IDENTIFICATION AND CONFIRMATION OF VOLATILES IN AQUEOUS AND BIOLOGICAL SPECIMENS BY HEADSPACE GAS CHROMATOGRAPHY

50.1 METHOD

This test method may be used to identify and confirm the presence of ethanol, methanol, isopropanol and acetone in aqueous and biological samples. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

The test method utilizes a headspace gas chromatograph (HSGC), equipped with an alcohol analysis capillary column and a flame ionization detector (FID). This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, and criteria for acceptance of volatile compounds.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Technical Management, and appropriately documented in the batch file.

50.2 PRINCIPLE

There is a direct relationship between the concentration of a volatile substance (e.g. ethanol) dissolved in a liquid (e.g., blood) and the concentration of the volatile substance in the vapor above the solution (headspace) for a given temperature, based on Henry's Law.

An aqueous or biological specimen is measured into a vial and then diluted with a measured volume of internal standard (n-propanol, 1-propanol). The vial is sealed with a septum-equipped airtight seal, incubated and pressurized, and a measured aliquot of the headspace is transferred to the gas chromatograph for analysis.

Ethanol is resolved from other volatiles, such as acetone, isopropanol and methanol. Identification is by comparison of retention times of observed analytes to those present in the calibrators. Quantitation is accomplished by multilevel calibration. Confirmation is performed on a separate instrument that is equipped with an analytical column with different selectivity.

50.3 SPECIMENS

The specimen volume is 0.2 mL. Specimens include whole blood, serum, plasma, urine, vitreous humor, tissue homogenate and aqueous samples.

NOTE: For Liquor and Cannabis Board sample testing, refer to the *Policy on Testing and Reporting Results for Liquor and Cannabis Board Samples (P48-3)*.

50.3.1 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion. The volume of n-propanol internal standard added is 2 mL for standard volume specimens and dilutions of specimens (diluted specimens are not brought to standard volume).

50.3.2 Analysis of larger specimen volumes must be approved and documented.

50.3.3 If volatiles other than ethanol, acetone, isopropanol or methanol are suspected in a specimen (e.g., inhalants such as difluoroethane), two additional samples of the specimen are tested in order to identify/confirm the presence of the volatile compound (for analysis by HSGC and gas chromatography – mass spectrometry). See *Identification/Confirmation of Inhalants in Aqueous and Biological Specimens by Headspace Gas Chromatography and Gas Chromatography – Mass Spectrometry (TCi12747)* for inhalants analysis.

NOTE: Ethanol and sevoflurane co-elute on the J&W DBALC2 capillary column. For specimens containing both ethanol and sevoflurane, ethanol confirmation must be performed on the J&W DBALC1 column only.

50.4 REAGENTS/MATERIALS AND EQUIPMENT

50.4.1 REAGENTS/MATERIALS

- Bleach, concentrated and approximate 10% solution in H_2O
- Deionized water, laboratory grade or better (DI H_2O)
- Disposable extraction tubes (12 x 75 mm recommended) with closures
- Headspace autosampler vials (10 mL), with crimp or screw caps

50.4.2 EQUIPMENT

- Agilent (Hewlett Packard) 6890 gas chromatograph equipped with either a J&W DBALC1 capillary column (30 m x 0.53 mm ID x 3 µm film thickness) or a J&W DBALC2 capillary column (30 m x 0.53 mm ID x 2 µm film thickness), or equivalent
- Agilent (Hewlett Packard) 7694/G1888 headspace autosampler, PAL RTC automated sampler, or equivalent
- Calibrated, adjustable piston pipettes with disposable polypropylene tips
- Cap crimper for headspace vials (manual or automatic)
- General-use equipment (sonic dismembrator, vortex mixer)
- Microlab® 600 Autopipette, Hamilton Automatic Diluter, or equivalent

50.5 INTERNAL STANDARD, CALIBRATORS AND CONTROLS

50.5.1 INTERNAL STANDARD

Internal standard is prepared and verified according to the *Procedure for the Verification of n-Propanol Internal Standard (PTis12501)*.

