Free Radical Research, March 2010; 44(3): 293–303

informa
healthcare

Formation of nitric oxide, ethyl nitrite and an oxathiolone derivative of caffeic acid in a mixture of saliva and white wine

UMEO TAKAHAMA[1], MARIKO TANAKA[1] & SACHIKO HIROTA[2]

[1]*Department of Bioscience, Kyushu Dental College, Kitakyushu 803-8580, Japan, and* [2]*Department of Nutritional Science, Kyushu Women's University, Kitakyushu 807-8586, Japan*

(Received date: 27 June 2009; in revised form date: 5 November 2009)

Abstract

Reactions of salivary nitrite with components of wine were studied using an acidic mixture of saliva and wine. The formation of nitric oxide (NO) in the stomach after drinking wine was observed. The formation of NO was also observed in the mixture (pH 3.6) of saliva and wine, which was prepared by washing the oral cavity with wine. A part of the NO formation in the stomach and the oral cavity was due to the reduction of salivary nitrite by caffeic and ferulic acids present in wine. Ethyl nitrite produced by the reaction of salivary nitrite and ethyl alcohol in wine also contributed to the formation of NO. In addition to the above reactions, caffeic acid in wine could be transformed to the oxathiolone derivative, which might have pharmacological functions. The results obtained in this study may help in understanding the effects of drinking wine on human health.

Keywords: *Ethyl nitrite, nitric oxide (NO), nitrite, oxathiolone derivative, saliva, stomach, wine*

Abbreviations: *AU, arbitrary unit; DTCS, N-(carboxythiocarboxy) sarcosine sodium salt.*

Introduction

Wine has health-promoting properties and beneficial effects on certain diseases, including heart disease and cancer, which in turn are attributed to the antioxidative function of phenolics in wine [1,2]. The phenolics include a phenylacetic and hydroxycinnamic acids (Figure 1A) and flavonoids. Recently, it has been reported that the ingestion of red wine results in an increase in the concentration of nitric oxide (NO) in the stomach and this increase has been proposed to be due to the reduction of salivary nitrite by phenolics in wine [3] (Figure 1B). The possibility of the formation of NO in the stomach by the reduction of salivary nitrite by dietary phenolics has also been reported [4–7]. The phenolics comprise quercetin, rutin, caffeic acid and chlorogenic acid [3–7]. Nitrite required for NO formation is produced in the oral cavity by the reduction of nitrate, which is ingested as a component of foods [8] and secreted into the oral cavity as a component of saliva, by nitrate-reducing bacteria [9–12]. The nitrite produced in the oral cavity is transformed into nitrous acid (pKa = 3.3) in the stomach so that it can be reduced to NO by phenolics [3–7]. NO formed in the stomach can increase the gastric mucosal blood flow and the mucus thickness [13,14]. When alcoholic beverages are ingested, in addition to NO, ethyl nitrite, which can function as a vasodilator [15], may also be produced in the stomach by the reaction of ethyl alcohol with nitrous acid (Figure 1C). The production of ethyl nitrite has been reported in the acidic mixture of saliva and ethyl alcohol [15,16].

Thiocyanate is also a component of saliva and functions as a substrate of salivary peroxidase producing an antimicrobial component hypothiocyanate ion ($OSCN^-$) in the oral cavity [17]. In the stomach,

Correspondence: Umeo Takahama, Department of Bioscience, Kyushu Dental College, Kitakyushu 803-8580, Japan. Email: takahama@kyu-dent.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715760903486057

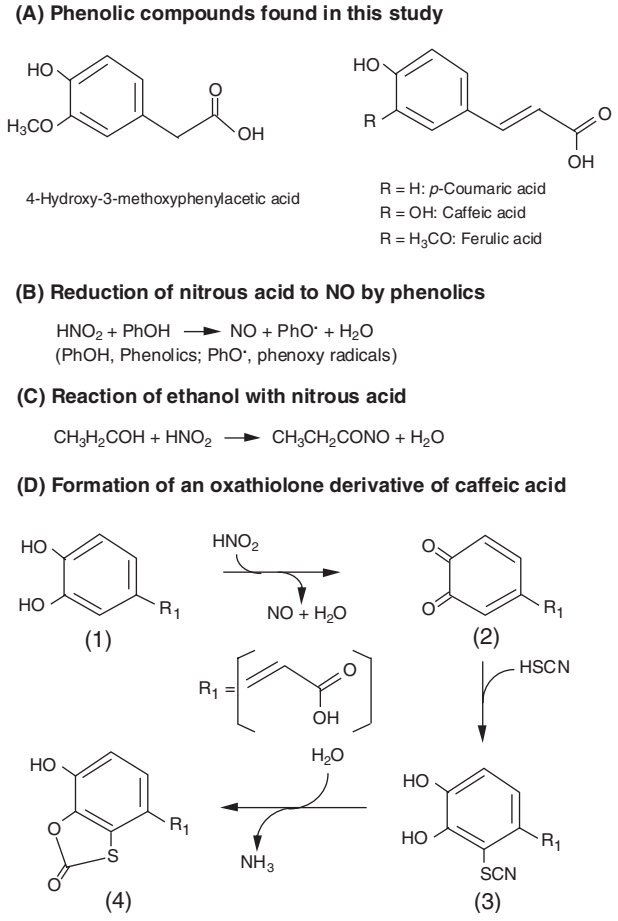

Figure 1. Chemical structures and reactions concerned in this study. (1), (2), (3) and (4) in (D) are caffeic acid, its *o*-quinone form, 2-thiocyanatecaffeic acid and oxathiolone derivative of caffeic acid, respectively.

SCN^- reacts with nitrous acid, producing nitrosyl thiocyanate (ONSCN) that can be reduced rapidly to NO by a component of gastric juice, ascorbic acid [18]. In addition, SCN^- can react with *o*-quinones, which are produced in the nitrous acid-dependent oxidation of *o*-dihydroxyphenolics such as chlorogenic acid and rutin, under acidic conditions [19,20]. The initial reaction products are 2-thiocyanate conjugates of chlorogenic acid and rutin that are transformed to the oxathiolone derivatives by hydrolysis [19,20]. Caffeic acid (Figure 1A), which has a catechol group, is present in wine [21–23]. This suggests that, in addition to NO, 2-thiocyanate conjugates of caffeic acid, which could transform to an oxathiolone derivative of caffeic acid, might be formed in the stomach after drinking wine (Figure 1D). The acidity of wine also led us postulate that NO, ethyl nitrite and an oxathiolone derivative of caffeic acid might be produced in the oral cavity after drinking wine.

The objectives of this study were to investigate the formation of ethyl nitrite and an oxathiolone derivative of caffeic acid in the stomach after the ingestion of wine, confirming the formation of NO in the stomach, and to investigate the formation of NO and ethyl nitrite in the mixture of saliva and wine in the oral cavity.

Materials and methods

Reagents and wine

N-(Dithiocarboxy)sarcosine sodium salt (DTCS) was obtained from Dojin (Kumamoto, Japan). 4-Hydroxy-3-methoxyphenylacetic acid, 3,4-dihydroxybenzaldehyde, *p*-coumaric acid, caffeic acid, ferulic acid, vanillin and ethyl nitrite were obtained from Wako Pure Chem. Ind. (Osaka, Japan). California white wine (table wine; pH ~3.3) was obtained from a local market. According to the information available from the label on the bottle, the alcohol concentration of this wine is 9.5% (v/v).

