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Reduction of Nitrous Acid to Nitric Oxide by Coffee Melanoidins and Enhancement of the Reduction by Thiocyanate: Possibility of Its Occurrence in the Stomach

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Reactions of nitrous acid with freeze-dried instant coffee and its methanol-insoluble melanoidin fractions were studied at pH 2 in the presence and absence of thiocyanate (SCN^-), simulating the mixture of coffee, saliva, and gastric juice. Coffee contained stable radicals, and the radical concentration increased by ferricyanide and decreased by ascorbic acid. This result indicates that the radical concentration was affected by the redox state of coffee and that the nature of the radical was due to quinhydrone structure that might be included in coffee melanoidins. Nitrite also increased the electron spin resonance (ESR) signal intensity at pH 2, suggesting that nitrite oxidized melanoidins producing nitric oxide (NO). The formation of NO could be detected by oxygen uptake due to the autoxidation of NO and using an NO-trapping agent. SCN^- largely enhanced NO formation in coffee and methanol-insoluble melanoidin fractions but only slightly in a methanol-soluble fraction, and the enhancement accompanied the consumption of SCN^- but did not accompany the formation of a stable ESR signal. The enhancement was explained by the reduction of NOSCN by melanoidins in methanol-insoluble fractions and that the consumption was due to binding of SCN^- to melanoidins during their oxidation by nitrous acid. The result obtained in this study suggests that when coffee is ingested, in addition to chlorogenic acid and its isomers, melanoidins can also react with salivary nitrite and SCN^- in the gastric lumen, producing NO.

KEYWORDS: Coffee; melanoidins; nitric oxide (NO); nitrous acid; oxygen uptake

INTRODUCTION

Coffee is a popular beverage in the world. After drinking coffee, its components are mixed with saliva and then gastric juice. The mixing with saliva in the oral cavity results in the oxidation of components in coffee by not only salivary peroxidase/H_2O_2 but also NO_2 that is generated by autoxidation of nitric oxide (NO) and by salivary peroxidase-catalyzed oxidation of nitrite and NO (*1*). The presence of nitrite and the production of H_2O_2, NO, and NO_2 in the human oral cavity have been reported (*1*). When the mixture of coffee and saliva is combined with gastric juice, components in coffee can be oxidized by nitrous acid ($pK_a = 3.3$) derived from salivary nitrite. This is deduced from the report that nitrous acid is reduced to NO by components in coffee (*2*). Chlorogenic acid and its isomers have been proposed as the reductants (*2*). *o*-Diphenols like quercetin and chlorogenic acid are known to be able to reduce nitrous acid to NO (*3–5*). In addition to NO, quinones of chlorogenic acid and nitrated chlorogenic acid are also produced in the mixture of coffee and nitrous acid (*2*). When a salivary component SCN^- is present in the reaction mixture, the quinone seems to be transformed to (*E*)-5′-{3-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl)acryloyloxy}quinic acid via 2-thiocyanatechlorogenic acid (*2*).

During the study of coffee-induced formation of NO in the presence of nitrous acid, the formation of NO seems to be faster than that expected from the concentration of chlorogenic acid and its isomers in coffee (*2*). This result suggests that, in addition to chlorogenic acid and its isomers, other components, which can reduce nitrous acid to NO, are also present in coffee. As the components, melanoidins are possible. This possibility is deduced from the reports that melanoidins, which have stable radicals in the molecules (*6, 7*), can react with O_2 (*7*) and can function as antioxidants (*8, 9*).

The objective of this study is the elucidation of reactions between nitrous acid and coffee melanoidins simulating the mixture of coffee, saliva, and gastric juice. Reactions between nitrous acid and melanoidins were studied by measuring electron spin resonance (ESR) spectra, O_2 uptake, NO formation, and absorption spectra in UV−visible regions. In addition, effects of SCN^- on the reaction between nitrous acid and melanoidins

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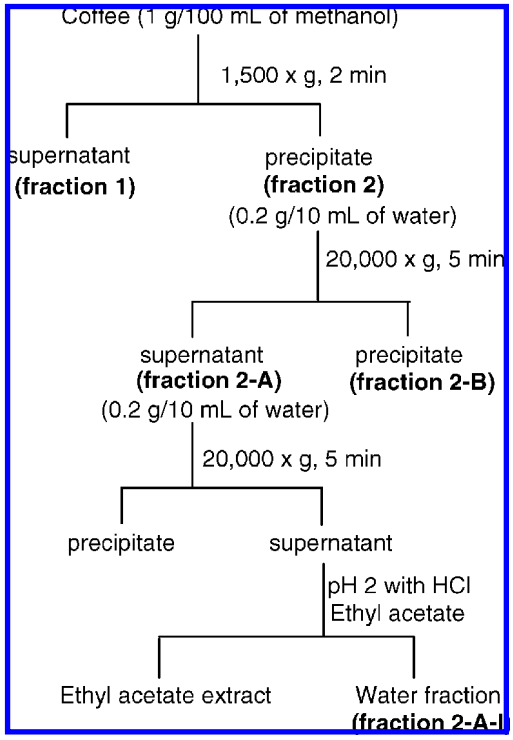

Figure 1. Preparation of various fractions of coffee.

were also studied as SCN^- affected the reaction between nitrous acid and chlorogenic acid in coffee (*2*). The elucidation of reactions between nitrous acid and melanoidins in coffee may be helpful to discuss functions of coffee and SCN^- in the stomach from the points of NO formation and scavenging of quinones that may be produced during the NO formation.

MATERIALS AND METHODS

Reagents. *N*-(Dithiocarboxy)sarcosine disodium salt (DTCS) was obtained from Dojin (Kumamoto, Japan). Chlorogenic acid, caffeic acid, and other reagents were from Wako Pure Chem. Ind. (Osaka, Japan). Instant coffee (freeze-dried) was obtained from a local market.

Fractionation of Coffee. One gram of instant coffee was suspended in 100 mL of methanol and stirred for 1 h at room temperature (about 25 °C). The suspension was centrifuged at 1500*g* for 2 min. The brown sediment was washed twice with 80 mL of methanol by centrifugation, and the methanol-insoluble fraction was dried. The yield was 0.43 g, and this sediment was referred to as fraction 2 (**Figure 1**). The supernatants obtained were combined, and methanol was evaporated in vacuo. The residue was referred to as fraction 1 (pasty and brown), and the yield was 0.50 g. Almost all of the free chlorogenic acid and its isomers was contained in this fraction.

Fraction 2 (0.2 g) was suspended in 10 mL of water and centrifuged at 20000*g* for 5 min. The supernatant, the color of which was brown, was dried with a rotary evaporator. The residue was referred to as fraction 2-A, and the yield was 96.5 mg. The sediment (beige in color) was washed twice with 10 mL of water by centrifugation, dried with a rotary evaporator, and referred to as fraction 2-B. The yield was 5 mg. The absorption spectra of fraction 2-B did not show any clear peaks in UV and visible regions. Fraction 2-A obtained from different preparations of fraction 2 was combined (0.2 g) and then suspended in 20 mL of water to centrifuge at 20000*g* for 5 min. The pH of the supernatant was adjusted to 2.2 by 1 M HCl to be extracted with 20 mL of ethyl acetate five times. The extracted water solution was concentrated with a rotary evaporator, and the concentrated pasty brown material was dried in vacuo over NaOH. This dried material was referred to as fraction 2-A-I, and the yield was 124 mg.

