Idaho State Police
Forensic Services
Toxicology Section

Section Four
Blood Volatiles Determination – Coeur D'Alene Laboratory

4.1 Quantitative Analysis for Ethanol and Qualitative Analysis for Other Volatiles by Dual Column Headspace Gas Chromatography

4.1.1 BACKGROUND

Fermented beverages such as beer and wine have been known and used by humans since prehistoric times.[6] Ethanol abuse is often manifest in driving under the influence (DUI) problems, which is a worldwide concern. The National Highway Traffic Safety Administration (NHTSA) estimates that alcohol was involved in 41% of fatal automobile crashes and 7% of all crashes in 1995.[2] Chronic alcoholism also contributes to ethanol related deaths. Ethanol consumed on a regular basis can lead to the development of alcoholic hepatitis which can progress into cirrhosis, liver failure, and death.[2,6,7] Chronic excessive ingestion of ethanol is directly associated with serious neurologic and mental disorders such as brain damage, memory loss, sleep disturbances and psychoses.[7] Alcohol is also involved in a high percentage of domestic disputes many of which result in injury and/or death.

Notwithstanding the public perception that ethanol is stimulatory, ethanol is classified as a *Central Nervous System Depressant.* Ethanol is a psychoactive drug that is similar in most respects to sedative-hypnotic compounds.[4] The first mental processes to be affected are those that depend on training and previous experience.[7] The individual's memory, concentration, and insight are dulled and subsequently lost. The person may become overly confident and exhibit uncontrolled mood swings and/or emotional outbursts.[7] The effects of ethanol and other central nervous system depressants is additive, resulting in more sedation and greater impairment of driving ability.[4]

Ethanol is rapidly and completely absorbed from the stomach, small intestine and colon. The mechanism of absorption is a simple diffusion process, that is, alcohol moving from a region of higher to a region of lower concentration.[2,4,6] Alcohol is soluble in both water and fat, a property that facilitates its diffusion through biological membranes.[4] The major amount of absorption takes place in the small intestine due to its large surface area, good blood supply and thin walled membrane. The time from the last drink to peak concentrations can range between 30 and 90 minutes, depending upon the individual's stomach contents.[4,7] Alcohol absorption is slowed by the presence of food in the stomach. The time period required for gastric emptying is a prime factor that contributes to the wide variety of absorption rates of ingested ethanol observed in different individuals and under different conditions.[2,7] Hence, the extent of absorption in the stomach and small intestine is a function of the

amount of ethanol at that site, the vascularity of the site and the surface area in contract with the blood supply.[2] Other factors that affect the absorption of ethanol include the type of beverage, the alcohol content and any disease state that affects normal gastric function.[2]

Upon absorption, ethanol is distributed to all the water containing regions of the body. Within the blood there can be significant differences between arterial and venous blood depending upon the absorption status of the individual.[2] In the absorptive phase, the arterial blood ethanol concentration exceeds the venous blood ethanol concentration. Analysis of venous blood therefore, underestimates the brain alcohol concentration of the individual at this point. When absorption is complete there is little difference in ethanol concentration between and arterial and venous blood.[2]

90 to 98 percent of ethanol is completely oxidized in the liver by reacting with the cofactor nicotinamide adenine dinucleotide (NAD) facilitated by alcohol dehydrogenase to produce acetaldehyde. Acetaldehyde is then acted upon by aldehyde dehydrogenase to form acetic acid. The amount of ethanol oxidized per unit time is roughly proportional to body weight and probably to liver weight. The remaining (unoxidized) alcohol is excreted unchanged in urine, expired air, saliva and sweat. The average elimination rate of ethanol is 0.015 g/dL/hour from men and 0.018 g/dL/hour for women.[2] In addition to gender, chronic abuse, ethanol use combined with prescription drugs and certain genetic factors can also influence the elimination rate.[2,6,7]

Methanol (wood alcohol) causes relatively little intoxication compared to ethanol.[2,6] Its harmful affects are due to the direct result of its metabolism to formaldehyde (embalming fluid) and subsequently to formic acid. These metabolites lead to the destruction of neural cells, particularly the optic nerve, which can result in blindness.[2,6]

4.1.2 **PRINCIPLE**

This method describes the analysis of aqueous samples for the presence of volatile compounds including methanol, ethanol, acetaldehyde, acetone, isopropanol and related compounds, via a headspace sampling gas chromatographic method. Samples, controls and standards are sealed into vials that contain an aqueous acetonitrile internal standard solution and heated by the headspace analyzer. As described in Henry's Law, in a closed container at a given temperature, a direct (proportional) relationship exists between the amount of a volatile substance dissolved in a liquid and the amount of the volatile substance in the headspace vapor above the solution. An aliquot of the vapor is injected into a gas chromatograph (GC) in a dual column configuration. The GC serves to separate out the components of the solution as a function of their chemical properties. The separated components are identified on the basis of the retention time determined for each of the columns. Quantitation is accomplished through area percent data obtained from a flame ionization

detector (FID). The quantitative result is based on a minimum of a three-point calibration curve, which uses the peak area ratio between the analyte and the internal standard.

