2º ENQFor - Encontro Nacional de Química Forense
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Ribeirão Preto - SP

Drugs of abuse and heavy alcohol use markers in hair samples: analytical methodologies and data interpretation in forensic toxicology

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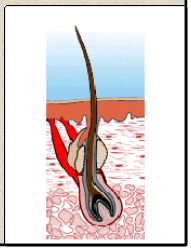
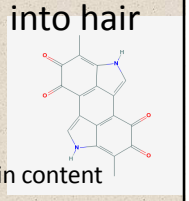
Xenobiotics incorporation into hair

- From blood stream during hair growth
- From sebum
- From sweat
- From environment (smoke)



Xenobiotics incorporation into hair

Basic drugs bind to **melanin**

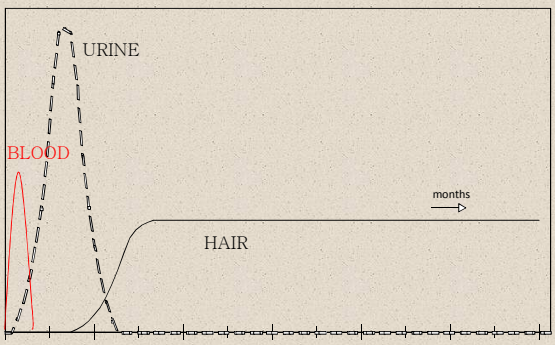


- Concentration dependent on melanin content (*Kronstrand et al., 1999*)
- Bias on hair color
- Positive linear relationship between total melanin content of hair and C_{max} of codeine, cocaine, and metabolites following controlled dosing (*Scheidweiler et al., 2005*)

Hair growth

- Head hair:
  - Anagen: 4-8 years, 0.6-1.4 cm/month
  - Catagen: 2 weeks
  - Telogen: 10 weeks
- Average growth rate in head hair: 1 cm/month

Surveillance window



Hair analysis

- Environmental Toxicology (trace elements/ pollutants)
- Nutrition (lack of elements)
- Disease diagnosis (Down syndrome, schizofrenia, …)
- Forensic Toxicology

Hair analysis in Forensic Toxicology

- Napoleon (*Smith et al. 1962*)

- John Keats (*Baumgartner et al. 1989*)

Season of mists and mellow fruitfulness
Close bosom-friend of the maturing sun
Conspiring with him how to load and bless
With fruit the vines that round the thatch-eaves run
…

Hair analysis in Forensic Toxicology

Drugs of abuse:

- Drivers license issue or re-issue
- Monitoring during addiction treatment
- Workplace drug testing
- Divorce / children custody
- Cause of death
- Unwilling sedation

Hair analysis: steps

- Segmentation
- Decontamination
- Cutting or pulverization
- Incubation
- Extraction of analytes from incubation solvent
- Instrumental analysis
  - Gas chromatography – mass spectrometry (GC-MS)
  - Liquid chromatography – tandem mass spectrometry (LC-MS-MS)

1. Sampling, shipping, storage
2. Decontamination
3. Hair disintegration and extraction
4. Screening tests
5. Criteria for MS analysis → **VALIDATION IDENTIFICATION**
6. Specific drug classes → **RECOMMENDED LLOQs**
7. Internal quality control
8. External quality control

Recommended Lower Limits of Quantification

MORPHINE **6-ACETYL MORPHINE**	**≤ 0.2 ng/mg**
COCAINE **COCAINE METABOLITES**	**≤ 0.5 ng/mg** **≤ 0.05 ng/mg**
AMPHETAMINES	**≤ 0.2 ng/mg**
THC **THC-COOH**	**≤ 0.1 ng/mg** **≤ 0.2 pg/mg**

SoHT, 2004

Hair analysis for drivers license regranting

- Depending on National (or local) Law
- As an example, in Florence, Italy:
  - Opiates
  - Buprenorphine
  - Cocaine
  - Cannabinoids
  - Amphetamines
  - Methadone

