1. Introduction

This procedure is primarily used to detect, identify, and quantitate ethanol and other volatile compounds (methanol, acetone and isopropanol) within biological matrices (e.g., blood, serum/plasm (S/P) and urine). The majority of samples being analyzed by this method are related to driving under the influence (DUI) investigations or Drug Faciliated Crimes (DFC).

2. Principle

This procedure involves the analysis of samples for volatile compounds, (e.g., methanol, ethanol, acetone, and isopropanol), utilizing a headspace-gas chromatographic (HS-GC) technique followed by detection using flame ionization (FID). Samples are typically in an aqueous environment, are sealed within air-tight vials, and are examined using a headspace analysis-type sampling technique. Volatile components within aqueous solutions, when placed in closed heated environments, reach an equilibrium between their vapors and their solutions. Vapor phases are analyzed with appropriate columns and detectors (e.g., HS-GC/dual FID, dual column technique). Volatile components of solutions are identified and can be subsequently quantitated using calibration graphs, reference standards, and internal standards.

3. Specimens

Any aqueous sample may be suitable for this analysis including, but not limited to: blood, serum/plasma and urine. Blood sample collection tubes containing proper anticoagulant and preservative (i.e., 'gray-tops' containing potassium oxalate and sodium fluoride) should be used. Containers for urine and other sample types should be within sealed, plastic (e.g., polypropylene) containers.

When available, 0.4 mL of specimen is used within this procedure (2 aliquots of specimen, 200 µL each). Specimens with elevated analyte concentrations may require dilution with water in order to ensure that results are within the linear range of the calibration graph used in the procedure (i.e., 0.01 g% to 0.40 g%). All liquid samples should be prepared using a 1:100 dilution prior to sampling of 200 uL.

If sample amount is limited then modifications to this procedure are allowed (e.g., analyzing only one aliquot, dilution) with approval of the Unit Lead or higher and will be documented within applicable case file(s). .

4. Equipment/Materials/Reagents

4.1 General laboratory glassware (e.g., Class A glassware)

4.2 Headspace autosampler vials (e.g., 20 mL) with appropriate seals/caps and crimper.

4.3 Pipettes (e.g., automatic pipettor-diluter), or equivalent

4.4 Vortex mixer

4.5 Headspace-gas chromatograph with flame ionization detectors (HS-GC dual(FID)), Agilent (or equivalent)

4.6 Capillary Column: (Rtx-BAC1; Restek 18003, or DB-BAC1 UI; Agilent 123-9334UI, 30m, 0.32mm i.d., 1.8 µm, or equivalent)

4.7 Capillary Column: (Rtx-BAC2; Restek 18002, or DB-BAC2 UI; Agilent 123-9434 UI, 30m, 0.32mm i.d., 1.2 µm, or equivalent)

4.8 Deionized water (DIW, Millipore, or equivalent)

4.9 Ethanol$_{(aq)}$ (80 mg/dL$_{(aq)}$; Certified Reference Material (CRM) Standard, Cerilliant E-030, or equivalent)

4.10 Multicomponent Mixture (MeOH, EtOH, Acetone, IPA ; 0.1 g%$_{(aq)}$; 100 mg/dL$_{(aq)}$; 1 mg/mL$_{(aq)}$; Certified Reference Material (CRM), Cerilliant A-056, or equivalent)

4.11 n-Propanol (NPA ; HPLC grade, or equivalent)

4.12 Volatile Mixture (MeOH, EtOH, Acetone, IPA ; in water
0.01 g%$_{(aq)}$, 0.025 g%$_{(aq)}$, 0.05 g%$_{(aq)}$, 0.2 g%$_{(aq)}$, 0.4 g%$_{(aq)(aq)}$;
Certified Reference Material (CRM), Cerilliant (A-127), or equivalent))

5. Preparation of Calibrators, Controls and Standard Stock Solutions

The externally-prepared ethanol positive control CRM at 0.080 g% (w/v)$_{(aq)}$ will be analyzed within batches. If this CRM is unavailable then the Lead Examiner (or higher) will be notified and, upon written approval, an equivalent positive control can be used. This will be documented in the notes within each batch.

