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Ethanol analysis from biological samples by dual rail robotic autosampler☆

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Abstract

Detection, identification, and quantitation of ethanol and other low molecular weight volatile compounds in liquid matrices by headspace gas chromatography–flame ionization detection (HS–GC–FID) and headspace gas chromatography–mass spectrometry (HS–GC–MS) are becoming commonly used practices in forensic laboratories. Although it is one of the most frequently utilized procedures, sample preparation is usually done manually. Implementing the use of a dual-rail, programmable autosampler can minimize many of the manual steps in sample preparation. The autosampler is configured so that one rail is used for sample preparation and the other rail is used as a traditional autosampler for sample introduction into the gas chromatograph inlet. The sample preparation rail draws up and sequentially adds a saturated sodium chloride solution and internal standard (0.08%, w/v acetonitrile) to a headspace vial containing a biological sample, a calibrator, or a control. Then, the analytical rail moves the sample to the agitator for incubation, followed by sampling of the headspace for analysis. Using DB-624 capillary columns, the method was validated on a GC–FID and confirmed with a GC–MS. The analytes (ethanol, acetonitrile) and possible interferences (acetaldehyde, methanol, pentane, diethyl ether, acetone, isopropanol, methylene chloride, *n*-propanol, and isovaleraldehyde) were baseline resolved for both the GC–FID and GC–MS methods. This method demonstrated acceptable linearity from 0 to 1500 mg/dL. The lower limit of quantitation (LOQ) was determined to be 17 mg/dL and the limit of detection was 5 mg/dL.
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Keywords: Ethanol; Robotic autosampler; Automation; Blood alcohol

1. Introduction

By far, ethanol is the most widely abused drug in the world, and thus, the most frequently encountered positive finding in forensic toxicology investigations [1–4]. This makes the need of reliable and robust analytical methods for ethanol vitally important to all laboratories conducting full-spectrum toxicological investigations. However, ethanol has several unique properties that produce analytical and interpretive challenges very different from those found for many other commonly encountered drugs. The high volatility of ethanol means that analysis must be conducted directly upon the available biological specimens without the extraction and isolation steps found in most drug analysis procedures. Owing to the nature of various laws regarding alcohol intoxication, the quantitative tolerances for ethanol analysis must be significantly tighter than is typical for the analysis of most routine drugs of abuse. Ethanol is also nearly unique in its ability to be generated *in situ* in poorly handled samples or as a natural result of post-mortem processes [5–8].

Ideally, any method for ethanol analysis should run quickly, require as little sample preparation as possible, use minimal sample volume, have high specificity, and show very good precision and accuracy in quantitation. There are several colorimetric and photometric methods available for ethanol analysis, most of which require minimal sample volume and can be rapidly conducted [9–15]. Further, the availability of commercial instrumentation for these tests allows for the assay to be performed with little or no sample preparation by lab analysts. Unfortunately, such tests typically have fairly poor specificity and show strong matrix-dependant performance, with negative consequences for assay accuracy. In short, while such tests may be appropriate for clinical toxicology laboratories, they fall short of the standards to which a forensic laboratory is held.

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Far more appropriate to the forensic analysis of ethanol in biological specimen are gas chromatographic methods. Since the volatility of ethanol precludes the use of classical extraction and isolation prior to analysis, either direct-injection analysis [16–21] or headspace sampling is typically employed [3,22–26]. Direct-injection is a paragon of minimized sample processing time, and is required by statute in some jurisdictions. But the direct-injection technique tends to be extremely abusive of equipment and often leads to excessive instrument downtime for maintenance. This leaves headspace sampling methods as the preferred choice from analytical and cost standpoints. Unfortunately, headspace-GC analysis, as classically performed, requires more sample preparation than other methods discussed above.