NO in expelled air

It has been reported that wine induces the production of NO in the stomach [3]. In this study, we, at first, performed experiments to confirm the above-mentioned result. Healthy volunteers (age range, 50–60 years) were fasted for 4 h after they had their lunch. During the fasting, they could consume only water whenever required. The voluntary regurgitation of air was collected in a gas-tight bag after an intake of 300 ml of carbonated water (Suntory Foods; Tokyo, Japan) under fasting conditions. Fifteen-to-thirty minutes after the collection of the expelled air, 150 ml of white wine was ingested. Carbonated water (300 ml) was given again 10–15 min after the ingestion of white wine to collect the air expelled from the stomach in the gas-tight bag. The protocol to collect the expelled air was designed according to the methods described in previous studies [3,24]. NO in the gas-tight bag was quantified using a nitrogen oxide detector tube (11L, Gastec; Ayase, Japan). The detector can detect (NO + NO_2) in the concentration range from 0.2–5 ppm.

Measurements of electron spin resonance spectra

Electron spin resonance (ESR) spectra were measured using an ESR spectrometer JE1XG (JOEL, Tokyo, Japan) at 25°C using a quartz flat cell (50 µl) under the following conditions [4,5]: microwave power, 10 mW; scanning speed, 5 mT/min; line width, 0.5 mT; and amplification, 250- or 1000-fold. NO produced in the mixture of nitrite and wine was trapped by $Fe(DTCS)_2$. The $Fe(DTCS)_2$ solution was prepared by adding 0.03 ml of 100 mM $FeCl_3$ to 1 ml of 10 mM DTCS that was dissolved in 0.1 M sodium phosphate (pH 7.6).

The formation of NO was studied in the following reaction mixtures (Figure 2). (A) White wine itself (pH 3.3; 0.25 ml) was incubated for 1 min after the

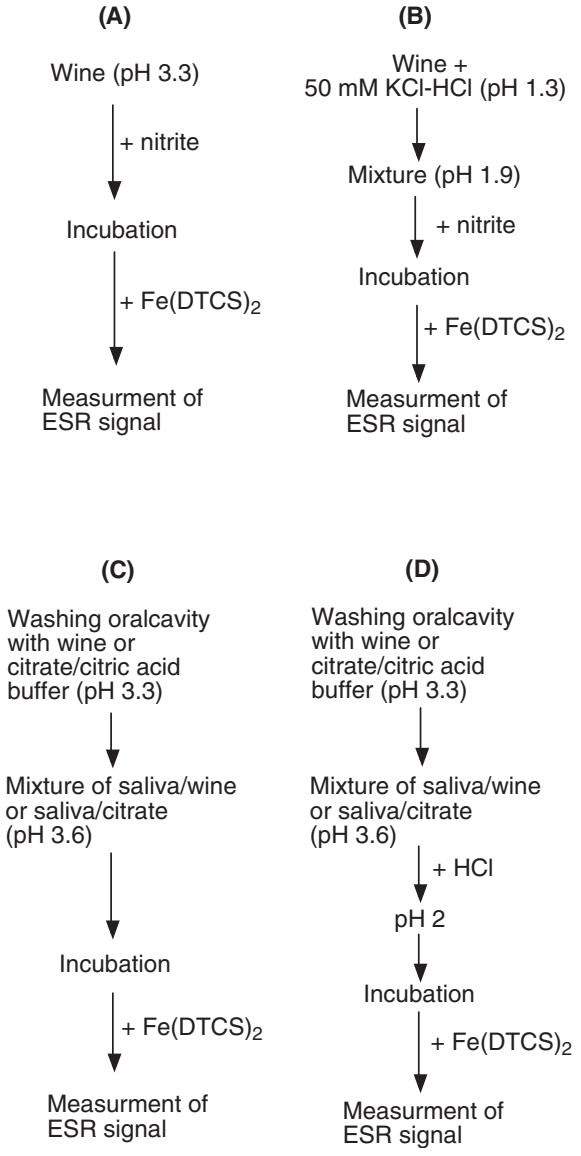

Figure 2. Procedures to prepare the mixtures for measurement of ESR spectra.

addition of various concentrations of $NaNO_2$ and then 0.25 ml of $Fe(DTCS)_2$ was added. After the addition of $Fe(DTCS)_2$, the pH was 6.6. (B) the white wine (0.125 ml) was mixed with 0.125 ml of 50 mM KCl-HCl buffer (pH 1.3). The pH of the mixture was 1.94. After the addition of $NaNO_2$, the mixture was incubated for 1 min and then $Fe(DTCS)_2$ (0.25 ml) was added. After the addition of $Fe(DTCS)_2$, the pH was 6.6. (C) The oral cavity was washed for 30 s with 5 ml of white wine and the wine was spat out into a beaker. The solution obtained by the washing should contain saliva and other components in the oral cavity. In the following sections, the spit obtained by the washing is referred to as the mixture of saliva/wine. The volume and the pH of the mixture of saliva/wine were ~5 ml and ~3.6, respectively. After keeping the mixture of saliva/wine for a further 30 s, 0.25 ml of $Fe(DTCS)_2$ was added to 0.25 ml of the mixture. The pH after the addition of the $Fe(DTCS)_2$ was 6.6. As a control, the oral cavity was washed for 30 s with 5 ml of 50 mM citric acid/sodium citrate buffer (pH 3.3). The volume of the mixture of saliva and citrate buffer (in the following, the mixture of saliva/citrate buffer) was ~5.6 ml and the pH was ~3.6. After keeping the mixture of saliva/citrate buffer for a further 30 s, 0.25 ml of $Fe(DTCS)_2$ was added to 0.25 ml of the mixture. After the addition of $Fe(DTCS)_2$, the pH was 6.3. (D) The oral cavity was washed with wine or citrate buffer as (C) and the pH of the mixtures of saliva/wine and saliva/citrate buffer was adjusted to 2 by adding a small volume of 1 M HCl simulating the mixture of saliva and gastric juice. The mixtures of pH 2 were incubated for 30 s and then $Fe(DTCS)_2$ (0.5 ml) was added to 0.25 ml of the each mixture of saliva/wine and saliva/citrate buffer (pH 2) to measure the ESR spectra. After the addition of $Fe(DTCS)_2$, the pH values were in the range of 6.6–6.8.

HPLC

Formation of ethyl nitrite and changes in the concentration of phenolics in wine were studied using a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm; particle size, 5 μm) combined with a spectrophotometric detector with a photodiode array (SPD M10A) (Shimadzu; Kyoto, Japan). The reaction mixture contained 1 mM $NaNO_2$ in wine (pH 3.3) or in the mixture of 0.5 ml of 50 mM KCl-HCl buffer (pH 1.3), the pH of which was 1.94. After incubation for 1 min, 10 μl of each reaction mixture was applied to the HPLC column described above. Ethyl nitrite was separated using a mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v) as the mobile phase and detected at 350 nm. Phenolics were separated using the mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v) as the mobile phase and detected at 280 nm. The flow rate of the mobile phases was 1 ml/min.

Products that were formed by the reaction of nitrite with caffeic acid were also studied using the above-mentioned HPLC system. The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v), and the flow rate was 1 ml/min. The reaction mixture contained 0.1 mM caffeic acid and 0.1 mM $NaNO_2$ in 50 mM KCl-HCl buffer (pH 2.0) or 0.1 mM caffeic acid and 0.2 mM $NaNO_2$ in 50 mM sodium citrate-HCl buffer (pH 3.3). After incubation for defined periods, an aliquot (20 μl) of each reaction mixture was applied to the HPLC column.

