1. PROTOCOL FOR THE ANALYSIS OF ETHANOL

PURPOSE

The Toxicology section is routinely asked to analyze evidence for the presence and quantity of ethanol. This protocol outlines the procedure to follow when performing analysis for the quantitation of ethanol in blood, serum and plasma samples and the qualitative identification of ethanol in urine and liquor/liquid samples.

PLAN

A. Instrumentation

(1) Agilent Gas Chromatograph equipped with:

(a) OpenLAB Software

(b) Dual capillary columns – Agilent J&W

- DB-ALC1 fused silica, 30m length, 0.32 mm i.d. stationary phase, 1.8 µm film thickness
- DB-ALC2 fused silica, 30m length, 0.32 mm i.d. stationary phase, 1.2 µm film thickness

(2) Agilent 7697A Headspace Autosampler

B. Solutions, Standards, Calibrators and Controls

(1) **HSGC Internal Standard Solution**
This standard consists of 0.015% v/v n-propanol and 0.5M ammonium sulfate in deionized water.

(a) Check water before use, verify no peaks present (acceptable peak area ≤0.25)

(b) Fill a 2L container half full of deionized water.

(c) Add 300 µl of n-propanol to the container.

(d) Add 132.08g ammonium sulfate to the container. Mix thoroughly until dissolved.

(e) Dilute to volume (2000ml) with deionized water. Invert and mix thoroughly.

(f) Check by HSGC prior to use, verify n-propanol peak area comparable to previous lot of IS (guideline: 100 to 150) and no other volatiles present.

(g) Record preparation and checks in HSGC Reagent Log Book

(h) Stability 1 year

(2) **Preparation of mixed volatiles standard**
Note: acetaldehyde must be pipetted cold. Boiling point is 20.8°C.

(a) Pipette into a 100ml volumetric flask:

- 25µl acetaldehyde
- 75µl methanol

- 25µl acetone
- 75µl ethanol
- 50µl isopropyl alcohol

(b) Add deionized water to 100ml mark

(c) Transfer to amber glass bottle(s) and cap

(d) Check prior to use to ensure separation and identification of all components

(e) Record preparation and check in HSGC Reagent Log Book

(f) Stability 1 year

(3) **Calibrators**

(a) 0.025%, 0.050%, 0.100%, 0.200%, 0.400% aqueous ethanol calibrators purchased from an independent vendor

(4) **Controls**

(a) 0.040%, 0.080% and 0.300% aqueous ethanol controls purchased from an independent vendor (different than the vendor for the calibrators).

(b) Whole blood control at a mid range concentration purchased from an independent vendor or prepared in house (stability 3 years) as follows:

- Negative whole blood is prepared as follows: Add 900mg of sodium chloride to a 100 mL volumetric flask. Dilute to volume (100mL) with deionized water. In a 200 mL volumetric add this 100mL 0.9% NaCl solution and 100 mL of red blood cells. Mix well gently by inversion for 10 minutes.

- Negative whole blood is tested by HSGC prior to addition of ethanol to demonstrate the absence of ethanol. If ethanol or another volatile is detected, prepare a new batch of negative whole blood.

- An interim 2% aqueous ethanol stock solution is prepared as follows: Fill a 100 mL volumetric flask approximately 1/3 full with deionized water. Place on analytical balance, tare, and gravimetrically add 2.00 grams of ethanol from an ethanol source with >99% purity (i.e. >198 proof). Record the weight. Dilute to volume with deionized water. Discard after use.

- Addition of preservatives, anticoagulants, and inhibitors: Fill a 200 ml volumetric flask 2/3 full with negative whole blood. Add 0.4 grams of Potassium Oxalate (KOX), 2.0 grams of Sodium Fluoride (NaF), 4.0 grams of Sodium Hydrosulfite (dithionite) ($Na_2S_2O_4$), and 10.0 mg of Pyrazole ($C_3H_4N_2$). Mix well by inversion, over a two hour period.

- Addition of ethanol: Volumetrically add 20.0 mL of the 2% aqueous ethanol stock to the blood prepared in the step above. Dilute to volume with negative whole blood. Mix thoroughly by inversion for 10 minutes.
- Propagation of aliquots: Pipette 1.1 mL of the blood control into 2.0 mL amber crimp cap vials. Crimp tightly and place in refrigerator.
- Assignment of value: Analyze at least 40 replicates on at least 2 different instruments and determine the average value. This will establish the target value for that batch of whole blood control.

(c) Prepare a negative control using DI water and internal standard.

C. Priming of Hamilton Microlab® Dispenser Diluters

Microlab 500A Series

(1) Verify that the pipettor is set up correctly. The left syringe should be set to a speed of 4, delivering 1000 µl of internal standard. The right syringe should be set to a speed of 2, delivering 100 µl of sample.

(2) Prime the instrument with internal standard solution for at least three cycles by depressing toggle switch labeled “step/prime” down into the “prime” position, to stop priming toggle the switch back to the middle position.

Microlab 600 Series

(1) Verify that the pipettor is set up correctly. Press Quick Start on screen, the left syringe should be set to deliver 1000 µl of internal standard. The right syringe should be set to deliver 100 µl of sample.

(2) Prime the instrument with internal standard solution for at least three cycles by pressing the ‘prime’ button, to stop priming press ‘prime’ button again. Before use, reset pipettor by pressing button twice on hand probe.

D. Sample Preparation

(1) Allow all calibrators, controls and samples to come to room temperature before starting.

