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Application News

SSI-LCMS-096

Liquid Chromatography Mass Spectrometry

Quantitation of Ethyl Glucuronide and Ethyl Sulfate in Urine using LC-MS/MS

LCMS-8045

Summary: A short, robust quantitative method for the analysis of ethyl glucuronide (EtG) and ethyl sulfate (EtS) using a Nexera LC coupled to LCMS-8045 triple quadrupole mass spectrometer was developed.

Background: EtG and EtS are metabolites of ethanol that are used to measure alcohol use. These metabolites are important to measure for zero tolerance treatment programs and rehab facilities.

Method: EtG and EtS standards and deuterated internal standards were purchased from Restek Corporation (Bellefonte, PA). A working internal standard (IS) of 100 ng/mL of EtG-d5 and EtS-d5 was prepared in 0.1% formic acid as the dilution solvent. Working solutions of EtG and EtS were prepared at 50, 100, 200, 500, 1000, 2500, and 5000 ng/mL in both pooled urine and synthetic urine (surine). In addition, four QC working solutions were prepared at 50 (LLOQ), 125 (LQC), 700 (MQC), and 4000 (HQC) ng/mL in both pooled urine and surine. The calibration and QC standards were prepared by aliquoting 50 µL of each working solution and diluting with 950 µL with working IS solution to yield a total of seven calibrators and 4 QC samples for both pooled urine and surine. The standards were vortexed and centrifuged for 10 minutes at 4000 rpm and the supernatant was injected onto the LCMS-8045. The structures for EtG and EtS are shown in **Figure 1**.

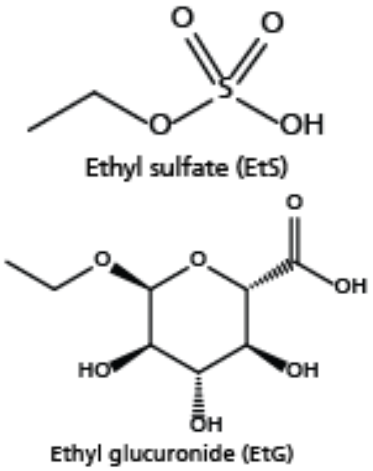

Figure 1. Chemical structures for ethyl sulfate (EtS) and ethyl glucuronide (EtG)

A Raptor EtG/EtS column from Restek (100 x 2.1mm, 2.7µm) was used with an UltraShield UHPLC precolumn filter (0.2 µm frit) and a linear binary gradient consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The gradient conditions are shown in **Table 1**. The column oven was at 30˚C and the injection volume was 10 µL.

Time (min)	%B
0.01	5
2.5	35
2.51	5
4	stop

Table 1. LC gradient conditions.

Negative mode electrospray ionization with multiple reaction monitoring (MRM) transitions were used for analysis on the LCMS-8045. The MRM transitions for each analyte and internal standard are shown in **Table 2**.

Analyte	MRM Transition	CE (V)
EtS	125.1>97.10	-17
	125.1>80.00	-30
EtS-d5	130.1>98.05	-19
EtG	221.2>75.10	-15
	221.2>85.00	-17
EtG-d5	226.2>85.00	-17

Table 2. MRM transitions for EtG, EtG-d5, EtS, EtS-d5.

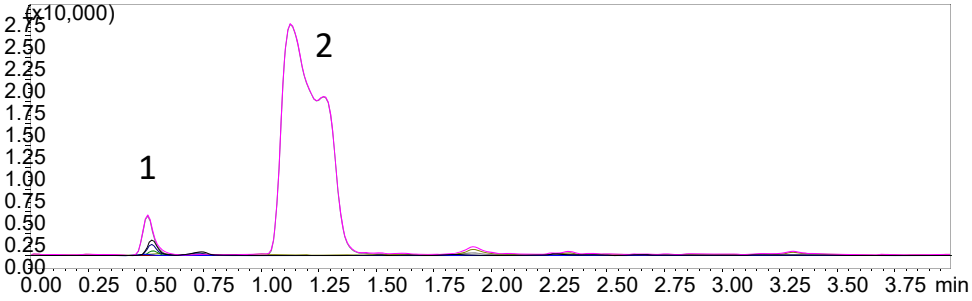

Figure 2. Chromatogram of double blank urine. Peak 1 and 2 show matrix interference peaks.