Modern instrumental technology can eliminate many of the manual sample processing steps that traditionally are required for headspace-GC ethanol analysis methods. New systems are available that combine liquid-handling sample preparation robotics with multi-function autosamplers, all of which can be directly mounted on an unmodified commercial gas chromatograph. We present the validation of an ethanol quantitation method on one of these systems in our laboratory. The only manual sample preparation steps consists of accessioning two 250 μL portions of biological sample into 10 mL headspace vials, immediately sealing the vials, and then loading them on the instrument. All further sample preparation and quantitative steps are fully automated on the GC–FID system. The vials for presumptive positive samples are transferred to a similarly equipped GC–MS (EI) system for qualitative confirmation of analyte identity. The method demonstrates excellent accuracy and precision, a wide linear range, a good lower limit of detection, and the unmatched specificity that comes from inclusion of mass spectral analysis. This method should prove most useful for any laboratory conducting large numbers of forensic blood alcohol determinations.

2. Experimental

2.1. Materials

The certified aqueous ethanol calibrators (25, 50, 80, 100, 200, 300, and 400 mg/dL) were purchased from Cerilliant Corporation (Round Rock, Texas). Other aqueous ethanol calibrators (10, 15, 20, 500, 750, 1000, 1250, and 1500 mg/dL) used in the study were made from 200 proof ethanol purchased from Aldrich (Milwaukee, Wisconsin). Negative control blood and positive control blood (77 mg/dL and 174 mg/dL) samples were purchased from Clinical Control International (Los Osas, California). Negative control urine was pooled from volunteers at the FBI Laboratory. Acetonitrile, methylene chloride, isopropanol, acetaldehyde and sodium chloride were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Pentane, *n*-propanol, and isovaleraldehyde were purchased from Aldrich (Milwaukee, Wisconsin), while diethyl ether was purchased from Burdick and Jackson (Muskegon, Michigan). De-ionized water (18 + MΩ grade) was obtained from an in-house Millipore purification system. Both the GC–FID and GC–MS instruments were equipped with DB-624 (30 m × 0.25 mm i.d., 0.25 μm film thickness) capillary columns purchased from Agilent Technologies (Wilmington, Delaware).

2.2. Procedure

Two-hundred and fifty microliters of calibrators, samples and control were placed in separate 10 mL headspace vials, immediately sealed with a silicone-septum cap, and then placed on the autosampler.

2.3. Instrumentation: HS–GC–FID

An Agilent (6890) GC equipped with a flame ionization detector (FID) was used for the quantitation of alcohol in biological samples. A dual rail autosampler purchased from Gerstel Inc. (Baltimore, Maryland), was interfaced with the GC–FID for sample preparation and sample introduction into the GC (Fig. 1). The analysis was performed isothermally at 40 °C with a constant pressure of 10.23 psi of nitrogen carrier gas. The inlet temperature was kept at 150 °C, while the detector was held at 250 °C. For the analysis, the inlet was set in a split mode with a split ratio of 10:1. The detector hydrogen, air, makeup gas (combined nitrogen and column flow) were 40, 430, 50 mL/min, respectively. The offset on the electrometer was set at 2.0. The sample acquisition time was 10 min.

2.4. Reagents addition parameter

A 1 mL aliquot of saturated salt solution (0.35 g/mL of sodium chloride in de-ionized water) was added to the headspace

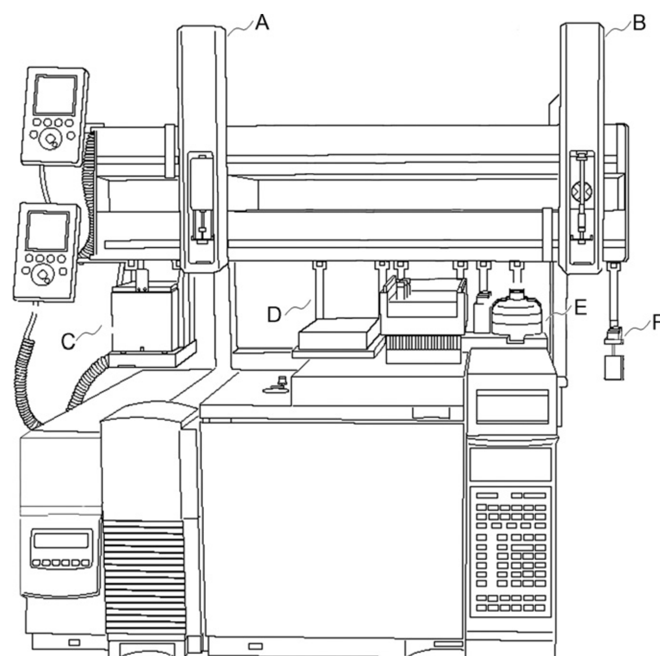

Fig. 1. Gas chromatography/mass spectrometer coupled to a dual rail multipurpose autosampler with (A) analytical rail; (B) preparation rail; (C) agitator; (D) sample holders with/without peltier cooler; (E) prep station reservoir; and (F) fast wash station.

