For Tox Method 029
Ethanol and Common Volatiles
By Headspace Gas Chromatography

Wisconsin State Laboratory of Hygiene:
Environmental Health Division, Forensic Toxicology Program

A. Principle

1. Samples are diluted with an ammonium sulfate solution containing 1-propanol as an internal standard. The dilution vials are placed in a headspace autosampler. After reaching equilibrium, the headspace vapor is examined by split injection dual-column gas chromatography with a flame ionization detector. Ethanol is separated, identified and quantitated. Other common volatiles are separated and identified.

B. Specimens

1. Whole blood collected with sodium fluoride (100 mg/10 mL) and potassium oxalate (20 mg/10 mL) is preferred for implied consent samples.
2. Unpreserved urine is acceptable. Random urine collection is permitted for indication of ethanol use. Special collection procedures are necessary for correlation with blood ethanol concentration. For additional information see Section L – Notes.
3. Post mortem blood should be collected with sodium fluoride (100 mg/10 mL)
4. Unpreserved blood, plasma, serum or vitreous are also acceptable specimens.
5. Tissue homogenates are acceptable.
6. The minimum sample volume for duplicate analysis is 0.5 mL.

C. Reagents

1. Ammonium sulfate, ACS certified
2. Ethanol, USP 200 proof
3. Methanol, ACS grade or better
4. Acetone, ACS grade or better
5. Isopropanol, ACS grade or better
6. 1-Propanol, ACS grade or better
7. High purity water (18.2 M ohm/cm or equivalent)
8. Internal standard solution (0.012 g/100 mL 1-propanol, 0.5 M ammonium sulfate): Fill a 10 L carboy to approximately 8 L with high purity water. Add 660 g of ammonium sulfate. Add 1.5 mL of 1-propanol. Add a large stir bar and

mix on a mixing plate for a minimum of 10 min. Add additional high purity water to fill the carboy to 10 L and continue mixing for another 30 min or more. Solution is stable for at least 6 months.

D. Supplies

1. 20 mL headspace glass vials with crimp top or screw cap PTFE-lined septa.

E. Equipment

1. Tube rotator and/or rocker plate
2. Hamilton 503 Diluter or equivalent which permits automatic dilution of sample with diluent in a 1:20 ratio
3. Automatic or hand operated crimper for headspace vials
4. Perkin-Elmer Autosystem XL Head Space Gas Chromatograph with Flame Ionization Detector
   a. Totalchrom Software
   b. Dual capillary columns:
      (i) Channel A (front column) Restek Rtx-BAC1 or equivalent.
      (ii) Channel B (back column) Restek Rtx-BAC2 or equivalent.
   c. TurboMatrix 110 Headspace Autosampler
   d. Headspace autosampler conditions:
      (i) Oven temperature at 60 C
      (ii) Needle temperature at 80 C
      (iii)Transfer line temperature at 90 C
      (iv)Equilibration time at 17.7 minutes
      (v) Pressurization time of 0.5 minute at transfer line pressure of 36.5 psi
      (vi)Withdraw time 0.2 min
      (vii)Inject time 0.02 min
   e. Gas Chromatograph conditions:
      (i) Oven isothermal temperature range of 45 – 50 C
      (ii) Inlet temperature at 150 C
      (iii)Detector temperatures at 220 C
      (iv)Carrier gas:
         (a) Helium, 5.0 Ultra High Purity
         (b) Flow rate at 9.0 to 10.0 mL/min
         (c) Flow rate parameters are determined for each column as necessary for optimal instrument performance.

F. Calibrators / Combination Volatile Solution

1. Use a previously unopened bottle of ethanol each time a set of calibrators is prepared. Prepare calibrators A, B, C and D by pipetting 1.0, 2.0, 4.0 and 8.0 mL respectively of ethanol into 1 liter volumetric flasks and dilute to volume with

high purity water. Prepare Calibrator L by pipetting 16 mL of Calibrator D into 1 liter volumetric flask and dilute to volume with high purity water. Calibrators will have the following concentrations:

Calibrator	Ethanol Concentration
A	0.079 g/100 mL
B	0.158 g/100 mL
C	0.316 g/100 mL
D	0.631 g/100 mL
L	0.010 g/100 mL

2. Combination volatile solution:
   a. Prepare a combination volatile stock solution by pipetting 8.0 mL of methanol, ethanol, acetone and isopropanol into a 1 liter volumetric flask and dilute to volume with high purity water. Pipette 16.0 mL of this stock solution into a 1 liter volumetric flask and dilute to volume with high purity water.
   b. Combination volatile solution will contain:

Volatile	Concentration
Methanol	0.010 g/100 mL
Ethanol	0.010 g/100 mL
Acetone	0.010 g/100 mL
Isopropanol	0.010 g/100 mL

3. When new sets of calibrators are prepared they are checked against the existing set of calibrators prior to actual use. In addition, the new set of calibrators is checked against NIST certified reference solutions (NIST SRM 1828b or equivalent) prior to use. All results must agree within 5% or 0.005, whichever is greater, of their respective target values in order to be deemed acceptable.
4. Calibrators and the combination volatile solution are stable for at least six months. Store in 60 mL glass screw top bottles. Label bottles with solution name, initials of preparer, preparation date and expiration date. Store one bottle of each at room temperature for use. Refrigerate remaining bottles; replace every 2 weeks or as needed.

G. Calibration

1. Analyze calibrators L, A, B and C using the internal standard method of calibration. Update the calibration table for Channel A (BAC1 column). The calibration curve must have an R^2 value of 0.995 or greater.
2. Reprocess results for calibrators L, A, B, C and D with the newly created calibration curve. Calibrators L and A must be within 0.005 of target values and calibrators B, C and D must be within 5% of target values for the calibration to be acceptable. Recalibrate if these conditions are not met.
3. The above instrument method conditions should yield the following approximate retention times:

Analyte	BAC1 RT	BAC2 RT
Methanol	0.68 min (0.53 RRT)	0.98 min (0.42 RRT)
Ethanol	0.83 min (0.65 RRT)	1.28 min (0.54 RRT)
Isopropanol	0.98 min (0.77 RRT)	1.48 min (0.63 RRT)
Acetone	1.18 min (0.92 RRT)	1.38 min (0.58 RRT)
1-propanol	1.28 min	2.36 min

Note that the retention order of isopropanol and acetone changes between BAC1 and BAC2.

H. Quality Control

1. The aqueous blank must demonstrate lack of interference.
2. The combination volatiles solution must demonstrate adequate separation of analytes and the appropriate change of elution order for the two columns (BAC1 and BAC2).
3. Blood and urine ethanol control materials are prepared in-house.
   a. See Addendum 1 for control material preparation procedure.
   b. Each analytical run will begin and end with a control material. A control material is analyzed for every 9 unknown specimens analyzed. A typical run will contain at least one observation of each control material. Shorter runs may contain proportionally fewer control materials.
   c. Control materials are run as a single analysis. Controls are acceptable if they are within 5 % or 0.005 of their target values, whichever is greater.

I. Procedure

1. Specimens are checked in as per Specimen Receipt-FOR TOX GENOP 001.doc.
2. Mix sample by inversion or on rotator.
3. Transfer internal standard solution to clean bottle and flush through diluter to remove bubbles from system.
4. Set the diluter to aspirate 0.250 mL of sample and dispense with 4.75 mL of internal standard solution.
5. Prepare a worksheet to indicate position of all calibrators, controls and specimens in the autosampler tray.
6. Prepare all calibrators, controls and samples in the order to be assayed.
7. Aspirate calibrator, specimen or control; wipe outside of diluter probe, dispense into labeled headspace vial and cap tightly.
8. Unknown specimens are analyzed in duplicate. Each duplicate analysis is made from a fresh individual dilution.
9. Cycle (rinse) the diluter between each dilution.
10. Check all sampler vials against worksheet when loading and unloading the autosampler.

J. Procedure for Instrument Setup and Use

1. Verify that the headspace autosampler and chromatograph are in standby condition. Verify that the flame ionization detectors are turned on.
2. Load the calibration sequence. Calibration sequence consists of calibrators A – L in order of increasing ethanol concentration. An aqueous blank follows the highest calibrator. The combination volatiles solution follows the blank.
3. Reprocess the calibration sequence to read the calibrator values from the newly created calibration curve. Identify and label methanol, acetone and isopropanol on each column by comparing with the RT/RRT from table in G above.
4. Verify that the instrument meets performance criteria as outlined in G and H above. See Addendum 2 if instrument fails to meet performance criteria.
5. Print GC chromatographic conditions and method report.
6. Print the Auto-Calibration report.
7. Load and run analytical sequence(s).
8. Record date, analyst initials, instrument and headspace injection count in the instrument log. Record the diluent lot number on the batch worksheet.