Evidence of surreptitious administration

- A 9 year old girl was assaulted
- Seven weeks later a hair strand was collected
- Segmented in 1 or 2 cm
- Each segment (about 20 mg) was analysed for diphenhydramine (LC-MS-MS, 2 transitions, validated method)

Kintz et al. 2007

Evidence of surreptitious administration

- Diphenhydramine: OTC drug, antihistaminic, sedative, antiemetic

Segment 0-1 cm	**37 pg/mg**
Segment 1-3 cm	**39 pg/mg**
Segment 3-5 cm	**33 pg/mg**

- Use of diphenhydramine as a drug-facilitated crime and subsequent impairment of a 9-year-old female victim

Kintz et al. 2007

Evidence of surreptitious administration

- 51 years old male admitted 9 times to hospital for drowsiness, ataxia, sedation, muscular weakness, and marked somnolence
- Hair strand of 7 cm, analysed in segments by LC-MS-MS (2 transitions)
- Alprazolam in 4 segments

0-1 cm	**71 pg/mg**
1-2 cm	**25 pg/mg**
2-3 cm	**8 pg/mg**
3-4 cm	**13 pg/mg**
4-5 cm	**< LOD (2 pg/mg)**
5-6 cm	**< LOD**
6-7 cm	**< LOD**

Kintz et al. 2008

Evidence of neonatal exposure

- Two twin female infants born at 34 weeks of gestation by caesarean section performed because of maternal preeclampsia.
- During preceding week pregnancy had been complicated by maternal hypertension. The mother reported use of venlafaxine during pregnancy.
- Both twins presented neonatal abstinence syndrome

Favretto et al. 2010

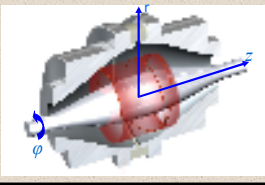
Evidence of neonatal exposure

- 2.5 mg hair washed twice with CH_2Cl_2
- Automatic pulverization with 145 µl of water, 20 µl of acetonitrile, 20 µl of 1 M trifluoroacetic acid, and 15 µl of IS working solution, 5 minutes.
- Double centrifugation
- 10 µl of supernatant injected directly into analytical column

Favretto et al. 2010

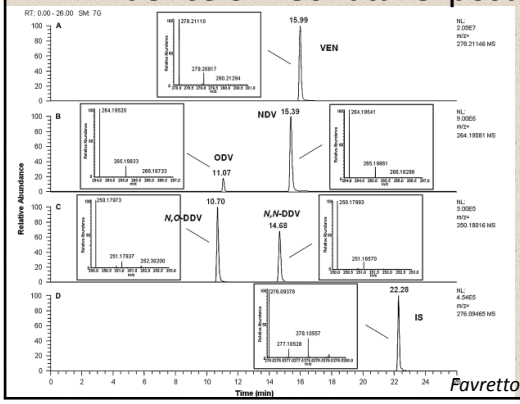
Evidence of neonatal exposure

- LTQ-Orbitrap MS in positive ESI mode
- AtlantisT3 analytical column by gradient at 100 µl/min
- Accurate mass measurements of MH^+ ions: *m/z* 278.21146 for venlafaxine



Favretto et al. 2010

Evidence of neonatal exposure



Favretto et al. 2010

Evidence of neonatal exposure

- HRMS permitted the detection of:
  - Venlafaxine (VEN)
  - O-desmethylvenlafaxine (ODV)
  - N,O-didesmethylvenlafaxine(N,O-DDV)
  - N,N-didesmethylvenlafaxine(N,N-DDV)
  - N-desmethylvenlafaxine (NDV)
- 10 ng/mg and 12 ng/mg of VEN in infants hair

Favretto et al. 2010

Drug facilitated sexual assault

- 61-year-old woman hospitalised for a minor surgical operation
- Local analgesia (mepivacain, ropivacain), general anaesthesia (propofol)
- On completion of operation fully awakened, was transferred to her ward
- The patient reached her ward with considerable delay and still unconscious
- Head and pubic hair collected 6 weeks later

