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Artifactual production of cyanide from coffee and tea drinks in the presence of nitrite

Faux positifs en cyanure dans le thé et le café en présence de nitrites

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Abstract – Introduction: In cases of unknown poisoning, forensic toxicologists are required to elucidate the cause of death, and one of the most noticeable poisons is cyanide. We report on the artifactual production of cyanide from coffee and tea drinks in the presence of nitrite, which is frequently adulterated to lead to the fault result in drug testing. **Methods:** Cyanide was measured by head-space gas chromatography using porous polymer capillary GS-Q column and a nitrogen-phosphorus detector. Samples were mixed with sodium nitrite in a glass vial under acidic conditions of 10% phosphoric acid or 0.6 M sodium phosphate buffer (pH 5.0). After incubation for 30 min at 50 °C, the resulting head-space was injected into the gas chromatograph. Hydrogen cyanide was eluted at 2.2 min, and the peak area was converted for measuring cyanide concentration in the liquid reaction mixture. From the 50 °C incubation mixture of commercial coffee drink (or tea drink) and nitrite (50 mM), several µg/mL and sub-µg/mL of cyanide was produced in the presence of 10% phosphoric acid and pH 5.0 buffer, respectively. Cyanide was also produced from the reaction mixture of nitrite and polyphenol compounds such as chlorogenic acid and catechins, which are included in coffee and tea drinks, but not from a monophenol mixture. The amount of cyanide produced was dependent on substrate concentrations of nitrite and polyphenol, pH, temperature and incubation time. **Conclusion:** Forensic toxicologists should pay attention to this type of artifactual production of cyanide.

Key words: Cyanide, false positive detection, polyphenol, nitrite

Résumé – Introduction : Dans les cas d'empoisonnement mystérieux, il est demandé aux médecins légistes de déterminer la cause du décès, et notamment la présence de cyanure. Nous avons examiné la production faussement positive de cyanure à partir de thé et de café en présence de nitrites, fréquemment employés pour fausser les tests de détection. **Méthodes :** La teneur en cyanure a été établie par chromatographie gazeuse d'espace de tête avec une colonne capillaire GS-Q sur polymère poreux et un détecteur azote-phosphore. Les échantillons ont été mélangés à du nitrite de sodium dans une fiole en verre, en acidifiant par 10 % d'acide phosphorique ou par un tampon de phosphate de sodium 0,6 M (pH 5,0). Après incubation à 50 °C pendant 30 min, l'espace de tête résultant était injecté dans le chromatographe. Le cyanure d'hydrogène est élué en 2,2 min, et l'espace de tête a été traité pour mesurer la concentration en cyanure dans le mélange liquide siège de la réaction. Dans le mélange café (ou thé) vendu dans le commerce et nitrite (50 mM) ayant incubé à 50 °C, moins de 1 µg/mL et plusieurs µg/mL de cyanure ont été produits en présence, respectivement, de 10 % d'acide phosphorique et du tampon à pH 5,0. Le cyanure a aussi pu être produit par réaction au mélange avec des nitrites ou des polyphénols tels que l'acide chlorogénique et les catéchines, qui sont contenus dans le thé et le café, mais pas à partir de monophénols. Les quantités de cyanure produites étaient proportionnelles aux concentrations en substrats nitrite et poliphénols, au pH, à la température et à la durée d'incubation. **Conclusion :** Les médecins légistes doivent avoir conscience de ce type de production faussement positive de cyanure.

