Differential Ion Mobility Spectrometry Tandem Mass Spectrometry

Improved Assay Selectivity and Sensitivity for Quantitative Determination of Total NNN, Total NNAL and 2-/3-HPMA in Human Urine

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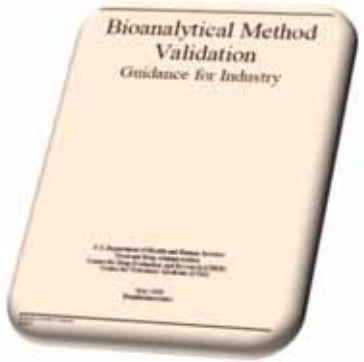
TBOE Assay Initiatives

- Align TBOE requirements with state-of-the-art technology, where applicable, to achieve next level LC-MS assay performance:
  - *Improved Selectivity > Lower Detection Limits > Higher Sample Throughput*
- Developed assays must adhere to the rigors of the FDA BMV 2018 guidelines:
  - *Matrix Factor, Specificity, Linearity, Recovery, Dilution Integrity*
  - *F/T, ST and LT Matrix Stability, Autosampler Stability*
  - *Precision and Accuracy (≥ 3 batches)*
  - *Deconjugation Efficiency*
  - *OTC Evaluation*



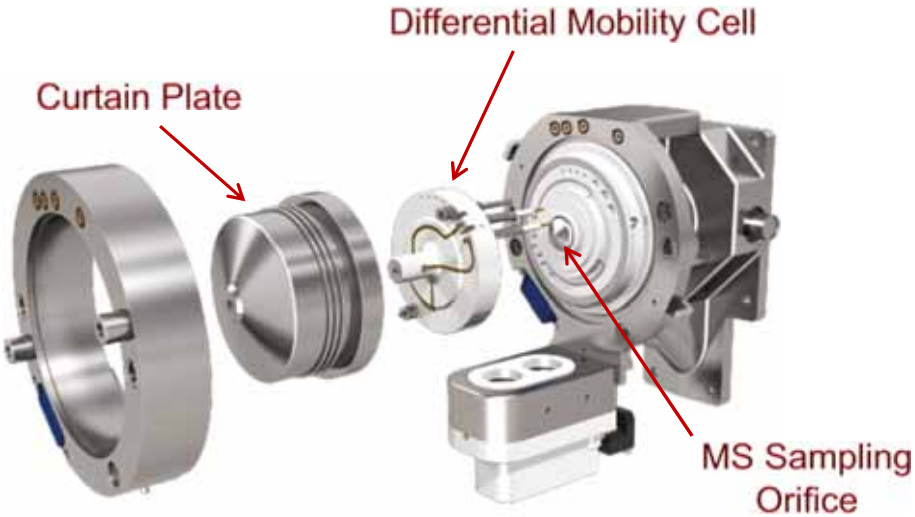
Total NNN / NNAL Assay Challenges in Urine

- Peer-reviewed literature LOQ's: 2.0 pg/mL (T-NNN) and 5.0 pg/mL (T-NNAL)
  - *60 – 400 fold extract concentration, low recoveries often reported*
  - *Emphasis on orthogonal sample preparation and chromatographic approaches (MIP, SPE, LLE)*
- Up to 30% of baseline smoker urine contains [T-NNN] < 2.0 pg/mL
- Achieving method detection limits < 2.0 pg/mL for T-NNN complicated by:
  - *Increased chemical baseline noise limiting signal-to-noise ratio (SNR)*
  - *Ionization suppression reducing analyte response (35 - 60%)*
  - *Co-extracted endogenous interference impacting selectivity*
- Can selectivity & sensitivity challenges be overcome when coupling Differential Ion Mobility Spectrometry with QQQ Tandem Mass Spectrometry?

Differential Ion Mobility Spectrometry (DMS)

Hardware Configuration

SCIEX TripleQuad 6500+



SelexION+: Rapid Interfacing to MS without Disruption of Vacuum

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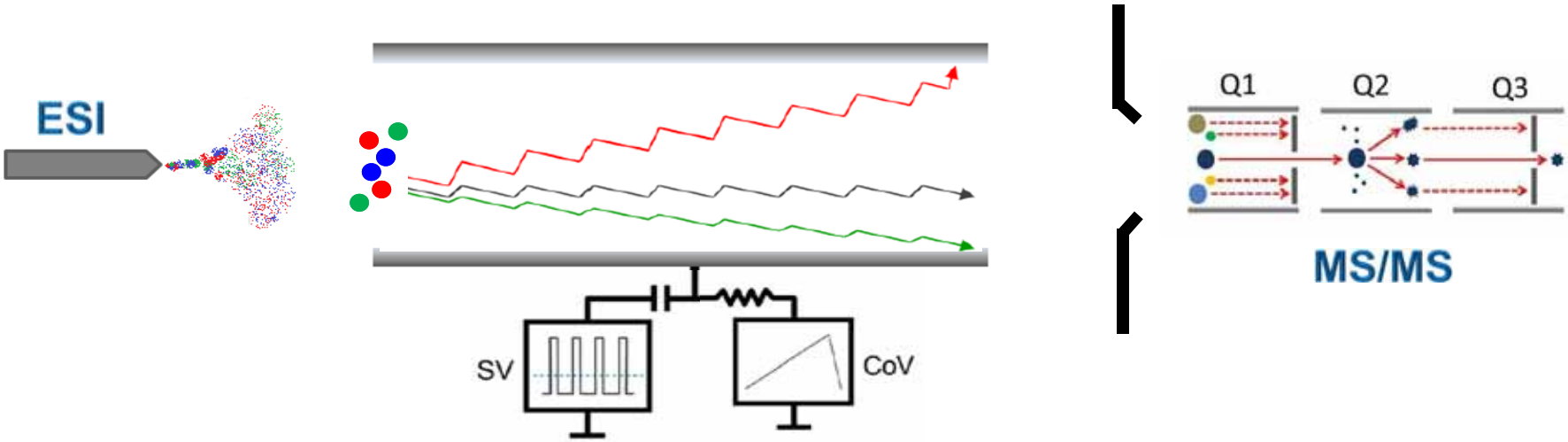
Principles of Separation by SelexION+ DMS

Separation Waveform and Compensation Voltage



Separation Waveform (SV)
Radially displaces ions towards one of the planar electrodes depending upon high and low field mobility characteristics

Compensation Voltage (CoV)
Restores the trajectory for a given ion to allow selective transmission through the DMS cell

