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 gm/kg.) The volume of sample is 0.2 mL and is diluted with 2.0 mL diluent. Samples with high concentrations of volatiles may be further diluted with water for re-analsyis to get the result within the limits of linearity for the assay. Alternatively, a 1:2 dilution can be using the diluter to dispense 0.1 mL of sample with 2.0 mL of diluent.

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D. *Calibration Standards:*

The headspace gas chromatograph is calibrated for each "batch". The calibration must be within 24 hours of the time the sample is analyzed for the results to acceptable. For analyses other than a complete batch (repeat samples, certification runs, etc) a previous calibration may be used, if there has been no change to the internal standard, at least one contemporary control is analyzed and the calibration is within 24 hours of the last sample analyzed. 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, a batch is defined as all samples run by one analyst, within a 24 hour period, using the same internal standard, with the same calibration, even if there is more than one sequence.

<u>Materials</u>

> Absolute ethanol, used within 6 months of the date it is first opened.
> 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 gm/100 mL	1 mL of ethanol in 1000 mL H_2O
0.158 gm/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 Log (see Appendix A).

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

E. *Internal Standard:*

The internal standard is prepared as follows:

> 20 gm 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 Log. Internal Standard expires 30 days after preparation.

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F.   *Controls:*

Commercially prepared controls are purchased for use in each assay. At a minimum, two (2) control levels are included in each batch. Reanalysis of some samples, a certification run or other limited run must include at least one control per 10 samples. 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:*

Non-ethanol-calibrating volatile standards are included in each batch in order to use the results to quantify the presence of acetone, isopropanol and/or methanol. The 0.02 standard is included to demonstrate that the method can accurately quantify low levels of alcohol for minor or zero tolerance cases.

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

> 0.02 gm/100 mL
>
> 1 mL absolute ethanol in 4 L H_2O
>
> 0.02 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 Log 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 acetone
> 1 mL ispropanol
> 1 mL methanol

> 1 mL ethanol
> 1 mL acetone
> 1 mL ispropanol
> 1 mL methanol

> in 2 L H_2O

> in 1 L H_2O

Preparation of the volatile Standards is documented in the Alcohol Standard Log (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.

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H.   *Equipment:*

Agilent (Hewlett Packard) Network Headspace Autosampler or equivalent
Agilent (Hewlett Packard) 6890 or 6890N gas chromatograph; equipped with a J&W
DBALC1 megabore (0.53 mm) 30 meter capillary column and/or a J&W DBALS2
megabore (0.53 mm) 30 meter capillary column or equivalent. (For information on the
columns, see Appendix C)
Computer System equipped with HP GC Chem Station
Compressed gases; air, nitrogen , hydrogen, helium
Autosampler vials
Cap Crimper
Hamilton Automatic Diluter

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 with the fan hood
on, 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 which test positive are re-analyzed using a second aliquot to
produce a duplicate result for compliance with WAC 448-14-020.

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 gm/100 mL
0.04 Volatile Mix
0.08 Volatile Mix

5) Auto-pipette 200 µL of blood, control, or standard solution into a 10 mL autosampler
vial. Add 2 mL of internal standard solution.   Seal the vial tightly and shake well
until homogeneous. Note: If other than 200 µL is alliquotted, this must be noted on
in the sample information in the sample log table.

6) Alternating controls (different levels) are repeated periodically throughout the run
followed by a blank. Each positive sample should be separated from a commercial

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control and a blank by no more than ten other samples. If all the unknown samples within a single analytical run are not aliquoted at once, the first aliquot is a control, followed by a blank.

7) Prepare the sample worklist of unknown samples, standards and controls. (Note: it is advisable to run an extra blank following any badly decomposed sample.) One or more headspace instruments may be controlled by a single 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. By convention, the subdirectory is numerical date (YYMMDD) followed by initial(s) of the analyst, if more than one subdirectory is run on a given day, a modifier is appended to the analyst's initials.

8) Select Sequence; Sequence Table. Enter the standards controls and unknown samples into the sequence table. The calibrators, 0.079 gm/100 mL, 0.158 gm/100 mL and 0.316 gm/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 or 70 samples may be included in one run dependent upon the headspace model.

9) Identify the method as BLDALCO (a modifier may identify the instrument). The injection number is 1. Print the sequence table for each instrument. (The method for the two volatile standards may be selected as VOLATILE.)

10) 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.

11) Select the HP6890 System monitor and click on the Vials icon to specify the number of vials in the run if analyzing on 6890. (This is not required on 6890N.) Click on the start icon. Repeat for the other instrument.

12) 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 on some models.

13) The GC methods for each instrument are found in Appendix D.

14) 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 biohazardous waste.

15) A sample chromatogram is found in Appendix E.

16) The results of the initial alcohol screen are reviewed.