4.1.**3** **SPECIMEN**

4.1.**3.1** The method can be applied to any aqueous samples or aqueous-homogenized material. This includes blood, bile, vitreous humor, gastric contents and tissue homogenates (1:4).

4.1.**3.2** Blood samples should be submitted in gray-top vacutainer tubes that contain 10% sodium fluoride (*IDAPA 11.03.01.012.02.b).* Blood samples submitted in non-compliant tubes will be analyzed; however, a qualifier will be placed on the analysis report indicating that the proper sodium fluoride requirement was not met.

4.1.**3.3** Urine samples should be submitted in Forensic Services issued containers however, any suitable urine specimen container is acceptable. A suitable container is both clean and leakproof (IDAPA **11.03.01**.*012.01.g*).

4.1.**3.4** Samples submitted via common carriers should include a containment system (i.e., biohazard bag with an absorbent sheet) in the advent of leakage.

4.1.**3.5** All other types of samples should be sealed and stored in appropriate airtight polypropylene containers. Tissues should be frozen until homogenization and analysis.

4.1.4 EQUIPMENT AND SUPPLIES

4.1.**4.1** Tube Rocker (Fisher Scientific or equivalent)

4.1.**4.2** Hewlett-Packard (HP) 5890 II Gas Chromatograph (GC)

4.1.**4.3** Columns

- 4.1.**4.3.1** HP INNOWAX (#19096N-123), 30 meter x 0.53 mm inner diameter, (ID), 1 µm film thickness (FT), or equivalent
- 4.1.**4.3.2** J&W DB 624 (#125-1334), 30 meter x 0.53 mm ID, 3 µm film thickness (FT), or equivalent

4.1.**4.4** Hamilton MICROLAB® 500A Series Auto Dilutor

4.1.**4.5** HP 7694 Headspace Sampler

4.1.**4.6** HP Chemstation Software, Revision A.06.03 [509]

4.1.**4.7** Sample Containers

4.1.**4.7.1** 10mL GC-Headspace vials (HP #5182-0838 or equivalent)
4.1.**4.7.2** Crimp caps (HP #9301-0721 or equivalent)
4.1.**4.7.3** Septa (HP #9301-0976 or equivalent)
4.1.**4.7.4** Hand Crimper (HP #9301-0720 or equivalent)

4.1.5 CONTROLS AND CALIBRATORS

4.1.**5.1** Whole Blood Alcohol Control (ToxiChem #2930-14 or equivalent)

4.1.**5.2** Aqueous Ethanol Standards (College of American Pathologists or equivalent)

College of American Pathologists (CAP) Aqueous Ethanol Controls	
Conc. (mg/dL)	Catalog #
0.040	ST011
0.100	ST017
0.200	ST018
0.300	ST019

4.1.6 REAGENTS

4.1.**6.1** Acetonitrile (Sigma # A-3396, Fisher Scientific #A996-1 or equivalent)
4.1.**6.2** Acetone (Fisher #A929-1 or equivalent)
4.1.**6.3** Acetaldehyde (Fisher #01004-250 or equivalent)
4.1.**6.4** Isopropanol (2-Propanol) (Fisher #A416-500 or equivalent)
4.1.**6.5** Methanol (Fisher #A454-1 or equivalent)
4.1.**6.6** Propanol (Fisher #A414-500 or equivalent)
4.1.**6.7** Mercuric Chloride (Fisher #M1551-50 or equivalent)

4.1.7 SAFETY CONCERNS

4.1.**7.1** Acetonitrile (methyl cyanide) is extremely poisonous. Refer to MSDS for further information.

4.1.**7.2** Blood samples should be processed according to toxicology safety guidelines (refer to toxicology training manual section 3.)

4.1.8 REAGENT PREPARATION

4.1.**8.1** Internal Standard Solutions

4.1.**8.1.1** 3% v\v Acetonitrile Stock

- Add 30mL acetonitrile.
- Add a pinch of mercuric chloride.

- QS to 1000 mL with DI water.

4.1.**8.1.2** 0.012% v\v Acetonitrile Working Solution
- Add 5mL of stock solution.
- QS to 1000 mL with DI water.

4.1.**8.2** Volatile Standard Mix Solution

4.1.**8.2.1** Add approximately 200 mL of DI water to a 1000mL volumetric flask.

