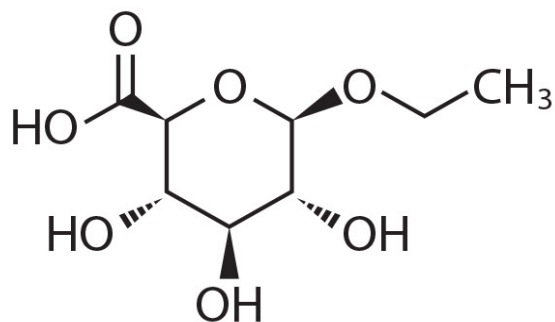


Quantitative Analysis of EtG and EtS in Urine Using Ion Exchange SPE and LC-MS/MS



EtG

UCT Part Numbers

CUQAX156

Clean-Up® 6 mL SPE cartridge
with 500 mg QAX

SLETGGDC20-3UM

Selectra® ETG guard column,
10 x 2.0 mm, 3 µm

SLETG100ID21-3UM

Selectra® ETG HPLC
column, 100 x 2.1 mm,
3 µm

SLDGRDHLDR

guard cartridge holder

Introduction:

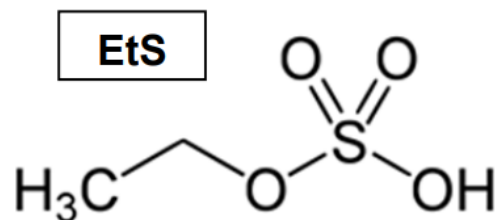
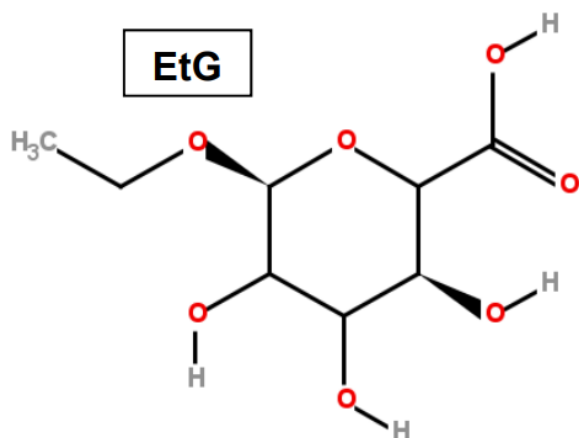
Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are conjugated ethanol metabolites formed in low amounts in the body following alcohol consumption. Compared with ethanol, EtG and EtS are excreted in urine for a prolonged time. Published literature indicates that EtG may be detectable for up to 80 hours after alcohol ingestion, while EtS is generally detectable for up to 24 hours after intake, making them both valuable as sensitive alcohol biomarkers. While these analytes can be good indicators for complied abstinence, their concentrations do not directly correlate with how much alcohol someone has actually consumed. That value is influenced by several contributing factors such as quantity and time frame when the alcohol was consumed, metabolic conversion rates of drinkers, and the ceiling affect EtG exhibits once the maximum threshold of EtG is measured.

The cutoff level for EtG confirmation is typically 500 ng/mL or higher; the EtS confirmation cut-off level is generally set at 100 ng/mL. Because of the sensitivity of both EtG and EtS testing, it is possible for exposure to alcohol from use of

personal hygiene products, foods containing alcohol, and cleaning or sanitizing products to result in a positive EtG and/or EtS test. Neither EtG nor EtS testing can distinguish between alcohol beverage consumption and incidental or unintentional alcohol exposure from the above mentioned extraneous sources. Thus, based on current published literature, it is recommended that levels of 1500 ng/mL for EtG and 100 ng/mL for EtS be used by monitoring programs when attempting to make determinations of drinking relapse (1).

This optimized SPE-based method uses strong anion exchange interactions (QAX) ideally constructed to extract acidic structures like that of EtG and EtS from urine. While several other SPE methods employ a dual elution scheme to optimally recover both analytes, this method utilizes a single elution step for both EtG and EtS that plays to the chemical nature of both analytes.





