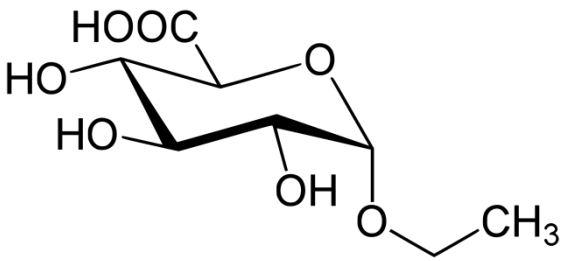
Quantitative Analysis of EtG and EtS in Urine Using FASt®ETG and LC-MS/MS

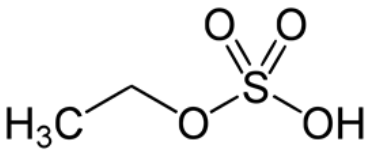
UCT Part Numbers:

CSFASETG203 - CLEAN SCREEN FASt® ETG, 200mg / 3mL tube
SLETG100ID21-3UM - Selectra® ETG HPLC column, 100 x 2.1 mm, 3µm
SLETGGDC20-3UM - Selectra®ETG guard column, 10 x 2.0 mm, 3 µm
SLGRDHLDR - guard cartridge holder

June 2015



Ethyl Glucuronide
pKa = 3.45



Ethyl Sulfate
pKa = (-) 2.08

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 use, making them both valuable as sensitive alcohol biomarkers. The detection of these metabolites has proven advantageous for zero tolerance treatment programs and abstinence enforcement where information regarding recent alcohol consumption is required. 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.

When analyzing chemical residues in a complex biological matrix, such as urine, a sample pre-treatment step is generally required to eliminate non-desirable matrix

components and/or concentrate the analyte(s) of interest. However, due to the highly polar nature of EtG and EtS (log P of 1.51 and 0.62, respectively), many labs turn away from traditional sample preparation procedures and instead use a dilute-and-shoot or simple filtration approach. These techniques do not adequately remove interferences from the sample and significant matrix suppression is commonly experienced during instrumental analysis. This is further complicated by the lack of retention of EtG/EtS on a traditional reversed phase HPLC column.

UCT has developed a line of products that allows for the efficient analysis of EtG and EtS in urine. By pairing UCT's FASt®ETG SPE cartridge with a Selectra®ETG HPLC column, cleaner extracts and lower LOD's/LOQ's can be achieved when comparing to a dilute-and-shoot method.

Procedure

Sample Dilution Ratio	Sample Volume	Dilution Volume*
1:1	500 µL	500 µL
1:4	200 µL	800 µL
1:9	100 µL	900 µL

* Diluent is 0.1% Formic Acid in D.I. H_2O.

1. Sample and diluents are added in an appropriately labeled tube. Add appropriate volume internal standard(s). It is recommended to use an internal standard volume of no more than 200 µL.

2. Set up extraction manifold with FASt®ETG cartridges and auto-sampler collection vials.

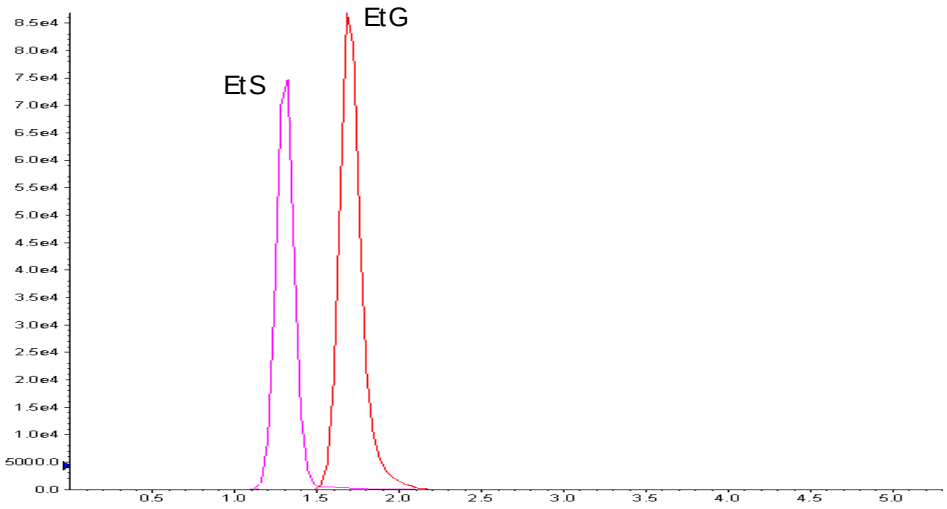
3. Pour sample into FASt®ETG cartridges and elute sample directly into auto-sampler vials.

