Blood Analysis for Ethyl Alcohol
By Headspace Gas Chromatography

PRINCIPLE

This procedure outlines a simple method for the detection and quantitation of ethanol in blood samples by "Headspace" gas chromatographic procedure. Static headspace offers a reliable, simple and accurate way to quantitate volatile compounds in a variety of liquid matrices. Practically, the sample and an internal standard are added to a vial and the vial sealed. The sealed bottle is then placed into the heated sample carousel of the Headspace Analyzer. The increased temperature causes the volatiles to be released from the solution into the "headspace" above the liquid. The headspace is then sampled and analyzed for the presence of the targeted analytes via gas chromatography.

g% = g/dl; e.g. 0.08g% = 0.08g of alcohol in 100ml of blood same as 0.08g/dl

ANALYTES DETECTED

Methanol, Ethyl Alcohol, Isopropanol and Acetone.

Definitions:

Alcohol – Any substance containing any form of alcohol, including Ethanol, Methanol, Propanol and Isopropanol. N.C.G.S. 20-4.01 (1a)

Alcohol Concentration – the concentration of Alcohol in a person, expressed as: Grams of Alcohol per 100 milliliters of blood. N.C.G.S. 20-4.01 (1b) a.

Chemical Analyst – A person granted a permit by the Department of Health and Human services under G.S 20-139.1 to perform chemical analyses. N.C.G.S. 20-4.01(3b).

MATERIALS

Control and Calibrators

0.05% n-Propanol in water (Internal Standard):
To a 1 L volumetric flask, add 0.05 mL (500 µL) of n-Propanol . Fill to the mark with DI water. Mix well, and transfer to reagent bottle and label with name of reagent, date of preparation, lot number -the date in numeric format, initials of preparer: 021212LN and expiration date.
Storage: Room temperature.
Expiration: 1 year

Ethyl Alcohol Calibrators [Cerilliant/Restek] or equivalent:

0.05 g%, 0.100 g%, 0.300 g% and 0.40 g%
Storage: Refrigerator
Expiration: as labeled

Multi Volatile Calibrator

Commercial Calibrator [Cerilliant Multi-component Alcohol Mix Cat # A-057 or equivalent]
e.g. 0.05 g% target concentration.

Mixed Volatile Control:

Commercial Control [Cerilliant Multi-component Alcohol Mix Cat # A-056 or equivalent]

e.g. Target Concentration: 100 mg%

Positive Control

0.08 g%, 0.15g%, or other controls available

Storage: Refrigerator

Expiration: see label

Prepare a 0.08g/100mL of Ethanol control

To a 100mL volumetric flask, add 100.5 ul of 200 proof ethanol and top with DI water. Keep the flask stoppered at all times. After verification, transfer to a container and label with reagent name, lot # and initials. Store in refrigerator; Aliquot and freeze.

Expiration date: as labelled for commercial or 1 year for in-house if frozen then 30-days after thawed.

This solution can be prepared in any amount provided the components ratio is kept constant.

Negative Blood:

(Lampire Bovine blood in NaF Cat# 7200815)

Storage: Refrigerator/Freezer

Expiration: 24 months if frozen, 30 days after thawed and stored in refrigerator.

PROCEDURE

Ethanol present in blood samples can be detected and quantified.

This method is used to determine ethanol in blood samples. The following compounds can also be determined by this method:

- Methanol
- Isopropanol
- Normal Propanol
- Acetone

APPARATUS AND MATERIALS

Heated headspace sample introduction apparatus, Agilent G1888 /GC 7890.

Chromatography Data System - Agilent Technologies GC ChemStation™ software (Optimized in accordance with vendor recommendation of revision changes).

Columns: Agilent J&W (DB-ALC1) 30m, 0.320 mm i.d., 1.8 µm film thickness or equivalent.

20 mL glass headspace vials, butyl stoppers and aluminum crimp tops.

Manual hand crimper for headspace vials.