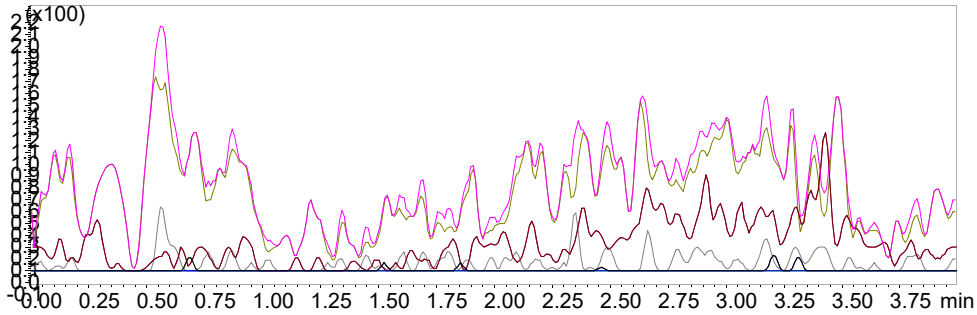

Figure 3. Chromatogram of double blank synthetic urine (surine).

Results and Discussion: In order to ensure that there were no interference peaks in matrix, a double blank of both urine and surine samples was run (**Figures 2** and **3**). The double blank has two known matrix peaks at 0.5 min and 1.2 min as shown. The surine sample did not show any matrix peaks. EtG elutes at 0.81 min and EtS elutes at 1.87 min which is baseline resolved from the matrix peaks.

This is shown in the chromatograms in **Figure 4** and **5** for the 100 ng/mL samples. The calibration curves for EtG and EtS in urine are shown in **Figures 6** and **7**, respectively. The green points represent the QC samples and red points are the calibration points. The accuracy for each QC ranged from 88-114% for EtG and 95-98% in EtS in the urine samples. For the surine samples, calibration curves are shown in **Figures 8** and **9**. The accuracy ranged from 88-99% for EtG and 91-102% for EtS. **Tables 3 and 4** show the results for each calibration curve.

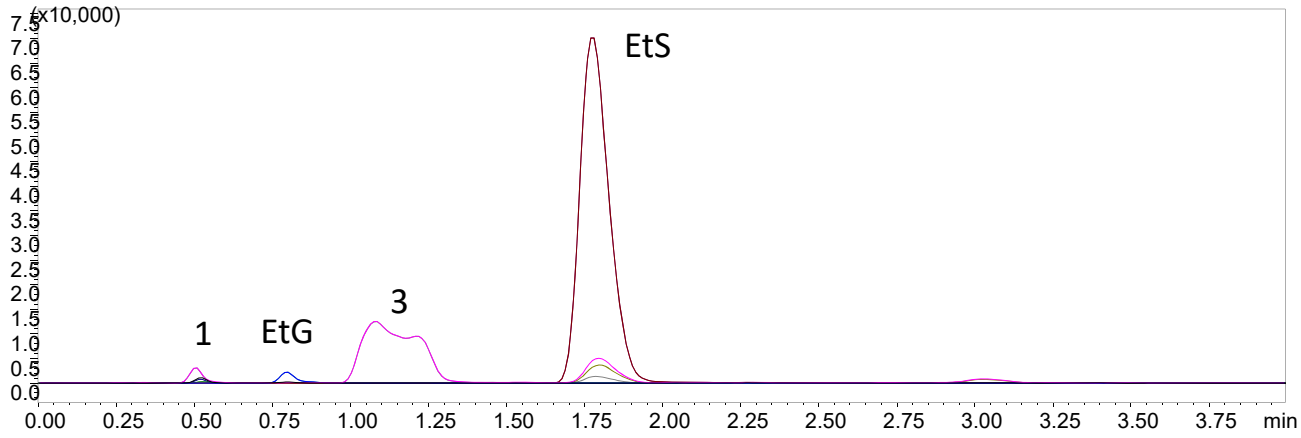

Figure 4. 100 ng/mL standard of EtG/EtS in Pooled Urine. EtG (peak 2), EtS (peak 4).