K. Results

1. Channel B (BAC2, back column) is used for confirmation analysis.
2. Results are reported from Channel A (BAC1, front column).
   a. Report to three decimal places.
   b. The mean of the two analyses is reported.
   c. The difference between the higher and lower of duplicate results must be less than or equal to 0.005, or 5% of the lower value, whichever is greater.
   d. Specimens that do not agree within these limits must be re-analyzed. Duplicate results that do not meet these criteria are not reported.
   e. Specimens with insufficient volume for duplicate analysis may be analyzed a single time. The report will document that an insufficient volume of specimen was received for duplicate analysis.
   f. Rounding rules: When the fourth decimal of a calculation (the average of duplicate assay results or a blood concentration calculated from a urine ethanol result) is "5", the reported result should be rounded to the nearest even number. (Examples: 0.1105 rounds to 0.110; 0.1115 rounds to 0.112.)
3. Procedure is linear to at least 0.630 g/100 mL.
4. The limit of quantitation for any specimen to be reported as positive is 0.010 g/100 mL. Specimens with results less than 0.010 g/100 mL are reported as "Not Detected."
5. All concentrations are automatically calculated by the Totalchrom software.
6. Review all chromatography for acceptability. Verify that ethanol is properly identified on each column. Identify and label any other volatiles noted.
7. Review all quality control data for acceptability, see Section H.

8. Quantitative specimen results must be bracketed (see Section H. 4(b)) by acceptable quality control results.
   a. Positive ethanol specimens that are not bracketed by acceptable control results must be reanalyzed.
   b. Specimens with "Not Detected" results need not be reanalyzed when a control result is unacceptable as long the confirmation analysis and chromatography are acceptable.
9. Transfer result data from instrument to worksheet on networked computer. Result means and duplicate acceptability criteria are determined electronically in the worksheet software.
10. Record specimen and QC results in the Laboratory Information System (LIS).
    a. Results below the lowest calibrator target concentration of 0.010 g/100 mL are reported as "not detected."
       (i) The actual numerical result of the lowest calibrator target concentration is recorded, even if it is below 0.010 g/100 mL
    b. Results equal to or above the highest calibrator target concentration of 0.631 g/100 mL are diluted and reanalyzed, or reported as "greater than 0.630 g/100 mL."
11. Add date of analysis and analyst and reviewer signature line comments in the LIS.
12. Determine whether testing is complete on each specimen or if drug testing is necessary. Add comments in the LIS as appropriate for status of drug testing.
13. Fill out Internal Drug Testing forms for each specimen that has a request for drug testing and compile a Drug List.
14. Reports are generated from the LIS.
    a. Implied Consent ethanol results are reported when completed. A copy of the request form, with chain of custody information completed by the analyst, is included with the report. If drug testing is performed a separate report is issued when all testing has been completed.
    b. Motor Vehicle Death ethanol results are reported when completed. A copy of the MVD request form, with chain of custody information completed by the analyst, is included with the report. If drug testing is performed a separate report is issued when all testing has been completed.
    c. Death investigation ethanol results are reported after all requested testing (alcohol and drug) has been completed.
    d. Implied Consent urine ethanol results are multiplied by 0.75 to obtain the corresponding blood ethanol concentration as set by Wisconsin statute 885.235 (2). Enter the free text comment in the ethanol test result comments area for the corresponding urine to whole blood conversion:
       "The urine ethanol concentration of 0.XXX g/100 mL corresponds to a <NEW LINE>
       whole blood ethanol concentration of 0.XXX g/100 mL"
15. A designated advanced analyst or laboratory supervisor will review: all chromatography; calibrator and control results; specimen results and reported

averages; and report comments. The reviewer will validate results in the LIS and print reports. Both the reviewer and analyst sign the worksheets and reports.

16. After the analyst and the reviewer have signed the reports, copy the reports and mail copies.
    a. Implied consent ethanol reports:
        (i) A photocopy of the request form is included on the back of the report.
        (ii) Copies of reports are mailed to submitters and subjects (unless the specimen was submitted directly by the subject).
        (iii) The original report and requisition form are retained by the Forensic Toxicology Program.
        (iv) Results and demographic data are electronically transmitted to the Wisconsin Department of Transportation.
    b. Motor Vehicle Death reports:
        (i) The original report and a copy of the MVD request form is mailed to the submitter.
        (ii) A copy of the report, and the original MVD request form, is retained by the Forensic Toxicology Program.
        (iii) A copy of the report and MVD request form is mailed to the Wisconsin Department of Transportation or Department of Natural Resources, as provided by statute.
    c. Death Investigation reports:
        (i) A copy of the report is retained by the Forensic Toxicology Program.
        (ii) As appropriate, a copy of the report is mailed to the consulting pathologist that conducted the autopsy and collected the specimen(s).
        (iii) The original report is mailed to the submitter (coroner or medical examiner).
    d. Report records for the Forensic Toxicology Program are retained according to the Records Disposition Authorization policy.
17. If necessary, volatile compounds other than ethanol are quantitated in a separate procedure. They are reported with the ethanol result, or with drug testing results, depending on the type of submission.