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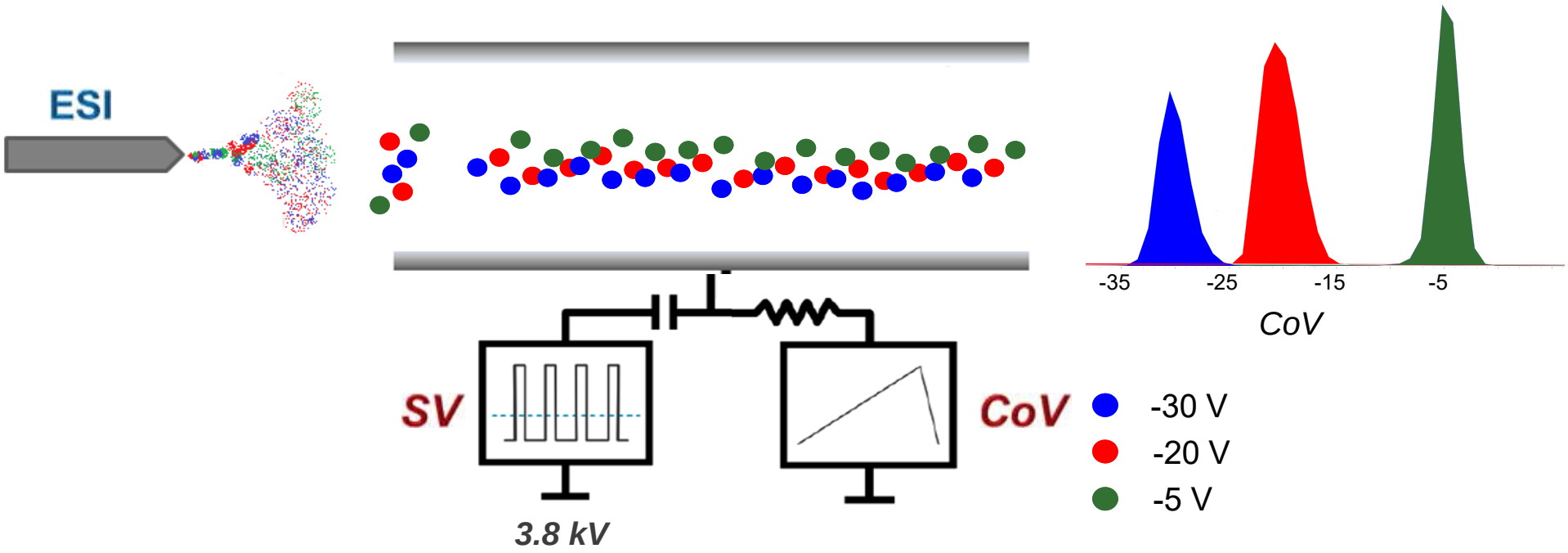
Principles of Separation by SelexION+ DMS

Ions Separated by Size, Shape and Charge State



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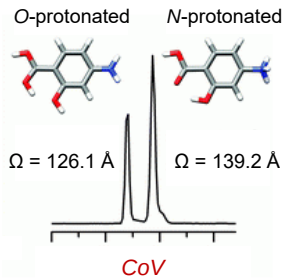
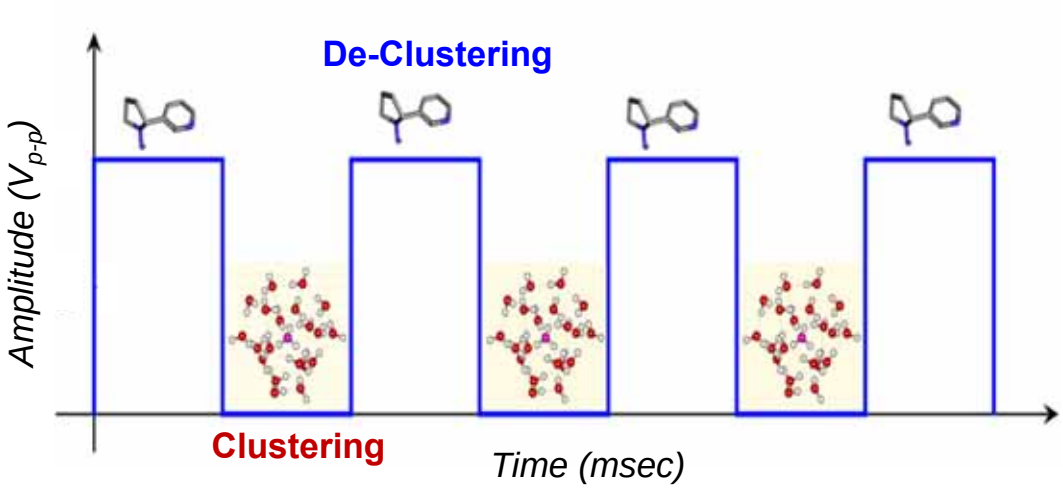
DMS Optimization

Chemical Modifiers – A Second Dimension

- SV and CoV
- Modifier type and conc.
- DMS temperature
- Resolution enhancement





NNN LOQ Response by LC-DMS-MS/MS

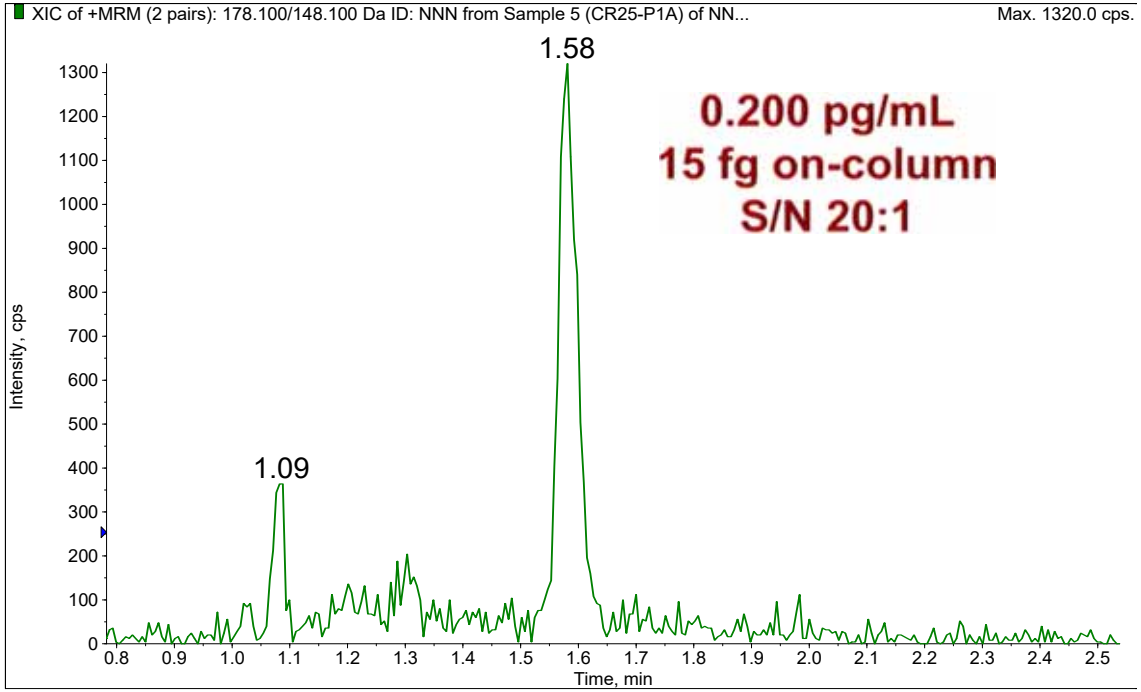
Extracted from Non-Smoker Urine

Sample Prep

- 1 mL urine
- Deconjugation (30 min)
- SCX-SPE + SLE
- Recon 0.20 mL
- 80% Avg. recovery

