Definitive EtG/EtS LC-MS/MS Analysis:

A Rugged 4-Min Method for High-Throughput Labs

By Justin Steimling and Frances Carroll

Abstract

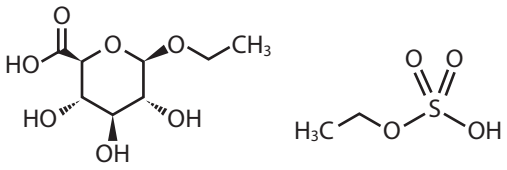
Methods for monitoring alcohol consumption biomarkers EtG and EtS are generally limited by poor retention and coelution with matrix interferences, as well as by long analysis times and short column lifetimes. The dilute-and-shoot EtG/EtS LC-MS/MS analysis developed here using the novel Raptor EtG/EtS column easily resolves EtG and EtS from matrix interferences, providing consistent, accurate results for high-throughput labs testing human urine samples for alcohol consumption.

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are important biomarkers of alcohol use. 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 analysis of EtG and EtS offers many advantages for abstinence monitoring, including a three-day detection window, good stability in properly stored specimens, and analytical specificity. However, EtG and EtS are both polar compounds, which makes them difficult to retain via reversed-phase chromatography. Both compounds are also very sensitive to matrix interferences, so accurate results can be difficult to obtain if retention is not sufficient to resolve EtG and EtS from the sample matrix. If matrix suppression occurs, low limits of detection may not be met. More important, if coeluting matrix components are isobaric to the compounds of interest, then accurate quantitation may be impossible.

Typical methods for EtG/EtS analysis have several shortfalls: poor retention and resolution of EtG and EtS from matrix components, long run times that limit sample throughput, and short column lifetimes. In this study, a simple dilute-and-shoot method was developed, validated, and applied to patient samples for EtG/EtS LC-MS/MS analysis in human urine. The method was developed and stringently tested on a Raptor EtG/EtS column, which features a novel stationary phase that was developed specifically for this critical application. This column was selected because it provides the retention characteristics that are needed to consistently elute the target analytes away from matrix interferences. In addition, it is rugged and long-lasting, which is beneficial to high-throughput labs.

Figure 1: Structures of EtG and EtS



Experimental

Standard and Sample Preparation

Human urine (alcohol free) was fortified with EtG and EtS in order to prepare calibration standards and QC samples. The concentration of calibration standards ranged from 50–5,000 ng/mL for both analytes. Four QC levels were prepared at 50, 150, 750, and 4,000 ng/mL.

Aliquots (50 µL) of urine were diluted with 950 µL of the working internal standard (100 ng/mL EtG-d5 and 25 ng/mL EtS-d5 in 0.1% formic acid in water). Samples were vortexed at 3,500 rpm for 10 seconds to mix followed by centrifugation at 3,000 rpm for 5 minutes at 10 °C. Additional double blanks were extracted for column equilibration.

LC-MS/MS Analysis

In order to ensure good response, peak shape, and retention time consistency, the analytical column was conditioned prior to use with 30 matrix injections that were run through the full gradient program. Instrument parameters for EtG/EtS LC-MS/MS analysis are shown below and the analyte transitions are given in Table I.

Analytical column: Raptor EtG/EtS, 2.7 µm, 100 mm x 2.1 mm (cat.# 9325A12)
Guard column: UltraShield UHPLC precolumn filter, 0.2 µm frit (cat.# 25809)
Mobile phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% Formic acid in acetonitrile
Gradient

Time (min)	%B
0.00	5
2.50	35
2.51	5
4.00	5

Flow rate: 0.5 mL/min
Injection volume: 10 µL
Column temp.: 35 °C
Ion mode: Negative ESI