The molecular weight of each fraction was estimated with a gel filtration column for HPLC [Shodex Asahipak GF-510 HQ (300 mm × 7.5 mm i.d.), Showa Denko, Tokyo, Japan; mobile phase, H_2O; flow rate, 0.6 mL/min] at about 25 °C using polyethyleneglycol with different molecular masses as reference. The values were below 400 kDa for fraction 1 and between 40 and 900 kDa for fractions 2, 2-A, and 2-A-1, indicating that fractions 2, 2-A, and 2-A-1 contained melanoidins, the molecular masses of which were higher than 400 kDa. It has been reported that coffee contains melanoidins, the molecular masses of which are larger than 100 kDa (*10*).

ESR Spectroscopy. ESR spectra of coffee were measured using a JE1XG (JEOL, Tokyo, Japan) at about 25 °C using a quartz flat cell (0.05 mL). The conditions of the measurement were as follows: microwave power, 1 mW; scanning speed, 2.5 mT/min; line width, 0.5 mT; and amplification, 2000-fold. The reaction mixture (0.5 mL) contained 10 mg of coffee or its fractions/mL, which were suspended in 50 mM KCl-HCl (pH 2.0). Various concentrations of $NaNO_2$, 1 mM NaSCN, 1 mM ascorbic acid, a grain of $NaBH_4$, and 1 mM $K_3Fe(CN)_6$ were added when required. ESR spectra were also measured using coffee (10 mg/mL) dissolved in 50 mM sodium phosphate buffer (pH 5.2) and 50 mM KCl-NaOH buffer (pH 12.5), respectively. It took 1.5−2 min to start the measurement of ESR spectra after the preparation of reaction mixtures.

The formation of NO was measured using the ESR spectrometer described above. NO produced was trapped by an NO-trapping reagent, and the ESR spectra were measured under the following conditions: microwave power, 10 mW; scanning speed, 5.0 mT/min; line width, 0.5 mT; and amplification, 500-fold. The reaction mixture (0.25 mL) contained 1 mg of coffee or its fractions/mL in 50 mM KCl-HCl (pH 2.0). After incubation for 5 s, the solution (0.25 mL), which contained 10 mM DTCS and 3 mM $FeCl_3$ in 50 mM sodium phosphate (pH 7.6) [$Fe(DTCS)_2$], was added to the reaction mixture. After the addition of $Fe(DTCS)_2$, the pH of the mixture increased to about 7.4. If NO is present, a stable radical NO-$Fe(DTCS)_2$ is produced (*11, 12*).

Oxygen Uptake. Oxygen uptake was measured at 25 °C using a Clark type oxygen electrode from Rank Brothers (Cambridge, United Kingdom). The reaction mixture (1 mL) contained 1 mg of coffee or its fractions/mL in 50 mM KCl-HCl (pH 2.0). Oxygen uptake was started by the addition of $NaNO_2$. When effects of concentration of coffee on the oxygen uptake were studied, the reaction mixture contained 1−5 mg of coffee/mL and 0.2 mM $NaNO_2$ in 50 mM KCl-HCl (pH 2.0).

Spectrophotometric Measurements. Changes in absorption spectra of coffee and its fractions were measured at about 25 °C using a UV-260 spectrophotometer (Shimadzu, Kyoto, Japan). The reaction mixture (1 mL) contained 0.2 mM $NaNO_2$ and 0.5 mg of coffee or its fractions/mL, which were suspended in 50 mM KCl-HCl (pH 2.0). The light-path length of the measuring beam was 4 mm.

Hydrolysis of Fraction 2-A. Fraction 2-A (10 mg) was suspended in 2 mL of 50 mM KCl-HCl (pH 2.0), and then, 1 mL of 2 M HCl was added. The suspension was incubated for 30 min at about 95 °C, cooled, and then extracted with 2 mL of ethyl acetate three times. The extracts were combined, and ethyl acetate was evaporated in vacuo. The residue was dissolved in 0.5 mL of methanol to apply to an high-performance liquid chromatography (HPLC) column.

HPLC was performed using a 150 mm × 6.0 mm i.d. Shim-pack CLC-ODS column (Shimadzu), and separated components were detected at 320 nm using a spectrophotometric detector with a photodiode array (SPD-M10Avp, Shimadzu). The mobile phase was a mixture of methanol and 5 mM KH_2PO_4 (1:3, v/v), the pH of which was adjusted to 3.0 by 1 M H_3PO_4 and the flow rate was 1 mL/min. The amounts of chlorogenic and caffeic acids were determined from the area of each peak. The above HPLC system was also used to determine the amount of free chlorogenic acid plus its isomers contained in coffee and its fractions.

Quantification of SCN^-. The reaction mixture (2 mL) contained 2 mg of fraction 2-A-1, 0.28 mM $NaNO_2$, and 0.1 mM NaSCN in 50 mM KCl-HCl (pH 2). After incubation for 20 and 40 min, the reaction mixture was filtered with a cellulose acetate filter (0.45 μm) (Advantec, Tokyo, Japan) to apply to the ODS-HPLC column described above. The mobile phase (flow rate, 1 mL/min) was a mixture of methanol and 25 mM KH_2PO_4 (1:4, v/v), the pH of which was adjusted to 3.0

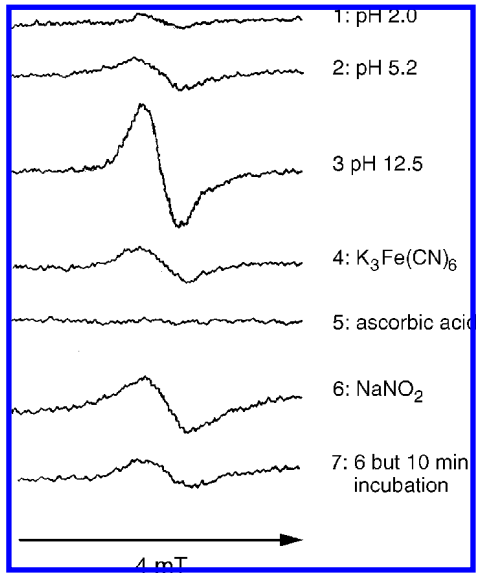

Figure 2. ESR spectra of coffee under various conditions. Trace 1, coffee (10 mg/mL, pH 2.0); trace 2, coffee (10 mg/mL, pH 5.2); trace 3, coffee (10 mg/mL, pH 12.5); trace 4, trace 1 + 1 mM $K_3Fe(CN)_6$; trace 5, trace 1 + 1 mM ascorbic acid or $NaBH_4$; trace 6, trace 1 + 0.5 mM $NaNO_2$ (1.5 min after the preparation of reaction mixture); and trace 7, as in trace 6 but 10 min after the preparation of reaction mixture.

with 1 M H_3PO_4. The concentration of SCN^- was determined from the peak area at 220 nm as described previously (*2*).

