Rapid Determination of Cyanide and Azide in Beverages by Microdiffusion Spectrophotometric Method

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Abstract
A rapid screening method was developed for the determination of the toxic volatile anions, cyanide and azide, in beverages. This method consisted of a microdiffusion extraction combined with spectrophotometry using the König cyanide reaction and ferric azide complex formation in conjugation with cerium azide oxido-reduction. The time required to achieve full recovery in the extraction of hydrogen cyanide and hydrazoic acid from samples was considerably shortened by increasing the diffusion temperature from 25°C to 40°C. The time required to achieve saturated color development in the König cyanide reaction was also shortened by increasing incubation temperature to 40°C. The interference in both azide color reactions was examined for volatile compounds. Cyanide interfered only in the case of ferric azide complex formation. Sulfide, sulfate, nitrite, and acetic acid interfered in both the color reactions. The established method gave a detection limit of 6 μM for cyanide and 0.5 μM for azide, and it required only 1 h to determine both anions. Cyanide and azide disappeared by evaporation from beverages during 25°C storage under open conditions in a pH-dependent manner as a function of their respective pKₐ values of 9.2 and 4.6.

Introduction
A widespread series of deliberate poisoning cases occurred in Japan during the second half of 1998 (1). The first case, the Wakayama curry poisoning case, occurred in late July, and 4 people died and more than 60 became seriously ill after eating arsenic-laced curry served at a community festival in the Sonobe district of Wakayama (2). In August, 10 employees of a wood-processing firm in Niigata became ill after drinking beverages made with sodium azide (NaN₃)-laced water in the electric kettle in the office. After that, four copycat crimes involving cases of sodium azide tampering occurred (2). The third event occurred in Nagano, and a resident there died after drinking cyanide-laced canned oolong tea (3). In addition, a number of attempted or fake cases of cyanide tampering of beverages have also been documented. Common articles of daily life such as commercial detergents have also been used to adulterate foods and drinks.

When poisoning cases occur, determination of the toxic substances is required for medical treatment of the casualties, as well as the subsequent criminal investigation. In Japan, forensic science laboratories (FSL) of the prefectural police headquarters and, when requested, the National Research Institute of Police Science (NRIPS), are often involved in the forensic investigations and perform analyses of samples from the victims and evidence samples taken from the crime scenes. After a simple preliminary examination, the causative toxic substances are typically identified by sophisticated instrumental analysis. Emergency medical units have also been known to examine samples (taken from a victim) by routine clinical analysis. Among the toxic substances, cyanide is an important compound and is a highly relevant candidate for testing. So far, several attempted suicides and accidental cases of sodium azide poisoning have been reported (4–9). After a number of homicides and cases of intentional injury by sodium azide tampering, azide has now become a target for initial inquiry in unknown poisoning cases in Japan (10). In general, sodium azide is routinely used for a variety of purposes, such as a preservative in laboratory use (11), and it has recently been included in automobile air bags as a combustion material (12). Azide has a hypotensive effect in humans (13), and its toxicity is derived from its ability to inhibit cytochrome oxidase activity (14). Azide and cyanide are weak acids when they exist in the acid form (pKₐ 4.8 and 9.31, respectively) and show a similar volatility (boiling points 26°C and 37°C, respectively) (13,15).

Laboratory analysis of cyanide consists of the following two procedures: extraction of hydrogen cyanide (HCN) from samples and the quantitation of the extracted cyanide. Aeration (16), distillation (17), microdiffusion (18,19), headspace (HS) (20–23), and HS-microextraction (24) were used as the extraction procedures. Titration (25), spectrophotometry (26), spectrofluorimetry (27), polarography (28), ion chromatography (IC) (29), high-performance liquid chromatography (HPLC) (30), and gas chromatography (GC) with electron-capture detection after conversion to cyanogen halide (20) and nitrogen-phosphorus detection (21–23) were used as the quan-
titration procedures. For the quantitation of azide, oxidoreductive titration (13,31–33), spectrophotometry using a ferric salt (34–36) or a cerium salt (37), IC (38–40), GC (41,42), and HPLC (8,9,43) have been reported. GC, after a pentfluorobenzoylation derivatization procedure, enables the simultaneous determination of azide, cyanide, and other anions (44,45). In the early stages of a poisoning, the more quickly the toxic substances are identified from evidence samples, the more effective is the applied medical treatment. In addition, the rapid determination of the causative poisons facilitates the early stage of the criminal investigation. Among the extraction methods now employed, microdiffusion is an ideal candidate for volatile substances. Spectrophotometric microdiffusion methods for the rapid determination of cyanide in the blood of a victim in emergency situations have already been reported (46,47).

