The Science Required For Successful Bioanalytical Methods

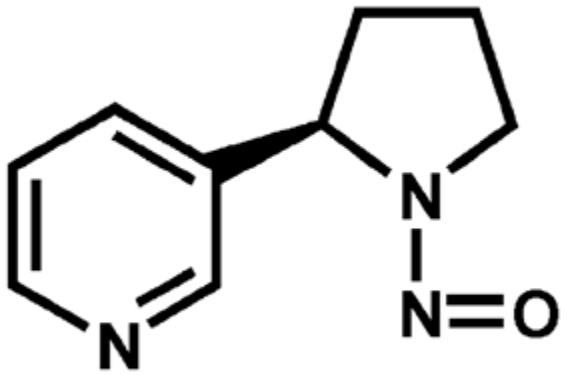
By: Ridha Nachi

TSRC, 2012

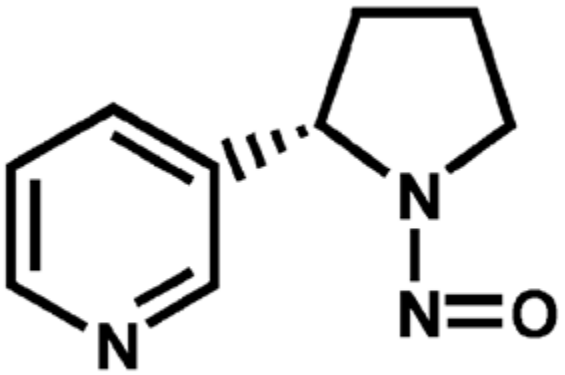
Scope Of Presentation

- Selectivity
- Sensitivity
- Matrix effect and accuracy. Are stable-labeled internal standards equivalent?

N'-nitrosonornicotine (NNN) Chemical Structures
Target LLOQ 0.75 pg/mL in human urine



