Cyanide and Azide Analysis in Biologicals and Foodstuffs

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1 INTRODUCTION

Cyanide is a rapidly acting poison that reacts with mitochondrial enzymes to cause an inhibition of cellular respiration that can lead to hypoxia and rapid death. The minimum adult lethal dose has been estimated as 100 mg for hydrocyanic acid and 200 mg for potassium cyanide. Industrial uses of cyanide salts (fumigants, insecticides and electroplating solutions) make this toxic anion available for suicidal purposes and criminal mischief.

Similar to cyanide, azide inhibits cellular respiration by irreversible binding to the heme cofactor. Sodium azide, the most commonly encountered azide salt, has numerous uses (automotive airbags, airplane escape chutes, preservative for biochemical reagents) and has been used for both suicidal and homicidal purposes. Sodium azide is acutely toxic, with an estimated adult lethal dose of 700 mg.

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☐ Quantitation
Matrices	Blood/Aqueous samples
Analytes	Cyanide, Azide
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 PRINCIPLE

This procedure for cyanide and azide is predicated on the condition that the analytes will be released as hydrogen cyanide gas (HCN) and hydrazoic acid (HN_3), respectively, upon acidification of the biological and/or food matrix.

For cyanide, screening is performed by headspace gas chromatography with selective nitrogen/phosphorus detection (HS-GC/NPD). Qualitative confirmation of cyanide and screening/confirmation of azide is performed by headspace gas chromatography-mass spectrometry (HS-GC/MS).

4 SPECIMEN CRITERIA

For cyanide, sample matrices can be comprised of biologicals (blood, tissue homogenate, or stomach contents) as well as common foodstuffs. For azide, blood and aqueous matrices are currently validated. Typically, 0.5 mL (or 1.0 gram of 1:1 homogenate) is used for these analyses.

5 EQUIPMENT

5.1 Equipment

Volumetric flasks (10, 25, and 100-mL)
Vortex mixer
Routine laboratory supplies, including pH paper, disposable glass pipets, spatulas, test tube racks, graduated cylinders, homogenizer, mortar and pestle, glass reagent bottles, etc.

5.2 Consumables

10-mL and/or 20-mL disposable headspace vials and caps
1.0 and 2.5 mL plastic syringes with needles

5.3 Instruments

A. Gas chromatograph equipped with an RT-QS-BOND (or equivalent) column, a nitrogen phosphorus detector and a headspace autosampler.

B. Gas chromatograph equipped with a DB-624 (or equivalent) column, a mass spectrometric detector and a headspace autosampler.

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	7 Pro SP 1
GC/MS	Enhanced Chemstation	E.02.02.1431
Autosampler	Gerstel Maestro 1	1.5.3.3/3.5
Data Analysis	Xcalibur	2.0.7 SP1

5.5 Chemicals/Reagents

5.5.1 Purchased

Acetonitrile (HPLC grade)
Deionized Water
1-Butanol (ACS grade)
Sulfuric acid
Sodium hydroxide

5.5.2 Prepared

5 N Sulfuric Acid

To a 100-mL graduated cylinder containing 70 mL deionized water, slowly add 12.5 mL concentrated sulfuric acid. Mix well and bring to 90 mL with deionized water. Store in glass at room temperature. Stable for at least 1 year.
5 N Sodium Hydroxide (approximate)
To a 100-mL beaker, add 60 mL deionized water and 20 g sodium hydroxide. Mix well to dissolve and let cool. Bring to volume with deionized water. Store in plastic containers at room temperature. Stable for at least 1 year.
0.4% Sodium Hydroxide (w/v, approximate)
To a 25-mL volumetric flask, add approximately 15 mL of deionized water. Add 0.1 g of sodium hydroxide. Dilute to the mark with deionized water and mix well. Store in plastic containers at room temperature. Stable for at least 1 year.

5.6 Standards/Controls

5.6.1 Purchased

Cyanide Stock Standard (Reagent grade)
Sodium Azide (Reagent grade)