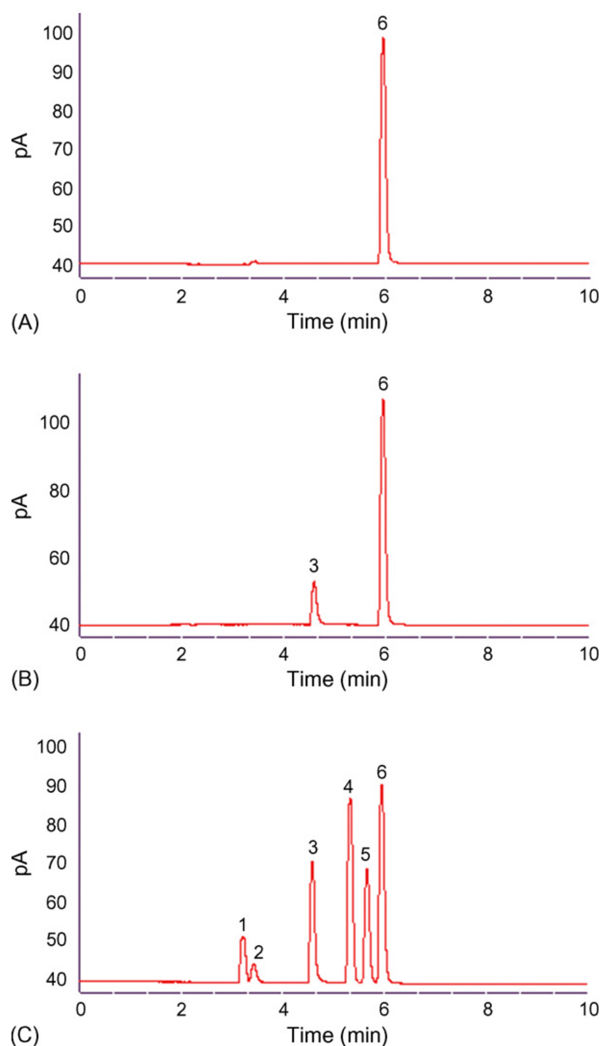

Fig. 2. Showing (A) aqueous blank; (B) 25 mg/dL calibrator; and (C) whole blood volatile control (ethanol 77 mg/dL) analyzed by GC/FID. The listed compounds are (1) acetaldehyde, (2) methanol, (3) ethanol, (4) acetone, (5) isopropanol, and (6) acetonitrile.

vials from a reservoir on the autosampler using a 1000 μL syringe. To ensure a good transfer of the solution, three fill strokes with a fill speed of 100 μL/s were used. A 250 μL aliquot of internal standard (0.08%, w/v) aqueous solution was made from acetonitrile) was transferred to the vial with a fill stroke count of 4 μL/s, and fill speed of 100 μL/s. Both the salt and internal standard was deposited in the vial at a depth of 13 mm from the top, well above the level of the sample in the vial. A fast wash station was installed as an accessory on the autosampler for washing the syringe before and after the transfer of each solution. At the time of washing, the needle is moved over to the fast wash station, which allows for washing both the outside of the needle and the inside of the syringe.

2.5. Headspace parameters

The samples were incubated for 30 min at 60 °C with an agitator speed of 250 rpm. The headspace analysis was performed with a 2.5 mL gas tight syringe that was heated at 70 °C. A 1 mL headspace aliquot was sampled for the analysis with a fill speed of 500 μL/s. To obtain homogenous sampling, a fill stroke count of 10 was used. The sample was injected into the GC at 1000 μL/s at an injector penetration depth of 25 mm. After each analysis, the syringe was flushed with helium for 2 min. The run cycle time was set at 14.8 min. Typical GC–FID data are displayed in Fig. 2.