Externally-prepared calibrator and control solutions will be used
For example, a typical batch will include the following (not in any particular order):

a) Externally-Prepared [5-Point] Volatile [CRM] Calibrator Solutions:
(0.010 g%, 0.025 g%, 0.050 g%, 0.20 g%, 0.40 g%)

b) Externally-Prepared Ethanol [CRM] Positive Control Solution (0.08 g%)

c) Externally-Prepared Volatile [CRM] Positive Control Solution (0.10 g%)

d) Negative Control Solution

e) Unknown Samples

New lots/batches of reagents, calibrator solutions, control solutions, and other types of solutions will be analyzed and validated for quality (and documented) prior to being used on casework samples for reporting purposes. These new lots/batches of solutions/material will typically be analyzed along with older (previously validated) reagents/solutions so that they can be quality-compared and validated as being acceptable for use.

Note: Additional positive controls (other than what are listed) and different external calibrator solutions can be used, if necessary, but lead examiner (or higher) approval is required prior to batch analysis.

Externally-Prepared Volatile Calibrator Solutions:

5.1 Externally-Prepared [5-Point] Volatile [CRM] Calibrator Solutions
(Volatile Mixture: $MeOH_{(aq)}$, $EtOH_{(aq)}$, $Acetone_{(aq)}$, $IPA_{(aq)}$)
(0.010 g% (w/v), 0.025 g% (w/v), 0.050 g% (w/v), 0.20 g% (w/v), 0.40 g% (w/v))

5.1.1 Carefully open (e.g., break top of glass ampoule), individually transfer liquid into autosampler vial, cap, and securely seal. Store refrigerated.

5.1.2 Manufacturer expiration dates are only valid for unopened solutions. When refrigerated, opened solutions are stable for one (1) month from date of opening.

Externally-Prepared Positive Control Solutions:

5.2 $Ethanol_{(aq)}$ [CRM] Positive Control Solution ($EtOH_{(aq)}$; 0.080 g% $(w/v)_{(aq)}$)

5.2.1 Carefully open (e.g., break top of glass ampoule), individually transfer liquid into autosampler vial, cap, and securely seal. Store refrigerated.

5.2.2 Manufacturer expiration dates are only valid for unopened solutions. When refrigerated, opened solutions are stable for one (1) month from date of opening.

5.3 Externally-Prepared Volatile [CRM] Positive Control Solution
($MeOH_{(aq)}$, $EtOH_{(aq)}$, $Acetone_{(aq)}$, $IPA_{(aq)}$; 0.10 g% $(w/v)_{(aq)}$)

5.3.1 Carefully open (e.g., break top of glass ampoule), individually transfer liquid into autosampler vial, cap, and securely seal. Store refrigerated.

5.3.2 Manufacturer expiration dates are only valid for unopened solutions. When refrigerated, opened solutions are stable for one (1) month from date of opening.

Internal Standard (I.S. or ISTD) Solutions:

5.4 n-Propanol [$NPA_{(aq)}$] Internal Standard Working Solution (0.011g% $(w/v)_{(aq)}$; 0.014 % $(v/v)_{(aq)}$)

5.4.1 Transfer 280 µL of n-propanol into a 2.0 L volumetric flask, dilute to volume with DIW, and mix well.

5.4.2 Transfer to an amber storage container and properly label.

5.4.3 This solution will be tightly capped and can be stored at room temperature. It is stable for one (1) year.

6. Procedure

6.1 Place all specimens and solutions at a room temperature environment for approximately thirty (30) minutes.

6.2 Label vials for calibrators and controls as seen below (variations are acceptable upon approval of the appropriate Lead Examiner (or higher)):

6.2.1 Blank DIW (no internal standard)

6.2.2 Negative Control (contains DIW and Internal Standard)

6.2.3 Volatile Calibrator (e.g., External [CRM] 0.010 g%)

6.2.4 Volatile Calibrator (e.g., External [CRM] 0.025 g%)

6.2.5 Volatile Calibrator (e.g., External [CRM] 0.050 g%)

6.2.6 Volatile Calibrator (e.g., External [CRM] 0.20 g%)

6.2.7 Volatile Calibrator (e.g., External [CRM] 0.40 g%)

6.2.8 Blank DIW (no internal standard)

6.2.9 Positive Control EtOH Solution (e.g., External Ethanol [CRM] 0.08 g%)

6.2.10 Volatile Positive Control (e.g., External 0.10 g%)

6.2.11 Evidentiary Samples ($X_i \ldots X_n$) ; will be made in duplicate.
Two (2) aliquots (200 µL) of each evidentiary item (i.e., blood, serum/plasma, urine, vitreous) will be placed into separate headspace vials.