Frison et al. 2003

Drug facilitated sexual assault

- SPME and GC-MS-MS (ion trap MS), monitoring 3 product ions per compound
- Validated method

	Pentobarbital (ng/mg)	Thiopental (ng/mg)
1 A Proximal	0.40	0.30
1 A Distal	n.d.	n.d.
1B Proximal	0.20	0.20
1 B Distal	n.d.	n.d.
2 Pubic	0.40	0.25

Frison et al. 2003

Drug facilitated sexual assault

- Lack of collection and toxicological analysis of traditional biological fluids in the immediacy of a DFSA in a healthcare setting
- Quali-quantitative results obtained from SPME and GC–MS-MS analysis of the victim head and pubic hair
- Document the use of the anaesthetic agent thiopental to sedate her quickly and deeply and commit sexual assault.

Frison et al. 2003

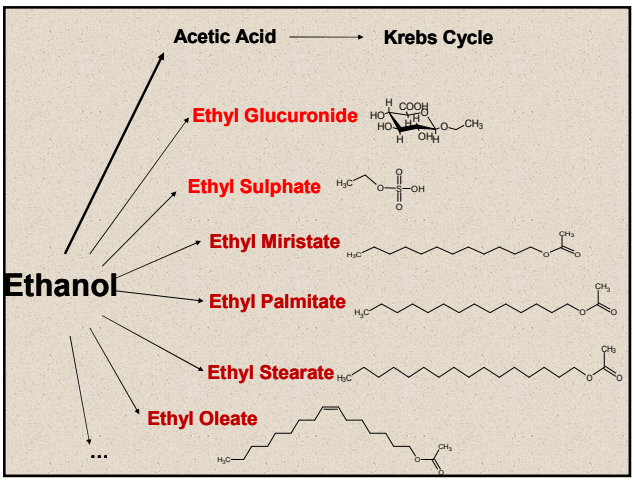
Pre-Columbian Mummies

- Chilean mummies, dated 2000 BC: benzoylecgonine (*Cartmell et al. 1991*)
- Eight pre-columbian mummies
  - Woman from Perù or Argentina
  - Child from Peruvian Chancay culture
  - Female skull with braided hair
- Positive to nicotine (57.5, 14.1, 11.4 ng/mg, *Musshoff et al. 2009*)

Egyptian Mummies

- Nine mummies dated from 1000 BC to 400 AD, included a priestess, Henut Taui
- Hair positive to:
  - THC
  - Cocaine
  - Nicotine

Balabanova et al. 1992

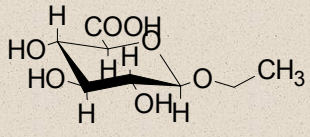


β-D-ethyl glucuronide

- Non-volatile
  water-soluble, slightly acidic
  direct metabolite of ethanol
- Indicative of ethanol intake for:
  - hours (serum)
  - days (urine)
  - months (hair)

Question:

Is there a relationship between the pattern of alcohol use and β-D-ethyl glucuronide (EtG) levels in hair?



Ethyl glucuronide in hair- HEtG

During 2000:

- Skopp *et al.* Alcohol and Alcoholism
- Alt *et al.* Alcohol and Alcoholism

Since 2004:

- LLOQs down to 2 - 3 pg/mg
- Fully validated methods
- Wider populations examined

Sample preparation

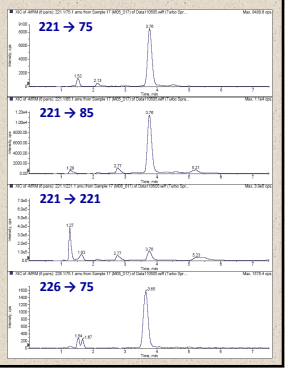
- Hair (50-100 mg, 3-5 cm, proximal segment)
- Washing: CH_2Cl_2, MeOH
- 1-2 mm scissor cut
- Overnight incubation in 700 µl H_2O (D_5-EtG)
- 2-hour ultrasonication
- 13000 rpm centrifugation
- Injection (8 µl)