Mots clés : Cyanure, faux positifs, polyphénol, nitrites

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1 Introduction

Forensic toxicologists rapidly and appropriately detect poisons and measure their concentrations in forensic samples in poisoning cases [1, 2]. Poisons can show post-mortem alteration such as artifactual production and degradation, and can be detected falsely. We elucidated the mechanisms of post-mortem alteration, particularly for volatile toxic substances. Post-mortem alteration can be divided at least into three parts [3]. Phase I occurs between death and sampling in casualties; or between adulteration and sampling in beverages, as exemplified for acrylonitrile poisoning [4], and blood carbon monoxide [5] and cyanide [6] in fire cases. Phase II occurs between sampling and analysis during the transportation and storage of samples, as exemplified for hydrogen sulfide and the metabolite thiosulfate in blood and urine [7] and lachrymators in water samples [8]. Phase III occurs during analysis, as exemplified for spontaneous production of cyanide from thiocyanate during oxyhemoglobin denaturation [9, 10]. Cyanide is a volatile poison; it should be determined preferentially in poisoning cases, but false detection has been observed [11, 12]. We reported that cyanide was artifactually produced from coffee drinks when isobutyl nitrite was adulterated into the coffee drink [13]. In this contribution, we report that cyanide is artifactually produced from coffee and tea drinks in the presence of nitrite under acidic conditions.

2 Materials and methods

2.1 Chemicals

Chemicals were of analytical grade. Potassium cyanide, sodium nitrite, chlorogenic acid hemihydrates, caffeic acid, tannic acid, pyrocatechol, *o*-methoxyphenol, (R)-(-)-epinephrine, phenol and chloramine T (sodium *N*-chloro-*p*-toluenesulfonamide) were purchased from Wako Pure Chemicals (Osaka, Japan). Cyanoline blue was purchased from Dojindo Laboratories (Kumamoto, Japan). (-)-epicatechin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were commercially available reagents. Polyphenols were dissolved with water to be subject to the reaction.

The following coffee and tea drinks used were purchased from Japanese markets. (I) Canned or PET-bottled coffee drinks: Georgia (Japan Coca Cola, Tokyo, Japan); Doutor Café au Lait (JT Foods, Tokyo, Japan); and Blendy, low-carbohydrates (40% reduced sugar; Ajinomoto General Foods, Tokyo, Japan). (II) Coffee beans: Nescafe Gold Blend Original (Nestle Japan, Kobe, Japan); Nescafe Koumibaisen soft mocha blend; and UCC Blend Everest Mountain Coffee (UCC Ueshima Coffee, Kobe, Japan). (III) PET-bottled oolong tea: Asahi Primary Tea Leaf oolong tea (Asahi Soft Drink, Tokyo, Japan); and Co-op Tekkannon oolong tea (Japanese Consumers' Cooperative Union, Tokyo, Japan). (IV) PET-bottled Japanese green tea: Iemon Kyoto Fukujen (Suntory Foods, Tokyo, Japan); and OOI Ocha (Itoen, Tokyo, Japan). (V) PET-bottled tea: Kocha straight tea (Itoen); and Lipton Limone lemon tea (Suntory Foods, Tokyo, Japan).

The water purification system was a Milli-Q gradient A-10 with an EDS polisher (Millipore, Bedford, MA, USA).

2.2 Incubation of samples with nitrite and determination of cyanide by head-space gas chromatography (HS-GC)

Sample solution (polyphenol dissolved with water, coffee or tea drink diluted with water) was mixed with sodium nitrite (final 50 mM), acidified by addition of phosphoric acid (final 10%) or sodium acetate (final 0.6 M, pH 5.0) (final liquid volume: 1 mL) in a Teflon-lined glass vial (8.7 mL; GL Science, Tokyo, Japan), and incubated at 50 °C for 30 min. Produced cyanide was quantified by HS-GC [14] under the following analytical conditions [15]. Gas chromatograph, HP6890 (Agilent Technologies, Tokyo, Japan) was used with a porous polymer capillary column of GS-Q (30 m × 0.53 mm i.d., J&W Scientific, Folson, CA, USA) and a nitrogen-phosphorus detector maintained at 250 °C. Column temperature was set at 140 °C. Flow rate of the carrier gas (helium) was 5 mL/min. Injection was done in split mode with a ratio of 5, and its temperature was maintained at 200 °C. Five hundred microliter of the vapor phase was introduced to the gas chromatograph by manual injection using a glass syringe. Potassium cyanide was dissolved with water to be >10 mg/mL, and stored in a refrigerator for use within two weeks. Stock cyanide solution was further diluted for preparing the calibration curve (0–40 µg/mL in liquid phase).

