

**FORENSIC TOXICOLOGY LABORATORY
OFFICE OF CHIEF MEDICAL EXAMINER
CITY OF NEW YORK**

**ETHANOL, METHANOL, 2-PROPANOL AND ACETONE
by
AUTOMATED HEAD SPACE ANALYSIS**

PRINCIPLE

This method is a modification of the one that has been used forensically for the determination of ethanol and other volatiles since 1964 when Machata first published his paper using this method.

This method determines the concentration of ethanol, 2-propanol, methanol and acetone in biological specimens such as blood, vitreous humor, and urine and tissue homogenates. Head space techniques are based on Henry's law which states that the ratio of a dissolved substance in an aqueous solution is dependent on temperature, pressure, and concentration in the aqueous medium. Volatile substances may be measured by analyzing the vapor (head space) which is in equilibrium with a liquid specimen. Biological fluids or tissue homogenates are placed into a vial with the appropriate internal standard and sealed with a septum. The vial is heated and allowed to stand until equilibrium is reached. A sample of the head space is removed from the sealed vial by an automated sampling device and injected into a gas chromatograph. Only volatile substances will reach the column, non-volatiles will remain in the sample. The volatiles are separated by the column and detected by a flame ionization detector, where hydrogen is introduced and volatiles are burned in an atmosphere of air. Burning organic matter in the flame releases electrons and negative ions which are collected on the anode, producing a change in electrical current proportional to the amount of burned material. The current is amplified, digitized and raw data collected on a computer. The data is processed by computer programs and can be displayed as a peak in a chromatogram. Components may be identified qualitatively by their retention time (length of time they are retained on the column). Quantitation is performed by computer programs, using the height ratios of the chromatographic peaks of interest and an internal standard (n-propanol), calculated from a least-squares equation derived from the four calibrators. The compounds are identified by their relative retention times on both columns and their quantitative amounts are determined by the peak-area ratios compared to that of the standard curve generated that day. See a representative chromatogram, Figure 1, page 26.

Automated head space gas chromatography employed in this laboratory uses a salting-out agent, ammonium sulfate, which is present in the diluents solution and is used to liberate volatiles from their matrix. The diluent solution also contains sodium dithionite to liberate the oxygen from the red blood cells.

SAFETY

The handling of all samples, reagents, and equipment is performed within the established laboratory safety guidelines detailed in the safety manual.

REAGENTS AND MATERIALS

All solutions are prepared at room temperature (20 - 30 °C).

1. **Ethanol** (ethyl alcohol, C₂H₅OH, fw 46.0)
Aldrich, 200 proof or equivalent
2. **Methanol** (methyl alcohol, CH₃OH, fw 32.04)
Fisher Scientific, certified or equivalent
3. **2-Propanol** (2-propanol, isopropyl alcohol, (CH₃CHOHCH₃, fw 60.10)
Fisher Scientific, certified or equivalent
4. **Acetone** (CH₃COCH₃) fw 58.08
Fisher Scientific, certified or equivalent
5. **1-Propanol** (n-propanol) (C₃H₇OH, fw 60.10)
Fisher Scientific, certified or equivalent
6. **Formaldehyde** solution (CH₂O) - 37% W/W, fw 30.03.
Fisher Scientific, certified or equivalent. **NOTE:** Contains 10-15% methanol.
7. **Ammonium sulfate** (fw 132.14)
Fisher Scientific, certified ACS grade or equivalent
8. **Sodium hydrosulfite** (Dithionite, Na₂S₂O₄, fw 174.10)
Fisher Scientific, certified ACS grade or equivalent
9. **Deionized water**
10. **Certified negative calf brain**
Previously found to be volatile free. Store frozen. Discard after 3 months.
11. **Hamilton Microlab 500 series pipettor-diluter**
12. **Vortex 2-genie, centrifuge (maximum 3000 r.p.m)**
13. **Ohaus portable advance electronic scale**
14. **Crimper, aluminum seals, rubber septa, 20mL headspace vials**
15. **Restek Resolution Control**
16. **N-butanol solution (C₄H₉OH), fw 74.12**
Fisher Scientific, certified or equivalent

PREPARATION OF CALIBRATORS AND CONTROLS

To determine the volume of alcohol or acetone used in a calibrator or control, the following formula is used:

$$\text{Density } (d) = \frac{\text{Mass } (m)}{\text{Volume } (v)} \quad \text{or} \quad \text{Volume} = \frac{\text{Mass } (m)}{\text{Density } (d)}$$

The density of methanol is 0.792, of ethanol 0.789, of 2-propanol 0.786, of acetone 0.791. Placing these values in the above equation, the volume of pure liquid for each calibrator and control can be calculated.

For the 0.05 g% calibrator (Cal 1, aqueous), the following volumes are used:

Methanol	63.0 µL in 100 mL of deionized water
Ethanol	63.0 µL in 100 mL of deionized water
Acetone	63.0 µL in 100 mL of deionized water

2-propanol **64.0 μL** in 100 mL of deionized water

For the 0.150 g% calibrator (Cal 2, aqueous), the following volumes are used:

Methanol **189.0 μL** in 100 mL of deionized water
Ethanol **190.0 μL** in 100 mL of deionized water
Acetone **190.0 μL** in 100 mL of deionized water
2-propanol **191.0 μL** in 100 mL of deionized water

For the 0.300 g% calibrator (Cal 3, aqueous), the following volumes are used:

Methanol **379.0 μL** in 100 mL of deionized water
Ethanol **380.0 μL** in 100 mL of deionized water
Acetone **379.0 μL** in 100 mL of deionized water
2-propanol **382.0 μL** in 100 mL of deionized water

For the 0.450 g% calibrator (Cal 4, aqueous), the following volumes are used:

Methanol **568.0 μL** in 100 mL of deionized water
Ethanol **570.0 μL** in 100 mL of deionized water
Acetone **569.0 μL** in 100 mL of deionized water
2-propanol **573.0 μL** in 100 mL of deionized water

For the 0.15 g% control (HQ, aqueous), the following volumes are used:

Methanol **189.0 μL** in 100 mL of deionized water
Ethanol **190.0 μL** in 100 mL of deionized water
Acetone **190.0 μL** in 100 mL of deionized water
2-propanol **191.0 μL** in 100 mL of deionized water

Note: In general, calibrators and controls are prepared in 100 mL volume. Larger amounts of a solution may be prepared by multiplying analyte volumes to adjust for larger final volumes, as long as the ratio of volatile to water is maintained. Thus, for the low control, the calculations yield approximately 12.6 μL per 100 mL. Because it is difficult to accurately add 12.6 μL of neat volatile to a solution, all low controls will be prepared at a volume of one liter. By multiplying the amount of volatile and water by a factor of 10, the concentrations remain 0.010 g%.

