

# LC-MS/MS Analysis of EtG and EtS in Dilute Urine on the TSQ Endura Triple Quadrupole MS

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## Key Words

EtG, EtS, ethyl glucuronide, ethyl sulfate, ion pairing, TSQ Endura, DHAA (dihexylammonium acetate)

## Goal

To develop a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for the forensic toxicological analysis of EtG and EtS in urine with limits of quantitation (LOQs) of 100 and 50 ng/mL, respectively, using only urine dilution as sample preparation.

## Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are long-term biomarkers for ethanol consumption. Although they are minor metabolites of ethanol, their longer half-lives make them useful for detection of past alcohol use in forensic settings. These compounds are highly polar, which makes them retain poorly on most reversed-phase HPLC columns and elute on or near the chromatographic solvent front. This results in poor peak shape and large matrix effects. Here an ion-pairing reagent was used to retain these compounds on an HPLC column long enough to move them off the solvent front. This enabled better peak shape and less matrix interference.

## Methods

### Sample Preparation

Equal volumes (25  $\mu$ L) of urine and internal standard (5,000 and 500 ng/mL of EtG- $d_3$  and EtS- $d_3$ , respectively) were mixed and then diluted with 450  $\mu$ L of water. For analysis, 30  $\mu$ L were injected into the HPLC-MS/MS.

### Liquid Chromatography

Chromatographic separations were performed under gradient conditions using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system and Thermo Scientific Dionex UltiMate 3000 OAS. The analytical column was a Thermo Scientific™ Hypersil GOLD™ column (50 x 3 mm, 5  $\mu$ m particle size, catalog #25005-053030). The column was maintained at room temperature. The injection volume was 30  $\mu$ L. Mobile phases A and B consisted of 5 mM dihexylammoniumacetate (TCI America™) ion-pairing reagent in Fisher Chemical™ water and acetonitrile, respectively. The flow rate was 1 mL/minute, and the total run time was 5 minutes.



Figure 1. UltiMate 3000 RSLC system and TSQ Endura mass spectrometer

## Mass Spectrometry

MS analysis was carried out on a Thermo Scientific™ TSQ Endura™ triple-stage quadrupole mass spectrometer equipped with a Thermo Scientific™ Ion Max NG source and heated electrospray ionization (HESI-III) probe (Figure 1). Table 1 shows the mass spectrometer source parameters.

Table 1. TSQ Endura MS source parameters

Parameter	Value
Spray Voltage	3500 V
Sheath Gas	60 Arb
Aux Gas	20 Arb
Sweep Gas	0 Arb
Ion Transfer Tube	380 °C
Vaporizer	475 °C
Divert Valve	1.2–2.5 min

Two selected-reaction monitoring (SRM) transitions were monitored for EtG, EtS and their deuterated internal standards to provide ion ratio confirmations (IRC). The scans were run in timed selected-reaction monitoring (t-SRM) mode with a cycle time of 0.25 seconds. In this mode, SRM transitions are given a retention time and window in which the mass spectrometer acquires the specified transitions only. This allows the instrument to maximize the amount of time spent acquiring each transition, while maintaining a consistent number of data points across the chromatographic peak.

Data was acquired and processed with Thermo Scientific™ TraceFinder™ software.

### Validation

Intra-assay precision and accuracy were determined by analyzing a calibration curve along with six replicates of quality control (QC) samples. Inter-assay precision and accuracy were determined by analyzing a calibration curve along with six replicates of QC samples on three different days. Matrix effects were evaluated by observing the internal standard signals in 23 different lots of human urine.

### Results

Both compounds were linear over a wide dynamic range. EtG was linear from 50 to 50,000 ng/mL, while EtS had a range of 25 to 50,000 ng/mL. Figure 2 shows representative calibration curves for both compounds. Figure 3 shows representative chromatograms for EtG and EtS at their respective LOQs.

