Ethanol Analysis by Headspace Gas Chromatography with Simultaneous Flame-Ionization and Mass Spectrometry Detection

Nicholas B. Tiscione*, Ilene Alford, Dustin Tate Yeatman, and **Xiaoqin Shan**
Palm Beach County Sheriff's Office, 3228 Gun Club Road, West Palm Beach, Florida 33406

Abstract

Ethanol is the most frequently identified compound in forensic toxicology. Although confirmation involving mass spectrometry is desirable, relatively few methods have been published to date. A novel technique utilizing a Dean's Switch to simultaneously quantitate and confirm ethyl alcohol by flame-ionization (FID) and mass spectrometric (MS) detection after headspace sampling and gas chromatographic separation is presented. Using 100 µL of sample, the limits of detection and quantitation were 0.005 and 0.010 g/dL, respectively. The zero-order linear range ($r^2 > 0.990$) was determined to span the concentrations of 0.010 to 1.000 g/dL. The coefficient of variation of replicate analyses was less than 3.1%. Quantitative accuracy was within ±8%, ±6%, ±3%, and ±1.5% at concentrations of 0.010, 0.025, 0.080, and 0.300 g/dL, respectively. In addition, 1,1-difluoroethane was validated for qualitative identification by this method. The validated FID-MS method provides a procedure for the quantitation of ethyl alcohol in blood by FID with simultaneous confirmation by MS and can also be utilized as an identification method for inhalants such as 1,1-difluoroethane.

Introduction

Ethanol is the most common analyte identified in forensic toxicology laboratories (1). Headspace gas chromatography with flame-ionization detection (HS-GC–FID) has become the gold standard for ethanol analysis because of its ease of automation, sensitivity, accuracy, and relative specificity. To enhance specificity, many HS-GC–FID procedures use dual-column confirmation, which involves injecting a single sample and splitting to two chromatographic columns of sufficiently different polarity to change retention and elution order of ethanol and other volatiles of interest (2,3).

Although desirable for increased specificity, relatively few mass spectrometric (MS) methods for ethanol analysis have been published to date. One published method determined the concentration of ethanol in blood specimens utilizing HS-GC–MS using *n*-propanol as the internal standard, but was only validated up to 0.2 g/dL of ethanol, required 1 mL of sample, and used a different instrument method to test for inhalants (4). A second published method required 250 µL of sample and utilized a GC–FID system to presumptively identify and quantitate ethanol followed by transfer of the vials and reanalysis on a separate GC–MS system for qualitative confirmation (5).

The aim of this study was to develop and validate a novel technique utilizing a Dean's Switch to simultaneously quantitate and confirm ethyl alcohol by FID and MS detection after HS sampling and GC separation. This method combines the simplicity and robustness of an HS-GC–FID quantitative procedure with the unequivocal confirmation generated through MS. Additional advantages which provide effectiveness and efficiency for routine blood alcohol analysis include a small sample volume of 100 µL, a demonstrated linear range of 0.010 to 1.000 g/dL, a single instrument used for quantitation and confirmation, and simultaneous analysis for ethanol and other volatiles that might be used as inhalants, such as 1,1-difluoroethane.

Materials

Human whole blood and urine used in validation were obtained from Utak Laboratories (Valencia, CA) and verified to be negative for all analytes. Commercially prepared aqueous ethanol standards at concentrations of 0.020, 0.025, 0.100, 0.200, and 0.500 g/dL were obtained from Cerilliant (Round Rock, TX). A whole blood volatiles standard containing methanol, ethanol, acetone, and isopropanol with target concentrations of 0.04, 0.08, 0.04, and 0.04 g/dL, respectively, was

* Author to whom correspondence should be addressed. Email: TiscioneN@pbso.org.

purchased from Cliniqa (catalog no. 93221, San Marcos, CA).

Additional reagents and consumable supplies used were as follows: ethanol, ethyl acetate, chloroform, acetonitrile, acetaldehyde, heptane, toluene, *n*-butyl acetate, 1-chlorobutane, pentane, and isoamyl alcohol (catalog nos., respectively, EX0278-1, EX0241-1, CX1054-1, AX0146-6, AX0025-4, HX0078-1, TX0737-1, BX1735-5, CX0914-1, PX0166-1, AX1440-3, EMD Chemicals, Gibbstown, NJ); methylene chloride (catalog no. 300-4, Burdick and Jackson, Muskegon, MI); hexanes (catalog no. 9262-03, J.T. Baker, Phillipsburg, NJ); *n*-propanol (catalog no. 41842, Alfa Aesar, Ward Hill, MA); isoflurane, sevoflurane, and desflurane (catalog nos., respectively, 1349003, 1612540, 1171900, U.S. Pharmacopeia, Rockville, MD); 1,1-difluoroethane (catalog no., 295264-100G, Aldrich, St. Louis, MO); deionized water (catalog no. W2-4, Fisher Scientific, Pittsburgh, PA); 20-mL glass round bottom headspace vials and 20-mm crimp-top seals (catalog nos., respectively, C4020-2, C4020-3A, National Scientific, Rockwood, TN); and 20-mm grey butyl stoppers (catalog no. 73827-21, Kimble Chase, Vineland, NJ).

Normal propanol internal standard was prepared at a concentration of 0.01% by volume (% v/v) in deionized water. One ethanol standard at 2.0 g/dL in whole blood and urine and two in deionized water were prepared. The 2.0 g/dL stock solution was then diluted with whole blood, urine, and deionized water to prepare four sets of ethanol standards, one in whole blood and urine and two in deionized water, at 1.000, 0.500, 0.300, 0.080, 0.025, 0.010, and 0.005 g/dL. Controls were also prepared in deionized water at 0.010 and 0.005 g/dL from a 2.0 g/dL stock solution.

Equipment included Reference® pipettes with disposable tips (Eppendorf, Westbury, NY), a Hamilton Microlab® 503A diluter/dispenser with a 1-mL reagent syringe and 100-µL sample syringe (Hamilton, Reno NV), and a manual crimper.

The instrumentation used for analysis was an Agilent (Palo Alto, CA) G1888 HS sampler with a 7890A series GC equipped with a Dean's Switch, FID, and 5975C series MS. The analytical column used was a DB-ALC1 (Agilent, Palo Alto, CA) fused-silica capillary column with dimensions of 30 m × 0.32-mm i.d. and a 1.8-µm film thickness. The Dean's Switch was configured using a 1:1 split ratio to the FID and MS according to the manufacturer's instructions using fused-silica capillary restrictors with dimensions of 1.06 m × 0.18 mm to the FID and 2.89 m × 0.18 mm to the MS. Helium was used as the carrier gas. All gases were ultra-high purity.