2.3 Spectrophotometric method for determining cyanide

Produced cyanide was quantified by the microdiffusion-spectrophotometric König method using the pyridine-pyrazolone reaction [16, 17]. Two milliliters of 0.1 M sodium hydroxide was added to the center well of the glass Conway cell (Shibata Scientific Technology, Tokyo, Japan). Sample solution (polyphenol dissolved with water) was mixed with sodium nitrite (50 µmol) at one side in the outer well, and 0.2 mL of 50% phosphoric acid or 3.0 M sodium acetate (pH 5.0) was added on the other side. After the cell was closed, sample and acid were mixed and incubated at 37 °C for 90 min. One milliliter of extracted solution in the center well was transferred to the test tube, chilled in ice, and mixed with 0.2 mL of chloramine T solution (0.5 mg/mL of chloramine T, 0.75 M sodium dihydrogen phosphate). Two minutes' later, 3 mL of pyridine pyrazolone solution (0.27 g of cyanoline blue dissolved in 20 mL of pyridine and 100 mL of water) was added. The mixture was allowed to stand at room temperature. The absorbance at 630 nm was measured at 40 min. Stock cyanide solution was diluted for preparing the calibration curve (0–1 µg/mL in the extracted mixture).

3 Results and discussion

We reported on the artifactual formation of cyanide from coffee drinks reacted with isobutyl nitrite [13]. This study was derived from the forensic investigation in one case of poisoning; a woman noticed a strange taste when opening canned coffee, and called the police. At first, cyanide (3.2 µg/mL) was detected from the forensic specimen (canned coffee drink).

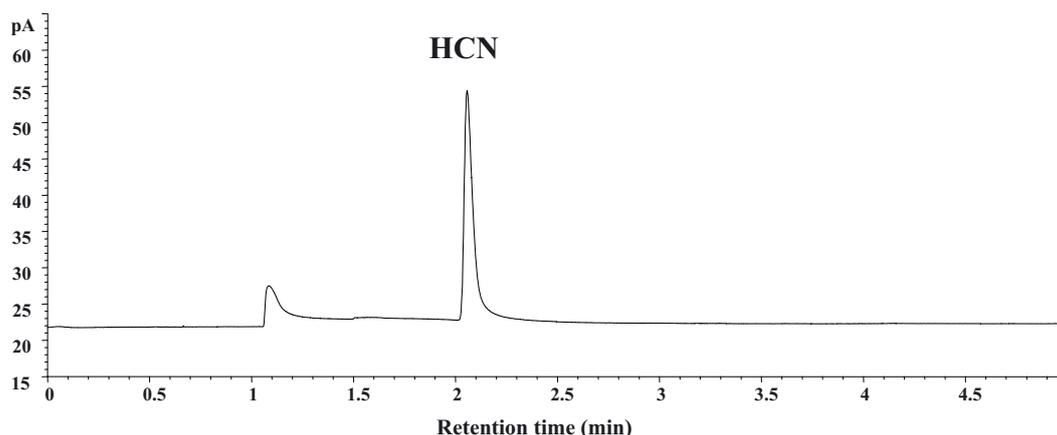


Fig. 1. Head-space gas chromatogram of hydrogen cyanide. Potassium cyanide (cyanide: 0.2 µg/mL in liquid phase) was incubated with phosphoric acid (10%) in the headspace vial at 50 °C for 30 min. The resulting vapor phase was injected into the gas chromatograph.

A remarkable level of alkaline or alkaline earth metal ions (possible counter-cation of cyanide) was not observed by capillary electrophoresis (CE), but considerable levels of nitrate and nitrite were detected by CE compared with the control coffee drink. Isobutyl nitrite and isobutyl alcohol were detected by gas chromatography-mass spectrometry (GC-MS). The reconstitution experiment revealed that isobutyl nitrite was readily degraded to isobutyl alcohol and nitrite, which was oxidized to nitrate, and some portions underwent oxidative or reductive reactions with organic compounds in the coffee to produce cyanide. In this article, we further elucidated artifactual production of cyanide from various types of coffee and tea drinks reacted with nitrite. Nitrite has been frequently used as adulterant for concealing evidence of drug abuse [18].