2.6. Instrumentation: HS–GC–MS

An Agilent (6890) GC equipped with a mass spectrometer (HP 5973) in the electron ionization mode was used for confirmation of the alcohol in biological samples. The analysis was performed isothermally at 50 °C with a constant pressure of 6.54 psi of helium carrier gas. The inlet, transfer line, quadrupole, and source were heated to 150, 260, 150, and 230 °C, respectively. For the analysis, the inlet was set in a split mode with a split ratio of 10:1. The mass spectrometer

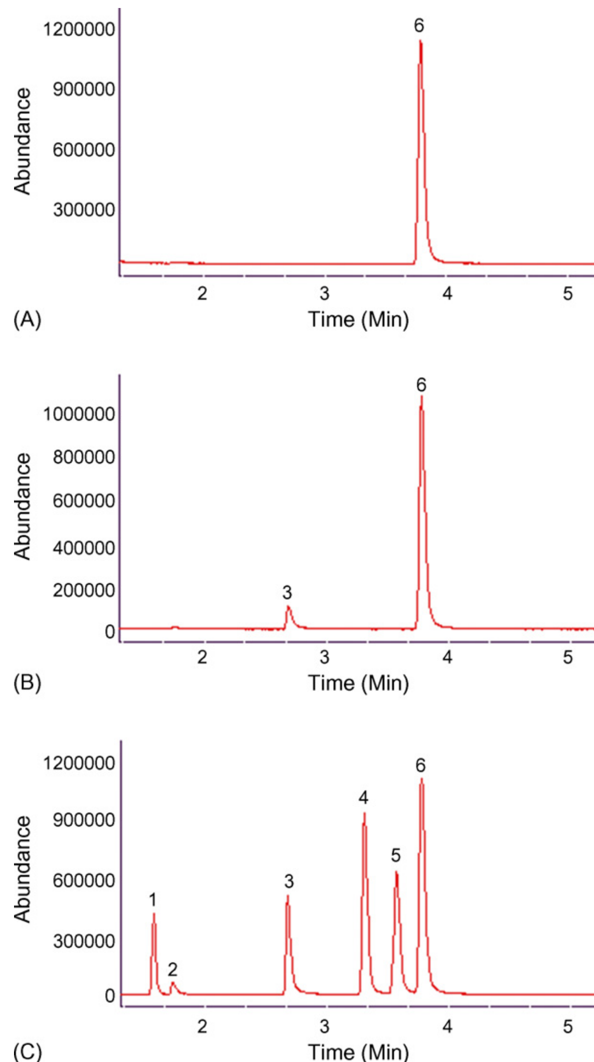

Fig. 3. Showing (A) aqueous blank; (B) 25 mg/dL calibrator, and (C) whole blood volatile control (ethanol 77 mg/dL) analyzed by GC/MS (EI). The listed compounds are (1) acetaldehyde, (2) methanol, (3) ethanol, (4) acetone, (5) isopropanol, and (6) acetonitrile.