Table I. Instrument Parameters

Instrument	Operating Parameters
Autosampler	
Handshake mode:	Headspace Wait
Sample temperature:	50°C
Loop temperature:	70°C
Transfer line temperature:	90°C
Injections per vial:	1 (Multi HS Extraction: Off)
Thermostat time:	20 min
Vial shaking:	Off
Vial pressure:	15 psi
Pressurization time:	0.15 min
Injection time:	0.50 min
Loop fill time:	0.15 min
Cycle time:	13.5 min
GC	
Inlet:	90°C, 5:1 split ratio in split mode
Helium carrier gas flow rate:	3 mL/min, constant flow mode
Oven temperature program:	35°C for 2 min, then 25°C/min to 90°C with a final hold time of 4.3 min
FID temperature:	300°C
MS transfer line temperature:	280°C
Dean's Switch (1:1 Split Ratio)	
FID restrictor:	1.060 m × 0.18 mm, 2 mL/min flow rate
MS restrictor:	2.890 m × 0.18 mm, 2 mL/min flow rate
FID	
Hydrogen flow rate:	40 mL/min
Air flow rate:	450 mL/min
Makeup flow rate:	50 mL/min, Constant column flow plus Makeup flow mode
Data rate:	10 Hz with autozero at 0 min
MS	
Tune file:	lomass.u, using Agilent Gain Tune followed by Low Mass Autotune
Acquisition mode:	Scan (20 to 200)
Threshold:	150
Sample #:	2^n, n = 4 (2.02 scans/s)
Electron multiplier mode:	Gain factor of 1.00
Source temperature:	230°C
Quadrupole temperature:	150°C

Method

Sample preparation

One-hundred microliters of sample (calibrators, controls, and case samples) was mixed with 1 mL of internal standard and placed in a 20-mL headspace vial with the Hamilton Microlab® 503A diluter/dispenser. The vials were then crimp sealed and placed on the instrument for analysis.

For routine casework analysis as well as the case comparison crossover study, one aqueous standard from Cerilliant at 0.020,

0.100, 0.200, and 0.500 g/dL was used to generate the linear (origin not included) calibration curve. To verify the calibration the whole blood volatiles control from Cliniqa with a target concentration of 0.08 g/dL ethanol and aqueous standards from Cerilliant at 0.025 and 0.300 g/dL were prepared in duplicate. One set of controls was analyzed prior to case samples and one set immediately after case samples. An internal standard blank negative control was also prepared with deionized water and analyzed after the 0.500 g/dL calibrator. All case samples were prepared in duplicate.

Instrumental analysis

All samples were analyzed on the HS-GC–FID–MS instrumentation described with a headspace oven temperature of 50°C. The HS loop and transfer line temperatures were set at 70°C and 90°C, respectively. Vial equilibration was set at 20 min. The vial pressurization was set at 15 psi for 0.15 min. Injection, loop fill, and loop equilibration times were set at 0.50, 0.15, and 0.05 min, respectively. Multi HS Extraction and vial shaking were set to off. The GC cycle time was set at 13.5 minutes. For the GC, a constant helium flow rate of 3 mL/min was used. The injection port temperature was maintained at 90°C with a 5:1 split injection of the headspace and a septum purge flow of 3 mL/min. The initial GC oven temperature of 35°C was held for 2 min and then ramped at 25°C/min to a final temperature of 90°C, which was held for 4.3 min. The total GC run time was 8.5 min/sample. Both restrictors were set at a constant helium flow of 2 mL/min. The FID temperature was maintained at 300°C with hydrogen, air, and constant column plus helium makeup pressures of 40, 450, and 50 psi, respectively. The FID signal was zeroed at 0 min with a data collection rate of 10 Hz. The MS transfer line was maintained at 280°C. The MS source and quadrupole were maintained at 230°C and 150°C, respectively. The MS electron multiplier voltage was set to a gain factor of 1 (tuned using Agilent Chemstation Gain Tune followed by Low Mass Auto Tune). The scan range was set at 20 to 200 with a threshold of 150 and a sample number of 4, which resulted in a scan rate of 2.02 scans/s. The instrument parameters are summarized in Table I. Quantitation was performed using the response ratio of the FID response of ethanol to *n*-propanol. A typical chromatogram from the FID signal and total ion chromatogram (TIC) from the MS of the Cliniqa whole blood control is presented in Figure 1. The corresponding mass spectra for each target compound and internal standard are presented in Figure 2.

Validation

The analysis of ethanol by HS-GC–FID–MS method was validated by evaluating headspace oven thermostat time, thermostat stability, sensitivity, linearity, matrix effects, carryover, repeatability, drift/bias, specificity, reportable range, and a crossover case comparison. The general validation scheme described previously (6,7), which has been used to validate numerous methods within the Toxicology Unit at the Palm Beach County Sheriff's Office (PBSO), was expanded to include evaluations of additional parameters specific to this type of analysis. All instrumental parameters were determined prior to the start of validation as part of method development and optimization with the exception of the HS oven thermostat time which was determined in the first step of validation as follows. The parameters are summarized in Table I.

Headspace oven thermostat time was evaluated by analyzing 30 Cerilliant ethanol standards at 0.100 g/dL with an incremented HS oven thermostat time. The standards were analyzed to evaluate thermostat times of 1 to 30 min incremented by 1 min for each successive standard.

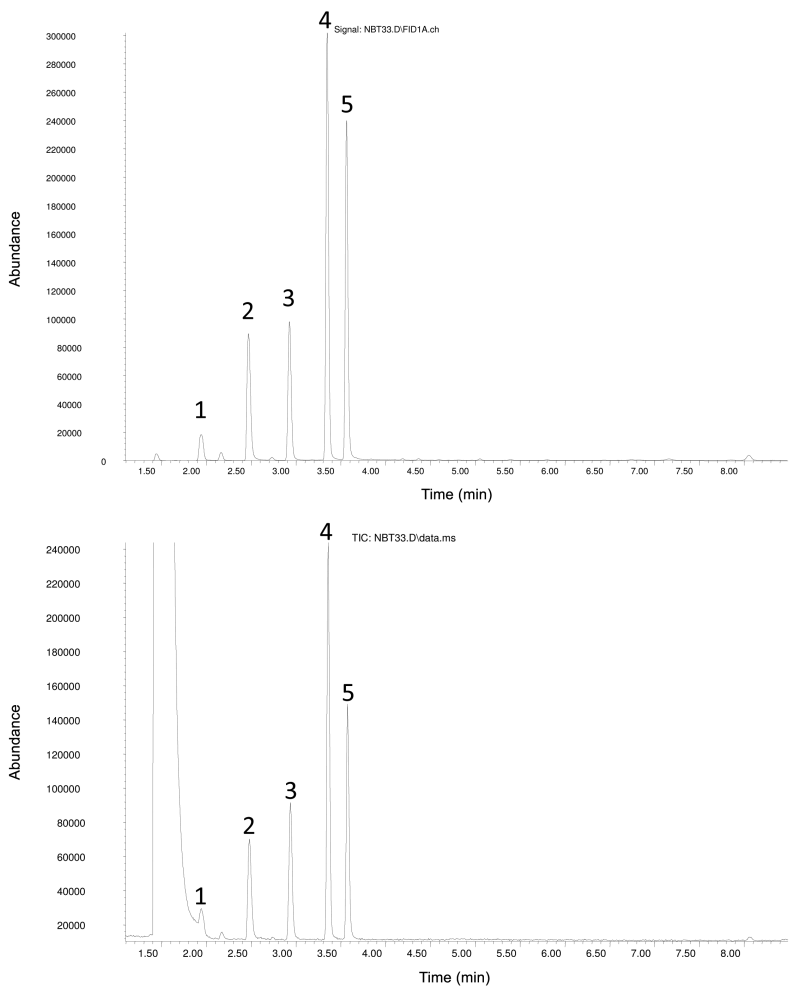

Figure 1. Clinqa whole blood volatiles control: FID signal (top) and MS total ion chromatogram (bottom). Peak identification: 1, methanol; 2, ethanol; 3, isopropanol; 4, acetone; and 5, *n*-propanol.