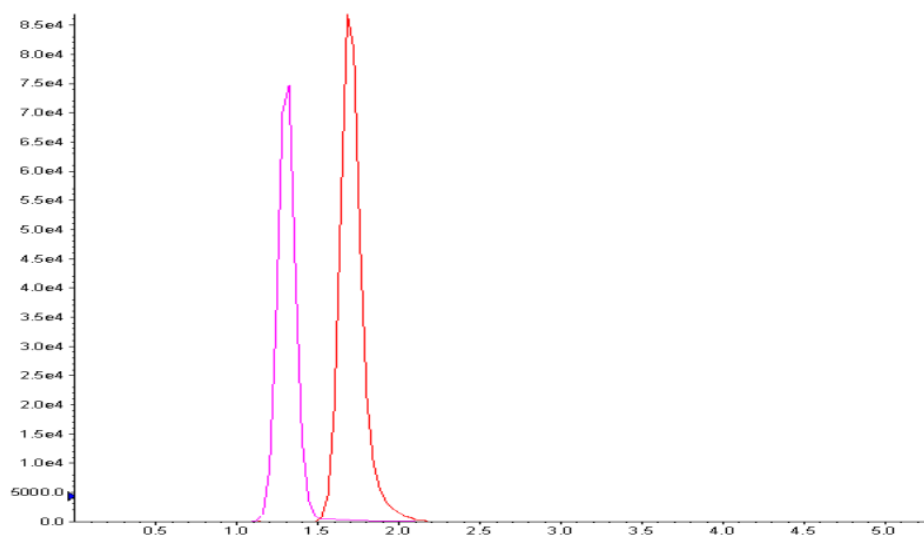
Sample Pretreatment

1. To 0.5 mL of urine sample containing deuterated analogues of EtG/EtS add 4.5 mL of D.I. H₂O.
2. Vortex for 30 seconds.

SPE Method

1. Precondition SPE column with 5 mL of MeOH followed by 5 mL of D.I. H₂O.
2. Apply sample to SPE column.
3. Wash SPE column with 5 mL of ACN followed by 5 mL of MeOH.
4. Dry column (10 minutes at full vacuum or pressure).
5. Elute EtG/EtS with 5 mL of 2% HCl in ACN (collect eluate at 1- 2 mL/min).
6. Evaporate to dryness at < 50°C.
7. Reconstitute sample in 100 µL of D.I. H₂O.

LC-MS/MS Method



LC-MS/MS Parameters				
System	AB Sciex API 4000 QTrap MS/MS with Agilent 1200 Binary Pump SL			
Column	UCT Selectra® ETG Column, 100 x 2.1 mm, 3 µm			
Guard Column	UCT Selectra® ETG guard, 10 x 2.1 mm, 3 µm			
Column Temperature	50 °C			
Flow Rate	0.3 mL/min			
Injection Volume	10 µL			
Mobile Phase A	0.1% formic acid in Water			
Mobile Phase B	0.1% formic acid in Methanol			
Gradient Program				
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)		
0	100	0		
2.5	100	0		
4.0	5	95		
6.0	5	95		
6.1	100	0		
11.0	100	0		
MRM transitions (ESI ⁻ , 50 ms dwell time)				
Compound	Rt (min)	Q1 ion	Q3 ion 1	Q3 ion 2
EtS-D5	1.28	130.1	97.8	79.7
EtS	1.31	125.1	95.8	96.9
EtG-D5	1.66	226.1	85.1	74.9
EtG	1.69	220.9	85.1	75.1