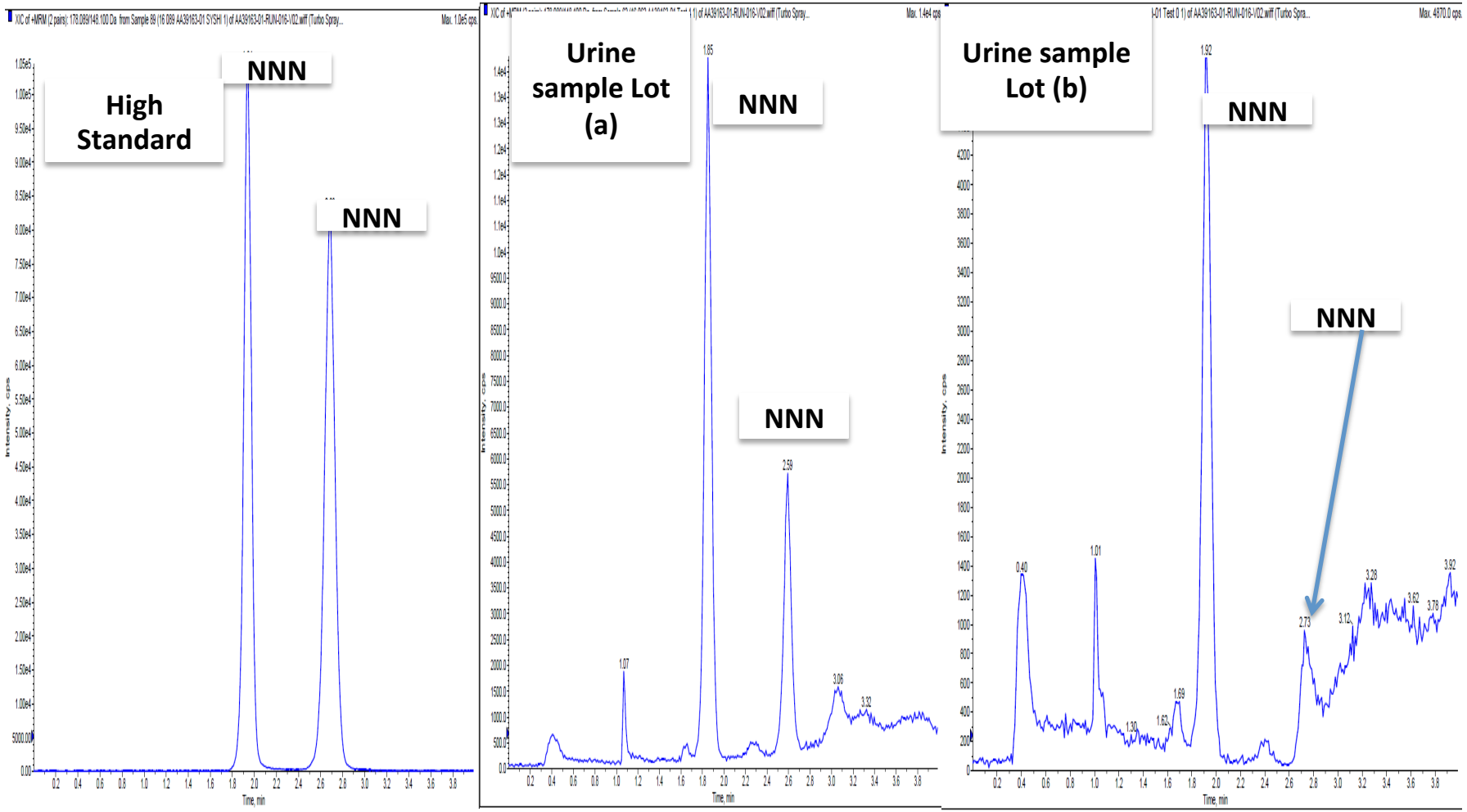
(*R*)-NNN



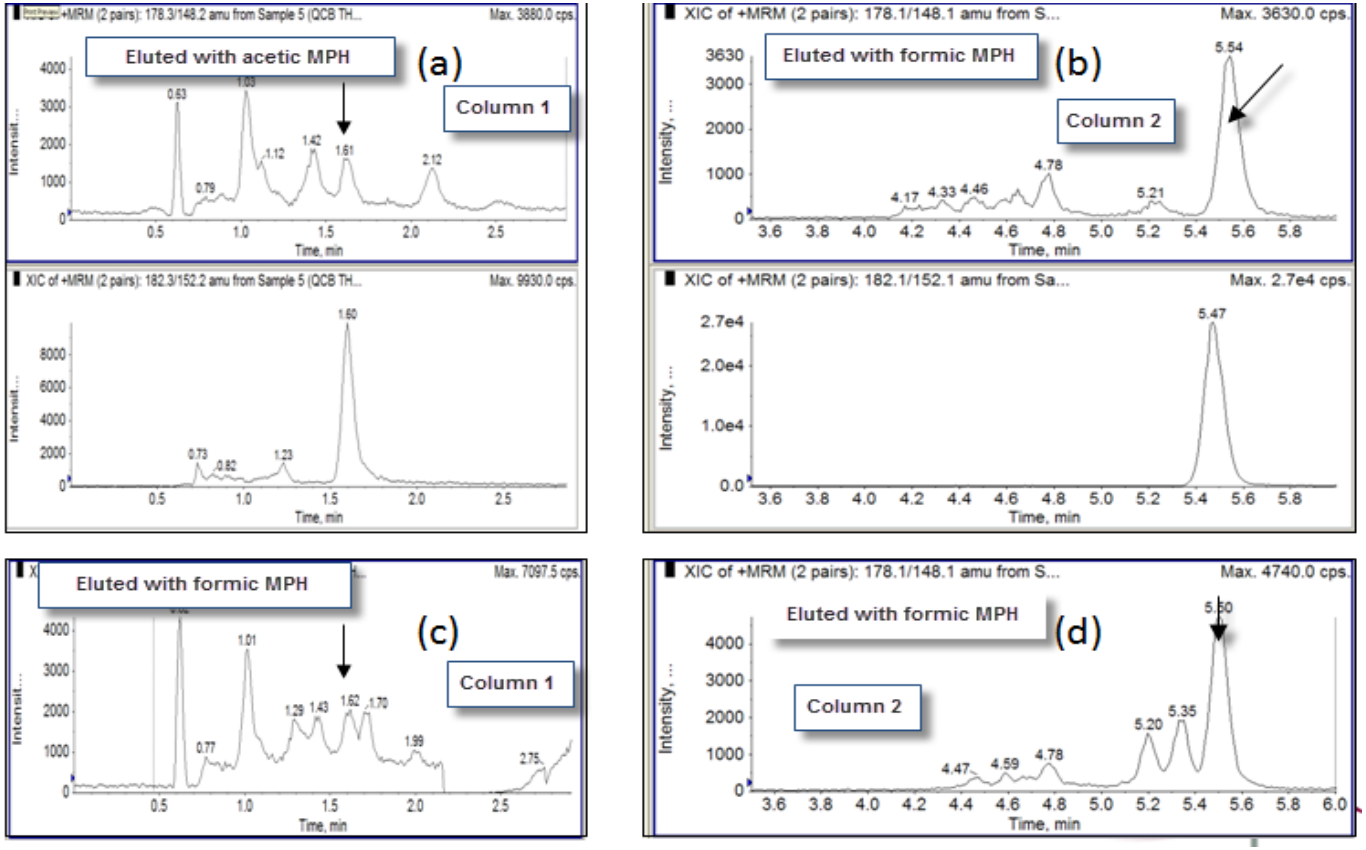
(*S*)-NNN

NNN Extracted Urine Sample