Linearity, matrix effects, sensitivity, carryover, and thermostat stability were evaluated by analyzing the ethanol standards prepared in whole blood, urine, and deionized water. One replicate of each of the 0.005 to 1.000 g/dL standards were analyzed in succession with a matrix matched internal standard blank prepared and analyzed after the 1.000 g/dL standard.

Linearity, sensitivity, and carryover were further evaluated by analyzing another set of ethanol standards prepared in deionized water, with calibrators prepared from 0.005 to 1.000 g/dL and controls prepared at 0.005 and 0.010 g/dL. One replicate of each of the 0.005 to 1.000 g/dL standards were analyzed in succession followed by a matrix matched internal standard blank and 10 replicates of each of the control levels at 0.005 and 0.010 g/dL. This was performed on three separate days by three different analysts.

The linearity of the typical calibration range that will be used for casework was also evaluated along with further evaluation of carryover. Throughout the validation process the typical calibrators used for casework (0.020, 0.100, 0.200, and 0.500 g/dL from Cerilliant) along with an internal standard blank analyzed after the 0.500 g/dL calibrator were analyzed 18 times on 18 different days by 4 different analysts.

Within-run and between-run repeatability was evaluated by analyzing 10 replicates of the 0.025, 0.080, and 0.300 g/dL prepared whole blood standards along with the typical calibrators utilized for casework and an internal standard blank analyzed after the 0.500 g/dL calibrator on four separate days by four different analysts. These levels were chosen to evaluate repeatability at the levels that will be used for routine casework. Also, the repeatability at the LOQ of the method, 0.010 g/dL, was evaluated by analyzing 10 replicates of a prepared 0.010 g/dL aqueous control along with prepared aqueous calibrators from 0.010 to 1.000 g/dL and an internal standard blank analyzed after the 1.000 g/dL calibrator.

Within-run repeatability was further evaluated along with an evaluation of drift/bias throughout a batch by analyzing 65 replicates each of the standards that will be used as controls for routine casework. These include Cerilliant aqueous standards at 0.025 and 0.300 g/dL and the Cliniqa whole blood volatiles control (verified to be 0.075 g/dL ethanol). The standards were analyzed immediately after an internal standard blank and the 4 calibrator standards from Cerilliant (0.020, 0.100, 0.200, and 0.500 g/dL) on three separate days.

Specificity was evaluated by analyzing common volatile solvents, inhalation anesthetics, and 1,1-difluoroethane (DFE). A Cliniqa whole blood volatiles standard was also analyzed and had standard target concentrations for methanol, ethanol, acetone, and isopropanol of 0.04, 0.08, 0.04, and 0.04 g/dL,

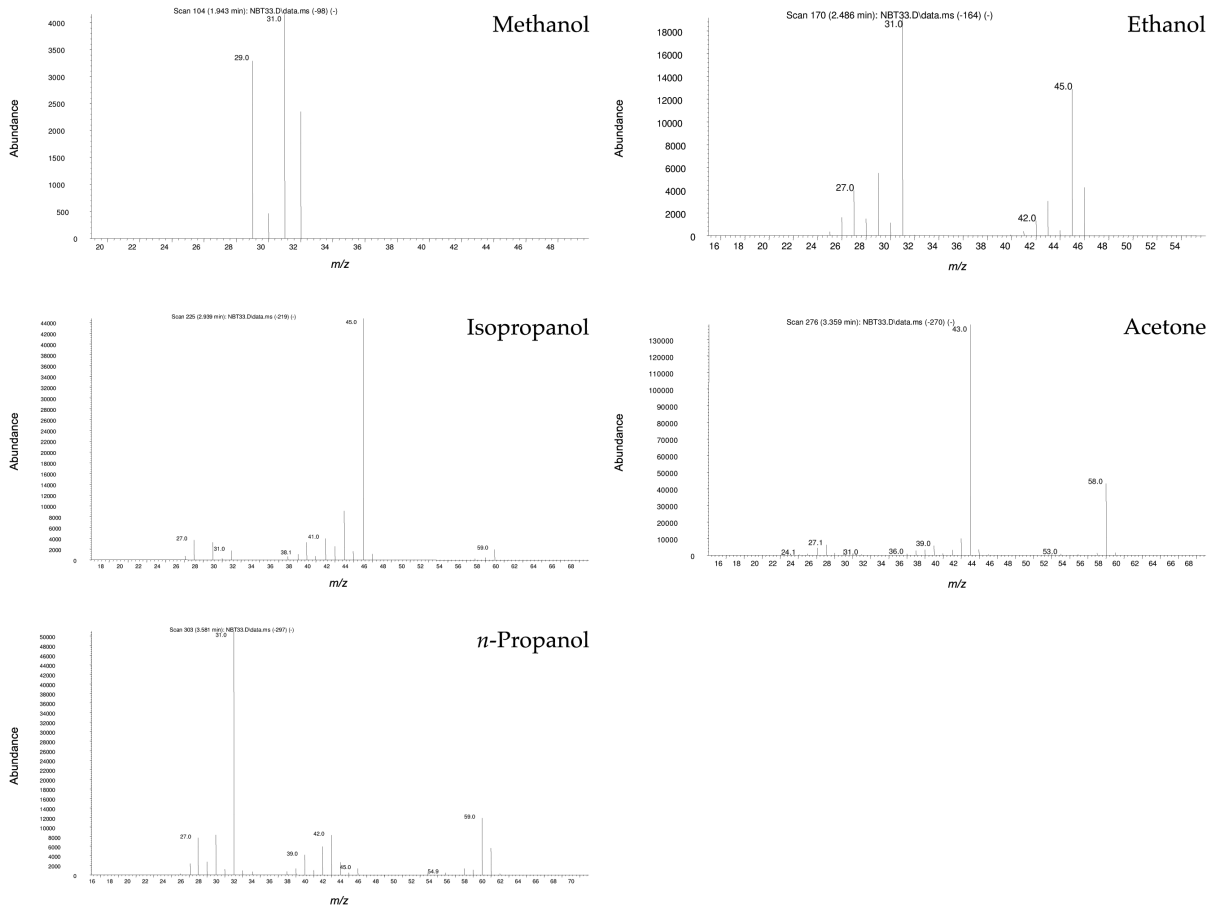

Figure 2. Scan mass spectra (20–200 scan range) for a Cliniqa whole blood volatiles control.

respectively. Volatile solvents used for analysis were prepared by adding 5 µL of ethyl acetate, chloroform, methylene chloride, acetonitrile, heptane, toluene, *n*-butyl acetate, 1-chlorobutane, pentane, and isoamyl alcohol to 10 mL of deionized water. The solutions of acetaldehyde and hexane were prepared by adding 2 and 10 µL, respectively, to 10 mL of deionized water. Isoflurane, sevoflurane, and desflurane aqueous solutions were prepared at 0.025, 0.10, and 0.10% v/v, respectively. DFE was prepared at 270, 27, and 2.7 µg/mL in deionized water. All prepared solutions along with the Cliniqa whole blood volatiles control were then prepared for analysis by diluting 100 µL with 1 mL of *n*-propanol internal standard as described.