0.01 g% control (LQ, aqueous), the following volumes are used:

Methanol **126.0 μL** in 1000 mL of deionized water
Ethanol **126.0 μL** in 1000 mL of deionized water
Acetone **126.0 μL** in 1000 mL of deionized water
2-propanol **126.0 μL** in 1000 mL of deionized water

Note: In tissue procedure 2 mL of tissue internal standard solution is added to 2 mL of control solution to yield a control for the tissues (brain method). Tissue calibrators use the same solutions as aqueous calibrators (cal 1, 2, 3 and 4).

Prepare the aqueous calibrators and controls by filling an appropriately sized volumetric flask $\frac{3}{4}$ full of deionized water. Add the indicated volume of each pure primary standard to the volumetric flask (for each volatile addition use an individual clean, dry syringe), swirl to mix, then q.s. to the line. Mix thoroughly. Validate each solution against the current lot of calibrator or control at least in triplicate, using one of the headspace instruments. After successful validation, aliquot into 5 mL headspace vials and cap with stoppers and appropriately colored aluminum caps. Label with lot number, date prepared, expiration date, and individual preparing the solution. Store at 2-8°C. The lot is stable for one year. As an individual vial is opened for use, it is dated (MM/DD/YY) and initialed. Discard within 7 days after opening.

Brain Matrix Control (0.08 g% target)

Cut and homogenize at least 100 g of negative validated calf brain.

Tare a blender for a homogenizer and place homogenized negative validated calf brain into the blender until there is exactly 100 g in the blender.

Add the following volumes of neat volatile to the negative calf brain in the blender. For each volatile addition use an individual clean, dry syringe.

Methanol	101.0 μL
Ethanol	101.0 μL
Acetone	101.0 μL
2-propanol	102.0 μL

Homogenize at low speed for approximately 3 minutes.

Weigh out 2.0 g of the brain matrix control into 20 mL headspace vials and aliquot the entire batch. Label the vials. The brain matrix control is stored frozen until removed for analysis.

The brain matrix control is validated using at least 6 aliquots (see tissue procedure) on a headspace instrument. The mean of the analyses becomes the target value for the control. The control must meet all the requirements established in the acceptance criteria guidelines. Each batch of control must be labeled with the lot number, date prepared, preparer's initials and expiration date.

Enter all validation data into the QC database.

VEC External Blood Control (variable target concentrations)

This is an assayed external blood control purchased through Clinical Controls International with a 0.036 g% target for methanol, 0.080 g% target for ethanol, 0.044 g% target for acetone and 0.041 g% target for 2-propanol. For each new lot of VEC control, the control is run at least 10 times, and the average of the 10 values for each analyte becomes the new target value. The acceptable control range is $\pm 10\%$.

INTERNAL STANDARD

Diluent solution

1. Weigh 100 grams of dithionite (sodium hydrosulfite) and transfer into a 1000 mL Erlenmeyer flask.

2. Place the flask on a hot plate and heat to 80 to 100 °C for approximately 15 minutes while stirring with a stir bar.
3. When no more bubbles are observed, de-gas the dithionite for 10 minutes
4. Allow the dithionite to equilibrate to room temperature.
5. Weigh 34.8 g of the previously heated dithionite in a weighing cup and transfer into a 2 liter volumetric flask.
6. Add approximately 1.5 L of distilled water and stir with stirring bar until the dithionite is completely dissolved.
7. Next, add 265 g of ammonium sulfate to the same flask and stir until completely dissolved.
8. Add 300 µL of n-propanol to one liter of diluent solution.
9. QS to 2 L mark.
10. Mix thoroughly. Inject diluent with internal standard on a headspace instrument. Assess the chromatogram for the presence of any interfering peaks. Normal peak heights for analytes in regular runs are 10^5 to 10^6 units, and the background noise peaks are 10^3 to 10^4 units. If the observed noise peak(s) height count is 10^4 or less, the observed noise peak(s) are considered not to be interfering peaks. If there are interfering peaks in the diluent, discard and prepare a new batch of diluent. Additionally, run a calibrator to ensure that the acetone peak height is greater than the isopropanol peak height. If the solution passes these validation injections, label with lot number, analyst initials, date of preparation and expiration date. Store at 2-8 °C. Discard after six months.

Internal standard solution for tissues

Add 600 µL of n-propanol to one liter of diluent solution. Mix thoroughly. Inject diluent with internal standard on a headspace instrument. Assess the chromatogram for the presence of any interfering peaks. If the solution passes the validation injection, label with lot number, analyst initials, date of preparation and expiration date. Store at 2-8°C. Discard after six months.

PROCEDURE FOR BIOLOGICAL FLUIDS

The following procedure is utilized for the qualitative and quantitative analysis of biological fluids (blood, vitreous, urine, gastric content and bile) for volatiles.

1. All reagents, samples, controls and calibrators must equilibrate to room temperature prior to sampling.
2. Prior to sampling, label the headspace vials to correspond to the order in which samples will be injected. Vials must be labeled such that the specimen type, the aliquot number, Tox number and any other factors unique to a given sample is clearly written on the headspace vial. Handwriting must be clear and legible.
3. Inspect the diluter/dispenser carefully. Make sure the reservoir is filled with enough internal standard solution to complete the batch. Assess the syringes for dirt or leaks, and the tubing for any indication of air bubbles, kinking or particulate matter. Maintain as necessary. If there are still problems, replace the tubing.
4. Prime the system by aspirating and dispensing an air sample 5 times.

5. Using the diluter/dispenser, aspirate 100 μ L of specimen (calibrator, control, or sample). Observe the tubing as the sample is aspirated. If blockage (clot) prevents sample rising in the tubing, or bubbles are aspirated, discard to waste and try again. Place the tip into the labeled headspace vial. Check that the sample information on the vial corresponds to the sample being pipetted, and then dispense the sample into the vial. 1.0 mL of internal standard solution will automatically be pipetted by the diluter/dispenser into the vial after the sample. Cap the vial with a rubber septum. Wipe the tubing with a tissue before and after each operation.
6. Repeat step 5 for each calibrator, control, and sample in the batch. The normal order as shown in the following table should be used:

Position #	Samples
1	Calibrator I 0.050 g%
2	Calibrator IR 0.050 g %
3	Calibrator II 0.150 g%
4	Calibrator III 0.300 g%
5	Calibrator IV 0.450 g%
6	Blank
7	0.150 g% Control
8	VEC
9	0.010 g% Control
10	Formaldehyde control
11-17	Specimens
18	VEC A
19-27	Specimens
28	VEC B
29-37	Specimens
38	VEC C
39-43	Specimens
44	VEC D
45	VEC E
46-54	Specimens
55	VEC F
56-64	Specimens
65	VEC G
66-74	Specimens
75	VEC H
76-84	Specimens
85	VEC I
86,87	Specimens
88	VEC J

7. A VEC control is analyzed after every ninth sample. The last sample in each batch is also a VEC control. **Note:** a batch may be greater than 44 samples. For batches greater than 44 the first sample placed on the auto sampler **MUST** be a VEC. VEC's will be placed in the sample table at every 10th sample and as the very last sample of the batch.
8. After calibrators, controls and samples are pipetted, place an aluminum seal on the vials and, with a crimper, seal each vial tightly. Recheck each vial to make sure the vial is correctly labeled.