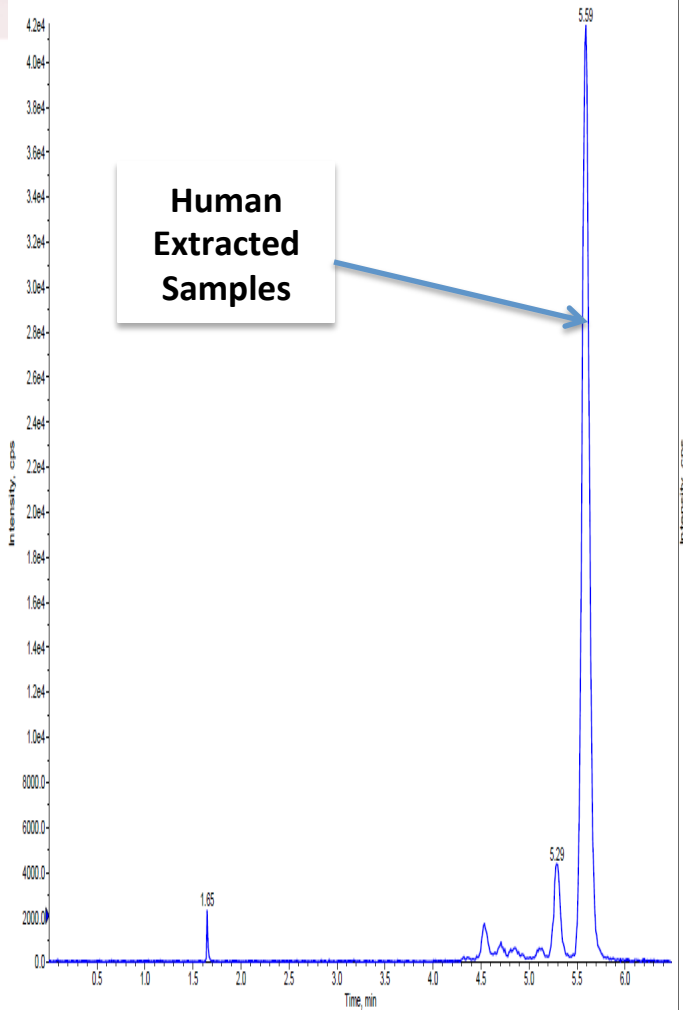
Chromatography on a cation-exchange column (with separation of NNN stereoisomers)

