

# The Application of Ethyl Glucuronide and Ethyl Sulphate in a Forensic Setting

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# Overview

- Alcohol biomarkers
- Application in forensic settings
  - Post mortem
  - Investigation of DFSA
- Limitations of EtG & EtS
  - False positives
  - Synthesis & degradation
- Alternative Matrices
  - Serum, vitreous, oral fluid, hair

# Ethanol Analysis

- The detection period is very short
  - BAC reduces by 10-25mg% per hour
  - A BAC of 80mg% can be 0 within a few hours
- \*\*Low sensitivity for recent drinking!\*\***
- Patients in detox could drink at times when testing was unlikely, due to the rapid excretion of alcohol

# Alcohol Biomarkers

“Alcohol biomarkers are physiological indicators of alcohol exposure or ingestion and may reflect the presence of an alcohol use disorder”

*Substance Abuse Treatment Advisory. Sept 2006, Vol 5, Issue 4.*

# Alcohol Biomarkers

## 2 Types:

- **Indirect** - Detect toxic effect of heavy alcohol use on organ systems & body chemistry

GGT, AST, ASL, MCV, CDT, 5HTOL

- **Direct** - Measure alcohol exposure or use  
(*Analytes of alcohol metabolism*)

PEth, FAEEs, EtG, EtS

# Use of Biomarkers

## **Clinical Settings:**

- Screening for alcohol problems
- Documenting abstinence
- Identifying relapse to drinking
- Motivating change in drinking behavior
- Evaluating interventions for alcohol problems
- Conditional liver transplantation

## **Forensic Settings:**

- Differentiation of anti-mortem consumption and post-mortem production of ethanol
- Establishing alcohol use after clearance
- Child custody cases
- Driving offences/Reinstating of driving licenses
- Conditional probation - threat of return to jail
- Loss of employment