6.2.12 Volatile Positive Control (e.g., External 0.08 g%)
Will be analyzed after each full set of twelve (12) evidentiary items or less (each replicate specimen vial is considered an evidentiary item). The frequency of the number of samples analyzed before a Volatile Positive Control is analyzed can be altered with the appropriate Lead Examiner's (or higher) permission.

6.2.13 Volatile Positive Control (e.g., External [CRM] 0.10 g%) ; next-to-last sample in batch.

6.2.14 Blank DIW ; last sample in batch.

Sample Preparation (Automatic Pipetting of Samples, Calibrators, and Controls):

Pipettor/Diluter Preparation/Priming:

6.3 Turn on the Pipettor/Diluter and check for bacterial contamination in solutions

6.4 Place the inlet tubing into the NPA Internal Standard working solution bottle making sure the end of the tubing is well below the level of the liquid.

6.5 Remove the dispenser probe from its holder and place the tubing from the probe in an empty waste container/flask.

6.6 Press the prime switch and liquid will be dispensed from the probe at this time. The system's lines will be primed. Continue to cycle until the bubbles have disappeared from the lines.

Sample preparation with Pipettor/Diluter:

6.7 Use the automated pipettor and place its probe into blank DIW, draw up 200 µL, dispense into a waste container, and perform the between-sampling rinse. Dry the probe tip with a clean wipe as needed.

6.8 Press the Pipette Activation Button (PAB) once to draw up 1 mL of the $NPA_{(aq)}$ (Internal Standard Working Solution) (This may occur automatically depending on automated pipettor programming).

6.9 Place the probe into the sample to be analyzed (i.e., negative control, calibrator, positive control, unknown sample) and press the PAB again to draw up 200 µL.

6.10 Place the probe inside the appropriately labeled autosample vial and press the PAB once to dispense.

6.11 Place the probe into a waste container and press the PAB once more to dispense the between-sampling rinse into the waste container. Dry the probe tip with a clean wipe as needed.

6.12 Place a headspace cap on the vial and crimp-seal tightly. Take this time to verify that the proper sample was transferred into the correspondingly labeled headspace vial.

6.13 Repeat the internal standard/sample dispensing into head-space vials for all calibrators, controls, and samples. This can be done according to the order stated earlier under this 'procedure' section.

Note: If automatic pipettor is not functioning then manual pipetting is permitted. Adequate adjustments will be performed by analysts so that this procedure can be followed. The appropriate Lead Examiner (or higher) can be sought for assistance, if necessary.

Setting-up Instrument with Samples:

6.14 Ensure air and hydrogen are flowing and ensure detectors (flame ionization detectors (FID) are operational).

6.15 The 0.1g% Multicomponent control is analyzed as a sample along with a negative control prior to casework. Both injections should demonstrate appropriate peaks and responses.The instrument must have passing QA/QC results prior to preparing and loading of samples.

6.16 Enter the sequence order for the method Batch Table.

6.17 Place the labeled headspace vials in the appropriate order within the instrument.

6.18 Enter the following dilution factors:
0.769 for urine, 0.862 for serum/plasma, and 1.000 for blood and vitreous samples.

6.19 Save the Batch Table to the day's date.

6.20 Prior to analyzing samples:
Print the sequence list. Check that the physical placement of the headspace vials and the vial positions within the instrument's sequence list match. Once the check has been completed place an indication of the sample check (e.g., 'sequence checked' or 'sequence verified') on the sequence page along with Analysts initials and date.