5.6.2 Prepared

Cyanide Stock Standard (0.2 mg/mL)
Prepared by adding 50 mg of potassium cyanide to a 100-mL volumetric flask containing 2 mL of 5 N NaOH. Dilute to volume with deionized water and mix thoroughly. Stable for at least one year.
Dilute Cyanide Stock Standard (25 µg/mL; needed for controls only)
See the Safety Section prior to handling cyanide. Prepared by adding 1.25 mL of Cyanide Stock Standard to a 10-mL volumetric flask containing 0.2 mL of 5 N NaOH. Dilute to volume with deionized water and mix thoroughly. Store in plastic at room temperature. Stable for at least one year.
Azide Stock Standard (1 mg/mL)
See the Safety Section prior to handling azide. Using a plastic spatula, weigh out 15.5 mg of sodium azide into a tared plastic weigh boat. Add sufficient 0.4% sodium hydroxide solution to dissolve the powder. Pour the solution into a 10-mL volumetric flask and dilute to the mark with 0.4% sodium hydroxide. Store in plastic containers at room temperature. Stable for 1 year.
Azide Working Standard (100 µg/mL)
See the Safety Section prior to handling azide. Add approximately 5 mL of 0.4% Sodium Hydroxide to a 10-mL volumetric flask. Add 1 mL of the Azide Stock Standard (1 mg/mL).

Dilute to the mark with 0.4% Sodium Hydroxide. Store in plastic containers at room temperature. Stable for 1 year.
Negative Control
Deionized water, blood, or an appropriate matrix blank is used as a Negative Control. Note that some materials are preserved with sodium azide, and would therefore be not be appropriate for use as an azide negative control. Verify the negative control matrix does not contain sodium azide prior to use.
0.04% Acetonitrile (v/v) (NPD Internal Standard)
Add 40 µL acetonitrile to about 90 mL deionized water in a 100-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store at room temperature in a tightly sealed glass or plastic container. Stable for 6 months.
0.1% 1-butanol (v/v) (GC/MS Internal Standard)
Prepared by dissolving 0.1 mL of 1-butanol in 50 mL of deionized water in a 100-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Stable for 6 months.
Cyanide Positive Controls – Qualitative (1 and 10 µg/mL)
Add 20 µL of the Dilute Cyanide Stock Standard to 0.5 mL of Negative Control (1 µg/mL) and 25 µL of the Cyanide Stock Standard to 0.5 mL of Negative Control (10 µg/mL). Prepare fresh on the day of analysis.
Azide Positive Controls (2 and 20 µg/mL)
Add 10 µL of Azide Working Standard to 0.5 ml of Negative Control (2 µg/mL) and 100 µL of Azide Working Standard to 0.5 ml of Negative Control (20 µg/mL). Prepare fresh on the day of analysis.

6 PROCEDURE

The selection of sample, vial, internal standard, acid, and headspace injection volumes may be adjusted depending upon the case need and autosampler configuration, so long as the ratios are kept proportionate. Analysis of smaller amounts of sample will impact limits of detection/quantitation.

Note: Sample preparation for all methods should be performed in a chemical fume hood.

6.1 Screening of Samples for Cyanide by GC/NPD

☐	Thoroughly homogenize solid or bulky specimens to ensure a representative aliquot is sampled. Tissue, stomach contents and food specimens are mixed (1:1) with deionized water prior to blending. Liquid samples (blood and beverages) can be directly assayed.		
☐	Measure and record the pH of all food specimens and stomach contents. Large quantities of inorganic cyanide salts may be indicated by an alkaline pH (>10).		
☐	Measure 0.5 mL of liquid sample or 1 gram of homogenate into a 10-mL headspace vial.		
☐	Control Preparation		
☐	Add 50 µL of 0.04% acetonitrile (Internal Standard)		
☐	Immediately cap		
☐	Using a 1.0 mL syringe, inject 0.5 mL of 5 N H_2SO4 into the vial through the cap septa.		
☐ ☐	Thoroughly vortex the sample to uniformly distribute the acid. *Remove acid residue from septum/cap with a water dampened wipe to reduce damage to the headspace sampling needle.*		
☐	Allow the sample to equilibrate at room temperature for 30 minutes.		
☐	Analyze headspace by GC/NPD (Section 7.1)		
☐	Upon completion of the analysis, check and record the pH of the sample matrix in the vial to ensure acidity (pH<2) for negative results. Replacing the GC inlet septa is recommended post-sequence due to remaining acid residue.		