50.5.2 CALIBRATORS

50.5.2.1 Ethanol calibrators are purchased as certified reference materials (CRMs), at concentrations of 0.01, 0.04, 0.10, 0.30 and 0.50 g/100mL. Ethanol calibrator CRMs are single use only, with aliquots taken from freshly opened ampoules.

50.5.2.2 Mixed volatile (MV) calibrators are purchased as multicomponent alcohol CRMs, and contain ethanol, methanol, isopropanol and acetone at concentrations of 10, 25, 50 and 100 mg/dL (100, 250, 500 and 1000 µg/mL). MV calibrator CRMs are single use only, with aliquots taken from freshly opened ampoules.

NOTE: The presence of ethanol in the multicomponent alcohol CRMs is for demonstration of chromatographic separation from other volatile compounds only. Batch acceptability and reporting of ethanol results are determined from the ethanol-only calibration and controls. Case specimen ethanol results from data processed using the mixed volatile method (from four-point mixed volatile calibration) are not reported.

50.5.2.3 The 10 mg/dL MV CRM serves as the MV SCREEN for the initial screening batch. A two-point calibration, using the MV SCREEN and origin, is used to obtain an estimated concentration for MV analytes in specimens to determine whether confirmation is needed. The MV CRM serving as the MV screen is not considered as single use only.

50.5.2.4 The source and lot number of each CRM is recorded in the *Volatiles Material Log (VolMat_Log)*. Each CRM lot must be verified prior to use, with verification documented on the *Volatile CRM Verification Worksheet* (*VolCRMVerWS)*.

50.5.3 CONTROLS

50.5.3.1 Ethanol CRM controls are purchased at concentrations of 0.025, 0.05, 0.15 and 0.40 g/100 mL. Whole blood matrix (WB) ethanol controls are purchased at low (0.08 g/100 mL) and high (0.20 g/100 mL) concentrations.

50.5.3.2 Two MV CRM controls are prepared by diluting purchased high concentration MV CRMs (2000 µg/mL and 4000 µg/mL) to target concentrations of 20 mg/dL and 80 mg/dL, respectively, for use on day of preparation only. See 50.6.4 for preparation of dilutions. If available, MV CRMs may be purchased at the target concentrations and used directly. An MV whole blood matrix control is also purchased (manufacturer targets vary by analyte).

50.5.3.3 The source and lot number of each CRM and whole blood matrix control is recorded in the *Volatiles Material Log (VolMat_Log)*, and each lot must be verified prior to use, with verification documented on the *Volatile CRM Verification Worksheet* (*VolCRMVerWS) or Volatile Whole Blood Control Verification Worksheet (VolWBVerWS)*.

50.5.3.3.1 Whole blood matrix controls are verified in-house, with replicates analyzed to obtain a reference value for each lot. The reference value is used to evaluate control performance for each batch.

50.6 SAMPLE PREPARATION

Equilibrate specimens and volatiles materials (n-propanol, calibrator/control CRMs, matrix controls) to room temperature prior to sample preparation. Ethanol and MV calibrator CRMs are single use only. If the batch includes quantitation of mixed volatile compounds, prepare MV CTRL 2 as described in 50.6.4.

50.6.1 Evaluate the specimens to ensure the blood (or other matrix) is mobile. If necessary, the specimen may be sonicated or homogenized. If sonication or homogenization is performed, this must be recorded on the worklist and care should be taken to minimize the amount of time the specimen is open.

50.6.2 Label a 10 mL headspace vial for each member of the test batch (blank, negative controls, calibrators, positive controls, specimen samples, etc.) The batch should be set up according to the following suggested sequence(s).

NOTE: It is recommended that a DI H_2O blank be run following decomposed specimens or where volatiles other than ethanol, methanol, isopropanol and acetone are expected (e.g., toluene, difluoroethane).