In this paper, we report on the expanded use of the microdiffusion method for the determination of cyanide and azide. Although the rapid screening test kits such as ion test paper (Cyantesmo paper, Macherey-Nagel and Cyano Check paper, Advantec Toyo) for the direct examination of water sample are commercially available, they have the disadvantages of interference from many coexisting compounds and semiquantitative nature. We selected spectrophotometry because from the standpoint of rapidity, simplicity, accurate quantitation, and economy, it is applicable to both emergency medical and forensic investigation as a preliminary screening method. The König method (48), which uses a pyridine-pyrazolone reagent for cyanide, was chosen because this method is the most frequently used in Japanese toxicological laboratories. We also adopted both the azide ferric complex reaction and the cerium oxidoreduction reaction for azide quantitation. Finally, we report on a simultaneous determination method for cyanide and azide, and also examined the stability of cyanide and azide in various commercially available beverages from the standpoint of detectability in leftover beverage samples that have been found in crime scenes.

**Experimental**

**Reagents**

Potassium cyanide (KCN), NaN₃, and p-toluenesulfonychloramide (chloramine T) were purchased from Wako Pure Chemical (Osaka, Japan). Diammonium cerium (IV) nitrate was from Kanto Chemical (Tokyo, Japan). Cyanoline blue (mixture of 1-phenyl-3-methyl-5-pyrazolone and 4,4'-bis(1-phenyl-3-methyl-5-pyrazolone)) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were of analytical grade. All aqueous solutions were prepared with deionized and distilled water.

**Microdiffusion**

Glass Conway cells (outer cell, height 8 mm, exterior diameter, 60 mm, interior diameter, 40 mm; center well, height 5 mm, exterior diameter 34 mm; Shibata Scientific Technology, Tokyo, Japan) were used for microdiffusion. Two milliliters of 0.1M sodium hydroxide (NaOH) was added to the center well of the cell. One milliliter of the sample solution was added to one side of the outer well. A glass plate coated with glycerol was then placed over the cells, leaving a small opening on the side opposite the sample, through which 0.5 mL of 10% (w/v) sulfuric acid (H₂SO₄) was added. The cell was closed rapidly, and the sample and acid were immediately mixed by gently tilting the cell. At the incubation for 30 min at 40°C in an air incubator, the lid was removed and the extracted solution in the center cell was removed with a pipette.

**Spectrophotometric method no. 1 (König reaction)**

The cyanide level was measured by the König reaction using the pyridine-pyrazolone reagent (26). Forty microliters of a solution of cyanide, after microdiffusion, was then pipetted into a 96-well microplate, and mixed with 15 μL of 1.0M potassium hydrogen phosphate solution, 5 μL of 5.0M NaOH solution, and 10 μL of 6.25 mg/mL chloramine T solution. After approximately 2 min, 0.12 mL of 0.27% (w/v) Cyanoline blue in pyridine/water (1.5, v/v) was added to the mixture. After the incubation for 10 min at 40°C in an air incubator, the absorbance of the solution was determined at 630 nm using a microplate reader (Spectramax 250, Molecular Device Co., Menlo Park, CA). The calibration standards (0–200μM) were also subjected to the same color reactions in parallel. Samples giving cyanide levels higher than 200μM were assayed again after appropriate dilution of the inner cell solution with 0.1M NaOH.

**Spectrophotometric method no. 2**

(ferric azide complex formation)

The azide level was measured as the azide ferric complex by spectrophotometry (35). Twenty microliters of the inner cell and 0.18 mL of 50M ferric trichloride in 10M hydrochloric acid (HCl) were pipetted into a 96-well microplate. After 5 min, the absorbance was determined at 450 nm using microplate reader. The calibration standards (0–10mM) are also subjected to the same color reactions in parallel. The samples giving azide levels higher than 10mM were assayed again after appropriate dilution of the inner cell solution with 0.1M NaOH.