Analysis

- Shimadzu Nexera X2
- SCIEX 6500+ TripleQuad
- SelexION+ DMS



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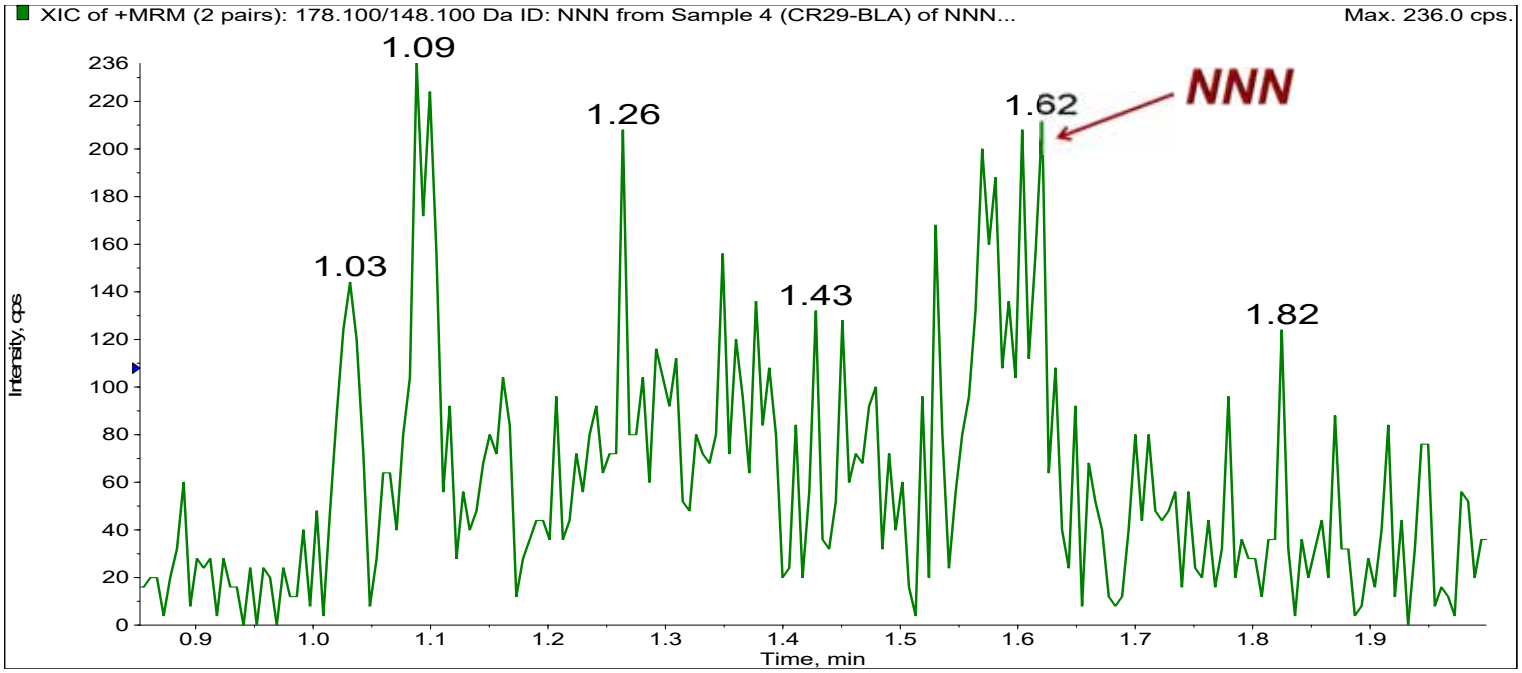
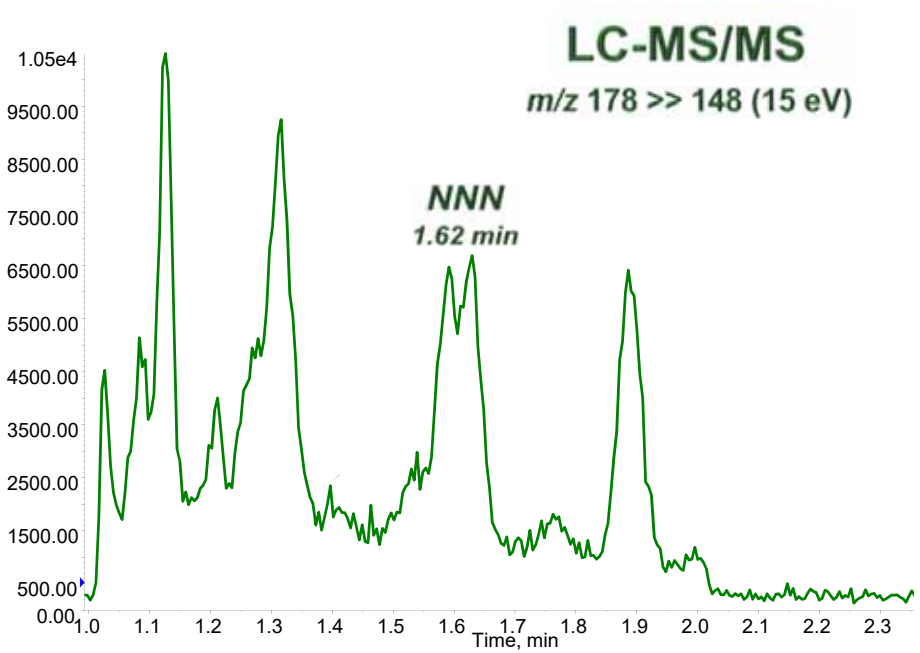
Assay Selectivity: Non-Smoker Urine

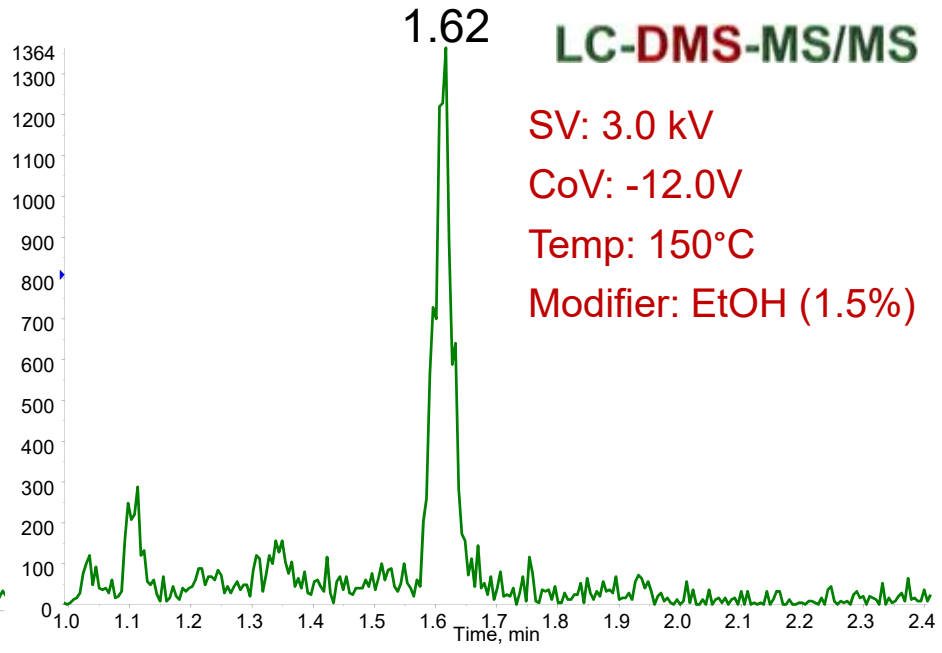
LC-DMS-MS/MS



Assay Selectivity / Sensitivity: Smoker Urine

MS/MS vs. DMS-MS/MS





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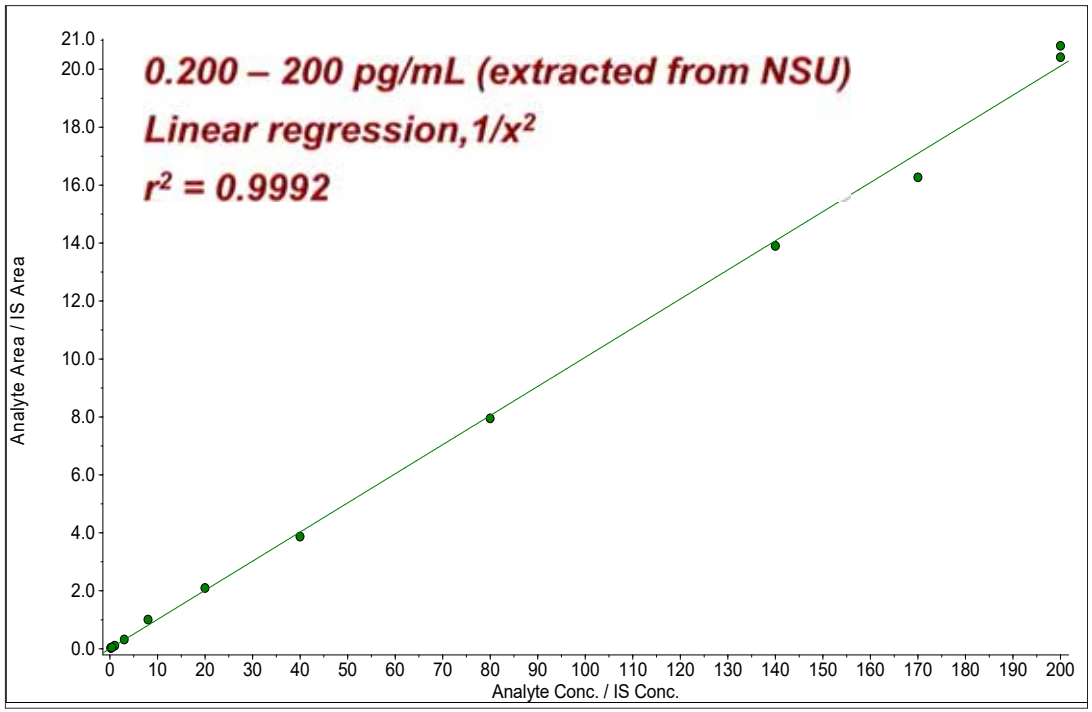
LC-DMS-MS/MS: Linearity of Response