50.6.2.1 Initial screen batch (first analysis for case specimens)

1	Blank (DI H_2O, no ISTD)	15	NEG CTRL
2	CAL 1 (0.01 g/100 mL)	16	Specimen #1
3	CAL 2 (0.04 g/100 mL)	17	Specimen #2
4	CAL 3 (0.10 g/100 mL)	18	Specimen #3
5	CAL 4 (0.30 g/100 mL)	19-28	Specimen #4 - #10
6	CAL 5 (0.50 g/100 mL)	29	CRM CTRL
7	NEG CTRL (DI H_2O, plus ISTD)	30	NEG CTRL
8	CTRL 1 (0.025 g/100 mL)	31-40	Specimen #11 - #20
9	CTRL 2 (0.05 g/100 mL)	41	CRM CTRL
10	CTRL 3 (0.15 g/100 mL)	42	NEG CTRL
11	CTRL 4 (0.40 g/100 mL)	43-52	Specimen #21 - #30
12	MV SCREEN (10 mg/dL)	53	MV CTRL WB
13	MV CTRL WB	54	CTRL WB High
14	CTRL WB Low	55	NEG CTRL

Insert a positive CRM CTRL and NEG CTRL after every 10 injections, with MV CTRL WB, CTRL WB High and NEG CTRL at the end of the run.

50.6.2.2 Confirmation batch – ethanol only; confirmation batches must be run on a different GC column than the initial screen.

1	Blank (DI H_2O, no ISTD)
2	CAL 1 (0.01 g/100 mL)
3	CAL 2 (0.04 g/100 mL)
4	CAL 3 (0.10 g/100 mL)
5	CAL 4 (0.30 g/100 mL)
6	CAL 5 (0.50 g/100 mL)
7	NEG CTRL (DI H_2O, plus ISTD)
8	CTRL 1 (0.025 g/100 mL)
9	CTRL 2 (0.05 g/100 mL)
10	CTRL 3 (0.15 g/100 mL)
11	CTRL 4 (0.40 g/100 mL)
12	CTRL WB Low
13	NEG CTRL
14	Specimen #1
15	Specimen #2
16	Specimen #3
17-26	Specimen #4 - #10
27	CRM CTRL
28	NEG CTRL
29-38	Specimen #11 - #20
39	CRM CTRL
40	NEG CTRL
41-50	Specimen #21 - #30
51	CTRL WB High
52	NEG CTRL

Insert a positive CRM CTRL and NEG CTRL after every 10 injections, with CTRL WB High and NEG CTRL at the end of the run.

50.6.2.3 Confirmation batch – mixed volatiles only (methanol, isopropanol, acetone); confirmation batches must be run on a different GC column than the initial screen.

1	Blank (DI H_2O, no ISTD)
2	MV CAL 1 (10 mg/dL)
3	MV CAL 2 (25 mg/dL)
4	MV CAL 3 (50 mg/dL)
5	MV CAL 4 (100 mg/dL)
6	NEG CTRL (DI H_2O, plus ISTD)
7	MV CTRL 1 (20 mg/dL)
8	MV CTRL 2 (80 mg/dL)
9	MV CTRL WB
10	NEG CTRL
11	Specimen #1
12	Specimen #2
13	Specimen #3
14	Specimen #4 - #10
15	MV CRM CTRL
16	NEG CTRL
17-26	Specimen #11 - #20
27	MV CTRL WB
28	NEG CTRL

Insert a positive MV CRM CTRL and NEG CTRL after every 10 injections, with MV CTRL WB and NEG CTRL at the end of the run.

50.6.2.4 Confirmation batch – ethanol and mixed volatiles (methanol, isopropanol, acetone); confirmation batches must be run on a different GC column than the initial screen.

1	Blank (DI H_2O, no ISTD)
2	CAL 1 (0.01 g/100 mL)
3	CAL 2 (0.04 g/100 mL)
4	CAL 3 (0.10 g/100 mL)
5	CAL 4 (0.30 g/100 mL)
6	CAL 5 (0.50 g/100 mL)
7	MV CAL 1 (10 mg/dL)
8	MV CAL 2 (25 mg/dL)
9	MV CAL 3 (50 mg/dL)
10	MV CAL 4 (100 mg/dL)
11	NEG CTRL (DI H_2O, plus ISTD)
12	CTRL 1 (0.025 g/100 mL)
13	CTRL 2 (0.05 g/100 mL)
14	CTRL 3 (0.15 g/100 mL)
15	CTRL 4 (0.40 g/100 mL)
16	MV CTRL 1 (20 mg/dL)
17	MV CTRL 2 (80 mg/dL)
18	MV CTRL WB
19	CTRL WB Low
20	NEG CTRL
21	Specimen #1
22	Specimen #2
23	Specimen #3
24-30	Specimen #4 - #10
31	CRM CTRL
32	NEG CTRL
33-42	Specimen #11 - #20
43	CRM CTRL
44	NEG CTRL
45-54	Specimen #21 - #30
55	MV CTRL WB
56	CTRL WB High
57	NEG CTRL

Insert a positive CRM CTRL and NEG CTRL after every 10 injections, with MV CTRL WB, CTRL WB High and NEG CTRL at the end of the run.

