ANALYSIS OF ALCOHOLS IN AQUEOUS AND BIOLOGICAL SAMPLES BY HEADSPACE GAS CHROMATOGRAPHY

A. *Introduction:*

Headspace Gas Chromatography is a useful and accurate method to analyze ethanol and other volatile substances in blood and other tissues. Use of two complimentary systems precludes co-elution of other volatiles interfering with ethanol quantification. Results are reported in units of grams of alcohol per 100mL of whole blood, as required by the Washington Administrative Code (WAC 448.14)

B. *Principle and Purpose*

There is a direct relationship between the concentration of a volatile substance (such as ethanol) dissolved in a liquid (such as blood) and the concentration of the volatile substance in the vapor above the solution for a given temperature based on Henry's Law. Headspace gas chromatography utilizes this principle to accurately quantify ethanol and other volatiles in biological fluids and tissues. The volatility of ethanol relative to the aqueous biological specimen is used to separate the volatile from the matrix. The solution is placed in an airtight container and the amount of volatile in the air space above the liquid is proportional to the concentration of the volatile liquid in the solution. Therefore, sampling the headspace of heated specimens and similarly treated ethanol calibrators allow calculation of the ethanol concentration in the specimen.

The headspace vapor is injected onto a capillary column. Separation of different volatiles takes place in the column according to the size of the analytes. A flame ionization detector (FID) is used; wherein a hydrogen/air flame burns at the jet tip and the column effluent exits through the jet into the flame. A constant electrical potential is maintained between the jet and the collector and the gap acts as a variable resistance. When just gas is flowing, this is monitored as baseline. As analyte molecules are ionized in the flame, the resistance decreases, more current flows and this amplified current is the detector response.

The unknown samples are diluted with a solution containing n-propanol as the internal standard and sodium chloride to increase the partial pressure of ethanol and n-propanol.

C. *Acceptable sample types and volumes:*

Serum, plasma, whole blood, vitreous humor, tissue homogenates, urine and aqueous solutions are appropriate samples for analysis. (Solid tissues are weighed, homogenized in deionized water and reported in g/kg.) The volume of sample is 0.2 mL and is diluted with 2.0 mL diluent. Samples with high concentrations may be further diluted with water for re-analysis to get the result within the limits of linearity for the assay.

Approved:

Barry K. Logan, PhD

Date: 5/21/2003

D · Calibration Standards:

The headspace gas chromatograph is calibrated each day it is used, using standards prepared in the Washington State Toxicology Laboratory. The accuracy of the calibration is verified against external quality control samples. See below. The standards are prepared once each week, at a minimum. Solution proportions are calculated based on ethanol density of 0.79g/mL.

For the purposes of this SOP, an assay is defined as all samples run within a 24 hr period, with the same calibration, even if there is more than one sequence.

Materials

Absolute ethanol
Water (deionized, or distilled)
1mL volumetric pipette, grade A
Volumetric flasks (250, 500, 1000mL), grade A
Plastic storage bottles

Using the grade A volumetric glassware, prepare the following:

Standard Concentration	Preparation
Blank	water only
0.079 g/100 mL	1 mL of ethanol in 1000 mL H_2O
0.158 g/100 mL	1 mL of ethanol in 500 mL H_2O
0.316 g/100 mL	1 mL of ethanol in 250 mL H_2O

Preparation of the Ethanol Calibration Standards is documented in the Alcohol Standard Logbook (see appendix A).

Standards are labeled, tightly sealed and refrigerated at 5 °C. when not in use. They are brought to room temperature before use.

E. *Internal Standard:*

The internal standard is prepared as follows:

10 g sodium chloride
0.3 mL n-propanol
diluted to 2 L water.

Mix thoroughly and store at room temperature in a sealed container.

Internal standard is stored at room temperature. Preparation of internal standard is documented in the Alcohol Standard Logbook. Internal Standard expires 30 days after preparation.

F. *Controls:*

Commercially prepared controls are purchased for use in each assay. At a minimum, two (2) control levels are included in each assay. See appendix B for a list of current

controls. After every 10 unknowns, one quality control sample followed by one blank is analyzed.

G. *Non-calibration Standards:*

A 0.02 g/100 mL standard is analyzed with each assay.

0.02 g/100 mL

1 mL absolute ethanol in 4 L H_2O

0.02 g/100mL Standard is tightly sealed, labeled and refrigerated at 5 degrees C. It is brought to room temperature before use.