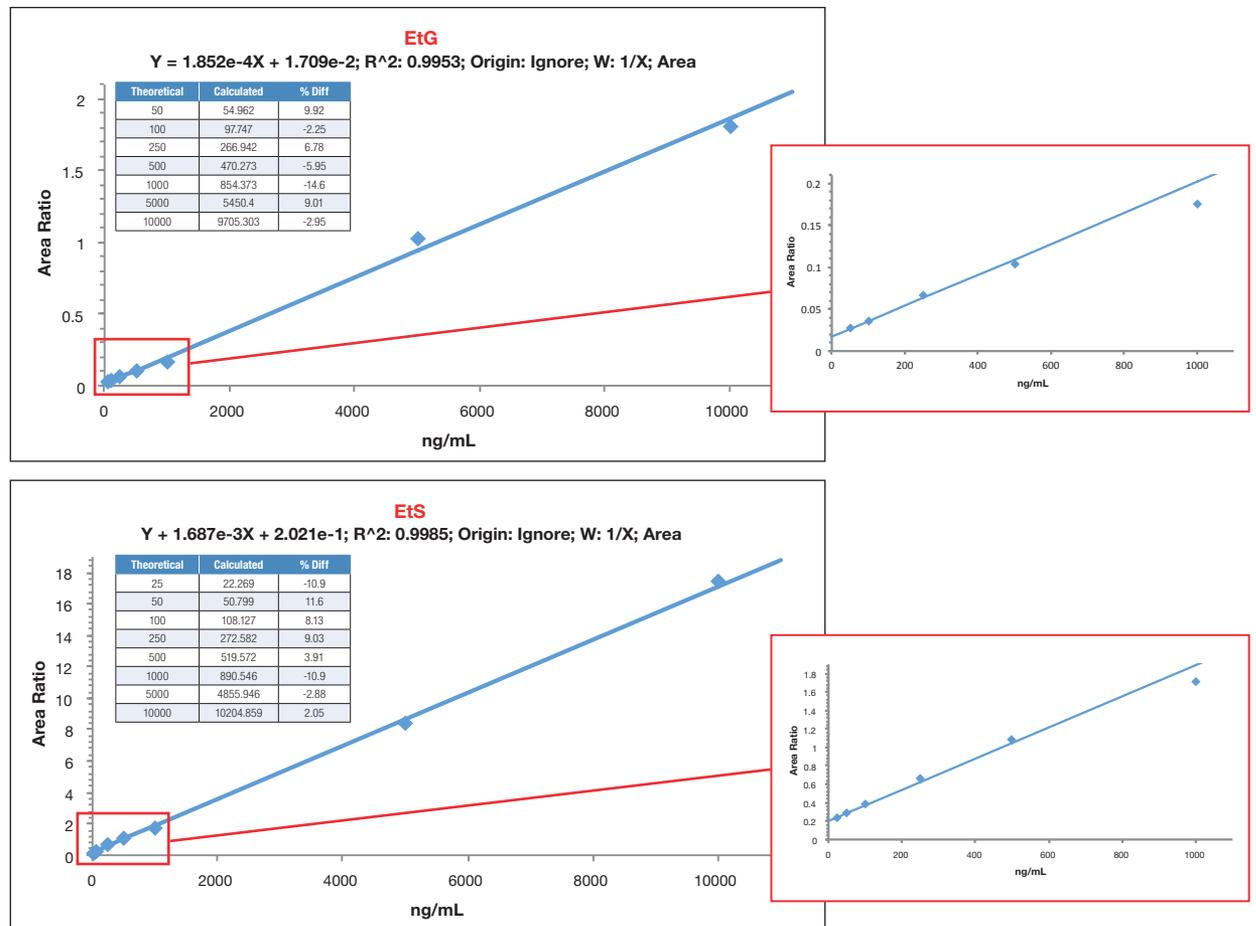


Figure 2. Representative calibration curves for EtG (top) and EtS (bottom) in urine