4. Cap vials and put directly onto LC/MS for analysis.

LC-MS/MS method

Instrument: Agilent 1200 Binary Pump SL
Detector: AB Sciex API 4000 Q Trap MS/MS
Column: UCT Selectra® ETG HPLC column, 100 x 2.1 mm, 3 µm
Guard Column: UCT Selectra® ETG, 10 x 2.0 mm, 3 µm
Column Temperature: 50 ° C
Column Flow Rate: 0.3 mL/min
Injection Volume: 10 µL
Gradient Program:

Time (min)	% Mobile Phase A (0.1% FA in water)	% Mobile Phase B (0.1% FA in ACN)
0	100	0
2.5	100	0
4.0	5	95
6.0	5	95
6.1	100	0
11.0	100	0

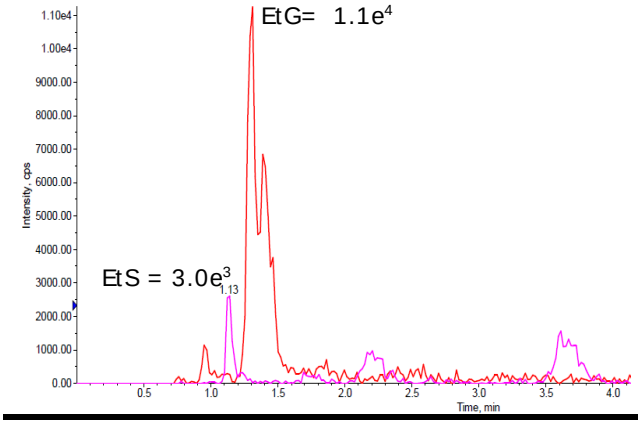


MRM transitions (ESI⁻, 50 ms dwell time)				
Compound	**Rt (min)**	**Q1 ion**	**Q3 ion 1**	**Q3 ion 2**
EtS-D5	1.28	130.1	97.8	79.7
EtS	1.31	125.1	95.8	96.9
EtG-D5	1.66	226.1	85.1	74.9
EtG	1.69	220.9	85.1	75.1