Preparation of an oxathiolone derivative of caffeic acid

The oxathiolone derivative of chlorogenic acid [(*E*)-5'-(3-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl) acryloyloxy)quinic acid] was prepared according to

the method reported previously [19]. One milligram of the compound was suspended in 2 ml of 2 M HCl and incubated for 30 min at ~92°C. After extraction of the acidic solution with ethyl acetate, the ethyl acetate fraction was analysed by HPLC using the ODS column described above. The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v). The results showed that the oxathiolone derivative of chlorogenic acid, which had a retention time of ~10.3 min and absorption peaks at 230 and 309 nm in the above mobile phase, was transformed to a new component. The new component had a retention time of 16.5 min and absorption peaks at 227 and 293 nm. The component was considered to be the oxathiolone derivative of caffeic acid because the quinic acid moiety of the oxathiolone derivative of chlorogenic acid would undergo hydrolysation during the incubation under acidic conditions, producing the oxathiolone derivative of caffeic acid.

Detection of 2-thiocyanatecaffeic acid

Mixed whole saliva was collected and filtered as described previously [20]. The mixture of whole saliva filtrate (0.5 ml), wine (0.5 ml) and 50 mM KCl-HCl buffer (pH 1.3, 1.0 ml) was prepared and the pH was determined to be 1.9. Caffeic acid (10 μM), sodium nitrite (0.1 mM) and NaSCN (0.5 mM) were added when required. After the incubation for 10 min at room temperature (~ 25°C), the mixture was extracted twice with 5 ml of ethyl acetate. The ethyl acetate extracts were combined and dehydrated with sodium sulphate. After the evaporation of ethyl acetate with a rotary evaporator, the residue was dissolved in 0.2 ml of the mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v). An aliquot (20 μl) of the solution was applied to the HPLC column described above to separate 2-thiocyanate caffeic acid. The mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v) was used as the mobile phase and the flow rate was 1 ml/min.

Results and discussion

Wine-induced increase in NO concentration in expelled air

The concentration of ($NO + NO_2$) in the air expelled from the stomach increased from 0.70 ± 0.51 ppm to 2.04 ± 1.48 ppm ($n=7$: $p=0.039$, paired t-test) by the ingestion of white wine. This result is consistent with a previous report that the ingestion of red wine increases the concentration of NO in the air expelled from the stomach [3]. In this study, NO might be mainly detected by the ($NO + NO_2$) detector tube because it is a primary reaction product of nitrite under the conditions of this study. The slow reaction of gaseous NO with gaseous O_2 may support that NO might be mainly detected by the nitrogen oxide detector tube used in this study; the half-life of 1 ppm of gaseous NO in the atmosphere was calculated to be ~56 h using the rate constant of 1.4×10^4 M^{-2} s^{-1} [25]. Even if NO_2 was produced by the reaction of NO with O_2, the concentration of NO_2 would be decreased by the reaction with NO leading to the formation of N_2O_3. The concentration of NO detected in the air expelled from the stomach was higher than 0.2 ppm and NO was not detected in the air exhaled by breathing in this study, suggesting that NO detected in this study was mainly from the stomach. It has been reported that the concentration of NO in the air exhaled by breathing is 4 ± 1 ppb and the air expelled from the stomach ranges from 0.6–2.6 ppm [24], supporting the idea that NO detected in this study was from the stomach.

Formation of NO in the mixture of saliva/wine

Figure 3 shows typical data for the formation of NO in the mixtures of saliva/citrate buffer and saliva/wine prepared by washing the oral cavity with citrate buffer and wine, respectively, as shown in Figure 2C. No clear ESR signal of NO-$Fe(DTCS)_2$ was detected when $Fe(DTCS)_2$ was added to the mixture of saliva/citrate

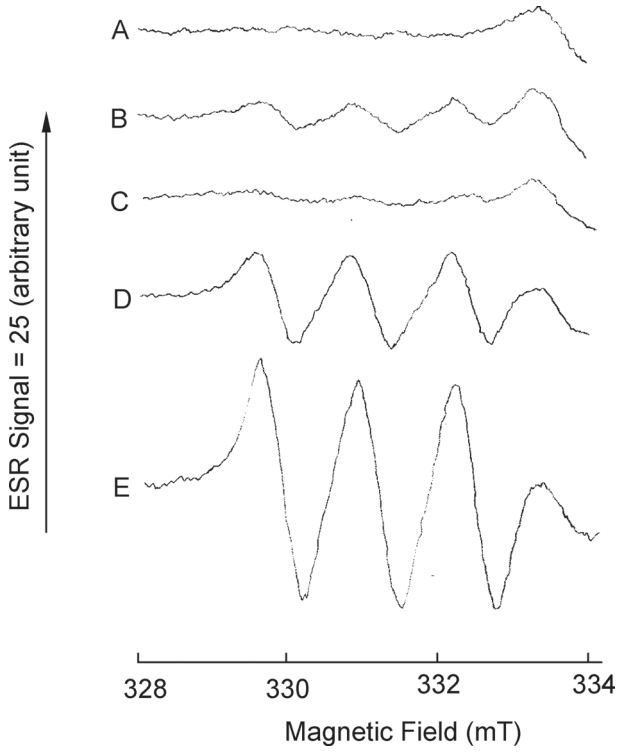

Figure 3. Concentration of NO in spit prepared after washing the oral cavity with acidic buffer or wine. (A) Washing with citrate/citric acid buffer; (B) washing with wine; (C) after washing with citrate/citric acid buffer, the pH of the spit was decreased to 2; (D) after washing with wine, the pH of the spit was decreased to 2; (E) as in (D) except for the addition of 0.1 mM $NaNO_2$ just after the decrease in pH. ESR spectra were started recording 2 min after the addition of $Fe(DTCS)_2$. Amplification, 1000-fold.

buffer (trace A), whereas the signal was detected when $Fe(DTCS)_2$ was added to the mixture of saliva/wine (trace B). The signal at 333.3 mT was from $Fe(DTCS)_2$ itself. When the pH of the mixture of saliva/citrate buffer was decreased to 2, as in Figure 2D, a very weak signal of NO-$Fe(DTCS)_2$ was detected by the addition of $Fe(DTCS)_2$ (trace C), whereas when the pH of the mixture of saliva/wine was decreased to 2, an intense signal was detected by the addition of $Fe(DTCS)_2$ (trace D). The signal intensity of NO-$Fe(DTCS)_2$ at pH 2 was stronger than that at pH 3.6, indicating that the formation of NO was faster at pH 2 than at 3.6. The addition of 0.1 mM nitrite to the mixture of saliva/wine (pH 2) resulted in an enhanced formation of NO-$Fe(DTCS)_2$ (trace E). The concentration of nitrite in the mixture of saliva/wine was estimated to be 0.06 mM by comparing the signal intensity before and after the addition of 0.1 mM nitrite (Figure 3, traces D and E). The result in Figure 3 indicates that NO was produced if wine was mixed with saliva in the oral cavity and that the production of NO could continue after swallowing the mixture into the stomach. Furthermore, the above-mentioned result suggests that salivary nitrite contributed to the formation of NO.

The intensity of ESR signal of NO-$Fe(DTCS)_2$ just after the addition of $Fe(DTCS)_2$ to the reaction mixture would reflect the concentration of NO in the mixture, and the signal intensity of NO-$Fe(DTCS)_2$ would be kept constant if NO production ceased after the addition of $Fe(DTCS)_2$. However, a slow increase in the intensity of the ESR signal was observed in trace B in Figure 3 after the addition of $Fe(DTCS)_2$, and the rate was ~0.1 arbitrary unit per min (AU/min). Such a slow increase in the intensity of the signal was also observed in trace E in Figure 3 and the rate was ~0.6 AU/min. The slow increase in the ESR signal will be discussed later.