Preparation of 0.02 mL standard is documented in the Alcohol Standard Logbook and expires 90 days after preparation.

Volatile standards – Two concentrations of commonly encountered volatiles are included as volatile standards in each assay. The two volatiles standards are prepared as follows:

0.04 Volatiles	0.079 Volatile standard
1 mL ethanol	1 mL ethanol
1 mL acetone	1 mL acetone
1 mL isopropanol	1 mL isopropanol
1 mL methanol	1 mL methanol
in 2 L H_2O	in 1L H_2O

Preparation of the volatile standards is documented in the Alcohol Standard Logbook (see appendix A).

Volatile standards are tightly sealed, labeled and refrigerated at 5 degrees C. They are brought to room temperature before use.

Volatile standards expire 90 days after preparation.

H. *Equipment:*

Agilent (Hewlett Packard) 7694 Headspace Autosampler or equivalent
Agilent (Hewlett Packard) 6890 gas chromatograph; one equipped with a J&W DBALC1 megabore (0.53 mm) 30 meter capillary column and another system equipped with J&W DBALC2 megabore (0.53 mm) 30 meter capillary column. (For information on the columns, see appendix C)
Computer System equipped with HP GC Chem Station
Compressed gases; air, nitrogen, hydrogen, helium BKC 7/24/03
Autosampler vials
Cap crimper
Hamilton Automatic Diluter

Approved:
Barry K. Logan, PhD

Date: 5/27/03

I. *Sample Handling:*

All biological samples must be treated as potentially infectious. The blood tube (vacutainer) is initially opened inside of a biological or chemical hood, to protect the analyst from potential aerosol hazard. Precautions should be used to limit exposure to blood and aerosols. The blood sample is inspected, to ensure that the blood is mobile. If the blood appears to be clotted, it may be necessary to homogenize the blood in a tissue homogenizer, prior to aliquoting. *NOTE: ALL TISSUE AND BLOOD HOMOGENIZATION MUST BE CONDUCTED INSIDE A BIOLOGICAL OR CHEMICAL HOOD.*

J. *Analysis:*

1) All unknown samples, standards and controls are analyzed in duplicate.

2) The following standards are used as calibrators:
0.079, 0.158, 0.316 g/100mL
Following the high standard, a blank is analyzed to verify the absence of carryover.

3) A minimum of two control samples is analyzed following the calibrators (and prior to the unknowns) to verify the calibration.

4) The following standards are included in the analysis:
0.02 g/100 mL
0.04 Volatile Mix
0.079 Volatile Mix

5) Auto-pipette 200 µL of blood, control, or standard solution into a 10mL autosampler vial. Add 2mL of internal standard solution. Seal the vial tightly and shake well until homogeneous.

6) Alternating controls (different levels) are repeated periodically throughout the run followed by a blank. Each positive sample should be separated from a commercial control and a blank by no more than ten other samples. If all the samples are not aliquoted at once, the first aliquot should be a control, followed by a blank.

7) Samples are analyzed in duplicate, once on each of two headspace systems, unless otherwise approved by the laboratory manager and or the State Toxicologist due to equipment limitations. (Under certain circumstances, the duplicates may be analyzed on the same instrument, using two different runs and two different calibrations. This is documented in a memorandum for record.)

8) Prepare the sample work list of unknown samples, standards and controls. (Note: it is advisable to run a blank following any badly decomposed sample.) Both alcohol instrument 1 and instrument 2 are controlled by MS Chem Station on one computer. (Back-up alcohol instrument, number 3, is controlled by a separate computer.) On the toolbar in Method and Run Control, select Sequence - Sequence Parameters. Identify the operator and establish a unique subdirectory for the data. The subdirectory should identify the date the analysis was started.

9) Select Sequence, Sequence Table. Enter the standards controls and unknown samples into the sequence table. The calibrators, 0.079 g/100 mL, 0.158 g/100 mL and 0.316 g/100 mL are identified in sample type as calibrators 1, 2 and 3, respectively. The blank following the high standard is identified as a Ctrl Samp in Sample Type as are all of the controls throughout the run. Each unknown and the other standards and blanks are identified as SAMP in the sample type. A maximum of 44 samples may be included in one run.

10) Be certain to identify the method as BLDALCO1 for instrument number 1 and BLDALCO2 for instrument number 2. The injection number is 1. Print the sequence table for each instrument. (The method for the two volatile standards may be selected as VOLATILE.)