Reaction of the drinks (polyphenol substrates) with nitrite was done in a sealed HS vial. The resulting vapor phase was subjected to GC. Reaction conditions were chosen 50 °C for 30 min, and the nitrite concentration was 50 mM. In consideration of the reactive species of nitrite and its pKa value (pH 3.37), the reaction was done in ionic (acetate pH 5.0) and protonated (10% phosphoric acid) conditions. A typical chromatogram is shown in Figure 1. Hydrogen cyanide was detected at 2.2 min. This method provides very sensitive determination of cyanide (limit of detection: about 0.5 ng/mL, *S/N* = 3) with a wide range linearity [15]. From the background nitrite solution (50 mM), the unknown peak was eluted just at the same position of hydrogen cyanide. This level of false-positive detection was about 0.3 µg/mL under 10% phosphoric acid, and about 0.03 µg/mL under 0.6 M acetate (pH 5.0) conditions, so this false-positive background nitrite level was subtracted from the measured values for the sample test. Cyanide was not ascertained from the background nitrite solution under acidic conditions by the microdiffusion-spectrophotometric König method.

Without nitrite, cyanide was not produced from the drinks irrespective of acidic conditions. Supplemented cyanide was quantitatively recovered from drink samples during the HS reaction without nitrite (data not shown). Cyanide was also stable under acidic conditions in the presence of nitrite (data not shown). Table I shows the production of cyanide from various types of coffee and tea drinks in the presence of nitrite.

Table I. Production of cyanide from coffee and tea drinks reacted with nitrite.

Drink	Phosphoric acid (10%)	Sodium acetate (0.6 M, pH 5)
Coffee: Georgia (20% v/v)	3.35	0.64
Coffee: Doutor Café au Lait (20% v/v)	1.74	0.62
Coffee: Blendy low carbohydrate (20% w/v)	3.41	0.63
Coffee bean: Nescafe Goldblend Original (0.15% w/v)	1.24	0.64
Coffee bean: Nescafe Koumibaisen (0.15%)	1.94	0.52
Coffee bean: UCC The Blend Everest Mountain (0.15%)	1.54	0.59
Oolong tea: Asahi Primary Tea Leaf (75% v/v)	2.89	0.50
Oolong tea: Co-op Tekkannon (75% v/v)	2.69	0.69
Japanese Tea: lemon Kyoto Fukujuen (75% v/v)	3.31	0.58
Japanese Tea: OOI Ocha (75% v/v)	3.91	0.70
Tea: Kocha straight tea (75% v/v)	3.90	0.67
Tea: Lipton Limone lemon tea (75% v/v)	3.70	0.71

Drink samples were incubated with 50 mM sodium nitrite under acidic conditions at 50 °C for 30 min in the head-space vial (total liquid phase, 1 mL) and the resulting vapor phase was introduced to the gas chromatograph. The values presented are the average of more than two determinations of the cyanide produced in the liquid phase (µg/mL).

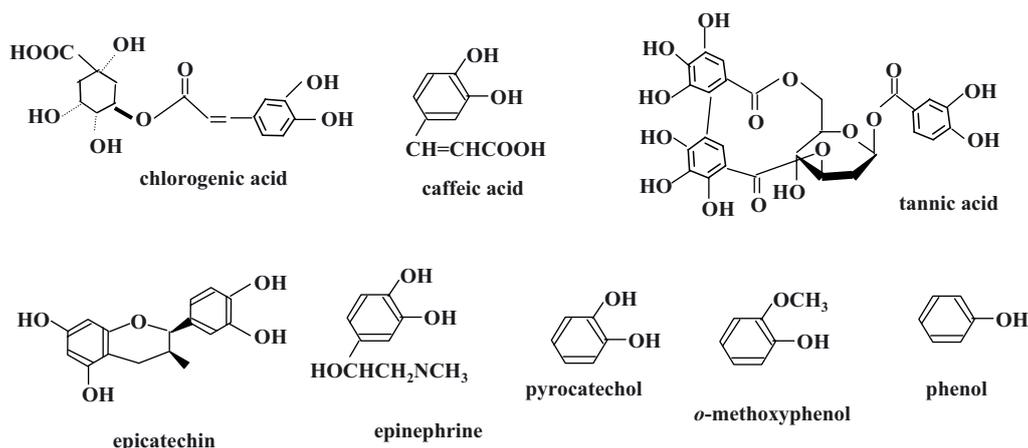


Fig. 2. Chemical structures of polyphenol compounds examined for cyanide production.