**Spectrophotometric method no. 3**

(ceurium reduction by azide)

The azide level was determined by spectrophotometry by oxidoreduction using the cerium reagent (37). Twenty microliter of the inner cell and 0.18 mL of 600μM diammonium cerium (IV) nitrate in 1.0M H₂SO₄ were pipetted into a 96-well micro plate. The absorbance was immediately determined at 390 nm using microplate reader. The calibration standards (0–6mM) are also subjected to the same color reactions in parallel. The samples giving azide levels higher than 6mM were assayed again after appropriate dilution of the inner cell solution with 0.1M NaOH.

**Routine spectrophotometric method**

The mentioned procedures for measuring color development using a microplate reader were modified by scaling up the reaction volume from 0.2 mL to 3.0 mL, and by measuring the absorbance of the color reaction solution by mean of a
U-best V-560DS spectrophotometer (Japan Spectroscopic, Tokyo, Japan) at the respective wavelengths. For the König cyanide assay, the mixture of chlorinated solution and pyridine-pyrazolone reagent was incubated at 40°C in a water bath.

A storage experiment for cyanide and azide in a beverage solution

The following six commercial beverages were used: canned Coca Cola (Japan Coca Cola Bottlers, Tokyo, Japan), a paper carton of orange juice drink (Sweet Orange Juice, reconstituted from concentrate to 100%, Gurico, Tokyo, Japan), a paper carton of sake drink (Miyakotaka, alcohol conc. 15–16%, Koyama Honke, Omiya, Japan), a canned draft beer (Ichiban Shibori, Kirin Beer, Tokyo, Japan), a canned coffee (Georgia Mocha/Kilimanjaro (50:50), Japan Coca Cola Bottlers), and a paper carton of milk (Rakuno Milk, Zenrakuren, Tokyo, Japan). Tea was extracted with 200 mL of hot water from 2 g of a tea pack (Lipton, Tokyo, Japan).

Twenty milliliters of beverage or a 100mM phosphate- or acetate-buffered solution was combined with 0.4 mL of a 500mM KCN solution or a 500mM NaN₃ solution in glass tube (30-mm i.d.) with or without closing the tube with a stopper and was left at 25°C. After the specified time, the aliquot was sampled, and the level of cyanide or azide was determined by the microdiffusion spectrophotometric method.

