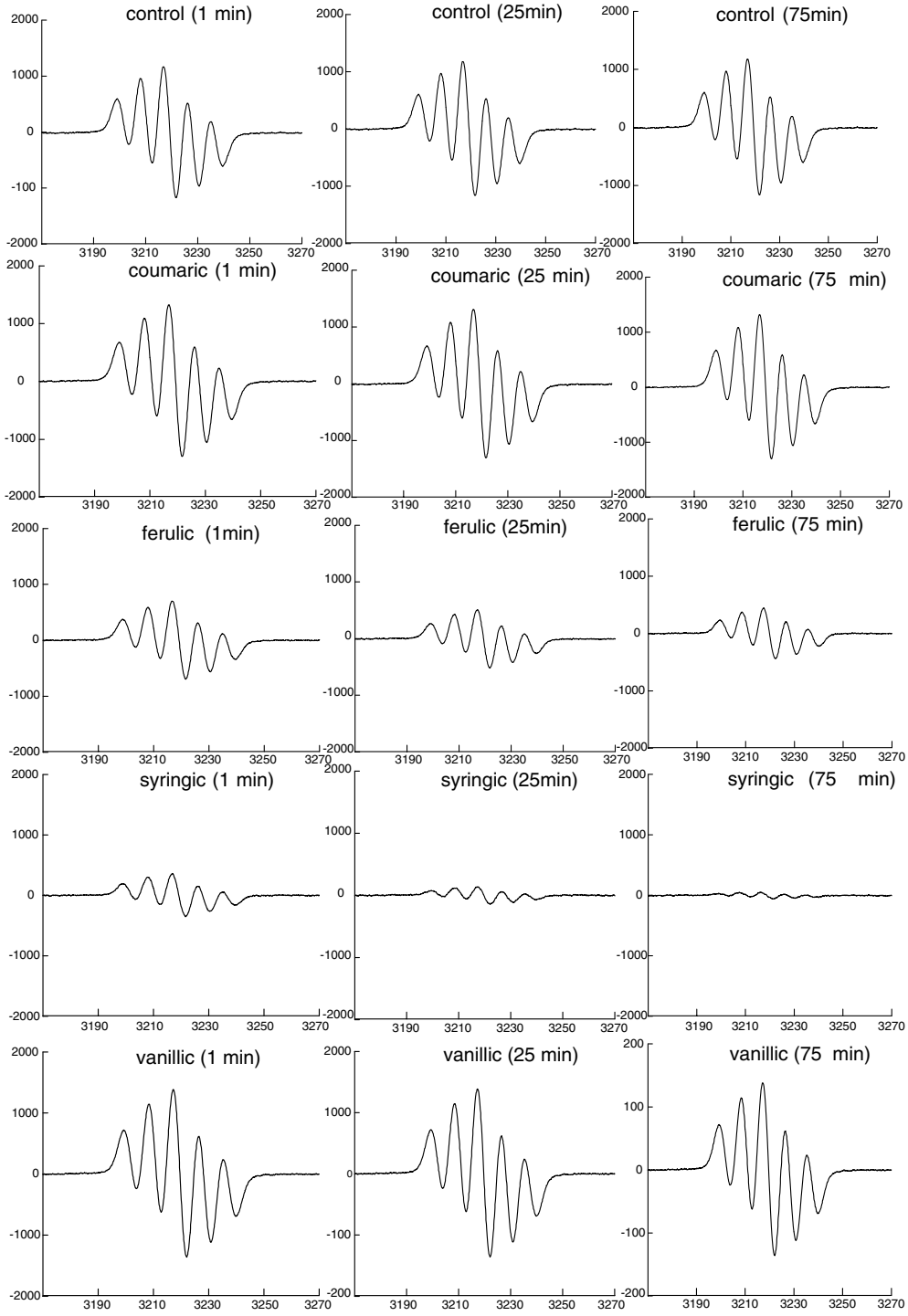

Fig. 6. DPPH· scavenging activities of phenolic acids determined by ESR. Coumaric, ferulic, syringic, and vanillic stand for 4-coumaric, ferulic, syringic, and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid showed no DPPH· scavenging activity under the experimental conditions. The final concentrations were 250 μM for DPPH· and 50 μM for phenolic acid in all reaction mixtures. ESR signals were recorded at 1, 25, and 75 min of each reaction at ambient temperature.

the phenyl ring increased the $ABTS^{\cdot+}$ scavenging capacity of 4-OH cinnamic acid derivatives, but decreased that of 4-OH benzoic acid derivatives. Structure–$ABTS^{\cdot+}$ scavenging capacity relationships of phenolic acids, including those tested in this study, have been discussed and summarized in detail in a previously published review article (Rice-Evans, Miller, & Paganga, 1996).