RESULTS

Characterization of Stable Radical in Coffee. Instant coffee (10 mg/mL) was suspended in buffer solutions with various pH values, and the suspensions were kept on ice until measurements. The suspension at pH 2 showed a weak and stable ESR signal with a line width of 0.6 mT (**Figure 2**, trace 1) as reported previously (*6, 7*). The signal intensity was increased when the pH was increased to 5.2 and 12.5 (traces 2 and 3). The addition of potassium ferricyanide (1 mM) at pH 2 resulted in the increase in ESR signal (trace 4), and the increased signal intensity decreased slowly (20% decrease during the incubation for 15 min). The ESR signal disappeared by the addition of ascorbic acid (1 mM) or sodium borohydride at pH 2 (trace 5). The result suggests that the signal intensity was dependent on the redox states of redox components contained in coffee.

The addition of 0.5 mM sodium nitrite to coffee at pH 2.0 increased the signal intensity about 4-fold (trace 6), and the increase in the intensity was dependent on the concentration of nitrite added (**Figure 3A**). The intensity of ESR signal, which was measured 10 min after the addition of 0.5 mM nitrite (trace 7), was smaller than that in trace 6. The time course of the decrease is shown in **Figure 3B**. Such increases and decreases in the signal intensity were also observed under anaerobic conditions (not shown), indicating that O_2 was not required for the nitrite-induced production of the radicals. Furthermore, the increases and decreases in ESR signal were also observed when 1 mM $NaNO_2$ was added to brown components (20 mg/mL), which had been prepared by heating solid chlorogenic acid and suspended in 50 mM KCl-HCl (pH 2).

Figure 4 shows ESR spectra of coffee (traces in **A**) and its various fractions (traces in **B**−**D**). No detectable ESR signal was observed in fraction 1, but a small signal appeared by the addition of 0.5 mM $NaNO_2$ (trace 2 in **B**). The ESR signal of fraction 2 was increased significantly by 0.5 mM $NaNO_2$ (trace 2 in **C**). A clear ESR signal was observed in fraction 2-A, and the intensity was also increased by 0.5 mM $NaNO_2$ (**D**). The signal intensity increased by nitrite decreased slowly as in **Figure 3B**. The ESR signal of fraction 2-A (trace 1 in **D**) was not affected by 1 mM ascorbic acid in contrast to the effect of ascorbic acid on the signal in coffee in **Figure 2** (data not shown). No ESR signals were observed in fraction 2-B.

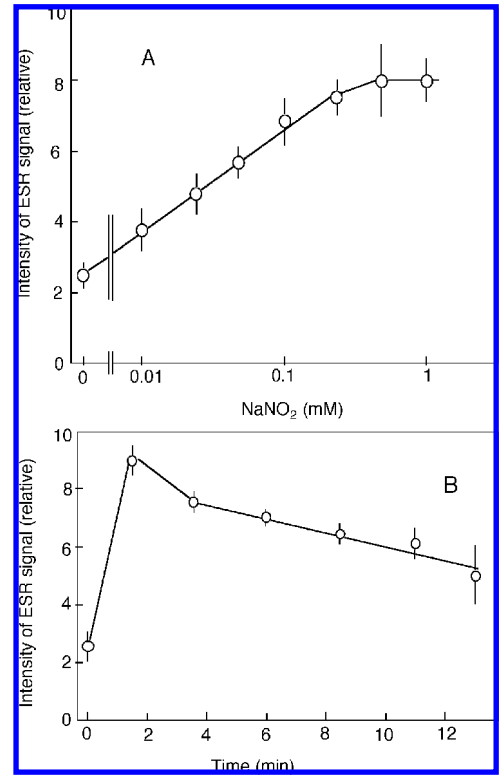

Figure 3. Effects of concentration of nitrite on ESR signals and decrease of the intensity. (**A**) Effects of concentration of nitrite of the ESR signal intensity. Signal intensities 2 min after the addition of nitrite were plotted. (**B**) Decrease in the intensity of ESR signal as a function of time. The signal was induced by the addition of 1 mM $NaNO_2$. Each data point represents an average of three experiments with SDs.

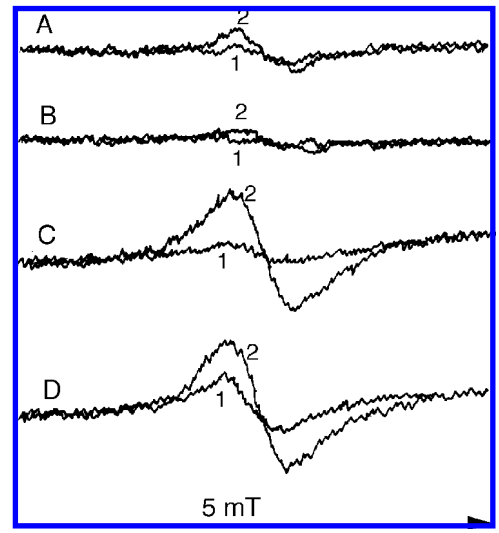

Figure 4. ESR signals of various fractions. (**A**) Coffee, (**B**) fraction 1, (**C**) fraction 2, and (**D**) fraction 2-A. Peaks: 1, no addition; and 2, 0.5 mM $NaNO_2$. The concentration of each component was 10 mg/mL.