A case comparison crossover study was conducted by reanalyzing case samples as well as proficiency samples that were analyzed with an established HS-GC–FID method that was in use for casework (8). The case samples were submitted to the laboratory in routine driving under the influence and sexual assault cases and were antemortem whole blood. The proficiency samples were from two cycles of the Florida Department of Law Enforcement Alcohol Testing Program (FDLE ATP) proficiency tests. Briefly, the HS-GC–FID method used a Perkin Elmer HS40XL headspace autosampler and AutosystemXL GC equipped with dual columns and dual FIDs. A single injection from the autosampler was split using a glass y-splitter onto a DB-ALC1 (30 m × 0.53 mm × 3 µm) and DB-ALC2 (30 m × 0.53 mm × 2 µm) column (Agilent). Sample preparation was identical with 100 µL of sample being diluted with 1 mL of 0.01% v/v *n*-propanol internal standard using the Hamilton diluter/dispenser. Quantitation was performed on channel A (DB-ALC1) by using the response ratio of ethanol to *n*-propanol. Channel B (DB-ALC2) was used for qualitative confirmation. The headspace vials, vial closures, internal standard, calibrators, and controls used were identical in both methods. Calibrators were aqueous ethanol standards from Cerilliant at 0.020, 0.100, 0.200, and 0.500 g/dL. Controls included an internal standard blank, the whole blood control from Cliniqa with a target ethanol concentration of 0.08 g/dL, and aqueous ethanol standards from Cerilliant at 0.025, and 0.300 g/dL. A total of 81 samples were compared between the two methods: 59 positive for ethanol and 22 negative for ethanol.

Table II. Sensitivity: Initial Evaluation in Three Matrices

Matrix	Concentration (g/dL)	Accuracy	S/N FID	S/N MS
0.005 g/dL Prepared standard				
Aqueous	0.0058	16.00%	24.1:1	3.5:1
Urine	0.0061	22.00%	39.3:1	3.8:1
Whole blood	0.0047	–6.00%	48.9:1	4.2:1
0.010 g/dL Prepared standard				
Aqueous	0.0106	6.00%	57.7:1	12.3:1
Urine	0.0108	8.00%	46.1:1	10.5:1
Whole blood	0.0091	–9.00%	55.8:1	10.0:1

Table III. Sensitivity: Repeatability Evaluation of Prepared Aqueous Standards

	Within-Run Day 1	Within-Run Day 2	Within-Run Day 3	Between-Run Days 1–3
Level (g/dL)	0.005	0.005	0.005	0.005
n	10	10	10	30
Mean	0.0052	0.0059	0.0056	0.0056
Minimum	0.0051	0.0058	0.0055	0.0051
Maximum	0.0052	0.0060	0.0057	0.0060
SD	0.00004	0.00005	0.00007	0.00030
CV	0.814%	0.799%	1.320%	5.485%
Accuracy	3.600%	18.000%	11.800%	11.133%
Level (g/dL)	0.010	0.010	0.010	0.010
n	10	10	10	30
Mean	0.0100	0.0107	0.0104	0.0104
Minimum	0.0099	0.0107	0.0104	0.0099
Maximum	0.0102	0.0109	0.0105	0.0109
SD	0.00010	0.00007	0.00004	0.00030
CV	0.995%	0.651%	0.405%	3.088%
Accuracy	–0.100%	7.400%	4.200%	3.833%

Results

Thermostatting oven temperature was set at 50°C to maximize partitioning of the volatiles into the headspace while not causing degradation of ethanol to acetaldehyde in whole blood specimens during equilibration (9). The equilibration time was evaluated from 1 to 30 min at 0.100 g/dL by plotting the instrument response in terms of peak area versus time. Equilibrium was reached for ethanol and the internal standard *n*-propanol at 10 min of thermostatting at 50°C. Vials should therefore be heated for at least 10 min to ensure equilibrium of the volatiles concentration between the liquid and headspace is achieved. Once the first vial is incubated, the instrument software is configured to have subsequent vials ready for injection at the conclusion of each analytical run. This causes the rate limiting step of sample analysis to be the analytical run time and not the thermostatting time. Therefore, 20 min was chosen as the set point for the method to be comparable to the existing HS-GC–FID procedure.

Ethanol has been shown to degrade while thermostatting whole blood samples at temperatures greater than 50°C (9,10). To prevent the oxidative loss of ethanol in whole blood while thermostatting for headspace analysis, addition of sodium dithionite as an inhibitor (10) or temperatures less than or equal to 50°C have been recommended (9). To verify that no ethanol degradation occurs through oxidative loss during ther-

mostatting at 50°C for 20 min without the use of sodium dithionite, prepared whole blood standards from 0.005 to 1.000 g/dL ethanol were analyzed. No detectable amounts of acetaldehyde (the product of oxidation of ethanol by oxyhemoglobin in whole blood) were present in whole blood standards up to 1.000 g/dL. Thermostatting at 50°C for 20 min without the addition of sodium dithionite did not cause degradation of ethanol present in whole blood samples to acetaldehyde.

To evaluate the limits of detection (LOD) and quantitation (LOQ), ethanol standards prepared in whole blood, urine, and aqueous matrices down to 0.005 g/dL were analyzed. The quantitative results obtained were within ± 22% of the target prepared concentration at 0.005 g/dL and within ± 9% at 0.010 g/dL for all matrices, as shown in Table II. Further evaluation of 10 replicates of prepared aqueous controls at 0.005 and 0.010 g/dL repeated on three days by three different analysts yielded quantitative results within ± 18% at 0.005 g/dL and within ± 8% at 0.010 g/dL, as shown in Table III. For ethanol quantitation, it has been recommended that the accuracy of all calibrators be within ± 10% (11). Quantitation and confirmation are performed simultaneously by FID and MS, respectively. The signal-to-noise ratio (S/N) of the 0.005 g/dL standard was greater than 24:1 by FID and greater than 3.5:1 by MS for all matrices as shown in Table II. Recommended S/N for the LOQ and LOD are 10:1 and 3:1, respectively (12). Considering the recommended accuracy and S/N ratio, the LOD by MS and LOQ by FID for ethanol were determined to be 0.005 and 0.010 g/dL, respectively. The observed sensitivity of the method was more than sufficient for routine casework, as routine calibration will be conducted down to 0.020 g/dL with a reporting limit of 0.010 g/dL.

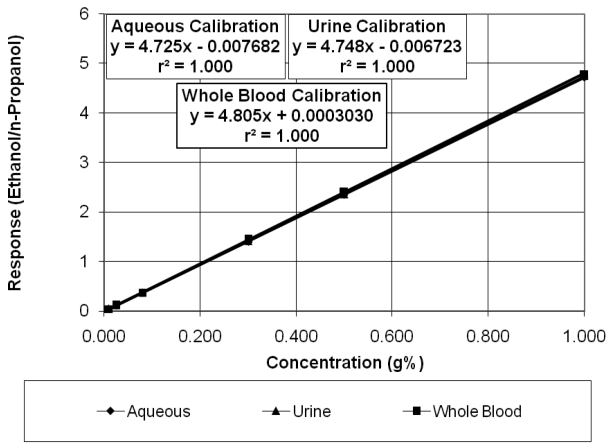

Figure 3. Ethanol calibration in three different matrices.