A cyanide level of several $\mu\text{g/mL}$ was produced under phosphoric acid conditions, and the level was lower under acetate (pH 5.0) conditions. The ratios of produced cyanide under phosphoric acid conditions compared with those under acetate (pH 5.0) conditions were 17–52%. Produced cyanide levels were not so varied with the same drink types. In the coffee “Nescafe Gold Blend Original”, increasing the amount of coffee contents raised the produced cyanide level, but this cyanide elevation was saturable (10 $\mu\text{g/mL}$ cyanide was produced in 3.7% coffee; data not shown).

In our previous article [13], we observed little production of cyanide from caffeine, carbohydrates and proteins when they reacted with nitrite under acidic conditions. Coffee and tea drinks contain various types of natural substances, and one of the active ingredients is polyphenol. Polyphenols have more than one phenol unit or building block per molecule, and are found in plants [19, 20]. In this contribution, several polyphenol compounds were examined to produce cyanide through the acidic reaction with nitrite (Fig. 2). Chlorogenic acid is included in coffee [21], and caffeic acid is produced from chlorogenic acid by hydrolysis at elevated temperatures. Tannic acid and catechins such as (-)-epicatechin are included in green tea, oolong tea and tea. (R)-(-)-epinephrine is produced and functions as a neurotransmitter in humans. Pyrocatechol is the simplest polyphenol. o-methoxyphenol is a methylated derivative of pyrocatechol. Phenol is a phenolic compound. Concentrations of target polyphenols were set at 0.75 mM except for tannic acid because the exact molecular weight of the latter was unknown; its concentration was set at 0.2 mg/mL. The polyphenols tested showed production of several $\mu\text{g/mL}$ cyanide under phosphoric acid conditions, and the level was lower under acetate (pH 5.0) conditions (Table II). The ratios of produced cyanide under phosphoric acid conditions compared with those under acetate (pH 5.0) conditions were from 18–68%. The only exception was caffeic acid, which produced a higher level of cyanide under acetate (pH 5.0) conditions. o-methoxyphenol showed lower production of cyanide (about one-third) than pyrocatechol. Phenol gave negligible cyanide production. As a stoichiometric consideration, about 10% molar level of the substrate pyrocatechol (0.75 mM) was converted to cyanide under phosphoric acid and excess nitrite (50 mM) conditions.

Table II. Production of cyanide from polyphenols reacted with nitrite.

	phosphoric acid (10%)	sodium acetate (0.6 M, pH 5)
chlorogenic acid 0.75 mM	2.79	0.52
caffeic acid 0.75 mM	2.86	4.46
tannic acid 0.2 mg/mL	3.39	0.81
(-)-epicatechin 0.75 mM	2.19	0.62
(R)-(-)-epinephrine 0.75 mM	1.82	1.23
pyrocatechol 0.75 mM	2.07	0.64
o-methoxyphenol 0.75 mM	0.63	0.21
phenol 0.75 mM	0.0	0.02

Target compounds were dissolved with water or methanol, and the part incubated with 50 mM sodium nitrite under acidic conditions at 50 °C for 30 min in the head-space vial (total liquid phase, 1 mL). The resulting vapor phase was introduced to the gas chromatograph. The values presented are the average of more than three determinations for the cyanide produced in the liquid phase ($\mu\text{g/mL}$).