PROCEDURE FOR TISSUES

The following procedure is utilized for the qualitative and quantitative analysis of tissue samples (i.e., brain, liver) for volatiles.

1. All reagents, samples, controls and calibrators must equilibrate to room temperature prior to sampling.
2. Prior to weighing, label the headspace vials to correspond to the order in which samples will be injected. Vials must be labeled with specimen type, aliquot number, Tox number and any other factors unique to a given sample. Handwriting must be clear and legible.
3. Pipette 2.0 mL of calibrator 1 solution into the headspace vial labeled br cal 1 using a 5 mL disposable pipet. Pipette 2.0 mL of tissue internal standard solution into the headspace vial. Immediately cap the headspace vial with a rubber septum. Final concentration is 0.050 g%.
4. Pipette 2.0 mL calibrator 1 into the headspace vial labeled br cal 1R (0.050 g %) using a 5 mL disposable pipet. Pipette 2 mL of tissue internal standard solution into the headspace vial. Immediately cap the headspace vial with a rubber septum.
5. Repeat step 3 using calibrator 2 into a suitably labeled vial. Final concentration is 0.150 g%.
6. Repeat step 3 using calibrator 3 into a suitably labeled vial. Final concentration is 0.300 g%.
7. Repeat step 3 using calibrator 4 into a suitably labeled vial. Final concentration is 0.450 g%.
8. Pipette 2.0 mL of distilled water and 2 mL of tissue internal standard solution into the vial labeled blank. Immediately cap the headspace vial with a rubber septum.
9. Pipette 2.0 mL of the 0.150 g% control into a headspace vial using a 5 mL disposable pipet. Pipette 2.0 mL of tissue internal standard solution into the vial labeled br 0.150 g% control. Immediately cap the headspace vial with a rubber septum.
10. Add 2.0 mL of tissue internal standard solution to a previously aliquotted and validated brain matrix control. Confirm the lot number and document on the volatile batch worksheet. Label vial QC and include lot number. Immediately cap the headspace vial with a rubber septum. The brain matrix control must completely thawed before use.

Note: *The most practical way to accomplish this is to thaw the frozen control overnight in the refrigerator.*

11. Pipette 2.0 mL of the 0.010 g% control into a headspace vial using a 5 mL disposable pipet. Pipette 2.0 mL of tissue internal standard solution into the headspace vial labeled br 0.010 g% control. Immediately cap the headspace vial with a rubber septum.
12. Retrieve the thawed brain (tissue) sample. Compare and confirm all data on the container against the data on the labeled headspace vial. If there are any questions regarding information on the container, consult the laboratory supervisor before aliquoting any samples. Next, under a biological hood, weigh out 2.0 g of specimen into the headspace vial. Add 2.0 mL of tissue internal standard solution to the specimen, and immediately cap the vial with a rubber septum.
13. For solid brain specimens, homogenize using the Polytron at low speed, for only a few seconds. Replace rubber septum, cap vials with an aluminum seal and, using a crimper, and seal each vial tightly. Mix the sealed specimen using the Vortex for approximately 15 - 30 seconds. Check each vial to make sure the labeling is correct. Tissues are done in duplicate, sample quantity permitting.

14. After every ninth sample, a brain matrix control is analyzed. The last sample in the batch is also an internal brain control.
15. Include a Restek Resolution Control in position #11, an n-butanol control in position #12 and an air blank in position #13 in the Batch.

INSTRUMENTATION

Instrument #1 & #2 (HS1,2)

Autosampler:	Agilent 7694 head space Sampler.
Gas Chromatograph:	Agilent 6890 Gas Chromatograph/Dual Flame Ionization Detector. (GC/FID)
Column:	RTX-BAC 1, 30 meters, 0.32 mm id, 1.8 micron film thickness, and RTX-BAC 2, 30 meters, 0.32 mm id, 1.2 micron film thickness.
Integrator:	Computer with Chemstation software.
Head space method:	HS1,2 blood.m (fluids) and HS1,2 brain.m (tissues).

INSTRUMENT SETUP

Information regarding the daily maintenance and standard operation of the GC/FID can be located in the Standard Operating Procedure Manual. At the beginning of the day, the nitrogen tank pressure is recorded in a logbook. The temperature of the oven, detector and injection port is checked. The detector is also checked to make sure it is lit.

The instruments are run with two columns connected to a “Y” connector (Restek part MTX) and connected to the injection port with a short length of deactivated fused silica tubing. The output of the injection port is split between the two columns (BAC 1 and BAC 2), and each column goes to a separate FID detector. Both chromatograms are collected in the same data file.

INSTRUMENT PRE-RUN PROCEDURE

The instruments are used for postmortem volatile screening and quantitation. All instruments are left on for twenty-four hours a day. If the instrument is shut down, consult the Standard Operation Procedure Manual for instrument setup.

1. Follow the laboratory safety guidelines when changing gas cylinders. Use proper body mechanics and always chain gas cylinders to hand truck. Inspect tank pressures and valves. Use snoot to check for leaks. When the air or nitrogen is below 500 psi, the tanks must be changed. Hydrogen generators are currently utilized. If a hydrogen tank is used, it must be changed if the hydrogen is below 200 psi. Air generators are currently utilized. If an air tank is used, it must be changed if the air is below 500 psi. Record all information in the Tank Pressure Log.
2. Check temperatures:
Instruments – HS1 and HS2

GC Oven Temperature	40 °C
Injector Temperature	150 °C
Detector Temperature	200 °C
Autosampler Oven	60 °C
Autosampler Sample Valve	100 °C
Autosampler Sample Line	100 °C

3. To ensure that the FID is lit:
 - a. The GC Detector display will indicate a signal greater than zero.
 - b. For a visual inspection, use a headspace vial to detect condensation at the detector.
 - c. If the FID is not lit, check to make sure regulator has been turned on. If problem persists, consult the SOP procedure manual or your supervisor.