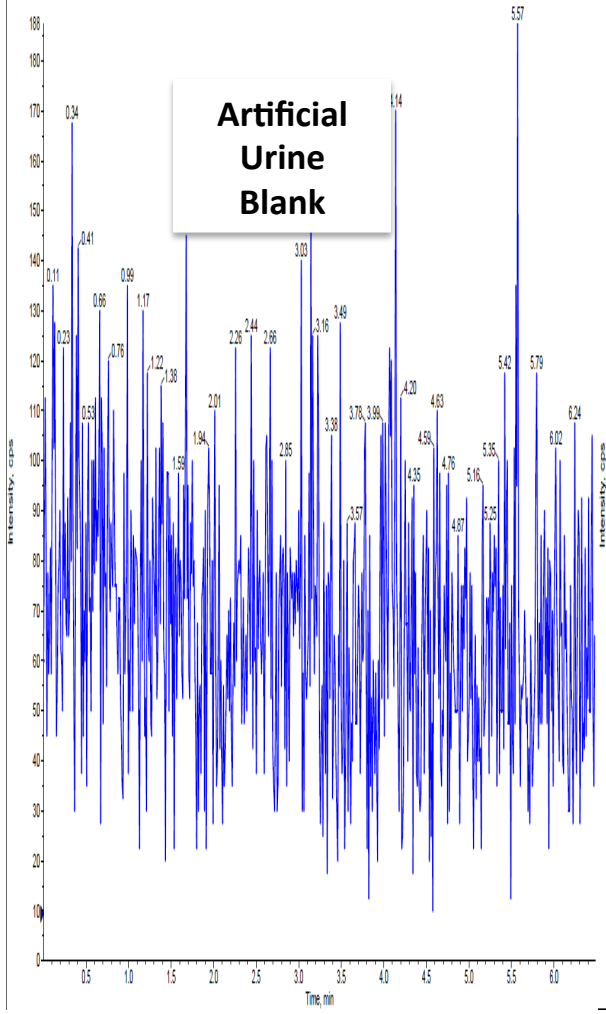


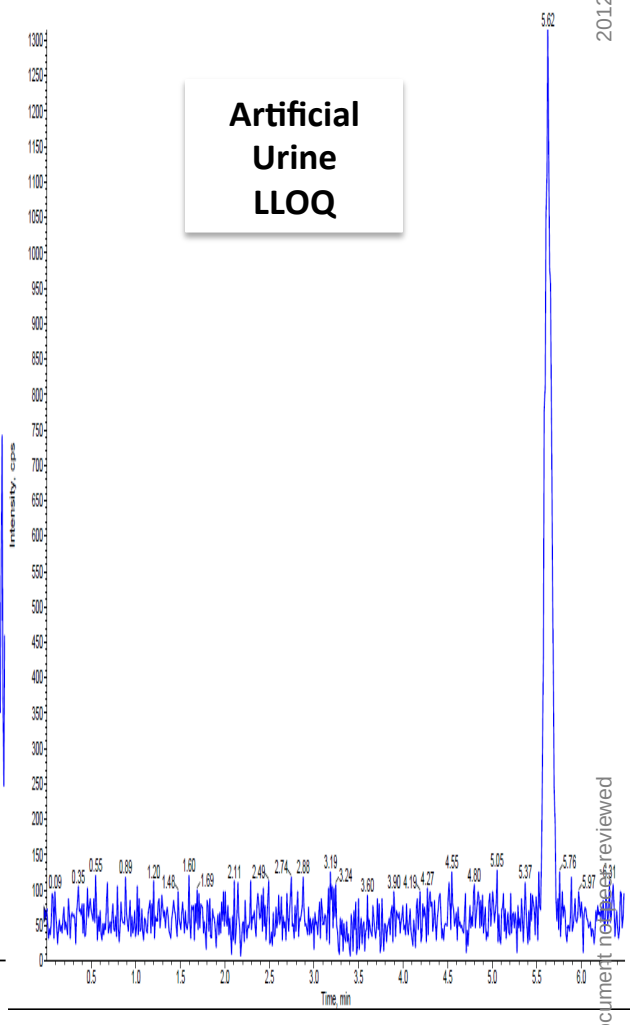
Two-dimensional chromatography of extracted urine samples on a Polar RP columns