4.1.**8.2.2** Add the following as indicated:
- 250µL acetaldehyde (0.02%w/v, 0.025%v/v)
- 250µL acetone (0.02%w/v, 0.025%v/v)
- 1000µL methanol (0.08%w/v, 0.10%v/v)
- 250µL isopropanol (0.02%w/v, 0.025%v/v)
- Pinch of mercuric chloride

4.1.**8.2.3** QS to 1000 mL with DI water.

4.1.9 DILUTOR PREPARATION

4.1.**9.1** Verify that there is sufficient internal standard (ISTD) solution to complete the number of specimens in run.

4.1.**9.2** Prime dilutor with ISTD. Bubble can be removed by first flushing the dilutor with acetone.

4.1.**9.3** Set syringe volumes

Reagent syringe: 2000µL

Sample syringe

Blood: 250µL

Urine: 250µL

4.1.10 ANALYSIS PROCEDURE

4.1.**10.1** Initial Processing of Specimens

4.1.**10.1.1** Open the sample submittal kit and remove the specimen's inner compartment. After inspecting and noting the condition of seals, open inner compartment (plastic tray or biohazard bag) and write laboratory number

on each blood/urine/vitreous humor specimen.

4.1.**10.1.2** When two blood/fluid samples are present, the samples should be labeled "1" and "2". Utilize sample "1" for analysis unless it contains insufficient sample.

4.1.**10.1.3** Place labeled specimen container on rocker for a minimum of 15 minutes to ensure thorough mixing.

4.1.**10.2** Preparation of Samples for Analysis

4.1.**10.2.1** Label two test vials with the laboratory number without the prefix.

4.1.**10.2.2** Add 2000 uL of internal standard (ISTD) to each vial.

4.1.**10.2.3** Addition of Sample in duplicate to vials.

4.1.**10.2.4** Blood or Vitreous Humor: Transfer 250µL into each of the vials.

4.1.**10.2.5** Urine: Transfer 250 uL into each of the vials.

4.1.**10.2.6** Seal test vials *immediately* with crimp caps.

4.1.**10.2.7** Between each sample, aspirate water 3x and dispense into waste rinse tubing. It is not necessary to rinse between duplicates.

4.1.**10.3** Preparation of Blank, Blood Control and Mixed Standard

4.1.**10.3.1** Blank

4.1.**10.3.1.1** Label a test vial with *blank.*

4.1.**10.3.1.2** Add 2000µL of DI water. Seal with crimp cap.

4.1.**10.3.2** Blood Control

4.1.**10.3.2.1** Label two test vials with *blood control.*

4.1.**10.3.2.2** Add 2000µL of internal standard (ISTD) to each vial.

4.1.**10.3.2.3** Transfer 250µL blood into each of the vials. Seal **immediately** with crimp caps.

4.1.**10.3.3** Mixed Other Volatiles Solution

4.1.**10.3.3.1** Label a test vial with *mixed volatiles.*

4.1.**10.3.3.2** Add 2000µL of internal standard (ISTD) to vial.

4.1.**10.3.3.3** Pipette 250µL mixed standard into each of the vials. Seal **immediately** with crimp caps.

4.1.10.4 Preparation of Standards

4.1.**10.4.1** Label vials for *low, 0.08* and *high* standards.

4.1.**10.4.2** Add 2000µL of internal standard (ISTD) to each vial.

4.1.**10.4.3** Pipette 250µL of appropriate ethanol concentration into each of the vials. Seal **immediately** with crimp caps.

4.1.**10.4.4** Establish ethanol calibration plot with a minimum of four calibration points. A 0.04, 0.10, 0.20 and 0.30g/100 mL. Additional points should be established as warranted.

4.1.**10.5** Preparation for Run

4.1.**9.5.1** Place prepared vials into headspace sampler carousel in the following order:

① Aqueous standards (.04, .10, .20, .30)
② Mixed standard.
③ Water blank
④ Blood control in duplicate.
⑤ Case samples in duplicate
⑥ Check standards (.04, .10, .20, .30)

4.1.**9.5.2** Open *Sequence* pull-down of HP ChemStation software.

4.1.**9.5.3** Into Sequence log table, enter the sample case numbers, ethanol standards, other volatiles mix, blank and controls.

4.1.**9.5.4** Active headspace sampler and run sequence.

4.1.**9.6** Headspace Analyzer and Gas Chromatography Parameters

4.1.**9.6.1** Refer to ***Method*** print-out for carrier pressure, vial pressure, oven temperature program, injector and interface temperatures.

4.2.**9.7** Run Acceptance Criteria

4.2.**9.7.1** Retention Time Criteria

4.2.**9.7.1.1** The presence of ethanol can be established if any difference between the retention time of a sample and

the mean retention time of standards, is within ±10%.