Table I: Analyte Transitions

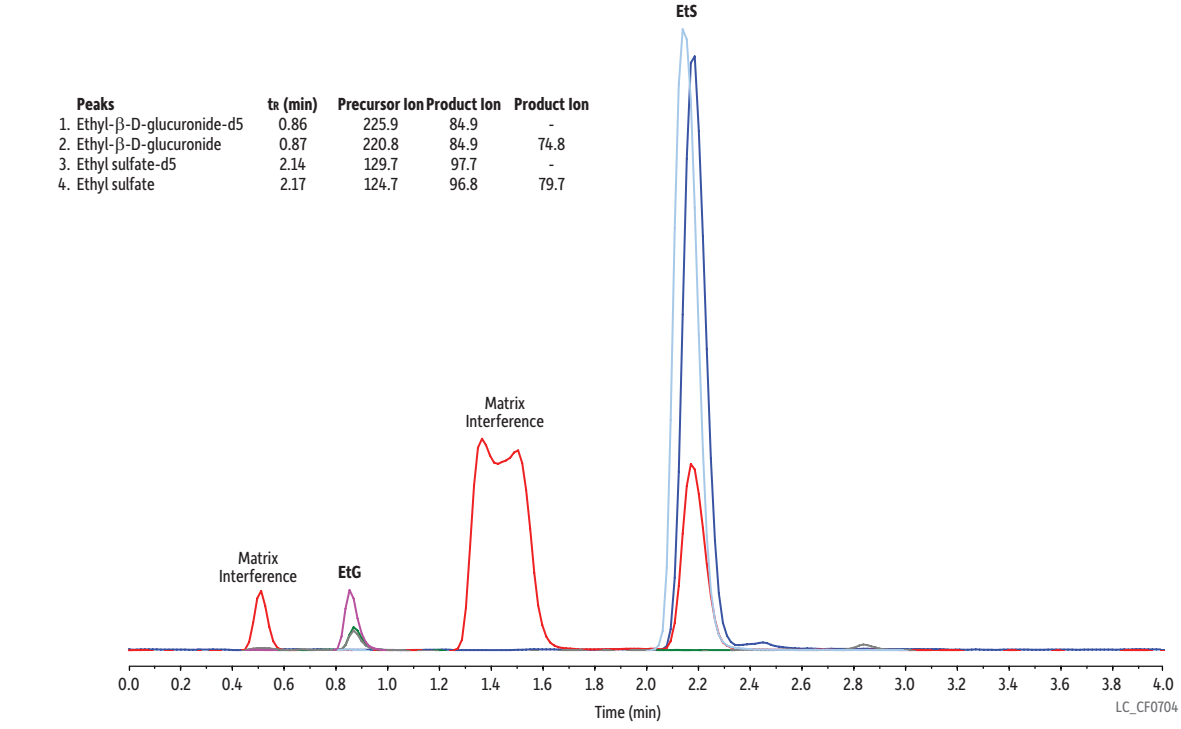
Analyte	Precursor Ion	Product Ion	Product Ion
EtG-d5	225.9	84.9	–
EtG	220.8	84.9	74.8
EtS-d5	129.7	97.7	–
EtS	124.7	96.8	79.7

Results and Discussion

Chromatographic Performance

A fast, four-minute EtG/EtS LC-MS/MS analysis was obtained from the direct (dilute-and-shoot) injection of supernatant (Figure 2). Both EtG and EtS are clearly resolved from matrix interferences, making accurate peak identification an easy task. As shown in Figure 3, low levels of quantitation can be obtained because matrix interferences are avoided.

Figure 2: Even with a fast, four-minute analysis time, the Raptor EtG/EtS column provides good retention of the target analytes so they elute well away from potential matrix interferences.



Peaks	t_R (min)	Precursor Ion	Product Ion	Product Ion
1. Ethyl-β-D-glucuronide-d5	0.86	225.9	84.9	-
2. Ethyl-β-D-glucuronide	0.87	220.8	84.9	74.8
3. Ethyl sulfate-d5	2.14	129.7	97.7	-
4. Ethyl sulfate	2.17	124.7	96.8	79.7