Politi et al. 2006

LC-ESI-MS-MS method

- C_{18} (100 × 3 mm, 3 µm id)
- 0.1% formic acid/ACN 99:1
- 200 µl/min
- 100 µl/min ACN post-column
- Negative electrospray ionization
- MRM transitions:

EtG:	*m/z*	221 → 75
		221 → 85
		221 → 221
D_5-EtG:		226 → 75
		226 → 85
		226 → 226



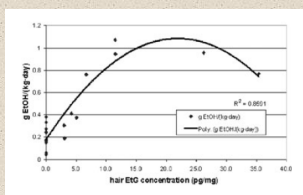
LC-ESI-MS-MS method

- Full validation (selectivity, linearity, accuracy, precision, matrix effect, stability, reproducibility)
- LLOQ: 3 pg/mg
- LOD: 2 pg/mg
- 2 MRM
- Surviving ion

Politi et al. 2006

Correlation EtG – ethanol daily intake

- Correlation between hair EtG and ethanol daily intake normalized by the individual body weight



Politi et al. 2006

Ethanol Daily intake - EDI

- *Politi et al. 2006*:
  - HEtG correlated with:
    - EDI
    - Body Mass Index
- *Appenzeller et al. 2007*:
  - Linear proportional relationship between HEtG and EDI
  - HEtG > 23 pg/mg corresponds to an EDI > 60 pg/mg

HEtG cut-offs

Suggested cut-offs:

Appenzeller *et al.* (2007)	23 pg/mg
Pragst and Yegles (2008)	25 pg/mg
Bendroth *et al.* (2008)	30 pg/mg
Kintz *et al.* (2008)	50 pg/mg

Factors possibly influencing EtG formation or incorporation: sex, age, gender, body mass index, cosmetic treatments, hygienic habits, *etc.*

Questions

Definition of a cut-off level for HEtG able to effectively discriminate a heavy drinking behaviour.

Evaluation of the influence of factors known to affect ethanol metabolism and/or the diagnostic power of other markers of ethanol use, and of issues possibly affecting substance incorporation into hair.

Study protocol

98 subjects:

– teetotallers, social drinkers, and heavy drinkers at the beginning of a withdrawal treatment

- EDI 2-week and 3-month ← Questionnaire, anonymous, self-administered
- HEtG, 3-cm prox ← LC-MS-MS
  – LLOQ: 3 pg/mg, full validation
  – 2 transitions per analyte

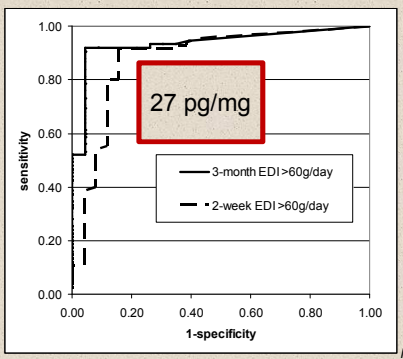
Morini et al. 2009

EDI and basic characteristics

Characteristics	3-month EDI <60 g/day (n=23)	3-month EDI ≥60 g/day (n=75)	p
gender (% females)	26 (n=11)	21 (n=12)	0.5839
age (years)	41 (31-52)	45 (40-54)	**0.0543**
BMI (kg/m^2)	23 (21-26)	23 (21-26)	0.5473
smokers (%)	55 (n=12)	83 (n=49)	**0.0085**
brown or black hair colour (%)	56 (n=13)	70 (n=50)	0.2203
hair treatment (%)	44 (n=7)	41 (n=16)	0.8537
n. shampoos/week	3 (2-6)	3 (2-4)	0.6461
wine as prevalent beverage (%)	83 (n=15)	67 (n=35)	0.2332

Morini et al. 2009

Receiver Operated Characteristic (ROC) curves



Morini et al. 2009

Sensitivity and Specificity (1)