The kinetics of cyanide production from chlorogenic acid and nitrite were investigated. Substrate concentrations significantly influenced cyanide production (Fig. 3). Cyanide was produced in a dose-dependent manner. Under the fixed conditions of 50 mM nitrite, cyanide production increased with chlorogenic acid concentration under acetate (pH 5.0) conditions to produce about 20 $\mu\text{g/mL}$ (0.8 mM) cyanide at about 40 mM chlorogenic acid. Cyanide production was complex under phosphoric acid conditions. Cyanide increased to a maximum at about 6 mM chlorogenic acid, then decreased, and again increased with high level of chlorogenic acid. The maximal cyanide concentration was about 10 $\mu\text{g/mL}$. In the fixed concentration of 0.75 mM chlorogenic acid, cyanide production increased with nitrite concentration to reach saturable levels. About 8 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ cyanide were produced at 300 mM nitrite under phosphoric acid and acetate (pH 5.0) conditions, respectively.

Figure 4A shows the time course of cyanide production. Levels of produced cyanide increased with time to 50 min and

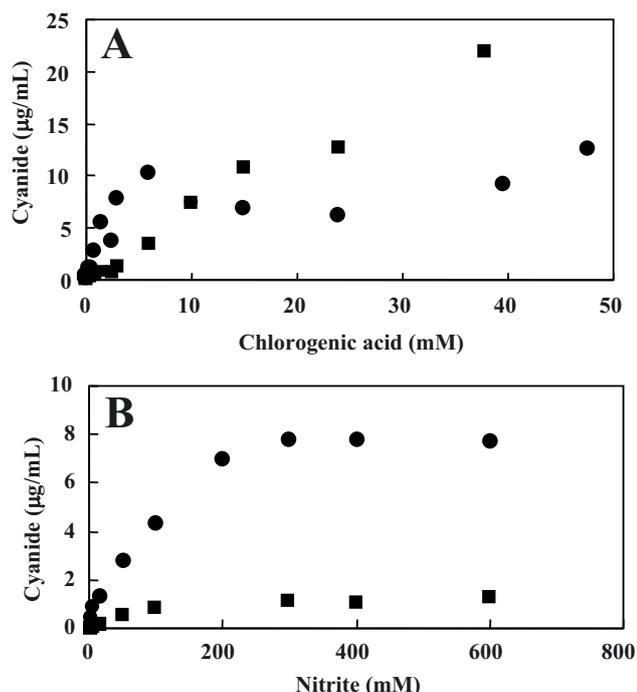


Fig. 3. Concentration dependence on cyanide production for the reaction of chlorogenic acid with nitrite. A: Effect of chlorogenic acid concentrations on cyanide production from 50 mM nitrite. B: Effect of nitrite concentrations on cyanide production from 0.75 mM chlorogenic acid. Reaction time and temperature were 30 min and 50 °C, respectively. Closed circle: 10% phosphoric acid conditions; closed square: 0.6 M acetate (pH 5.0) conditions.

100 min under phosphoric acid and acetate (pH 5.0) conditions, and then reached a plateau. Figure 4B shows the temperature dependence of cyanide production. Levels of produced cyanide increased significantly with temperature increase. Produced levels were negligible at temperatures lower than room temperature.

Figure 5 shows the effect of pH on cyanide production. Higher levels of cyanide were produced at lower pH. Produced cyanide levels were negligible at pH > 6. Levels of produced cyanide in phosphate-buffered solution were almost identical with those in acetate-buffered solution. We adopted the two pH conditions. For higher pH conditions (pH 5.0), we used acetate buffer because of the better buffering action of acetate.

Production of cyanide from chlorogenic acid was also ascertained by the microdiffusion-spectrophotometric König method. From the reaction mixture of 0.75 mM chlorogenic acid and 50 mM nitrite under 10% phosphoric acid and 0.6 M acetate (pH 5.0) conditions at 37 °C for 90 min, 0.94 µg/mL and 1.3 µg/mL of cyanide were extracted, respectively. Elucidation of the molecular mechanism of cyanide production from non-nitrogenous polyphenols with nitrite will be pursued in a later study.