The Agilent Headspace Sampler Parameters are as follows:

Matrix	Blood
Matrix Boiling Point	100.0 °C
	Temperature (°C)
Sample Oven	60
Sample Valve	100
Transfer Line	100
	Time (minutes)
GC Cycle	4
Sample Equilibration	5
Vial Pressurization	0.15
Loop Fill	0.15
Loop Equilibration	0.15
Sample Inject	1
Oven Stabilization	0.5
Shaking	
Agitation	Low
Parameter	
Oven Step	No
Equilibration Time	No
Mode	
Extractions	1
Puncture Mode	Single

4. Proceed with the sample analysis.

SAMPLE ANALYSIS

Instrument 1/HS1

Instrument 2/HS2

When Chemstation is opened, the **Openlab ECM Login** screen appears, Enter HS1 or HS2 as appropriate for username and the current password. Verify that Account field is production and

Domain field is Built-In. If Chemstation is already running, it may be necessary to log out and log in again. Using the Chemstation software, at the top toolbar under **ECM**, select **Logon to ECM**. Follow the instructions above to log on.

1. On the top toolbar, under **Sequence**, select **Load Sequence**. Select the corresponding model sequence template to be loaded. For bloods on HS1 select HS1model.s; for tissues select HS1modelbrain.s. For bloods on HS2 select HS2bloodmodel.s; for tissues select HS2brainmodel.s. In the same dialog box, verify that the correct sequence template Model is loaded under File Name. Click on Select.
2. In the top toolbar, under **Sequence**, select **Edit Sequence**.
At the top of the screen under Data Path, click on Browse. Under **Select Data Path**, click on the **msdchem** folder. Then click on either **folder 1** (for HS1) or **2** (for HS2) and then click on the **Data** folder to highlight it. In lower left of dialog box, select **Make New Folder**. A folder with the name New Folder is created under DATA. Right click on **New Folder** and **Rename** or double click to highlight the folder name and change the entry. Rename the file using the format HSNMMDDYYx, where N = 1 or 2, depending on instrument, MM = month, DD = day, YY = year and x = a letter indicating the batch being run, e.g. hs2041111a. Click **OK** which will take you back to the sequence table.
3. In the Sample column verify the correctness of pre-loaded entries. Starting at the first empty field, enter sample or QC information. For samples this would include aliquot number, laboratory number, specimen source, dilution if any (i.e., 2-11-2432 fem). If the sample has been diluted, enter the appropriate dilution factor in the Multiplier column. For QC samples use an appropriate designator. Enter the next sample in the batch in the next open field down the column.
4. In the **Type** column, select the corresponding sample type for each vial: Sample, Blank, Calibration or QC.
5. In the **Vial** column, click in the cell with the number 1, hold down the left mouse button and drag to the last vial number in the sequence (cells will be highlighted). Right click and select **Fill Column and Increment**. Verify that the vial numbers are correct.
6. In the **Method** column, verify that the correct method is loaded in the first cell. Then click on the first cell containing the method name, hold down the left mouse and drag to the last vial number in the sequence (cells will be highlighted). Right click and select **Fill Column No Increment**. Verify that the method for each vial is correct.
7. In the **Data File** column, in the cell corresponding to the first vial, enter the data file name in the format hsNMMDDx001, where N = instrument number, MM = month, DD = day, and x = a letter indicating the batch being run, i.e. hs20411a001a. Click on this cell, hold down the left mouse and drag to the last vial number of the sequence (the cells will be highlighted). Right click and select **Fill Column and Increment**. Verify that the data file information for each vial is correct.
8. In the **Comment** column, enter any additional information for the vials.
9. In the **Multiplier** column, enter any sample dilutions.
10. In the **Level** column, verify levels 1, 2, 3 and 4 are entered for calibrators 1, 2, 3 and 4.
11. Verify **No Update** is selected for all vials under **Update Rf** and **Update Rt**.

12. Review the information typed for the sequence. Correct any information as needed. Verify that the Data Path is C:MSDCHEM\1 or 2\Data\current sequence name. Verify that the Method Path is C:MSDCHEM\1 or 2\METHODS. Then Click **ok**.
13. On the Method and Run toolbar, under **Sequence**, select **Run Sequence**. In the dialog box under **Sequence Comment** enter the initials of the individual who has sampled the specimens followed by a slash (/) and the initials of the person who has entered the sequence in the **Operator Field** (e.g., EH/EH both pipetted the specimens and created the sequence). Under **Data File Directory** verify that the data file path is C:MSDCHEM\1 or 2\DATA\batch.
14. Under **Sequence**, select **Save Sequence As**. Under File Name, type in the name of the folder that the batch will be saved to under DATA, HS (1 or 2)MMDDYYx (e.g. hs2041211e.s) Select **Save**. The extension “.s” will automatically be added.
15. Under **Sequence** select **Print Sequence**. Verify that **Brief Format** is selected and click on **OK**. The sequence will be printed. Apply preprinted labels with the control values, and the preprinted labels for documenting verification of process steps to the printed sequence list.
16. Load vials into the appropriate autosampler positions as indicated by the order on the printed sequence list. Check vial information against the sequence list and ensure that the vial is inserted in the correct numeric position in the autosampler as indicated on the sequence list. Document correct positioning by dating and initialing the appropriate line on the sequence list.
17. Under Sequence select **Run Sequence**. Verify that the Sequence comments and Data Field information are correct (i.e. verify that the proper sequence is loaded. If not, load the proper sequence). Click on **Run Sequence**.
18. On the Agilent head space sampler front panel, press **Zone Temps**. Check that the oven, loop, and transfer line are set to the correct temperature by pressing the up or down arrow to cycle through the setpoints. Press **Event Times**. Cycle through the GC cycle, sample equilibration, pressurization, fill, loop equilibration and inject times, checking that they are at the correct setpoints. Click **Vial Parameters**. Press the up arrow and verify the position of the first vial to be injected (usually 01). Change if necessary. Press the down arrow. The screen should show last vial. Enter the position of the last vial to be injected for this run. Hit down arrow to display **Shaking**. It should be “low”. Change if necessary. Press **Enter**. Press **Start** on the head space sampler front panel to start the head space sampler.
19. After the batch is finished, unload the vials. Compare the vial information to the sequence list as they are removed, to verify that the correct vial was in the correct position. Date and initial the sequence list when this is completed. Annotate discrepancies if necessary.

POST-RUN PROCEDURE

Reports including chromatography are generated using Chemstation software. Quantitative values are determined by a linear regression analysis of the calibrators injected. All positive results are compared to the calibration curve and a resultant concentration is obtained for samples and controls.

Data Review – First Level Review

All processing and review are performed on a processing computer.

DATA TRANSFER AND PROCESSING

Save Method to ECM

1. After the batch finishes, the data files will be in the **Data** subdirectory on the local chemstation and also automatically transferred to ECM. At the top toolbar under **ECM** click on **Save Method to ECM**.
2. Click on the HSGC, the correct instrument name (HS1 or HS 2) folder, the appropriate month and batch to which the method will be saved. Click **OK** to save the method to the batch subdirectory in **ECM**.

