HALO®

TECHNICAL REPORT: AMT_TR_TOX_25

TITLE: IMPROVED RETENTION OF ETHANOL METABOLITES ETHYL GLUCURONIDE AND ETHYL SULFATE USING A POSITIVELY CHARGED STATIONARY PHASE UNDER LOW IONIC STRENGTH CONDITIONS

MARKET SEGMENT: CLINICAL/TOXICOLOGY

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ABSTRACT

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are important biomarkers for ethanol consumption, but their high polarity often results in poor retention on conventional reversed-phase columns under acidic conditions. This report demonstrates how a positively charged stationary phase (PCS) improves retention and resolution of EtG and EtS under low ionic strength conditions (formic acid), reducing co-elution with early-eluting matrix components and enhancing method robustness for LC-MS/MS analysis.

INTRODUCTION

EtG and EtS are highly polar metabolites, making them challenging to retain on traditional reversed-phase columns, especially under acidic conditions commonly used for LC-MS compatibility. On uncharged stationary phases, these analytes often elute near the column void volume (t_0), where matrix interferences are most prevalent. This can compromise sensitivity and selectivity, particularly in complex biological matrices.

The use of a positively charged stationary phase introduces electrostatic interactions that complement hydrophobic retention, increasing analyte retention without requiring high ionic strength buffers. This approach maintains MS-friendly conditions while improving separation robustness. Additionally, mass spectrometry (MS) detection was employed throughout this study due to the poor UV absorbance of EtG and EtS, allowing for sensitive and selective quantification.

EXPERIMENTAL

All experiments were performed using LC-MS/MS detection with multiple reaction monitoring (MRM) transitions for each analyte. The full panel of 11 compounds included: Methadone, Norfentanyl, Oxycodone, Oxymorphone, Buprenorphine, Fentanyl, Hydrocodone, Hydromorphone, Alprazolam, EtG, and EtS. These compounds were selected for their clinical and toxicological relevance and varying hydrophobicity.

Chromatographic separations were performed using HALO® PCS Phenyl-Hexyl, Phenyl-Hexyl, PCS C18, and C18 columns (2.1 × 100 mm, 2.7 µm). For most separations the mobile phase A consisted of water with 0.1% formic acid, and mobile phase B was methanol with 0.1% formic acid. Gradient and isocratic conditions were tested, with flow rate, temperature, and injection volume optimized for each experiment.

MRM transitions for all compounds are listed in Table 1 and final concentrations for the full panel are listed in Table 2.

Name	Polarity	Precursor	Product Ion (1)	Product Ion (2)	Focus Voltage	Ion (1) Collision Energy	Ion (2) Collision Energy
Alprazolam	+	309.1	280.9	204.9	3.5	-35	-35
Buprenorphine	+	468.3	55.1	414.2	3.5	-35	-35
EtG	-	221.1	75	85	-2.5	16	20
EtS	-	125.1	97	80	-2.5	20	40
Fentanyl	+	337.3	188	105.1	3.5	-35	-35
Hydrocodone	+	300.1	199	128	3.5	-35	-35
Hydromorphone	+	286.2	184.9	156.9	3.5	-35	-35
Methadone	+	310.2	264.9	105.1	3.5	-35	-35
Norfentanyl	+	233.1	84.1	33	3.5	-35	-35
Oxycodone	+	316.2	298	169	3.5	-35	-35
Oxymorphone	+	302.1	227.2	198.2	3.5	-35	-35

Table 1. List of MRM transitions and polarity used to collect MS data.

Panel	
Compound	Concentration (ng/mL)
Methadone	20
Norfentanyl	40
Oxycodone	200
Oxymorphone	40
Buprenorphine	200
Fentanyl	10
Hydrocodone	12
Hydromorphone	40
Alprazolam	5
EtG	1000
EtS	100

Table 2. List of final concentrations for the DoA panel.

Results and Discussion

On a conventional C18 column under formic acid conditions, EtG and EtS exhibit minimal retention, eluting near t_0. This is due to their high polarity and lack of hydrophobic character, combined with the absence of ion-pairing agents in low ionic strength mobile phases. This early elution increases the risk of co-elution with matrix components, leading to ion suppression and poor reproducibility.