50.6.3 If running mixed volatiles confirmation, prepare the MV CTRL 1 (20 mg/dL) and the MV CTRL 2 (80 mg/dL) by diluting the high concentration MV CRMs. Alternatively, MV CRMs at the target concentrations may be purchased and used directly.

a. Using a calibrated pipette, follow table below and prepare in a clean, labeled 12 x 75mm tube. Cap the tube and briefly vortex mix.

Final Concentration	CRM	DI H_2O µL	Amount of CRM µL
CTRL 1 20mg/dL	2000 µg/mL	900	100
CTRL 2 80mg/dL	4000 µg/mL	800	200

b. The solution is for use on day of preparation only. The tube is labeled with the initials of the person preparing the solution, the identity and the concentration. The preparing scientist will record the preparation information on their batch worklist or instrument sequence comments (pipette ID(s), lot number of multicomponent alcohol CRM used to prepare solution). Subsequent analysts using the prepared control

solution will record the initials of the preparing scientist and the date prepared on their batch worklist or sequence.

50.6.4 Add approximately 2.2 mL DI H_2O into the vial labeled blank, cap and seal the vial tightly.

50.6.5 Using the auto-pipettor, aliquot 200 µL of the calibrators, controls or specimens and 2 mL of the n-propanol solution into the respectively labeled headspace vials, cap and seal the vials tightly.

50.6.6 Rinse the pipette tip with DI H_2O and/or dilute bleach rinse followed by DI H_2O rinse, as necessary, when aliquoting the batch.

50.7 REINJECTION

50.7.1 If necessary, reinjection of samples may be performed. The reason(s) for reinjection (e.g., extraneous peak in chromatogram, instrument stoppage, interfering peak carrying over from a decomposed sample) will be documented in the batch.

50.7.2 The original calibration for the batch must be valid in order to perform partial batch reinjection. See Appendix A for reinjection sequence information.

50.8 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – BLDALCO (The method name may contain a numeric suffix to differentiate between instruments; for example BLDALCO8 for instrument 8.)
- Calibration curve – linear, equal weighting, origin excluded
- Updating calibrator (retention times ±2%) – Calibrator 5 for ethanol, MV Calibrator 4 for MV analytes
- Printed reports for each vial in the batch are generated for review, along with the batch sequence and calibration table(s) applied to the batch.

50.9 CRITERIA FOR BATCH ACCEPTANCE

Technical review of the batch is conducted by an authorized reviewer, using the criteria below.

50.9.1 The blank shall be devoid of any significant peaks[1].

50.9.2 Calibrators and Calibration Curves

50.9.2.1 Chromatographic peaks for ethanol, MV analytes and n-propanol shall appear symmetrical (i.e., no co-elution, split peaks, or shoulders).

[1] Peaks appearing in the blank, calibrators, or positive and negative controls that are fully resolved from any volatile compound or internal standard are considered extraneous and not significant.

50.9.2.2 Retention times for ethanol and n-propanol shall be within ±2% of those in ethanol calibrator 5 and MV analytes shall be within ±2% of MV calibrator 4. These are inclusive ranges.

50.9.2.3 Quantitative results for ethanol in each calibrator shall be within ±10% of the nominal value. Result comparisons will use values to three decimal places in units of g/100 mL. These are inclusive ranges.