Nitrite-induced formation of NO in wine

Salivary nitrite seemed to contribute to the NO formation in the mixture of saliva/wine. Next, we studied the effects of nitrite on the formation of NO in wine itself. No NO-$Fe(DTCS)_2$ was detected in the mixture of wine and $Fe(DTCS)_2$, the pH of which was 6.6. After mixing $Fe(DTCS)_2$ with wine, 1 mM nitrite was added and then the ESR spectra were measured. The ESR signal of NO-$Fe(DTCS)_2$ was not detected immediately after the addition of nitrite, but the signal intensity increased slowly during the incubation at the rate of 0.2 AU/min. There were three possibilities for the slow increase in the ESR signal intensity of NO-$Fe(DTCS)_2$: (i) self-decomposition of nitrite producing NO, (ii) the reduction of nitrite to NO by the reductants in wine, and (iii) the decomposition of ethyl nitrite that was produced by the reaction of nitrite with ethyl alcohol in wine. Possibility (iii) can be excluded for the slow increase because the reaction between nitrite and ethyl alcohol producing ethyl nitrite does not proceed when the pH is higher than 4 [15].

Nitrite (0.2 or 1 mM) was added to 25 mM citrate/citric acid buffer (pH 3.3). After incubation for 1 min, $Fe(DTCS)_2$ was added. A small ESR signal of NO-$Fe(DTCS)_2$ was observed but the subsequent increase in the signal of NO-$Fe(DTCS)_2$ was not observed (Figure 4, upper panel, closed circles). When nitrite was added to 50 mM KCl-HCl buffer (pH 2.0) followed by $Fe(DTCS)_2$, a weak ESR signal of NO-$Fe(DTCS)_2$ was observed and the intensity of the signal of NO-$Fe(DTCS)_2$ did not increase subsequently (Figure 4, lower panel, closed circles).

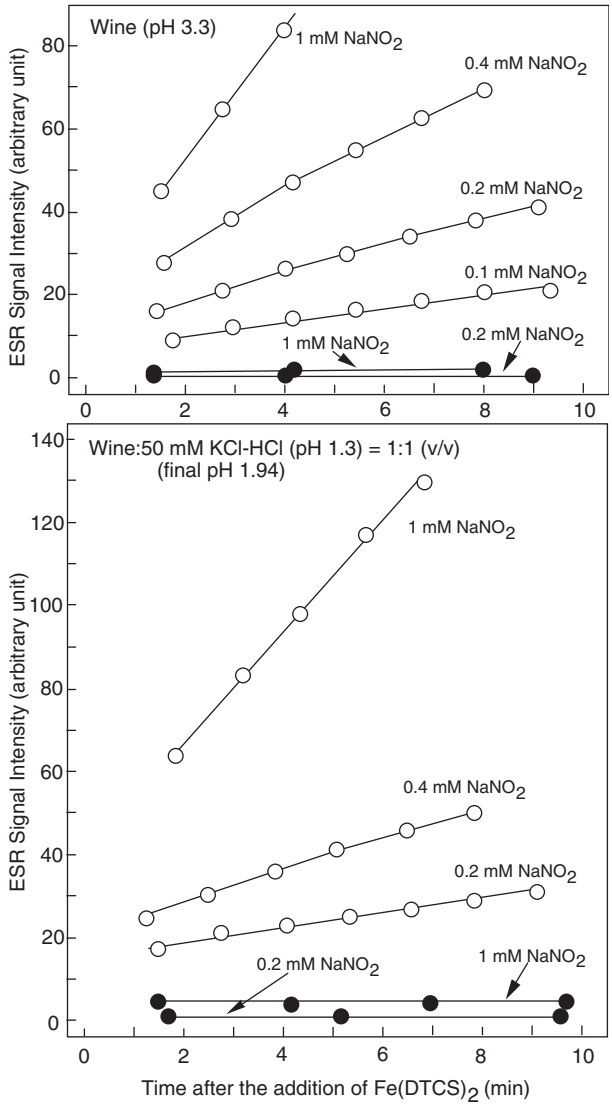

Figure 4. Time courses of nitrite-induced formation of NO-$Fe(DTCS)_2$ in wine. Upper panel. ●, 25 mM citrate/citric acid buffer (pH 3.4); ○, wine itself (pH 3.3). Lower panel. ●, 50 mM KCl-HCl buffer (pH 2.0); ○, mixture of wine and 50 mM KCl-HCl buffer (pH 1.3) (1:1, v/v). Concentrations of nitrite shown in the figure are the concentrations before the addition of $Fe(DTCS)_2$. Amplification, 250-fold.

The results indicate that the conversion of nitrite to NO by self-decomposition could proceed at pH values of 2 and 3.3 and that the self-decomposition producing NO did not proceed after the addition of $Fe(DTCS)_2$, excluding possibility (i) and remaining possibility (ii) for the slow formation of $NO\text{-}Fe(DTCS)_2$ that was observed by the addition of 1 mM nitrite to the mixture of $Fe(DTCS)_2$ and wine, the pH of which was pH 6.6.

Wine was incubated for 1 min after the addition of nitrite, and then mixed with $Fe(DTCS)_2$, as shown in Figure 2A. The mixing of the wine with $Fe(DTCS)_2$ resulted in the detection of the ESR signal of $NO\text{-}Fe(DTCS)_2$ (Figure 4, upper panel, open circles), indicating the production of NO in the nitrite-containing wine before the addition of $Fe(DTCS)_2$. Time courses of the increase in the ESR signal intensity after the mixing of $Fe(DTCS)_2$ with the nitrite-containing wine showed slow increases in the intensity of the ESR signal of $NO\text{-}Fe(DTCS)_2$ following the initial rapid increase. The initial intensity in the ESR signal of $NO\text{-}Fe(DTCS)_2$, which could be estimated by the extrapolation of the slow increase to time zero and would reflect the concentration of NO just before the addition of $Fe(DTCS)_2$, was dependent on the concentration of nitrite added. The rate of the slow increase was also dependent on the concentration of nitrite added, and the rates were 1.1, 3.2, 6.7, and 14.2 AU/min in the presence of 0.1, 0.2, 0.4, and 1.0 mM nitrite, respectively. When nitrite was added to the mixture of wine and 50 mM KCl-HCl buffer (pH 1.3), followed by the addition of $Fe(DTCS)_2$ as in Figure 2B, the intensity of the ESR signal of $NO\text{-}Fe(DTCS)_2$ increased, as shown in the lower panel of Figure 4. The result was essentially the same as the result shown in the upper panel. The initial signal intensity of $NO\text{-}Fe(DTCS)_2$ was dependent on the concentration of nitrite added and rates of the slow increase were 1.7, 3.7 and 13.5 AU/min in the presence of 0.2, 0.4 and 1.0 mM nitrite, respectively.

The signal of $NO\text{-}Fe(DTCS)_2$ detected just after the mixing of $Fe(DTCS)_2$ with the nitrite-containing wine may reflect the concentration of NO in the reaction mixture as described above, but the slow increase in Figure 4 remains to be elucidated. The rate of the slow increase of $NO\text{-}Fe(DTCS)_2$, which was observed after the mixing of $Fe(DTCS)_2$ with wine that was supplemented with 1 mM nitrite, was ~14 AU/min (Figure 4), whereas the rate of the formation of $NO\text{-}Fe(DTCS)_2$ after the addition of 1 mM nitrite to the mixture of $Fe(DTCS)_2$ and wine, which might proceed by possibility (ii), was 0.2 AU/min (see above). Since ethyl nitrite could be produced under the former experimental conditions but not under the latter experimental conditions and ethyl nitrite could decompose producing NO [15,16], the slow increase in the intensity of the ESR signal of $NO\text{-}Fe(DTCS)_2$ in Figure 4 could be explained by possibility (iii), namely, the decomposition of ethyl nitrite.