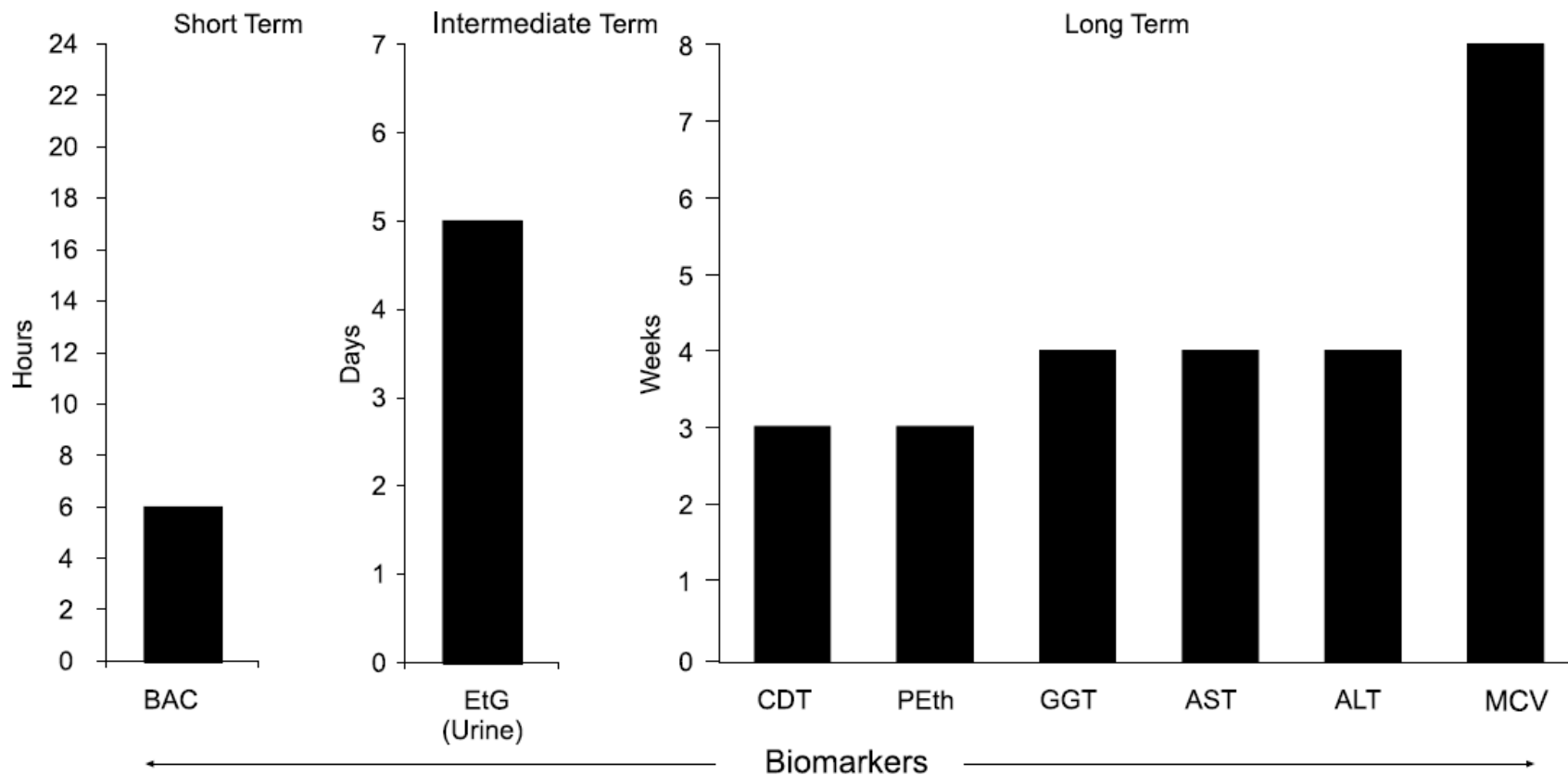
# Current EtOH Biomarkers

Marker	Abbreviation	Type of drinking	False positives
Ethanol	EtOH	Under the influence	Foods
Ethyl Glucuronide Ethyl Sulfate	EtG & EtS	Recent drinking	Hygiene products, cosmetics, foods
5-Hydroxytryptophol	5HTOL	Recent drinking	Further investigation required
Carbohydrate-Deficient Transferrin	CDT	Riskful drinking	Iron deficiency, hormonal status in women, carbohydrate-deficient glycoprotein syndrome, fulminant hepatitis C and severe alcohol disease
Phosphatidyl Ethanol	PEth	Riskful drinking	None likely but still unknown due to paucity of research
Gamma Glutamyl Transferase	GGT	Chronic abuse/organ damage	Liver and biliary disease, smoking, obesity, and medications inducing microsomal enzymes
Aspartate & Alanine Amino Transferase	AST & ALT	Chronic abuse/organ damage	See GGT Excessive coffee consumption can lower values
Mean Corpuscular Volume	MCV	Chronic abuse/organ damage	Liver disease, haemolysis, Bleeding disorders, anaemia, folate deficiency, and medications reducing folate

Due to their relative strengths and weaknesses biomarkers are often used in combination, i.e. GGT & CDT