Calibrant (pg/mL)	Back-Calculated Conc. (pg/mL)	% Nominal
0.200	0.199	99.3
0.400	0.402	100.6
1.00	1.007	100.7
3.00	3.12	104.1
8.00	8.18	102.3
20.0	19.98	99.9
40.0	38.64	96.6
80.0	80.14	100.2
140	136.6	97.6
170	167.3	98.4
200	200.8	100.4



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Validated Between-Run P&A

3 Batches, 6 QC's / Concentration (pg/mL) / Batch

STATS	LOQ-QC (0.200)	Low-QC (0.600)	Mid-QC (100.0)	High-QC (150.0)
Mean	0.194	0.577	97.8	152.1
% C.V.	6.6	4.6	4.9	3.7
% Nominal	97.0	96.1	97.8	101

Deconjugation Efficiency QCs

Ten NSU Donors Fortified with NNN:*N*-Gluc (1:1 molar ratio), Extracted in Triplicate

STATS	Low-QC (0.60 pg/mL)	High-QC (150 pg/mL)
Mean	0.55	141
% C.V.	4.2	3.7
% Nominal	92.5	94.2

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Short Term Stability In Smoker Urine

Literature Indicates Increased [NNN] in S/T Stability Assessments at RmT

Subject Number	Initial Conc* (pg/mL)	1 F/T + 20h	
		RmT	4ºC
1	0.33	0.32	0.32
2	1.40	1.35	1.40
3	0.31	0.28	0.28
4	0.55	0.54	0.50
5	1.00	1.08	1.09

*measured by DMS-MS/MS

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Short Term Stability In Smoker Urine

Reported Increase Due to Isobaric Interference? Re-analyze by LC-MS/MS (no DMS)

Subject Number	Initial Peak Area Ratio	20h, RmT	% Deviation
1	0.0386	0.0352	-9.0
2	0.1318	0.1422	7.9
3	0.0568	0.0639	12.5
4	0.0564	0.0620	9.9
5	**0.1508**	**0.2319**	**53.8**

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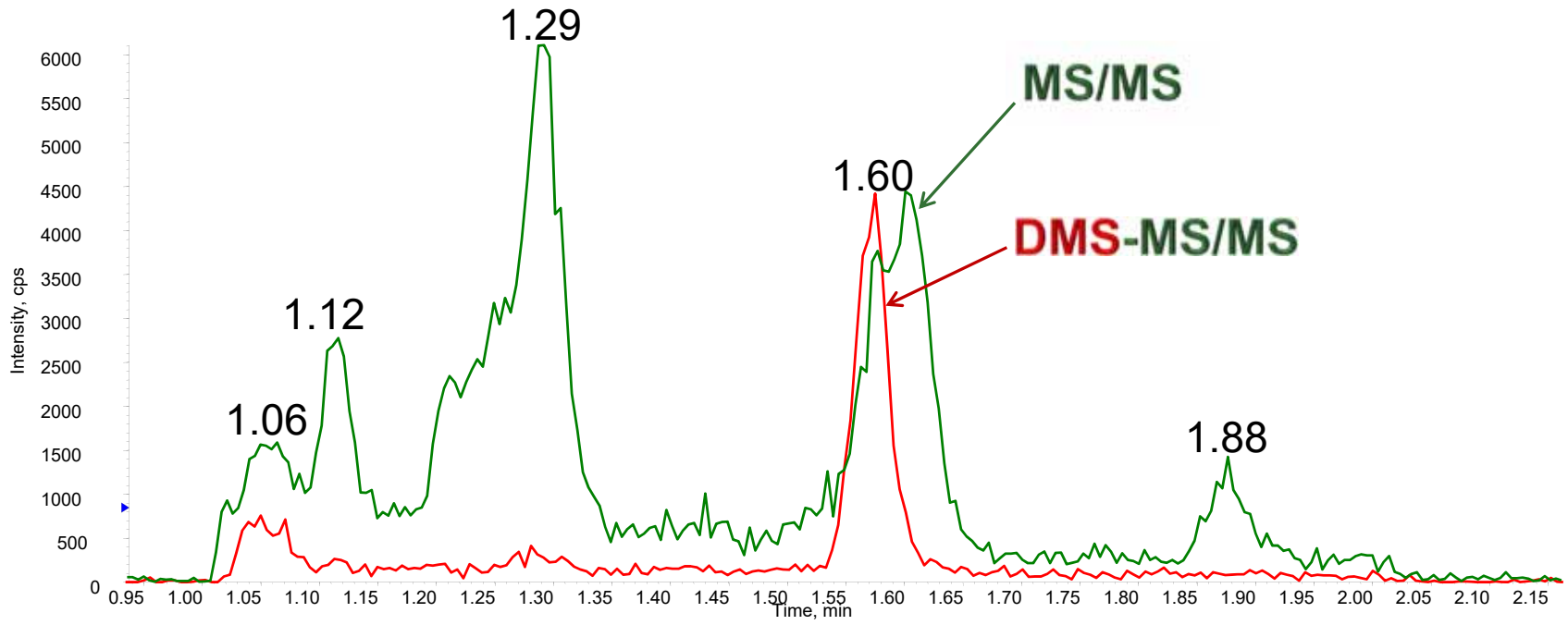
Short Term Stability in Smoker Urine

20h RmT, Subject 5: MS/MS vs. DMS-MS/MS



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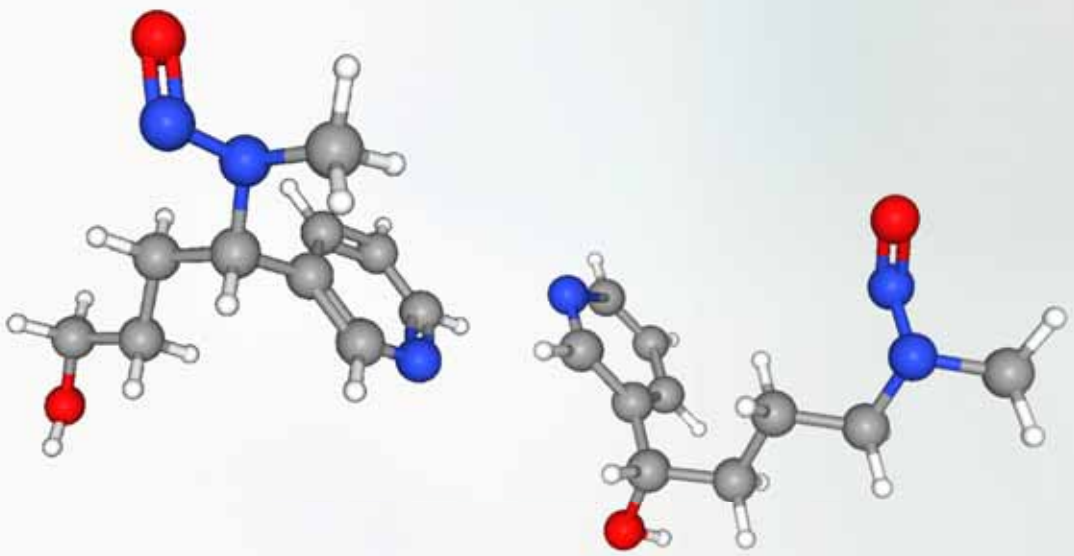
Validation Results: Stability