In the present study, syringic acid exhibited stronger $O_2^{\cdot-}$ scavenging activity than vanillic acid, while ferulic

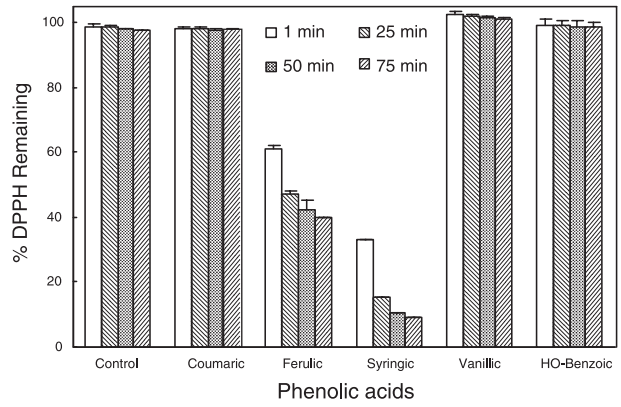

Fig. 7. DPPH· scavenging activities of phenolic acids at different reaction times. Coumaric, ferulic, syringic and vanillic stand for 4-coumaric, ferulic, syringic and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid had no DPPH· scavenging activity under the experimental conditions. The DPPH· scavenging activities of phenolic acids are expressed as % DPPH· remaining. The vertical bars represent the standard deviation of each data point ($n = 3$).

acid had greater $O_2^{\bullet-}$-scavenging capacity than an 4-coumaric acid (Fig. 3), suggesting that an additional OCH_3 in the ortho position to the hydroxyl group on the phenyl ring also increased the $O_2^{\bullet-}$ scavenging capacity. The unshared pair of electrons of *o*-OCH_3 in the *p*-orbital, as shown in the resonance structure III in Fig. 9a and b, stabilizes the phenyl radical through electron delocalization and electron donation. It was also noted that ferulic acid had significantly greater capacity than vanillic acid in reacting with and quenching the $O_2^{\bullet-}$ in the system. This indicates that 4-OH cinnamic acid derivatives may have stronger $O_2^{\bullet-}$-scavenging activity than their corresponding 4-OH benzoic acid derivatives. This may be explained by the additional possible resonance structures of the resulting phenoxyl radicals of 4-OH cinnamic acids (Fig. 9a and b). This is supported by the observation of a previous study conducted by Natella et al. (1999). Natella et al. (1999) evaluated the activities of benzoic acid and cinnamic acid derivatives in quenching peroxyl radical and suppressing LDL oxidation induced by either 2,2′-azobis(amidinopropane) dihydrochloride or Cu^{2+}, and concluded that the propenoic side chain could stabilize the phenoxyl radical by resonance and enhance the antioxidant activity of the phenyl ring. In addition, it needs to be emphasised that both xanthin oxidase inhibitors and superoxide anion scavengers may result in reduced amounts of $O_2^{\bullet-}$ in the reaction mixture (Cos et al., 1998). This study examined the total reduction of $O_2^{\bullet-}$ ESR signals, which could not provide information about whether these phenolic acids may also have enzyme inhibitory activities.

ESR determination showed that only ferulic and vanillic acids had significant capacity to form a chelating complex with Cu^{2+} among the tested phenolic acids under the experimental conditions (Fig. 8). Both ferulic