# Biomarker Detection Windows

**Exhibit 2: Windows of Assessment for Various Alcohol Biomarkers**



BAC=Blood alcohol concentration

*Substance Abuse Treatment Advisory. Sept 2006, Vol 5, Issue 4.*

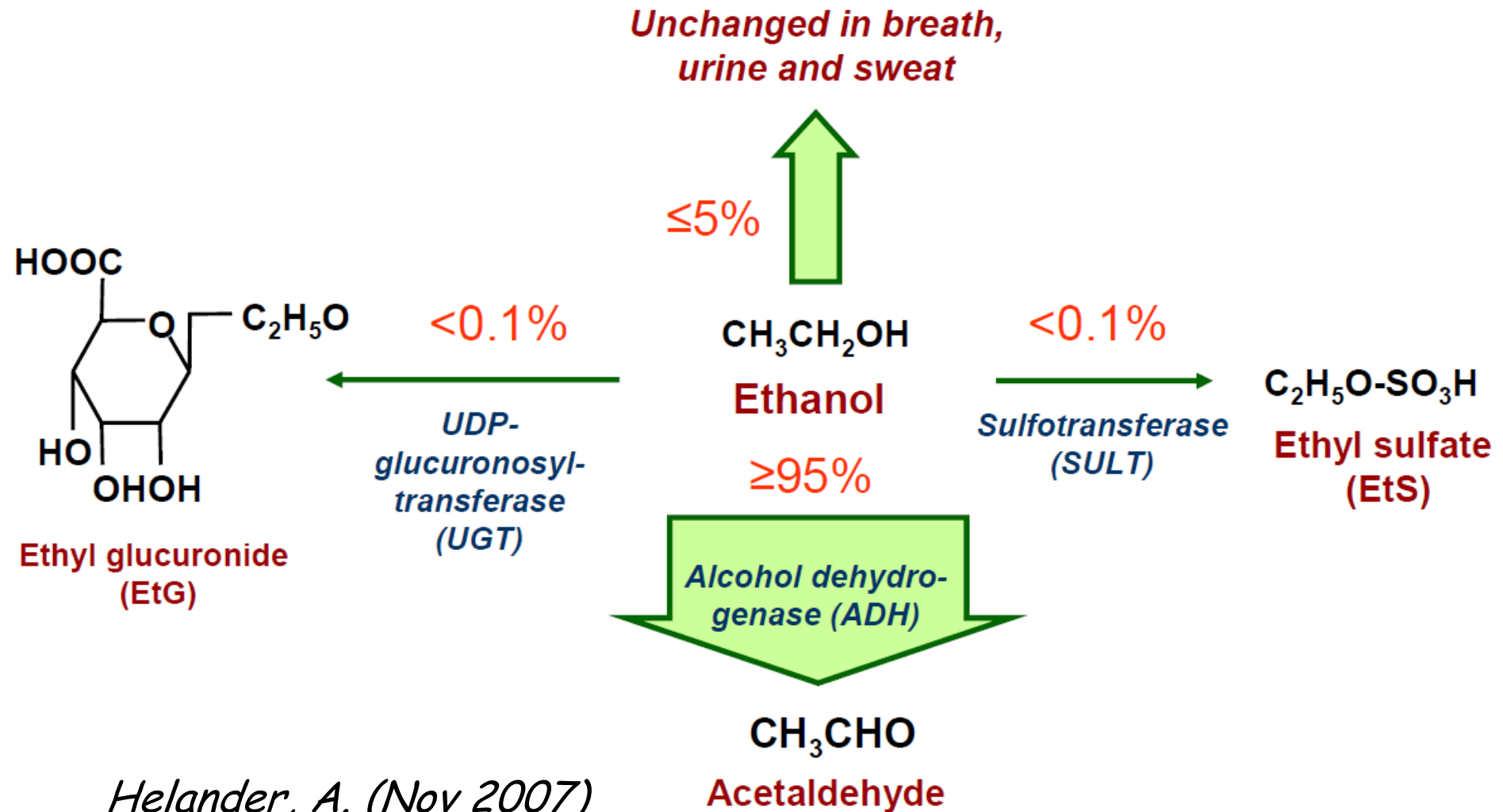


# EtG & EtS

- Direct
- Non-volatile
- Water soluble
- Present only if ethanol is consumed
- Not dependant on chronic alcohol consumption
- Less likely than traditional biomarkers to be influenced by:
  - Age
  - Gender
  - Medication
  - Non-alcohol related diseases
- Do not accumulate during chronic alcohol intake

Their specificity and sensitivity exceed those of all other known ethanol markers

# Ethanol Metabolism



Helander, A. (Nov 2007)

# Post-mortem Cases

- PM production of EtOH is a well known and documented phenomenon
- Caused by yeast/bacterial fermentation of sugars
- Typically low (<50mg%)
- May exceed 150mg% if the conditions for production are optimal:
  - Prolonged delay between death and sampling
  - Humidity and warm temperatures
  - Location of the body
  - Trauma
  - Diabetics
  - Urinary tract infections

# Post-mortem Cases

- Inhibited by correct sample storage and preservation (>1% fluoride)
- **BUT:**
  - Significant concentrations of EtOH may already have been formed prior to sampling
- Comparison of BL, UR and VH EtOH concentrations can help to identify fermentation
- Generally, fermentation is assumed if UR and VH negative
- **BUT:**
  - Ur only available in ~50% cases
  - Coroners reluctant to collect VH

Int J Legal Med  
DOI 10.1007/s00414-008-0245-3

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CASE REPORT

# **Was a child poisoned by ethanol? Discrimination between ante-mortem consumption and post-mortem formation**

**Brice M. R. Appenzeller • Marc Schuman •  
Robert Wennig**

Received: 22 November 2007 / Accepted: 18 April 2008  
© Springer-Verlag 2008

# Case Study 1

- 91 year old female
- Suffered with:
  - Parkinson's disease & limited mobility
  - Depression
  - Previous suicide/self harm attempts
- Facing forced eviction and relocation to unsatisfactory accommodation
- Found suspended from the hanging rail of wardrobe by her dressing gown cord

# Case Study 2

- A normally fit and well 45 year old male
- Found dead face down in bed, gripping his pillow
- A small amount of blood was coming from his mouth
- The cause of death was found to be aspiration but the reason for this occurrence was unknown
- The Coroner recorded an open verdict

# Case Study 3

- 61 year old male
- Found dead on his back, next to his bed
- Wound to the back of his head
- Vomit was found in the toilet
- Neighbours not seen him for ~10 days
- Police notified due to build up of post
- The TV was still on
- TV listing magazine open at a date 9 days previous to his discovery



# Case Study Samples

- Case 1 & 2
  - Unpreserved femoral blood
  - Unpreserved urine
- Case 3
  - Fluoride preserved femoral blood
  - Fluoride preserved urine