Assessment	Conditions	% Nominal	
		Low-QC	High-QC
Short-Term	29h, 22°C	106	97.9
Long-Term	On-going	N/AP	N/AP
Freeze-Thaw	4 cycles	110	96
Autosampler	166h, 4°C	102	101



Determination of Total NNAL by LC-MS/MS with Ion Mobility Separation

NNAL LOQ Response by LC-DMS-MS/MS

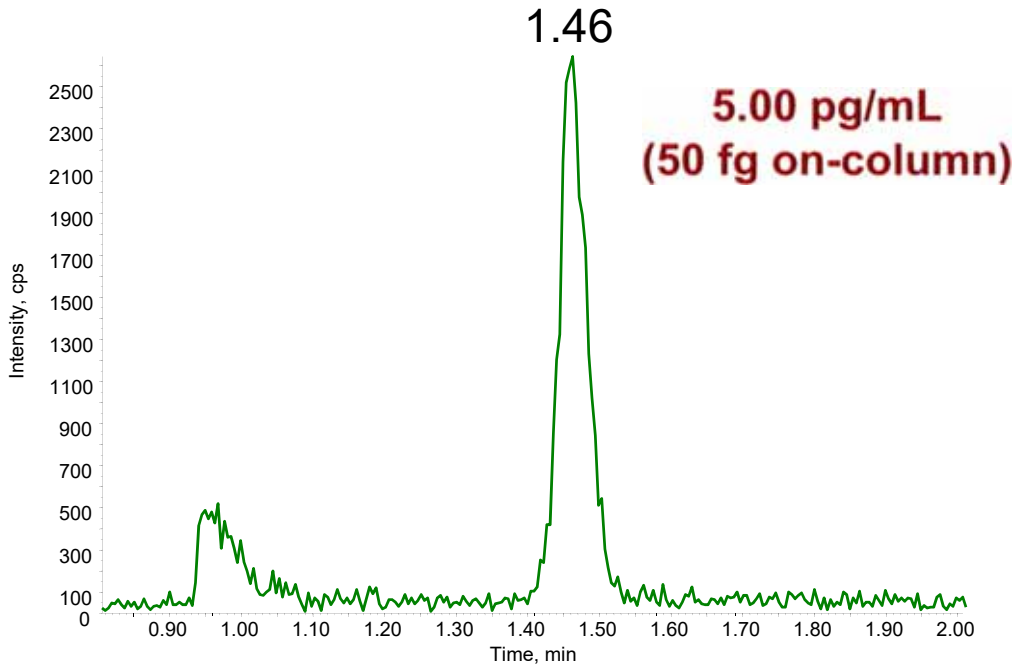
Extracted from Non-Smoker Urine

Sample Prep

- 150 µL Urine
- Deconjugation (30 min)
- SCX-SPE
- Recon 250 µL (1.7x DF)
- 92% Avg. recovery

Analysis

- Shimadzu Nexera X2
- SCIEX 6500+ TripleQuad
- SelexION+ DMS



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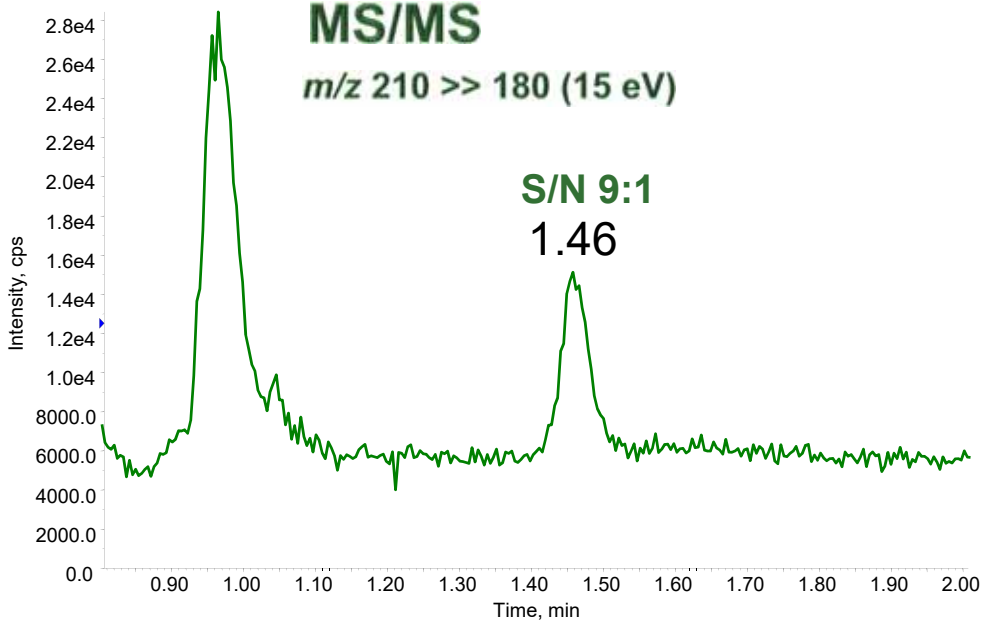
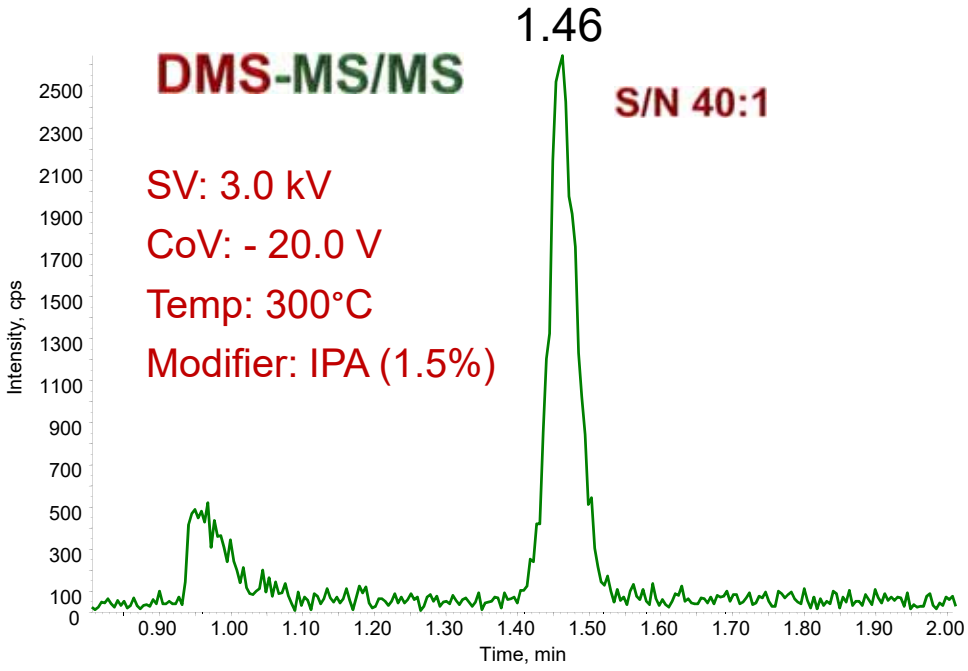
Assay Sensitivity Gains with Ion Mobility