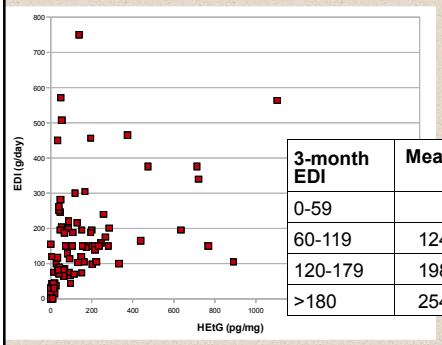
Characteristics	n	SE	SP	*AUC*
2-week EDI (≥60 g/day)	98	0.92	0.85	*0.88*
3-month EDI (≥60 g/day)	98	0.92	0.96	*0.94*
Females	42	0.90	0.91	*0.91*
Males	56	0.93	1.00	*0.95*
Age 20-34	11	0.75	1.00	*0.75*
Age 35-49	42	0.88	1.00	*0.88*
Age ≥ 50	29	0.95	0.86	*0.95*

Morini et al. 2009

Characteristics	n	SE	SP	*AUC*
BMI<25	52	0.91	1.00	*0.96*
BMI≥25	30	0.88	0.86	*0.83*
Non-smokers	20	0.90	1.00	*0.98*
Smokers	61	0.90	0.92	*0.89*
Hair colour blonde/grey	31	1.00	0.90	*0.96*
Hair colour brown/black	63	0.90	1.00	*0.95*
Hair treatments	32	0.88	1.00	*0.87*
No hair treatments	23	0.91	0.89	*0.96*
1-2 shampoos/week	35	0.88	1.00	*0.94*
3 or more shampoos/week	41	0.90	0.92	*0.90*
Prevalent beverage beer	19	0.91	0.93	*0.92*
Prevalent beverage wine	50	0.88	1.00	*0.92*

Morini et al. 2009

Correlation EDI- HEtG



3-month EDI	Mean (Std Dev)	N
0-59	9.6 (20.7)	23
60-119	124.8 (176.0)	25
120-179	198.7 (181.4)	18
>180	254.5 (345.3)	32

Morini et al. 2009

EtG in Hair and Racial Bias

No correlation found between
EtG in hair
and melanin content

(*Appenzeller et al. 2007. Ethyl glucuronide concentration in hair is not influenced by pigmentation*)

Correlation EDI- HEtG: conclusions

When a fully validated analytical method providing adequate sensitivity and selectivity of detection is used:

- HEtG is able to ascertain chronic heavy drinking (EDI of 60 g/day or higher within the last 3 months) with high sensitivity (0.92) and specificity (0.96)
- A 27 pg/mg cut off presented the best test performance in accordance with the results of previous studies .

Morini et al. 2009

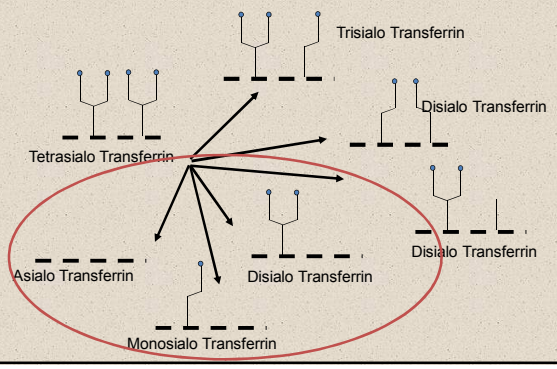
Correlation EDI- HEtG: conclusions

• None of the examined factors was found to significantly affect HEtG performance.

• Further studies on larger groups of individuals are required in order to establish if the differences observed for some factors (e.g. BMI, smoke, and hair treatments), as well as associations between factors, may reach statistical significance.

Morini et al. 2009

Carbohydrate Deficient Transferrin CDT



CDT: sensitivity and specificity

WHO/ISBRA Collaborative Study:

	Men	Women
Sensitivity (SE)	60%	29%
Specificity (SP)	92%	92%

as compared to patient's interview

- Low CDT serum levels when < 20 years
- High serum CDT with low BMI (< 20 kg/m^2)

Conigrave et al. 2002

Questions

Is hair EtG a better indicator than CDT?

What is sensitivity (SE) and specificity (SP) for EtG in hair and CDT in serum as markers of chronic heavy alcohol use?