6.2 Confirmation of Samples for Cyanide, and Screening/Confirmation of Samples for Azide by Headspace GC/MS

☐	Thoroughly homogenize solid or bulky specimens to ensure a representative aliquot is sampled. Tissue, stomach contents and food specimens are mixed (1:1) with deionized water prior to blending. Liquid samples (blood and beverages) can be directly assayed.		
☐	Measure and record the pH of all food specimens and stomach contents. Large quantities of inorganic cyanide or azide salts may be indicated by an alkaline pH (>10).		
☐	Measure 0.5 mL of liquid sample or 1 gram of homogenate into a 10-mL headspace vial.		
☐	Control Preparation		
☐	Add 50 µL of 0.04% acetonitrile (Internal Standard)		
☐	Immediately cap		
☐	Using a 1.0 mL syringe, inject 0.5 mL of 5 N H2SO4 into the vial through the cap septa.		
☐ ☐	Thoroughly vortex the sample to uniformly distribute the acid. *Remove acid residue from septum/cap with a water dampened wipe to reduce damage to the headspace sampling needle.*		
☐	Allow the sample to equilibrate at room temperature for 30 minutes.		
☐	For cyanide analysis, analyze the headspace by GC/MS using the instrumental parameters in Section 7.2.		
☐	For azide analysis, analyze the headspace by GC/MS using the instrumental parameters in Section 7.3.		
☐	Upon completion of the analysis, check and record the pH of the sample matrix in the vial to ensure acidity (pH<2) for negative samples. Replacing the GC inlet septa is recommended post-sequence due to acid residue.		
	Repeat examinations as necessary to confirm target analytes.		

7 ANALYTICAL PARAMETERS

7.1 GC/NPD Analysis of Hydrogen Cyanide

7.1.1 Headspace Sampler Parameters

Syringe	2.5 ml-HS	**tray type**	VT32-10/20
oven / syringe temp.	45ºC / 55ºC	**Fill/injection volume**	2500 / 250 µl
flush time	4.0 min	**fill speed / strokes**	500 µl /sec / 5
incubation time	5.0 min		
agitator speed	250 rpm	**injection speed**	1000 µl / sec
agitator on/off time	10 s / 1 s	**Inj./Vial penetration**	40 /25 mm

7.1.2 Gas Chromatograph and NPD Parameters

Oven Parameters		**Inlet and Carrier Parameters**		**NPD Parameters**	
temperature	110ºC	inlet temperature	150ºC	temperature	225ºC
ramp	4 ºC/min	purged packed inlet pressure	10.894 psi	makeup flow	3.5 mL/min
final temperature	130 ºC	carrier gas	N_2	air flow	60 mL/min
final hold time	5 min	carrier mode	constant flow	hydrogen flow	3.0 mL/min
Column Parameters		carrier flow	1.5 mL/min	electrometer	on
type	RT-QS-BOND				
length	30 m				
internal diameter/film	320 µm /10 µm				

7.2 GC/MS Analysis of Hydrogen Cyanide

7.2.1 Headspace Sampler Parameters

Syringe	2.5ml-HS	**tray type**	VT32-10/20
oven / syringe temp.	45ºC / 55ºC	**Fill/injection volume**	2500 / 1000 µl
flush time	4.0 min	**fill speed / strokes**	500 µl /sec / 5
incubation time	5.0 min		
agitator speed	300 rpm	**injection speed**	1000 µl / sec
agitator on/off time	10 s / 1 s	**Inj./Vial penetration**	40 / 22 mm

7.2.2 *Gas Chromatograph Parameters*

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	60ºC	**inlet temperature**	150ºC	**type**	DB-624
hold 1	1.5 min	**injection mode**	split	**length**	30 m
ramp 1	50ºC/min	**carrier gas**	ultrapure helium	**internal diameter**	0.25 mm
temperature 2	120ºC	**carrier mode**	constant pressure	**film thickness**	1.4 µm
hold 2	2.5 min	**carrier pressure**	6.54 psi		
total run time	5.7 min	**split ratio**	10:1		

7.2.3 *Mass Spectrometer Parameters*

ionization mode	electron ionization(+)	**source temperature**	230ºC
solvent delay	1.5 min (approximate)	**transfer line temperature**	260ºC
scan mode	SIM	**quadrupole temperature**	150ºC
group 1 (1.65 – 2.7 min)	m/z 12, 26, 27	**multiplier offset**	+200 V
group 2 (2.7 – 10.2 min)	m/z 31, 41, 56		

7.3 GC/MS Analysis of Hydrazoic Acid

7.3.1 *Headspace Sampler Parameters*

Syringe	2.5 mL	**Injection Volume**	1000 µL
Syringe Temperature	55°C	**Injection Speed**	1000 µL / s
Flush Time	240 s	**Pullup Delay**	0 s
Incubation Temperature	45°C	**Fill Volume**	2500 µL
Incubation Time	10.00 min	**Fill Strokes**	5
Agitator On/Off Time	10 s / 1 s	**Fill Speed**	500 µL / s
Agitator Speed	300 rpm	**Pre/Post Inj. Delay**	0/0 s
Inj./Vial Penetration	40 / 22 mm	**Sample Tray Type**	VT32-10/20