MS/MS vs. DMS-MS/MS; 5.00 pg/mL





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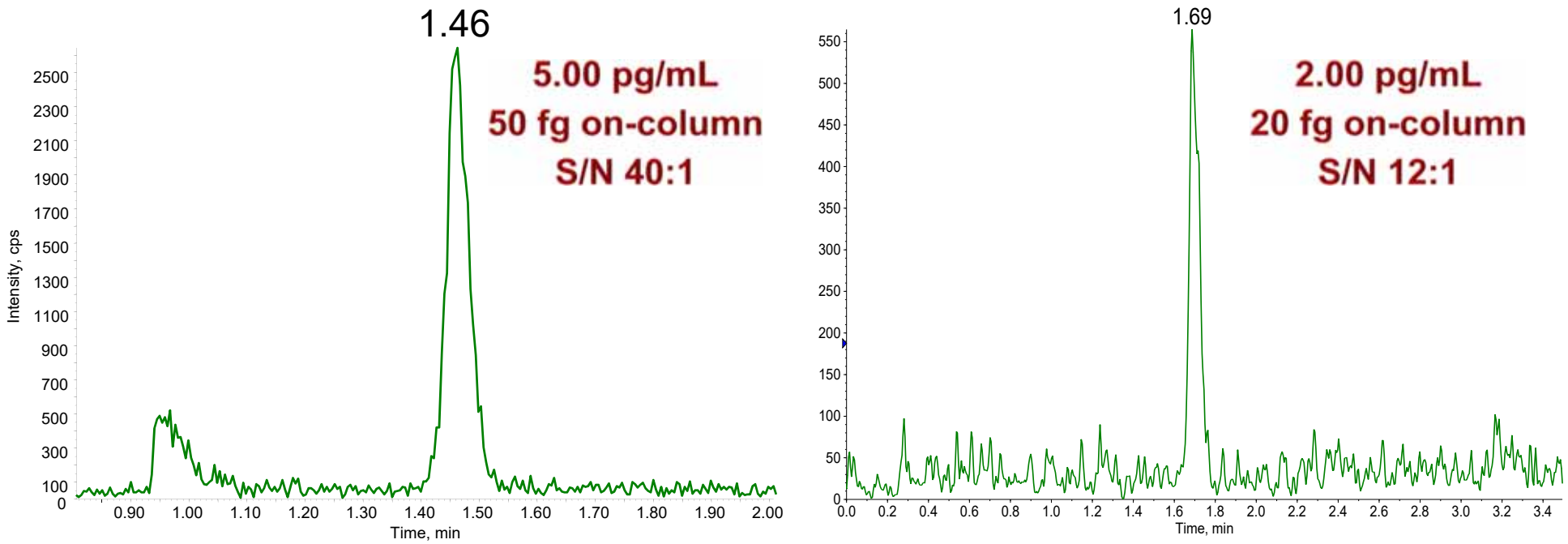
Assay Sensitivity Gains with Ion Mobility

DMS-MS/MS; 5.00 pg/mL *vs* 2.00 pg/mL



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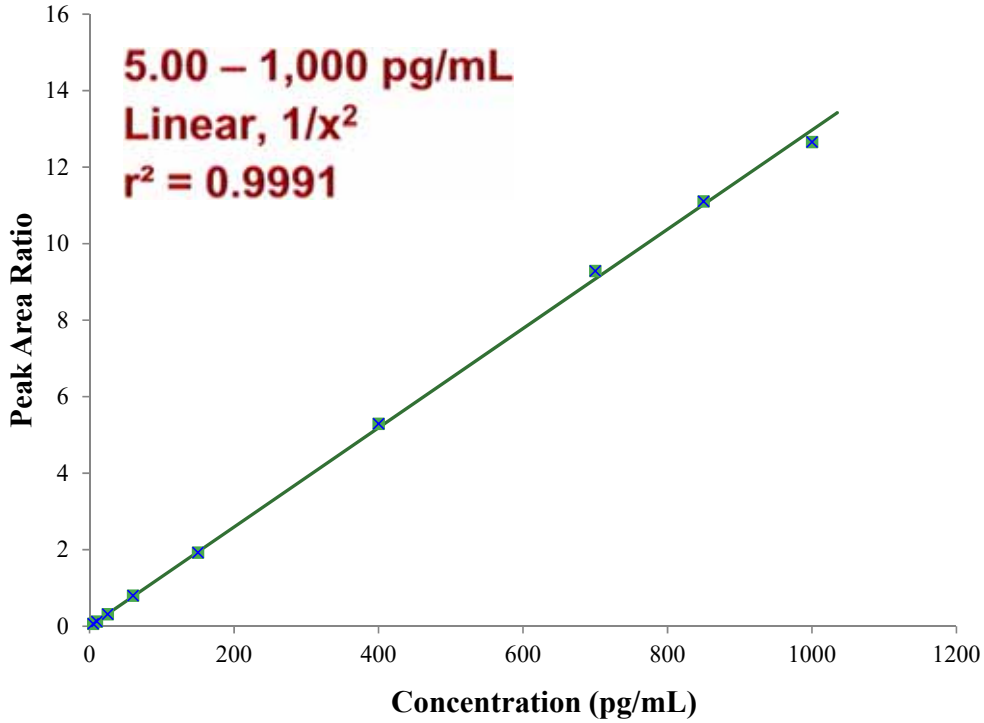
Linearity of Response: Calibration Curve

Nominal (pg/mL)	Back-Calculated Conc. (pg/mL)	% Nominal
5.00	5.05	100.9
10.0	9.98	99.8
25.0	23.99	96.0
60.0	60.01	100.0
150	147.18	98.1
400	404.30	101.1
700	695.58	99.4
850	880.51	103.6
1000	1011.51	101.2



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Validated Between-Run P&A

3 Batches, 6 QC's / Concentration (pg/mL) / Batch

STATS	LOQ-QC (5.00)	Low-QC (15.0)	Mid-QC (500)	High-QC (750)
Mean	5.09	16.06	501.0	765.1
%C.V.	8.4	3.9	4.4	4.8
% Nominal	101.8	107.1	100.2	102.0

Deconjugation Efficiency QCs

10 Donors Fortified with NNAL:*N*-Gluc:*O*-Gluc (4:3:3 molar ratio), Extracted in Triplicate

STATS	Low-QC (15.0 pg/mL)	High-QC (750 pg/mL)
Mean	16.1	752.6
% C.V.	5.4	3.4
% Nominal	107.5	100.4

Validation Results: Stability

Assessment	Conditions		% Nominal	
			Low-QC	High-QC
Short-Term	27h, 22°C		101	107
Long-Term	41d	-20°C	96.4	103
		-80°C	105	103
Freeze-Thaw	4 cycles		96.4	103
Autosampler	181h, 4°C		105	108

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NNN / NNAL Matrix Effect Summary

20 Urine Donors, Extracted in Triplicate

TBOE	Low QC			High QC		
	AMF	ISMF	ISNMF	AMF	ISMF	ISNMF
NNN	0.90 -1.01	0.87 -1.04	***0.97-1.06***	0.93 -1.02	0.93 -1.05	***0.98 -1.03***
NNAL	0.86 -1.04	0.89 -1.04	***0.97-1.06***	0.87-1.05	0.86-1.03	***0.97-1.04***