4.2.**9.7.2** Quantitation Criteria

The quantitative amount of ethanol can be reported if the following criteria are met:

4.2.**9.7.2.1** The results from duplicates must be within 0.01 of each another.

- If this precision requirement is not met, the sample is reanalyzed.

4.2.**9.7.2.2** Results obtained from an aqueous alcohol standard must be within ±10% of target or 0.01 whichever is greater.

4.2.**9.7.2.3** Human blood alcohol controls must be within ±10% of the manufacturer's target value, as determined by referee laboratories. Refer package insert for values.

4.2.**9.8** Reporting of Results

4.2.**9.8.1** Calibration

The multi-level calibration curve is established through the comparison of the ratios of the calibrator ethanol peak areas to their respective internal standard peak areas. Based upon this curve, the ratios of sample ethanol peak area to internal standard peak area will yield a quantitative determination of the percent alcohol contained in the specimen.

4.2.**9.8.2** Blood Results

Blood results are reported as grams of ethanol per 100 cc of whole blood *(IDAPA 11.03.01.012.03.)*.

4.2.**9.8.3** Urine Results

Urine results are reported as grams of ethanol per 67 mL of urine *(IDAPA 11.03.01.012.04.)*.

- Results of alcohol analysis of urine specimens shall be accompanied by the following warning statement about the questionable value of urine alcohol results.

- *"Urine alcohol results may be of questionable value."*

4.2.**9.8.4** Vitrous Humor Results
Blood results are reported as grams of ethanol per 100 cc of blood.

4.2.**9.8.5** Other Volatile Substances
Since this method only allows for the qualitative identification of volatile compounds such as toluene and methanol, only the presence of these compounds is noted on the report.

4.2.10 QUALITY ASSURANCE

4.2.**10.1** Specimen Integrity

4.2.**10.1.1** Blood and Vitreous Specimens
Blood or vitreous samples are to be stored under refrigeration while at the laboratory.

4.2.**10.1.2** Urine Specimens
Urine samples are frozen until preparation of analysis. Samples being processed will be stored refrigerated until after alloquot(s) are removed. As soon as practical, the sample should be frozen in long-term storage freezers.

4.2.**10.2** Validity of Results Safeguard

4.2.**10.2.1** Analyses are conducted in duplicate.

4.2.10.3 Proficiency Testing Requirements

4.2.**10.3.1** As required under IDAPA 11, Title 03, Chapter 1, Forensic Services participates in approved alcohol proficiency testing on a continuous basis.

4.2.**10.3.2** U.S. Department of Transportation, National Highway Traffic Safety Administration (NHTSA), administers blood alcohol proficiency testing.

4.2.**10.4** Monitoring of Human Blood Controls

4.2.**10.4.1** The results obtained from analysis of the human blood alcohol control is tracked utilizing the EXCEL spreadsheet program. Data plots should be maintained in the quality control binder for alcohol determinations along with the package insert from the human alcohol controls.

4.2.**10.5** Peer Review

4.2.**10.5.1** Case files are peer reviewed prior to release.

4.2.11 REFERENCES

1. Stafford, D.T., *Chromatography. in:* Principles of Forensic Toxicology, edited by Barry Levin, pp. 93-101, 103-114, AACC Press, 1999.

2. Levine, B., *Alcohol. in:* Principles of Forensic Toxicology, edited by Barry Levin, pp. 170-184, AACC Press, 1999.

3. Caplan, Y.H., *The Determination of Alcohol in Blood and Breath. in:* Forensic Science Handbook, edited by Richard Saferstein, pp. 594-648, Prentice-Hall New Jersey, 1982.

4. Julien,R.M., *Central Nervous System Depressants: Alcohol and the Inhalants of Abuse, in:* Primer of Drug Action, pp. 64-92, Freeman-New York, 1998.

5. Saker, E.G. Screening and Quantitation by Head Space Technique of Some of the Vapors Most Commonly Found in Forensic Toxicology, in: Current Approaches in Forensic Toxicology, Chapter 11, SOFT Meeting, 1994.

6. Perrine, D.M., *Depressants: Alcohol, Benzodiazepines, Barbiturates, in:* The Chemistry of Mind-Altering Drugs, pp. 113-129, ACS, Washington, DC, 1996.

7. Hobbs, W.R., Rall, T.W. and Verdoorn, T.A., *Drugs Acting on the Central Nervous System - Hypnotics and Sedatives; Ethanol, in:* Goodman and Gilman's The Pharmacological Basis of Therapeutics, pp. 361, 386-393, McGraw-Hill, 1996.

8. Hamilton County Coroner's Office Toxicology SOP Manual

9. Minnesota Department of Public Safety, Ethanol in Specimens by Headspace Gas Chromatography SOP.