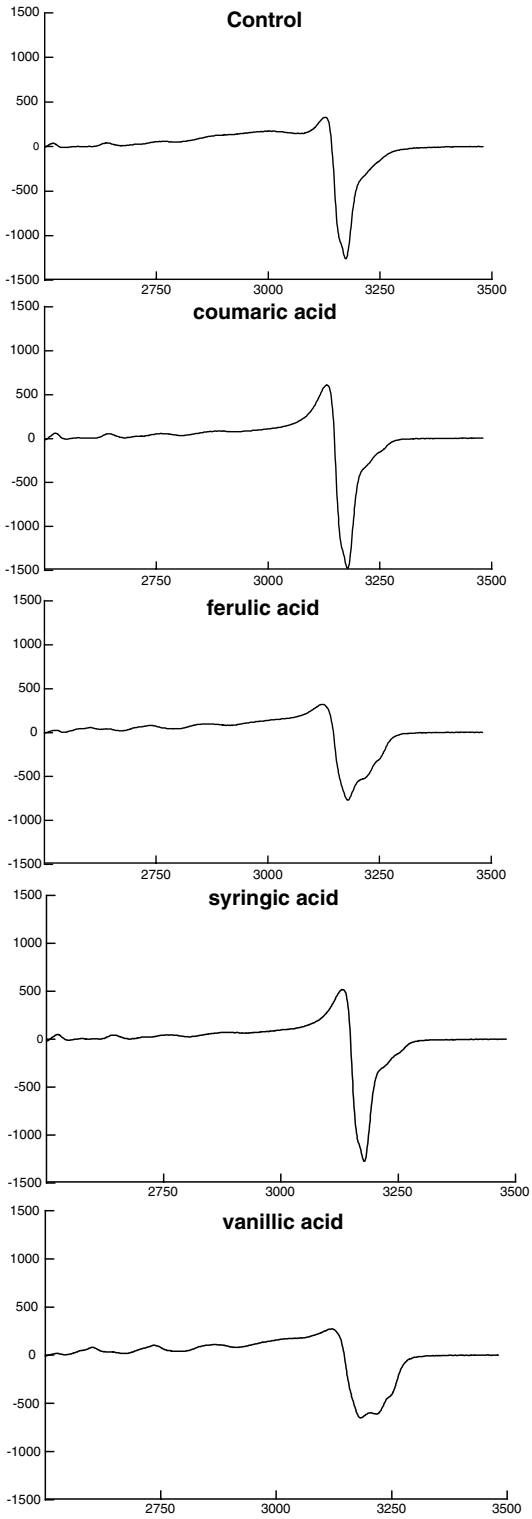

Fig. 8. Interaction between Cu^{2+} and individual phenolic acids measured by ESR. The final concentrations were 5 mM for each phenolic acid and 1 mM for copper chloride ($CuCl_2$). The ESR spectrum was recorded at 1 min of reaction at 77 K.

and vanillic acids contain OCH_3 in the ortho position to the hydroxyl group (Fig. 1), suggesting that the presence of a hydroxyl and an OCH_3 in the ortho position