CAL 1 (0.01 g/100 mL): 0.009 – 0.011 g/100 mL
CAL 2 (0.04 g/100 mL): 0.036 – 0.044 g/100 mL
CAL 3 (0.10 g/100 mL): 0.090 – 0.110 g/100 mL
CAL 4 (0.30 g/100 mL): 0.270 – 0.330 g/100 mL
CAL 5 (0.50 g/100 mL): 0.450 – 0.550 g/100 mL

50.9.2.4 Quantitative results for MV analytes in each calibrator shall be within ±10% of the nominal values. Result comparisons for MV CAL 1-2 will use values truncated to one decimal place, in units of mg/dL. For MV CAL 3-4, the truncated whole number values are used. These are inclusive ranges.

MV CAL 1 (10 mg/dL): 9.0 – 11.0 mg/dL
MV CAL 2 (25 mg/dL): 22.5 – 27.5 mg/dL
MV CAL 3 (50 mg/dL): 45 – 55 mg/dL
MV CAL 4 (100 mg/dL): 90 – 110 mg/dL

50.9.2.5 The calibration curves for ethanol and MV analytes shall have R^2 ≥0.99.

50.9.2.6 Evaluation of ethanol performance in the MV calibrators is not used in determine the acceptability of a batch for reporting of ethanol (NOTE in 50.5.2.2)

50.9.3 Controls

50.9.3.1 Negative controls

50.9.3.1.1 Ethanol shall not be present in the negative control(s) at or above 0.0025 g/100 mL (LOD). MV analytes shall not be present at or above 5 mg/dL (LOD). Identification is based on an acceptable retention time match and an integrated, symmetrical peak.

50.9.3.1.2 All negative controls for an analyte must meet these criteria in order to report results for that analyte from the batch.

50.9.3.1.3 Failure to meet criteria for one analyte does not invalidate the acceptability of another analyte.

50.9.3.2 Positive controls

50.9.3.2.1 Positive controls shall meet those criteria in 50.9.2.1 and 50.9.2.2.

50.9.3.2.2 Quantitative results for ethanol CRM controls shall be within ±7.5% of their nominal values. Result comparisons will use values to three decimal places, as appear on the data report. These are inclusive ranges.

CTRL 1 (0.025 g/100 mL): 0.023 – 0.027 g/100 mL
CTRL 2 (0.05 g/100 mL): 0.046 – 0.054 g/100 mL
CTRL 3 (0.15 g/100 mL): 0.139 – 0.161 g/100 mL
CTRL 4 (0.40 g/100 mL): 0.370 – 0.430 g/100 mL

50.9.3.2.3 Quantitative results for MV CRM controls shall be within ±7.5% of their nominal values. Result comparisons will use values truncated to one decimal place for MV CTRL 1 and truncated whole number values for MV CTRL 2.

MV CTRL 1 (20 mg/dL): 18.5 – 21.5 mg/dL
MV CTRL 2 (80 mg/dL): 74 – 86 mg/dL

50.9.3.2.4 Quantitative results for the ethanol and MV whole blood controls shall be within ±10% of the reference value obtained from in-house replicate analysis of the lot number used in the batch.

50.9.3.2.5 All positive controls for an analyte must meet these criteria in order to report quantitative result for that analyte from the batch.

50.9.3.2.6 Failure to meet criteria for one analyte does not invalidate the acceptability of another analyte.

50.10 METHOD PERFORMANCE

- Limit of detection (LOD): ethanol: 0.0025 g/100 mL, MV analytes: 5 mg/dL
- Dynamic range: ethanol 0.01 – 0.50 g/100 mL, MV analytes 10 – 100 mg/dL
- Upper limit of linearity: ethanol: 0.60 g/100 mL, MV analytes: 400 mg/dL

50.11 REPORTING

50.11.1 Blood ethanol results are reported according to the procedure found in the *Policy on Reporting of Blood Alcohol Results (P46-1)*. Limit of quantitation is 0.01 g/100mL for ante mortem samples and 0.02 g/100mL for post mortem samples.

50.11.2 Mixed Volatiles

50.11.2.1 Positive results for acetone, isopropanol and methanol are reported from the initial mixed volatile screen if values are ≥ 8 mg/dL, provided that the subsequent confirmation test (analyzed on a different GC column) yields a value ≥ the LLOQ (10 mg/dL).

50.11.2.2 Quantitative results for acetone, isopropanol and methanol are reported as the whole integer, truncated result in units of mg/dL, to two significant figures.