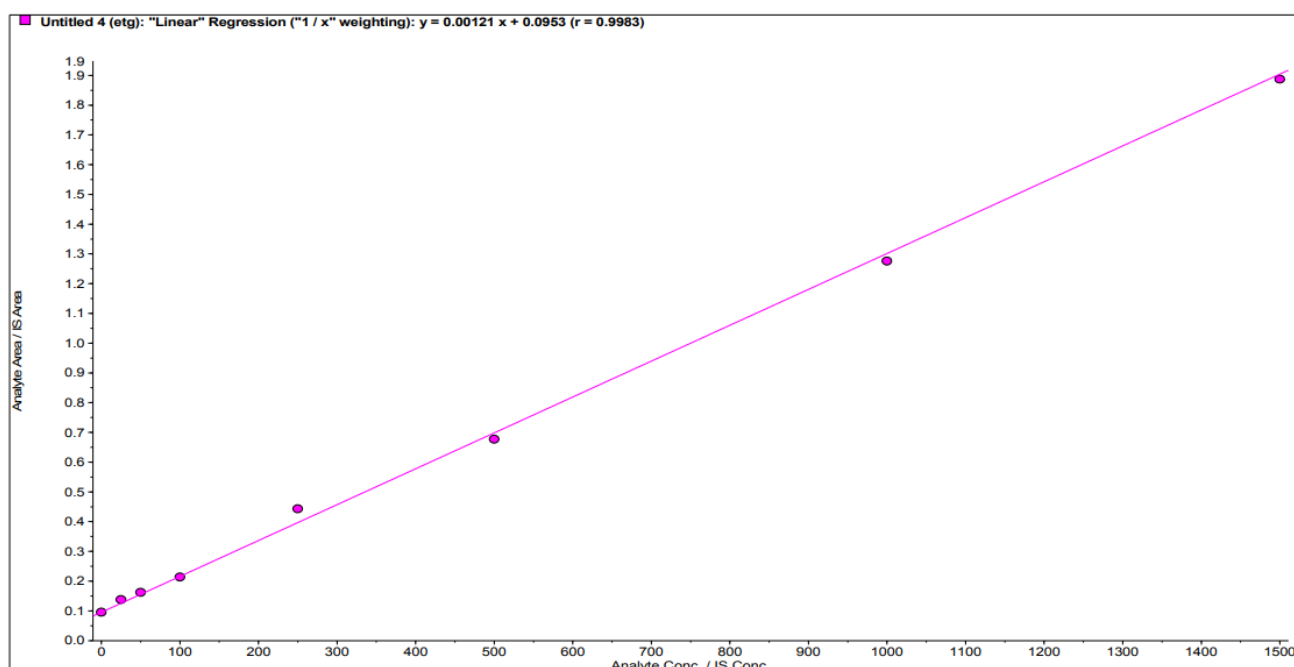
Results:

Excellent recoveries were achieved with EtG at 97.9% and EtS at 84.9%. The extraction efficiency was evaluated by fortifying samples at two concentrations (100 ng/mL and 500 ng/mL). RSD values were less than 11% (n=5 at each concentration). Matrixmatched calibration curves were used for quantification with R² values ranging from 0.9983 to 0.9998 over the concentration range (50 - 1500 ng/mL). The limits of detection and quantification for this method were determined to be 25 ng/mL and 50 ng/mL, respectively for EtG/EtS.



Recovery and RSD% from Urine Spiked at 2 Levels

Compound	Spiked at 100 ng/mL		Spiked at 500 ng/mL	
	Recovery %	RSD% (n=5)	Recovery %	RSD% (n=5)
EtG	86.2	4.8	97.9	10.9
EtS	95.1	6.5	84.9	5.9
Overall Mean	90.65	5.65	91.4	8.4

Matrix-Matched Calibration Curve of EtG ($R^2=0.9983$)

Discussion:

Several variables were assessed during method optimization. The first was the wash solvents and volumes of choice that functioned to remove as much matrix as possible without compromising the recovery of either EtG or EtS. Four solvents schemes obtained from established literature were evaluated:

Wash 1) 3 mL D.I. H₂O; 3 mL MeOH

Wash 2) 5 mL D.I. H₂O; 5 mL MeOH

Wash 3) 3 mL ACN; 3 mL MeOH

Wash 4) 5 mL ACN; 5 mL MeOH

While the D.I. H₂O and MeOH wash did function to clean the sample well without significant loss of analytes, the switch from the 3 mL volumes to 5 mL volumes resulted in an almost a 10% loss of EtG. EtS was not compromised. This is most likely due to the high polarity of EtG (logP: -1.61) and the fact that it is not as strongly retained on the QAX column as EtS, allowing it to be more susceptible to eluting in a polar wash. The ACN and MeOH wash combination produced the best overall recoveries for EtG and EtS, where the increased wash volume actually enhanced the recoveries for EtG. See table below for actual recoveries.



Recovery (%) from Urine Based on 4 Wash Solvent Schemes (n=3)

Compound	Wash 1	Wash 2	Wash 3	Wash 4
EtG	88.4	79.8	75.1	97.9
EtS	87.3	89.7	93.1	84.9

Discussion:

Lastly, the solvent of choice for elution of EtG and EtS from the QAX column was assessed. Initially, 5 mL of 5% Formic Acid in MeOH (pH ~ 4) was utilized. While this solvent worked well to elute EtG (pKa= 3.45), it was not strongly acidic enough to withdraw EtS (pKa= -2.1) from the strong anion exchange functionality of the column. In an attempt to improve EtS recovery, 2% HCl in ACN (pH ~ 2) was used. This produced excellent recoveries for both compounds. From there, MeOH was substituted for ACN as a more economic alternative and recoveries were again evaluated. Contradictory to the thought that MeOH could be interchanged and produce comparable results, EtG proved to be very unstable in this solvent, not even being recovered in post extraction spikes. It is unclear whether this is a solubility or stability related issue. EtS recovery improved by about 10% with this solvent change. Lastly, a dual solvent scheme was attempted utilizing 2.5 mL of 5% Formic Acid in MeOH followed by 2.5 mL of 2% HCl in ACN. EtG was minimally recovered, which could again be due to solubility or stability issues in the presence of MeOH and HCl. EtS recovery dropped by about 40% which is most likely due to the decrease in volume of 2% HCl in ACN from 5mL to 2.5 mL. For convenience and favorable recoveries of both compounds, 5mL of 2% HCl in ACN was chosen as the final elution solvent. See table below for actual recoveries.

Recovery (%) from Urine Based on 5 mL Elution Solvent of Choice

Compound	5% Formic Acid in MeOH	2% HCl in ACN	2% HCl in MeOH	50:50 2.5 mL 5% Formic Acid in MeOH; 2.5 mL 2% HCl in ACN
EtG	94.6	97.9	None Recovered	16.9
EtS	2.4	84.9	94.2	56.4

Conclusion:

- 1) A strong anion-exchange SPE method was successfully developed for the extraction, cleanup, and quantification of ethanol metabolites EtG and EtS from urine.
- 2) HPLC separation of extremely polar alcohol biomarkers was successfully conducted on UCT's Selectra® ETG column in under 3 minutes.
- 3) Because the majority of co-eluting matrix interferences found in urine are acidic and one is actually exploiting the acidic nature of EtG/EtS within this extraction, it can be anticipated that a residual amount will still remain within the final eluate. It is strongly recommended to use matrix-matched calibration curves and include isotopically labeled internal standards to facilitate any remaining matrix that is not removed via the SPE procedure.



References:

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- [2] Journal of Analytical Toxicology, Vol. 32, November/December 2008, 778-780
- [3] Journal of Analytical Toxicology, Vol. 33, May 2009, 204-207
- [4] Determination of urinary ethyl glucuronide and ethyl sulfate by LC/MS/MS for clinical research, MASCL 2014, Poster 19
- [5] LC-MS/MS Analysis of Ethyl Glucuronide and Ethyl Sulfate in Urine: A Comparison of Sample Preparation Techniques, SOFT 2014

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