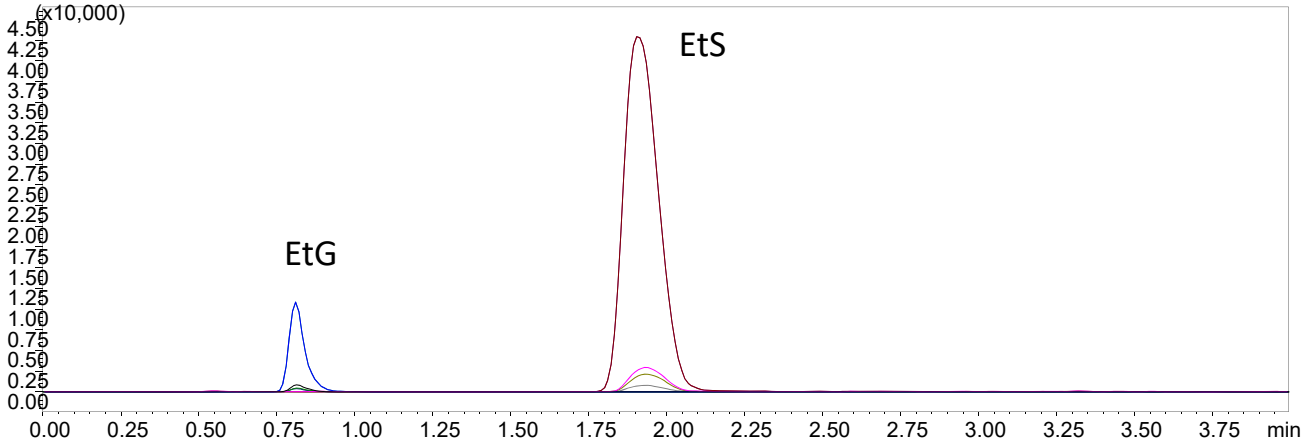

Figure 5. 100 ng/mL standard of EtG/EtS in Surine. EtG (peak 2), EtS (peak 4).

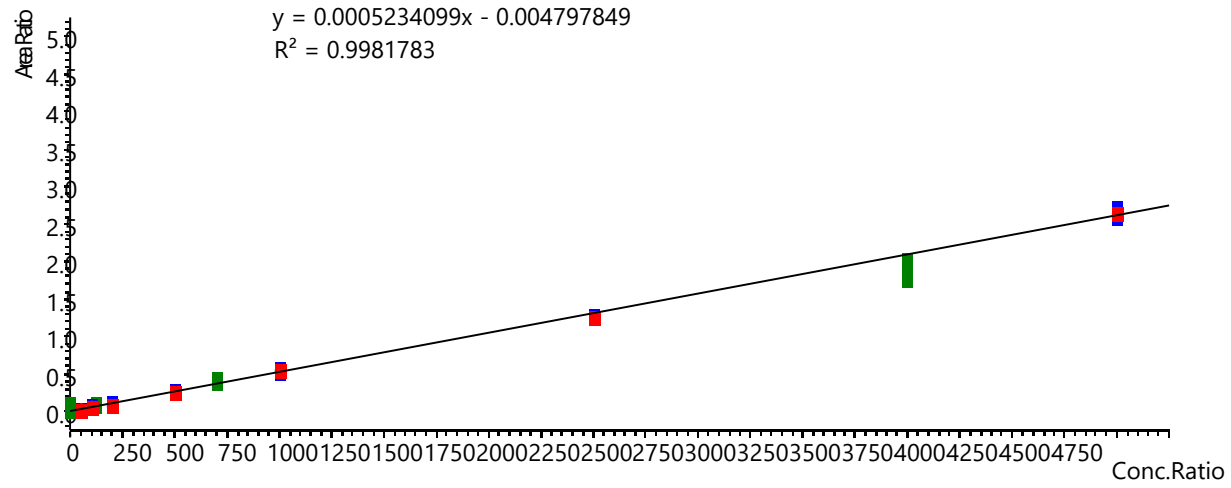

Figure 6. Calibration curve for EtG in urine. Red points are calibration points, Green points are QC samples.