# Analytical Approach

- **Ethanol Analysis:**

- Head space GC-FID (dual column) on a Shimadzu GC 2014 coupled to a HTA, HT200H headspace auto sampler



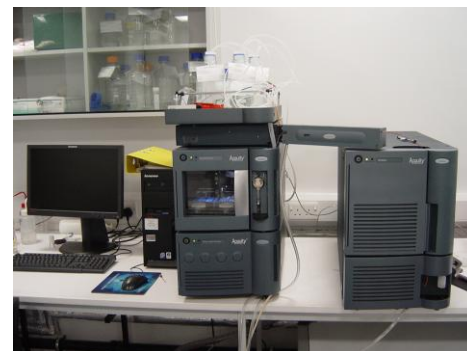
- **EtG Screening:**

- Microgenics DRI<sup>®</sup> EtG Enzyme Immunoassay on the Olympus AU400 platform



- **EtG & EtS Confirmation:**

- Waters<sup>®</sup> ACQUITY UPLC<sup>®</sup> System coupled to a Waters ACQUITY<sup>®</sup> TQD



# Microgenics EtG Assay

- **Reagent Type**
  - DRI® Ethyl Glucuronide Assay (EtG-mAb)
- **Qualitative**
  - 500ng/mL or 1000ng/mL Cut off
- **Semi-Quantitative**
  - 0, 100 (LLOQ), 500, 1000, 2000 (ULOQ) ng/mL
- **Nominal QC Values**
  - 375, 625, 750, 1250ng/mL

**\*\*No marked x-reactivity with other urinary glucuronides\*\***

# EtG & EtS Confirmation

## Sample Preparation:

- **Urine:** 1:20 diln after centrifugation
- **Blood:** LLE (dcm/diethyl ether/hexane mix)

## LC Conditions:

- **Column:** Waters® Acquity UPLC HSS C18 (2.1 x150mm, 1.8µm)
- **Column Temp:** 50°C
- **Flow Rate:** 400µL/min
- **MP:** A: dH2O + 0.05% FA B: ACN **Gradient:** 1-100% B (2.5min)
- **Injection Vol:** 10µL

## MS Conditions:

- **MS:** Waters® TQ Detector
- **Ionisation Mode:** ESI Negative
- **Acquisition Mode:** MRM
- **Run Time:** 4 mins

Compound	Precursor ion (m/z)	Product ion (m/z)
EtG	<b>221*</b>	<b>85</b>
	221	75
EtS	<b>125</b>	<b>97</b>
	125	125
EtG-D5	<b>226</b>	<b>85</b>
EtS-D5	<b>130</b>	<b>98</b>

Table 1. MRM conditions used for EtG, EtS and internal standards \***Bold** transitions used as the quantifier ion

# Case Study Results

Case Report	Ethanol (mg%)		EtG (ng/mL)		EtS (ng/mL)
	Blood	Urine	DRI-EA	UPLC/MS/MS*	UPLC/MS/MS*
1	99	ND	ND	ND	ND
2	157	ND	ND	ND	ND
3	103	13	ND	ND	ND

ND = None detected \* = Blood and urine

**\*\*Blood EtOH likely to have resulted from bacterial fermentation\*\***

# Case Study 4

- 27 year old male died suddenly
- Poorly controlled IDDM

## Toxicology:

- STA negative
- **Blood:** Ethanol 491 mg/dL
- **Urine:** Ethanol not detected
- **CSF:** Ethanol not detected
- **Vitreous Humour:** Ethanol insufficient sample
  - Beta-hydroxybutyrate >5000 umol/L
  - Glucose 85.4 mmol/L
  - Urea 26.1 mmol/L
  - Creatinine 366 umol/L

### EtG

DRI® Assay: >2000ng/mL

UPLC/MS/MS: None detected

### EtS

UPLC/MS/MS: None detected

Origin of false positive ???

BHB, glucose, urea, creatinine

Cause of death: Diabetic ketoacidosis

## *SHORT REPORT*

# **Misleading results of ethanol analysis in urine specimens from rape victims suffering from diabetes**

A. W. Jones,<sup>1</sup> A. Eklund,<sup>1</sup> A. Helander<sup>2</sup>

<sup>1</sup>*Department of Forensic Chemistry, University Hospital, Linköping, Sweden*

<sup>2</sup>*Department of Clinical Neuroscience, Karolinska Institutet & Hospital, Stockholm, Sweden*