Results and Discussion

Improvement in the spectrophotometric methods for cyanide and azide

In the König cyanide color development using pyridine-pyrazolone reagent, the formation of a blue color indicates the presence of cyanide (26). As shown in Figure 1, more than 25 min was required to reach maximal color development at 25°C. Increasing the incubation temperature to 40°C shortened the time to 10 min, after which the absorbance remained nearly constant. This result is consistent with literature observations (46,47). As shown in Figure 2, the calibration curve was linear (γ = 0.9998) with respect to cyanide concentrations ranging from 0 to 200μM in the inner well solution. The within-day repeatability (40μM, n = 8) was 3.5% (relative standard deviation (RSD)), and the detection limit was 3μM (S/N = 3). Only equimolar thiocyanate gave a positive interference (49). However, thiocyanate can be removed in the microdiffusion process because of its nonvolatility. Neither NaN₃, sodium sulfide (Na₂S), sodium sulfate (Na₂SO₃), nor sodium nitrite (NaNO₂) showed a König color development. Neither 40% ethanol nor 0.65mM of acetic acid interfered with the color development. Ethanol is presented at high levels in alcoholic beverages, and acetic acid in vinegar beverages. Acetic acid (100mM) interfered with the color development, which can be attributed to a pH decline in the König reaction (49). Therefore, ethanol and volatile anions which may be adulterated and which can be extracted by microdiffusion, such as azide, sulfide, sulfite and nitrite, represent negligible interferences in the König color reaction, except for high levels of acetic acid.
The ferric azide complex gave red color under acidic conditions. As shown in Figure 3, the color development of the ferric complex in the presence of NaN₃ was pH-dependent. As the pH increased, the absorbance at 450 nm of both the azide-blank solution (yellow color) and the azide solution (red color) increased, but the difference in both absorbance values did not shift in parallel with pH. We adopted a 10mM HCl solution, for which the pH of the reaction solution is approximate 2.1. A calibration curve was linear ($y = 0.9998$) with respect to azide concentration over the range of 0 to 10mM in the inner well solution (Figure 4). The within-day repeatability (5.0mM, $n = 8$) was 2.3% (RSD), and the detection limit was 0.25mM (S/N = 3). The color development in the ferric solution, as measured at 450 nm, was not specific for azide (Table I). KCN reacted with the ferric molecules, giving the same red color development, which was equivalent to approximately 1/12 of the azide in terms of molar level. Na₂S caused a white precipitate and thus disturbed the spectrophotometric determination. Ethanol (50%) did not interfere with color development. Although a low level (3.2mM) of acetic acid did not, a high level (80mM) suppressed color development to a significant degree, which could be attributed to a decline in pH. Na₂SO₃ and NaNO₂ showed complicated phenomena. When sulfite or nitrite was added to an acidic ferric solution in the absence of NaN₃, the color of the solution immediately became red (a rise in the absorbance at 450 nm) in a concentration dependent manner, and the red color faded and the absorbance decreased to a lower value than that of the blank solution soon after (5 min). When sulfite or nitrite was added in the presence of NaN₃, the color of the solution immediately became red (a rise in the absorbance at 450 nm), and the red color faded in a concentration-dependent manner soon after (5 min). Sulfite and nitrite are capable of forming a red-colored ferric complex, which immediately causes an oxido-reduction reaction between ferric molecules and sulfite (or nitrite). Sulfite or nitrite is oxidized to sulfate or nitrate, and a portion of the yellow-colored ferric (III) molecules are converted to colorless ferrous (II) molecules. Azide may be also oxidized by sulfite or nitrite (33). Complicated reactions between ferric (III) molecules and azide and sulfite (nitrite) proceed within a few minutes. Azide is not oxidized in the presence of low levels of Na₂SO₃ or NaNO₂, resulting in complete ferric azide complex formation. With high levels of Na₂SO₃ or NaNO₂, part of the added azide is oxidized by sulfite or nitrite to nitrogen and nitric oxide (11), thus decreasing the azide level leads to a lowering in the color formation. Therefore, volatile anions, which do not give rise to high color levels, and ethanol can be regarded as compounds that do not interfere in the color reaction, except for cyanide and sulfide and high levels of sulfite, nitrite, and acetic acid.

![Figure 4. Calibration curve for azide relative to color development of the ferric azide complex reaction. Absorbance at 450 nm was plotted against the azide concentration.](https://academic.oup.com/jat/article-abstract/25/4/228/762431)

![Figure 5. Calibration curve for azide in a fading solution of cerium. Twenty microliters of NaN₃ in 0.1M NaOH was mixed with 180 mL of 600mM diammonium cerium (IV) nitrate in 1M H₂SO₄. Absorbance at 390 nm was plotted against the azide concentration.](https://academic.oup.com/jat/article-abstract/25/4/228/762431)
The addition of azide to a cerium solution led to a change of the yellow color (ceric (IV)) to colorless (cerous (III)). A color change in the cerium azide reaction was not so drastically influenced by pH as was the case for the ferric azide complex formation. Under optimized conditions, the calibration curve was linear ($y = 0.9995$) with respect to azide concentration over the range of 0 to 7 mM in the inner well (Figure 5). The within-day repeatability (5.0 mM, $n = 8$) was 2.7% (RSD), and the detection limit was 0.5 mM ($S/N = 3$). The reaction of the cerium reagent with azide is based on the oxidation of azide by ceric (IV) molecules. Accordingly, volatile reducing substances could interfere in the reaction. As shown in Table II, even 200 mM KCN did not interfere in the color reaction. Na$_2$S, Na$_2$SO$_3$, and NaNO$_2$, all reducing compounds, caused the same color fading. Ethanol (50%) did not interfere in the color reaction. High levels (80 mM) of acetic acid suppressed the color development significantly, although low levels (0.64 mM) did not. Therefore, ethanol and cyanide can be regarded as not interfering with the color reaction, except for sulfide, sulfate, and nitrite and high levels of acetic acid.

### Improvement in microdiffusion conditions relative to cyanide and azide

The extraction of HCN and hydrazoic acid (HN$_3$) is an important process in isolating them from interfering compounds in complicated-matrix samples. If the determination method omits the extraction process, potentially interfering reactions can lead to mistaken findings for certain toxic substances. In the widespread series of poisoning cases in 1998 in Japan, there were several cases of mistaken detection in the early stages of the investigations. In the Wakayama arsenic curry poisoning case, a false-positive finding of cyanide was reported during the first week of the investigation (50).