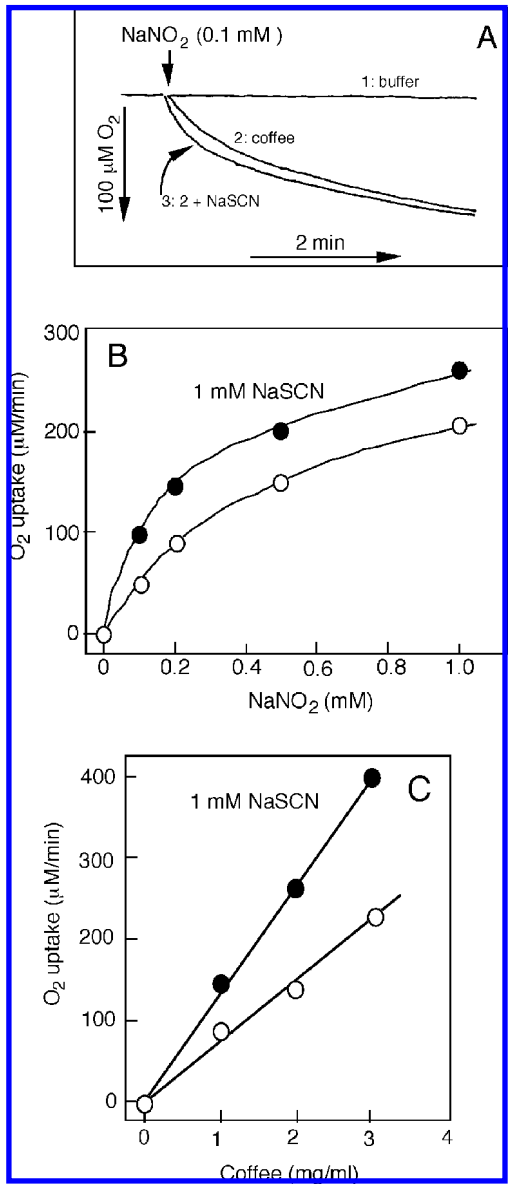

Figure 5. Effects of SCN^- on nitrite-induced oxygen uptake by coffee. (**A**) Time courses of oxygen uptake. Trace 1, 50 mM KCl-HCl (pH 2.0); trace 2, 1 mg of coffee/mL of 50 mM KCl-HCl (pH 2.0); and trace 3, trace 2 plus 1 mM NaSCN. Where indicated by a downward arrow, 0.1 mM $NaNO_2$ was added. (**B**) Effects of concentration of $NaNO_2$ on oxygen uptake. The reaction mixture (2 mL) contained 2 mg of coffee in 50 mM KCl-HCl (pH 2.0). No addition, ○; and 1 mM NaSCN, ●. (**C**) Effects of concentration of coffee on oxygen uptake. The reaction mixture (2 mL) contained 0.2 mM $NaNO_2$ and various amounts of coffee in 50 mM KCl-HCl (pH 2.0). No addition, ○; and 1 mM NaSCN, ●. Each data point represents the average of two experiments. In **Figure 4B**,**C**, the initial rate of oxygen uptake was plotted.

As a salivary component, SCN^- affected nitrite-induced oxygen uptake and NO formation as shown below; the effects of SCN^- on the formation of radical were studied, but any effects of SCN^- on the increase and decrease of the ESR signal were not observed.

Oxygen Uptake. Figure 5A shows time courses of nitrite-induced oxygen uptake in 50 mM KCl-HCl buffer (pH 2.0) under various conditions. When nitrite (0.1 mM) was added to the buffer solution, practically no oxygen uptake was observed (trace 1). Oxygen uptake (initial rate, 52 μM/min) was observed when nitrite was added to acidic coffee solution (1 mg/mL)

Table 1. Rates of Nitrite-Induced Oxygen Uptake and Amounts of Chlorogenic Acid and Its Isomers in Coffee and Its Various Fractions[a]

	coffee	fraction 1	fraction 2	fraction 2-A	fraction 2-B
	oxygen uptake (μM mL^{-1} of coffee or its fraction min^{-1})[b]				
0.2 mM $NaNO_2$	75	94	34	33	2
+ 1 mM NaSCN	116	105	53	58	2
	equivalent to chlorogenic acid (nmol mg^{-1} of coffee or its fraction)[c]				
chlorogenic acid and its isomers	64	113	8	4	not determined

[a] Data represent the averages of two experiments. [b] The reaction mixture for the measurement of oxygen uptake contained 0.2 mM $NaNO_2$ and 1 mg of coffee or its fraction in 1 mL of 50 mM KCl-HCl (pH 2.0). Initial rates of nitrite-induced oxygen uptake were presented as data. [c] Amounts of chlorogenic acid plus its isomers in coffee and fractions 1 and 2 were determined using their water suspension, and the amount in fraction 2-A was determined using ethyl acetate extracts as described in the text.

(trace 2), and the oxygen uptake was enhanced by SCN^- (102 μM/min) (trace 3). The enhancement was observed for several seconds after the addition of nitrite. The concentration of chlorogenic acid plus its isomers in 1 mg of coffee/mL has been estimated to be equivalent to 0.06−0.07 mM chlorogenic acid (*2, 13*). The rate of oxygen uptake in the presence of 0.1 mM chlorogenic acid and 0.2 mM $NaNO_2$ was about 5 μM/min, and SCN^- (1 mM) did not enhance the oxygen uptake. This result indicates that chlorogenic acid alone did not contribute to the oxygen uptake observed in coffee. Potassium ferricyanide (1 mM), which increased the ESR signal intensity, did not induce the oxygen uptake in coffee suspended in 50 mM KCl-HCl buffer (pH 2.0) (not shown), indicating that the oxygen uptake was not due to the shift of redox levels of redox components contained in coffee.

Figure 5B shows effects of the concentration of nitrite on oxygen uptake. As the concentration was increased, the rate of the oxygen uptake increased in the presence and absence of SCN^-. The difference in rates of oxygen uptake between the presence and the absence of SCN^- seemed to be constant, and the value was calculated to be 53 ± 9 μM O_2/mg of coffee/min (mean ± SD, $n = 4$). The rate of oxygen uptake as a function of concentration of coffee was nearly linear in the presence and absence of SCN^- (**Figure 5C**), and the difference in value between the presence and the absence of SCN^- was 52 ± 3 μM O_2/mg of coffee/min (mean ± SD, $n = 3$).

Nitrite-induced oxygen uptake was also studied in fractions 1, 2, 2-A, and 2-B (**Table 1**). The initial rate of the oxygen uptake in fraction 1 was slightly enhanced by 1 mM SCN^-, and the enhancement of the oxygen uptake in fraction 2 was larger than that in fraction 1. SCN^- also largely enhanced the oxygen uptake in fraction 2-A. No effects of SCN^- were observed in the oxygen uptake in fraction 2-B. **Table 1** also includes amounts of chlorogenic acid plus its isomers, and the data indicate that chlorogenic acid and its isomers did not significantly contribute to oxygen uptake in fractions 2 and 2-A.

Formation of NO. NO-$Fe(DTCS)_2$ was formed when $Fe(DTCS)_2$ was added to the mixture of nitrite and coffee (**A**) or fraction 2-A (**B**) (**Figure 6**, traces 1). Its formation was enhanced by SCN^- when $Fe(DTCS)_2$ was added after the incubation of coffee or fraction 2-A for 5 s (traces 2). No clear effects of SCN^- were observed in coffee and fraction 2-A when $Fe(DTCS)_2$ was added after the incubation for 1 min.

Components of Fraction 2-A. Fraction 2-A was suspended in methanol and centrifuged at 20000*g* for 5 min. In the supernatant, no chlorogenic acid and its isomers were detected. The ethyl acetate extract of fraction 2-A (5 mg/mL), which was

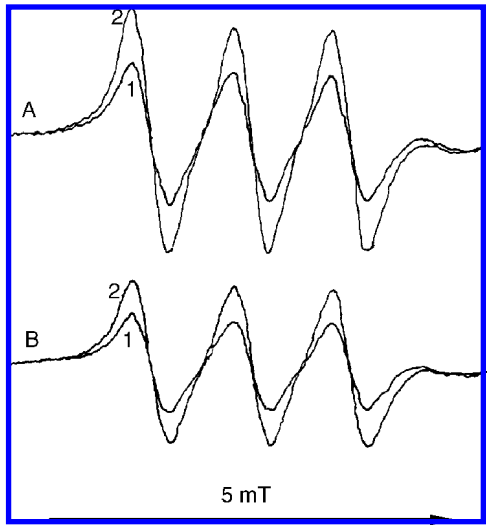

Figure 6. Effects of SCN^- on nitrite-induced production of NO. The reaction mixture contained 1 mg of coffee or fraction 2-A/mL in 50 mM KCl-HCl (pH 2.0). The reaction mixture (0.25 mL) was incubated for 5 s after the addition of 0.2 mM $NaNO_2$, and then, 0.25 mL of $Fe(DTSC)_2$ was added to measured the ESR signal of NO-$Fe(DTCS)_2$. (**A**) Coffee and (**B**) fraction 2-A. Trace 1, no addition; and trace 2, 1 mM NaSCN.