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**SUMMARY.** We report appreciably high concentrations of ethanol (82 and 102 mg/dL) in specimens of urine collected from two victims of date rape. Both girls (aged 15 and 18 years) suffered from diabetes mellitus, but adamantly denied drinking any alcohol before or after the incident. The presence of glycosuria and high risk of fungal infections in female diabetics suggests that ethanol was produced in vitro by fermentation after voiding. Making a routine test for sugar in the urine and ensuring that the sampling tubes contain sufficient sodium or potassium fluoride to inhibit glycolysis are recommended practices. A specific marker for post-sampling synthesis of ethanol might also be used such as the 5HTOL/5HIAA ratio. © APS/Harcourt Publishers Ltd 2000

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# Drug Facilitated Sexual Assault

- Late presentation of victims → Loss of evidence
- Many of the drugs implicated in sexual crimes have a narrow detection window: alcohol is no exception!
- 39% (n=391) presented within 12hr post incident (Scott-Ham & Burton. *J Clin Forensic Med* (2005/06))
- Many cases hinge on consent
- An individual is not legally capable of providing consent when incapacitated with alcohol or drugs
- Alcohol, not drugs, appears to pose the biggest "date rape" risk



# Ethanol & DFSA

• EtG & EtS could be used to establish alcohol consumption even after the complete elimination of alcohol

Ethanol (mg%)	EtG (mg/L) Immunoassay	EtG (mg/L) UPLC/MS/MS	EtS (mg/L) UPLC/MS/MS	Time post Incident (hrs)
174	171.7	184.4	42.8	4.5
126	1301.0	1751.7	294.0	8
<10	113.1	144.2	39.1	30
<10	Below cut-off	Below cut-off	0.2	18
<10	176.6	254.7	37.5	8.5
<10	0.5	1.0	0.9	38
<10	6.1	10.5	1.9	1
55	54.7	75.6	19.9	6.5
209	62.1	82.8	28.4	6.5
175	96.9	108.8	32.5	2

# Ethanol Interactions

## Enhanced sedative effect:

- Analgesics
- Anti-depressants
- Anti-histamines
- Anti-muscarinics
- Anti-psychotics
- Hypnotics
- Muscle relaxants

## Disulfiram-like reaction:

- Anti-bacterials
- Cytotoxics

flushing, throbbing in head and neck, throbbing headache, breathing difficulty, nausea, copious vomiting, sweating, thirst, chest pain, palpitations, tachycardia, hypotension, syncope, uneasiness, **weakness**, vertigo, **blurred vision** and **confusion**

# Limitations

- No correlation between EtG/EtS and BAC
- EtG/EtS concentrations are highly influenced by diuresis (*EtG-100 - standardised to creatinine of 100mg/100mL*)
- EtG/EtS does not differentiate between alcohol exposure and consumption at lower levels
- It is not known if other factors influence an individuals biomarker response to alcohol, e.g.
  - Genetics (Oriental)
  - Gender (↓ glucuronidation in females)
  - Age
  - Disease
  - Medication etc

# Limitations

## Degradation:

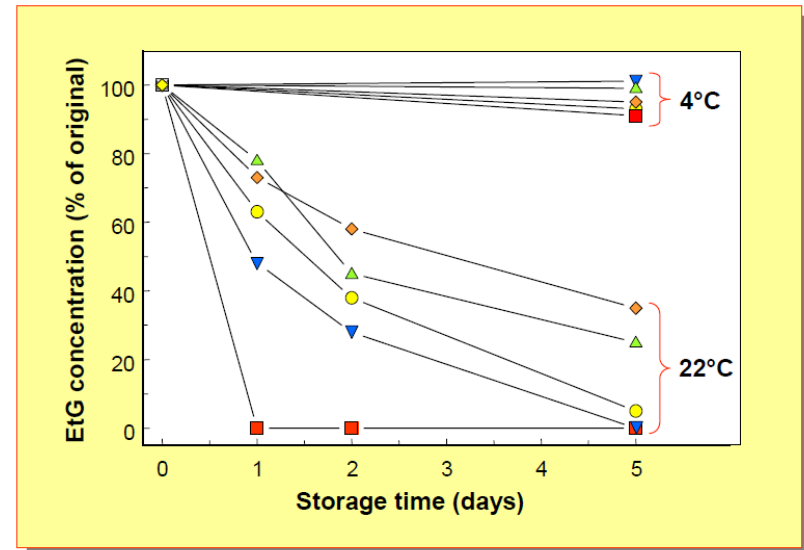
- Bacterial  $\beta$ -glucuronidases can breakdown of EtG
- $\beta$ -glucuronidase, but not sulfatase, activity is prominent in *E. coli*

- Case study:

- Urine Ethanol: 279mg%
- EtG: <100ng/mL

• Hydrolysis reduced by correct storage & preservation

• EtS can be degraded!  
(Halter, *et al*, 2009)



Helander A, Dahl H. *Clin Chem* 51:1728-1730, 2005.

