Ethanol Analysis Using Headspace Gas Chromatography-Flame Ionization Detection

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1.0 Scope

1.1 The analysis of ethanol in blood and liquid samples is performed using headspace (HS) sampling on a dual column gas chromatograph with a flame ionization detector (GC-FID).

2.0 Theory

2.1 Ethanol analysis is based on the theory of Henry's law, which states that at a constant temperature, the concentration of alcohol in a liquid (e.g., blood) is equal to the concentration of alcohol in the vapor or gas above the solution (headspace) in a closed container.

A portion of the sample is placed into a vial and then diluted with internal standard (n-propanol). The vial is closed with an airtight seal. Once the vial is on the instrument, it is heated, pressurized, and a portion of the headspace is injected on the instrument (GC). The GC separates the components within a solution based on their affinity for the column, and the FID detects those components producing a response that can be measured.

3.0 Reagents and Equipment

3.1 Reagents

3.1.1 Deionized (DI) Water

3.1.2 n-propanol

3.2 Equipment

3.2.1 Pipettes

3.2.2 Volumetric flask

3.2.3 Analytical balance

3.2.4 Homogenizer

3.2.5 Vortex mixer or rocker

3.2.6 Gas Chromatograph/Headspace Sampler

4.0 Instrumentation

4.1 Parameters

4.1.1 Capillary columns: Agilent DB-ALC1 and DB-ALC2, 30 m x 0.320 mm x 1.8 µm (DB-ALC1) or 1.2 µm (DB-ALC2) thickness (or equivalent). Using a 10:1 split injection, the column flow rates are approximately 7 mL/min with a pressure of 24 psi.

4.1.2 HS-GC: Agilent 7697A-7890A (BAC1) and Agilent 7697A-7890B (BAC2)

GC Oven Temp	40°C hold for 4 minutes
Total Run Time	4 minutes
HS Oven and Loop Temperatures	70°C
Equilibration Time	7 minutes

HS Transfer Line Temperature	90°C
Detector Temperature	250°C
Detector Flows	H_2: 30 mL/min Air: 400 mL/min N_2: 25 mL/min

4.1.3 Method: BAC.M

4.2 Maintenance

4.2.1 Routine maintenance shall include checking the gas pressure levels and conducting a performance check (PC). Maintenance will be documented on the Headspace GC-FID Maintenance Log (LAB-TOX-04-00).

4.2.2 Other maintenance or repairs should be performed as needed. Preventative maintenance (PM) shall be performed annually by an external party. After maintenance or repair a verification shall be performed prior to performing casework. The verification shall meet acceptance criteria as specified in 5.4.4.

5.0 Verification

5.1 Verifications shall be performed after any maintenance, repair, or changes in the method parameters, excluding changes made to data acquisition. Changes made to data acquisition shall require a validation to be performed. The verification run must meet acceptance criteria defined in 5.4.4. If the acceptance criteria is not met, appropriate actions will be taken to remedy the issue. Verification runs shall be documented in the maintenance log.

5.2 If any maintenance, repairs, or changes to data acquisition significantly impact the retention time of ethanol (e.g. installing a new GC column), the mean retention time of calibrators in the verification shall be used to determine the new retention time of ethanol.

5.3 The calibrator data obtained from a verification performed after annual preventative maintenance is completed shall be used to update the retention time of ethanol in the method.

5.4 A major or minor verification shall be performed dependent upon the extent of the maintenance, repair or changes made to the method.

5.4.1 A minor verification will consist of one analysis of calibrators and at least three replicates of low QC, high QC, blood QC 1, and blood QC 2.

5.4.2 A major verification will consist of three analyses of calibrators and at least three replicates of low QC, high QC, blood QC 1, and blood QC 2.

5.4.3 Deviations from these verification plans and/or additional experiments may be performed depending on the purpose of the verification.