Results

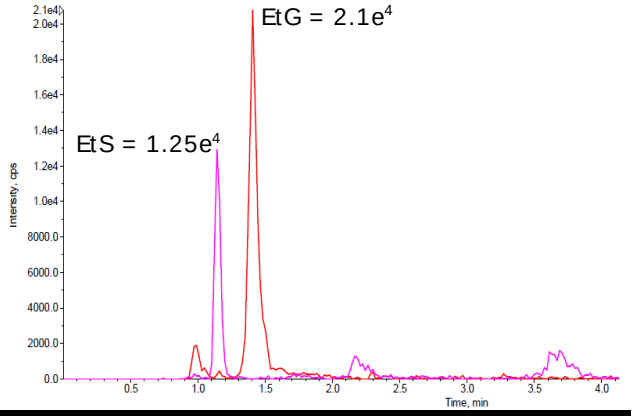
Dilute & Shoot 1:1

50 ng/mL

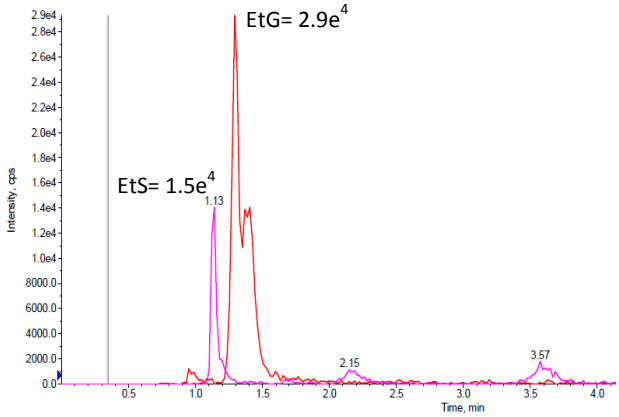


FASt® ETG 1:1

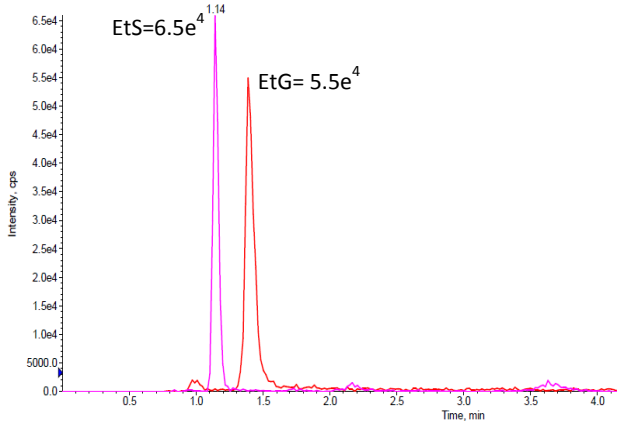
50 ng/mL



750 ng/mL



750 ng/mL

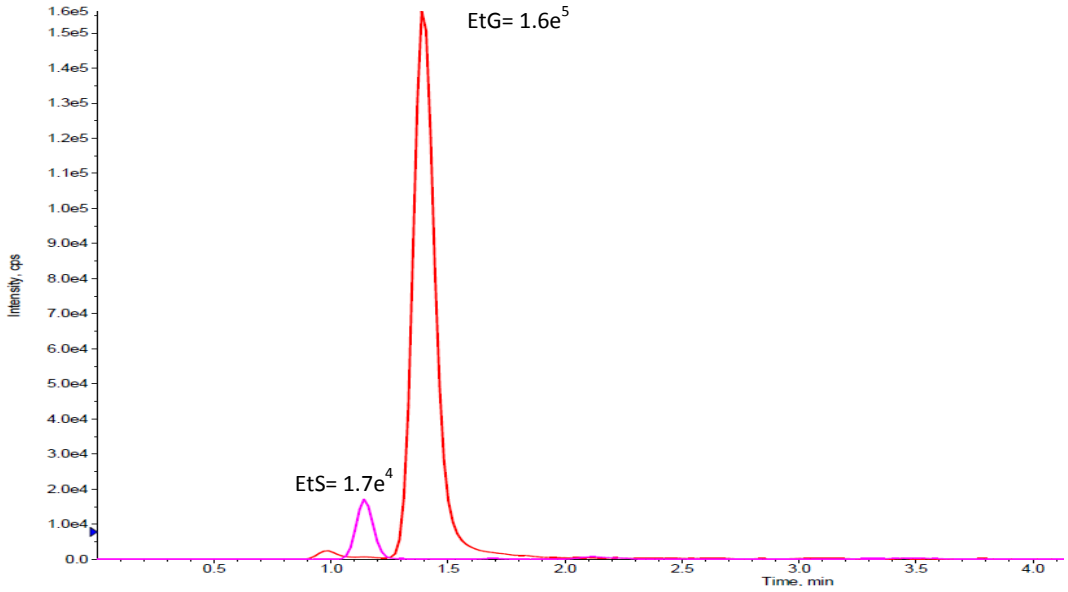


Samples were prepared by spiking stated concentrations into 1mL of urine. These samples were then diluted 1:1 with 0.1% formic acid in water. The chromatograms on the left represent the 1:1 dilute and shoot samples, while the chromatograms on the right represent samples that were filtered through UCT's FAST ETG columns. The filter and shoot preparation resulted in both improved chromatography and reduced analyte suppression.