Auto-dilutor (Hamilton Microlab Diluter) or calibrated pipettes

Ethyl Alcohol Internal Standard (0.05% n-Propanol)

Ethanol, 200 proof ACS grade

Calibration standards used

- 0.05, 0.10, 0.30, 0.40 % Ethanol or equivalent,
- Multicomponent Alcohol (0.05 or 0.10 %) Cerilliant Standards or equivalent
- Commercial control (Utak or equivalent)
- A known Target concentration e.g. . 0.08,0.15 % (in blood and/or DI water).
- Additional calibration may be used upon approval from the supervisor.

Deionized Water

Negative Blood

Kim Wipes

Bleach solution

Syringes – All Teflon with leur lock fitting for auto-diluter

0.25 mL Hamilton Gas Tight #1725

2.5 mL Hamilton Gas Tight # 1002

Calibrated pipettes, 1.0 mL and 0.25 mL for manual preparation

SAMPLE HANDLING

All evidence submission shall be according to current Lab evidence policies. This policy mandates specific requirements that shall be adhered to, *in addition*, to the policies outlined in the Lab Quality Manual (QM4.4) and Policy Manual (PM4.1).

All evidence shall be submitted to the receiving analyst and custody transferred within the PLIMS/Property Control System. The receiving analyst shall verify contents and assure that the evidence submitted is consistent with the description. In the event of a discrepancy, the analyst will note the discrepancy in PLIMS, email the case officer and return custody of the item to Property Control for correction.

Blood kits received should correspond to the evidence requested for analysis. BAC will be analyzed on grey top tubes preferably. Blood samples in which the blood cells have been separated from the liquid (including serum and/or plasma) *see calculation on page 8 (calculations – Serum and plasma).* Make note of any abnormalities in the packaging of the blood tubes. The name on the tube should match the name in PLIMS. Report will be generated using the name on the Blood tube.

Each sample will be run in duplicate. The appropriate calibration standards and controls will be run with every batch.

After the analysis is complete and no repeating of analysis is necessary, the evidence can then be properly resealed and labeled per the "Quality Manual" and returned to the property and evidence, releasing the chemist from custodial responsibility of the evidence.

INITIAL SETUP

Label a series of 20 mL headspace vials as follows: Ethanol calibrators, Multicomponent Alcohol Calibrator, Negative Control-1, Positive Controls-1, Mixed Volatile control, samples, Negative Control #2 and Positive Controls #2. [*See page 5 for preferred position allocation of run sequence for case samples].*

All samples will be run in duplicate; however samples with a small volume may be run in singlet. Record all information on the Headspace load list.

Check volume of Internal Standard and settings on the Hamilton Pipettor/Dilutor.

Press the left "SIZE" button. The display will indicate the size of the syringe on the left side of the instrument. It should read "1000", and the syringe should be a 1.0 mL (1000 µL) Hamilton syringe. If the display is incorrect, press the "increase" or "decrease" arrows until the display indicates "1000".

Press the right "SIZE" button. The display will indicate the size of the syringe on the right side of the instrument. It should read "1000", and the syringe should be a 1.0 mL (1000 µL) Hamilton syringe. If the display is incorrect, press the "increase" or "decrease" arrows until the display indicates "1000".

Press the left "VOLUME" button. The display will indicate the volume (in µL) of internal standard solution the pipettor will deliver when the button is pushed. It should read "1000". If the display is incorrect, press the "increase" or "decrease" arrows until the display indicates "1000".

Press the right "VOLUME" button. The display will indicate the volume of sample (in µL) the pipettor will deliver when the button is pushed. It should read "250". If the display is incorrect, press the "increase" or "decrease" arrows until the display indicates "250".

ALIQUOTING

Retrieve appropriate specimen(s) from the refrigerator. Place all specimen tubes on the tube rocker (or manually invert several times) to produce a homogenous sample. Allow all tubes to come to room temperature for approximately 30 minutes. Verify tubes have reached ambient temperature by touch.

Place tip of pipettor into the calibrator, controls or sample container and activate the sample withdrawal by pushing the switch. This will pull 250 uL of the sample into the pipettor tip.

Wipe the tip of the pipettor, place the tip in the appropriately labeled headspace vial and press the trigger once. This will dispense 250 µL of sample or calibrator into the vial, followed by 1 mL of the internal standard. The internal standard is diluted with sufficient water that it acts as a wash solution, clearing the lines of sample. If sample is still visible, rinse until clear.