5.4.4 Verification acceptance criteria will be as follows:

5.4.1.1 Bias: % bias ≤5% for target concentrations >0.05 g/100mL

5.4.1.2 Within-run precision: %CV ≤10%

5.4.1.3 Between-run precision (major verifications): %CV ≤10%

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6.0 Calibration

6.1 Certified Reference Materials (CRM) from an external source are used as calibrators for the ethanol calibration. Once a CRM is opened, the contents will be transferred to a labeled container, sealed, and stored in the refrigerator.

6.1.1 **Storage**: Refrigerator **Discard**: CRM expiration date

6.2 The following ethanol calibrator concentrations will be used to establish the calibration curve:

Level 1: 0.010 g/100mL
Level 2: 0.025 g/100mL
Level 3: 0.050 g/100mL
Level 4: 0.100 g/100mL
Level 5: 0.200 g/100mL
Level 6: 0.400 g/100mL
Level 7: 0.500 g/100mL

7.0 Controls

7.1 Negative Control

7.1.1 The negative control is used to monitor potential carryover from the highest calibrator. It consists of 100 µL of DI water and 1000 µL of internal standard.

7.2 Low Quality Control (Low QC) – 0.080 g/100mL

7.2.1 The Low QC is a quality control from an external source. The contents of the vial may be transferred to a labeled container and capped.

7.2.2 Storage: **Storage**: Refrigerator **Discard**: CRM expiration date

7.3 High Quality Control (High QC) – 0.400 g/100mL

7.3.1 The High QC is a quality control from an external source. The contents of the vial may be transferred to a labeled container and capped.

7.3.2 Storage: **Storage**: Refrigerator **Discard**: CRM expiration date

7.4 Whole Blood Controls (Blood QC A and Blood QC B)

7.4.1 Blood QC A is a purchased control at a low ethanol concentration.

7.4.2 Blood QC B is a purchased control at a high ethanol concentration.

7.4.3 Storage: **Storage**: Refrigerator **Discard**: CRM expiration date

7.5 DI Water Control

7.5.1 The DI water control is used to monitor potential contamination from the environment throughout the sampling process. It consists of 100 µL of DI water added to a headspace vial and left uncapped for the duration of the sampling process. Internal standard is added at the end of sampling.

7.6 Calibrators and controls should be from different manufacturers. If a standard is not available from a different manufacturer, then a different lot from the same manufacturer should be used.

8.0 Internal Standard (IS)

8.1 0.01% n-propanol Internal Standard

8.1.1 1% n-propanol Internal Standard Stock Solution (%w/v)

8.1.1.1 Weigh 1 g of n-propanol in a 100 mL volumetric flask and bring to volume with DI water.

8.1.1.2 **Storage**: Refrigerator **Discard**: 6 months

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8.1.2 0.01% n-propanol Internal Standard (%v/v)
8.1.2.1 Add 10 mL of the 1% IS stock solution to a 1000 mL volumetric flask and bring to volume with DI water.
8.1.2.2 **Storage**: Room temperature **Discard**: 6 months

9.0 Verification of Whole Blood Controls and Internal Standard
9.1 Whole Blood Controls – Establishing Target Concentration of New Lot
9.1.1 Run a minimum of 12 replicates over more than one run to establish the mean. Current verified whole blood controls shall be run along with the new controls.
9.1.2 The established mean must be within ±20% of the manufacturer's mean.
9.2 Internal Standard
9.2.1 Prepare two negative samples with the newly prepared internal standard. These samples should be added to the end of a casework run.
9.2.2 Compare the area of the new internal standard to the area of the current internal standard in the negative control. The internal standard area of the newly prepared lot should be within ±20% of the current lot.

10.0 Performance Check (PC)
10.1 The PC is a quality control check performed prior to analysis to ensure the instrument is performing as expected.
10.1.1 As part of the performance check, the following tasks will be performed: gas cylinder levels are checked, a performance check sequence is run, and the maintenance log is filled out.
10.2 A performance check sequence consists of an air blank and a sample spiked using a certified reference material at the calibrator level 1 (0.01g/100mL).
10.3 The air blank must be clear of peaks of interest (i.e., no analytes of interest identified by both FIDs).
10.4 The performance check sample must meet the acceptance criteria for peak symmetry and retention time for ethanol and internal standard (see 13.3.1).