Retrieve Batch from ECM

1. At the processing computer, click on Processing Data Analysis. Log on using your OCME network username and password.
2. On Enhanced Data Analysis screen, click on ECM at the top toolbar and select Retrieve entire sequence from ECM.
3. This will open up Openlab ECM screen. Select HSGC, then HS1 or HS2, the month, and, finally the batch to be retrieved. The status line at the bottom of the screen will show the progress as the batch is being retrieved. The batch will be downloaded to the following location: C:\msdchem\1\ECM\Retrieve\”batch name”

Load Method and Batch

1. On the left screen under the C drive, open C:\msdchem\1\ecm\retrieve
2. Under retrieve, click on the batch that was retrieved. When all files have been downloaded to the processing computer, verify that the appropriate method is present in the batch.
3. To load the method, right click on the method under the batch being processed and select load. This will bring up “Be sure changes are saved. Load now?” Click yes. If the method is not present, load the method by retrieving the method from ECM. Save the method to the correct batch subdirectory.
4. Click on any file in the batch to load it.

Perform Batch Calibration

Under enhanced data analysis:

1. On the top toolbar, click on Tools
2. Under Tools click on **Dolist** This will bring up Qlist Configuration window.

3. Under the Qlist Configuration window. In the left hand window are various options. In the **Selected Options** window on the right, the only program listed should be **Quant No Report**. If any other option(s) is present, click to highlight it and select remove. If **Quant No Report** is not present in the **Selected Options** box, choose **Quant No Report** from the left side by clicking on it and then select Add. Finish by selecting **OK**.
4. Under the **Multiple File** screen, verify that the correct batch is loaded. If the correct batch is not loaded, load the correct batch by selecting **Change Path**, then selecting the batch to be loaded. Select **OK**. Verify that the path for the data files is correct.
5. Note: correct path is C:\msdchem\ecm\retrieve\batch
6. In the left "available data file" screen, highlight calibrator files (2,3,4, and 5) and select right arrow to load **Files Selected for Processing** window.
7. Select Process.
8. After the files have been processed, select **File** on the command line. Click on **load data file** to set path. Highlight and click on data file to be loaded. Select Ok.
9. Note: the second data file (cal r) will usually be loaded first.
10. In the first icon line, click on **Qedit** (16th from left). A dialog box appears. Review results by double clicking on each analyte to be reviewed (an "x" next to the analyte name is displayed if the program automatically detected the analyte) or click start for an automated run through the list of analytes. During data review, review all chromatograms to determine if the software has correctly integrated all peaks, and correctly assigned them. If there is an error in integration, either failure to integrate or inappropriate baseline, manually integrate the peak. Draw the most scientifically correct baseline you are able to for the particular peak in the chromatograph. Check to see that the peaks are reasonably Gaussian in shape. If not, notify supervisor and make note of the problem. When completed, click Exit.
11. Note: If formaldehyde is detected in any calibrator, remove by selecting **QDel**, then selecting **Yes** to **Delete Compound #4**. This will bring up **Save changes to quantitation report**, select **Yes**.
12. In the second line of icons, select "**up arrow**" (17th from left) to update the appropriate level in cal table. The **Select Update Options** window appears. Choose **Update one level** and **OK**.
13. This brings up the question, **Files has previously been quantitated, requantitate now**. Select **no**.
14. On **Update Calibration** screen, select **Update Level**. Then click on **Responses** and **Replace** and on **Retention Times** and **Replace**. Then under **Existing level ID**, select the cal level to update and click on **Do Update**. Click **Yes** to the question **Formaldehyde not found**. Click **OK** to the warning **Update only 10 of 12 compounds**.
15. This will bring up edit compounds screen. Select OK at the bottom of the screen.
16. This brings up **Current changes will be saved. Proceed?** Select **OK**. Under File, select next data file, then repeat step 6. (Alternatively, to access the next available file in order, click on the Load Next Data File icon (second from left, top)).
17. After the last cal data file has been updated on the **Edit Compound** screen, select the **Calibration** tab and review calibration results for all compounds. In the left side box at the "vial" icons, expand the n-propanol IS and n-propanol IS#2 files representing results for both columns). Click on each compound. The plot of the curve for that compound will be

displayed along with statistical data for the curve to the right. Check that the r^2 is greater than 0.98 for all analytes.

18. If the calibration results are okay, then click OK. This brings up **Current changes will be saved, proceed**. Click **OK**.
19. Save the current method to the correct batch subdirectory in the C drive. Then save the method to ECM by selecting: ECM\HSGC\HS1 or HS2\month\batch.
20. Print the calibration table for the current batch by clicking **Calibrate** on the command line. Select **List, Calibrate Report** and click OK. The Calibration report will print to the screen. Review the r^2 values, then right click on the screen report to print it.
21. Click on close report icon (top icon line, #14)

Batch Processing

1. After the method for the batch has been calibrated, to process the batch select Tools on the command line.
2. Select Dolist and OK.
3. The program required is "Quant No Report". Remove other programs from Selected Options on the right and add "Quant No Report" to this box. Select OK.
4. The next dialog box contains all files in the batch. Highlight all files in the "Available Data Files" window and transfer to the "File Selected for Processing" window.
5. Select Process to process the data files

Data Review

1. To review the processed data files on the "Data Analysis" screen, click on File in the command line.
2. Select the first data file to be reviewed.
3. Select Qedit to review the results. During data review, review all chromatograms to determine if the software has correctly integrated all peaks, and correctly assigned them. If there is an error in integration, either failure to integrate or inappropriate baseline, manually integrate the peak. Draw the most scientifically correct baseline you are able to for the particular peak in the chromatograph. Check to see that the peaks are reasonably Gaussian in shape. If not, notify supervisor and make note of the problem.
4. After the results are reviewed, click on the "Next Data File" icon and Qedit. Repeat until the last data file has been reviewed.
5. When reviewing sample results, data from both columns are to be reviewed and any major discrepancies or unidentified peaks noted.
6. If peak assignments are incorrect, either due to failure to automatically integrate, or miss-assignment of peaks, correct these failures.
7. When all samples have been reviewed and any corrections made, select Tools from the command line.
8. Under Tools select Dolist and edit the programs to delete Quant No Report and add Summary Quant w/o Calculations.

9. Select OK. This will bring up the Multiple Files Screen with the listed data files. Highlight all the data files and transfer them to the "Files Selected for Processing" window. Select Process to print reports for the data files.
10. Save the data files to ECM. Select ECM on the command line, then Save multiple files to ECM. Highlight all the data files in left window, and, using the right arrow, transfer them to the right window. Select Process. This will upload all the data files, making a new revision in ECM.
11. After the data files have been saved to ECM, under File on the command line select Exit. This brings up Be sure changes are saved, Exit now. Select yes. This will close the Processing Data Analysis screen.