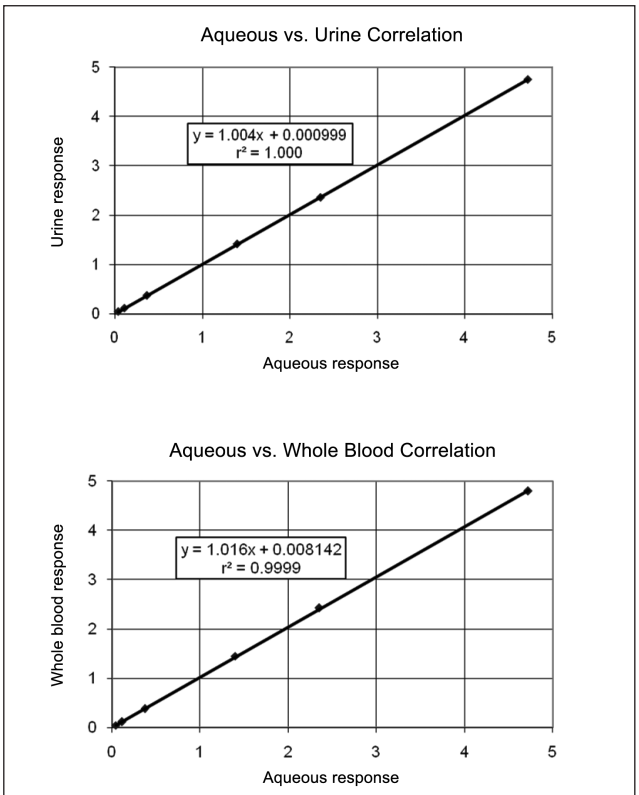

Figure 4. Correlation of aqueous standards to urine and whole blood.

Linearity was evaluated by analyzing the prepared ethanol standards. The method was shown to be linear with a coefficient of determination (r^2) of 1.000 for all matrices from 0.010 to 1.000 g/dL. The linear (not including the origin) calibration curves that were generated for the aqueous, urine, and whole blood standards are displayed in Figure 3. Linearity was further verified by analyzing a second set of ethanol standards prepared in deionized water from 0.005 to 1.000 g/dL by three different analysts on three different days. All three calibration curves from 0.010 to 1.000 g/dL yielded an r^2 of 1.000. The typical calibration range used for casework (0.020 to 0.500 g/dL) was also evaluated throughout the validation process by analyzing the Cerilliant calibrators at concentrations of 0.020, 0.100, 0.200, and 0.500 g/dL 18 times on 18 different days by 4 different analysts. Each calibration curve yielded an r^2 of 1.000. The concentration of all calibrators in all experiments was within 0.005 g/dL or 5% (whichever is larger) of the target concentration. The method was shown to be linear from 0.010 to 1.000 g/dL and is suitable for the typical calibration used for casework (0.020 to 0.500 g/dL).

The effect of various matrices on the partitioning of ethanol and other volatiles has been documented (13–16). Techniques to minimize or eliminate these matrix effects include changing equilibration time and temperature, the use of salting-out agents, specimen dilution, and use of different internal standards as well as modifying a multitude of GC conditions. A significant sample dilution (5:1 or greater) was one technique studied that was shown to effectively eliminate this matrix effect (13). To verify the elimination of a matrix effect at an 11:1 sample dilution, standards prepared in three matrices (deionized water, urine, and whole blood) over a concentration range of 0.005 to 1.000 g/dL were analyzed using the procedure. The calibration curves generated from each matrix were virtually identical when comparing the slope, y-intercept, and r^2 as shown in Figure 3. Correlation between the instrument responses for whole blood and urine standards were also com-

pared to aqueous standards by plotting the instrument response (ethanol/*n*-propanol response ratio) and evaluating the coefficient of determination of the resulting curve. Good correlation was observed for both blood (r^2 = 0.9999) and urine (r^2 = 1.000) as compared to aqueous (Figure 4). There were no observed matrix effects between water and whole blood or water and urine standards. Therefore, aqueous ethanol standards may be used as calibrators and controls when analyzing whole blood or urine samples. As described in the Sample preparation section, aqueous standards will be used for calibrators and controls along with a whole blood control for routine casework.

Matrix-matched internal standard blank controls were prepared and analyzed immediately after the 1.000 g/dL prepared standard for each of the three matrices. The possibility of carryover was evaluated for both the sampling process with the Hamilton dilutor/dispenser as well as during analysis by the instrument. No carryover of ethanol due to either sampling or analyzing matrix matched blank samples immediately after a 1.000 g/dL ethanol standard for any of the matrices studied (human whole blood, human urine, deionized water) was observed. Carryover was further evaluated by analyzing a second set of ethanol standards prepared in deionized water from 0.005 to 1.000 g/dL along with an internal standard blank analyzed after the 1.000 g/dL standard by three different analysts on three different days. No carryover of ethanol was observed. Also, throughout the validation process Cerilliant calibrators at concentrations of 0.020, 0.100, 0.200, and 0.500 g/dL along with an internal standard blank analyzed after the 0.500 g/dL calibrator were analyzed 18 times on 18 different days by 4 different analysts. No carryover of ethanol was observed. In PBSO casework, no sample has ever had a concentration greater than 0.500 g/dL. Therefore calibrators, controls, and case samples may be run consecutively without blanks on the instrument or additional rinsing of the dilutor/dispenser in between. For routine casework, as described in the sample preparation section, at least one internal standard blank will be prepared and analyzed after the 0.500 g/dL calibrator for each batch of cases.

The headspace autosampler used in this method has 70 vial positions. An internal standard blank followed by four calibrators was analyzed along with 65 replicates of each of the controls that will be routinely used for casework (0.025 and 0.300 aqueous from Cerilliant and 0.08 whole blood from Cliniqa). Data was evaluated limiting the number of vials to 45 (40 replicates with 1 internal standard blank and 4 calibrators) compared to the full 70 vials (65 replicates with 1 internal standard blank and 4 calibrators). Positive drift/bias was observed as the sequence progressed when running 65 replicates and was significant at 0.300 g/dL, causing the minimum and maximum observed concentrations to be outside the Florida Administrative Code (FAC) precision requirement of ±0.01 g/dL for legal blood alcohol determinations in the state of Florida (17). By limiting the total batch size to 45 (40 replicates) the drift was mitigated and acceptable minimum and maximum ranges were observed at all three levels. The data are presented in Table IV. To address this for casework, no more than 40 vials will be analyzed in any one batch of samples, with two replicates of each positive level of quality control material analyzed. One replicate at each positive level will be analyzed prior to case samples and one replicate at each positive level will be analyzed after case samples.

Replicates of prepared whole blood ethanol standards were also analyzed to verify the precision and accuracy of the method when analyzing whole blood. As mentioned previously, the stated criterion in the FAC for precision is that replicate samples must agree within ± 0.01 g/dL. Through the authors' validation and experience with the HS-GC–FID ethanol quantitation procedure, acceptable criteria for accuracy was established as within ± 0.005 g/dL or ± 5% (whichever is larger) of the true value. Recommended accuracy of quality control materials is ± 20% (or ± 30% at or near the concentration of the LOQ) for most drugs (9), but ± 10% may be more appropriate for ethanol. The 0.025, 0.080, and 0.300 g/dL whole blood prepared standards were analyzed a total of 40 times each (10 replicates of each level by four different analysts on four different days) to evaluate within-run and between-run precision and accuracy. These data are presented in Tables V and VI. Additionally, as described, 65 replicates of each of the controls that will be routinely used for casework (0.025 and 0.300 aqueous from Cerilliant and 0.08 whole blood from Clinqa) were analyzed. The data from the 65 replicates for within-run precision and accuracy was limited to 40 replicates as described in the drift/bias evaluation shown in Table IV. Repeatability at 0.010 g/dL, the LOQ of the method, was evaluated by analyzing 10 replicates of a prepared aqueous standard on

