

**FORENSIC TOXICOLOGY LABORATORY
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**ANALYSIS OF MISCELLANEOUS VOLATILE COMPOUNDS
by
HEAD SPACE GAS CHROMATOGRAPHY**

PRINCIPLE

This method uses headspace gas chromatography (HSGC) for analysis of miscellaneous volatile compounds in biological specimens such as blood, serum, vitreous humor, urine, tissue homogenates and gastric content. Head space techniques are based on Henry's law of physics, 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 the liquid specimen. Biological fluids or tissue homogenates are placed into a vial, sealed and allowed to stand until equilibrium is reached. A sample of the head space is removed from the sealed vial by syringe and injected into a gas chromatograph. Volatile analytes are separated by the column and detected with a flame ionization detector. Analytes are identified qualitatively by their relative retention time. Quantitation is performed by computer programs, using the height ratios of the chromatographic peaks of interest and an internal standard, calculated from a least-square equation derived from three calibrators.

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

1. 20 mL headspace vials
2. Rubber septa
3. Aluminum seals
4. Crimper
5. Deionized water
6. Standards as appropriate (highest purity available). All standards are validated prior to use.
7. n-Propanol

8. Ammonium sulfate (fw 132.14) Fisher Scientific, certified ACS grade or equivalent
9. Sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$, fw 174.10) Fisher Scientific, certified ACS grade or equivalent

CONTROLS AND CALIBRATORS

The solubility of the target compound in water and the analytical response of the headspace gas chromatography must be determined before controls and calibrators are prepared.

Aqueous controls and calibrators are prepared for compounds sufficiently soluble in water. Qualitative controls are prepared for compounds not readily soluble in water.

INTERNAL STANDARD DILUENT SOLUTION

1. Weigh 100 grams of sodium 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 methanol bubbles are observed, de-gas the flask for 10 minutes
4. Allow sodium hydrosulfite solution to equilibrate to room temperature.
5. Weigh 34.8 g of the previously heated sodium 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 sodium dithionite is completely dissolved.
7. Next, add 265 g of ammonium sulfate to the same flask and stir until completely dissolved.
8. QS to 2 L mark.
9. Analyze diluent solution 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.

INTERNAL STANDARD SOLUTION FOR LIQUID SAMPLES

Add 300 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 this validation injection, label with lot number, analyst initials, date of preparation and expiration date. Store at 2-8 $^{\circ}\text{C}$. Discard after six months.

INTERNAL STANDARD SOLUTION FOR TISSUE SAMPLES

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 $^{\circ}\text{C}$. Discard after six months.

PROCEDURE FOR BIOLOGICAL FLUIDS

The following procedure is utilized for the qualitative/quantitative analysis of biological fluids (blood, serum, vitreous humor, urine, gastric content and bile) for miscellaneous volatiles which are water soluble.

Note: For compounds which are not readily miscible with water and for gases, headspace vials containing only target reference standards or samples are analyzed.

1. All reagents, samples, controls and calibrators (not included in initial screen) must equilibrate to room temperature prior to sampling.
2. Prior to sampling, label the headspace vials. Vials must be clearly and legibly labeled to indicate the specimen type, the aliquot number, specimen ID number and any other factors unique to a sample.
3. Inspect the diluter/dispenser carefully. Using the diluter/dispenser, aspirate 100 μL of specimen (calibrator, control, or sample). Place the tip into the labeled headspace vial. Check that the sample information on the vial corresponds to the sample being pipetted, then dispense. The sample will be pipetted into the vial, followed by 1 mL of internal standard solution. Cap the vial with a rubber septum. Wipe the tubing with a tissue before and after each pipetting. (Skip step for compounds not readily miscible in water and gases)
4. Repeat step 3 for each calibrator, control and sample in the batch.
5. The normal order as shown in the following table should be used for screen sequences:

Position #	Samples
1	Air Blank
2	Air Blank
3	Matrix Blank(s) Control
4	Qualitative Control
5	Air Blank
6	Specimen(s)

6. The normal order as shown in the following table should be used for quantitative sequences:

Position #	Samples
1	Air Blank
2	Air Blank
3	Calibrator 1
4	Calibrator 2
5	Calibrator 3
6	Air Blank
7	Sample Matrix Blanks
8	Qualitative Control
9	Air Blank
10	Specimen(s)

PROCEDURE FOR TISSUES

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

Note: For compounds which are not readily miscible with water and for gases, headspace vials containing only target reference standards or samples are analyzed.

- All reagents, samples, controls and calibrators must equilibrate to room temperature prior to sampling.

8. Prior to sampling, label the headspace vials. Vials must be clearly and legibly labeled to indicate the specimen type, the aliquot number, specimen ID number and any other factors unique to a given sample.
9. Under a bio-hazard hood, weigh out 2 g of specimen. Add 2 mL of tissue internal standard to the specimen and immediately cap the headspace vial with a rubber septum.
10. For quantitative analysis, pipet 2 mL of calibrator 1, 2 and 3 along with appropriate controls into separate headspace vials. Pipet 2 mL of tissue internal standard into the headspace vials. Immediately cap headspace vial with a rubber septum

INSTRUMENTATION

Instrument #1	Agilent 7694-6890N Network GC System.
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 Agilent Chemstation software.
Head space method:	HS1VOLSCREEN.M
Instrument # 2	Agilent 6890 Series GC System.
Autosampler:	Agilent 7694 head space autosampler.
Gas Chromatograph:	Agilent 6890 Series GC System/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 AGILENT Chemstation software.
Head space method:	HS2VOLSCREEN.M

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 tank pressures are recorded in a logbook. The temperature of the oven, detector and injection port is checked. The FID detectors are also checked to make sure they are lit.