No significant hydrolysis of EtS in random unpreserved UR samples stored at RT for 1 year (Rana & Ross, 2010)

# Limitations

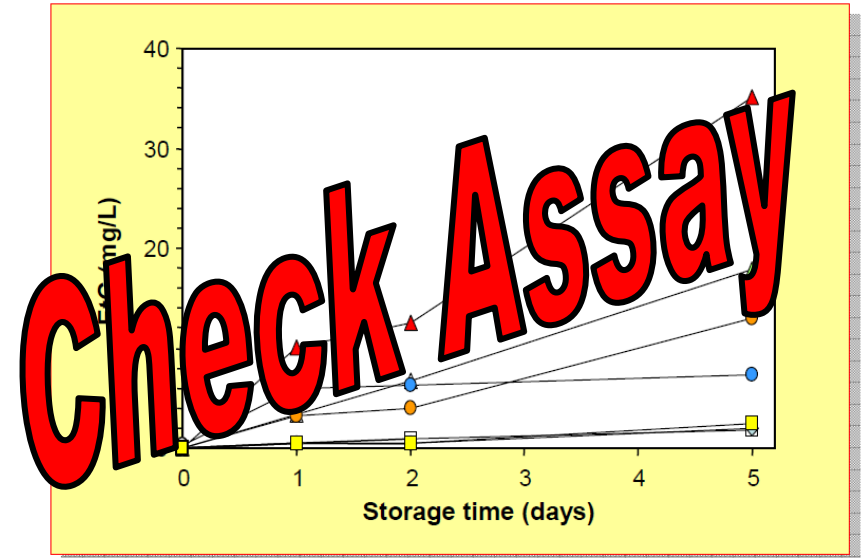
## Synthesis:

- EtG can be produced by *E. coli* if ethanol is present or produced *in-vitro*

- Production of EtG may not be prevented by optimising storage conditions

- No evidence to suggest EtS is subject to *in-vitro* synthesis

- EtS appears to be a more reliable marker than EtG



Helander A, Olsson I, Dahl H. *Clin Chem* 53:1855-1857, 2007.

EtS increased to 250% in one sample after a year (Rana & Ross, 2010)

# False Positives

- Health & hygiene products:

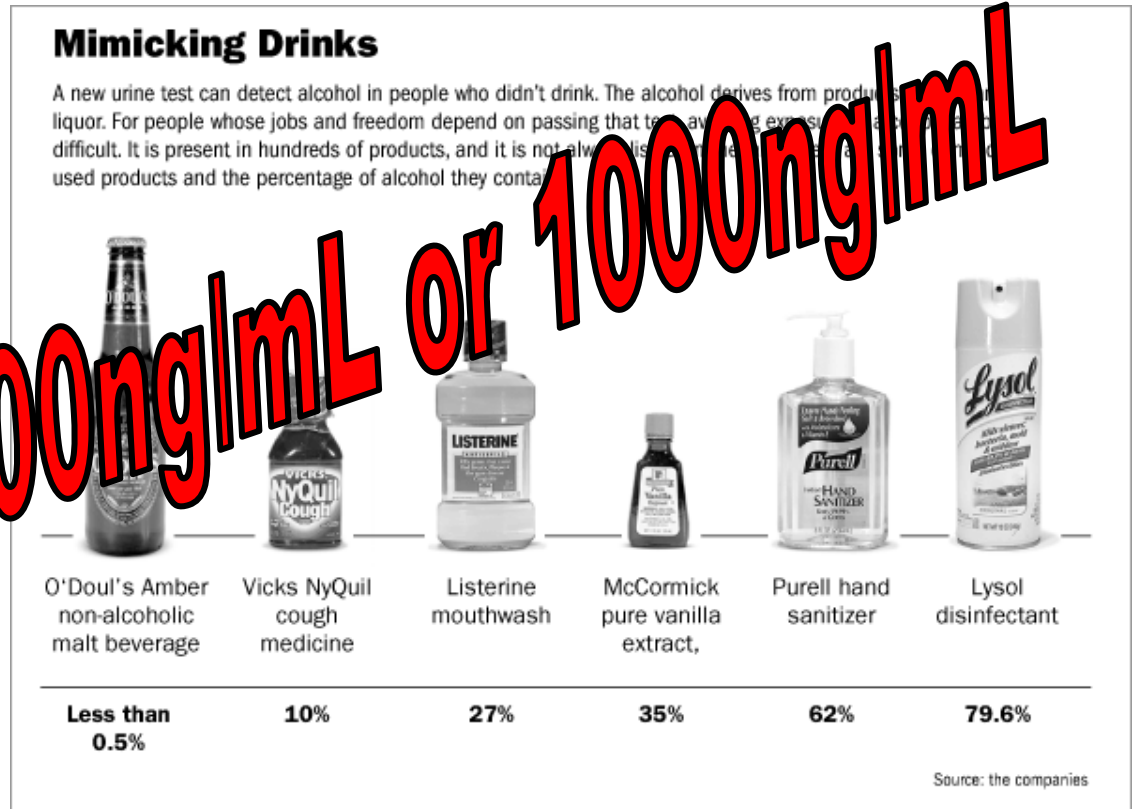
- Mouthwash
- Perfume
- Hand sanitisers
- Disinfectant
- Cold medicine