Cap headspace vial with septum stopper/aluminum seal and crimp the seal with the crimper.

Repeat above steps until all calibrators, controls and samples have been aliquoted.

The following is the preferred position allocation of run sequence for case samples.

Ethanol 50 mg/dl – Level 1 calibrator

Ethanol 100 mg/dl – Level 2 calibrator

Ethanol 300 mg/dl – Level 3 calibrator

Ethanol 400 mg/dl – Level 4 calibrator

MultiVolatile Alco mix 500 mg/dl – Level 5 calibrator

Blank Whole Blood (Neg. Control) 1

Ethanol 80 mg/dl – Pos. control 1

Ethanol 150 mg/dl – Pos. control 1

Mixed –Volatile 1000 mg/dl Pos. control

Case Samples {up to 20 vials *(10 samples)*}.

Blank Whole Blood (Neg. Control) 2

Ethanol 80 mg/dl – Pos. control 2

Ethanol 150 mg/dl – Pos. control 2

GC AND DATA ACQUISITION PARAMETERS

Headspace/Agilent 7890 GC/FID

Method

GC Method:	BLD_ALC.M
Column:	30 m x 0.53 mcm DB_ACL1 or equivalent
Oven Program:	40ºC (Isothermal)
Detector Temperature:	280ºC
Headspace Oven:	75ºC
Loop Temperature:	90ºC
Transfer Line:	100ºC

Note: Final oven hold may vary-to achieve full elution of internal standard

Place all of vials in the carousel of the Headspace Analyzer, beginning with position. The order will be as listed in *Initial Set-Up*.

Batch Creation

See PLIMS manual for further details.

In order to analyze a "batch" (i.e one or more samples with a controls and calibrators), a batch must be created in PLIMS and exported to the instrument.

In PLIMS, go to Batch Create and create a new worklist by selecting the items to be analyzed in that batch. Once the batch has been selected hit "Update and Print" to create the worklist number.

Once the worklist has been created, go to "Batch Results" and load the new worklist. Export the worklist so it will be sent to ChemStation.

Go to ChemStation and enter "Sequence" and then "Sequence Parameters", and enter the data directory as the current date, i.e., 053112, and exit. When the instrument asks if you would like to create this directory, say, "Yes".

Under "Sequence" go to "Import worklist" to import the worklist just created which can be found: c:\labsave\xmlseq. This will import the batch of samples as well as all the controls and calibrators.

Exit, and save the sequence as the current date, i.e., 053112.s, or 070412.s.

Print Sequence List and compare against "Load list".

Enter "Run Control", and hit "Run Sequence" or go to sequence table and hit "Run".

After completion of a batch run, verify identity/location of each vial on the auto sampler against the sequence list prior to removal from the auto sampler. Document verification by initialing the sequence list printed.

Calibration

The instrument is calibrated with each batch.

After the instrument has completed data collection for the calibrator(s), go into the "Data Analysis" screen.

Load the data file for the 0.050 g% ethanol calibrator.

From the "Calibration" menu at the top of the window, enter "Calibrate/Recalibrate". The instrument will display a parameters screen.

On the parameters screen, click on the button for "Replace" under the "Recalibration" header.

Make sure that the "Level" is "1". NOTE: Level 2 for 0.10 g%, Level 3 for 0.30 g%, Level 4 for 0.40 g% calibrators.

Click on "OK".

The instrument will give you a message "Calibration peak(s) missing. Calibrate the identified peak(s)?" Click on "OK".

Repeat the steps above for all remaining calibrators.

Go to "File" and "SAVE-Method", this will save the calibration.

The instrument is now calibrated for ethanol.

Data Processing

Select Calibration Table Window and highlight Ethanol.

Make sure the printer is set to "Black ice Color Plus" if printing to PLIMS or to the associated printer

Select Calibration Curve Window and Print [File/Print].

Go to Data Analysis, Report, Specify Report and Select Printer. Then "OK".

Click on the "Report Icon". Load Signal "1". Select Signal 1 and Print.

Repeat the above until all runs are printed.