4 Conclusion

We elucidated the artifactual production of cyanide from coffee and tea drinks when they reacted with nitrite under

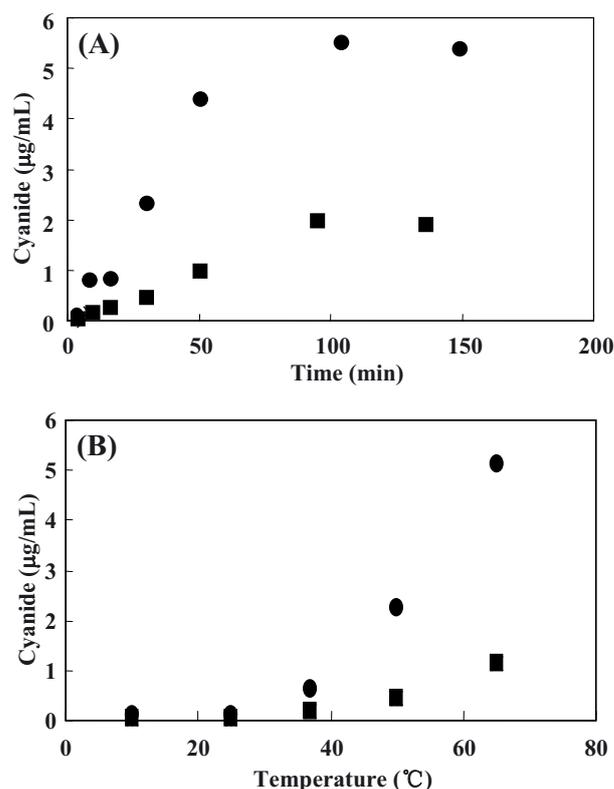


Fig. 4. Time course and temperature dependence of cyanide production for the reaction of chlorogenic acid with nitrite. Substrate concentrations were 0.75 mM for chlorogenic acid and 50 mM for nitrite, respectively. (A) Time course, temperature 50 °C. (B) Temperature dependence, reaction time 30 min. Closed circle: 10% phosphoric acid conditions; closed square: 0.6 M acetate (pH 5.0) conditions.

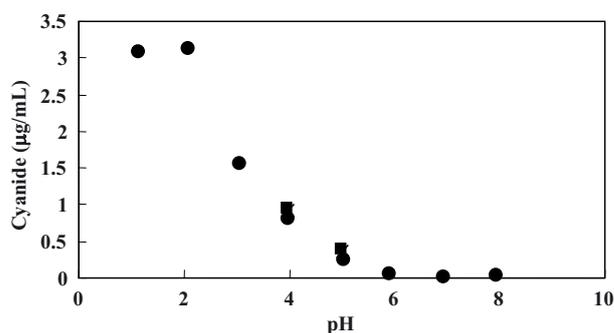


Fig. 5. pH dependence on cyanide production for the reaction of chlorogenic acid with nitrite. Reaction time and temperature were 30 min and 50 °C, respectively. Substrate concentrations were 0.75 mM for chlorogenic acid and 50 mM for nitrite, respectively. Acidic buffer concentrations were 0.6 M for phosphate (closed circle, pH adjusted by sodium hydroxide) and for acetate (closed square, pH adjusted by sodium hydroxide), respectively.

acidic conditions. This cyanide production could be ascribed to polyphenols. Levels of produced cyanide were of the order of several µg/mL from commercially available coffee and tea drinks with high levels of nitrite (50 mM, 2.3 mg/mL) under strong acidic (phosphoric acid) conditions at an elevated temperature (50 °C) during 30 min; the levels were sub-µg/mL under higher pH. The lethal dose of cyanide in humans is about

100 mg, so a level of 0.5 mg/mL seems lethal for drinking about 200 mL of beverages. A lower level of cyanide (several $\mu\text{g/mL}$) should also be considered because the toxicity of cyanide may disappear through phase-II alteration. Nitrite has been adulterated for destroying evidence of drug abuse [18], so it is possible that nitrite is used in crime cases. If nitrite is adulterated into coffee and tea drinks which the solution pH is weakly acidic [16], cyanide is artifactually produced, just like the case of adulteration by isobutyl nitrite [13]. Even in an emergency situation in poisoning cases, forensic toxicologists should consider the behaviors of poisons in bodies and in the forensic specimen, and not be fooled by false detection of cyanide.

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