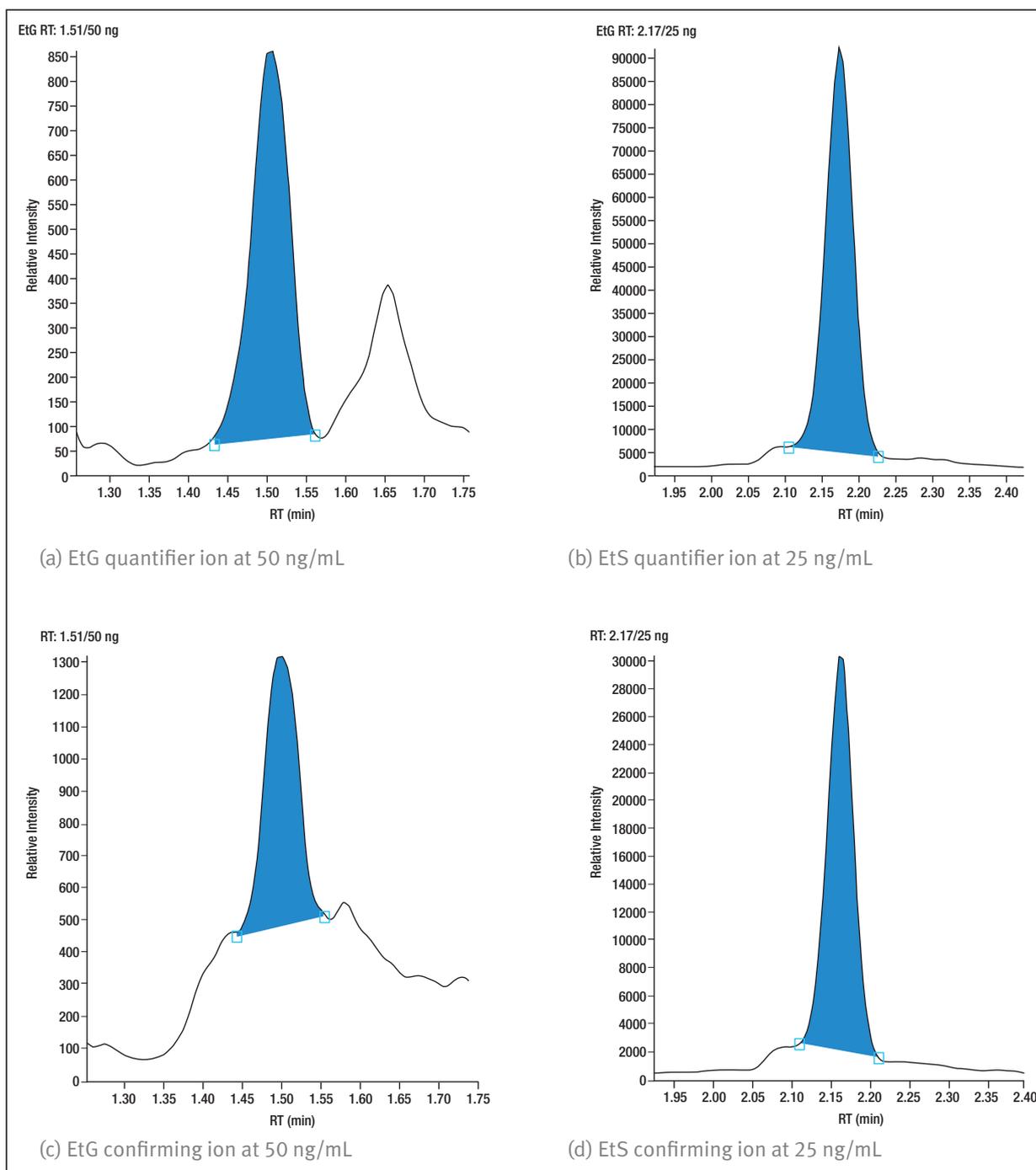


Figure 3. Representative chromatograms showing quantifier (a and b) and confirming (c and d) ions for EtG and EtS at 50 and 25 ng/mL, respectively

Table 2 shows the inter-assay precision and accuracy for EtG and EtS at 50 and 100 ng/mL.