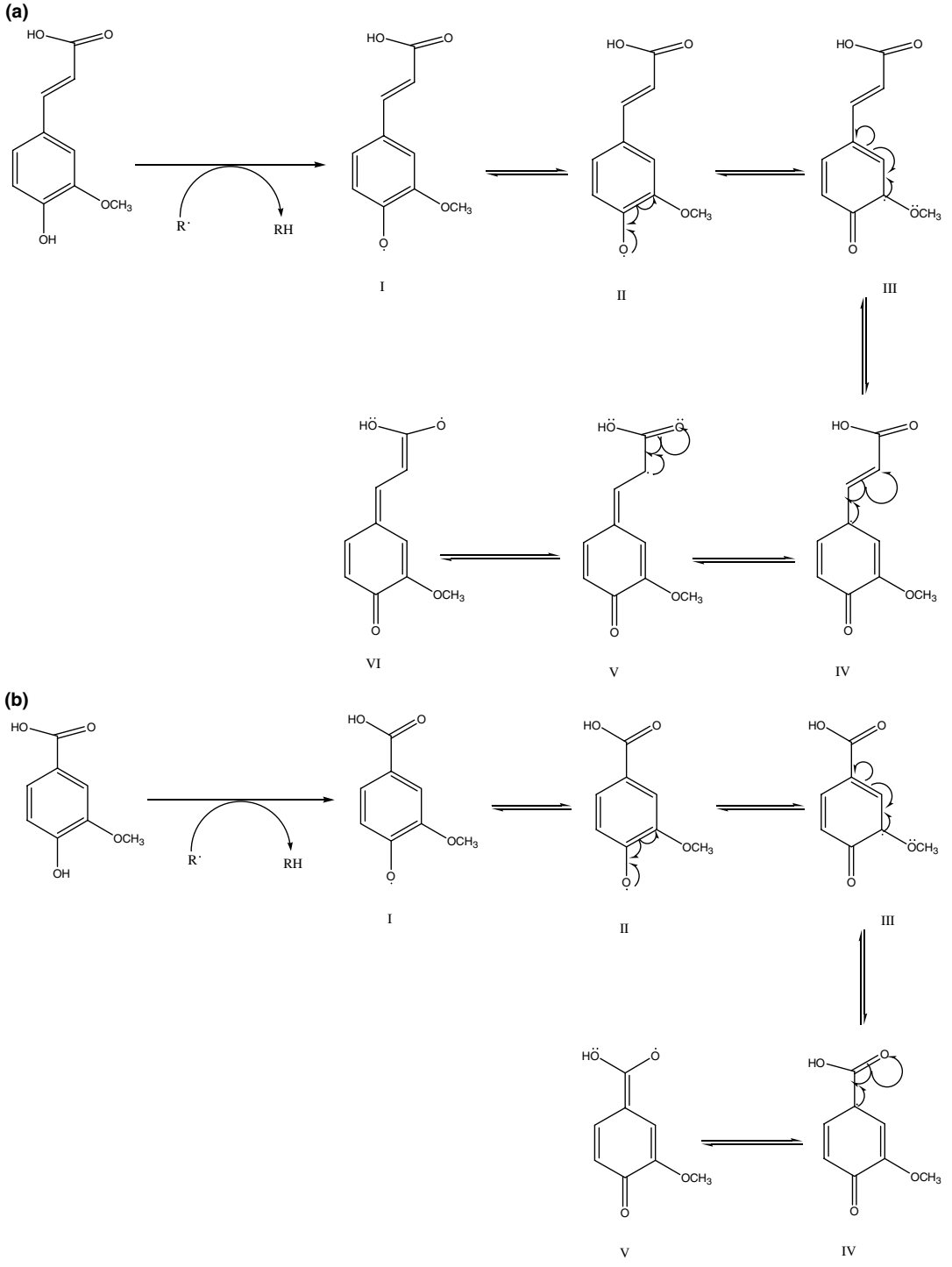

Fig. 9. Phenoxyl radical formation and possible resonance structures for cinnamic and benzoic acid derivatives. Phenoxyl radical is initially formed at the 4-OH group by abstraction of the hydroxyl H atom, regardless of the carbon skeleton of the molecule. (a) represents the formation and resonance structures of 4-OH cinnamic acid derivatives, including ferulic and 4-coumaric acids. For resonance structure III, the unshared pair electrons in the P-orbital of the 3-OCH_3 stabilizes the unpaired electron on C3 of the phenyl ring. For resonance structure VI, the unshared pair electrons in the P-orbital of the hydroxyl group stabilizes the unpaired electron on the O-atom of the carboxylic group. (b) represents the formation and resonance structures of 4-OH benzoic acid derivatives, including 4-OH benzoic, vanillic and syringic acids. For resonance structure III, the unshared pair electrons in the P-orbital of the 3-OCH_3 stabilizes the unpaired electron on C3 of the phenyl ring. For resonance structure V, the unshared pair electrons in the P-orbital of the hydroxyl group stabilizes the unpaired electron on the O-atom of the carboxylic group. Cinnamic acid derivatives may have six possible resonance structures for the phenoxyl radical, whereas benzoic acid derivatives have five possible resonance structures.

might produce the formation of a phenolic acid–Cu^{2+} chelating complex. This was different from the observation by Natella and others that only the phenolic acids with two hydroxyl groups in the ortho position showed Cu^{2+} chelating activity, measured by a spectrophotometric acid (Natella et al., 1999). This might be explained by the greater sensitivity of the ESR method and the weaker capacity of OCH_3 to interact with Cu^{2+} due to the steric effect. Interestingly, syringic acid with two ortho OCH_3 groups showed no ability to form a chelating complex with Cu^{2+}, suggesting that addition of the second ortho OCH_3 on the phenyl ring dramatically eliminated the chelating capacity of the phenolic acid. This might partially be explained by the change in the spatial arrangement of the hydroxyl group and the *o*-OCH_3 because of steric exclusion, and possible interruption of electron delocalization between p- and π-orbitals.

In summary, five phenolic acids commonly present in wheat grain and fractions significantly differed in their abilities to react with and quench $HO^{\cdot}$, $O_2^{\cdot -}$, $ABTS^{\cdot +}$ and $DPPH^{\cdot}$. These phenolic acids also differed in their capacity to form chelating complexes with transition metals. Both substituents on the phenyl ring and the conjugated carbon skeleton may influence the antioxidant properties of phenolic acids. The presence of an additional methoxyl group in the ortho position of the hydroxyl group enhances the radical-scavenging capacity of phenolic acids against $O_2^{\cdot -}$, $ABTS^{\cdot +}$ and $DPPH^{\cdot}$, but not their $HO^{\cdot}$-scavenging activity. In addition, the presence of the first methoxyl group in the ortho position to the hydroxyl group on the phenyl ring may produce the capacity to form chelating capacity against transition metals, but introduction of a second methoxyl group to the ortho position of the hydroxyl group may eliminate the chelating capacity of the phenolic acid.

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