Methods

Healthy volunteers (teetotalers n=5, social drinkers n= 16) and alcoholics at the beginning of an in-patient or an out-patient treatment (n= 65)

Hair samples (n=86, 3-cm proximal segment) for EtG determination

Serum (n=86) for CDT determination by immunonephelometry (n=30) or by HPLC (n=56)

Morini et al. 2009

Hair EtG and CDT

Marker	***Cut-off* (EDI 60 g/*die*)**	**EDI 2-weeks SE**	**EDI 2-weeks SP**	**EDI 3-months SE**	**EDI 3-months SP**
HEtG	27 pg/mg	1.00	0.93	1.00	1.00
CDT*	2.5%	0.44	0.93	0.47	1.00
CDT*	2.2%	0.50	0.86	0.53	0.92

* CDT by immunonephelometry, Dade Behring N-Latex

Morini et al. 2009

Hair EtG and CDT

Marker	***Cut-off* (EDI 60 g/*die*)**	**EDI 2-weeks SE**	**EDI 2-weeks SP**	**EDI 3-months SE**	**EDI 3-months SP**
HEtG	27 pg/mg	0.96	0.70	0.98	0.89
CDT*	2.5%	0.50	0.70	0.51	0.78
CDT*	2.2%	0.63	0.60	0.62	0.56

* CDT by HPLC.

Morini et al. 2009

Hair EtG and CDT: conclusions

- At detecting an ethanol daily intake of 60 g/day or higher Hair EtG provides equal selectivity of CDT (by both immuno-nephelometry and HPLC)
  - within the last 2 weeks
  - within the last 3-months
- Hair EtG proved to be about 2 times more sensitive than either of the two CDT methods

Morini et al. 2009

SOCIETY OF HAIR TESTING

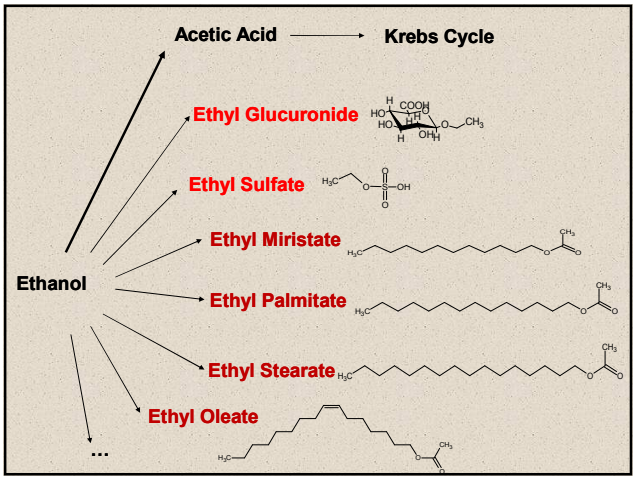
Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption

1. Alcohol is a legal compound in many countries and is consumed in much higher amounts in comparison to other drugs of abuse and by a much higher portion of the population. Compared to other substances, the detection of chronic excessive alcohol consumption by hair analysis has some specific characteristics.
2. Currently, according to the World Health Organization and a literature survey, chronic excessive alcohol drinking corresponds to a consumption higher than 60 g of pure ethanol per day during since several months.
3. For clinical and forensic purposes, there is a need to establish chronic excessive alcohol consumption.

SOCIETY OF HAIR TESTING

Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption

5. After absorption, a small fraction of ethanol is conjugated with glucuronic acid during phase II metabolism to form EtG.
6. EtG is a polar water soluble substance, stable but sensitive to cosmetic treatment and whose incorporation is not biased by natural hair color.
7. Either gas or liquid chromatography coupled to (tandem) mass spectrometry with deuterated EtG as internal standard should be used to test for EtG in hair.
8. The cut-off for EtG in hair to strongly suggest chronic excessive alcohol consumption is proposed at 30 pg/mg scalp hair measured in the 0-3 cm proximal segment.