Table IV. Within-Run Precision and Drift/Bias Evaluation Data

	0.025 g/dL Cerilliant Aqueous		0.080 g/dL Cliniqa Whole Blood		0.300 g/dL Cerilliant Aqueous	
	n = 65	*n* = 40	*n* = 65	*n* = 40	*n* = 65	*n* = 40
Concentration (g/dL)	0.0250	0.0250	0.0755	0.0755	0.3000	0.3000
Mean	0.0265	0.0265	0.0737	0.0735	0.3040	0.3026
Minimum	0.0260	0.0260	0.0724	0.0724	0.2998	0.2998
Maximum	0.0270	0.0269	0.0745	0.0742	0.3100	0.3062
SD	0.00017	0.00018	0.00043	0.00033	0.00249	0.00149
CV	0.6352%	0.6649%	0.5785%	0.4480%	0.8176%	0.4934%
Accuracy	5.8585%	5.8700%	–2.3515%	–2.6589%	1.3369%	0.8625%

Table V. Within-Run Precision Data of Prepared Whole Blood Standards

	0.025 g/dL Prepared Whole Blood				0.080 g/dL Prepared Whole Blood				0.300 g/dL Prepared Whole Blood			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Concentration (g/dL)	0.0258	0.0258	0.0258	0.0258	0.0796	0.0796	0.0796	0.0796	0.2980	0.2980	0.2980	0.2980
n	10	10	10	10	10	10	10	10	10	10	10	10
Mean	0.0253	0.0251	0.0253	0.0250	0.0789	0.0786	0.0785	0.0778	0.2972	0.2960	0.2964	0.2938
Minimum	0.0251	0.0250	0.0252	0.0249	0.0786	0.0784	0.0782	0.0776	0.2963	0.2952	0.2945	0.2928
Maximum	0.0254	0.0253	0.0254	0.0251	0.0791	0.0788	0.0788	0.0780	0.2977	0.2967	0.2979	0.2951
SD	0.00009	0.00011	0.00008	0.00005	0.00019	0.00015	0.00018	0.00012	0.00042	0.00047	0.00105	0.00064
CV	0.373%	0.420%	0.325%	0.189%	0.235%	0.188%	0.233%	0.154%	0.143%	0.160%	0.354%	0.217%
Accuracy	–1.938%	–2.713%	–1.822%	–3.101%	–0.892%	–1.231%	–1.382%	–2.249%	–0.285%	–0.661%	–0.544%	–1.403%

three different days by three different analysts and is shown in Table III. The coefficient of variation (CV) for within-run and between-run precision for all levels was less than 3.1%. The largest observed difference between the minimum and maximum of the replicates was 0.007 g/dL at the 0.300 g/dL level. Quantitative accuracy was within ±8%, ±6%, ±3%, and ±1.5% for the 0.010, 0.025, 0.080, and 0.300 g/dL concentrations, respectively. All quantitative results were within the stated acceptable ranges (±0.005 or ±5%, whichever is larger).

Table VI. Between-Run Precision Data of Prepared Whole Blood Standards

	0.025 g/dL Prepared Whole Blood	0.080 g/dL Prepared Whole Blood	0.300 g/dL Prepared Whole Blood
Concentration	0.0258	0.0796	0.2980
n	40	40	40
Mean	0.0252	0.0785	0.2958
Minimum	0.0249	0.0776	0.2928
Maximum	0.0254	0.0791	0.2979
SD	0.00016	0.00043	0.00142
CV	0.6415%	0.5507%	0.4794%
Accuracy	–2.3934%	–1.4384%	–0.7232%

Several volatiles having similar properties to ethanol were prepared and analyzed along with internal standard blanks prepared with three different matrices (deionized water, urine, and whole blood) to verify the specificity of the method for ethanol and the internal standard *n*-propanol. The list of compounds studied along with the retention times for each detector is presented in Table VII. Because of the design of the Dean's Switch, even when configured for a 1:1 split ratio, the retention times for each detector will be slightly different. The grade of hexanes used in the experiment was > 95% *n*-hexane and cyclohexane was not detected. The prepared desflurane solution yielded a single peak that was tentatively identified as pentafluoroethane by a spectral library match (NIST 2008 database) which appeared to be a breakdown product from desflurane. No intact desflurane was detected. None of the compounds studied interfered with ethanol or *n*-propanol. There were no matrix interferences observed in the internal standard blanks prepared in three different matrices. The combination of headspace sampling, GC (DB-ALC1 column), and dual detection by FID and MS provided for the specific identification of the target, ethanol, and the internal standard, *n*-propanol, as well as the other volatiles studied.

Table VII. Retention Times of Volatiles Evaluated for Specificity

	Retention Time (min)	
Volatile	FID	MS
1,1-Difluoroethane	1.638	1.647
Desflurane (breakdown product)	1.936	1.951
Methanol	1.939	1.943
Acetaldehyde	2.160	2.173
Ethanol	2.467	2.478
Sevoflurane	2.733	2.750
Pentane	2.733	2.741
Isopropanol	2.924	2.939
Isoflurane	2.972	2.988
Methylene chloride	3.169	3.186
Acetone	3.345	3.359
Acetonitrile	3.348	3.359
n-Propanol	3.561	3.573
Hexanes (*n*-Hexane)	3.836	3.853
Chloroform	4.067	4.091
Ethyl acetate	4.495	4.511
1-Chlorobutane	4.700	4.709
Heptane	5.055	5.063
Isoamyl alcohol	5.894	5.911
Toluene	6.438	6.454
n-Butyl acetate	8.125	8.142

Eighty-one whole blood samples were analyzed in duplicate by this method, 59 of which had quantitative ethyl alcohol results obtained by the HS-GC–FID ethanol quantitation procedure. All qualitative results were identical. All quantitative results showed good correlation (quantitative values agreed

within ±0.0068 g/dL). The difference in duplicate analysis of the same case sample was within ±0.0042 g/dL for the HS-GC–FID–MS procedure and within ±0.0045 for the established HS-GC–FID procedure. Twenty-two case samples negative for ethyl alcohol did not show any interference with the target analytes. The method showed excellent correlation with the established HS-GC–FID method for quantitative ethyl alcohol determination in proficiency and case samples.

Estimation of Uncertainty

An estimation of uncertainty of measurement was conducted to meet requirements set forth as part of the ASCLD/LAB-International accreditation program which currently accredits forensic science laboratories to the ISO/IEC 17025:2005 (18) and the 2011 ASCLD/LAB-International Supplemental Requirements (19).

The estimation of uncertainty of measurement was determined for this procedure based on an internationally agreed upon approach to estimating and expressing measurement uncertainty (20–22). The approach involves the development of an uncertainty budget identifying all potential sources of uncertainty including both Type A and Type B uncertainty. Type A uncertainty is defined as uncertainty derived by statistical analysis of experimental data. Type A uncertainty results from the repeatability of control data which is scattered in a random fashion due to the laws of chance and thus has a normal Gaussian-shaped distribution. For the determination of the Type A component, repeatability was calculated using data from a 0.025 g/dL control analyzed both throughout validation and in over four months of routine casework. Type B uncertainty is that uncertainty that cannot be evaluated by a statistical means. Type B uncertainty results from the inherent biases in measuring systems and quantitative analytical methods such as uncertainty associated with calibration, weighing, pipetting, and sampling. Sources of Type B uncertainty taken into account included the accuracy of reference material and precision of the sampling device (the Hamilton diluter/dispenser) utilized during sample preparation. Precision of the diluter/dispenser associated with the sampling of calibrators, internal standard, and samples was included.