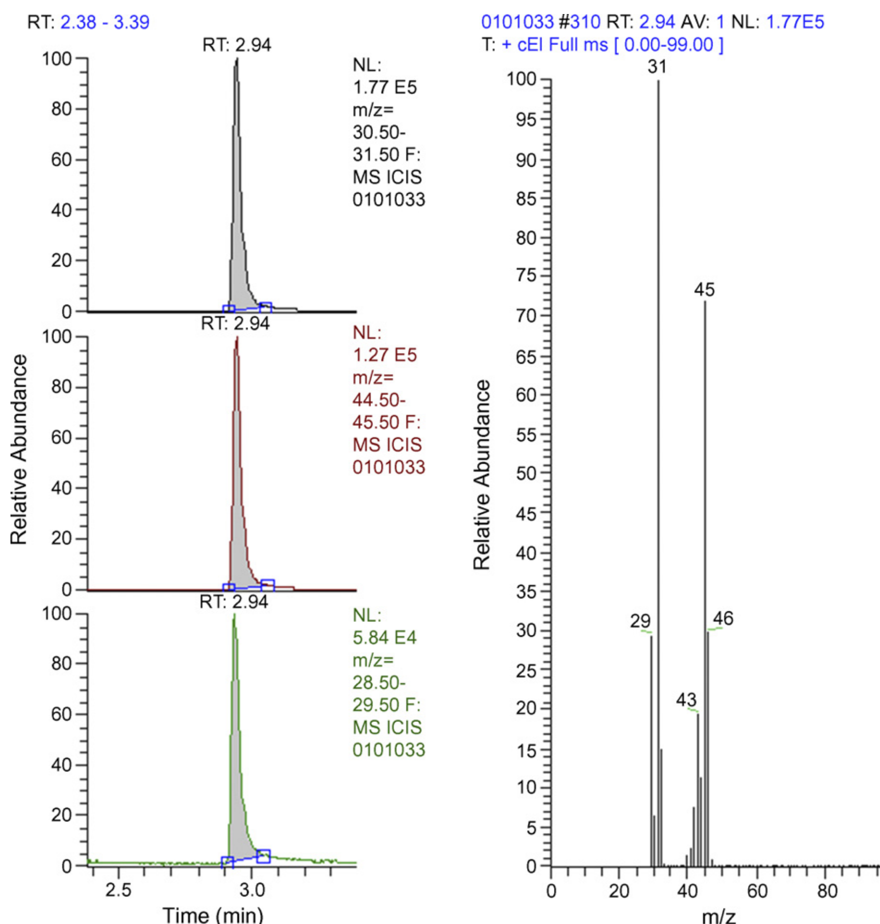

Fig. 4. Spectrum and extracted diagnostic ion traces (31, 45 and 29, respectively) for 25 mg/dL ethanol in blood.

was operated in the full scan mode over a mass range of *m*/*z* 29–100 with a threshold of 10 and sample rate of 4.96 s^{-1}. The sample acquisition time was 9.5 min. Headspace parameters were as described for the GC–FID experiments, except that the agitator speed was 250 rpm, the fill stroke count was 5, and the cycle time was 13 min. Typical GC–MS data are shown in Figs. 3–5.

3. Results and discussion

For this study, fourteen sample sets were analyzed using a seven-point linear calibration curve and the data were compared to historical data of twenty-three sample sets. The averaged line equation was determined to be $y = 0.00819\ (\pm 0.001)x - 0.0285$ ($\pm$ 0.038), at three standard deviations. A typical calibration curve is shown in Fig. 6. All studies were performed with de-ionized water serving as negative control and bi-level whole blood positive volatile controls analyzed in quadruplicate. Analysis of multiple negative blood and urine controls showed negligible contribution to the signals for analytes of interest. All calibrators and controls were within ±10% of target values. Blanks were placed between samples to evaluate the possibility of sample carryover. No carryover was demonstrated within the experimental range of 0–1500 mg/dL in all three matrices (de-ionized water, urine, whole blood).

The sensitivity of the method (average slope of several calibration curves) was 0.00819 and the selectivity demonstrated no matrix interference for all three matrices. The within-run accuracy was determined to be −3.8% (relative) at 77 mg/dL and −6.51% (relative) at 174 mg/dL. The between-day precision of bi-level positive controls was 4.16% RSD at 77 mg/dL and 2.9% RSD at 174 mg/dL.

Three methods were utilized to determine the most conservative limit of detection (LOD) and limit of quantitation (LOQ): serial back dilution, 3.3 times (for the LOD) or 10 times (for the LOQ) the standard deviation (SD) of the *y*-intercept divided by the slope, and 3 SD (Δx) of the uncertainty boundary (for the LOD) or 10 SD (Δx) of the uncertainty boundary (for the LOQ) of the calibration curve (Table 1). The LOD and LOQ were determined to be 5 mg/dL and 17 mg/dL, respectively. The upper limit of linearity (ULOL) was determined by serial escalation method. The ULOL was determined to be 1500 mg/dL in all three matrices. Method performance parameters are summarized in Table 2.