11) Open the top of the headspace autosampler. Place the autosampler vials in the numbered positions according to the positions identified in the sequence log table.

12) Select the HP7694 System monitor and click on the Vials icon to specify the number of vials in the run. Click on the start icon. Repeat for the other instrument.

13) Return to the Sequence Table for in Instrument Method & Run Control and select "Run Sequence" for each instrument. Note that the headspace and the software must be started independently.

14) The GC methods for each instrument are found in appendix D.

15) At the conclusion of the run, it advisable to review the data before removing the remove the vials from the autosampler. Autosampler vials are discarded in biohazzardous waste.

16) A sample chromatogram is found in appendix E.

K. *Quality Control and Data Review:*

1) Ensure that the blank following the high standard does not have any peaks present. Ensure that all blanks are devoid of peaks (although those following a decomposed sample may have peaks.)

2) Verify the presence of the internal standard in each analysis. To ensure sensitivity the ISTD area must be at least 2000. Low ISTD area counts may be indicative of a clogged injector needle.

3) Verify that each control is properly identified and quantifies within $\pm$ 0.01 g/100 mL of the target value. Verify that the other standards quantify within $\pm$ 0.01 g/100 mL of their respective target value.

i. If any quality control values are out of range or any of the blanks following a quality control are positive, determine if it is due to incorrect placement of the sample vials in the autosampler. This can be corrected only before any vials are removed from the autosampler, as follows:
   a) Compare the written numbers on the autosampler vials in each

position with the sample ID on the sequence log table.
b) If there is a mismatch, the samples can be moved to the correct position and a partial sequence may be analyzed.
c) Line through the incorrect data, initial and date and indicate the corrective action taken.
d) Document the QC failure and the corrective action in the QC Out of Range Log, appendix F.

ii. If one quality control per analytical run is out of range but within +/- 0.02 g/100 mL of the target value, all positive samples within 10 samples of the failed QC may be realiquotted and reanalyzed.
a) Use the same calibration. It must be within 24 hours of the original calibration time.
b) Use the same internal standard.
c) Include a positive control and a blank at the beginning and end of the partial sequence.

iii. If more than one QC is out of range or the partial sequence in "i" does not resolve the problems, the entire run is realiquotted and rerun, including recalibration of the instrument(s).

4) Verify that for each unknown sample, the duplicate results agree to within ± 0.01 g/100 mL from the mean (inclusive). Report the average of the two values, rounding to two decimal places, using the mathematical rules of rounding. If the duplicate results are not within ± 0.01 g/100 mL, the sample is rediluted and reassayed on two instruments. It may be necessary to homogenize the sample before reanalysis.
i. Include one quality control sample and a blank at the beginning or end of the realiquotted sequence, insuring that all samples are within 10 samples of a control.
a) If the original calibration is within 24 hours and there have been no changes to the internal standard, recalibration is not necessary.
b) If the original calibration is outside of 24 hours or there has been a change in the internal standard, recalibration is required.

5) Examine each unknown for the presence of other peaks in the chromatogram. If other peaks are identified, determine if they are one of the volatiles found in the volatile mix. If they are, they may be quantified in the offline method.
i. In the Offline Mode of Instrument Method & Run Control Panel, load the volatile method.
ii. Identify the file, which contains the 0.04 volatile standard, and load it. On the toolbar, select Calibration. Select recalibrate, select level 1 and click on replace. Identify and load the 0.079 volatile standard, and update as calibrator #2.
iii. Identify any unknown with volatiles and load the files. Select Generate Report. The printout will quantify acetone, methanol, isopropanol (as well as ethanol). See appendix G for a printout of the 2 calibrators and an unknown recalculated with the volatile method.

6) If the extraneous peaks are not acetaldehyde and are not identified as one of the other volatiles, run the sample (or the previous sample if there is an indication that it is a late peak from a previous sample) on instrument #3, toluene method, in an attempt to

identify the volatile. Refer to the interfering substance manual in the laboratory for assistance with identification of unknown peaks. It may be necessary to heat the vial and inject the vapor on GCMS #5 for identification. To quantify any volatile, appropriate standards must be concurrently analyzed with the sample and the contemporary standards filed in the folder with the sample data.

7) Samples with concentrations greater than 0.400g/100mL must be diluted appropriately and re-analyzed.