By contrast, the PCS phase introduces a permanent positive surface charge, which interacts with the negatively charged functional groups of EtG and EtS (carboxylate and sulfate moieties). This electrostatic attraction significantly increases retention, pulling the analytes away from the void region and improving separation from matrix interferences.

Retention Behavior and pH Study

A range of pH conditions was tested using ammonium acetate (pH 4.5, 5, 5.5, 6.7) and ammonium formate (pH 4). Across all four columns tested (PCS Phenyl-Hexyl, PCS C18, Phenyl-Hexyl, and C18), only minimal changes in retention for EtG and EtS were observed, with a slight increase at pH 4. These results confirmed that formic acid conditions are necessary to achieve sufficient retention for EtG and EtS, particularly on PCS phases. Non-charged phases consistently failed to provide adequate retention, making them unsuitable for these analytes. To the right are the testing conditions for 3 different pH values tested with data presenting the retention changes across pH.

TEST CONDITIONS:

Column: HALO 90 Å PCS C18, 2.7 µm, 2.1 x 100 mm
HALO 90 Å C18, 2.7 µm, 2.1 x 100 mm
HALO 90 Å PCS Phenyl-Hexyl, 2.7 µm, 2.1 x 100 mm
HALO 90 Å Phenyl-Hexyl, 2.7 µm, 2.1 x 100 mm

Mobile Phase A: 10mM Ammonium Acetate, pH - 6.72
10mM Ammonium Acetate, pH – 5.06
10mM Ammonium Formate, pH –3.97
Water + 0.1% Formic Acid

Mobile Phase B: Methanol
Methanol + 0.1% Formic Acid

Gradient:

Time	%B
0.0	3
2.0	3
4.5	95
5.0	95
5.1	3
10.0	3

Flow Rate: 0.4 mL/min.
Back Pressure: 241 bar
Temperature: 30 °C
Injection: 1µL (125ng/mL EtS, 2.5µg/mL EtG)
Sample Solvent: Methanol
LC System: Shimadzu Nexera X2
MS System: Shimadzu 8060nx Triple Quad

MS Conditions:

Polarity: Negative mode
Nebulizing Flow: 3 L/min.
Heating Gas Flow: 15 L/min.
Interface Temperature: 400 °C
Desolvation Temperature: 650 °C
Drying Gas Flow: 3 L/min.
DL Temperature: 250 °C
Heat Block Temperature: 400 °C

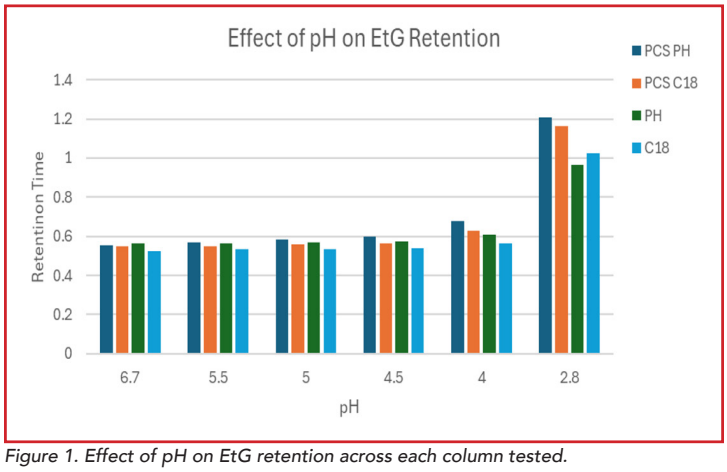

Figure 1. Effect of pH on EtG retention across each column tested.

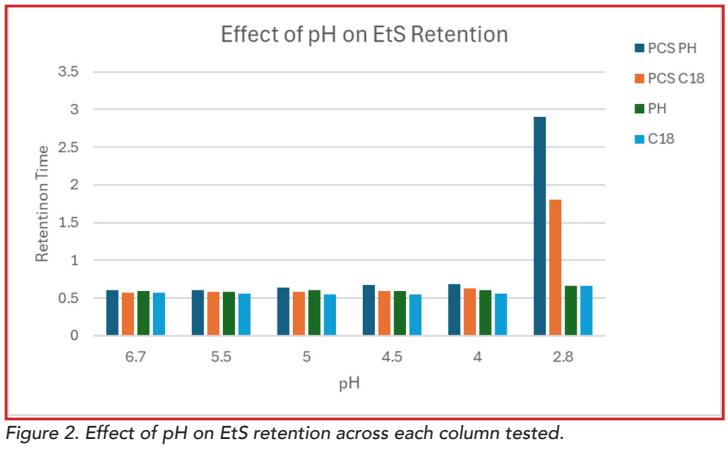

Figure 2. Effect of pH on EtS retention across each column tested.