Taking the above-mentioned discussion into consideration, the slow formation of $NO\text{-}Fe(DTCS)_2$ after the addition of $Fe(DTCS)_2$ to the acidic mixture of saliva/wine (see description related to Figure 3) can be explained as below. The concentrations of nitrite in the mixtures of saliva/wine were estimated to be 0.06 and 0.16 mM in traces B and E, respectively (Figure 3). If the slow formation of $NO\text{-}Fe(DTCS)_2$ proceeded by possibility (ii), namely, by the reduction of nitrite to NO by the reductants in wine after the addition of $Fe(DTCS)_2$ to the mixture of saliva/wine, the rates of the slow formation of $NO\text{-}Fe(DTCS)_2$ in traces B and E can be calculated to be 0.012 and 0.032 AU/min, respectively, using the rate of $NO\text{-}Fe(DTCS)_2$ formation in the presence of 1 mM nitrite (0.2 AU/min, see above). The calculated rates were much smaller than the rates of the slow formation of $NO\text{-}Fe(DTCS)_2$ observed by the addition of $Fe(DTCS)_2$ to the mixture of saliva/wine (0.1 and 0.63 AU/min in traces B and E, respectively). The result suggests that the slow formation of $NO\text{-}Fe(DTCS)_2$ observed after the addition of $Fe(DTCS)_2$ to the mixture of saliva/wine might also be explained by possibility (iii) (production of NO by the decomposition of ethyl nitrite) but not by possibility (ii) (reduction of nitrite to NO by the reductants in wine). Since ethyl nitrite seemed to contribute to the slow formation of $NO\text{-}Fe(DTCS)_2$, we studied the nitrite-induced formation of ethyl nitrite using wine.

Nitrite-induced formation of ethyl nitrite and oxidation of hydroxycinnamic acids in wine

Figure 5 shows the nitrite-induced formation of ethyl nitrite in the mixture of wine and 50 mM KCl-HCl buffer (pH 1.3). During the incubation for 1 min, a new peak was observed at a retention time of ~8.2 min (compare Figures 5A and B). When wine itself was incubated with 1 mM nitrite for 1 min the new peak was also observed (data not shown). The retention time and the absorption spectrum (Figure 5C) were identical to those of standard ethyl nitrite, supporting the nitrite-induced formation of ethyl nitrite in wine as in the acidic mixture of ethyl alcohol and nitrite [15,16]. The concentration of ethyl nitrite in wine decreased slowly with a half-life of ~7 min. Since it has been reported that ethyl alcohol can react with nitrite producing ethyl nitrite when the pH is lower than 4 [15], the above-mentioned result suggests that, after the ingestion of wine, ethyl alcohol in wine can be transformed to ethyl nitrite in the oral cavity and in the stomach. The detection of ethyl nitrite in the blood after the administration of ethyl alcohol has been reported [26]. If ethyl nitrite is produced in the

oral cavity and in the stomach after drinking wine, it might also be detectable in the blood.

The formation of ethyl nitrite during the incubation for 1 min accompanied the disappearance of peak X (compare Figures 5A and B) and the absorption spectrum of peak X suggested that peak X might be constituted of hydroxycinnamic acids. The concentration of the hydroxycinnamic acid-like components was also decreased by the addition of 1 mM nitrite to wine (pH 3.3), although the decrease in the concentration in wine (half-life, ~7 min) was slower than that in the mixture of wine and 50 mM KCl-HCl (pH 1.3). One of the reasons for the difference in the rate between the pH values might be the difference in the concentration of nitrous acid that could contribute to oxidation of hydroxycinnamic acids.

Hydroxycinnamic acids in wine were separated by HPLC using the mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v) as the mobile phase. Six peaks were separated when the mixture of wine and 50 mM KCl-HCl (pH 1.3) was analysed (Figure 6).

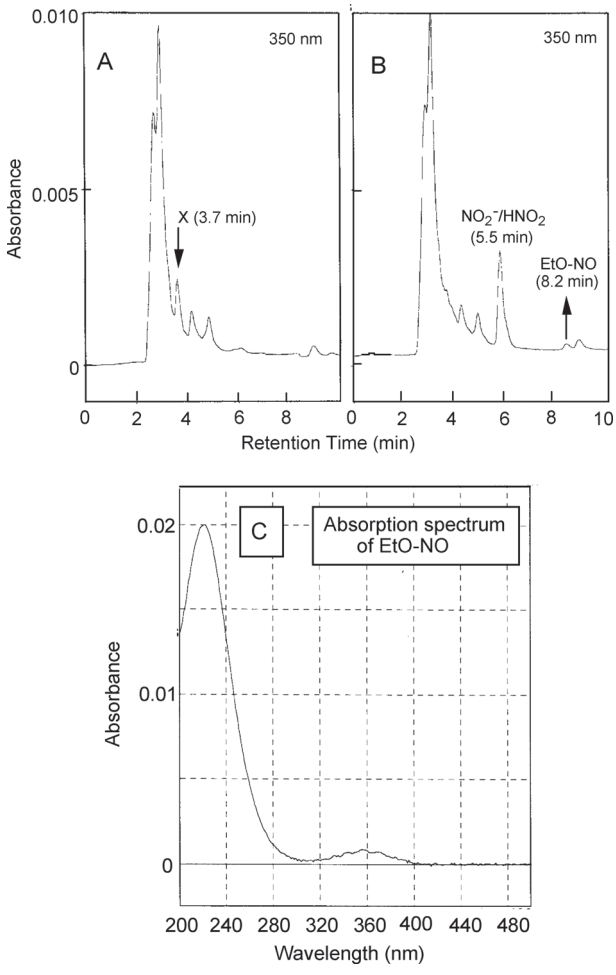

Figure 5. Nitrite-induced formation of ethyl nitrite in wine. Wine (1 ml) was mixed with 1 ml of 50 mM KCl-HCl buffer (pH 1.3) and then nitrite was added. (A) without nitrite; (B) 1 min after the addition of 1 mM $NaNO_2$; (C) absorption spectrum of ethyl nitrite (EtO-NO in (B)). X in (A), a component disappeared by nitrite.

The components of peaks 2, 5 and 6 were identified to be caffeic, *p*-coumaric and ferulic acids (Figure 1A), respectively, by comparing the retention times and absorption spectra with their standard compounds, and their concentrations were estimated to be 10, 4.5 and 1.5 μM, respectively. The presence of these hydroxycinnamic acids in wine has been reported [20–23]. It is clear from Figure 6 that 1 mM nitrite decreased the concentration of caffeic (peak 2) and ferulic acid (peak 6) and the concentrations of caffeic and ferulic acids after 1-min incubation were less than 10% of their initial concentrations. The component of peak 1, the concentration of which was not decreased by nitrite, was identified to be 4-hydroxy-3-methoxyphenylacetic acid (Figure 1A) by comparing the retention time and absorption spectrum with the standard compound. The components of peaks 3 and 4 could not be identified, although nitrite decreased the concentration of the component of peak 4. When 1 mM nitrite was added to the wine itself, the concentration of caffeic and ferulic acids decreased slowly, and their concentrations after 20-min incubation were ~10% of their initial concentrations (data not shown).