Table 2. Inter-assay precision and accuracy for quality controls of EtG and EtS

	50 ng/mL		100 ng/mL	
	%Bias	%RSD	%Bias	%RSD
EtG	-0.253	12.0	3.31	6.94
EtS	-1.04	8.80	-1.99	5.67

Figure 4 displays the internal standard recovery compared to that of the calibrators.

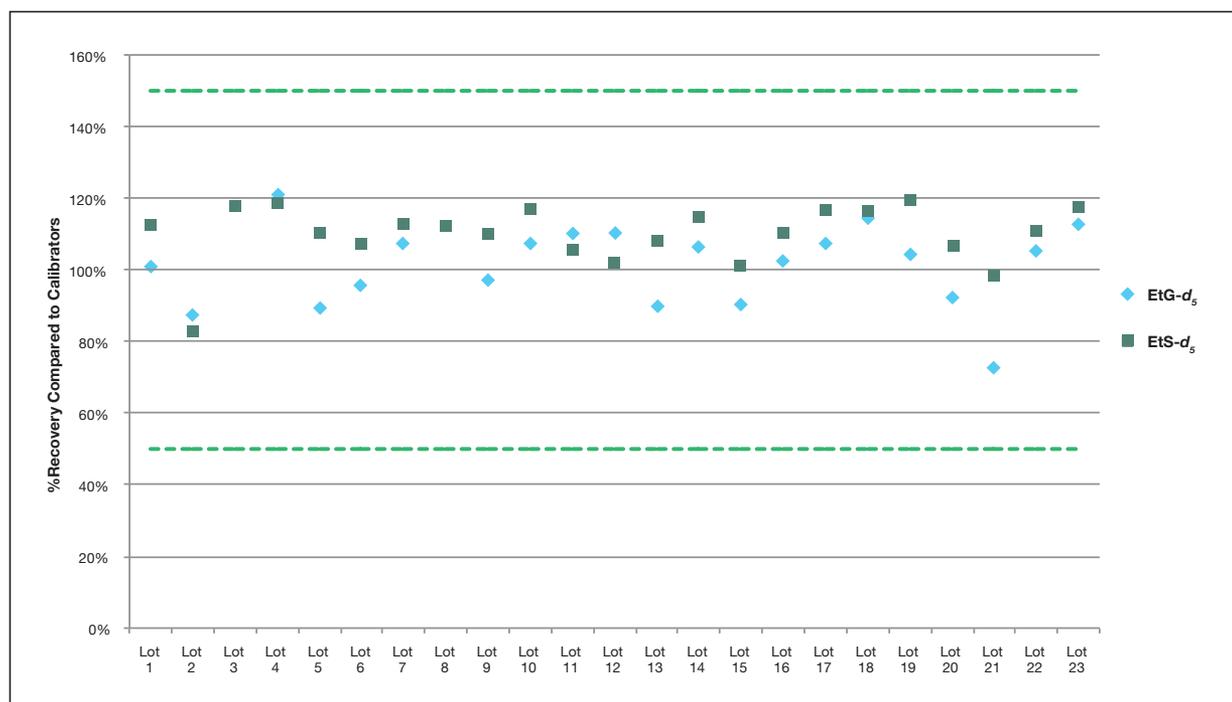


Figure 4. Internal standard recovery in 23 lots of urine compared to calibrators

## Conclusion

- This method gives limits of detection in urine of 50 ng/mL for EtG and 25 ng/mL for EtS while still maintaining a wide dynamic range up to 50,000 ng/mL.
- An ion-pairing reagent helps chromatographically separate the compounds from interferences on the solvent front, thereby improving limits of detection.
- The TSQ Endura MS is a robust system that provides accurate results within 5% and good precision all the way down to the LOQ.
- This method is suitable for forensic toxicology use.

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