EtG and EtS have minimal retention under all pH conditions but once formic acid as a modifier is introduced to the mobile phase, retention increases for both compounds. EtS retention increases significantly (5 fold) for the PCS Pheny-Hexyl phase. Interestingly, the retention flips for the uncharged phases. For standard Phenyl-Hexyl and C18 the retention of EtS is stagnant even with formic acid where EtG increases almost 2 fold.

Gradient Hold Experiments

To further improve retention, experiments were conducted using a 0% methanol hold at the beginning of the gradient compared to a 3% hold. The 0% hold introduced greater retention for EtS, providing more separation from the t_0 region and improving robustness in dirty sample matrices. Among all columns tested, PCS Phenyl-Hexyl consistently provided the highest retention for both EtG and EtS.

TEST CONDITIONS:

Column: HALO 90 Å Phenyl Hexyl, 2.7 µm, 2.1 x 100 mm
Mobile Phase A: Water + 0.1% Formic Acid
Mobile Phase B: Methanol + 0.1% Formic Acid

Gradient:

Time	%B
0.0	3 or 0
2.0	3 or 0
4.5	95
5.0	95
5.1	3
10.0	3

Flow Rate: 0.4 mL/min.
Back Pressure: 241 bar
Temperature: 30 °C
Injection: 1µL (125ng/mL EtS, 2.5µg/mL EtG)
Sample Solvent: Methanol
LC System: Shimadzu Nexera X2
MS System: Shimadzu 8060nx Triple Quad

MS Conditions:

Polarity: Negative mode
Nebulizing Flow: 3 L/min.
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Interface Temperature: 400 °C
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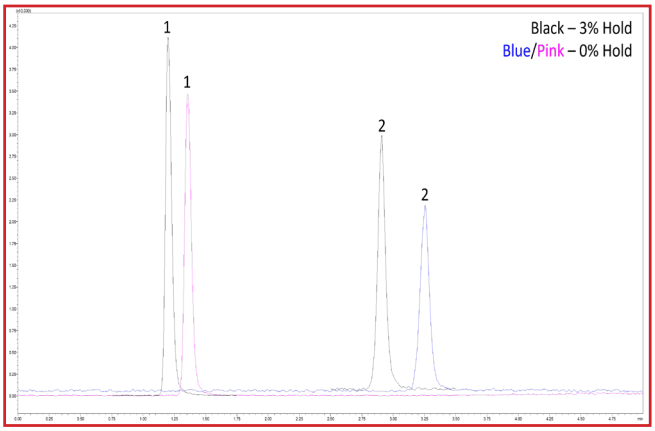

Figure 3. A comparison of EtG (Peak 1) and EtS (Peak 2) separated on the HALO® PCS Phenyl-Hexyl column. A reduced organic hold (0%) was incorporated to increase retention of both compounds to limit any potential interactions with dirty samples.

With the testing at 0% and 3% holds it seemed unnecessary for our testing to run a gradient. By switching to isocratic conditions, we could gather more reproducible peak statistics.

Isocratic Separation of EtG and EtS

An isocratic method was developed using the PCS Phenyl-Hexyl column under 3% methanol conditions. Isocratic separations are advantageous for their simplicity, repeatability, and ease of method transfer. They eliminate gradient-related variability and are particularly beneficial for routine testing where consistent retention times are critical. The PCS Phenyl-Hexyl column demonstrated excellent stability and reproducibility under these conditions.