11.0 Sample Preparation
11.1 Prior to sampling, allow calibrators, controls, and case samples to come to room temperature.
11.2 Thoroughly mix all calibrators, controls, and case samples by inverting or rocking.
11.3 Prepare DI water control and leave open for the duration of the sampling process. After sampling is completed, add internal standard, cap, and crimp.
11.4 Samples shall be prepared in the same order in which they will be analyzed. Case samples will be analyzed in duplicate. Each sample will be aliquoted once, the order will be reversed, and the sample will be aliquoted a second time.
11.5 Using a repeater, pipette 1000 µL of IS into headspace vials. Aliquot 100 µL of calibrator, control, or case sample into the appropriately labeled and barcoded headspace vial, cap and crimp. Aliquoting occurs one case sample at a time.
11.6 Case samples that have multiple items containing different colored top blood tubes, preference will be given in the following order based on top color: gray>lavender>pink>tan>royal blue>red.

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11.7 Any unusual sample conditions shall be noted in the case file. If a sample contains clots, the sample will be homogenized using a homogenizer.

11.8 Headspace vials are loaded onto the HS-GC autosampler and the sequence injected using the BAC.M method.

11.9 If necessary, dilutions may be performed using DI water.

11.9.1 When alcoholic beverages are analyzed, a dilution is required.

11.9.2 Examples of common dilutions are as follows:

Specimen Type	Dilution	Sample Volume (µL)	DI Water Volume (µL)
Blood	1:2	500	500
Beer	1:20	50	950
Wine or unknown	1:50	20	980
Liquor	1:100	10	990

11.9.3 A 100 µL aliquot from the dilution is analyzed. For unknown liquor samples, perform a 1:50 dilution.

12.0 BAC Sequence Example

Calibrators (low to high)
Negative Control
High QC
Low QC
Blood QC A
Samples 1 to 10
Blood QC B
Samples 11 to 20
Blood QC A
Samples 20 to 11
Blood QC B
Samples 10 to 1
Blood QC A
High QC
Low QC
DI Water Control

12.1 The sequence should be verified and documented by any licensed analyst prior to analysis. Due to the limited staff of the BCCL, a sequence verification may not be possible. To combat this issue, the barcodes that are placed on each vial will be read by the instrument. The barcode identification number from the instrument and the barcode typed in the sample name must match. If the typed case sample barcode number and the barcode number from the instrument do not match, that sample shall be analyzed again in a separate batch. Known samples (e.g. calibrators and controls) do not have to be repeated if the barcodes do not match.

13.0 Acceptance Criteria

13.1 The calibration curve is calculated by the instrument software. Each ethanol concentration is determined by linear regression ($y = mx + b$) and is based on the ratio of the peak area of ethanol divided by the peak area of the internal standard.

13.2 Calibrators and Controls

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13.2.1 The R^2 of the calibration curve shall be 0.99 or greater.
13.2.2 Retention time shall be within ±1% of the average of all calibrators.
13.2.3 Average ethanol concentrations from FID1 and FID2 ≤0.05 g/100mL shall be within 10% of the target concentration. Average ethanol concentrations from FID1 and FID2 >0.05 g/100mL shall be within 5% of the target concentration.
13.2.4 Negative, air blank, and DI water controls shall be clear of significant peaks of interest (i.e., area counts >10% of the LOQ). All other quality controls shall meet acceptance criteria to report ethanol quantitatively.
13.2.5 If the concentration of a calibrator does not meet acceptance criteria, non ethanol case sample results may be reported. If a control does not meet acceptance criteria, the batch may be partially acceptable. Control failures will be handled on a batch by batch basis.