6.21 Print the instrument method and include both the method and the sequence printouts with the batch documents.

6.22 Begin the sequence and analyze the samples.

7. Instrumentation

Instrumental Parameters:

HS-GC dual (FID):
The following parameters can be used. Some values may change due to slight variability (e.g., flow rates). Significant differences must be approved by the appropriate Lead Examiner (or above) prior to changing.

Autosampler	Parameters		Autosampler	Parameters
GC Cycle Time	6.5 min.		Sample Vial Penetration	15 mm
Sample Volume	0.5 mL		Sample Vial Penet. Speed	50 mm/sec
Incubation Time	5 min.		Sample Aspiration Rate	12 mL/min
Incubation Time Increment	0 min.		Sample Post Asp. Delay	1 sec.
Heat Agitator	On		Inlet Penetration Depth	45 mm
Incubation Temperature	50 C		Inlet Penet. Speed	50 mm/sec
Heat Syringe	On		Pre-Inject Time Delay	0.5 sec.
Pre-Injection Flush Time	5 sec.		Inject Flow Rate	10 mL/min.
Agitator Speed	250 rpm		Post Injection Delay	0.5 sec
Agitator On Time	5 sec.		Flush Time	10 sec.
Agitator Off Time	5 sec.		Continuous Flush	Off
GC	**Parameters**		**Front Inlet**	**Parameters**
Run Time	6.5 min.		Mode	SPLIT
Post Run Time	0 min.		Split Ratio	10:1
Oven Equilibration Time	0 min.		Split Flow	36.575 mL/min.
Max. Oven Temperature	250 C		Heater	250 C
Initial Temperature	44 C		Pressure	5.8565 psi
Hold Time	6.5 min.		Total Flow	43.232 mL/min.
Post Run Temperature	100 C		Septum Purge Flow	3 mL/min.
Carrier Gas	H_2		Gas Saver	On

HS-GC dual (FID) (Cont'd):

Column Information		
Column Names	Col. #2 (BAC1)	Col. #3 (BAC2)
Film Thickness	1.8 µm	1.2 µm
Column Length	30 m	30 m
Inner Diameter	320 µm	320 µm
Column Max Temp	260°C	260°C
Pressure (initial)	5.2 psi	5.2 psi
Pressure (post run)	3.8 psi	3.8 psi
Flow	2.1193 mL/min.	2.1516 mL/min.
Ave. Velocity	40.3 cm/sec.	40.606 cm/sec.
Holdup Time	1.2407 min.	1.2314 min.
Detector	**FID2 (Back Detector)**	**FID1 (Front Detector)**
Temperature	250 C	250 C
Sampling Rate	20 Hz (Signal #1)	20 Hz (Signal #1)
Makeup Gas	N2	N2
Makeup Flow	25 mL/min.	25 mL/min.
H_2 Flow	25 mL/min.	25 mL/min.
Air Flow	400 mL/min.	400 mL/min.

8. Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. Retention time (chromatographic characteristic), peak shape, and signal-to-noise (aka: integrable peaks) are used as the basis for peak detection. In most cases all of the criteria below will be met in order to detect the appropriate volatile analytes within specimens. Only analytes found within evidentiary specimens of batches will need to be evaluated. For example, if twenty-five (25) unknown samples are analyzed and ethanol is the only volatile analyte detected at reportable limits, then the other volatiles which are in the controls and calibrators need not be processed nor evaluated.

8.1 Chromatography

Chromatographic peaks will possess good chromatographic quality (i.e., Gaussian peak shape, reasonable peak width, distinguishing signal-to-noise). In order for a chromatographic peak to be deemed acceptable it will compare favorably to corresponding chromatographic peaks within known samples (i.e., positive controls). The retention time of a chromatographic peak in a case sample should be within ±0.1 minute of the retention time obtained from analysis of a calibrator or positive control.

8.2 Batch Acceptance

8.2.1 The batch must have completed with no unexplained interruptions and no unexplained errors. Consult the Lead Examiner (or higher) if unsure whether to continue processing the batch.

8.2.2 All calibrator solutions that have been analyzed during a batch (and their resulting data) will be included in data processing and sample evaluation. No calibrators within a batch can be selectively eliminated from the resulting calibration graph without AD approval and will be documented appropriately within the batch.