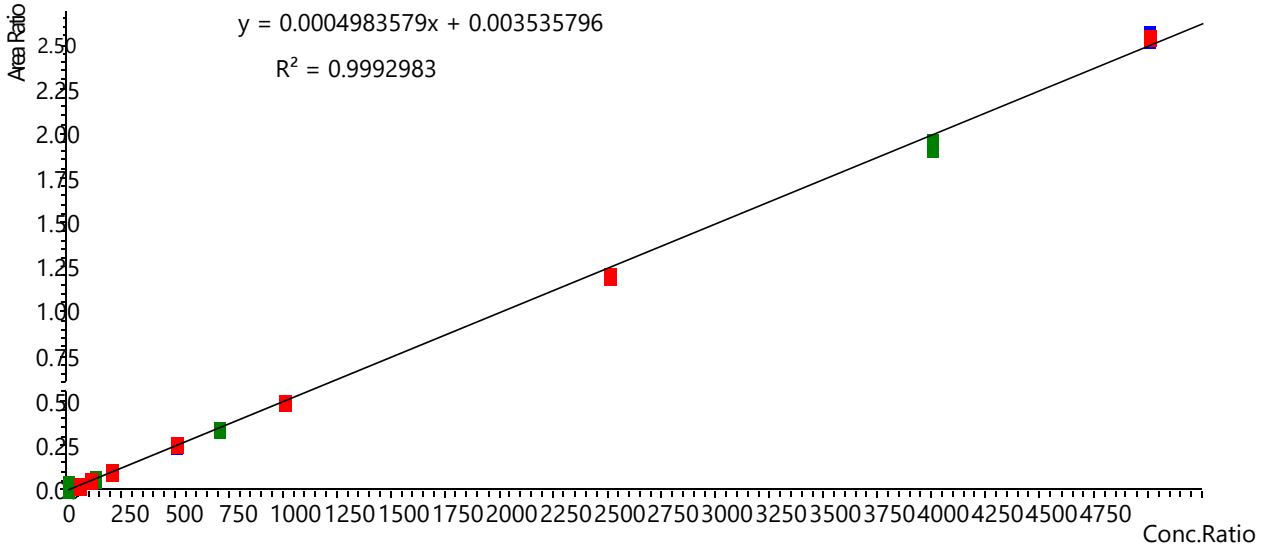

Figure 7. Calibration curve for EtS in urine. Red points are calibration points, Green points are QC samples.

QC	Spiked EtG/ EtS	Avg. EtG in Urine	% Accuracy	Avg. EtS in Urine	% Accuracy
LLOQ	50ng/mL	44.27	88.53	47.67	95.35
LQC	125ng/mL	142.81	114.25	123.03	98.43
MQC	700ng/mL	745.83	106.55	675.64	96.52
HQC	4000ng/mL	3691.14	92.28	3855.76	96.39

Table 3. Average calculated levels of EtG and EtS and %Accuracy for LLOQ, LQC, MQC and HQC samples in urine.

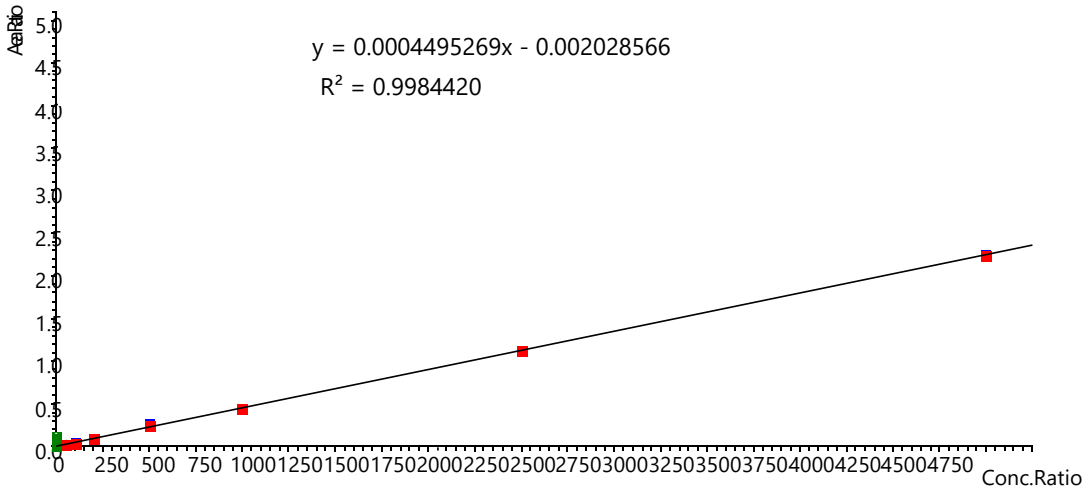

Figure 8. Calibration curve for EtG in surine.