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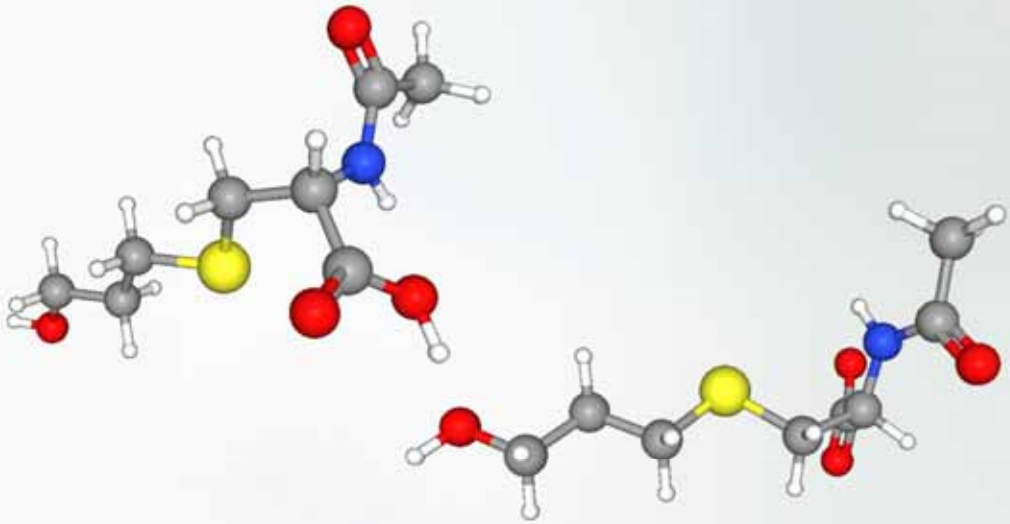
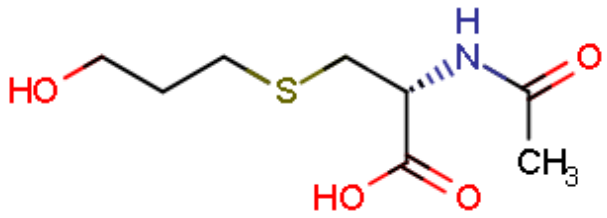


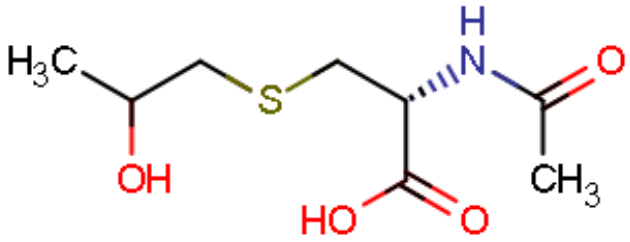
Determination of HPMA by LC-MS/MS with Ion Mobility Separation

2 & 3-HPMA Assay Challenges in Urine

- Donor-dependant degradation in urine observed for 2-HPMA and 3-HPMA during short-term stability at RmT (unreported in literature)
- Endogenous interference impacts selectivity for 3-HPMA
- Chemical noise impacts achievable S/N at LOQ for 2-HPMA
- Chromatographic resolution required between structural isomers



3-HPMA



2-HPMA

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2 & 3-HPMA LOQ Response by LC-DMS-MS/MS

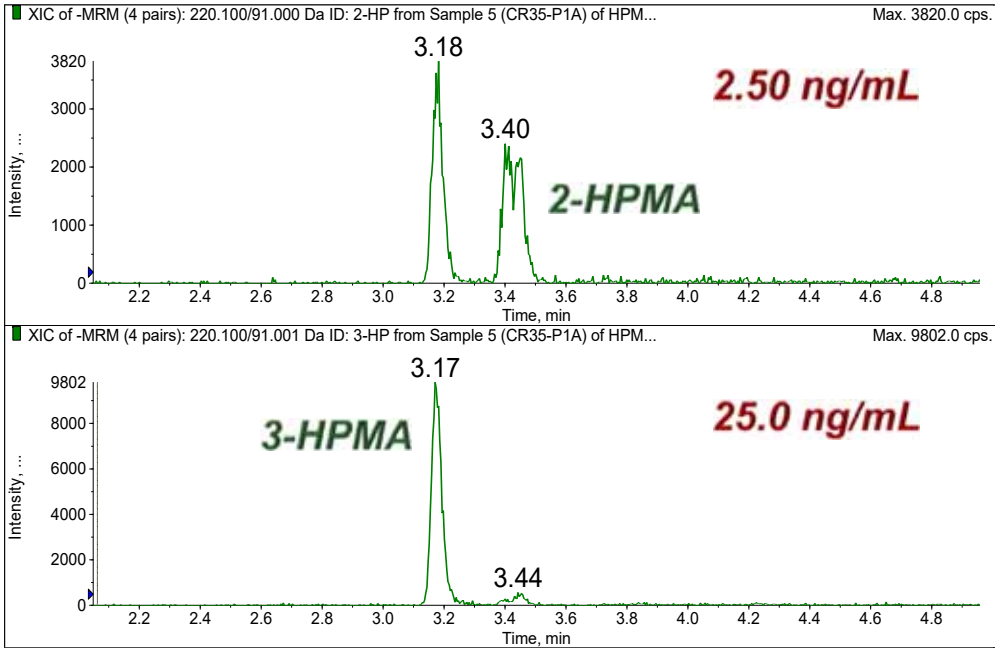
Extracted from Charcoal Stripped Urine

Sample Prep

- 25 µL urine (stabilized)
- SAX-SPE, Recon 400 µL
- 83% Avg. recovery (3-HPMA)
- 86% Avg. recovery (2-HPMA)

Analysis

- Shimadzu Nexera X2
- SCIEX 6500+ TripleQuad
- SelexION+ DMS



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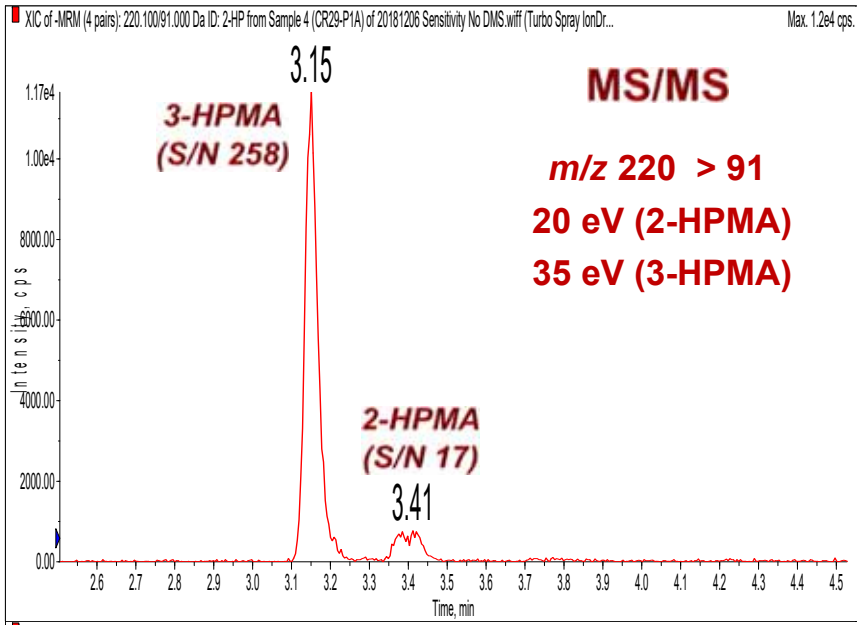
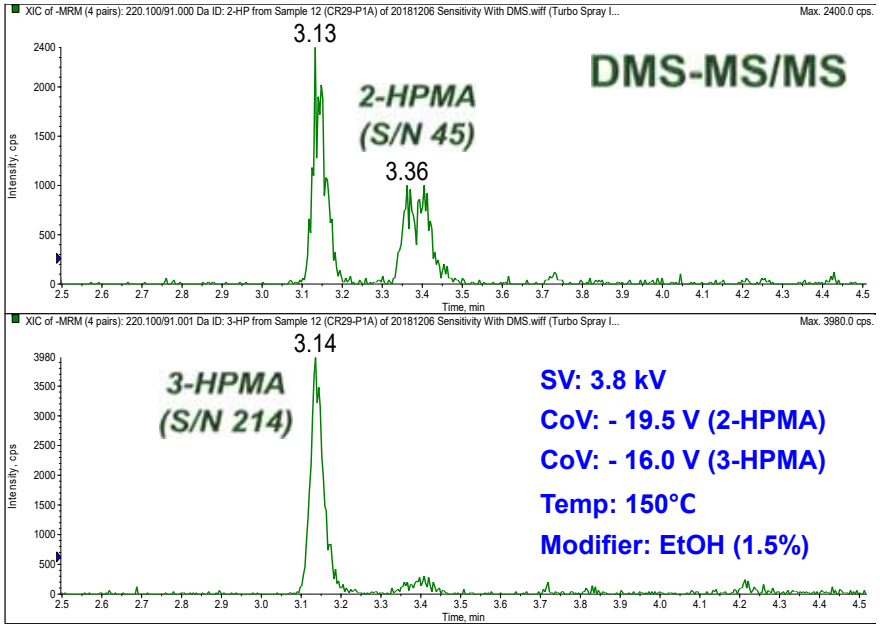
Sensitivity Gains by LC-DMS-MS/MS