- Foods:

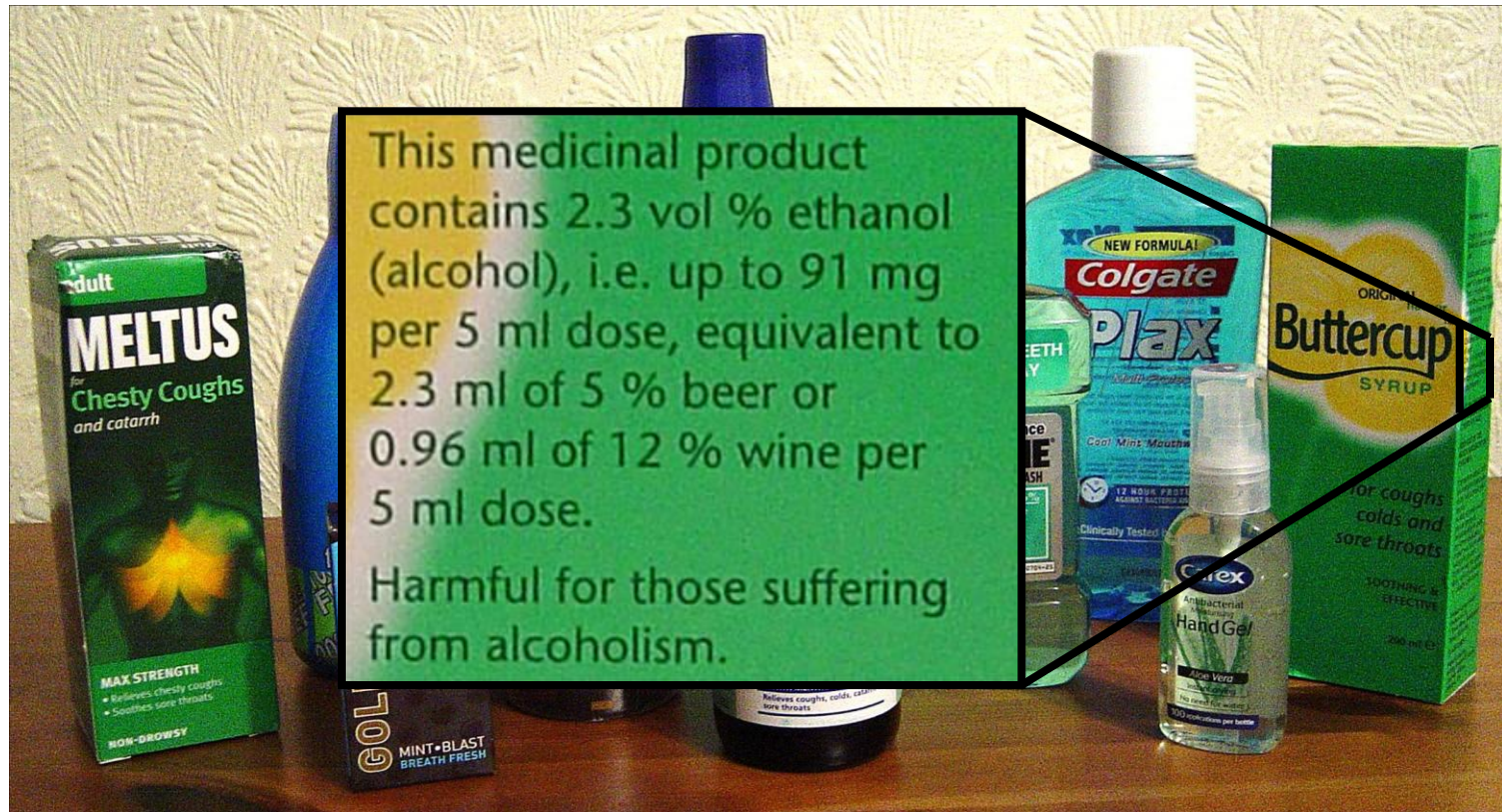
- Pastries
- Balsamic vinegar
- Ripe fruit
- Vanilla extract
- Ice cream
- Non-alcoholic beer