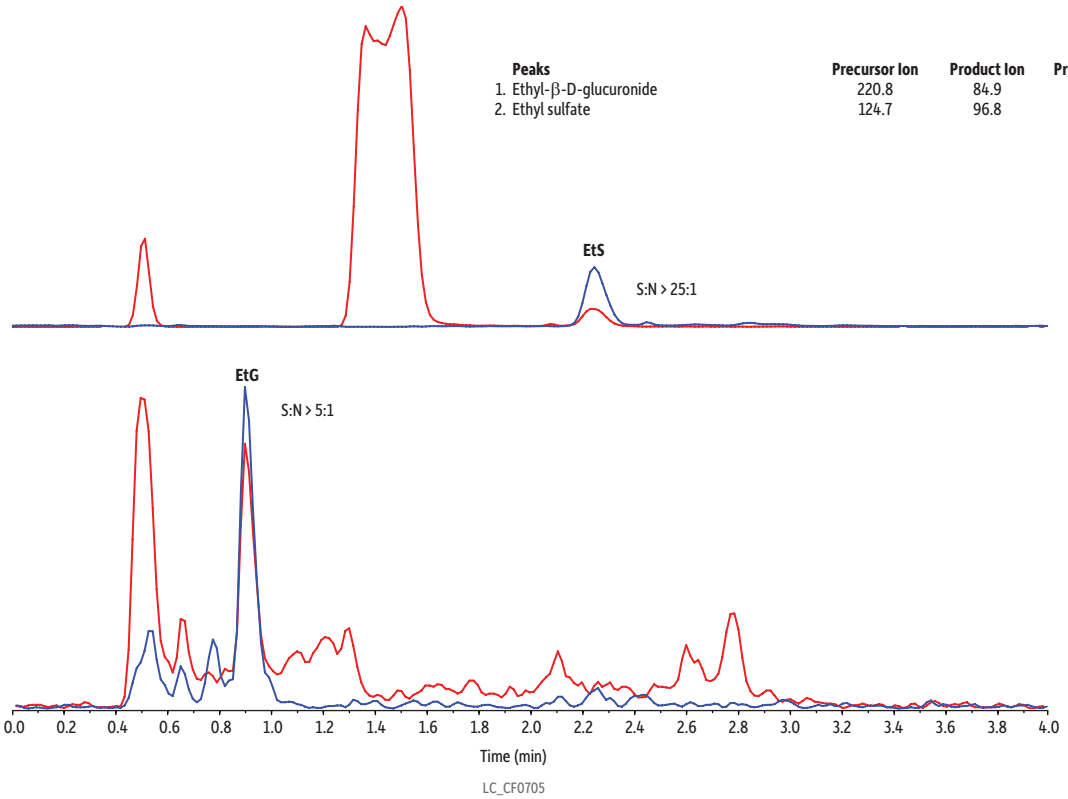
Column
Dimensions: Raptor EtG/EtS (cat.# 9325A12)
Particle Size: 100 mm x 2.1 mm ID
Pore Size: 2.7 µm
Guard Column: 90 Å
Temp.: UltraShield UHPLC precolumn filter, 0.2 µm frit (cat.# 25809)
Sample
Diluent: 35 °C
Conc.: 0.1% Formic acid in water
A 500 ng/mL standard was prepared in urine. 50 µL of the standard was diluted with 950 µL of a working internal standard (25 ng/mL EtS-d5/100 ng/mL EtG-d5 in 0.1% formic acid in water). The sample was vortexed at 3,500 rpm for 10 seconds to mix. The sample was then centrifuged at 3,000 rpm for 5 minutes at 10 °C. The autosampler needle was adjusted to inject from the supernatant.
Inj. Vol.: 10 µL

Mobile Phase
A: 0.1% Formic acid in water
B: 0.1% Formic acid in acetonitrile

Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	95	5
2.50	0.5	65	35
2.51	0.5	95	5
4.00	0.5	95	5

Detector MS/MS
Ion Mode: ESI-
Mode: MRM
Instrument HPLC
Notes **Reference Standards**
Ethyl-β-D-glucuronide (cat.# 34101)
Ethyl-β-D-glucuronide-d5 (cat.# 34102)
Ethyl sulfate sodium salt (cat.# 34103)
Ethyl sulfate-d5 sodium salt (cat.# 34104)

Figure 3: Low levels of quantitation can be met with this EtG/EtS LC-MS/MS analysis method because matrix interferences are avoided. (50 ng/mL LLOQ sample)



Column	Raptor EtG/EtS (cat.# 9325A12)
Dimensions:	100 mm x 2.1 mm ID
Particle Size:	2.7 µm
Pore Size:	90 Å
Guard Column:	UltraShield UHPLC precolumn filter, 0.2 µm frit (cat.# 25809)
Temp.:	35 °C
Sample	
Diluent:	0.1% Formic acid in water
Conc.:	A 50 ng/mL standard was prepared in urine. 50 µL of the standard was diluted with 950 µL of a working internal standard (25 ng/mL EtS-d5/100 ng/mL EtG-d5 in 0.1% formic acid in water). The sample was vortexed at 3,500 rpm for 10 seconds to mix. The sample was then centrifuged at 3,000 rpm for 5 minutes at 10 °C. The autosampler needle was adjusted to inject from the supernatant.
Inj. Vol.:	10 µL