8.2.3 All applicable analytes of interest within positive controls, as well as internal standards, will be identified during data processing.

8.2.4 To report results on a specimen, the internal standard response must be within +/- 10% of a calibrator or control.

Note: Analytes of interest are considered those compounds that are being reported.

8.2.5 Quantitation of the headspace-GC data may be performed automatically by the software. While quantitation is normally performed using BAC1 column data if applicable and if necessary, GC data processing using BAC2 data can be performed. Acceptability of data using BAC2 data will be at the discretion of the appropriate FSE2 (or higher) and will be documented in case notes.

8.2.6 Quantitative calculations are based on comparisons of chromatographic peak areas between analytes and the internal standard (i.e., n-propanol).

8.2.7 Peak area response ratios from the calibrant solutions are calculated and compiled to create a linear calibration graph (y-axis is Area Ratio and x-axis is ethanol

concentration). A best-fit line (y = mx+b) is created from the calibration graph which does not include the origin as a point.

8.2.8 Calibrator and control solution data should be evaluated prior to analyzing evidentiary samples to ensure that quality control measures have been satisfied and the calibration graph is acceptable.

8.2.9 Calibration Linearity: All calibration lines are considered acceptable if their associated linear regression coefficient of determination (R^2) are $\geq$ 0.99. The calibration lines for each analyte are independently evaluated as follows:

8.2.10 If a calibration line is not acceptable then the batch is rejected for that particular analyte and case samples will not be quantitatively reported until a new calibration line is acquired and deemed acceptable.

8.2.11 Troubleshooting, instrument evaluation, and/or preparation of new calibration solutions, if necessary, will be done prior to analysis of samples.

8.2.12 Calibration Check (EtOH, MeOH, Acetone, IPA): Quantitative results of the Externally-Prepared Volatile Calibrator Solutions [CRM] must be within +/- 20% of its target value. Results which do not meet this criteria will be recorded in the appropriate Quality Control (QC) chart spreadsheet.

8.2.13 Calibration Check (EtOH-only): Quantitative results of the External Ethanol [CRM] ; 0.080 g%) Positive Control Solution must be within +/- 5% of its target value (range: 0.076 g% – 0.084 g%). Results from the CRM data are recorded in the appropriate Quality Control (QC) chart spreadsheet.

8.2.14 Calibration Checks (MeOH, EtOH, Acetone, IPA): Quantitative EtOH results within the Volatile Positive Control Solution must be within +/- 5% of its target value (i.e., 0.10 g% ; range: 0.095 g% – 0.105 g%) and quantitative MeOH, acetone, and IPA results within the Volatile Positive Control Solution must be within +/- 10% of their target values (i.e., 0.10 g% ; range: 0.090 g% – 0.110 g%). Results from these data are recorded in the appropriate Quality Control (QC) chart spreadsheet.

8.2.15 Other than the n-propyl alcohol (NPA internal standard), no significant peaks will be present in the negative control sample. A significant peak is an analyte of interest with an extrapolated concentration $\geq$ 0.005 g% .
{Blank and Carryover Check}

Note: Significant peaks are considered peaks which are automatically integrated and which impact the quality of the results.

8.2.16 Column (BAC1) Sample Replicate Evaluation (for EtOH only):

8.2.16.1 The difference between the BAC1 column results for each aliquot replicate should be within +/- 5% of the mean value (using 3 decimal places). If the above criteria is not met, seek the appropriate FSE2 (or higher) guidance to determine if samples need to be re-analyzed or if acceptance of current value is allowed – document in batch case notes.

8.2.17 Dual Column Evaluation:

8.2.17.1 Any reportable analyte must be able to be identified by retention times (i.e., peak times compared to the internal standard) on both GC columns.

8.2.17.2 All reportable analyte GC peaks should be totally resolved.

8.2.17.3 In order to be reportable, analytes must have quantitative values from both GC columns (BAC1 and BAC2) that agree within +/- 10 % of each other.