All of the contributing uncertainties were combined in a mathematical model that best represents their interactions in the measurement process. The model yields an estimated combined measurement of uncertainty ($U_{combined}$) for the entire procedure. An expanded uncertainty of measurement ($U_{expanded}$) at a specified confidence level was determined by multiplying the combined uncertainty by the coverage factor (k_{corr}) associated with the amount of historical data available. The expanded measurement of uncertainty ($U_{expanded}$) was calculated by multiplying the combined measurement of uncertainty ($U_{combined}$) by the correction factor of 3.23 (degrees of freedom equal to 60) (23). The combined measurement of uncertainty ($U_{combined}$) associated with this method for quantitative analysis of ethanol in whole blood was ±2.01%. The expanded measurement of uncertainty ($U_{expanded}$) associated with this method

Table VIII. Select Variables Examined During Method Development

Description	Conclusion
HS Vial Size: 10 vs. 20 mL	No appreciable difference in analyte response.
HS Vial Shaking: On vs. Off	No appreciable difference in analyte response.
Split Ratio for Split GC Injection: splitless vs. 2:1 vs. 5:1 vs. 10:1	Split injection was required to minimize the air peak on the MS and yield suitable chromatography. 5:1 yielded optimum response and resolution with comparable peak width to existing HS-GC–FID method.
GC Column Flow Rate: 1 vs. 2 vs. 3 mL/min	3 mL/min yielded best chromatography while still within capabilities / requirements of maximum flow rate for Dean's Switch and MS.
GC Temperature Program: 35 and 40°C isothermal vs. temperature program	35°C gave best separation of methanol from the air peak on MS. Temperature program necessary for late eluters to exit column in < 10 min. Incorporated initial hold for 2 min at 35°C into temperature program.
FID Data Sampling rate: 50 vs. 10 Hz	No appreciable difference in peak shape or quantitation. 10 Hz yielded comparable data rates to existing HS-GC–FID method.
Electron Multiplier: Gain Factor of 15 vs. 1	High Gain Factor increased abundance, but did not lead to an increase in sensitivity.

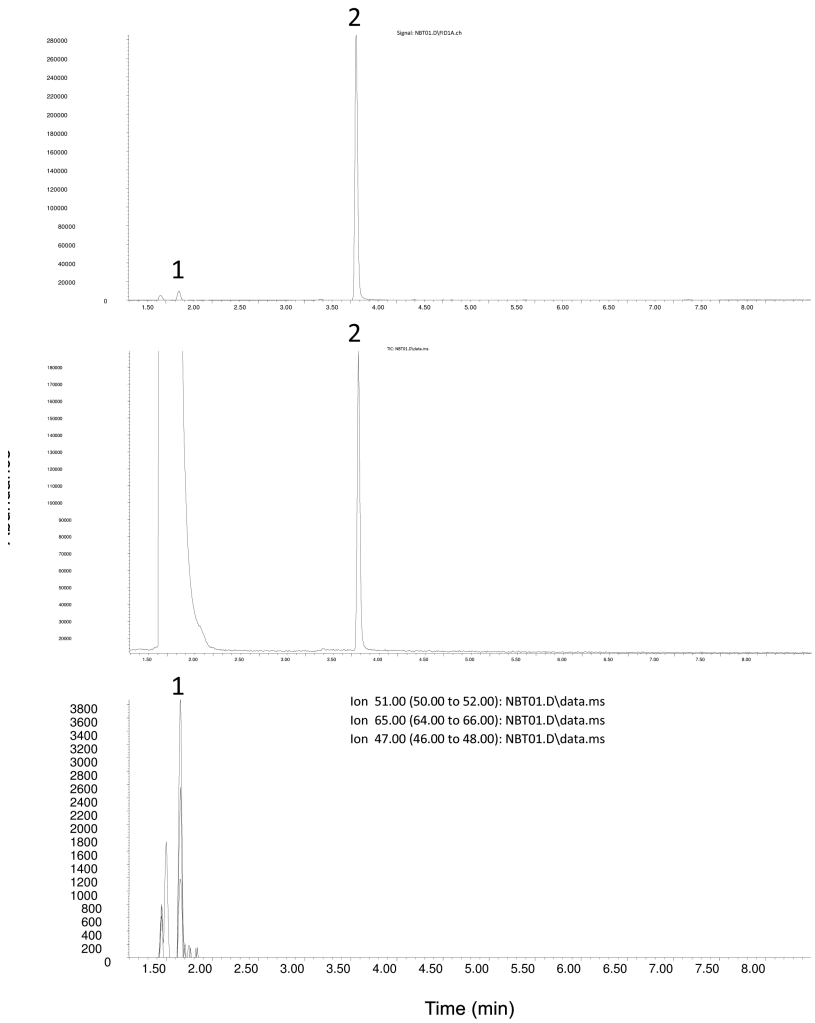

Figure 5. 1,1-Difluoroethane control in deionized water: FID signal (top), MS total ion chromatogram (middle), and MS extracted ion chromatogram (bottom). Peak identification: 1, 1,1-difluoroethane and 2, *n*-propanol.

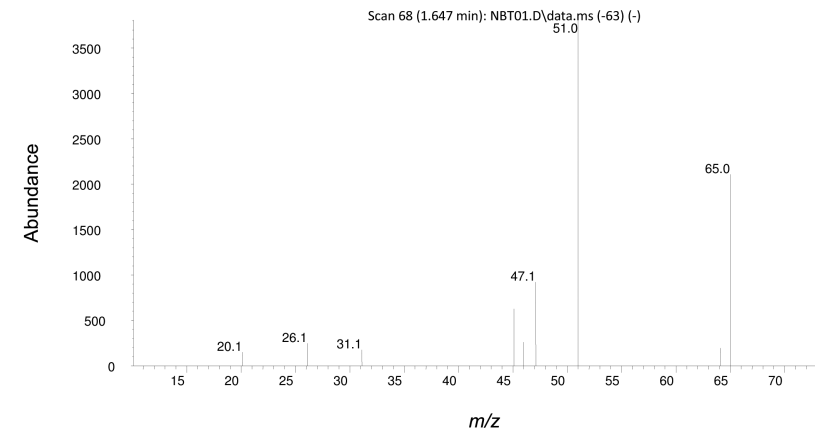

Figure 6. Scan mass spectrum for 2.6 µg/mL 1,1-difluoroethane control in deionized water.

was ±6.49% at a confidence level of 99.8%.

Discussion

This laboratory had an existing validated method for ethanol analysis by dual column HS-GC–FID, as described in the Validation section. One goal in the development of the HS-GC–FID–MS method was that the same sample preparation could be used (sample volume requirements and internal standard) to obtain similar instrument performance. For the HS-GC–FID method, the LOD and LOQ was 0.005 and 0.020 g/dL, respectively. The zero-order linear range (r^2 = 0.9998) was validated to cover the range of 0.020 to 1.000 g/dL. Quantitative accuracy was within ± 6.4%, ± 1.3%, and ± 1.1% at control concentrations of 0.044, 0.080, and 0.300 g/dL, respectively. The CV of replicate analyses for these three control levels was less than 5%. Therefore, the HS-GC–FID–MS method did obtain similar instrument performance as the HS-GC–FID method. Various settings on the HS-GC–FID–MS were evaluated during method development to achieve similar performance. A summary of select variables examined is presented in Table VIII.