Microdiffusion is the rate-limiting step in the total determination procedure. As shown in Figure 6, the extraction of HCN from the outer well to the inner well in a Conway cell required over 2 h at 25°C for completion. Raising the diffusion temper-

### Table II. Effect of Volatile Compounds on the Absorbance of Ceric Solutions in the Absence and Presence of Azide

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Azide 0mM</th>
<th>Azide 5.0mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td></td>
<td>0 (0.725)*</td>
<td>-0.455</td>
</tr>
<tr>
<td>Cyanide</td>
<td>1.0mM</td>
<td>0.000</td>
<td>-0.454</td>
</tr>
<tr>
<td></td>
<td>5.0mM</td>
<td>-0.001</td>
<td>-0.454</td>
</tr>
<tr>
<td></td>
<td>200mM</td>
<td>0.001</td>
<td>-0.453</td>
</tr>
<tr>
<td>Sulfide</td>
<td>1.0mM</td>
<td>-0.097</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10mM</td>
<td>-0.409</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50mM</td>
<td>-0.669</td>
<td>-</td>
</tr>
<tr>
<td>Sulfite</td>
<td>3.1mM</td>
<td>-0.051</td>
<td>-0.421</td>
</tr>
<tr>
<td></td>
<td>13mM</td>
<td>-0.359</td>
<td>-0.420</td>
</tr>
<tr>
<td></td>
<td>50mM</td>
<td>-0.359</td>
<td>-0.461</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.8mM</td>
<td>-0.271</td>
<td>-0.455</td>
</tr>
<tr>
<td></td>
<td>3.2mM</td>
<td>-0.482</td>
<td>-0.499</td>
</tr>
<tr>
<td></td>
<td>50mM</td>
<td>-0.490</td>
<td>-0.434</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50%</td>
<td>-0.001</td>
<td>-0.462</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.64mM</td>
<td>-0.005</td>
<td>-0.452</td>
</tr>
<tr>
<td></td>
<td>80mM</td>
<td>-0.013</td>
<td>-0.283</td>
</tr>
</tbody>
</table>

* The color solution was measured by microplate reader at 390 nm. Values are represented as the increase of absorbance against the blank solution.

* Absorbance of the blank solution.
ature to 40°C shortened the time for reaching full recovery to 15 min, and the recovery then remained nearly constant. This result is consistent with other literature reports (46,47). As shown in Figure 7, the extraction of HNO₃ from the outer well to the inner well took over 1 h at 25°C. Increasing the diffusion temperature to 40°C shortened the time for completion to 30 min, and the recovery then remained nearly constant.

As a result of this finding, the optimized condition was set to be 30 min microdiffusion at 40°C, a 5 min color reaction using ferric trichloride, an immediate reaction using diammonium cerium (IV) nitrate, and a 10 min color reaction using pyridine-pyrazolone reagent after a 2 min chlorination reaction. Under these analytical conditions, the within-day repeatability (n = 6) was 2.54% (RSD) for the König method (10mM in canned coffee drink solution), 2.15% for the ferric azide complex method and 2.31% for cerium method (10mM). The recovery was 101.2% for 5.0mM cyanide, 98.6% and 96.8% for 5.0mM azide using the ferric trichloride and the cerium reagents, respectively. If an analyst wishes to examine cyanide and azide more rapidly, a 10 min microdiffusion at 40°C and 5 min color reaction can be recommended as a semiquantitative method.