Mobile Phase

A: 0.1% Formic acid in water

B: 0.1% Formic acid in acetonitrile

Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	95	5
2.50	0.5	65	35
2.51	0.5	95	5
4.00	0.5	95	5

Detector	MS/MS
Ion Mode:	ESI-
Mode:	MRM
Instrument	HPLC

Notes

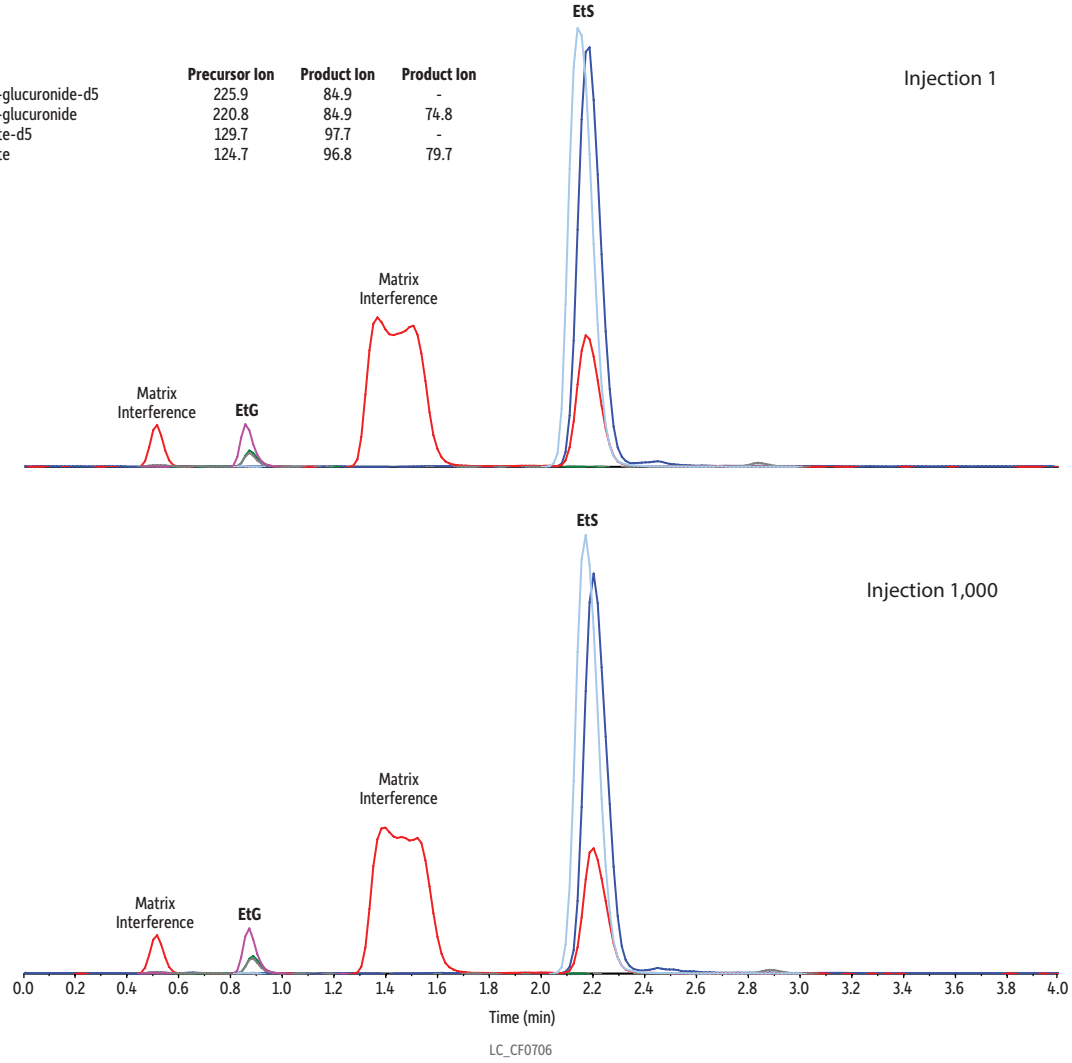
Reference Standards

Ethyl-β-D-glucuronide (cat.# 34101)
Ethyl-β-D-glucuronide-d5 (cat.# 34102)
Ethyl sulfate sodium salt (cat.# 34103)
Ethyl sulfate-d5 sodium salt (cat.# 34104)

Column Robustness

After 1,000 sample injections on a Raptor EtG/EtS column, all chromatographic peaks maintained the initial peak shape, retention time, and response (Figure 4). The maximum system pressure also remained at the same level, indicating no column clogging had occurred. This demonstrates the reliability of the column and method, which is particularly beneficial for high-throughput laboratories that need to minimize downtime for column changes.