Batch Clean Up

Select my computer. Find the batch on the C drive at C:\msdchem\1\ecm\retrieve\batch. Right click on the batch to be deleted and select delete. **Do not delete a batch that has not been successfully uploaded to ECM.**

QC-QA database entry

The control values for each specimen run are entered as follows:

1. On the network drive, open Microsoft Excel and locate the proper month or tissue file in the network directory T:\AlcoholQC\XXXX, where XXXX is the current year. Load by double-clicking on the appropriate file name. The spreadsheet will open.
2. Enter all values for the controls into the proper column of the chart. This includes data from the 0.15 g% and 0.01 g% controls and all VEC or brain matrix control data. Initial in the appropriate space for each line of entry
3. After all relevant data have been entered, save the file, and then also execute a "save as" to the corresponding file with a .bckp extension.

SECOND LEVEL REVIEW

The Second Level Reviewer will review the processed data in its entirety according to the acceptance criteria. The second level reviewer will also ensure the following;

1. Check chromatography of all injections. Examine the peak shape and note if any peaks show non-Gaussian shape. Identify unresolved peaks and peaks with shoulders on either side. Address any noted problems before proceeding. Consult a supervisor about any unusual events.
2. Check that all significant peaks in the chromatogram are integrated. If the printout allows, check if the baseline used to integrate is appropriate. The analyst should consult with a supervisor about any unusual events, such as the presence of overload peaks

3. Check that all components of each calibrator are present and that each peak is properly assigned. Correct any errors. If any calibration compounds are not detected, or if unexpected multiple peaks are detected for a calibration compound, the analyst must consult with a supervisor.
4. Check the blank for significant peaks that might co-elute with a compound of interest. There is some room for judgment in this step, but the analyst should consult with a supervisor if there are ANY questions. A blank may be scheduled for mass spectrometry to discover the identity of peaks in a blank, but this step must have no bearing on the decision whether a blank is acceptable or not. If any significant peaks are present in a blank for the batch, the run must be rejected.
5. Review QC samples, determine if the controls meet all acceptance criteria and verify the concentration of the components. If the batch fails, ensure that the sample chromatograms are annotated, listing the reason for failure. Notify a supervisor and / or the QC manager.
6. Ensure the following information is annotated on the first calibrator's chromatogram or sequence list:
 - a. Calibrators and blank reviewed and accepted (or not accepted). Any QC or calibrator failures are annotated on the proper chromatograms.
 - b. Any deviations from the SOP are noted or any comments are stated in a concise but detailed fashion.
 - c. Initial(s) and date
 - d. Regression coefficient (r^2) of the four point calibration curve of each component present in any case in the batch must be greater than or equal to 0.98. Notify a supervisor about any errors and make sure they are corrected before proceeding.
8. Make sure that each control has the appropriate target concentration range label.
9. Review calibrators, controls, and blanks; ensure all acceptable criteria are met.
10. Initial and date the results.

Note: By initialing and dating this document, the analyst certifies that a complete and accurate review was done.

11. Ensure the master copy of the sequence list includes, (from top to bottom): Calibrators, all QC samples and blanks, r^2 report, sequence list, internal standard recovery form and the lot sheet form. It is the responsibility of the analyst to verify that the master copy is legible and is an accurate copy of the originals, with no information cut off at the margins.
12. Retrieve the case file for each sample in the batch and associate the case file with the corresponding chromatogram(s).
13. Review the case's initial chromatogram, if applicable. Determine if the results are consistent with all the other applicable case data to date. For repeat analysis, it is important to compare the first chromatogram result with the second chromatogram result. The ratio of peak to internal standard for both chromatograms should be examined. If the results are

consistent, report the results on the Result Summary Sheet. Date and initial both the chromatogram and the Result Summary Sheet. If the results are not consistent, the inconsistency must be resolved. Consult a supervisor about rescheduling the sample, or other appropriate corrective action.

14. If the chromatogram shows overloaded peaks, or poor recovery of the internal standard, then repeating the sample may be necessary. If the problem is an overload, re-schedule with appropriate dilutions. In cases of poor recovery, the sample, its control, blank, and a calibrator may be re-injected once. If the criteria as listed in batch review are still not met, the sample is re-scheduled. If a case has an unknown or unidentified peak not observed in previous screening results, the case must be run by GCHS/MS for identification of that peak. All chromatograms must be attached to the case file. Consult with the supervisor if there is any question.
15. Submit the case file for third level review.

Note: Do not discard any sample paperwork.

THIRD LEVEL REVIEW (FINAL REVIEW)

The third and final level review can only be performed by the Laboratory Manager. He/she will review the data for the entire case according to all established criteria. They will ensure that screening, confirmatory and quantitative analysis on the case have been completed and reported accurately. As needed, they will also schedule additional analysis and contact the Medical Examiner on the case to discuss any findings and / or review case history.

ACCEPTANCE CRITERIA

1. Acceptable peak shape. Peaks must be symmetrical.
2. Satisfactory baseline resolution of all peaks of interest in the volatile mixture is required.
3. All analytes in the 0.010 g% control must be integrated.
4. Correlation coefficient equal to or greater than 0.980.
5. Blank must have no peaks for the target analytes. In cases where there are interfering and/or carry over peaks the run shall be brought to the attention of one of the toxicology managers for advisement.
6. All calibrators must quantitate to within $\pm 10\%$ of target value for all analytes. If a result is outside this range then any case positive for this analyte must be repeated.
7. Internal standard (IS) response comparable to the response for the matrix calibrators (internal standard recovery/response shall be $\pm 30\%$ of the selected internal standard).
8. The positive 0.150 g% control must quantitate within $\pm 10\%$ of established target mean for all analytes.
9. The external control (VEC) must quantitate within $\pm 10\%$ of established target mean for all analytes.

10. For the VEC control, positive samples are reported if bracketed by an acceptable VEC controls for the positive analytes. Any samples that are not bracketed by an acceptable control must be repeated.
11. Replicate analyses of a sample must agree within ± 0.008 g% for concentrations < 0.10 g% or $\pm 7.5\%$ for concentrations ≥ 0.10 g% of the mean of the quantitative results. If a result is outside this range, it must be repeated.