Back in PLIMS go back to "Batch Result" and pull up the appropriate worklist. Click on "Import" and all the data, controls, and calibrators will be imported as associated to the selected worklist. The QA/QR rules will automatically run on the data and reject any values out of range.

If the batch passes all QA/QC rules click on "Ready for Review" to create results packets that can be reviewed along with the report.

CALCULATIONS

The ChemStation will calculate the concentration of the volatiles and print a report. Should a manual calculation be required, the following formula should be used. Use the data from the multi standard and the unknown, and insert the appropriate values in the following formula:

$$\text{Concentration in unknown sample} = \frac{(Avp)(Aiss)(Cs)}{(Avs)(Avisp)}$$

Where

Avp = Area counts of the sample volatile of interest
Aiss = Area counts of the internal standard on the multi std injection
Cs = Concentration of the analyte in the multi standard
Avs = Area counts of the multi volatile of interest
Avisp = Area counts of the internal standard on the sample injection

Example:

In a hypothetical injection of the multi-volatile calibrator, the area counts for acetone are 19982, for the internal standard are 22300. The concentration of acetone in the multi is 0.024g%. In our hypothetical sample, the acetone counts are 34113 and the internal standard counts are 20013. Thus,

Avp = 34113 Cs = 0.024 Avs = 19982 Aiss = 22300 Avisp = 20013

$$\text{Concentration} = \frac{(34113)(22300)(0.024)}{(19982)(20013)} = 0.045 \text{ g\% Acetone}$$

Serum and Plasma:

Serum is obtained by allowing blood to clot while Plasma is the liquid separated from whole blood with an anticoagulant. For alcohol determination, the concentration in both Serum and Plasma are the same. The average ratio of Serum/Plasma Alcohol concentration to whole blood is approximately 1.18 (Garriot's Medicolegal Aspects of Alcohol *5th edition, page 207*)
Example:
To convert Serum alcohol to whole blood equivalent:
Convert units to g/100mL.
mg/dl = mg/100 mL
mg/100mL = g/100 mL
1000
120 mg/dL = 0.120 g/dL (serum alcohol concentration).

$$\text{Whole blood Alcohol concentration} = \frac{\text{Serum/plasma Alcohol concentration}}{1.18}$$

$$= \frac{0.120}{1.18} = 0.101 \text{ g/100mL} = \text{average whole blood equivalent.}$$

QUALITY CONTROL

For equipment maintenance and validation see Drug ID SOP 5.6, 5.7

In order for a run to be acceptable:

Chromatography

In general, all peaks should be symmetrical in appearance, without shoulder or excessive tailing.

There must be baseline separation between analyte(s) and internal standard peaks.

The retention times of the ethanol peak and the n-Propanol internal standard peak must be within 0.02 minutes of the retention times of the same peaks in the calibrator.

Calibration

If more than one calibrator fails to meet quality control, the curve is invalidated and must be repeated.

The concentrations in the controls must be within 10% of the target.

The ethanol concentration in the negative control must be less than 0.005 g%.

Calibrators must back-calculate within ±5% or 0.005g/dL, whichever is greater, of known values. BAC control chart form (Levy Jennings Plots) should be noted after each run.

The concentrations of the duplicate injections for a sample must each be $\pm$ 5% of the mean of the two injections.

The correlation coefficient of the calibration line [R2] must be >0.995.

LINEARITY

The linearity of the method is validated on an annual basis.

The curve will contain six data points, ranging from 0.01 g% to 0.50 g%. A linear regression analysis (least squares) will be performed on these data. An acceptable correlation coefficient for the range will be 0.995 or greater. The LOD/LOQ will be the lowest standard, which is within +10% of the target and the ULOL will be the highest standard that falls within +10% of the target and shows no carryover.

The LOD/LOQ and ULOL of the method shall be determined on an annual basis.

REPORTING

The results shall be truncated to two digits after the decimal point [e.g. 3.254 to 3.25 g%]. Values below 0.02 g% [truncated] should be repeated as negative. Samples with values above the upper end for linearity, >0.50 g%, may be repeated after diluting and re-assayed.

See sample dilution table below.