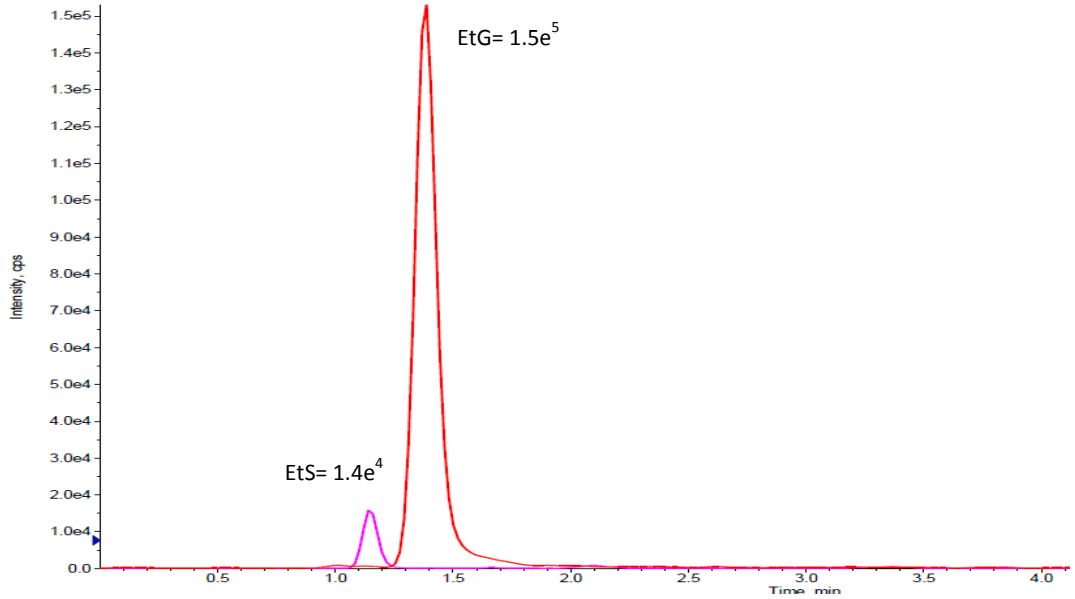
* ETS's second major transition was used for relative comparison purposes for these graphs to compensate for ETG's overall lower intensity in signal.

Method Reproducibility: FASt® ETG 1:1 (spiked urine sample)