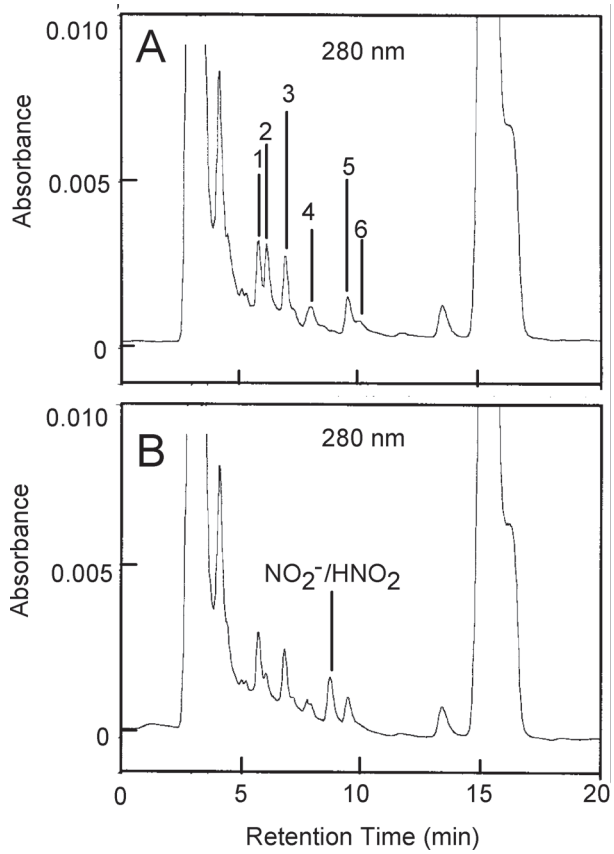

Figure 6. Nitrite-induced decrease in the concentrations of components in wine. Wine (1 ml) was mixed with 1 ml of 50 mM KCl-HCl buffer (pH 1.3). (A) Before the addition of nitrite; (B) 1 min after the addition of 1 mM nitrite.

The nitrite-induced formation of NO accompanying the decrease in concentration of the hydroxycinnamic acids suggests that NO can be formed by the hydroxycinnamic acid-dependent reduction of nitrous acid when wine is mixed with saliva and when the pH of the mixture is decreased to ~2 in the stomach. In addition to the hydroxycinnamic acids, catechins and flavonols [22,23] might also contribute to the reduction of nitrous acid after the ingestion of wine. In this study, nitrite-induced nitration and nitrosation of the phenolics in wine were not investigated.

Reactions of nitrous acid with caffeic acid in the presence of SCN⁻

It has been reported that caffeic acid reacts with nitrite under acidic conditions and some of the products have been identified [27]. In this study, it was shown that nitrite could react with caffeic acid in wine. Since a salivary component SCN^- can affect the reactions between nitrous acid and *o*-diphenolics [17,18], the effects of SCN^- on the reaction between nitrous acid and caffeic acid were investigated. When caffeic acid was incubated with nitrite at pH 2 in the absence of SCN^-, peak p-1 was detected (Figure 7, upper panel). The component of peak p-1 was identified to be 3,4-dihydroxybenzaldehyde by comparing the retention time and absorption spectrum with the standard compound, and the concentration increased as a function of incubation time. The formation of 3,4-dihydroxybenzaldehyde in an acidic mixture of nitrite and caffeic acid [27] and also in the mixture of iron and a wine-like medium [28] has been reported.

When caffeic acid was incubated with nitrite at pH 2 in the presence of SCN^-, in addition to peak p-1, p-2 and p-3 were detected and their retention times were ~11.5 and 16 min, respectively (Figure 7, upper panel). The absorption spectra of peak p-2 (maxima, 240 and 296 nm) and peak p-3 (maxima, 227 and 293 nm) (Figure 7, middle panel) resembled 2-thiocyanatechlorogenic acid (maxima, 240 and 311 nm) and the oxathiolone derivative of chlorogenic acid (maxima, 230 and 311 nm) [19], respectively. The concentration of the component of peak p-2 decreased accompanying the increase in the concentration of the component of peak p-3 (Figure 7, lower panel). Such kinetics has been observed for the formation of the oxathiolone derivative of chlorogenic acid from 2-thiocyanatechlorogenic acid [19]. The retention time and absorption spectrum of the component of peak p-3 were identical to those of the component obtained by acid hydrolysis of the oxathiolone derivative of chlorogenic acid, namely, (*E*)-5'-(3-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl)acryloyloxy) quinic acid. Therefore, we identified that the component of peak p-3 was the oxathiolone derivative of

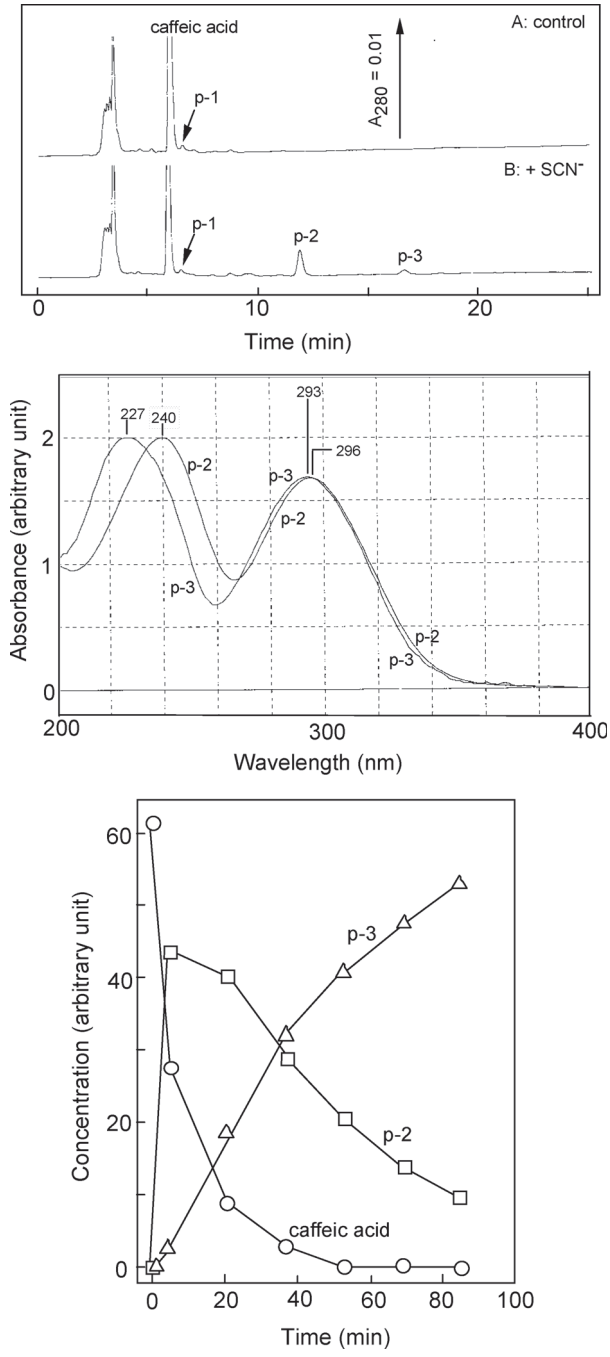

Figure 7. Effects of SCN^- on nitrite-induced transformation of caffeic acid. The reaction mixture (1 ml) contained 0.1 mM caffeic acid and 0.1 mM $NaNO_2$ in 50 mM KCl-HCl buffer (pH 2.0). Upper panel: HPLC after incubation for 10 min. (A) without SCN^-; (B) 1 mM NaSCN. Middle panel: Absorption spectra. p-2, spectrum of peak p-2; p-3, spectrum of peak p-3. Lower panel: Change in the concentration of components as a function of incubation time. ○, caffeic acid; □, component of peak p-2; △, component of peak p-3.

caffeic acid [(7-hydroxy-2-oxo-benz[1,3]oxathiol-4yl) acrylic acid], which might be formed by hydrolysis of 2-thiocyanatecaffeic acid of peak p-2. The formation of the components corresponding to peaks p-2 and p-3 could not be detected when 0.1 mM caffeic acid was incubated with 0.2 mM nitrite plus 1 mM NaSCN

in 50 mM citrate-HCl buffer (pH 3.3). One of the reasons for the failure of the detection at pH 3.3 may be the much lower concentration of HSCN (pKa ≈ 0.8) that can react with quinones producing a thiocyanate conjugate (Figure 1D).