Evaluation of the established method
From the qualitative aspect, the blue color development in the König reaction indicates the presence of cyanide, whereas a red color development with the ferric reagent indicates the presence of azide and a high level of cyanide. If cyanide is not detected in the König reaction, the ferric color reaction indicates the presence of azide conclusively. If cyanide is detected in the König reaction, the presence of azide can be confirmed by the cerium reaction, although the naked eye can not clearly distinguish the fading of the yellow color, especially at low levels. Oxidizable gases, hydrogen sulfide (H₂S), sulfur oxide (SO₂), and nitric acid (HNO₂), severely interfere with both the ferric azide formation and the cerium oxido-reduction methods (Tables I and II). H₂S can be detected if a commercial sulfur product is adulterated. In Japan, a commercial sulfur product such as a mixture of lime and sulfur has been used as agricultural chemicals and bath supplements, and there have been several suicide cases as the result of taking such a product (51). SO₂ can be detected if Na₂SO₃, which is used in photographic film development, is adulterated. HNO₂ can be detected if NaNO₂, which is used for a bleaching reagent, is adulterated. However, in Japan, only a few poisoning cases involving sulfite and nitrite (51) have been reported. As for sulfide, its presence can be detected by a strong sulfur smell, or it can be specifically detected by an additional spectrophotometric method involving methylene blue formation (48). This method, which consists of mixing 0.17 mL of the inner well solution with 0.02 mL of 0.2% p-aminodimethylaniline in 10N H₂SO₄ and 0.01 mL of 0.33% ferric trichloride in 0.36N H₂SO₄ and measuring absorbance at 670 nm after a 15 min incubation, can quantitate sulfide below 25µM in beverages. Nitrite is not evaporated in the form of HNO₂ but as nitric oxide, so there is little possibility of interference by nitrite. As an alternative choice, capillary electrophoresis permits the rapid and simple simultaneous determination of various kinds of anions for the extracted solution without interference by oxidizable volatile anions (52).

We examined the appropriateness of the established method for measuring a mixture of cyanide and azide. As shown in Table III, cyanide was quantitated accurately for the solution by the König method. The cerium method also accurately determined the level of azide. Although the ferric method gave an artifactually raised absorbance value, the correction from the cyanide data obtained by König method permits the accurate quantitation of azide.

When cyanide or azide is positive in the established method, more specific analytical methods should be performed on the remaining extracted solution in the inner well of the Conway

Table III. Determination of a Mixture of Cyanide and Azide*

<table>
<thead>
<tr>
<th>Method</th>
<th>Absorbance observed (B.G.)</th>
<th>Cyanide (mM) calculated (recovery)</th>
<th>Azide (mM) calculated (recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>König</td>
<td>0.385 ± 0.014 (0.009 ± 0.000)</td>
<td>5.16 ± 0.19mM (103.2 ± 3.7%)</td>
<td></td>
</tr>
<tr>
<td>Ferric</td>
<td>0.757 ± 0.019 (0.386 ± 0.088)</td>
<td>5.58 ± 0.14mM (111.6 ± 2.8%)</td>
<td></td>
</tr>
<tr>
<td>Ferric</td>
<td>corrected†</td>
<td>5.16 ± 0.13mM (103.2 ± 2.6%)</td>
<td></td>
</tr>
<tr>
<td>Cerium</td>
<td>0.539 ± 0.012 (0.754 ± 0.054)</td>
<td>5.15 ± 0.11mM (103.0 ± 2.2%)</td>
<td></td>
</tr>
</tbody>
</table>

* A solution containing 5.0mM KCN and 5.0mM NaN₃ was subjected to the established microdiffusion spectrophotometric method. The concentration of cyanide in the inner well was measured by the König method, and that of azide was measured by both ferric and cerium methods. The result was an average of five determinations plus or minus the standard deviation.

† The observed absorbance value for the mixture solution was subtracted by the calculated value corresponding to the contribution of cyanide which level was measured by König method.
cell for the confirmation of the presence. HS-GC is effective for cyanide identification (21,22), and GC–MS after pentafluorobenzylolation is effective for azide identification (44,45).

Stability of cyanide and azide in beverages

Little information on the stability of cyanide and azide in beverages is available in the literature. Nishioka et al. (53) reported on the oxidative breakdown of cyanide in water under alkaline conditions during a 10-day period, the time span of which would not be encountered in actual poisoning cases. We have extensively examined the stability of cyanide and azide in beverage samples within a short time span, assuming real poisoning cases where a fatal dose of cyanide salt or NaN₃ is adulterated into beverages. In order to investigate the behavior of cyanide in beverages during storage at 25°C, KCN was added to various beverages to concentrations where the cyanide level (10mM) would be fatal when one bottle was ingested and stored at 25°C in atmospheric conditions. As shown in Figure 8, the pH of the solution remained weakly acidic for each sample, although the pH values had increased to a small extent compared to the original drink solution. Cyanide disappeared rapidly from all samples tested. After 6 h, cyanide levels were reduced by about 50%, and after 2 days only trace levels of cyanide could be detected. It is probable that cyanide disappears by evaporation, in the form of HCN under the experimental con-