INSTRUMENT PRE-RUN PROCEDURE

The Agilent 7694-6890N headspace autosampler/GC/FID and 6890-7694 Headspace autosampler Series GC Systems 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.

Note: 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 snoop to check for leaks. Tank must be changed when nitrogen is below 500 psi,.

Note: 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.

Set following temperatures:

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

Oven Program

Setpoint

Initial Temp.	40 °C
Initial Time	7 min.

Ramps

#	Rate	Final temp. (°C)	Final time (min.)
1	20	200	3.00
2	0.0 (off)		
Post temp.	200 °C		
Post time	0.00 min		
Run time	18 min		

The Agilent Headspace Sampler Parameters are as follows:

Matrix	
Matrix	Blood
Matrix Boiling Point	100.0 °C
	Temperature (°C)
Sample Oven	60.0*
Sample Valve	100.0*
Transfer Line	100.0*
	Time (minutes)
GC Cycle	25.0
Sample Equilibration	5.0
Vial Pressurization	0.15
Loop Fill	0.15
Loop Equilibration	0.15
Sample Inject	1.0
Oven Stabilization	0.5
Shaking	
Agitation	Low
Parameter	
Oven Step	No
Equilibration Time	No
Mode	
Extractions	1
Puncture Mode	Single

SAMPLE ANALYSIS

Instrument 1/HS1 Agilent 6890N Network G.C System/Agilent Headspace Autosampler 7694
Instrument 2/HS2 Agilent 6890 Series G.C System/Agilent Headspace Autosampler 7694

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 says production and

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

1. On the Method and Run toolbar, under **Sequence**, select **Load Sequence**. Select the corresponding model sequence template to be loaded. On HS1 select HS1volscreen.m. On HS2 select HS2volscreen.m. In the same dialog box, verify that the correct sequence template Model is loaded under File Name. Click on Select.
2. In the Method and Run 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. The 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 HS (1or2) MMDDYYx, where 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 sample log 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 and 3 are entered for calibrators 1, 2 and 3.
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\Sequence.
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 **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. 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 on the network 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

All processing and review are performed on a processing computer. The Chemstation software is configured for dual column processing.

DATA TRANSFER AND PROCESSING

SAVE METHOD TO ECM

1. On the Method and Run toolbar, under **Sequence** After the run 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.

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. On the status line at the bottom of the screen that 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.
4. Click on any file in the batch to load it.

PERFORM BATCH CALIBRATION

Note: Proceed to Batch Processing if there are no analytes to be quantitated in the batch.

Under enhanced data analysis:

1. On the command line 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 command programs. 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.

Note: correct path is C:\msdchem\ecm\retrieve\batch

5. In the left "available data file" screen, highlight calibrator files (2,3,4) and select right arrow to load **Files Selected for Processing** window.
6. Select Process.
7. 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.

Note: the second data file (cal r) will usually be loaded first.

8. 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 (shown by an "x" next to the analyte name) 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.

*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**.*

9. In bottom line on 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**.
10. This brings up the question, “Files has previously been quantitated, requantitate now?”. Select **NO**.
11. 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**.
12. This will bring up edit compounds screen. Select **OK** at the bottom of the screen.
13. 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)).
14. 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 R² meets criteria.
15. If the calibration results are okay, then click **OK**. This brings up **Current changes will be saved, proceed**. Click **OK**.
16. 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.
17. 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 r2 values, then right click on the screen report to print it.
18. Click on close report icon (top icon line, #14)

*Note: the calibration report may be printed when not in ECM by selecting **Run ECM Explorer**. Then right click on method and right click on review history. Click on **Calrpt.txt**.*

BATCH PROCESSING

1. 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.
4. After the results are reviewed, click on the "Next Data File" icon and Qedit. Repeat until the last data file has been reviewed. 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.
5. When reviewing sample results, data from both columns is 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,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. After the reports have completed printing, save the method to ECM by selecting: ECM\HSGC/HS(1or2)\month\batch.
11. After the method has been saved, save the data results 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.
12. 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

1. 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.

ACCEPTANCE CRITERIA

1. Acceptable peak shape. Peaks must be symmetrical.
2. All analyte correlation coefficients ≥ 0.98 for quantitative analysis.
3. Positive controls must perform within $\pm 10\%$ of target.
4. All analytes in qualitative control(s) must be detected.
5. The blank must have no interfering peaks in the target areas of interest.
6. Satisfactory baseline resolution of all peaks of interest is required.

REPORTING

1. Qualitative analysis: if a significant peak is found in a sample(s) with a retention time within $\pm 2\%$ of the reference, the sample is reported as "detected" for the compound.
2. Quantitative analysis: all quantitative results 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 be within $\pm 10\%$ of each other.

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Agilent Headspace Sampler Manuals

Agilent 6890 Series II Gas Chromatograph Operating Manual and Reference Manual

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