17) All samples with reportable levels of ethanol or other volatiles are re-analyzed on a second headspace system, unless otherwise approved by the laboratory manager and or the State Toxicologist due to equipment limitations. (Under certain circumstances,

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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.)

18) The vials for the duplicate test are selected and a chain of custody is generated by scanning the bar-code on the vial. Each selected sample is aliquotted, as above, and analyzed on the second headspace as detailed above.

19) The two results are reviewed for compliance with 448-14-020 such that the two duplicate ethanol results must be within 0.01 gm/100 mL of the mean of the two results.

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 following control samples are devoid of peaks. (Blanks following decomposed samples may have peaks.)

2) Verify the presence of the internal standard in each analysis. To ensure sensitivity the ISTD area must be at least **900**. 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 gm/100 mL of the target value. Verify that the other standards quantify within $\pm$ 0.01 gm/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 analyzed.

   ii. If one quality control per analytical run is out of range but within +/- 0.02 gm/100 mL of the target value, all positive samples within 10 samples of the failed QC are realiquotted and reanalyzed.
      a) Use the same calibration, if it is 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, including standards and controls and rerun.

4) For any Out-of-Range Quality Control, clearly document the QC failure and the corrective action taken on the QC Out of Range Log, Appendix F and indicate which

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samples were re-analyzed along with the failed controls. This is reviewed, monthly, by the ~~technical lead~~. QA manager *Amos 3/19/07*

5) For any data which was re-analyzed due to control failure or other reason, draw one line through the failed data with a brief comment as to the reason for failure, initial and date the annotation and maintain this in the corresponding case folder.

6) Verify that for each unknown sample, the duplicate results agree to within ± 0.01 (% BAC) gm/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 gm/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.

7) 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, in duplicate.
    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.
    IV. *Report volatiles in mg/dL*   *Amos 3/19/07*

8) 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) by Headspace GC, 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. Appendix H lists of some commonly identified volatiles and their relative retention times on each currently used instrument.

9) It may be necessary to heat the vial and inject the vapor for analysis by GCMS for identification. To quantify any volatile, appropriate standards must be concurrently

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analyzed with the sample and the contemporary standards filed in the folder with the sample data.

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

11) Place all chromatograms into the respective files. Initial standards and all controls (and subsequent blanks) are filed with the first sample of the run. Include all chromatograms in the file, even if the sample is reanalyzed. Line through unacceptable data, initial and date and add a brief explanation for the reanalysis.

12) 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 the contemporary calibrators and update the calibration curve.
    ii. Identify the files 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 0.019 g/100 mL or less shall be reported as negative.

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

Ante-mortem samples collected prior to death are reported using the rules for living subjects.

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

BAC (g%)	*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

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	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 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 (Helwett 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.

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. This replaces the headspace protocol dated 7/04, which remains in effect.

_____   10/10/2006
Barry K. Logan Ph.D.   Date
Washington State Toxicologist

Reviewed By: _____   Date: ____10/10/06____
             Ann Marie Gordon
             Laboratory Manager

Approved: Date:
_____   10/10/2006
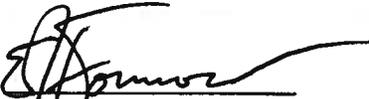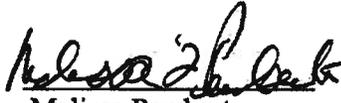Barry K. Logan, PhD

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

Reviewed By: _____ Date: __10/11/06__
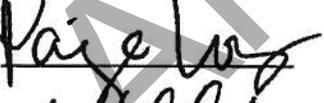Edward Formoso
Quality Assurance Manager

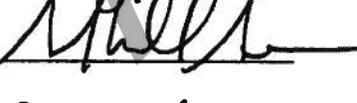Reviewed By: _____ Date: __10-11-06__
Melissa Pemberton
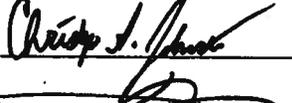Supervisor

Reviewed By: _____ Date: __10-11-06__
Brian Capron
Supervisor

Reviewed By: __Brian E Akins__ Date: __10-10-2006__

Reviewed By: _____ Date: __10-10-2006__

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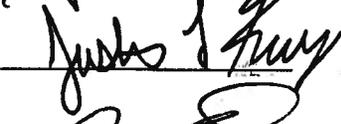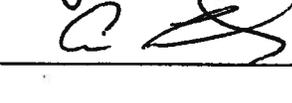
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Approved: _____ Date: __10/10/2006__
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Reviewed By: _____ Date: _10/12/2006_

Reviewed By: _____ Date: _10-18-2006_

Reviewed By: _____ Date: _2/20/07_

Reviewed By: _____ Date: _3/29/07_

Reviewed By: _Brittany Ball_ Date: _3/29/07_

Brianna Peterson 05/31/07

Approved: _____ Date:

Barry K. Logan PhD _10/6/2006_

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