DFE was also validated for qualitative identification by this method. Standards were evaluated in human whole blood, human urine and deionized water matrices over a concentration range of 2.6–52 µg/mL. DFE did elute with the air peak on the MS, but good quality scan spectra was obtained with subtraction. The FID signal, MS TIC, and MS extracted ion chromatogram for the 2.6 µg/mL control in deionized water are presented in Figure 5. The corresponding scan mass spectrum is presented in Figure 6. For qualitative analysis, sufficient correlation was observed for urine (r^2 = 1.0) and blood (r^2 = 0.98) when compared to aqueous standards. Using 100 µL of sample the LOD was ≤ 2.6 µg/mL. Reported levels in death cases for DFE in blood and urine range from 30 to 192 µg/mL (24–28). The CV of replicate within-run and between-run analyses was less than 2% and 8%, respectively. Because the same procedure was used, specificity data from the ethanol validation was used to confirm

that volatiles listed in Table VII did not interfere with 1,1-difluoroethane. Further work will be conducted to evaluate the performance of this method with other volatiles that might be used as inhalants such as toluene, 1,1,1,2-tetrafluoroethane, and chlorodifluoromethane.

Conclusions

A novel method has been validated for the quantitation of ethanol by HS-GC–FID combined with simultaneous confirmation by MS. Through validation, this method was shown to possess all the hallmark characteristics of a solid analytical method. The method was shown to possess excellent sensitivity, selectivity, repeatability, robustness, linearity, and ease of use. This method can be used for routine alcohol analysis to provide reliable quantitative data combined with unquestionable confirmation of ethanol and many other volatiles potentially present in blood and urine specimens.

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References

1. B. Levine and Y.H. Caplan. Alcohol. In *Principles of Forensic Toxicology*, 2nd ed., B. Levine, Ed. AACC Press, Washington, D.C., 2006, pp 169–184.
2. R. Firor, C.-K. Meng, and M. Bergna. Static headspace blood alcohol analysis with the G1888 network headspace sampler. Application note from Agilent Technologies, 2004.
3. GC Analysis of Blood Alcohols using RTX-BAC1 and RTX-BAC2 columns and a Perkin Elmer HS 40 headspace autosamper. Application note from Restek Corporation. http://www.restek.com/pdfs/59598A.pdf (accessed January 2011).
4. I.A. Wasfi, A.H. Al-Awadhi, Z.N. Al-Hatali, F.J. Al-Rayami, and N.A. Al Katheeri. Rapid and sensitive static headspace gas chromatography–mass spectrometry method for the analysis of ethanol and abused inhalants in blood. *J. Chromatogr. B* **799:** 331–336 (2004).
5. C.L. Morris-Kukoski, E. Jagerdeo, J. Schaff, and M.A. LeBeau. Ethanol analysis from biological samples by dual rail robotic autosampler. *J. Chromatogr. B* **850:** 230–235 (2007).
6. N.B. Tiscione, X. Shan, I. Alford, and D.T. Yeatman. Quantitation of benzodiazepines in whole blood by electron impact-gas chromatography–mass spectrometry. *J. Anal. Toxicol.* **32:** 644–652 (2008).
7. N.B. Tiscione, X. Shan, I. Alford, and D.T. Yeatman. Quantitation of opioids in whole blood by electron impact-gas chromatography–mass spectrometry. *J. Anal. Toxicol.* **35:** 99–107 (2011).
8. X. Shan, N.B. Tiscione, I. Alford, and D.T. Yeatman. A study of blood alcohol stability in forensic antemortem blood samples. *Forensic Sci. Int.* doi: 10.1016/j.forsciint.2011.04.012
9. L. Kristoffersen, L.-E. Stormyhr, and A. Smith-Kielland. Headspace gas chromatographic determination of ethanol: the use of factorial design to study effects of blood storage and headspace conditions on ethanol stability and acetaldehyde formation in whole blood and plasma. *Forensic Sci. Int.* **161:** 151–157 (2006).
10. D.S. Christmore, R.C. Kelly, and L.A. Doshier. Improved recovery and stability of ethanol in automated headspace analysis. *J. Forensic Sci.* **29:** 1038–1044 (1984).
11. SOFT/AAFS Forensic Laboratory Guidelines – 2006. http://www.soft-tox.org/files/Guidelines_2006_Final.pdf (accessed May 2010).
12. A.J. Jenkins. Forensic drug testing. In *Principles of Forensic Toxicology*, 2nd ed., B. Levine, Ed. AACC Press, Washington, D.C., 2006, pp 37–38.
13. M.T. Watts and O.L. McDonald. The effect of biological specimen type on the gas chromatographic headspace analysis of ethanol and other volatile compounds. *Am. J. Clin. Pathol.* **87(1):** 79–85 (1987).
14. J. Schuberth. A full evaporation headspace technique with capillary GC and ITD: a means for quantitating volatile organic compounds in biological samples. *J. Chromatogr. Sci.* **34:** 314–319 (1996).
15. B. Kolb. Multiple headspace extraction—a procedure for eliminating the influence of the sample matrix in quantitative headspace gas chromatography. *Chromatographia* **15:** 587–594 (1982).
16. M. Markelov and J.P. Guzowski, Jr. Matrix independent headspace gas chromatographic analysis. The full evaporation technique. *Anal. Chim. Acta* **276:** 235–245 (1993).
17. Florida Administrative Code, Chapter 11D-8.002 (11). https://www.flrules.org/gateway/chapterhome.asp?chapter=11D-8 (accessed January 2011).
18. ISO/IEC 17025. General Requirements for the Competence of Testing and Calibration Laboratories: International Organization for Standardization/International Electrotechnical Commission, 2005.
19. 2011 Supplemental Requirements for the Accreditation of Forensic Science Testing Laboratories: ASCLD/LAB-International.
20. ISO/IEC. Guide 98-Guide to the Expression of Uncertainty in Measurement. International Organization for Standardization/International Electrotechnical Commission, Geneva, Switzerland, 1995.
21. ANSI/NCSL, Z540-2-1997, U.S. Guide to the Expression of Uncertainty in Measurement, 1st ed., October 1997.
22. T.M. Adams. G104—A2LA Guide for Estimation of Measurement Uncertainty In Testing. http://www.a2la.org/guidance/est_mu_testing.pdf (accessed October 2010).
23. MedCalc Values of the t-distribution (two-tailed). http://www.medcalc.org/manual/t-distribution.php (accessed October 2010).
24. Z. Xiong, J. Avella, and C.V. Vetli. Sudden death caused by 1,1-difluoroethane inhalation. *J. Forensic Sci.* **49:** 627–629 (2004).
25. L.A. Broussard, T. Brustowicz, T. Pittman, K.D. Atkins, and L. Presley. Two traffic fatalities related to the use of difluoroethane. *J. Forensic Sci.* **42:** 1186–1187 (1997).
26. J. Avella, J.C. Wilson, and M. Lehrer. Fatal cardiac arrhythmia after repeated exposure to 1,1-difluoroethane. *Am. J. Forensic Med. Pathol.* **27:** 58–60 (2006).
27. T. Hahn, J. Avella, and M. Lehrer. A motor vehicle accident fatality involving the inhalation of 1,1-difluoroethane. *J. Anal. Toxicol.* **30:** 638–642 (2006).
28. K. Sakai, K. Maruyama-Maebashi, A. Takatsu, K. Fukui, T. Nagai, M. Aoyagi, E. Ochiai, and K. Iwadate. Sudden death involving inhalation of 1,1-difluoroethane (HFC-152a) with spray cleaner: three case reports. *Forensic Sci. Int.* **206:** e58–e61 (2011).