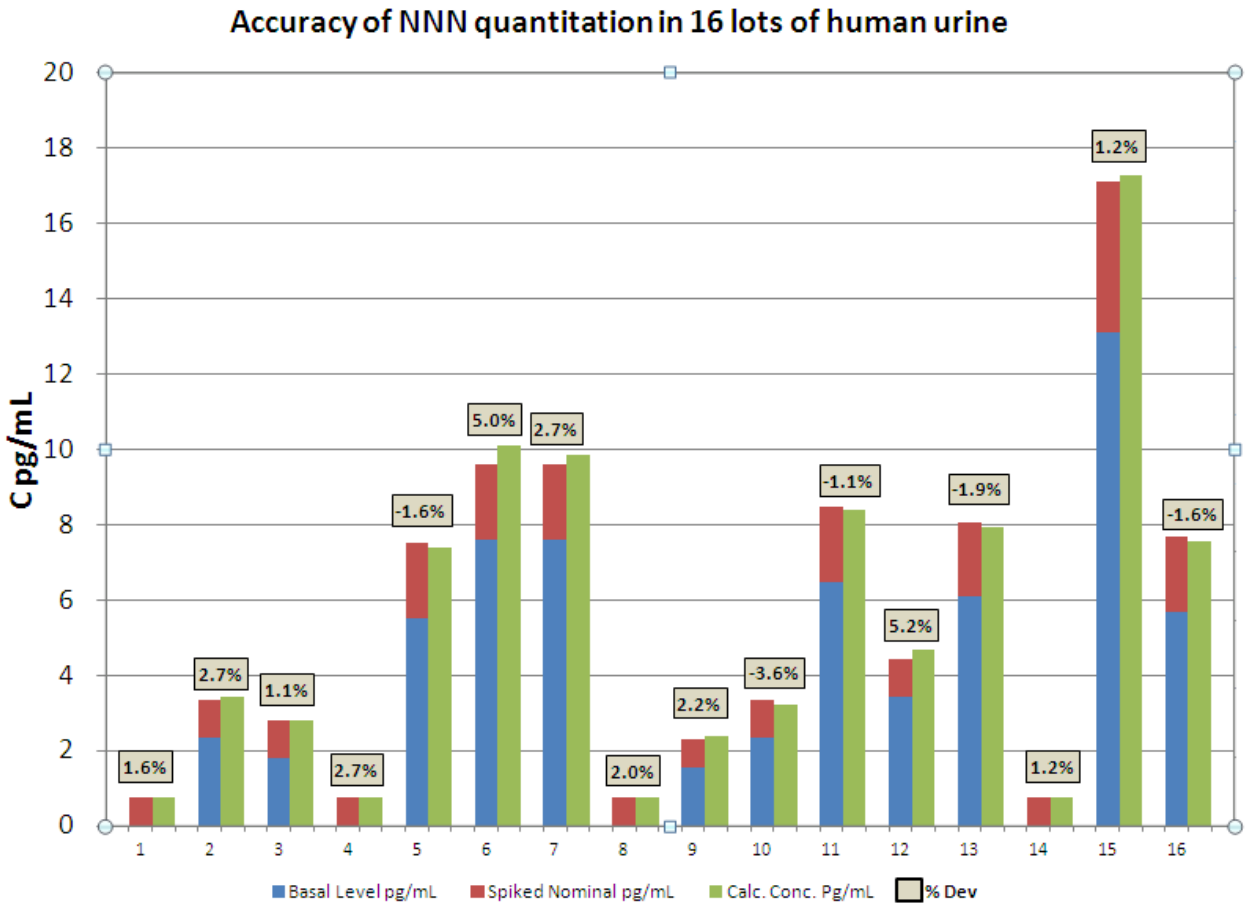
NNN Urine Extracted LLOQ (0.75pg/mL)