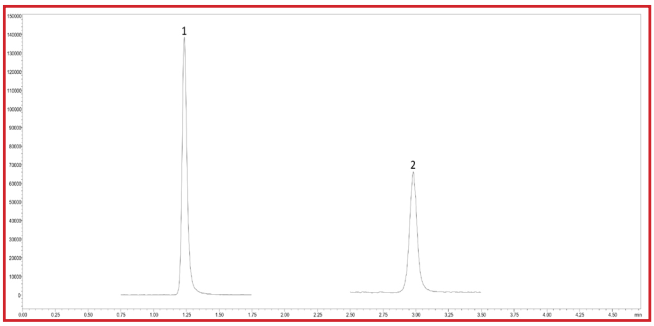

Figure 4. An isocratic separation of EtG (1) and EtS (2) performed on the HALO® PCS Phenyl-Hexyl.

A great way to confirm that a method is robust and reliable is to perform calibration curves. Calibration curves for EtG and EtS were generated under isocratic conditions to assess method linearity and column performance. Excellent linearity was observed, demonstrating the robustness and reproducibility of the HALO® PCS Phenyl-Hexyl column. Calibration curves are essential for quantitative analysis, as they validate the method's accuracy and precision across the expected concentration range. Strong linearity also reflects the column's stability and suitability for routine use.

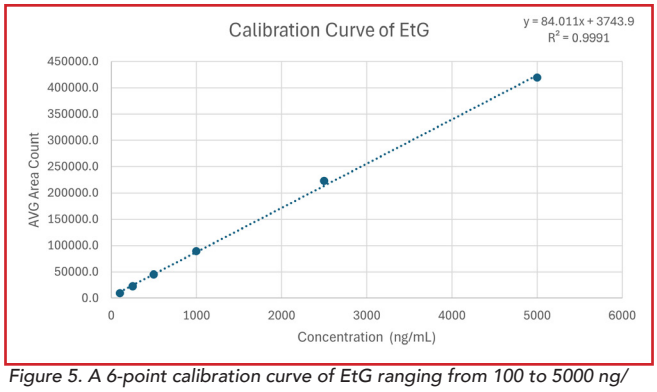

Figure 5. A 6-point calibration curve of EtG ranging from 100 to 5000 ng/mL, with findings of high reproducibility for the HALO® PCS Phenyl-Hexyl column.

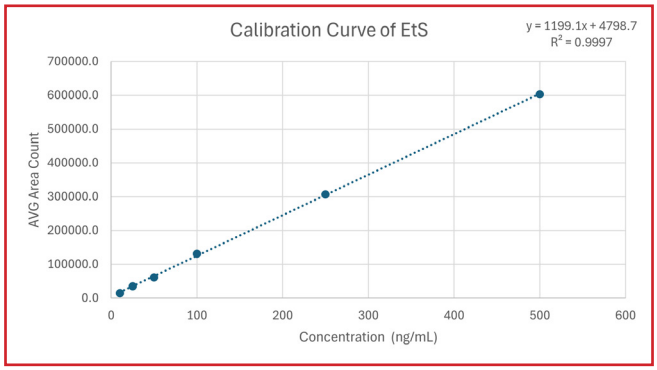

Figure 6. A 6-point calibration curve of EtS ranging from 10 to 500 ng/mL, with findings of high reproducibility for the HALO® PCS Phenyl-Hexyl column.

Multi-Analyte Panel Testing

A full panel of 11 compounds was analyzed to simulate realistic clinical and toxicological testing scenarios. The panel was first run on the PCS Phenyl-Hexyl column with a gradient hold to shift analytes away from t_0. A second run used a rapid gradient (0% to 100% in 5 minutes) to evaluate throughput. Finally, the panel was run on a PCS C18 column to compare elution behavior. The PCS C18 column showed increased retention for early eluters like oxymorphone, hydromorphone, and EtG, but reduced retention for EtS. These results provide users with options depending on their analyte panel and retention needs.