13.3 Chromatography
13.3.1 Peak symmetry for ethanol and internal standard shall be between 0.5 and 2.0 for all samples.
13.3.2 All peaks shall be resolved from any interferences. If any case sample ethanol peak is not resolved or not symmetrical, the sample shall be repeated.

13.4 Internal Standard
13.4.1 Internal standard recovery for case samples shall be within 10% of the average internal standard area of the calibrators.

13.5 Case Samples
13.5.1 Results are calculated using the average of the four results obtained from aliquot 1 and 2 on FID1 and FID2.
13.5.2 Ethanol results from FID1 and FID2 for each aliquot shall be within 10% for concentrations ≤0.05 g/100mL and 5% for concentrations >0.05 g/100mL.
13.5.3 Results will be truncated to three decimal places. All four values shall be within ±10% of the average if the truncated result is ≤0.05 g/100mL or within ±5% of the average if the truncated result is >0.05 g/100mL.
13.5.4 In order to report positive results, case samples must be bracketed by acceptable controls. Any cases samples not bracketed by acceptable controls will be repeated.

13.6 Repeated Case Samples
13.6.1 Case samples above the calibration curve shall be repeated with a dilution. Any positive case samples that do not meet the above acceptance criteria shall be repeated.
13.6.2 If a case sample fails to meet acceptance criteria after two analyses with the same issue (e.g., peak symmetry fails) the sample shall be reported as unsuitable for BCCL ethanol analysis.

14.0 Reinjection
14.1 Reinjection of known samples (i.e. calibrators and controls) may be performed to complete a sequence if the instrument stops. All acceptance criteria shall be met in order to use reinjected data for reporting.
14.2 Reinjection of unknown samples shall not be allowed. Any punctured case samples not bracketed by an acceptable control shall be repeated in a separate batch. If a run stops,

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the run can be resumed at the previous bracketing control followed by any unpunctured case samples.

15.0 Carryover

15.1 If a case sample is greater than 1.000 g/100mL, any positive case sample immediately following the highly concentrated sample shall be repeated.

15.2 In validation, the BCCL has demonstrated that no carryover was observed following a highly concentrated known sample of 1.000 g/100mL.

16.0 Reporting

16.1 Ethanol results will be reported in g/100mL.

16.1.1 Ethanol is the only volatile being tested within the BCCL, other volatiles will not be evaluated.

16.1.2 For serum/plasma samples, the whole blood concentration is calculated first by applying the UM followed by the conversion factor range of 1.04 to 1.26 (19.2). In order to be conservative, the lower end of the concentration range will be divided by the high conversion factor of 1.26 and the higher end of the concentration range will be divided by the low conversion factor of 1.04.

16.1.2.1 The conversion factor is applied to serum and plasma samples to account for the higher water content of serum and plasma compared to whole blood.

16.1.3 For serum and plasma samples, the report shall contain a comment stating the sample type and its equivalent whole blood concentration range (e.g. Item x.x was serum/plasma. The equivalent whole blood concentration was calculated to be xx.xx – xx.xx grams of ethanol per 100 milliliters of blood by applying the uncertainty of measurement and a conversion factor range of 1.04 to 1.26.)

16.2 Reported results shall be the average of the four results obtained from aliquot 1 and 2 on FID1 and FID2, truncated to three decimal places. Concentrations shall be reported when the average value is greater than or equal to the method LOQ of 0.01 g/100mL. Results below the LOQ shall be reported as none detected.

16.3 For clotted samples requiring homogenization, the report shall state that the sample was homogenized prior to analysis.

17.0 Uncertainty of Measurement (UM)

17.1 The UM is evaluated annually. See UM SOP for how the calculation is performed.

17.2 The UM shall be rounded to three decimal places and reported in the same units as the BAC (g/100mL).

18.0 Retrograde Extrapolation and Widmark Equation Calculations

18.1 Requests for Performing Calculations

18.1.1 A request shall be made in order for retrograde extrapolation and/or Widmark calculations to be performed. These types of requests should be submitted, performed, and technically reviewed prior to trial. The request must be made on LAB-TOX-05-00 (Retrograde Extrapolation and Widmark Calculation Request Form). The analyst will use the information provided on the request form (LAB-TOX-05-00) to perform the calculations on LAB-

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TOX-06-00 (Retrograde Extrapolation and Widmark Calculation Worksheet). All request and worksheets relevant to retrograde and Widmark calculations shall be placed in the case file.