8) Place all chromatograms into the respective files. Initial standards and controls are filed with the first sample of the run and the subsequent controls are filed with the adjacent sample. Include all chromatograms in the file, even if the sample is reanalyzed although unacceptable data should be lined through, initialed and dated with a brief explanation as to the reason for the reanalysis.

9) If it is necessary to reprint a chromatogram, note that the sample is always recalculated when it is printed, based upon the most recent calibration curve. If the instrument has not been used since the sample was run, the sample may be reprinted. If it has, take the following action:

i. Load each contemporary calibrator and update the calibration curve.
ii. Identify the files which need to be reprinted.
iii. Load each file and generate the report. Print the contemporary calibrators.
iv. DO NOT REPRINT A RESULT WITHOUT RELOADING THE CONTEMPORARY CALIBRATOR.
v. Include the reprinted calibrators with the reprinted data in the file or note in which file it can be located.

L. *Interpretation of results:*

1) Post mortem samples: Blood alcohol results of 0.019g/100mL or less shall be reported as negative.

2) Samples drawn from living subjects: Blood alcohol results of 0.009g/100mL or less shall be reported as negative.

3) The following clinical effects and symptoms are associated with various blood alcohol levels (Caplan, 1982).

BAC (g/100mL)	*clinical effects and symptoms*
0-0.06	no apparent influence by ordinary observations; Slight changes detectable by special tests
0.03-0.12	euphoria, sociability, decreased inhibitions, diminished attention, judgement and control, loss of efficiency in performance tests
0.09-0.25	emotional instability, loss of critical judgement, decreased sensitory response, impaired memory and comprehension, some muscular incoordination, decreased reaction time.
0.18-0.30	disorientation, mental confusion, dizziness, loss of emotional control, impaired balance, muscular incoordination, slurred speech decreased pain perception
0.27-0.40	apathy, inertia, marked decrease to stimuli and advanced muscular incoordination, vomiting, incontinence, sleep or stupor
0.35 and above	partial or complete unconsciousness, coma, respiratory distress, circulatory failure, possible death

The signs and symptoms reported at all levels may significantly impair driving regardless of their severity or detectability. Note that tolerance to alcohol such as that present in alcoholics or conditioned drinkers can cause these effects to be less obvious in some individuals.

M. *References:*

Y.H. Caplan in "Forensic Science Handbook vol. 1." R. Saferstein (ed.) Prentice Hall , 1982.

"Goodman and Gilman's the Pharmacological Basis of Therapeutics", McMillan publishing, 7th ed., 1985

Agilent (Hewlett Packard) 7694 Headspace Autosampler instruction manual

Agilent (Hewlett Packard) 6890 Gas Chromatograph manual

James C. Garriott "Analysis for Alcohol in Postmortem Specimens" in Medicolegal Aspects of Alcohol J. Garriott (ed.) Lawyers and Judges Publishing Co. 3rd edition 1996.

Approved:

Barry K. Logan, PhD

Date: 5/27/03

STATEMENT OF STATE TOXICOLOGIST -

In my capacity as Washington State Toxicologist, and by my authority outlined in RCW 46.61.506, I have reviewed this protocol and find it to be proper and adequate in form and substance for the purpose it was intended. I therefore approve and authorize its use. This protocol replaces all previous headspace GC analysis protocols and ethanol standard preparation protocols.

Barry K. Logan Ph.D.
Washington State Toxicologist

Date: 5/27/03

Reviewed By: _____ Date: 5/27/03
Ann Marie Gordon
Laboratory Manager

Reviewed By: _____ Date: 5/30/03
Edward Formoso
Supervisor

The following toxicologists have read the Headspace GC Protocol and agree to follow this procedure as it is written. Any deviations from the procedure must be documented in writing and approved by the laboratory manager and/or the State Toxicologist.

Reviewed By: _____ Date: 5-29-03

Reviewed By: _____ Date: 5-29-03

Reviewed By: _____ Date: 5/29/03

Reviewed By: _____ Date: 5/29/03

Reviewed By: _____ Date: 5/29/03

Reviewed By: _____ Date: 5/30/03

Reviewed By: _____ Date: 5/30/03

Approved: _____ Date: 5/27/03
Barry K. Logan, PhD

Reviewed By: _____ Date: 6/2/03

Reviewed By: _____ Date: 6/5/03

Reviewed By: _____ Date: 6-9-03

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Approved: _____ Date: 5/27/03

Barry K. Logan, PhD