An interference study was performed to evaluate the potential interference of several known volatiles with similar retention times when analyzed via HS–GC–FID (Table 3). Volatiles were added to various negative matrices (de-ionized water, urine, and blood) with and without the addition of ethanol. All volatiles demonstrated good baseline separation from each other and did

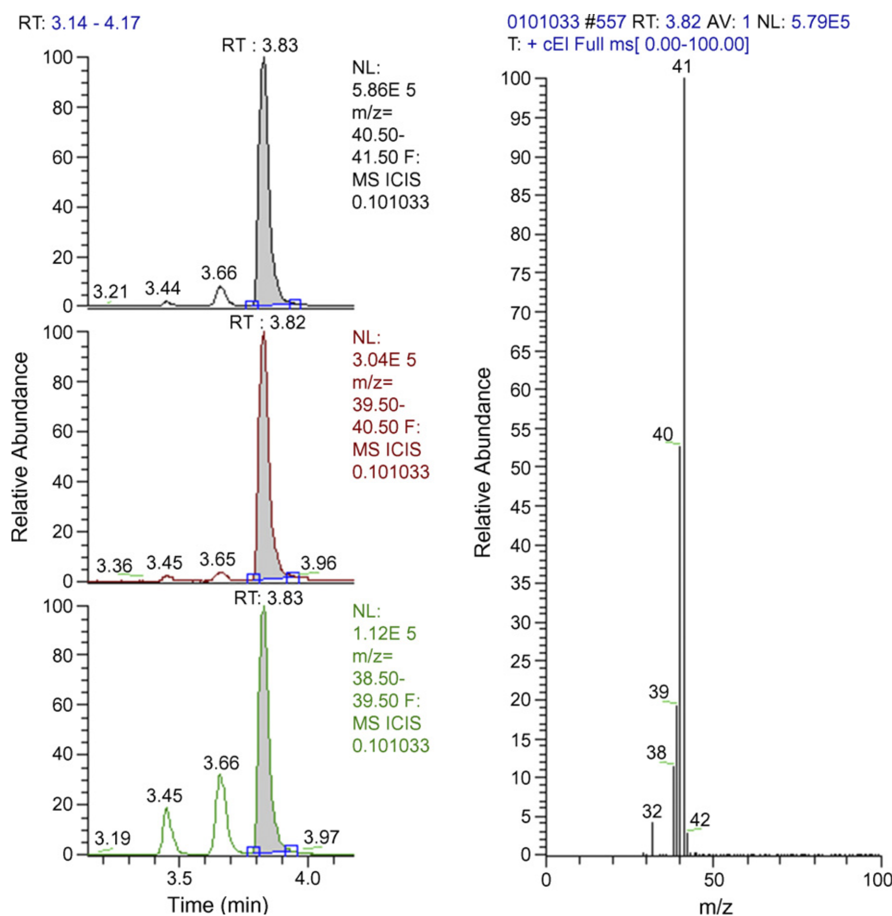

Fig. 5. Spectrum and extracted diagnostic ion traces (41, 40, and 39, respectively) for 80 mg/dL acetonitrile (internal standard) in blood.

not interfere with the proper identification and quantitation of ethanol.

HS–GC–FID acceptance criteria: in using this HS–GC–FID method, a specimen or control result is valid when the retention time of the suspected peak is within ±2% of positive control. Further, the chromatography of all peaks must have good fidelity, reasonable peak shape, width, and resolution. All samples qualitatively screened and quantitated via HS–GC–FID were qualitatively confirmed via HS–GC–MS.

Table 1
Methods used in determining the limit of detection and limit of quantitation

	Water (mg/dL)	Urine (mg/dL)	Blood (mg/dL)
Limit of detection			
Serial back dilution	2.5	2.5	2.5
3.3 *x* SD *y*-intercept/slope	5.1		
Δx 3SD (calibration data)[a]	4.7		
Limit of quantitation (LOQ)			
Serial back dilution	10	17	15
10 *x* SD *y*-intercept/slope	15		
Δx 10SD (calibration data)[a]	17		

[a] Using $\Delta x = x\sqrt{(\Delta m/m)^2 + (\Delta b/y - b)^2}$ and solve for when; Δx 3SD = x as the LOD; Δx 10SD = x as the LOQ.