Ferulic acid was readily oxidized and nitrosylated by nitrite under acidic conditions and the products including vanillin have been identified [29,30]. In this study, the production of vanillin was confirmed in the acidic mixture of ferulic acid and nitrite, but SCN^- had no significant effects on the formation of the reaction products.

Formation of 2-thiocyanatecaffeic acid in the mixture of saliva and wine

The components corresponding to peaks p-2 and p-3 were produced in the mixture of caffeic acid, nitrite and SCN^- in the buffer solution at pH 2. Then the formation of 2-thiocyanatecaffeic acid (p-2) was examined in the mixture of saliva/wine/acidic buffer at pH 1.94 (Figure 8). The mixture was incubated for 10 min and extracted with ethyl acetate, but the component corresponding to peak p-2 in could not be detected in the extract. The addition of 10 μM caffeic acid to the mixture seemed to result in the formation of the component (an arrow in Figure 8A), which was confirmed by the addition of 10 μM caffeic acid together with 0.1 mM nitrite and 0.5 mM NaSCN to the mixture (an arrow in Figure 8B). The retention time (11.5 min) and absorption spectrum (240 and 296 nm) of the component were identical to those of peak p-2 in Figure 7. If 2-thiocyanatecaffeic acid was formed in the mixture of saliva/wine at pH 2, the component would also be formed in the stomach after drinking wine and its formation would be enhanced by increases in the concentrations of caffeic acid, nitrite and SCN^-. The concentration of caffeic acid in wine can increase to ~60 μM depending on the grapes used to produce wine [21–23] and the salivary concentrations of nitrite and SCN^- can increase to ~1 and ~2 mM, respectively [8,31,32]. Although 2-thiocyanatecaffeic acid was detected in the mixture of saliva/wine/acidic buffer, the oxathiolone derivative of caffeic acid could not be detected. The detection failure was due to the presence of a component in wine, which absorbed UV light strongly and had a retention time similar to that of the oxathiolone derivative (Figures 7 and 8).

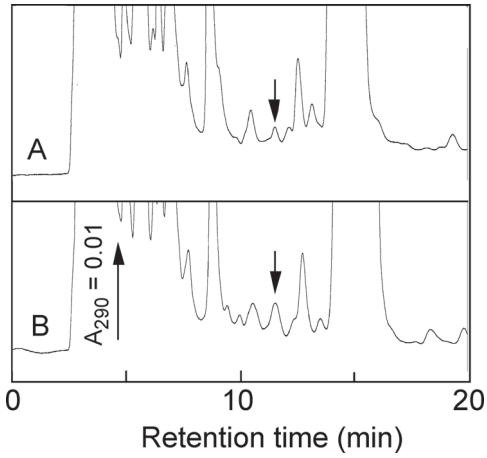

Figure 8. Formation of a thiocyanate conjugate of caffeic acid in mixture of saliva and wine. The reaction mixture (2 ml) contained 0.5 ml of saliva, 0.5 ml of wine and 1 ml of 50 mM KCl-HCl buffer (pH 1.3). (A) 10 μM caffeic acid; (B) A plus 0.1 mM $NaNO_2$ and 0.5 mM NaSCN. Downward arrows, thiocyanate conjugate of caffeic acid.

Thiocyanate conjugates, which transform to components with an oxathiolone moiety, are formed in the acidic mixtures of saliva and coffee [19] and saliva and dough prepared from buckwheat flour [20]. In this study, caffeic acid in wine was suggested to be transformed to the oxathiolone derivative of caffeic acid in the stomach via 2-thiocyanatecaffeic acid. The compounds with an oxathiolone moiety have been reported to have some pharmacological functions such as anti-fungal, bacteriostatic and cytostatic activities [33,34]. In the compounds, 6-hydroxy-1, 3-benzoxathiol-2-one is used for the treatment of acne [31] and some compounds with an oxathiolone moiety have been indicated to be able to inhibit inhibitory kappaB kinase beta [35] and carbonic anhydrase [36]. In addition, compounds with an oxathiolone moiety are also reported to have alkyl radical scavenging activity [37].

Concluding remarks

In this study, it was shown that the mixture of saliva/wine could produce NO under pH conditions of 2 and 3.6, indicating that the ingestion of wine resulted in the formation of NO in the oral cavity as well as in the stomach. In addition to NO, ethyl nitrite seemed to be produced in the oral cavity as well as in the stomach. Two pathways are possible for the formation of NO after the ingestion of wine. One is the reduction of nitrite by phenolics that are contained in wine and the other is the decomposition of ethyl nitrite producing NO and ethyl radicals. It has been reported that NO formed in the stomach can increase the activities of the stomach [13,14,38]. Therefore, the results obtained in this study indicate that the use of wine as an aperitif can be beneficial. The increased formation of NO after drinking wine may also result in the inhibition of the growth of microorganisms [39–41] and the increase in mucosal blood flow and mucus thickness [13,14] in the stomach. These actions of NO may aid in preventing stomach ulcers and gastritis [42,43]. Ethyl nitrite formed in the stomach may be transported from the stomach to the whole body and may function as a vasodilator [15]. Oxathiolone

derivatives are produced in the stomach but not in the oral cavity after the ingestion of phenolics and, at present, their functions have not been fully elucidated.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Teissedre PL, Landrault T. Wine phenolics: contribution to dietary intake and bioavailability. Food Res Int 2000;33: 461–467.

[2] Serafini M, Laranjinha JA, Almeida LM, Maiani G. Inhibition of human LDL lipid peroxidation by phenol-rich beverages and their impact on plasma total antioxidant capacity in humans. J Nutr Biochem 2000;11:585–590.

[3] Gago B, Lundberg JO, Barbosa RM, Laranjinha J. Red wine-dependent reduction of nitrite to nitric oxide in the stomach. Free Radic Biol Med 2007;43:1233–1242.

[4] Takahama U, Oniki T, Hirota S. Oxidation of quercetin by salivary components. Quercetin-dependent reduction of salivary nitrite under acidic conditions producing nitric oxide. J Agric Food Chem 2002;50:4317–4322.

[5] Takahama U, Yamamoto A, Hirota S, Oniki T. Quercetin-dependent reduction of nitrite to nitric oxide under acidic conditions and interaction between quercetin and ascorbic acid during the reduction. J Agric Food Chem 2003;51: 6014–6020.

[6] Takahama U, Tanaka M, Hirota S. Interaction between ascorbic acid and chlorogenic acid during the formation of nitric oxide in acidified saliva. J Agric Food Chem 2008;56: 10406–10413.

[7] Peri L, Pietraforte D, Scorza G, Napolitano A, Fogliano V, Minetti M. Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a new biological function for polyphenols with a catechol group? Free Radic Biol Med 2005;3:668–681.