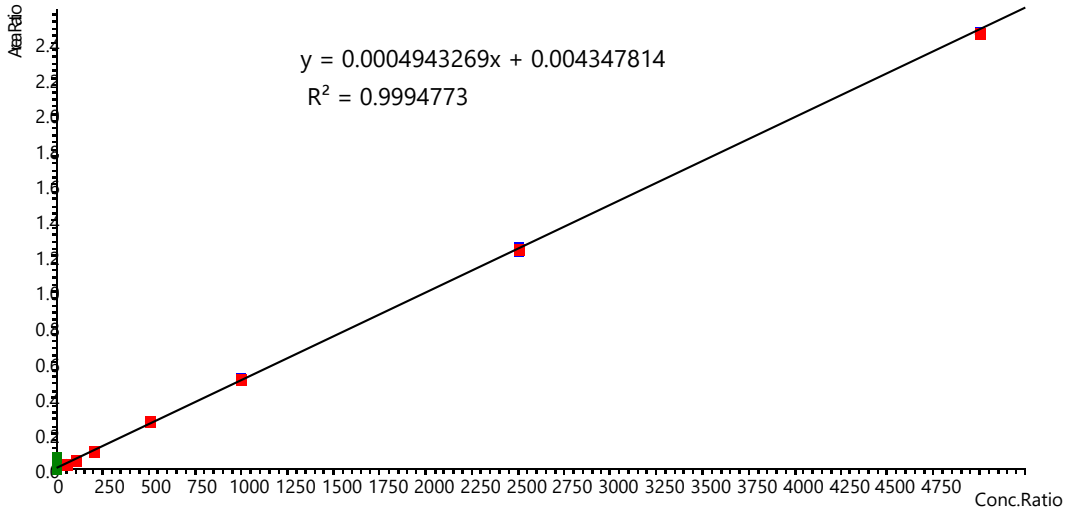

Figure 9. Calibration curve for EtS in surine.

QC	Spiked EtG/ EtS	Avg. EtG in Surine	% Accuracy	Avg. EtS in Surine	% Accuracy
LLOQ	50ng/mL	44.0	88.1	45.9	91.8
LQC	125ng/mL	119.6	95.7	123.9	99.1
MQC	700ng/mL	670.4	95.8	716.4	102.3
HQC	4000ng/mL	3953.1	98.8	4066.5	101.7

Table 4. Average calculated levels of EtG and EtS and %Accuracy for LLOQ, LQC, MQC and HQC samples in surine.

In addition to running calibration standards, two UTAK controls (level 1 and level 2) and two positive in-house urine samples (56045 and 56043) were analyzed with this method. The chromatograms for the UTAK controls are shown in **Figures 10** and 11 and the in-house positive samples are shown in **Figures 12** and **13**. The calculated concentrations for the UTAK standards are listed in **Table 5**. The calculated concentrations for the in house positive samples are listed in **Table 6**. One can clearly see that the matrix effects are fully resolved from the analyte peaks and the amount found in each sample can be easily quantified using the calibrations.