Extracted from Charcoal Stripped Urine





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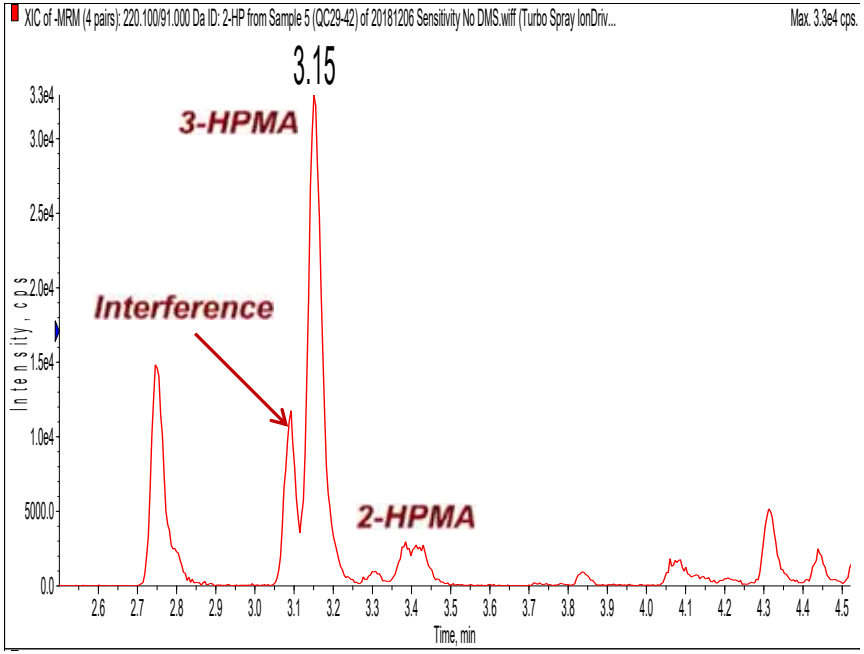
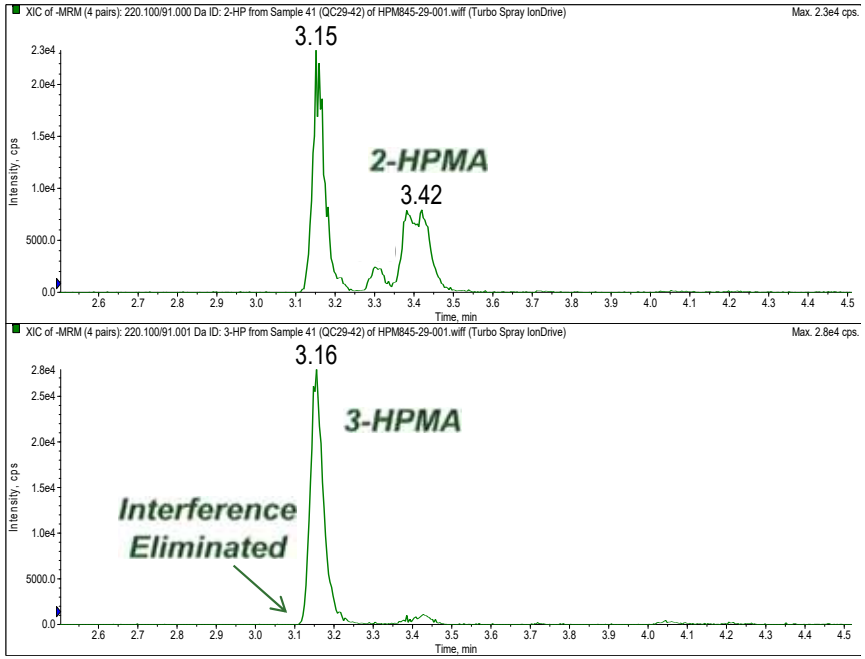
Assay Selectivity in Smoker Urine

MS/MS



DMS-MS/MS



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Validated Between-Run P&A: 2-HPMA

2.50 – 750.0 ng/mL, Linear regression $1/x^2$, $r^2 = 0.9972$

STATS	LOQ-QC (2.50)	Low-QC (7.50)	Mid-QC (375.0)	High-QC (562.5)
Mean	2.60	7.41	381.1	561.2
% C.V.	8.4	5.2	3.0	3.0
% Nominal	104.2	98.8	101.4	99.8

3 Batches, 6 QC's / Concentration (ng/mL) / Batch

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Validated Between-Run P&A: 3-HPMA

25.0 – 7,500 ng/mL, Linear regression $1/x^2$, $r^2 = 0.9971$

STATS	LOQ-QC (25.0)	Low-QC (75.0)	Mid-QC (3750)	High-QC (5625)
Mean	25.4	72.6	3806.7	5585.2
% C.V.	7.2	6.3	3.3	4.0
% Nominal	101.4	96.8	101.5	99.3

3 Batches, 6 QC's / Concentration (ng/mL) / Batch

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Validation Results: Stability

Assessment	Conditions		% Nominal			
			3-HPMA		*2-HPMA*	
			Low-QC	High-QC	Low-QC	High-QC
Short-Term	98h, 4°C		94.9	92.0	94.8	92.7
Long-Term	28d	-20°C	90.0	95.7	91.0	100
	15d	-80°C	90.6	93.6	91.7	95.5
Freeze-Thaw	2 cycles		93.1	90.4	94.0	91.4
Autosampler	167h, 4°C		93.0	93.4	94.3	99.3

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In Summary

- **DMS** technology successfully implemented as an orthogonal separation strategy to overcome analytical challenges for T-NNN, T-NNAL and 2/3-HPMA through:
  - *Reduction of Baseline Noise Response >> Improved S/N >> Lower LOQs*
  - *Elimination of Co-extracted Interference >> Reduced Sample Prep / Chromatographic Complexity*
- Robust 200 fg/mL LOQ obtained for NNN allowing a more accurate assessment of baseline response in smoker urine; 2 pg/mL feasible for NNAL
- Novel enzyme implemented for rapid deconjugation of NNN/NNAL; 16h >> 30m
- All three assays successfully validated and suitable for regulatory submission

Acknowledgements

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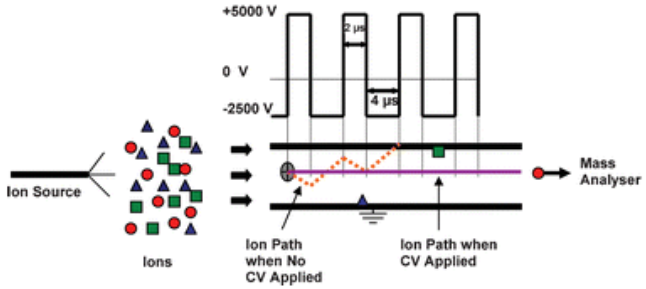

Q&A