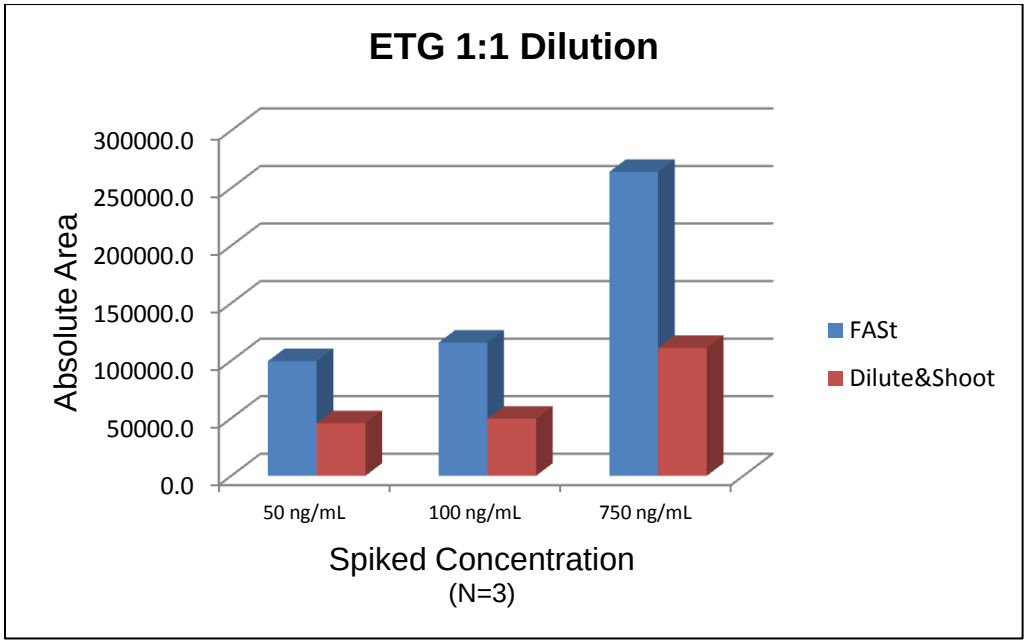
Injection 1

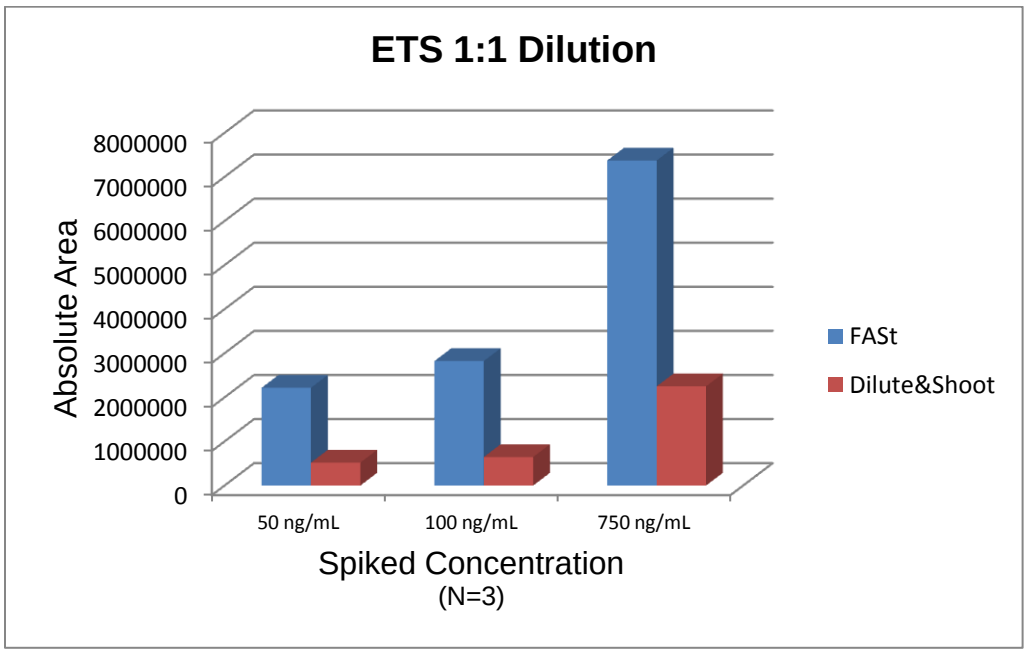


Injection 50



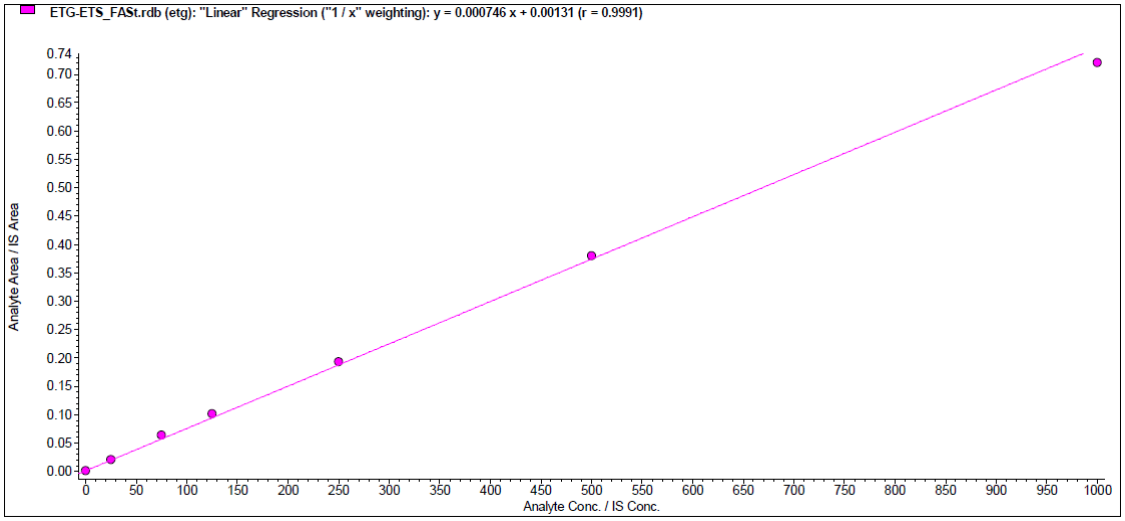
One of the main problems associated with the analysis of EtG/EtS is the deterioration of signal response over time due to matrix buildup on the analytical column and instrument source. To assess the efficiency of UCT's FASt® ETG column and method, a patient sample was monitored over the course of 50 injections. The above chromatograms compare the absolute peak heights of ETG & ETS on the initial injection versus the 50th, illustrating no significant loss in signal.



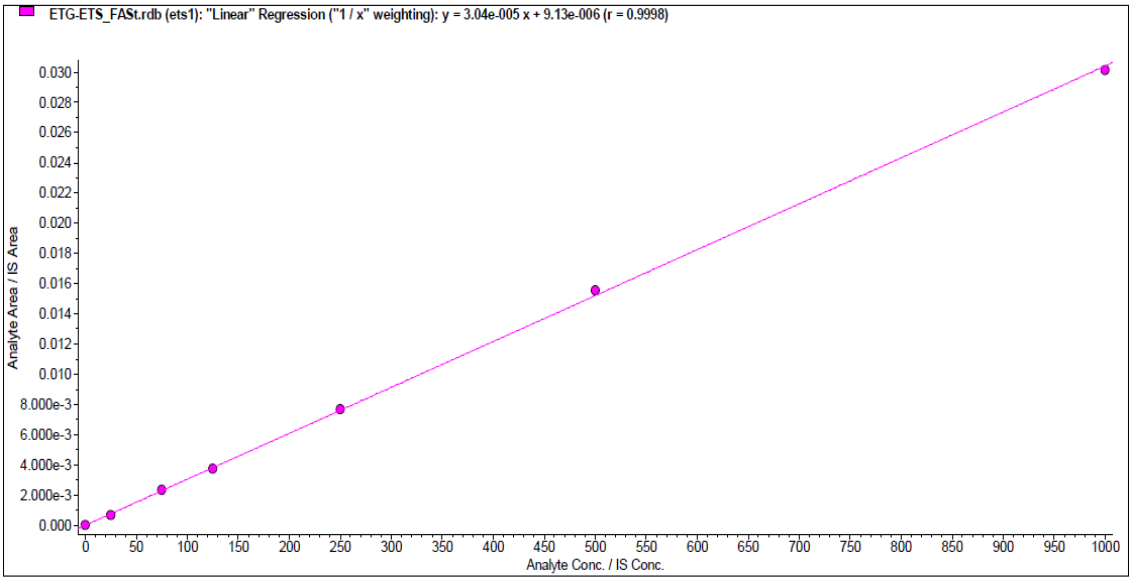


The above graphs compare the absolute peak areas of ETG & ETS utilizing the dilute and shoot method versus a filter and shoot method employing UCT's FASt® ETG column.

Calibration Curve of EtG using FASt®ETG 1:1 (R^2= 0.9991)



Calibration Curve of EtS using FASt®ETG 1:1 (R^2= 0.9998)



Recovery from Urine Spiked at 3 levels

Analyte	Recovery (n= 3)		
	50 ng/mL	100 ng/mL	750 ng/mL
EtG	76%	91%	101%
EtS	103%	102%	109%

Discussion

Overall sample volume is a limiting factor when using a dilute and shoot approach for routine analysis. One must compromise between obtaining acceptable limits of detection/quantitation and prolonged instrument down time due to the buildup of endogenous matrix materials.