18.1.1.1 Retrograde extrapolation and Widmark calculations shall not be performed on the stand.

18.1.2 When performing retrograde extrapolation calculations, the lower and upper range shall be rounded to three decimals.

18.1.3 When performing Widmark calculations to estimate the BAC, the lower and upper range shall be rounded to two decimal places.

18.1.4 When performing Widmark calculations to determine the number of drinks (amount of alcohol in the body), the lower and upper range shall be rounded to one decimal place.

18.1.5 When performing Widmark calculations to estimate the number of standard drinks, the result shall be a whole number range. This range will encompass the calculated results (e.g., if N= 2.8 and 4.5, the range shall be reported as 2 to 5 standard drinks) to be conservative.

18.2 Retrograde Extrapolation

18.2.1 Retrograde extrapolation is used for estimating a person's BAC at an earlier point in time.

18.2.2 Equations and Calculations

18.2.2.1 $BAC = [(BAC_k - UM) + (t\ x\ e_{low})]\ to\ [(BAC_k + UM) + (t\ x\ e_{high})]$

- BAC= estimated blood alcohol concentration at the time of the stop in g/100mL
- BAC_k= known blood alcohol concentration at the time of blood draw in g/100mL
- UM= uncertainty of measurement associated with BAC_k in g/100mL
- t= time between the stop and blood draw in hours
- e_{low}= elimination rate of 0.010 g/100mL/hour
- e_{high}= elimination rate of 0.025g/100mL/hour

18.2.3 Facts

18.2.3.1 The following facts are needed in order to perform retrograde extrapolation calculations:

- BAC_k
- Time of the stop
- Time of blood draw
- Time since last drink

18.2.3.2 All of the above facts must be provided in order to perform a retrograde extrapolation calculation.

18.2.4 Assumptions and Limitations

18.2.4.1 Retrograde extrapolation calculations can only be performed if absorption is complete and an individual is undergoing elimination at the time of the stop. Absorption is considered complete if the time between the last drink and the stop is at least 2 hours.

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18.2.4.2 Retrograde extrapolation calculations shall not be performed on a BAC_k less than 0.02 g/100mL.

18.2.4.3 The elimination rates of 0.010 g/100mL/hour (low) and 0.025 g/100mL/hour (high) shall be used to obtain a BAC range. It is assumed that most individuals fall within this range regardless of age, sex, and drink experience. There are extreme cases (i.e., alcoholics, ultra-rapid metabolizers, and extreme liver or dietary conditions) where individuals may have an elimination rate below 0.010 g/100mL/hour or above a 0.025 g/100mL/hour.

18.2.4.4 First pass metabolism shall not be considered.

18.2.4.5 The rate of absorption of ethanol may be slowed if there is food in the stomach, delaying the peak BAC. For this reason, retrograde shall not be performed if the time since last drink and the stop is less than 2 hours. The low to high range of elimination includes both fasting and fed individuals however conditions such as gastroesophageal reflux disease (GERD) may increase the alcohol absorption in individuals to more than 2 hours. For this reason, GERD will not be considered.

18.2.4.6 Many factors may slow the rate of absorption of ethanol such as type of food (carbohydrates containing fructose and amino acids containing glycine), trauma, shock, and mass blood loss.

18.2.4.7 Gastric surgery and liquor (drinks with higher ethanol concentrations) increase the absorption rate of ethanol.

18.2.4.8 Drugs that affect the rate of gastric emptying may cause changes in the ethanol absorption rate.

18.3 Widmark

18.3.1 Widmark is used for estimating the blood alcohol concentration based on the amount of alcohol consumed or how much alcohol an individual has in the body at a given blood alcohol concentration.