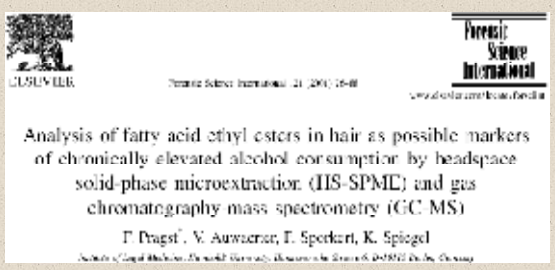


Fatty acid ethyl esters
FAEE

- Goodman and Deykin. 1963. P*roc Soc Exp Biol Med.* **Fatty acid ethyl ester formation during ethanol metabolism in vivo.**
- Lange and Sobel. 1983. *J Clin Invest.* **Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol.**
- De Pergola et al. 1991. *Alcohol Clin Exp Res.* **The metabolism of ethyl esters of fatty acids in adipose tissue of rats chronically exposed to ethanol.**

FAEE

Hair FAEE



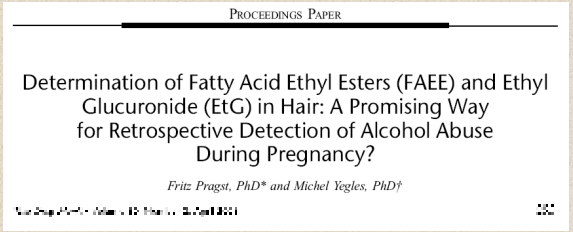
Analysis of fatty acid ethyl esters in hair as possible markers of chronically elevated alcohol consumption by headspace solid-phase microextraction (HS-SPME) and gas chromatography mass spectrometry (GC-MS)

T. Pragst*, V. Auwaerter, T. Sporkert, K. Spiegel

Hair FAEE

- Zimmermann and Jackson, 2010.
  Ther Drug Monit.
  Gas chromatography tandem mass spectrometry for biomarkers of alcohol abuse in human hair

Hair FAEE

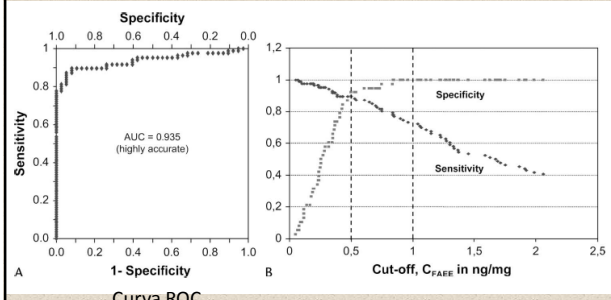


PROCEEDINGS PAPER

Determination of Fatty Acid Ethyl Esters (FAEE) and Ethyl Glucuronide (EtG) in Hair: A Promising Way for Retrospective Detection of Alcohol Abuse During Pregnancy?

Fritz Pragst, PhD and Michel Yegles, PhD†*

FAEE nel capello



Curva ROC

Pragst et al. 2008

SOCIETY OF HAIR TESTING

Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption

Question

Is it possible to determine hair FAEE by gas chromatography – single quadrupole mass spectrometry with respect to analytical selectivity and sensitivity?

Study design

- 60 volunteers (teetotallers, social, and heavy drinkers)
- Hair strand
- Questionnaire:
  - Alcohol daily consumption habits
  - Pathological conditions and pharmaceuticals continuously used
  - Hair treatments

Politi et al. 2011

Sample preparation

- Double washing in MeOH of hair, 3-cm proximal segment
- Finely scissor cut
- Addition of internal standard (alpha-colestan, 50 ng)
- Overnight incubation with 0,5 ml DMSO + 4 ml n-hexane

Politi et al. 2011

Sample preparation

Incubation medium on aminopropylic SPE cartridges:

- cartridges conditioned with 3 ml CH_2Cl_2 + 3 ml n-hexane
- n-hexane load
- elution of FAEE with 3 ml n-hexane + 3 ml CH_2Cl_2
- n-hexane and CH_2Cl_2 evaporated to dryness
- re-constitution in n-hexane (50 µl)