Example: an isopropanol value of 67.8 mg/dL is obtained.

- The result is truncated to the whole integer value of 67 mg/dL (two significant figures) and reported.

50.11.2.3 Mixed volatile results may be reported as positive only, provided two tests have been performed, on two different GC columns, from individual samplings of the specimen.

50.11.3 When multiple dilutions of specimens are analyzed, the smallest dilution within the dynamic range is reported.

50.11.4 Liquor and cannabis board sample results are reported according to the *Policy on Testing and Reporting Results for Liquor and Cannabis Board samples (P48-3)*.

50.11.5 Quantitative results are reported for blood, plasma, serum, urine, vitreous and Liquor and Cannabis Board samples. Results for other specimen types are reported qualitatively (e.g., spleen squeeze, tissue homogenate).

50.12 DOCUMENTATION AND REVIEW

50.12.1 In the event that a sequence is started on one day and completes after midnight, the date the sequence began will be the date of testing (if full batch reinjection is necessary, the reinjection date is used as the date of testing). Analysts will place the data reports, sequence, calibration tables/curves and batch worklist in a batch file.

50.12.2 The batch file will be forwarded to a reviewer for technical review, which will include, but is not limited to, the following:

- The batch file contains all data reports, the sequence and calibration tables/curves and the batch worklist.
- All dates are correctly documented
- Calibrator and control expiration dates have not been exceeded

- All pages of the record are labeled with the batch identifier
- Calibrator and control values are within acceptable ranges and the batch meets criteria for acceptance in 50.9.
- The testing scientist's initials are on the first page of the sequence, calibration table/curves printout and all data reports.

50.12.3 The reviewer will initial and date the first page of the sequence, calibration table/curves printout and all data reports. The reviewer will also sign and date the batch worklist and QC form, indicating that the batch file is complete and the above procedures have been reviewed.

50.12.4 Upon completion of the technical review, the batch file is returned to the testing scientist.

50.12.5 The final batch file shall contain the calibration table and curves and all relevant sequence tables and chromatograms. Case sample chromatograms are filed in their respective case files, with copies of the batch worklist and QC form(s).

50.13 REFERENCES

- Agilent 7697 Headspace Autosampler (or current model) Operating and Service Manual.
- Agilent 6890 Gas Chromatograph (or current model) Manual (Operating manual 1 and 2).

Appendix A

Reinjection

Should reinjection of a known sample (blank, calibrator, positive or negative control) be necessary, this may be done as a single injection. Consideration should be given to any specimens bracketed in the original sequence by a control that requires reinjection.

Reinjections of unknown specimens must be bracketed by both positive and negative controls.

Examples:

a. If the specimen(s) contains only ethanol, the reinjection sequence is as follows:

1. CTRL (CRM or matrix)
2. NEG CTRL
3. Specimen (up to 10 injections)
4. CTRL (CRM or matrix)
5. NEG CTRL

b. If the specimen(s) contains only mixed volatiles, the reinjection sequence is as follows:

1. MV CTRL (CRM or matrix)
2. NEG CTRL
3. Specimen (up to 10 injections)
4. MV CTRL (CRM or matrix)
5. NEG CTRL

c. If the specimen(s) contains ethanol and mixed volatiles, the reinjection sequence is as follows:

1. MV CTRL (CRM or matrix)
2. CTRL (CRM or matrix)
3. NEG CTRL
4. Specimen (up to 10 injections)
5. MV CTRL (CRM or matrix)
6. CTRL (CRM or matrix)
7. NEG CTRL

If the specimen is negative upon initial injection, the reinjection sequence in c. is used.

If the initial injection of the specimen has no results (e.g., due to clogged needle, loose cap), the reinjection sequence in c. is used. For a screening batch, injections for vials 1 and 5 may consist of either MV SCREEN or matrix control.

Reinjected controls are subject to those evaluation criteria described in 10.9.3.

LIST OF CHANGES

Revision Date	Description	Page Number
10/2/23	Method validation and procedure reviewed and test method approved 9/30/2023. In use for evidentiary analysis 10/2/23. Method TCv12750 replaces method TCv12710.	All