18.3.2 Equations and Calculations

18.3.2.1 Widmark

$$BAC = \frac{a}{p \; x \; V_{d\,(high)}} \; to \; \frac{a}{p \; x \; V_{d\,(low)}}$$

or

$$a = \; BAC \; x \; p \; x \; V_{d\,(low)} \; \; to \; \; BAC \; x \; p \; x \; V_{d\,(high)}$$

- BAC= blood alcohol concentration in g/100mL
- a= amount of alcohol in grams (g)
- p= weight of indivdual in grams
- Vd (low)= volume of distribution low limit (0.45L/kg = 0.0045mL/g)
- Vd (high)= volume of distribution high limit (0.81L/kg = 0.0081mL/g)

18.3.2.2 Number of Standard Drinks (N)

$$N = \frac{(BAC \; x \; p \; x \; V_d)}{14} \quad or \quad N = \frac{a}{14}$$

18.3.2.2.1 Conversions

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- 1 fluid ounce (oz) = 29.6 mL
- 1 pound (lb) = 454 g
- 1 mL ethanol = 0.789 g ethanol
- 1 standard drink = 14 g ethanol which is equivalent to: 5 oz wine (12% alcohol), 12 oz beer (5% alcohol), and 1.5 oz (80 proof or 40% alcohol)

18.3.3 Facts

18.3.3.1 The following facts are needed in order to perform Widmark and standard number of drink calculations:

- BAC or number of drink(s)
- Weight (lb)

18.3.3.2 All of the above facts shall be provided in order to perform a Widmark calculation or determine the standard number of drinks.

18.3.4 Assumptions

18.3.4.1 Individuals generally fall within a V_d range of 0.45 and 0.81 L/kg, regardless of sex and body mass index (BMI).

19.0 References

19.1 Caplan, Yale H., Goldberger, Bruce A., eds. Garriott's Medicolegal Aspects of Alcohol, 6th ed. Tucson, AZ: Lawyers & Judges Publishing Company, Inc. 2015.

19.2 Charlebois, RC, Corbett, MR, and Wigmore, JG. Comparison of ethanol concentrations in blood, serum, and blood cells for forensic application. Journal of Analytical Toxicology. 1996;20:171-178

19.3 Agilent 7697A Headspace Sampler Getting Started, 1st edition.

19.4 Agilent 7697A Headspace Sampler Operation Manual.

19.5 Agilent 7890B Gas Chromatograph Getting Started, 2nd edition.

19.6 Agilent 7890A Gas Chromatograph Operating Guide.

19.7 Agilent 7890B Gas Chromatograph Operating Manual.

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Date	Section	Previous	Changed to	Reason	By whom
11/3/2023	13.6.2	If a case sample fails to meet acceptance criteria after two analyses with the same issue (e.g., peak symmetry fails) the sample shall be reported as unsuitable for BCCL ethanol analysis.	If a case sample fails to meet acceptance criteria after two analyses with the same issue (e.g., peak symmetry fails) the sample shall be reported as unsuitable for BCCL ethanol analysis.	Removed highlight from (e.g., peak symmetry fails)	KB/MH
11/7/2023	11.4	Samples shall be prepared in the same order in which they will analyzed.	Samples shall be prepared in the same order in which they will be analyzed	Updated grammatical errors	KB/MH
	15.1 and 15.2	15.1 If a case sample is greater than 1.0 g/100mL, any positive case sample immediately following the highly concentrated sample shall be repeated. 15.2 In validation, the BCCL has demonstrated that no carryover was observed following a highly concentrated known sample of 1.0g/100mL.	15.1 If a case sample is greater than 1.000 g/100mL, any positive case sample immediately following the highly concentrated sample shall be repeated. 15.2 In validation, the BCCL has demonstrated that no carryover was observed following a highly concentrated known sample of 1.000 g/100mL.		
	16.0			Changed font to coincide with the rest of the SOP	

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