L. Notes

1. The presence of methanol in post-mortem specimens may indicate contamination of the specimen with formaldehyde or other external source. Verify the possibility of contamination by contacting the submitter on all methanol-positive specimens. If no source of methanol contamination is identified a separate methanol analysis is conducted and reported.
2. Acetone may be seen in the blood or urine of diabetic or fasting subjects. The ingestion of isopropanol will produce both acetone and isopropanol in the sample.
3. Check all ethanol-positive urine specimens for the presence of glucose with a Ketodiastix reagent strip. Record the Ketodiastix result on the sample submission

form (implied consent specimens) or internal tracking form (MVD, death investigation specimens).

4. If a urine specimen is glucose-positive, store at room temperature for at least two days and re-analyze for ethanol concentration. If the ethanol concentration has increased by 5% or more, the ethanol result is not reported and an appropriate comment is placed on the report.
5. For implied consent urine ethanol specimens collected under Wisconsin statute 343.305, the subject must first void his/her bladder before collecting the specimen to be tested. The specimen to be tested is collected when the subject is again able to produce a urine specimen, usually within 30 minutes, in order to obtain a specimen which will reflect the circulating blood ethanol concentration. Collection instructions are included in the collection kits provided by the Forensic Toxicology Program.

M. Related Notes, Documents and References

1. For Tox GENOP 001 – Specimen Receipt
2. Phoenix Police Department Crime Laboratory – "Protocol for the Analysis of Ethanol" Effective Date 01/20/07.
3. Procedure as modified at the Wisconsin State Laboratory of Hygiene, Madison, Wisconsin.
4. This procedure is conducted in accordance to: the Forensic Toxicology QA/QC Manual; the WSLH Chemical Hygiene Plan, Environmental Health Division; the Blood Borne Pathogens Exposure Control Plan; and the American Board of Forensic Toxicology Accreditation Program guidelines.

N. Revision History

Revision #	Date	History
1.0	7-3-2008	Original Issue
1.1	8-14-2008	MVD reporting change; Acceptable result criteria clarification; Minor language updates
1.2	2-11-2009	Use of Calibrators L, A, B and C for calibration curve, D as linearity check

O. Signature Page – Approval and Annual Review

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P. ADDENDUM 1

PREPARATION OF ETHANOL CONTROLS

1. Whole blood and urine control pools are prepared by the Toxicology Section, Wisconsin State Laboratory of Hygiene, Madison, Wisconsin for use in the internal quality assurance program for the determination of blood and urine ethanol by headspace gas chromatography.
2. Outdated whole blood is obtained from the American Red Cross. The blood is mixed by rotator or inversion for at least one hour and filtered through cheesecloth. An aliquot of the pool material is analyzed to ensure that it is alcohol-free.
3. Alcohol-free urine is collected from volunteers, pooled and filtered through cheesecloth. An aliquot of the pool material is analyzed to verify that it is alcohol-free.
4. A stock ethanol solution is prepared by diluting 20 mL of 95% ethanol to 500 mL with high purity water. This solution will contain 3.016% ethanol weight/volume or 3.016 g/100 mL.
5. Pools are stored in 5 mL (50 x 16 mm) polypropylene vials with push tops (Sarstedt). Vials are labeled with appropriate pool number or color-coded caps for identification.
6. Into 1000 mL volumetric flasks, pipette desired amount of stock ethanol solution to yield target value (see chart below) and dilute to volume with either whole blood or urine. Pools should be mixed gently by inversion and left to equilibrate for at least one hour with frequent mixing during that time.
7. Target values are calculated as follows:

$$\frac{(\text{mL of stock ethanol solution}) \times (3.016)}{1000} = \text{ethanol concentration}$$

   For example, if 40 mL of stock solution is diluted to 1000 mL with urine or blood, then:

$$\frac{(40)(3.016)}{1000} = 0.121 \text{ g/100 mL} \quad (0.121\% \text{ weight/volume})$$

8. After equilibration of pool material, it is dispensed in 5 mL aliquots into the prepared vials. It is mixed well by inversion prior to dispensing.
9. Cap all vials tightly and store in freezer at temperature of -20 to -25°C. Frozen pool samples are stable for a minimum of 10 months.
10. Pools are assayed in 20 different analytical runs to establish mean values and standard deviations. The coefficient of variation should be within 5% and the mean value within 5% of the predicted target value. The established mean value will then become the official control value.