The data generated with the new automated method were compared to the historical manual method. The averaged line equation (3 SD) for the historical manual method was $y = 6.5(\pm 0.9)x - 0.005\ (\pm 0.018)$. The historical manual methods accuracy was −5.7% and the precision was 2.5% RSD, with both relative at 160 mg/dL. The historical method's LOD and LOQ were 3 mg/dL and 11 mg/dL, respectively. The manual headspace method's linearity was established as the calibration curve of 25–300 mg/dl. Comparison of the perfor-

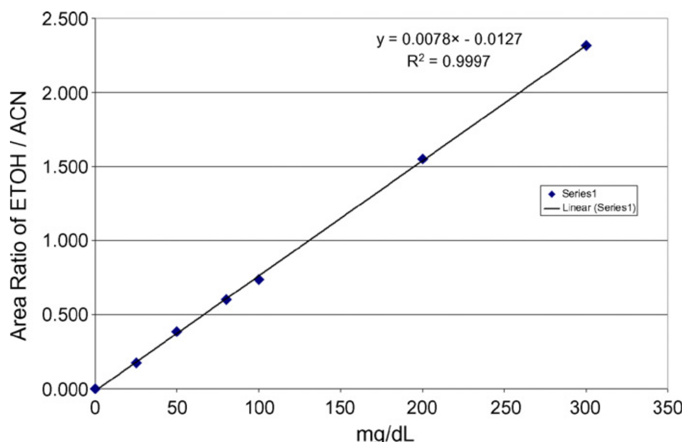

Fig. 6. A typical standard curve of ethanol.

Table 2
Critical method performance parameters

Accuracy	−3.8% at 80 mg/dL	$n = 52$
	−6.5% at 170 mg/dL	$n = 51$
Intraday %RSD	4.1% at 80 mg/dL	$n = 52$
	2.9% at 170 mg/dL	$n = 51$
Interday %RSD	2.9% at 80 mg/dL	$n = 12$
	2.3% at 170 mg/dL	$n = 11$
Linearity	25–1500 mg/dL	
Sensitivity	0.00819 (mg/dL)$^{-1}$	
LLOQ	17 mg/dL[a]	
Limit of detection	5 mg/dL[b]	(FID)
	8 mg/dL	(MS)

[a] Using Δx 10SD method.
[b] Using Δx 3SD method.

mance characteristics between the new automated method and the old manual method demonstrated good agreement. Both demonstrated LOQ's lower than the lowest non-zero calibrator (25 mg/dL), and both statistically equivalent, forensically insignificant LOD's.

HS–GC–MS acceptance criteria: in using this HS–GC–MS method, a specimen is valid if the retention time of the suspected peak is within ±2% of the positive control. The chromatography of all peaks must have good fidelity, reasonable peak shape, width, and resolution. Ethanol is identified by ions *m*/*z* 31, 45, 29, while acetonitrile (internal standard) is represented by ions *m*/*z* 41, 40, 39. Ratio matching tolerances were determined as follows: ion ratio (relative to base peak) in the known spectrum >50% are within 10% absolute, ion ratios in the known spectrum between 25% and 50% of the base peak are within 20% relative, and ion ratios in the known spectrum <25% of the base peak are within 5% absolute. Based upon ion matching criteria, the LOD for this analysis was determined to be 8 mg/dL. Below 8 mg/dL, the relative abundance of the diagnostic *m*/*z* 29 ions diverges significantly from the value observed at higher concentrations, which precludes proper identification.

Table 3
Retention times of volatiles using HS–GC–FID

List of volatiles	Retention time (min)
Acetaldehyde	3.259
Methanol	3.457
Pentane	4.413
Ethanol	4.619
Diethylether	4.801
Acetone	5.369
Isopropanol	5.704
Acetonitrile	5.996
Methylene chloride	6.368
n-Propanol	8.789
Isovaleraldehyde	9.622

4. Conclusion

The fully automated instrumental method used for this study eliminates many of the time consuming, manual, sample processing steps used by other headspace GC ethanol methods, thereby minimizing the chance of error. Interfacing a fast wash station to the autosampler provide versatility for the method when processing larger sample batches because the system is no longer restricted to fixed wash and waste reservoirs. Furthermore, a large wash station facilitates thorough washing of the outside of the needle and inside of the syringe and thus, eliminates any possibility of carry-over. This method demonstrates excellent accuracy and precision, a wide linear range, a good lower limit of detection, and unmatched specificity that comes from inclusion of mass spectral analysis. The analytical results demonstrate the feasibility of this technique for high throughput and fully automated analysis, without sacrificing accuracy and precision.

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