suspended in 1 mL of 50 mM KCl-HCl (pH 2.0), contained chlorogenic acid and its isomers equivalent to 3.8 nmol of chlorogenic acid per mg of fraction 2-A (average of two experiments). No caffeic acid was detected in the extract. The result indicates that chlorogenic acid and its isomers, which could not be extracted with methanol, were contained in fraction 2-A. The ethyl acetate extract of fraction 2-A, which had been hydrolyzed in 1 M HCl, contained chlorogenic acid and its isomers equivalent to 4.8 nmol of chlorogenic acid per mg of fractions 2-A (average of two experiments). This extract also contained caffeic acid equivalent to 7.0 nmol/mg of fraction 2-A (average of two experiments). Chlorogenic acid and caffeic acid were identified by comparing their retention times (9.5 and 15.4 min for chlorogenic acid and caffeic acid, respectively) and absorption spectra with the standard compounds. The isomers of chlorogenic acid were identified by their retention times and absorption spectra (*2*). The amount of chlorogenic acid plus its isomers was also measured using ethyl acetate extract of acidic suspension of fraction 2-A-I (pH 2). The value was 1.2 nmol/mg (average of two experiments). As fraction 2-A-I was contaminated less with chlorogenic acid plus its isomers than fraction 2-A, fraction 2-A-1 was used for spectrophotometric studies.

Spectrophotometric Studies of Fraction 2-A-I. Figure 7A shows absorption spectra of coffee and fraction 2-A-I at pH 2.0. The large difference between the spectra might be attributed to the difference in content of chlorogenic acid and its isomers.

Figure 7B shows difference spectra of coffee in the absence of SCN^-. By the addition of nitrite, absorbance at 255 and 400 nm increased and absorbance at 290 and 325 nm decreased. The absorbance decrease may be due to the oxidation of chlorogenic acid and its isomers, and the absorbance increase may be due to the nitration and the formation of their quinones as reported previously (*2*). SCN^- inhibited the absorbance increase around 400 nm without significantly affecting the absorbance increase at 255 nm (**Figure 7C**). The SCN^--dependent inhibition of the absorption increase at 400 nm may be due to the inhibition of nitration and quinone formation (*2*). The absorption at about 310 nm decreased at the initial period

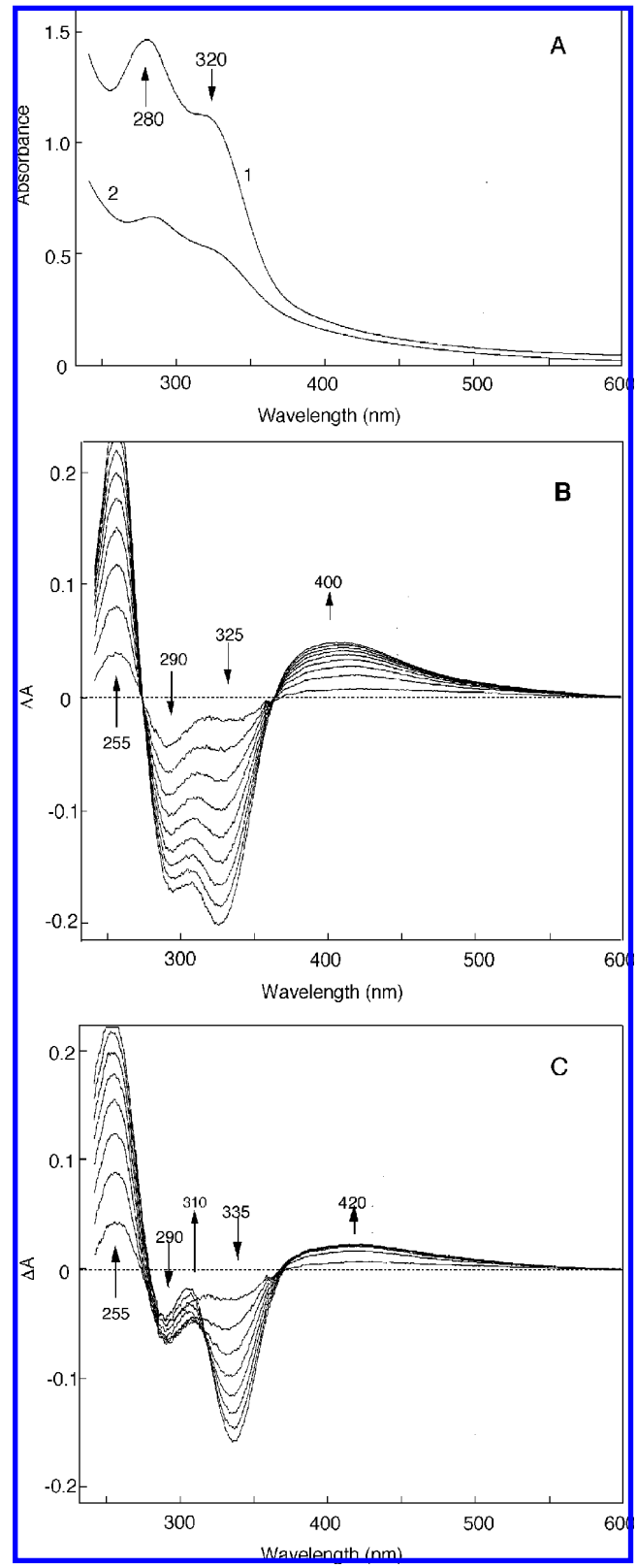

Figure 7. Absorption spectra and nitrite-induced changes in absorption spectra of coffee. (**A**) Absorption spectra. Trace 1, coffee (0.5 mg/mL, pH 2.0); and trace 2, fraction 2-A-I (0.5 mg/mL, pH 2.0). (**B** and **C**) Difference spectra of coffee: (**B**) 0.2 mM $NaNO_2$; and (**C**) 0.2 mM $NaNO_2$ + 1 mM NaSCN. Scanning was repeated every 1.33 min from 600 to 240 nm.

of incubation and then increased after the addition of nitrite in the presence of SCN^- (**Figure 7C**). This absorbance increase