Figure 4: Rugged Raptor EtG/EtS columns provide reliable results for EtG/EtS LC-MS/MS analysis allowing more samples to be analyzed between column changes.



Peaks	Precursor Ion	Product Ion	Product Ion
1. Ethyl-β-D-glucuronide-d5	225.9	84.9	-
2. Ethyl-β-D-glucuronide	220.8	84.9	74.8
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Column
Dimensions: 100 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: UltraShield UHPLC precolumn filter, 0.2 µm frit (cat.# 25809)
Temp.: 35 °C

Sample
Diluent: 0.1% Formic acid in water
Conc.: A 500 ng/mL QC sample was prepared in urine. 50 µL of the sample was diluted with 950 µL of a working internal standard (25 ng/mL EtS-d5/100 ng/mL EtG-d5 in 0.1% formic acid in water). The sample was vortexed at 3,500 rpm for 10 seconds to mix. The sample was then centrifuged at 3,000 rpm for 5 minutes at 10 °C. The autosampler needle was adjusted to inject from the supernatant.
Inj. Vol.: 10 µL

Mobile Phase
A: 0.1% Formic acid in water
B: 0.1% Formic acid in acetonitrile

Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	95	5
2.50	0.5	65	35
2.51	0.5	95	5
4.00	0.5	95	5

Detector MS/MS
Ion Mode: ESI-
Mode: MRM
Instrument HPLC
Notes
Reference Standards
Ethyl-β-D-glucuronide (cat.# 34101)
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Linearity

Using linear 1/x weighted regression for EtG and EtS, both compounds showed good linearity with r^2 values of 0.999 or greater over a calibration range of 50–5,000 ng/mL. Accurate calibration down to 50 ng/mL allows low-level positive results to be reported with confidence.

Accuracy & Precision

Precision and accuracy analyses were performed on three different days. The method accuracy was demonstrated to be within 6.3% of the nominal concentration for all QC levels for both EtG and EtS. The %RSD was 1.28–9.19% and 4.01–6.82% for intra- and inter-run, respectively, at the QC LLOQ. The %RSD was 0.675–7.78% and 1.00–4.99% for intra- and inter-run, respectively, at the QC low, mid, and high levels, indicating good method precision for EtG/EtS LC-MS/MS analysis (Table II).

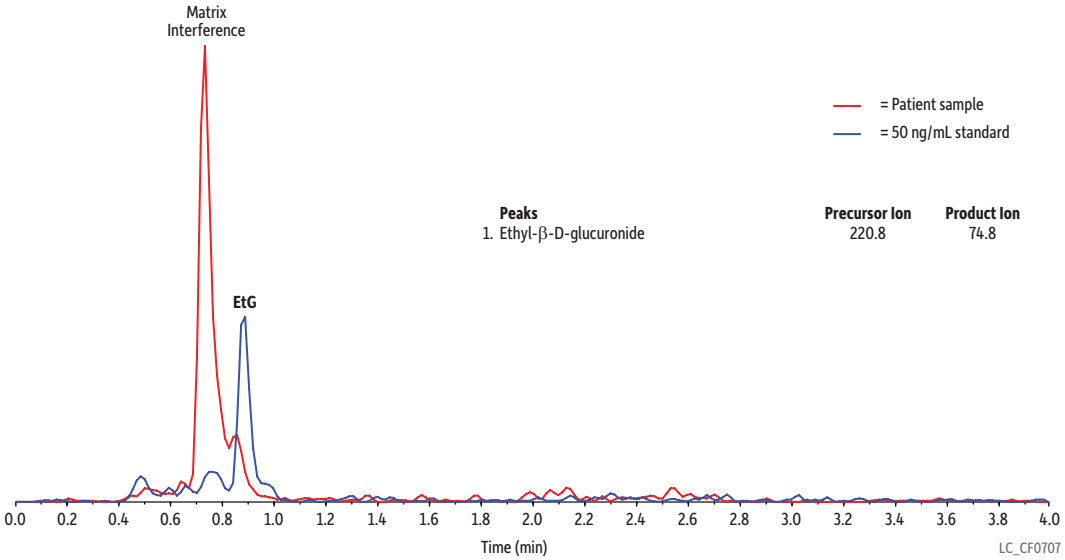
Table II: Accuracy and Precision of QC Samples