REPORTING

1. Chromatograms, along with a copy of the sequence worksheet are entered in each appropriate case file. The sequence worksheet in the case file will indicate the analyst who loaded/ removed the samples from the headspace and whether the batch has met the minimum acceptable criteria of the laboratory. All controls must be entered in the QC-QA database. In the event a batch has not met the minimum established requirement, an explanation must accompany the failed batch and the chromatogram(s) must be filed with the relevant cases. The case must be rescheduled, and the appropriate aliquot number cited.
2. Quantitative results from the BAC-1 column are reported. The BAC-2 column is used to substantiate the presence of analytes. Any significant discrepancies between the BAC-1 and BAC-2 results must be resolved by additional analysis or discussion with a supervisor. For very low concentrations, if an analyte returns a positive result on the BAC-1 column but not on the BAC-2 column, the BAC-1 column result is ignored and the analyte reported as "not detected".
3. All negative cases are reported on the toxicology report sheet. Ethanol and methanol are reported as positive if greater than or equal to 0.010 g%. Acetone and 2-propanol are reported as positive if equal to or greater than 0.001 g%. Results are entered on the toxicology report to the thousandth of one percent (e.g. 0.128 g %), initialed and dated.
4. All final quantitative results for ethanol and methanol are reported to the hundredth of one percent after the mean is obtained (e.g. result #1 = 0.128, result #2 = 0.126, mean = 0.127, reported as 0.12 g %). Replicate results must agree within ± 0.008 g% for concentrations < 0.10 g% or $\pm 7.5\%$ for concentrations ≥ 0.10 g% of the mean.
5. For DUI/DUID cases (EP cases) blood ethanol shall be reported to the second decimal place as found, for example 0.253 g% will be reported as Blood ethanol 0.25 g%. Additionally the range of concentrations calculated at the 99.7 % confidence level shall be reported. As of April 4, 2014 the confidence level is 7.44 % at the 99.7 % confidence level, $k=3$. In the example just given, the report will read:

Blood: Ethanol 0.25 g%

Measured Blood Concentration 0.253 g%

0.233g% to 0.271g% blood ethanol concentration range at the 99.7% confidence level

6. For DUI/DUID cases (EP cases) where serum or plasma has been submitted as the analytic specimen, the serum/plasma ethanol shall be reported to the second decimal place as

found, for example 0.253 g% will be reported as serum ethanol 0.25 g%. Additionally the range of concentrations calculated for the measured serum/plasma at the 99.7 % confidence level shall be reported. The calculated whole blood ethanol is then calculated by multiplying by 0.9 per New York Codes, Rules and Regulations, Title 10, Part 59.2. As of April 4, 2014 the confidence level is 7.44 % at the 99.7 % confidence level, k=3. In the example just given, the report will read:

Serum: Ethanol 0.25 g%

Measured Serum Concentration 0.253 g%

0.233g% to 0.271 g% blood ethanol concentration range at the 99.7% confidence level

Calculated Blood ethanol: 0.22 g%

7. For DUI/DUID cases (EP cases) where urine has been submitted as the analytic specimen, the urine ethanol shall be reported to the second decimal place as found, for example 0.253 g% will be reported as serum ethanol 0.25 g%. Additionally the range of concentrations calculated for the measured serum/plasma at the 99.7 % confidence level shall be reported. The calculated whole blood ethanol is then calculated by multiplying by 0.75 per New York Codes, Rules and Regulations, Title 10, Part 59.2. As of April 4, 2014 the confidence level is 7.44 % at the 99.7 % confidence level, k=3. In the example just given, the report will read:

Urine: Ethanol 0.25 g%

Measured Urine Concentration 0.253 g%

0.233g% to 0.271 g% blood ethanol concentration range at the 99.7% confidence level

Calculated Blood ethanol: 0.18 g%

TROUBLESHOOTING

There are an infinite number of problems which can occur with the instruments. The problems vary from software to hardware type problems. This troubleshooting section describes the most common problems.

SYMPTOMS	PROBABLE CAUSES	SUGGESTED ACTION
1. No peaks	Sample vial empty	Check that the proper vial is sampled and the vial contains sample.
2. No peaks	Leaks in the system	Check system for leaks with snoop.
3. No peaks	Needle clogged/broken	Check condition of needle/replace if necessary.
4. No peaks	Sample condensed in the loop and transfer line	Check that the sample loop and transfer line temperature are higher than the oven temperature.
5. No peaks	Head space not connected to GC	Make sure transfer line is hooked up to GC and inlet in an appropriate manner.
6. Poor chromatography	Sample contaminated	Steam clean the system
7. Unwanted peaks present	a) Dirty vials/septa b) Carry over from previous sample c) Contaminated GC system	a) Bake the vials and septa in oven. b) Wash vials/septa and then dry them in oven. c) Steam clean the head space sampler.
8. Vial handling problem	a) No vial in that location) Arm movement out of adjustment	a) Check that vial is loaded in the proper locations in sample tray. b) Adjust the arm movement. c) Power down the unit.
9. Vial jam problems	a) Vial remains in robotic arm.	a) Remove the vial if necessary. b) Power down the unit, lift the horizontal loader, pull down the vial manually, power up the unit.
10. Sampling sequence won't start.	a) Head space sampler not ready. b) Oven stabilization time not reached.	a) Check head space sampler set points b) Check that vials are loaded into the proper vial locations.

PROCEDURE FOR PIPETTING CLOTTED SPECIMENS

1. For clotted blood specimens for which the standard pipetting of the specimen is not able to be performed, the below procedure is to followed:
2. For blood specimens which contain small clots and can be poured, a small volume of specimen (approximately 1 mL) is poured into a micro tissue grinder and homogenized. The specimen is then pipetted from the grinder.
3. For blood specimens which cannot be poured due to major clotting of the specimen, a small portion of the specimen (approximately 1 - 2 gram) is removed (cut) and placed into the micro tissue grinder, homogenized and pipetted from the grinder.

Notes:

- a. *If after homogenizing in the grinder, the blood specimen is still not able to be satisfactory pipetted, the specimen is indicated to be unsuitable for alcohol analysis.*
- b. *If the specimen contains less than 20 ml (20 gram) of specimen, the remaining specimen after homogenization is returned to the sample container.*

FORMALDEHYDE

Formalin is employed as a convenient source of formaldehyde and is often used to preserve and embalm anatomical specimens.

Reagents

0.150 g% Formaldehyde Control (w/v)

Given the fact that a 37% formalin solution has a specific gravity of 1.08 and the needed amount (mass) = 0.150 g, then volume required is $150/(1.08 \times 0.37) = 375 \mu\text{L}$ in 100 mL of distilled water.

1. Add 50 mL of distilled water (reagent 8) to a clean dry 100 mL volumetric flask.
2. Add 375 μL of formaldehyde (reagent 10) to the volumetric flask containing 50 mL of distilled water.
3. Cap volumetric flask and mix gently for 30 seconds.
4. Q.S. to 100 mL with distilled water and mix.
5. Validate control and assign lot number. Label with lot number, date prepared, preparer, and expiration date. Store at 2-8°C. Stable for one year. As an individual vial is opened for use, it is dated (MM/DD/YY) and initialed. Discard within 30 days after opening.