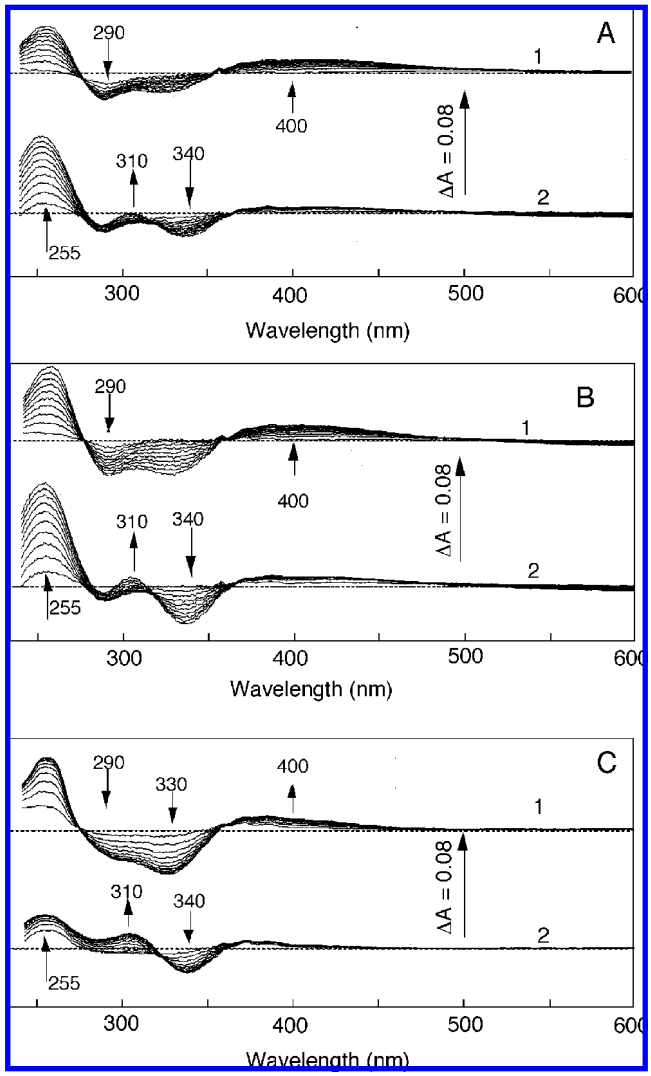

Figure 8. Nitrite-induced changes in absorption spectra of fraction 2-A-I and chlorogenic acid. (**A**) Difference spectra of fraction 2-A-I (0.5 mg/mL, pH 2.0); (**B**) difference spectra of fraction 2-A-I + 8 μM chlorogenic acid (pH 2.0); and (**C**) 8 μM chlorogenic acid (pH 2.0). Traces in 1, 0.2 mM $NaNO_2$; and traces in 2, 0.2 mM $NaNO_2$ + 1 mM NaSCN. Scanning was repeated every 1.33 min from 600 to 240 nm.

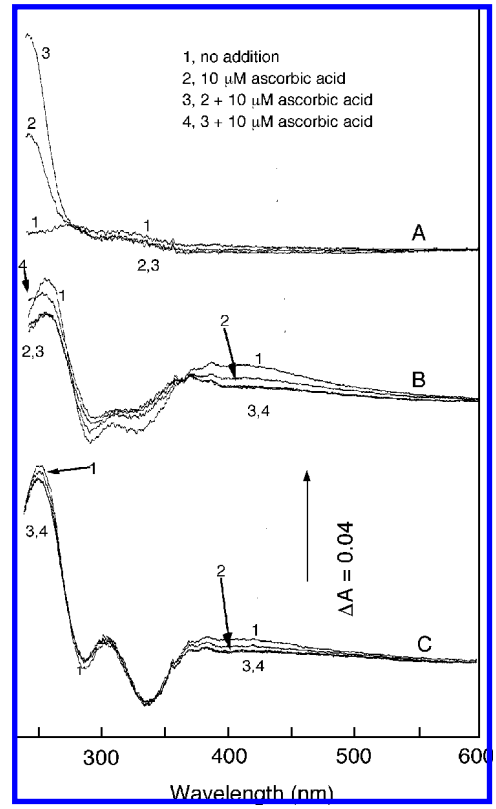

Figure 9. Ascorbic acid-dependent reduction of oxidation products of fraction 2-A-I. (**A**) Untreated fraction 2-A-I (0.5 mg/mL, pH 2.0). Trace 1, difference spectrum immediately after the recording of a reference spectrum; and traces 2 and 3, difference spectrum after the addition of 10 μM ascorbic acid successively. (**B**) Fraction 2-A-1 (0.5 mg/mL, pH 2.0) that was treated with 0.2 mM $NaNO_2$ for 15 min. Trace 1, difference spectrum before and after the treatment; and traces 2−4, difference spectra after the addition of 10 μM ascorbic acid successively. (**C**) Fraction 2-A-1 (0.5 mg/mL, pH 2.0) that was treated with 0.2 mM $NaNO_2$ and 1 mM NaSCN for 15 min. Trace 1, difference spectrum before and after the treatment; and traces 2−4, difference spectra after the addition of 10 μM ascorbic acid successively.

may be attributed to the formation of 2-thiocyanatechlorogenic acid and its isomers (*2*). It has been reported that 2-thiocyanatechlorogenic acid has absorption peaks at 240 and 311 nm in methanol and 25 mM KH_2PO_4 (pH 3) (1:2, v/v) (*2*).

Figure 8 shows difference spectra of fraction 2-A-I that contained chlorogenic acid and its isomers equivalent to 0.6 μM chlorogenic acid. In the presence of 0.2 mM $NaNO_2$ but not SCN^-, absorbance increased at 255 and 400 nm and decreased in the wavelength range from 280 to 350 nm (A-1). When chlorogenic acid (8 μM) was added to fraction 2-A-I, not only absorbance decrease in a wavelength range from 280 to 350 nm but also absorption increases at 255 and 400 nm were enhanced (B-1). The difference spectra of 8 μM chlorogenic acid at pH 2 (C-1) showed absorption increases at about 255 and 400 nm as A-1 and B-1, and the difference spectra in the wavelength range from 280 to 350 nm (C-1) did not show a clear peak of absorbance decrease at 290 nm as A-1 and B-1. The failure of observation of the peak at 290 nm in C-1 indicates that the absorption decrease at 290 nm in fraction 2-A-I (A-1) was due to oxidation of components other than chlorogenic acid and its isomers. The initial rate of the absorbance decrease around 330 nm in B-1 was slower than that in C-1, indicating that fraction 2-A-I inhibited the absorbance decrease around 330 nm due to oxidation of chlorogenic acid.

SCN^- significantly affected the difference spectra in fraction 2-A-I enhancing and inhibiting absorption increases at 255 and 400 nm, respectively (A-2). Another peak of absorption increase was observed at about 310 nm. The absorption increase at 255 nm during nitrite-dependent oxidation of chlorogenic acid (C-2) was smaller than that in fraction 2-A-I (A-2). The result indicates that the absorbance increase at 255 nm in fraction 2-A-I in the presence of SCN^- (A-2) was due to the oxidation of components other than chlorogenic acid and its isomers. The nitrite-induced absorbance changes in the presence of SCN^- accompanied the decrease in concentration of SCN^-. The amount decreased during initial 20 min was 16.8 ± 1.2 μM (n = 4) and that during 20 and 40 min was 11.6 ± 3.3 μM (n = 4). No detectable decrease in concentration of SCN^- was observed in the reaction mixture that contained 0.28 mM nitrite and 0.1 mM NaSCN but not fraction 2-A-I in a buffer solution at pH 2.