[8] Pannala AS, Mani AR, Spencer JPE, Skinner Y, Bruckdorfer KR, Moore KP, Rice-Evans CA. The effect of dietary nitrate on salivary, plasma and urinary nitrate metabolism in humans. Free Radic Biol Med 2003;34:576–584.

[9] Palmerini CA, Palombari R, Perito S, Arienti G. NO synthesis in human saliva. Free Radic Res 2003;37:29–31.

[10] Zetterquist W, Pedroletti C, Lundberg JO, Alving K. Salivary contribution to exhaled nitric oxide. Eur Respir J 1999;13: 327–333.

[11] Doel JJ, Hector MP, Amirtham CV, Al-Anzan LA, Benjamin N, Allaker RP. Protective effect of salivary nitrite and microbial nitrate reductase activity against caries. Eur J Oral Sci 2004;112:424–428.

[12] Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. Eur J Oral Sci 2005;113:14–19.

[13] Björne H, Petersson J, Phillipson M, Weitzberg E, Holm L, Lundberg JO. Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. J Clinic Invest 2004;113: 106–114.

[14] Whittle BJ, Lopez-Belmonte J, Moncada S. Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. Br J Pharmacol 1990;99:607–611.

[15] Gago B, Nyström T, Cavaleiro C, Rocha BS, Barbosa RM, Laranjinha J, Lundberg JO. The potent vasodilator ethyl nitrite is formed upon reaction of nitrite and ethanol under gastric conditions. Free Radic Biol Med 2008;45:404–412.

[16] Takahama U, Tanaka M, Hirota S. Transformation of ethyl alcohol to ethyl nitrite in acidified saliva: possibility of its occurrence in the stomach. Arch Biochem Biophys 2008; 475:135–139.

[17] Tenovuo J. Nonimmunoglobulin defense factors in human saliva. In: Tenovuo JO, editor. Human saliva: Clinical chemistry and microbilology. Vol II. Boca Raton, FL: CRC Press; 1989. p. 55–91.

[18] Licht WR, Tannenbaum SR, Deen WM. Use of ascorbic aicd to inhibit nitrosation: kinetic and mass transfer consideration for an *in vitro* system. Carcinogenesis 1988;9:365–372.

[19] Takahama U, Tanaka M, Oniki T, Hirota S, Yamauchi R. Formation of thiocyanate conjugate of chlorogenic acid in coffee under acidic conditions in the presence of thiocyanate and nitrite: possible occurrence in the stomach. J Agric Food Chem 2007;55:4169–4176.

[20] Takahama U, Tanaka M, Hirota S, Yamauchi R. Formation of an oxathiolone compound from rutin in acidic mixture of saliva and buckwheat dough: possibility of its occurrence in the stomach. Food Chem 2009;116:214–219.

[21] Caccetta RA-A, Croft KD, Beilin LJ, Puddey IB. Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect *ex vivo* lipoprotein oxidizability. Am J Clin Nutr 2000;71:67–74.

[22] Soleas GJ, Dam J, Carey M, Goldberg DM. Toward the fingerprinting of wines: cultivar-related patterns of polyphenolic constituents in Ontario wines. J Agric Food Chem 1997;45:3871–3880.

[23] Kallithraka S, Salacha MI, Tzourou I. Changes in phenolic composition and antioxidant activity of white wine during bottle storage: accelerated browning test versus bottle storage. Food Chem 2008;113:500–505.

[24] Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurement in expelled air. Gut 1994;35:1543–1546.

[25] Hisatsne IC, Zafonte L. Kinetic study of some third-order reactions of nitric oxide. J Phys Chem 1969;73:2980–2989.

[26] Deng XB, Bludeau P, Deitrich RA. Formation of ethyl nitrite *in vivo* after ethanol administration. Alcohol 2004;34: 217–223.

[27] Napolitano A, d'Ischia M. New insights into the acid-promoted reaction of caffeic acid and its esters with nitrite: decarboxylation drives chain nitrosation pathways toward novel oxime derivatives and oxidation/fragmentation products thereof. J Org Chem 2002;67:803–810.

[28] Lutter M, Clark AC, Prenzler PD, Scollary GR. Oxidation of caffeic acid in a wine-like medium: production of dihydrobenzaldehyde and its subsequent reactions with (+)-catechin. Food Chem 2007;105:968–975.

[29] Rousseau B, Rosazza JPN. Reaction of ferulic acid with nitrite: formation of 7-hydroxy-6-methoxy-1,2-(4*H*)-benzoxazin-4-one. J Agric Food Chem 1998;46:3314–3317.

[30] Kikugawa K, Hakamada T, Hasunuma M, Kurechi T. Reaction of *p*-hydroxycinnamic acid derivatives with nitrite and its relevance to nitrosoamin formation. J Agric Food Chem 1983;31:780–785.

[31] Galanti LM. Specificity of salivary thiocyanate as marker of cigarette smoking is not affected by alimentary sources. Clin Chem 1997;43:184–185.

[32] Tsuge K, Kataoka M, Seto Y. Cyanide and thiocyanaide levels in blood and saliva of healthy adult volunteers. J Health Environ 2000;46:343–350.

[33] Lius V, Sennerfeldt P. Local treatment of acne with thioxolone. Lakartidningen 1979;76:39–41.

[34] Konieczny MT, Konieczny W, Sabisz M, Skladanowski A, Wakiec R, Augustynowicz-Kopec E, Zwolska Z. Synthesis of isomeric, oxathiolone fused to chalcones, and comparison of

their activity toward various microorganisms and human cancer cells line. Chem Pharm Bull 2007;55:817–820.
[35] Kim BH, Roh E, Lee HY, Lee IJ, Ahn B, Jung SH, Lee H, Han SB, Kim Y. Benzoxathiole derivative blocks lipopolysaccharide-induced nuclear factor-kappaB activation and nuclear factor-kappaB-regulated gene transcription through inactivating inhibitory kappaB kinase beta. Mol Pharmacol 2008;73: 1309–1318.
[36] Innocenti A, Maresca A, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors; thioxolone versus sulfonamides for obtaining isozyme-selective inhibitors? Bioorg Med Chem Lett 2008;18:3938–3941.
[37] Povalishev VN, Polozov GI, Shadyro OI. Effects of α-tocopherol and related compounds on reactions involving various organic radicals. Bioorg Med Chem Lett 2006;16:1236–1239.
[38] Desai KM, Sessa WC, Vane JR. Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food and fluid. Nature 1991;351:477–479.

This paper was first published online on Early Online on 25 January 2010.

[39] Benjamin N, O'Driscoll F, Dougall H, Duncan C, McKenzie H. Stomach NO sysnthesis. Nature 1994;368:502.
[40] Yu H, Sato EF, Nagata K, Nishikawa M, Kashiba M, Arakawa T, Kobayashi K, Tamura T, Inoue M. Oxygen-dependent regulation of the respiration and growth of *Escherichia coli* by nitric oxide. FEBS Lett 1997;409:161–165.
[41] Xu J, Xu X, Verstraete W. The bactericidal effect and chemical reactions of acidified nitrite under conditions simulating the stomach. J Appl Microbiol 2001;90:523–529.
[42] Larauche M, Anton PM, Gracia-Villar R, Theodorou V, Frexinos J, Bueno L, Fioramonti J. Protective effect of dietary nitrate on experimental gastritis in rates. Br J Nutr 2003; 89:777–786.
[43] Jansson EA, Petersson J, Reinders C, Sobko T, Bjorne H, Phillipson M, Weitberg E, Holm L, Lundberg JO. Protection from nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers by dietary nitrate. Free Radic Biol Med 2007;42:510–518.