- Others

- Automotive fuel



# False Positives



# False Positives

Journal of Analytical Toxicology, Vol. 34, March 2010

## Levels of Ethyl Glucuronide and Ethyl Sulfate in Oral Fluid, Blood, and Urine After Use of Mouthwash and Ingestion of Nonalcoholic Wine

Gudrun Høiseith\*, Borghild Yttredal, Ritva Karinen, Hallvard Gjerde, and Asbjørg Christophersen

*Norwegian Institute of Public Health, Division of Forensic Toxicology and Drug Abuse, Oslo, Norway*

- Nonalcoholic wine containing 3mg/L EtG and 1.5mg/L EtS.
- All samples negative for EtG.
- Urine positive for EtS at concentrations up to 2.15mg/L.
- Bioavailability of EtS > EtG.



# Alternative Matrices

## Hair

- Hair testing for alcohol is a relatively recent and developing science.
- Alcohol cannot be detected directly in hair.
- EtG and fatty acid ethyl esters (FAAEs) can be.
- EtG is believed to be incorporated into the hair mainly through sweat.
- FAAEs are incorporated through the sebum glands.

## Disadvantages

- Inter individual differences in sweating, hair length etc.
- Susceptible to being 'washed out' (EtG).
- Loss through cosmetic treatment i.e. bleaching (FAEEs)
- Incorporation through alcohol containing hair products (EtG & FAEEs).
- Incorporation through atmospheric exposure to alcohol (FAEEs).

# Alternative Matrices

## Hair

**LB Richmond v B & W & B & CB [2010] EWHC 2903 (Fam)**

*Care proceedings seeking to establish whether a parent had consumed alcohol, and if so, to what extent.*

## Judgement

- 1) Hair tests should only be part of the evidential picture.
- 2) EtG and FAEs should be used.
- 3) >30pg/mg EtG in the proximal 3cm of hair is consistent with excessive consumption.
- 4) No cut offs have been agreed for 1cm segments of hair.
- 5) The tests are not designed to differentiate between abstinence and social drinking.

*Also see SOHT Consensus document 2011*

# Alternative Matrices

## Oral Fluid

- 1<sup>st</sup> Validated Method for EtG in OF (Hegstad et al, 2009)
  - EtG in OF extends the window of detection by 'several hours'.
- 2<sup>nd</sup> Study of EtG in OF (Høiseth et al, 2010)
  - Detection window of EtG in OF only a 'few hours' longer than EtOH and is limited additional value.
- 1<sup>st</sup> Report of EtS in OF (Moore et al, 2010)
  - Volunteers dosed to reach a BAC of 80mg% in 60-90 mins.
  - EtG was not detected in any OF samples.
  - EtS was detected up to 18 hrs after drinking started and 8 hrs after EtOH was 0.

# Alternative Matrices

## Oral Fluid

### Advantages:

- 1) Non invasive sampling.
- 2) Supervised collection.
- 3) Increased window of detection of EtG (?) and EtS.
- 4) False positives unlikely.

### Disadvantages:

- 1) Concentrations of EtG in OF are <1% of those in BL (OF:BL 0.029).
- 2) Variations in pH.
- 3) Effect of saliva stimulation collection devices.
- 4) Variable sample volume.
- 5) Normalise to IgG or amylase.
- 6) OF a less controlled medium than BL.

# Alternative Matrices

- **Post-mortem Blood & Vitreous**

Post-mortem blood & vitreous EtG & EtS quantitation by LC/MS/MS (Jenkins *et al*)

- BL & VH comparable
- Good discrimination where PM BL alcohol <100mg%
- Viable alternative matrix to BL - less susceptible to bacterial contamination

- **Serum/Blood**

Serum/whole blood concentration ratio for EtG & EtS (Høiseth *et al*)

- Higher concentrations of EtG & EtS in SM than BL
- EtG SM/BL ratio 1.69 (1.33-1.90)
- EtS SM/BL ratio 1.30 (1.08-1.47)
- No correlation between absolute concentration in SM and BL and SM/BL ratio

# Conclusion

- EtG & EtS have a place in the forensic setting.
- Their limitations must be considered.
- EtS appears to be a more reliable marker than EtG.
- EtG & EtS are formed by different metabolic pathways and therefore simultaneous determination can increase sensitivity in detecting recent ethanol consumption.

# Acknowledgements

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