Figure 9 shows effects of ascorbic acid on the absorption spectra of fraction 2-A-I. In untreated fraction 2-A-I, no clear absorbance changes were observed on the addition of ascorbic acid except for the absorption increase at 240 nm by ascorbic

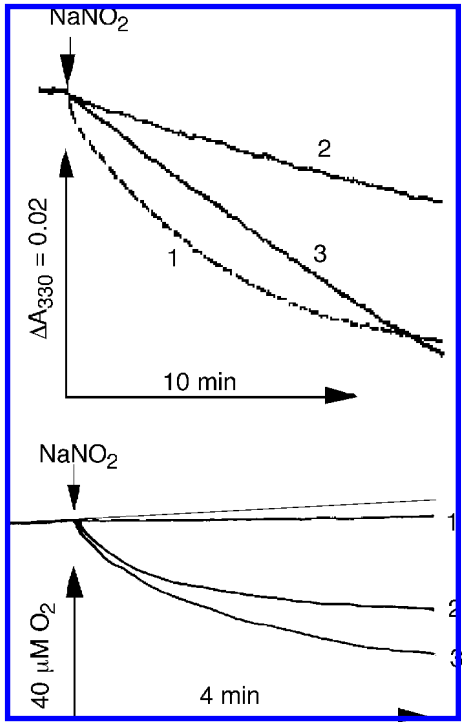

Figure 10. Interaction between chlorogenic acid and fraction 2-A-I. Upper traces, absorbance change. The reaction mixture (1 mL) contained 0.5 mg of fraction 2-A-I and/or 10 μM chlorogenic acid and 0.2 mM $NaNO_2$ in 50 mM KCl-HCl (pH 2). Lower traces, oxygen uptake. The reaction mixture (2 mL) contained 2 mg of fraction 2-A-I and/or 10 μM chlorogenic acid and 0.2 mM $NaNO_2$ in 50 mM KCl-HCl (pH 2). Trace 1, chlorogenic acid; trace 2, fraction 2-A-I; and trace 3, chlorogenic acid and fraction 2-A-I.

acid itself (**A**). This result coincides with the effects of ascorbic acid on the ESR signal of fraction 2-A. When ascorbic acid was added to fraction 2-A-I that had been treated for 15 min with nitrite, absorption decreases (around 255 and 400 nm) and increases (between 280 and 360 nm) were observed (**B**). Smaller absorbance changes were observed when ascorbic acid was added to fraction 2-A-I that had been treated for 15 min with nitrite plus NaSCN (**C**). The result suggests that ascorbic acid could reduce the oxidation products formed in fraction 2-A-I. No clear absorption increase due to ascorbic acid itself was observed at 240 nm in **B** and **C**. This might be due to the oxidation of ascorbic acid by not only the oxidation products but also nitrous acid.

Interaction between Chlorogenic Acid and Fraction 2-A-I. Figure 10 (upper traces) shows nitrite-induced absorption decreases of chlorogenic acid and fraction 2-A-I. The absorption decrease in the presence of chlorogenic acid slowed down (trace 1), whereas the absorbance decrease in the presence of fraction 2-A-I was nearly linear during the incubation (trace 2). In the mixture of chlorogenic acid and fraction 2-A-I, the two components seemed to affect each other in the absorbance decrease at 330 nm (trace 3). **Figure 10** (lower traces) shows nitrite-induced oxygen uptake. Slow oxygen uptake was observed in the presence of 10 μM chlorogenic acid (trace 1), and the rate of the oxygen uptake in the presence of fraction 2-A-I was decelerated rapidly (trace 2). Chlorogenic acid (10 μM) enhanced the oxygen uptake of fraction 2-A-I and prevented the deceleration, and the degree of the enhancement seemed to be larger than the oxygen uptake in trace 1 (trace 3). The result in **Figure 10** suggests that there were interactions between chlorogenic acid and melanoidsins during the reduction of nitrous acid.

DISCUSSION

The radical concentration of coffee was increased by ferricyanide and decreased by ascorbic acid and sodium borohydride (**Figure 2**). This result indicates that quinone/hydroquinone complexes (quinhydrone structure) participate in the formation of the stable radical. The increase in ESR signal intensity as the increase in pH supports the presence of quinhydrone structure in coffee (*14–16*). The quinhydrone structure may be produced by the oxidation of chlorogenic acid and its related compounds during the roasting of coffee beans. The reports that coffee melanoidins contain phenolic groups (*17, 18*) and have stable radicals (*6, 7*) support the presence of quinhydrone structure in melanoidins. The presence of caffeic acid moieties in fraction 2-A further supports the presence of quinhydrone structure in coffee melanoidins.

The radical intensity of coffee increased by nitrite. This result suggests that nitrite oxidized *o*-diphenol groups derived from caffeic acid moieties or its equivalents, increasing the concentration of quinhydrone structure in melanoidins. The oxidation of *o*-diphenol groups may accompany the formation of NO:

$$2HNO_2 + XH_2 \rightarrow 2NO + X + 2H_2O \quad (1)$$

where XH_2 is a diphenol group and X is a quinone form of the diphenol group. A nitrite-induced increase in the ESR signal was dependent on the concentration of nitrite when the concentration of nitrite was low but nearly constant when the concentration of nitrite was high (**Figure 3**). This result suggests that a limited number of *o*-diphenol groups might contribute to the increase in the formation of quinhydrone structure. Although chlorogenic acid can reduce nitrous acid to NO (*2, 3*), the radical species detected here seems not to be chlorogenic acid radicals because phenoxyl radicals in solutions are, in general, unstable under neutral and acidic conditions and have hyperfine structures (*3, 19, 20*). We tried to detect radicals of chlorogenic acid using reagent chlorogenic acid, but no ESR signals could be detected when 1 mM chlorogenic acid was mixed with 0.2 mM $NaNO_2$ at pH 2.

The intensity of the ESR signal decreased slowly after the treatment with nitrite, suggesting that reductants, which did not contribute to the formation of quinhydrone structure, reduced quinone moieties in quinhydrone structures. ESR signals of fractions 2 and 2-A were also increased by nitrite, and the increased signal intensity decreased during incubation (**Figure 4**). Such increases and decreases were also observed in brown components prepared from solid chlorogenic acid by heating. The results can also be explained by the oxidation of *o*-diphenol groups and the reduction of oxidized *o*-hydroxyl group by unknown reductants in melanoidins. No ESR signal was observed in fraction 1 in the absence of nitrite (**Figure 4**), suggesting that the signals in fractions 2 and 2-A were derived from methanol-insoluble melanoidins with high molecular masses.

Oxygen uptake was observed by the addition of nitrite to coffee at pH 2. The uptake can be explained by the autoxidation of NO produced by reaction 1 (*21–23*):

$$2NO + O_2 \rightarrow 2NO_2 \ (k = 2.8 - 11.6 \times 10^6\ M^{-2}\ s^{-1}) \quad (2)$$

It has been reported that rates of oxygen uptake in NO-generating systems depend on the formation of NO if the oxygen uptake is mainly due to the autoxidation of NO (*22, 23*). The

increase in the rate of the oxygen uptake became smaller as a function of the concentration of nitrite (**Figure 5B**). This result may be explained by the saturation of the rate of the formation of NO as discussed for a nitrite-dependent increase in the ESR signal in **Figure 3**. A linear increase in the oxygen uptake as a function of the concentration of coffee (**Figure 5C**) can be explained by the increase in the rate of the formation of NO. NO_2 produced by reaction 2 can be scavenged by chlorogenic acid, its isomers, and melanoidins (*8, 9, 24*).