TEST CONDITIONS:

Column: HALO 90 Å PCS Phenyl-Hexyl, 2.7 µm, 2.1 x 100 mm
Mobile Phase A: 0.1% Formic Acid in Water, pH- 2.8
Mobile Phase B: 0.1% Formic Acid in Methanol
Gradient:

Time	%B
0.0	0
2.0	0
5.0	100
5.5	100
5.6	0
7.5	0

Flow Rate: 0.4 mL/min.
Back Pressure: 215 bar
Temperature: 30 °C
Injection: 1µL
(Final Mix – Range of Concentrations)
Sample Solvent: H_2O

PEAK IDENTITIES:

1. Oxymorphone
2. Hydromorphone
3. ETG
4. Oxycodone
5. ETS
6. Hydrocodone
7. Norfentanyl
8. Fentanyl
9. Buprenorphine
10. Methadone
11. Alprazolam

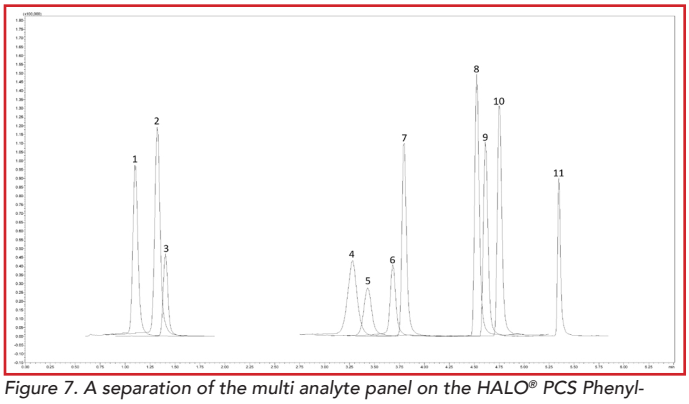

Figure 7. A separation of the multi analyte panel on the HALO® PCS Phenyl-Hexyl column.

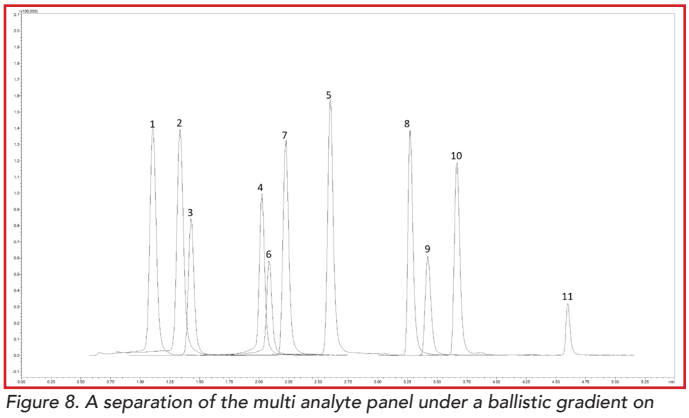

Figure 8. A separation of the multi analyte panel under a ballistic gradient on the HALO® PCS Phenyl-Hexyl.

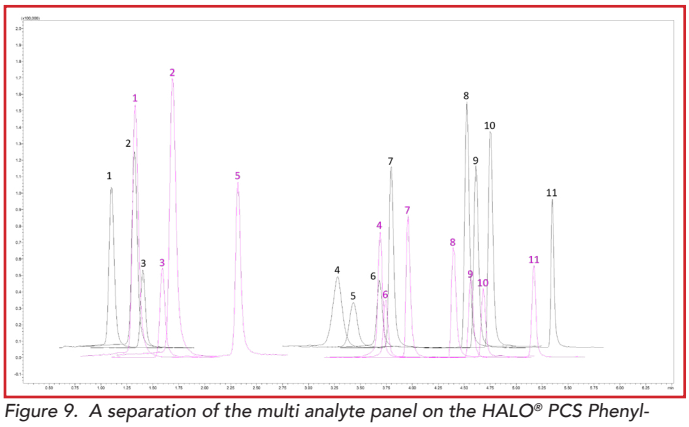

Figure 9. A separation of the multi analyte panel on the HALO® PCS Phenyl-Hexyl (Black) and the HALO® PCS C18 (Pink).