7.3.2 *Gas Chromatograph Parameters*

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temp 1	40ºC (1.5 min)	**inlet temperature**	150ºC	**type**	DB-624
temp 2	110ºC @ 15ºC/min (0 min)	**injection mode**	split	**length**	30 m

temp 3	250 ºC @ 35ºC/min (4 min)	**carrier gas**	ultrapure helium	**internal diameter**	0.25 mm
Eq. Time	0.2 min	**carrier mode**	constant pressure	**film thickness**	1.4 µm
Run Time	14.167 min	**carrier pressure**	5.463 psi	**split ratio**	10:1

7.3.3 Mass Spectrometer Parameters

ionization mode	electron ionization(+)	**source temperature**	230ºC
solvent delay	1.9 min (approximate)	**transfer line temperature**	260ºC
scan mode	SIM (high resolution)	**quadrupole temperature**	150ºC
group 1	m/z 15, 29, 43 (65ms dwell) m/z 45, 58 (15ms dwell)	**multiplier offset**	+200 V

8 DATA ANALYSIS

8.1 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed calibrator or extracted positive control. In most cases, all of the below should be met in order to identify cyanide or azide within a biological specimen.

8.1.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs.

Additionally, the following two criteria should be met.

8.1.1.1 Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of a calibrator or extracted Positive Control of cyanide.

8.1.1.2 Signal-to-Noise

To justify the existence of a peak, its signal-to-noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10-fold greater than that for any observed peak at a similar retention time in a Negative Control or blank injected just prior to that sample.

8.1.2 Mass Spectrometry

The mass spectrum of the analyte of interest should compare favorably that of a reference standard, extracted calibrator, or an extracted Positive Control. See TOX-104 for further guidance.

8.1.3 Batch Acceptance

No analytes of interest should be detected in a Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected. See TOX-101 for further guidance.

8.2 Calculations

See the TOX-101 for guidance.

9 REPORTING

9.1 Measurement Uncertainty

Refer to CHEM-100 and TOX-101 for guidance.

9.2 Report Phrases

As this procedure generates hydrogen cyanide and hydrazoic acid which are then detected by GC/NPD or GC/MS, reported positive results will include language stating the limitations of this technique. Suggested language includes, but is not limited to:

> *The identification of hydrogen cyanide in the Item 1 blood sample is indicative of exposure to a cyanide containing compound. Various cyanide compounds will produce hydrogen cyanide under acidic conditions, including sodium cyanide and potassium cyanide.*
>
> *The identification of hydrazoic acid in the Item 1 blood sample is indicative of exposure to an azide containing compound. Various azide compounds will produce hydrazoic acid under acidic conditions, including sodium azide and lead azide.*

9.3 Interpretation

Postmortem blood specimens may contain endogenous cyanogenic sources that render uncertain the toxicological significance of amounts less than 0.2 µg/mL. Also, it is not uncommon to find trace amounts of cyanide in the blood of smokers.

10 CORRECTIVE MEASURES

Refer to TOX-101 for guidance.

11 PERFORMANCE CHARACTERISTICS

11.1 Cyanide Analysis

11.1.1 Limit of Detection

GC/NPD: 0.65 µg/mL (water) 0.50 µg/mL (blood)
GC/MS: 1.0 µg/mL

11.2 Azide Analysis

11.2.1 Limit of Detection

GC/MS: 2 µg/mL in blood and aqueous matrices

12 LIMITATIONS

12.1 Interferences

Azide will interfere with the NPD detection of cyanide but is chromatographically resolved from cyanide on the GC/MS, and so will not cause a false positive result. However, positive cyanide results must be confirmed by GC/MS analysis. Grossly decomposed or putrefied samples may affect both detection limits.

13 SAFETY

Take standard precautions for the handling of chemicals and biological materials. Acids liberate hydrogen cyanide gas and care must be taken to isolate acid solutions from cyanide sources. Collect all cyanide waste as a separate waste stream.

Similarly, acids also liberate toxic hydrazoic acid, which then constitutes an inhalational hazard. Additionally, sodium azide in its powder form constitutes an explosion hazard. Handling of the powder is performed with plastic spatulas and plastic weigh boats. All manipulations of the solid material must take place within a functioning fume hood. For storage of the powder and the solutions, keep azide materials segregated from acids and metals. For disposal, do not discard sodium azide down the sink or mix with acidic waste. Collect all sodium azide waste as a separate hazardous waste stream.

Refer to the *FBI Laboratory Safety Manual* for guidance.

14 REVISION HISTORY

Revision	Issued	Changes
04	02/11/2022	Document reformat. Updated language in Sections 1 and 2. Removed reference to TOX-103. Updated cyanide analysis to qualitative only. Added language in procedure to specify wiping of septa. Updated hazardous waste disposal language.