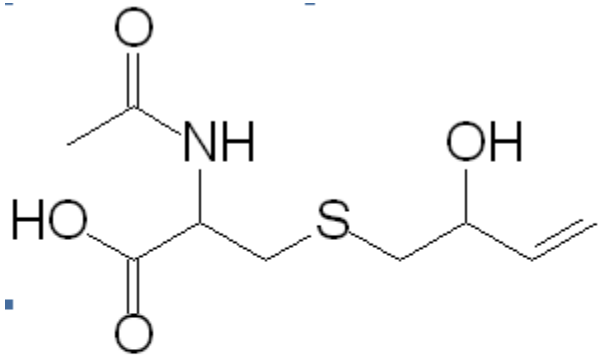


Accuracy of NNN Quantitation in Human Urine



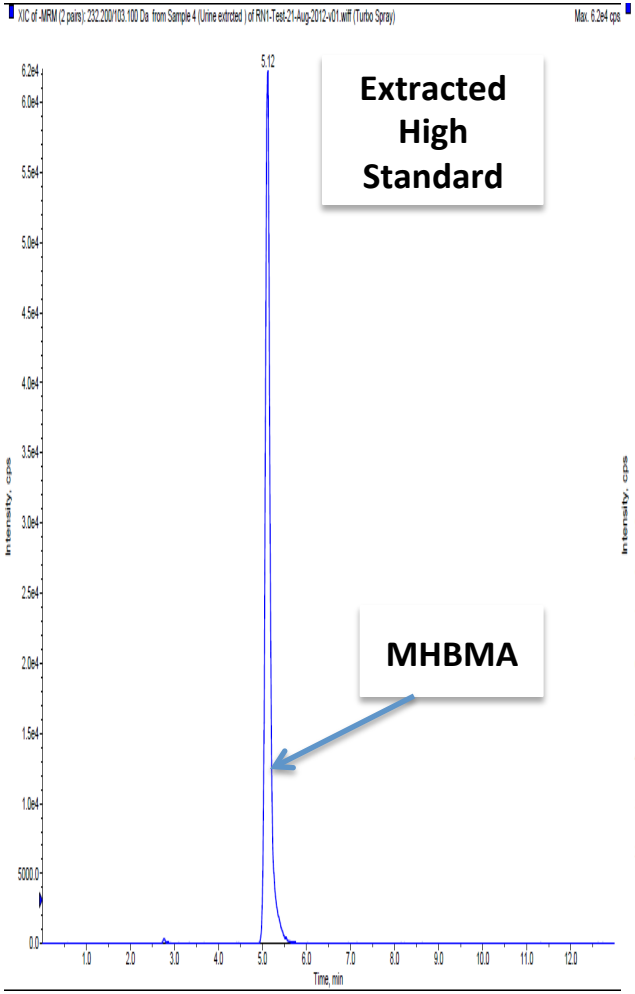
Monohydroxy-3-Butenyl Mercapturic Acid (MHBMA) In Human Urine