DILUTION TABLE

Dilution	Amount of Specimen (ml)	Amount of DI Water/Matrix (ml)
1:2	1	1
1:4	1	3
1:5	1	4
1:10	1	9

DOCUMENTATION

The case file will contain all documentation related to a request for analysis and all communications about the case.

CASE FILE CONTENTS

Administrative Documentation (if available)

Laboratory Request Forms (if available)

Laboratory work sheet (if available)

Case related conversations/correspondence (if any)

The file in PLIMS shall contain, upon completion, a copy of the issued report, any addendum/supplemental reports, and corrected/amended reports.

Each page within the case file should have at a minimum the complaint and/or PLIMS Lab # for the case and the analyst's initials.

The laboratory case files shall be maintained in secured areas of the Crime Laboratory.

UNCERTAINTY OF MEASUREMENT

General

Estimation of uncertainty shall be determined where the testing contains measurement results that are quantitative, reported and may reasonably be expected to be used, by an immediate or extended customer (anyone in the judicial process) to determine, prosecute or defend the type or level of criminal charge(s).
The uncertainty measurement will include at a minimum the identification and assessment of the major sources of uncertainty in the procedure which are of importance to the process. This may include the methods, instrument/equipment, special environmental conditions, types of evidence tested, the reference standards used and the operator. The sources of uncertainty will be classified as Type A or Type B components.
An uncertainty budget table shall be completed to include both Type A and Type B components of uncertainty for the process of determining blood alcohol concentration. Uncertainty budget should be re-evaluated on an annual basis. The actual uncertainty budget table and calculations/data will be maintained in separate binder located in the Chemistry Section of the laboratory.
Calculations used to estimate the uncertainty should be rounded up to be conservative.
The units of uncertainty values should be measured (converted) in the same units.
The reported UOM shall not exceed two significant figures.
The 99.7% confidence interval will be used for reporting measurement of uncertainty as it pertains to determining blood alcohol concentration.

Identification of Uncertainty Components:

Staff:		Procedure:		Facility:		Calibrators:		Internal Standard:		QC :		Instrumental Analysis:	
knowledge	A	Vortex samples	A	temperature	A	Ref. Material COA	B	purity	B	Ref. Material COA	B	Calibration curve	A
experience	A	Diluting with	A	humidity	A	Cal.	A	solvent	A	QC	A	Integration	A

		IS				uncertainty				uncertainty		parameters	
training	A	Sealing HS vials	A	barometric pressure	A	Volume error	A	Pipetting technique	A	Volume error	A	Injection volume	A
Compliance with SOP	SOP			vibrations	A	pipetting	A	Reading meniscus	A	pipetting	A	Gas flow	A
Multiple staff performing	A			limitations of workspace size	A							Instrument method	A
Operator technique	A											Process algorithm	A
distraction	A												
Mood of operator	A												

Definitions for Uncertainty of Measurement

Type A evaluation of uncertainty is a method by statistical analysis of a series of observations.

Type B evaluation of uncertainty is a method by means other than the statistical analysis of a series of observations.

The mean is defined as the sum of the measured values divided by the total number of values: $X = \frac{\Sigma x_i}{n}$

Where X is the mean,
x_i is the different measured values,
n is the number of measured values

The standard deviation measures how closely the data are clustered about the mean. The standard deviation is defined as

$$s = \sqrt{\Sigma (x_i - X)^2 / n-1}$$

where: s is the standard deviation
X is the mean,
x_i is the different measured values
And n is the number of measured values

The confidence interval is an expression stating that the true mean, μ, is likely to lie within a certain distance of the measured mean, X. The confidence interval of μ is given by:

$$\mu = X \pm \frac{ts}{\sqrt{n}}$$

where s is the measured standard deviation,
n is the number of measured values, and
t is a number from the Student's t table for a certain confidence interval.

Sometimes the values are more likely to fall near the average than further away. This is typical of a *normal* or *Gaussian* distribution. This is graphically represented by a bell curve. Normal distribution uses the above equations for determining measured standard uncertainty.