REPORTING

Concentrations of ethanol, methanol, isopropanol and acetone in excess of 1.000 gm % must be repeated on dilution.

If the case specimen has a peak with the same retention time as formaldehyde in the formaldehyde control, the sample is positive for formaldehyde.

All negative cases are reported as "Formaldehyde not detected". If formaldehyde alone is detected, report "Formaldehyde detected". When methanol is detected along with formaldehyde, report "Components of embalming fluid detected" or "Components of formalin detected".

CONTROLS FOR DECOMPOSED SAMPLES

When either ethyl acetate, methyl ethyl ketone and/or n-butanol are detected in the initial blood screen, the blood sample and other samples for the case are repeated on GC HS2, along with Restek Resolution Control in position #11, a qualitative n-butanol control in position #12, followed by an air blank in position #13.

Reagents

Restek Resolution Control, catalog #36256

0.150 g% n-butanol control (w/v)

Given that n-butanol has a specific gravity of 0.81 and the needed amount (mass) – 0.150 g, the volume required is $150/(0.81) = 185 \mu\text{l}$ in 100 ml of distilled water

1. Add 40 ml of distilled water (reagent 8) to a clean dry 100 mL volumetric flask.
2. Add 185 μL of n-butanol to the volumetric flask containing 50 mL of distilled water.
3. Cap volumetric flask and mix gently for 30 seconds.
4. Q.S. to 100 mL with distilled water and mix.
5. Validate control and assign lot number. Label with lot number, date prepared, preparer and expiration date. Store at 2-8 degrees C. Stable for one year.

Reporting

When ethyl acetate, methyl ethyl ketone and/or n-butanol are detected, they are reported as detected.

CONFIRMATION OF DECOMPOSITION COMPOUNDS

When decomp compounds (ethyl acetate, methyl ethyl ketone and or n-butanol) are detected on initial blood screening, the compounds are confirmed by reinjecting the blood samples on either HS1 using the method HS1BLOODEXTEND or on HS2 using the method HS2BLOODEXTEND.

Instrumentation

Except for a GC Cycle time of 9 minutes and an Autosampler GC Cycle time of 9.5 minutes, all instrument parameters are the same as outlines in the Automated Headspace SOP.

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REPRESENTATIVE CHROMATOGRAMS

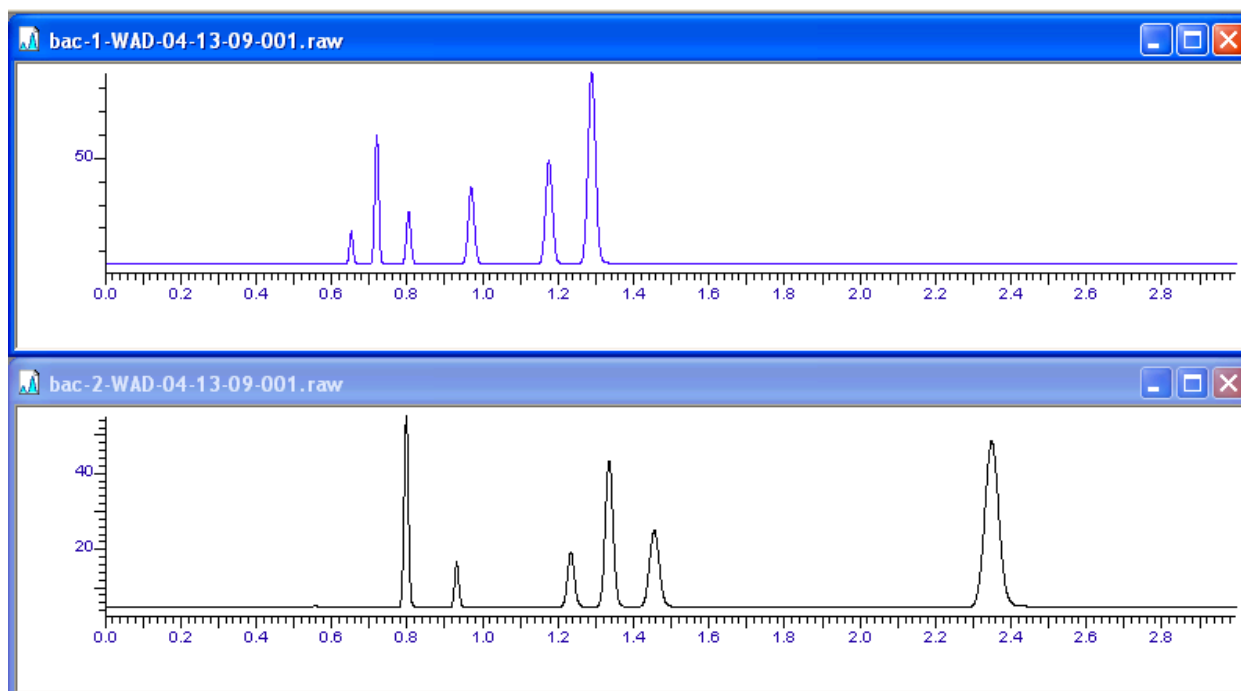


Figure 1.

Mixed volatiles chromatograms. Elution orders BAC-1: Methanol, acetaldehyde, ethanol, isopropanol, acetone, n-propanol. BAC-2 acetaldehyde, methanol, ethanol, acetone, isopropanol, n-propanol.

Uncontrolled

REVISION HISTORY

- Ver 12.01.11
1. Clarification as to the number of samples per batch.
 2. Defining the upper limit of quantification.
 3. Defined confirmatory testing when methanol is detected.
 4. Minor correction of spelling errors.
- Ver 12.01.12
1. Addition of the 0.450 % calibrator.
 2. Inclusion of the formaldehyde QC in each batch.
 3. Calibration concentrations are no longer halved for tissues.
 4. Final tissue results are no longer doubled.
 5. Modification of the preparation of the formaldehyde qualitative control.
- Ver 09.30.13
- Additional discretion given to toxicology managers when there is evidence of carry over.
- Ver 04.20.14
1. The acceptance criteria have been modified for ethanol concentrations ≥ 0.100 and for those concentrations < 0.100 .
 2. An uncertainty budget has been included in the reporting of DUI/DUID cases for ethanol.
 3. The preparation of the internal standard has been modified.
 4. The reporting of ethanol in DUI/DUID cases is specified.
- Ver 04.20.2015
1. Inclusion of retention time markers for decomposition products.
- Ver 09.14.2015
1. Inclusion of internal standard recovery and first, second and third level review.