	QC LLOQ (50 ng/mL)			QC Low (150 ng/mL)			QC Mid (750 ng/mL)			QC High (4,000 ng/mL)		
Analyte	**Average Conc. (ng/mL)**	**Average Accuracy (%)**	**%RSD**	**Average Conc. (ng/mL)**	**Average Accuracy (%)**	**%RSD**	**Average Conc. (ng/mL)**	**Average Accuracy (%)**	**%RSD**	**Average Conc. (ng/mL)**	**Average Accuracy (%)**	**%RSD**
EtG	51.2	102	6.82	143	95.2	4.99	749	99.8	3.68	3,949	98.7	2.04
EtS	46.9	93.7	4.01	143	95.6	1.42	762	102	1.00	3,958	98.9	1.59

Selectivity

Complex biological matrices, such as urine, can vary across patients. All samples analyzed in this study contained the isobaric interference for EtS shown in Figure 2. However, the sample shown in Figure 5 contained an unusual isobaric interference for EtG. When overlaid with an LLOQ standard, this sample demonstrated the exceptional retention of the Raptor EtG/EtS column, which allowed even less common and more closely eluting matrix interferences to be resolved, preventing the reporting of false positives.

Figure 5: Even closely eluting matrix interferences are well resolved on the Raptor EtG/EtS column (overlay of LLOQ EtG standard and patient sample with EtG interference).



Column	Raptor EtG/EtS (cat.# 9325A12)
Dimensions:	100 mm x 2.1 mm ID
Particle Size:	2.7 µm
Pore Size:	90 Å
Guard Column:	UltraShield UHPLC precolumn filter, 0.2 µm frit (cat.# 25809)
Temp.:	35 °C
Sample	
Diluent:	0.1% Formic acid in water
Conc.:	50 µL of the sample or standard was diluted with 950 µL of a working internal standard (25 ng/mL EtS-d5/100 ng/mL EtG-d5 in 0.1% formic acid in water). The sample was vortexed at 3,500 rpm for 10 seconds to mix. The sample was then centrifuged at 3,000 rpm for 5 minutes at 10 °C. The autosampler needle was adjusted to inject from the supernatant.
Inj. Vol.:	10 µL

Mobile Phase	
A:	0.1% Formic acid in water
B:	0.1% Formic acid in acetonitrile

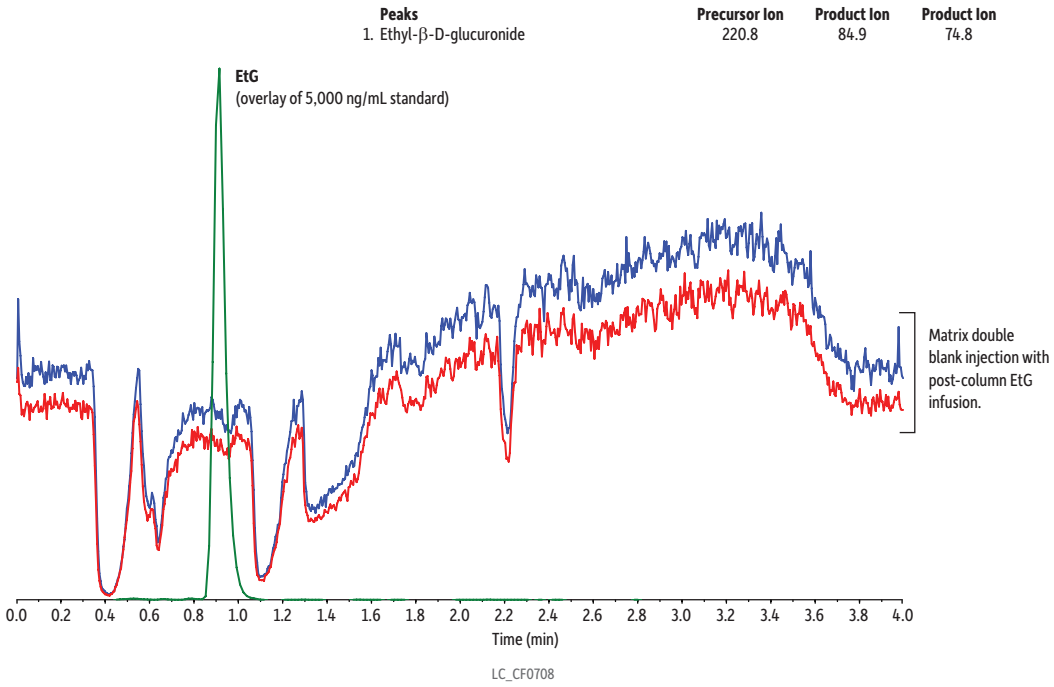
Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	95	5
2.50	0.5	65	35
2.51	0.5	95	5
4.00	0.5	95	5