Figure 9. Effect of pH on cyanide disappearance in the buffered solution. In a glass tube, 20 mL of 100mM buffer solution (acetate, pH 3.6–4.5; phosphate, 5.6–8.1) and 0.4 mL of 500mM KCN solution were added and allowed to stand under open conditions at 25°C. At the specified times a 1.0-mL aliquot was removed and extracted by microdiffusion, and the inner well solution was diluted 100-fold with 0.1M NaOH, and the cyanide concentration was measured by the König method. The measured concentration is converted to the percentage recovery of cyanide remaining in the tube and plotted against the pH of the mixture.

Figure 10. The change of concentrations of azide in beverages under closed conditions during 25°C storage. In the glass tube, 20 mL of beverages and 0.4 mL of 500mM NaN₃ solution were added and allowed to stand with closing the tube at 25°C. At the specified times, an aliquot was removed, and the azide concentration was measured by microdiffusion combined with the ferric azide complex method. The measured concentration is converted to the percentage recovery of azide remaining in the tube. Each value represents the mean of three determinations plus or minus the standard deviation.

Figure 11. Effect of pH on azide disappearance in a buffered solution. In the glass tube, 20 mL of 100mM buffer solution (acetate, pH 3.6–5.6; phosphate, 6.0–8.1) and 0.4 mL of 500mM NaN₃ solution were added, and allowed to stand without closing the tube at 25°C. At the specified times, an aliquot was removed, and the azide concentration was measured by microdiffusion combined with the ferric azide complex method. The measured concentration is converted to the percentage recovery of azide remaining in the tube and plotted against the pH of the mixture.
ditions, because the pK_a of cyanide is 9.31 and the boiling point of HCN is 26°C (15). As shown in Figure 9, the pH dependence of cyanide disappearance is clear. Below pH 10, the behavior for cyanide disappearance showed a similar pattern and was independent of pH. Above pH 10, the disappearance of cyanide was retarded considerably.

As for stability of azide in beverages, no information exists in the literature. In order to investigate the behavior of azide in beverage solutions during storage at 25°C, NaN_3 was added to various beverages to a level where the azide level (10 mM) would be toxic with the drinking of one bottle and stored at 25°C under closed conditions. As shown in Figure 10, within such a time span (14 days), no change in azide level was observed under closed conditions where the evaporation loss of HN_3 was prevented, indicating the chemical stability of azide in beverages that consist of protein, minerals, carbohydrates, organic acids, and alcohol. Figure 11 shows changes in the concentrations of azide in a system which is open to the atmosphere. Azide disappeared rapidly from Coca Cola, orange juice, and sake, and the pH values of those samples, after NaN_3 addition, were below 5. Azide disappearance was moderate in coffee and milk where the pH of the solution was around 6.5, and even after 5 days, azide was easily detected. Azide did not disappear from an aqueous solution with a pH of 7.4. As shown in Figure 12, azide disappearance was pH dependent. Above pH 5, the concentration of azide did not decrease, even after 2 days. Below pH 4.5, azide disappearance was rapid, and after 1 day, the detection level was very low. It is probable that azide disappears by evaporation as HN_3 under those experimental conditions because the pK_a of azide is 4.6 and the boiling point of HN_3 is 36°C (13).

Our experimental results indicate that residual cyanide and azide in beverages is low because HCN and HN_3 rapidly evaporate under the pH conditions where a cyanide salt or NaN_3 was adulterated in beverages. Common beverages are weakly acidic. Therefore, in the poisoning cases, left-over drink samples found on the scene or in the stomach should be evaluated as having been lowered by evaporation, in comparison with the original level, immediately after adulteration.

**Conclusions**

The established screening method using microdiffusion and spectrophotometry permits the simultaneous determination of cyanide and azide below toxic dose levels in beverage samples within 1 h and does not require special equipment except for a spectrophotometer, water bath incubator, and air incubator, which can be found in general clinical testing laboratories. Cyanide and azide disappeared by evaporation from beverage samples in a pH-dependent manner. Although nearly half of the added anions could be detected by established methods after 2 h incubation at 25°C, they were not detectable after 1 day.

**References**


Manuscript received June 26, 2000; revision received September 27, 2000.