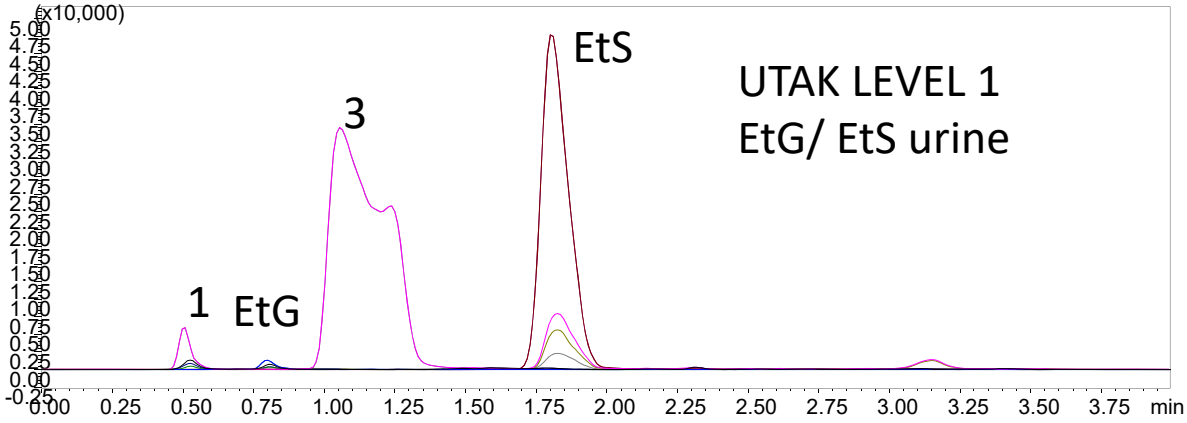

Figure 10. Chromatogram for UTAK Level 1 standard. Peaks 1 and 3 are matrix peaks.

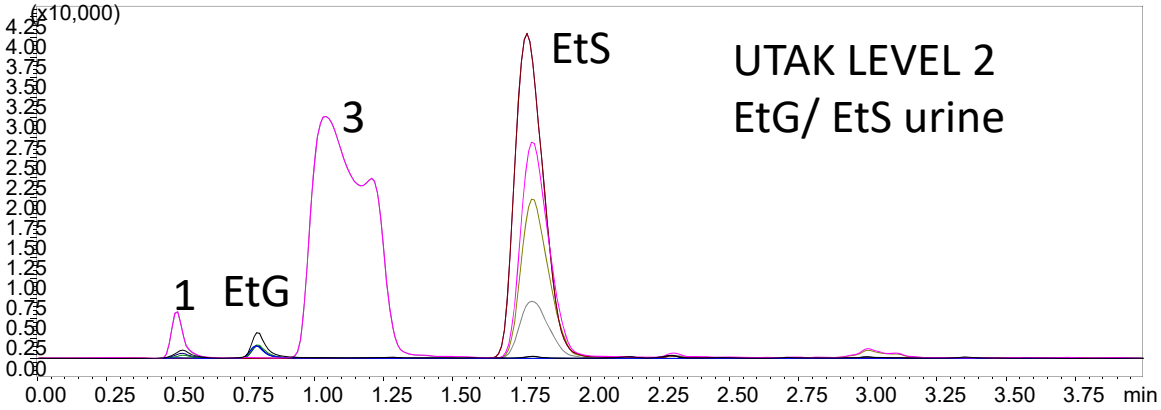

Figure 11. Chromatogram for UTAK Level 2 standard. Peaks 1 and 3 are matrix peaks.

	Level 1		Level 2	
	Target Value	Reference Value	Target Value	Reference Value
EtG	500ng/mL	481 ng/mL	2000 ng/mL	1870 ng/mL
EtS	200ng/mL	199 ng/mL	800 ng/mL	753ng/mL

Table 5. Calculated reference values for UTAK samples for EtG and EtS.