Politi et al. 2011

GC-MS

- Agilent 7890A/5975C Inert MSD with autosampler 7683B
- Capillary fused silica column HP-5MS (30 m x 0,25 mm x 0,25 µm methyl silicone 5% diphenyl silicone)
- Splitless mode injection - Helium 1 ml/min
- Injector: 300°C
- T at 100°C for 2 min; 12°C/min up to 200°C; 8°C/min up to 300°C, final isotherm for 3 min
- Interface : 300°C

Politi et al. 2011

GC-MS

Ethyl Linolenate

Ethyl Linoelate

Ethyl Arachidonate

Methyl Stearate

…

Politi et al. 2011

Validation

- Specificity (non significant FAEE)
- Linearity: 0.01; 0.05; 0.1; 0.2; 0.5 ng/mg
- LLOQ: 0.01 ng/mg
- Accuracy and precision
- Recovery

Politi et al. 2011

Ethanol daily consumption and FAEE

- Questionnaire
  - Ethanol daily consumption: 0 – 246 g/day
  - median: 13 g/day
- SPE-GC-MS analysis
  - FAEE tot: 0.02 – 10.78 ng/mg
  - median: 0.66 ng/mg

Politi et al. 2011

Ethanol intake and FAEE: Results

	FAEE < 0.5 ng/mg		FAEE > 0.5 ng/mg		SE	SP
	EtOH < 60g/day	EtOH > 60g/day	EtOH < 60g/day	EtOH > 60g/day		
All samples (n=60)	19	0	28	13	100%	40%
Excluding lotions (n=49)	19	0	17	13	100%	52%
Excluding lotions, hormonal treatments (n=40)	19	0	9	13	**100%**	**70%**

Politi et al. 2011

Ethanol intake and FAEE: Results

- False negatives: 0 → Sensitivity 100%
- True negatives: 19
- False positives: 28
  - 11 EtOH-containing trichological lotions
  - 8 hormonal based contraceptives
- True positives: 13

Politi et al. 2011

Ethanol intake and FAEE: Results

- False positives: 9 → Specificity 70%
- Tentative reasons:
  - Individual genetic variability in FAEE formation?
  - Alcohol use not reported in questionnaire?
  - Not reported hair lotion use?
  - …?

?

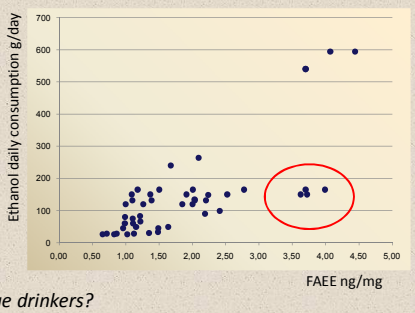
Politi et al. 2011

Method – Alcohology Unit

- 53 volunteers upon admission to an Alcohology Unit treatment
- Hair strand for FAEE analysis, 3-cm prox
- Alcohol daily consumption habits estimated by the Alcohology Unit operator during anamnesis

Politi et al. 2011

Results – Alcohology Unit



- *Binge drinkers?*

Politi et al. 2011

Hair FAEE: conclusions

- GC-MS is a valid technique for hair FAEE analysis
- Factor to be taken into account:
  - Use of trichological lotions
  - Use of hormonal contraceptives ?
  - Binge drinking ?

Politi et al. 2011

FINAL CONSIDERATIONS

- When hair analysis is aimed at forensic purposes, always:
  - VALIDATION
  - IDENTIFICATION
  - INTERPRETATION

FINAL CONSIDERATIONS

- Refer to specific Guidelines:
  - Guidelines for European workplace drug and alcohol testing in hair. Agius R, Kintz P; European Workplace Drug Testing Society. *Drug Test Anal.* 2010; 2: 367-76.
  - Recommendations for hair testing in forensic cases. Society of Hair Testing. *Forensic Sci Int.* 2004; 145: 83-4.
  - Guidelines for Drugs of Abuse Testing for Forensic Purposes. Group of Italian Forensic Toxicologists (GIFT): http://www.gtfi.it/index-en.html
- Proficiency Tests

Thank you!