When the measurements are quite evenly spread between the highest and the lowest values, a *rectangular* distribution is produced. The standard uncertainty for a rectangular distribution is:

$$\frac{a}{\sqrt{3}}$$

Where: a is the semi-range (or half-width) between the upper and lower limits.

Estimating the Uncertainty of Measurement

The factors to be considered in determining the uncertainty of the measurement are: pipette use, uncertainty associated with preparing the internal standard, preparing quality control samples, preparing blood alcohol samples, and preparing calibrators, repeatability, uncertainty associated with the manufactured standards, instrument variations, and analyst pipetting technique differences.

The Chemistry section relies on the use of quality controls to establish the historical standard deviation for blood alcohol concentration cases.

The quality controls are documented for each batch on the Headspace Volatiles Loadlist.

Use of quality controls eliminates most of the uncertainty associated with pipet use, preparation of internal standard, preparation of QC standards, preparation of samples, preparation of calibrators, and analyst pipetting technique differences.

The use of an internal standard for quantitative analysis minimizes other sources of uncertainty including instrument variations. Therefore this is not included in the uncertainty calculation.

Example of a budget table for Blood Alcohol Analysis by Headspace:

Component	**Value**	**Units**	**Distribution**	**Type**	**Divisor**	**Degrees of Freedom (n-1)**	**Standard Uncertainty**	**Relative Contribution**
Process Reproducibility	0.0015	g/dL	normal	A	1	24	0.0015	40 %
Positive Control UOM from COA	0.0033	g/dL	normal	B	2	Infinite	0.0016	45 %
Calibrator UOM from COA	0.0011	g/dL	normal	B	2	Infinite	0.0005	15 %
	Combined Standard Uncertainty (Uc)						0.0023	100 %

Calculation of Combined Uncertainty

The combined uncertainty will be determined when more than one standard uncertainty is shown to be a major contributor to the uncertainty of measurement.
The combined uncertainty will be calculated using the Root Sum Squares (RSS) method. The RSS equation is defined as the square root of the sum of the squares of the data points.

$$U_{combined} = \sqrt{u_1^2 + u_2^2 + u_3^2}$$

Calculation of Expanded Uncertainty

The expanded uncertainty is used as a measure of uncertainty that defines a confidence interval about the measurement result. It is the combined uncertainty multiplied by the confidence factor (k).

$$U_{expanded} = U_{combined}(k) \text{ where } k = 3, \text{ at the 99.7\% confidence level}$$

For the BAC example, the expanded uncertainty at the 99.7 % confidence level is:

$$U_{expanded} = 0.0023 \text{ g/dL } (3) = 0.0069 \text{ g/dL}$$

Recording and Reporting

The measurement of uncertainty will be recorded in the case notes to the third decimal place and on the report to the second decimal place as required by the state of North Carolina.

e.g. Blood alcohol analysis with an average concentration of 0.1480g/dL $\pm$ 0.06 g/dL on the worksheet; the Report should have the following recorded:

Results: 0.14g/dL $\pm$ 0.006 g/dL.

A statement will be added to the report, ‘All ± values are reported at a confidence interval of 99.7 %.’

REFERENCES

James C. Garriott. Medicolegal Aspects of Alcohol. 5th Ed.

Randall C. Baselt. Disposition of Toxic Drugs and Chemicals in Man. 2nd Ed.

B.L. Glendening and R.A. Harvey; "A Simple Method using Headspace Gas Chromatography for Determination of Blood Alcohol by Gas Chromatography." J For Sci 14:136-145 (1969).

Richard Saferstein; Criminalistics- An Introduction to Forensic Science.

R.M. Anthony, D.A. Sutheimer, and I. Sunshine; "Acetaldehyde, Methanol and Ethanol Analysis by Headspace Gas Chromatography." J Anal Tox 4:41-42 (1980).

American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) manual.

Scientific Working Group for Forensic Toxicology (SWGTOX) Recommendations

ASCLD/LAB-*International* Guidance on Measurement Traceability, May 2013.

ASCLD/LAB-*International* Guidance on Measurement Traceability-Measurement Assurance, May 2013.

ASCLD/LAB-*International* Guidance on the Estimation of Measurement Uncertainty – Overview, May 2013.