11. Target Values for Pools

a. The following chart indicates the final target concentration of the pool as a function of the amount of stock ethanol solution (3.016 g/100 mL) diluted to a final volume of 1000 mL.

mL of Stock	Ethanol Conc.	mL of Stock	Ethanol Conc.
10.0	0.030	66.0	0.199
12.0	0.036	68.0	0.205
14.0	0.042	70.0	0.211
16.0	0.048	72.0	0.217
18.0	0.054	74.0	0.223
20.0	0.060	76.0	0.229
22.0	0.066	78.0	0.235
24.0	0.072	80.0	0.241
26.0	0.078	82.0	0.247
28.0	0.084	84.0	0.253
30.0	0.090	86.0	0.259
32.0	0.097	88.0	0.265
34.0	0.103	90.0	0.271
36.0	0.109	92.0	0.277
38.0	0.115	94.0	0.283
40.0	0.121	96.0	0.289
42.0	0.127	98.0	0.296
44.0	0.133	100.0	0.302
46.0	0.139	102.0	0.308
48.0	0.145	104.0	0.314
50.0	0.151	106.0	0.319
52.0	0.157	108.0	0.326
54.0	0.163	110.0	0.332
56.0	0.169	112.0	0.338
58.0	0.175	114.0	0.344
60.0	0.181	116.0	0.350
62.0	0.187	118.0	0.356
64.0	0.193	120.0	0.362

P. ADDENDUM 2

MAINTENANCE AND TROUBLESHOOTING

1. It is recommended that the O-rings in the headspace autosampler be replaced every 2000-2500 injections or as needed to maintain acceptable performance.
2. If the combination volatile solution fails to adequately separate constituent peaks on a routine basis, replacing the column may resolve the issue.
3. Refer to the Perkin-Elmer Autosystem XL Head Space Gas Chromatograph owner's manual for detailed information on the above maintenance and other potential procedures.
4. Hamilton Diluters are maintained according to the owner's manual.
5. All performed maintenance is recorded in the maintenance log.

Diluter and GC Maintenance Log

It is recommended that the GC should be baked out monthly (45°C to 200°C at 10°C/min, hold 5 min)

It is recommended that the HS needle seals be changed approximately every 2000 injections

Recommended weekly diluter maintenance inclu 1. Place the diluent tubing into the **bleach** container and run bleach through the diluter at least 3 times.

2. Place the diluent tubing into the container of **rinse** solution and run the "Prime" method on the electronic diluter at least twice or prime the diluter at least 20 times.

Date / Initials		Alc1		Alc2		Front Diluter		Back Diluter		Comments
		GC bakeout	HS needle seals	GC bakeout	HS needle seals	Weekly Bleach	Weekly Rinse	Weekly Bleach	Weekly Rinse	
16-Jul-09	TN									Pneumatic arm failure on **Alc2**. Low pressure on pneumatics message on HS unit. Resumed run 7/17/09. No further maintenance required.
17-Jul-09	TN					X	X	X	X	
27-Jul-09	TE					X	X	X	X	
30-Jul-09	DM/KD		X							Replaced needle seals at 2156 injections. GC column pressure OK.
30-Jul-09	DM									INSTALLED NEW BAC2 COLUMN IN ALC1 BACK. CONDITIONED AT 45 TO 200, 5/MIN, HOLD FOR 60 MIN.
31-Jul-09	KD				X					Replaced needle seals at 2055 injections. GC column pressure OK.
31-Jul-09	TE / TN					X	X	X	X	
7-Aug-09	TE					X	X	X	X	
13-Aug-09	TN							X	X	
17-Aug-09	TE					X	X	X	X	
27-Aug-09	TN		X							Replaced needle seals at 2021 injections. GC column pressure OK.
28-Aug-09	TE					X	X	X	X	
1-Sep-09	RP	X								
1-Sep-09	KD			X	X					Replaced needle seals at 2155 injections. GC column pressure OK.
4-Sep-09	TN/TE					X	X	X	X	
18-Sep-09	RP	X	X							Replaced needle seals at 2123 injections. GC column pressure OK.
21-Sep-09	TE					X	X	X	X	
25-Sep-09	RP			X	X					Replaced needle seals at 2132 injections. GC column pressure OK.
25-Sep-09	TE					X	X	X	X	
2-Oct-09	TE					X	X			
6-Oct-09	TE							X	X	
7-Oct-09	RP/DM									Alc2 - Vial jammed in oven. Cleaned oven.
9-Oct-09	TN					X	X	X	X	
15-Oct-09	DM				X					Replaced needle seals at 2031 injections. GC column pressure OK.
16-Oct-09	DM					X	X	X	X	Replaced sample syringe on diluter #4575