(2) Whole blood samples should be mixed thoroughly before pipetting. Plasma/serum and urine samples require no preparation. In the case of samples in Serum Separation tubes (SST’s) or samples for toxic vapor analysis an additional vial, with internal standard and DI water only, should follow each of the case sample vials to ensure no possible carryover from toluene. Clotted samples must be THOROUGHLY homogenized before pipetting or alternatively centrifuged and reported as plasma/serum.

(3) Prepare the following in sequence: calibrators in duplicate; a mixed volatile standard (without toluene); a negative control; enough ethanol whole blood controls to bracket up to five samples in duplicate throughout the batch; aqueous controls bracketing every five subject samples (pipetted in sequence throughout the batch) and subject samples in duplicate with the following procedure:

(a) Insert probe tip into the sample vial and push the button on the probe tip holder once.

(b) Remove probe tip from sample container and extend tip into the headspace vial designated for the sample.

(c) Push the button on the probe tip once to automatically dispense the preset volumes of sample and internal standard into the headspace vial.

(d) Tightly crimp cap onto the vial.

(e) Rinse pipette tip with DI water between each sample.

(4) Preparation of Liquor Samples; Prior to pipetting, samples will be diluted as follows:

(a) Beer should be diluted 1:20 with DI water.

(b) Wine should be diluted to 1:50 with DI water.

(c) Spirits and other specimens should be diluted 1:100 (or other appropriate dilution) with DI water.

(5) Urine samples may be analyzed for ethanol if no blood sample is available. If positive for ethanol report qualitatively and test the urine sample for glucose using a "Keto-Diastix" or equivalent. Test both positive and negative controls, recording the lot number of the controls as well as the "Keto-Diastix."

E. Instrument Setup and Documentation

(1) Upload sequence to instrument and print

(2) Load samples onto autosampler according to sequence and have it verified by another analyst prior to unloading

(3) Complete Blood Alcohol Analysis Face Sheet

(4) Refer to Tox-Trn-Apx7 and Tox-Trn-Apx8 for detailed instructions

F. Quality Assurance Checks for analytical result acceptability:

(1) Check linearity of calibration. Calibration curve must have $R^2 \geq 0.99$

(2) Check standards and controls for accuracy and precision. Standards and controls must be within ±5% of the target values.

(3) Ensure ethanol is identified on both channels

(4) Check duplicates for precision. The difference between duplicate samples must be within ± 5% of the average of the two results (or ± 0.005 grams per 100mL for results where the average is <0.025 grams per 100mL).

(5) Check resolution of the mixed volatile standard.

(6) Check for the absence of ethanol in negative control.

(7) File Face Sheet, printouts for calibration and controls in appropriate electronic file/file cabinet

G. Instrument and Quality Control Corrective Actions

The following actions are not intended to cover all possible scenarios, but are provided to address some infrequently experienced occurrences. Unforeseen future issues will be addressed as they arise.

(1) **Issue:** Calibration failure due to any of the following; any individual calibrator value exceeding ± 5%; linear regression analysis where R^2<0.99; or ethanol not present in any of the calibration chromatograms. **Correction:** Re-pipette the calibrators and/or entire batch if applicable.

(2) **Issue:** Negative control contains ethanol or any other volatile, properly identified per reporting guidelines. **Correction:** Prepare a valid negative control and re-pipette and analyze entire batch.

(3) **Issue:** Not all components of the mixed volatile standard are properly identified on one or both of the GC columns. **Correction:** Update the retention times in the method and reprocess the entire batch, or update the retention times and re-start the entire batch.

(4) **Issue:** A quality control sample, either blood or aqueous control has exceeded ± 5%. **Correction:** Re-pipette and analyze any case samples bracketed by the out of tolerance control.

(5) **Issue:** An individual case sample has duplicates which exceed the ± 5% or ± 0.005 grams per 100mL requirement. **Correction:** Re-pipette and analyze the individual case sample.

(6) **Issue:** Communication or other error during the batch. **Correction:** Visually evaluate and document what caused the failure if possible, press the stop button on the autosampler display if necessary. When the vials have been unloaded from the oven and the autosampler is in standby, restart the batch from the computer with the vial # the batch had stopped on. Samples which were thermostatted in the oven for an extended period of time (i.e. overnight) will be re-pipetted and analyzed.

(7) **Issue:** If on an individual result or multiple samples in a batch any of the following are observed: ethanol and/or internal standard retention time shift, or area counts are abnormally high or low (i.e. exceeds by 50% or more the average area count observed throughout the batch). **Correction:** Visually inspect the vials associated with the affected chromatogram for abnormalities, e.g. loose vial cap, etc. Re-pipette and analyze the affected case samples.

H. Conclusions

(1) Any average result between 0.025 and 0.400 grams per 100mL report the result truncated to the third digit and the measurement uncertainty associated with the result.

(2) If either result is less than 0.005 grams per 100mL it will be reported as “no ethyl alcohol detected.”

(3) Any average result between 0.005 and 0.025 grams per 100mL will be reported as “ethyl alcohol detected <0.025 grams per 100mL”.

(4) Any average result greater than 0.400 grams per 100mL will be reported as “ethyl alcohol detected > 0.400 grams per 100mL”.

(5) For liquor cases report results qualitatively but convert w/v to v/v, (v/v) = (w/v) / 0.789 x dilution, record amount on worksheet.

(6) Qualitatively report volatile compounds other than ethanol and acetaldehyde, only if identified on all four chromatograms, the retention time matches the mixed volatile

standard within ± 1%, and the concentration is ≥0.005 g/100mL. Do not report toluene in samples from Serum Separation tubes (SST's) since they contain toluene.