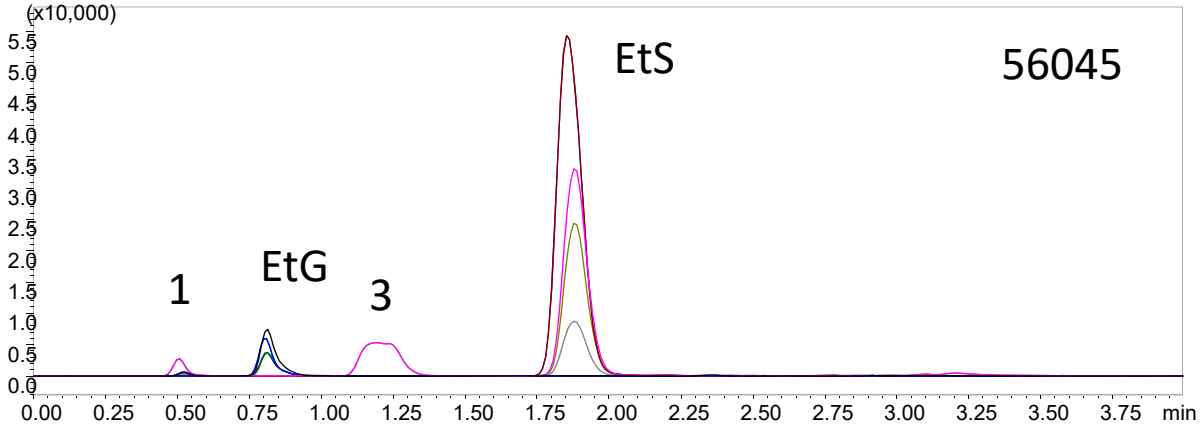

Figure 12. Chromatogram for 56045 positive in-house sample. Peaks 1 and 3 are matrix peaks.

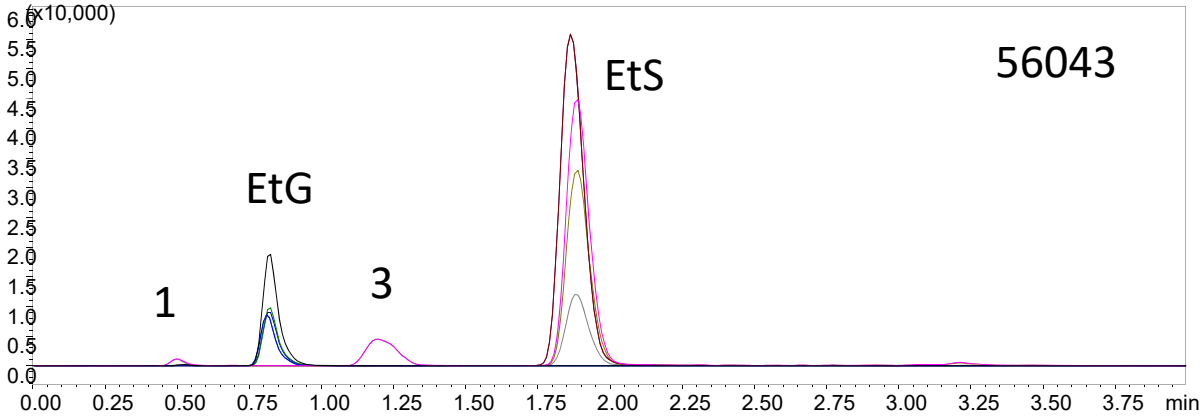

Figure 13. Chromatogram for 56043 positive in-house sample. Peaks 1 and 3 are matrix peaks.

Sample ID	Mean EtG Conc in Urine. ng/mL	Mean EtS Conc in Urine. ng/mL
56045	1461.3	845.0
56043	2275.4	1152.2

Table 6. Calculated values for positive in-house samples for EtG and EtS.

Conclusion: An rapid, accurate and robust method was developed using the LCMS-8045 triple quadrupole mass spectrometer to measure EtG and EtS in urine and surine samples. The accuracy values were all between 82-116% and the calibrations were able to successfully measure EtG and EtS in unknown samples. The ability to be able to fully resolve the urine matrix peaks from EtG and EtS allows for a simple and quantifiable method for analyzing these two biomarkers.

Acknowledgements: We would to thank Restek Corporation for their assistance with providing the standards, column and results for this application note.

Consumables used in this application

Part Number	Description
REST-9325A12	Raptor EtG/EtS. 2.7 µm x 100 x 2.1 mm
REST-25809	UltraShield UHPLC precolumn filter, 0.2 µm frit
REST-34101	Ethyl-β-D-glucuronide (EtG)
REST-34102	Ethyl-β-D-glucuronide-d5 (EtG-d5)
REST-34103	Ethyl Sulfate sodium salt (EtS)
REST-34104	Ethyl Sulfate-d5 sodium salt (EtS-d5)
220-91545-49	0.1% Formic acid in water (1L)
220-91545-47	0.1% Formic acid in acetonitrile (1L)
220-34001-01	Vials, LC, LabTotalKit, 100/pk
220-91239-40	EtG/EtS Analysis Startup Kit – RUO – Reference Material Version
220-91239-41	EtG/EtS Analysis Startup Kit – RUO – Certified Reference Material Version

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