8.2.18 Reporting Limit (RL):

8.2.18.1 The reporting limit for volatiles will be 0.02 g%. Anything below 0.020 g% but greater than or equal to 0.010g% will be reported as "Detected less than 0.02 g%"

8.2.18.2 Blood samples with ethanol concentrations greater than the highest calibrator solution (e.g., > 0.40 g%) will be reanalyzed using appropriate dilutions if accurate quantitations above that highest calibrator's concentration are needed. Information regarding how the dilutions were prepared will be noted within the batch paperwork. If unsure of the proper dilution consult the Unit Lead (or higher).

8.2.18.3 For urine samples with converted (i.e., blood-equivalent) ethanol concentrations ≥ 0.30 g%, appropriate dilutions and re-analyses are necessary if accurate quantitations above that highest calibrator's concentration are needed. Notes describing how the dilutions were prepared will be in the batch paperwork. If unsure of the proper dilution then consult the Unit Lead (or higher).

8.2.18.4 For serum/plasma samples with converted (i.e., blood-equivalent) ethanol concentrations ≥ 0.34 g%, appropriate dilutions and re-analyses are necessary if accurate quantitations above that highest calibrator's concentration are needed. Notes describing how the dilutions were prepared will be in the batch paperwork. If unsure of the proper dilution then consult the Unit Lead (or higher).

8.2.18.5 Based on toxicological significance, samples with other volatile analytes that are found to be outside the calibration ranges will be assessed to determine if they need to be diluted and repeated or if they can simply be reported either qualitatively or greater than the highest calibrator. The Unit Lead (or higher) will be consulted for such instances and notes will be kept within batch documentation.

8.2.18.6 Other causes where samples are not able to be repeated (e.g., limited sample) will be noted within batch and casefile paperwork. The appropriate FSE2 or higher will be consulted on how to phrase report so that such instances are captured within the report.

8.2.18.7 Samples which contain significant carry-over peaks (i.e., contamination into the next sample) may need their samples re-analyzed. Consult Unit Lead (or higher) when these situations occur. Documentation in case files will occur to explain rationale of decision of whether to re-analyze or not.

8.2.18.8 If some of the repeated ethanol positive control values are found to be outside their designated acceptable limits then casework samples that were analyzed prior-to and directly-after (i.e., those analyzed in-between bracketed controls) will be re-analyzed, if possible. Consult the appropriate Unit Lead (or higher) for guidance, if necessary.

8.2.18.9 Appropriate corrective action(s) may need to be done before casework samples are reported if multiple control values are found to be unacceptable (e.g., repeat entire batch after performing instrument QA/QC). Consult the appropriate Unit Lead (or higher) for guidance, if necessary.

8.2.18.10 Difluoroethane (DFE) and other commonly abused inhalants may be detected by this method. They may be reported qualitatively if a peak is observed in both sample aliquots on both columns. The relative retention times shall be within 0.02 min of a reference standard.

9. Reporting of Results

The following criteria are used as guidelines in determining how to report results using this procedure.

9.1 For evidentiary biological samples where volatile analytes were quantitated and were within the reportable limits (RL):

9.1.1 The reported value will be the lower of the two (2) BAC1 column replicate concentrations.

9.1.2 Whole blood concentration numbers will be truncated to two (2) decimal digits.

9.1.3 Urine sample ethanol concentration values will be converted to whole blood equivalent ethanol values (i.e., BAC equivalent) using a conversion ratio of 1.3–to–1 (i.e., 0.769).

9.1.4 Serum/Plasma sample ethanol concentration values will be converted to whole blood equivalent ethanol values (i.e., BAC equivalent) using a conversion ratio of 1.16–to–1 (i.e., 0.862).

9.2 Ethanol concentration values within non-biological samples will not have any conversions performed and will be reported qualitatively (i.e Detected)

9.3 The uncertainty value must have the same number of decimal digits as the measured quantity, therefore measurement uncertainty will be calculated using the three (3) decimal place concentration and listed on the final report in addition to the truncated concentration.

10. Safety

This procedure is carried out in a laboratory environment and standard safety procedures appropriate for such an environment will be utilized, including gloves, safety glasses, and protective clothing (e.g., lab coat). Biological specimens will be handled using universal precautions and will be treated as biohazardous. Potentially contaminated items and surfaces will be cleaned prior to use. Refer to Safety Manual for further guidance

11. References

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