UCT's FASt® ETG column utilizes standard size exclusion principles combined with a sorbent chemistry that adsorbs and removes the primary matrix compounds found in urine samples, allowing for up to 0.5mL of sample to be used. Due to the polar nature of these compounds, a 100% aqueous solution was used for dilution. This permits the samples to be loaded directly onto the instrument following filtration without compromising chromatographic peak shape and/or resolution.

Conclusions

1. Utilizing a dilute and shoot method increases the risk of instrument down time and shortens analytical column life. By integrating UCT's FASt® ETG column into routine sample prep, it helps to eliminate unwanted matrix and allows for cleaner samples to be injected onto instruments.

2. HPLC separation of extremely polar alcohol biomarkers was successfully conducted on UCT's Selectra® ETG column.

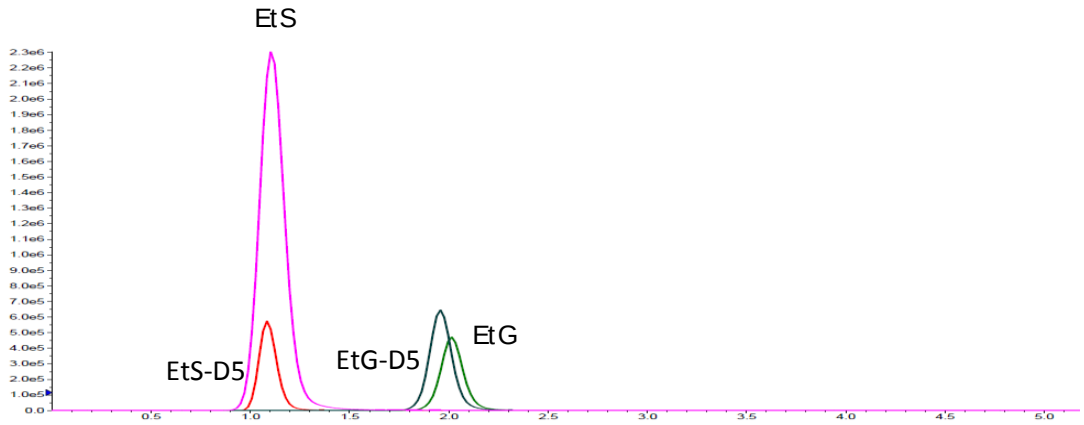
3. Due to the fact that the majority of matrix interferences found in urine are acidic, it can be anticipated that a residual amount will remain in the final eluate when using either a dilution or dilution and filtration method. Removal of these compounds can be better achieved when using a traditional solid phase extraction procedure, however, this not ideal for most high-throughput labs due to time and cost involved. It is strongly recommended to use matrix-matched calibration curves, which include isotopically labeled internal standards to compensate for any remaining matrix that is not removed.

Addendum

Alternative LC-MS/MS: Shorter Overall Run Time

Instrument: Agilent 1200 Binary Pump SL
Detector: AB Sciex API 4000 Q Trap MS/MS
Column: UCT Selectra® ETG HPLC column, 100 x 2.1 mm, 3 µm
Guard Column: UCT Selectra® ETG, 10 x 2.0 mm, 3 µm
Column Temperature: 30 ° C
Column Flow Rate: 0.3 mL/min
Injection Volume: 10 µL
Gradient Program:

Time (min)	% Mobile Phase A (0.1% FA in water)	% Mobile Phase B (0.1% FA in ACN)
0	100	0
1.5	100	0
1.7	0	100
2.7	0	100
3.0	100	0
6.0	100	0



To reduce the analytical run time, the ramp to organic was shortened along with the overall hold at 100% MPB. The initial parameters were developed as a protection mechanism to flush the column of any remaining organic matrix adhering to the bonded phase. While this new method still has a highly organic column wash in place, it has been significantly decreased in order to produce an even higher throughput assay.

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

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