Detector	MS/MS
Ion Mode:	ESI-
Mode:	MRM
Instrument	HPLC
Notes	**Reference Standards**
	Ethyl-β-D-glucuronide (cat.# 34101)
	Ethyl-β-D-glucuronide-d5 (cat.# 34102)
	Ethyl sulfate sodium salt (cat.# 34103)
	Ethyl sulfate-d5 sodium salt (cat.# 34104)

Matrix Effect

Samples prepared in matrix show approximately 80% (EtG) and 100% (EtS) of the signal obtained when samples are prepared in solvent across all QC levels, demonstrating that matrix effect is minimal. The sensitivity and consistency of the EtG response at the LLOQ is only possible because retention on the Raptor EtG/EtS column was optimized so that EtG elutes outside the zones of matrix suppression (Figure 6). Because the optimal EtG elution time was established here during method development, clinical labs adopting the method can be confident that matrix suppression has already been minimized. This, in combination with the established calibration range and LLOQ demonstrates that the method provides excellent sensitivity for EtG, which is generally harder to detect at low levels than EtS.

Figure 6: EtG elutes outside the matrix suppression zones (double blank injection with post-column EtG infusion).



Column	Raptor EtG/EtS (cat.# 9325A12)
Dimensions:	100 mm x 2.1 mm ID
Particle Size:	2.7 µm
Pore Size:	90 Å
Guard Column:	UltraShield UHPLC precolumn filter, 0.2 µm frit (cat.# 25809)
Temp.:	35 °C
Sample	
Conc.:	50 µL of urine was diluted with 950 µL of 0.1% formic acid in water. The sample was vortexed at 3,500 rpm for 10 seconds to mix. The sample was then centrifuged at 3,000 rpm for 5 minutes at 10 °C. The autosampler needle was adjusted to inject from the supernatant. A post-column infusion of 1,000 ng/mL EtG was made.
Inj. Vol.:	10 µL

Mobile Phase	
A:	0.1% Formic acid in water
B:	0.1% Formic acid in acetonitrile

Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	95	5
2.50	0.5	65	35
2.51	0.5	95	5
4.00	0.5	95	5

Detector	MS/MS
Ion Mode:	ESI-
Mode:	MRM
Instrument	HPLC

Sample Analysis

The robustness of the method was further tested by monitoring five patient samples with positive results for both EtG and EtS in the lower end of the linear range across multiple instrument platforms and nine lots of Raptor EtG/EtS columns. The precision of the results (n=9) was found to range from 3.24–11.2% for both analytes over multiple days and sample preparations, indicating excellent robustness and ease of method transfer (Table III). High-throughput labs in particular will benefit from implementing this rugged EtG/EtS LC-MS/MS analysis method as highly consistent performance is seen across a wide range of scenarios.

Table III: Inter-Run Precision of Patient Samples Across Multiple Instrument Platforms and Nine Column Lots

Patient Sample	Average EtG Concentration (ng/mL)	%RSD	Average EtS Concentration (ng/mL)	%RSD
A	216	6.13	78.0	3.56
B	1,167	4.81	300	3.24
C	98.2	9.76	82.4	4.01
D	319	8.33	233	3.63
E	247	11.2	163	8.70

Conclusion

It was demonstrated that the Raptor EtG/EtS column is excellent for the rapid and accurate analysis of EtG and EtS in human urine. Isobaric matrix interferences are easily resolved, preventing issues with peak identification and quantitation. In addition, superior sensitivity for EtG is achieved because it elutes outside the zones of matrix suppression that are typically observed with dilute-and-shoot assays. With a fast and simple sample preparation procedure and only four minutes of chromatographic analysis time, the EtG/EtS LC-MS/MS method established here provides accurate, high-throughput monitoring of alcohol consumption.

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