Re-equilibration Time Optimization

To determine the minimum re-equilibration time required for consistent retention, a series of experiments were conducted with decreasing re-equilibration times: 10 min, 5 min, 2.5 min, 1 min, and 0.5 min. Retention time shifts were observed at 1 min and below, indicating that a minimum of 1.9 minutes (equivalent to 3.74 column volumes) is necessary for stable performance on a 2.1 × 100 mm column using Fused-Core® technology. Reducing re-equilibration time is particularly advantageous with superficially porous particles because their unique structure, featuring a solid core and thin porous shell, allows for faster mass transfer and shorter diffusion path lengths. This means the stationary phase can return to equilibrium more quickly than fully porous particles, enabling higher sample throughput without sacrificing chromatographic stability. This reduction in downtime translates directly into improved productivity for high-throughput laboratories, where even **small-time** savings per run can significantly increase daily sample capacity.

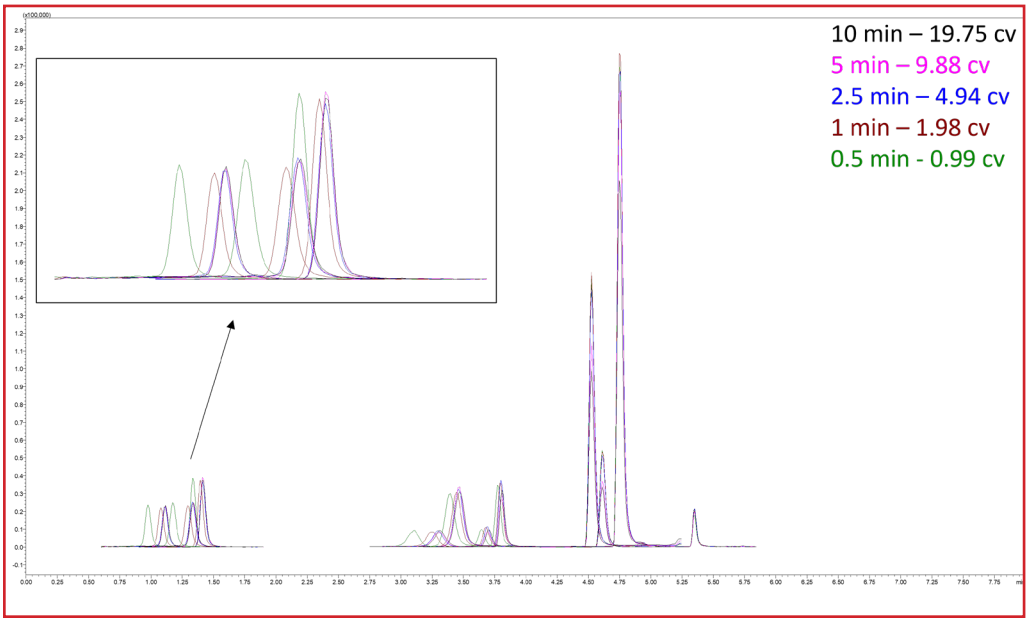

Figure 10. Mass spectra displaying the effect of re-equilibration time on retention stability.

TEST CONDITIONS:

Column: HALO 90 Å PCS Phenyl-Hexyl, 2.7 µm, 2.1 x 100 mm

Mobile Phase A: 0.1% Formic Acid in Water, pH- 2.8

Mobile Phase B: 0.1% Formic Acid in Methanol

Gradient:

Time	%B
0.0	0
2.0	0
5.0	100
5.5	100
5.6	0
Variable	0

Flow Rate: 0.4 mL/min.

Back Pressure: 241 bar

Temperature: 30 °C

Injection: 1µL (125ng/mL EtS, 2.5µg/mL EtG)

Sample Solvent: Methanol

LC System: Shimadzu Nexera X2

MS System: Shimadzu 8060nx Triple Quad

MS Conditions:

Polarity: Negative mode
Nebulizing Flow: 3 L/min.
Heating Gas Flow: 15 L/min.
Interface Temperature: 400 °C
Desolvation Temperature: 650 °C
Drying Gas Flow: 3 L/min.
DL Temperature: 250 °C
Heat Block Temperature: 400 °C

CONCLUSION:

The HALO® PCS Phenyl-Hexyl column provides superior retention and separation of EtG and EtS under low ionic strength, MS-compatible conditions. Isocratic separations offer enhanced repeatability, while re-equilibration optimization ensures high throughput without compromising performance. The column also supports robust multi-analyte testing, making it ideal for clinical and forensic applications. Calibration curve results confirm the column's reliability and efficiency for quantitative LC-MS/MS analysis.