It has been reported that SCN^- enhances the oxygen uptake induced by ascorbic acid/nitrous acid systems (*21*) but not phenolics/nitrous acid systems (*3, 4*). The SCN^--dependent enhancement has been explained by the ascorbic acid-dependent reduction of NOSCN (*21, 25*):

$$HNO_2 + SCN^- + H^+ \rightarrow NOSCN + H_2O \quad (3)$$

$$NOSCN + AH_2 \rightarrow NO + SCN^- + H^+ + AH^\bullet \quad (4)$$

where AH_2 and $AH^\bullet$ are ascorbic acid and monodehydroascrobic acid radical, respectively. SCN^- enhanced nitrite-induced oxygen uptake in coffee for several seconds after the addition of nitrite (**Figure 5**). The result indicates that the amount of reductants, which could reduce NOSCN, were limited, and the amount was calculated from the enhancement of oxygen uptake in **Figure 5**, postulating that the enhancement was observed for 5 s. The value was equivalent to 4.9 ± 1.2 nmol of O_2/mg of coffee/mL (mean $\pm$ SD, $n = 7$). The presence of reductants, which could reduce NOSCN, was supported by SCN^--dependent enhancement of the formation of NO in coffee/nitrite systems (**Figure 6**). The enhancement of oxygen uptake and NO formation by SCN^- was also observed in methanol-insoluble fractions (**Table 1** and **Figure 6**), suggesting that melanoidins with high molecular mass participated in the SCN^--dependent enhancement of NO formation. No clear effects of SCN^- were observed on the nitrite-induced changes in the intensity of ESR signal of quinhydrone structure. This result suggests that the component, which had been oxidized by NOSCN, did not contribute to the formation of stable radicals.

Products formed during nitrite-induced oxidation of coffee (**Figure 7**), chlorogenic acid and fraction 2-A-I (**Figure 8**), were compared. It became clear by the comparison that the absorption decrease at about 290 nm in fraction 2-A-I was mainly due to the oxidation of melanoidins. The increase in absorption around 400 nm of fraction 2-A-I, which contained 0.6 μM chlorogenic acid (**Figure 8**, A-1), was larger than that of 8 μM chlorogenic acid (**Figure 8**, C-1), indicating that the absorption increase around 400 nm in fraction 2-A-I was also due to the oxidation of melanoidins. The addition of ascorbic acid to fraction 2-A-I, which had been treated with nitrite, resulted in the decrease in absorbance around 400 nm and the increase in absorbance in the wavelength range from 280 to 360 nm (**Figure 9B**). This result suggests that *o*-quinone groups in melanoidins were reduced to *o*-diphenol groups by ascorbic acid. It has been reported that quinones and quinhydrone structures are reduced by reducing agents such as sodium borohydride decreasing the absorption in visible region (*14*).

SCN^- clearly affected the products formed during nitrite-induced oxidation of coffee (**Figure 7**), chlorogenic acid, and fraction 2-A-I (**Figure 8**). By comparing nitrite-induced absorbance changes between chlorogenic acid and fraction 2-A-I in the presence of SCN^-, it became clear that the absorbance increase at 255 nm in fraction 2-A-I (**Figure 8**, A-2) was due to the oxidation of melanoidins as the absorbance increase was much faster than that in chlorogenic acid (**Figure 8**, C-2). Although SCN^- inhibited nitrite-induced absorbance increase at 255 nm of chlorogenic acid, the reagent enhanced the absorbance increase in fraction 2-A-I (compare **A** and **C** in **Figure 8**). The result suggests that the inhibited and enhanced absorbance increase at 255 nm might be due to the conjugation of SCN^- to *o*-quinone of chlorogenic acid and *o*-quinone groups in melanoidins, respectively, which were formed during nitrite-induced oxidation of chlorogenic acid and fraction 2-A-I. This idea coincides with a previous report that SCN^- reacts with the quinone of chlorogenic acid producing 2-thiocyanatechlorogenic acid (*2*). The decrease in concentration of free SCN^- during the incubation of fraction 2-A-I with SCN^- in the presence of nitrite supports the formation of SCN^- conjugate in melanoidins. The decrease in concentration of SCN^- was not due to the reaction of SCN^- with chlorogenic acid and its isomers contained in fraction 2-A-I, as the concentration of SCN^- decreased (about 30 μM during 40 min of incubation) was larger than the concentration of the dihydroxycinnamic acids (equivalent to about 3.6 μM chlorogenic acid).

In the mixture of chlorogenic acid and melanoidin fraction, chlorogenic acid and melanoidins seemed to interact (**Figure 10**). A possible interaction is melanoidin-dependent reduction of oxidation intermediates of chlorogenic acid. This idea was deduced from the result that fraction 2-A-I inhibited the oxidation of chlorogenic acid and that chlorogenic acid enhanced the oxidation of fraction 2-A-I. On the other hand, the enhancement of nitrite-induced oxygen uptake of fraction 2-A-I by chlorogenic acid suggests that chlorogenic acid reduced melanoidins in fraction 2-A-I that could reduce nitrous acid. Further studies are required to elucidate the precise mechanism of the interaction between the monomer and the polymer.

In this study, it becomes clear that *o*-diphenol groups in melanoidins of high molecular mass could reduce nitrous acid producing NO under acidic conditions like the mixture of saliva and gastric juice. Accompanying the reaction, the quinhydrone structure seemed to be produced and quinones in the structure seemed to be reduced by reductants in melanoidins without generating stable radicals. The reaction between melanoidins and nitrous acid was enhanced by SCN^-, and the enhancement was observed as the increase in rates of NO formation. Furthermore, SCN^- seemed to be incorporated into melanoidins by reacting with the *o*-quinone groups that had been produced by nitrous acid-dependent oxidation of *o*-diphenol groups. The interaction between chlorogenic acid and melanoidins was observed, and the interaction seemed to be due to the reduction of oxidation intermediates of chlorogenic acid by melanoidins and vice versa. If the formation of NO in the stomach increases after coffee drinking, the NO may contribute to the inhibition of the growth of microorganisms suppressing respiratory electron transfer reactions (*26–28*). In addition, coffee melanoidins also have antimicrobial activity (*29–31*). The NO formed may also contribute to the regulation of mucosal blood flow, mucosal formation, and gastric mobility (*32–36*). On the other hand, the NO formed can react with O_2 in the stomach, producing NO_2 and N_2O_3. If toxic quinones and NO_2 are scavenged by SCN^- and antioxidants in coffee in the mixture of coffee, saliva, and gastric juice, concentrations of NO and O_2 can be increased and decreased, respectively, inhibiting the formation of N_2O_3 in the stomach after coffee drinking.

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