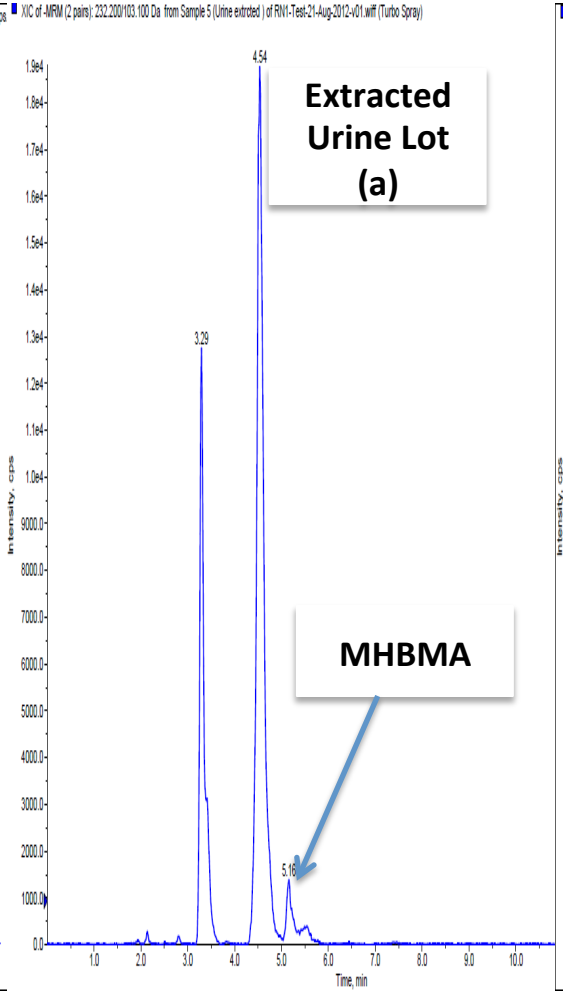
Mixed mode SPE.
Anion exchange chromatography.
ESI Negative Ion mode ionization.

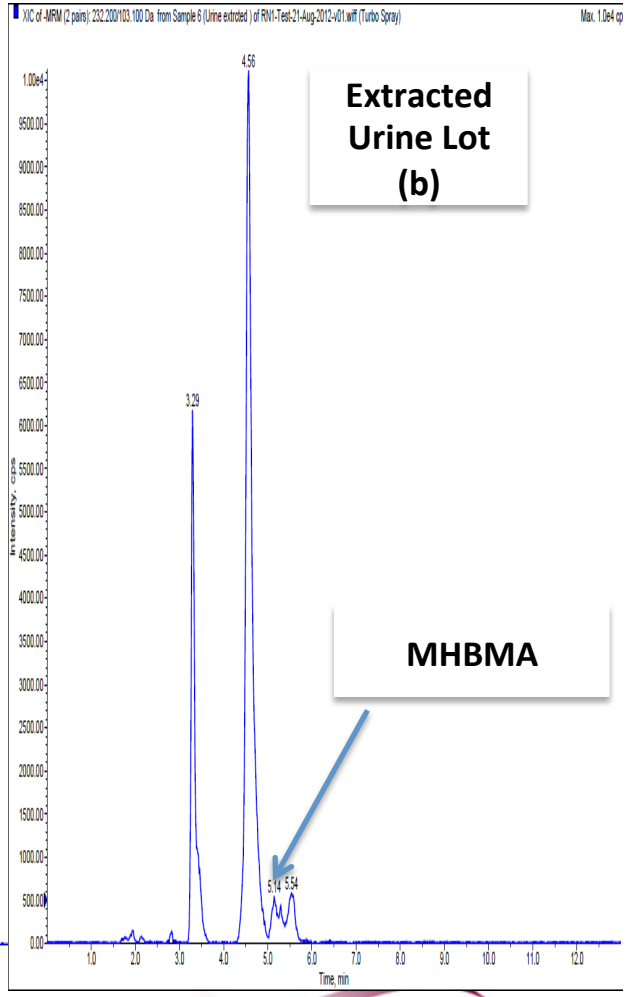


MHBMA
$C_9H_{15}NO_4S$
232.28 Da

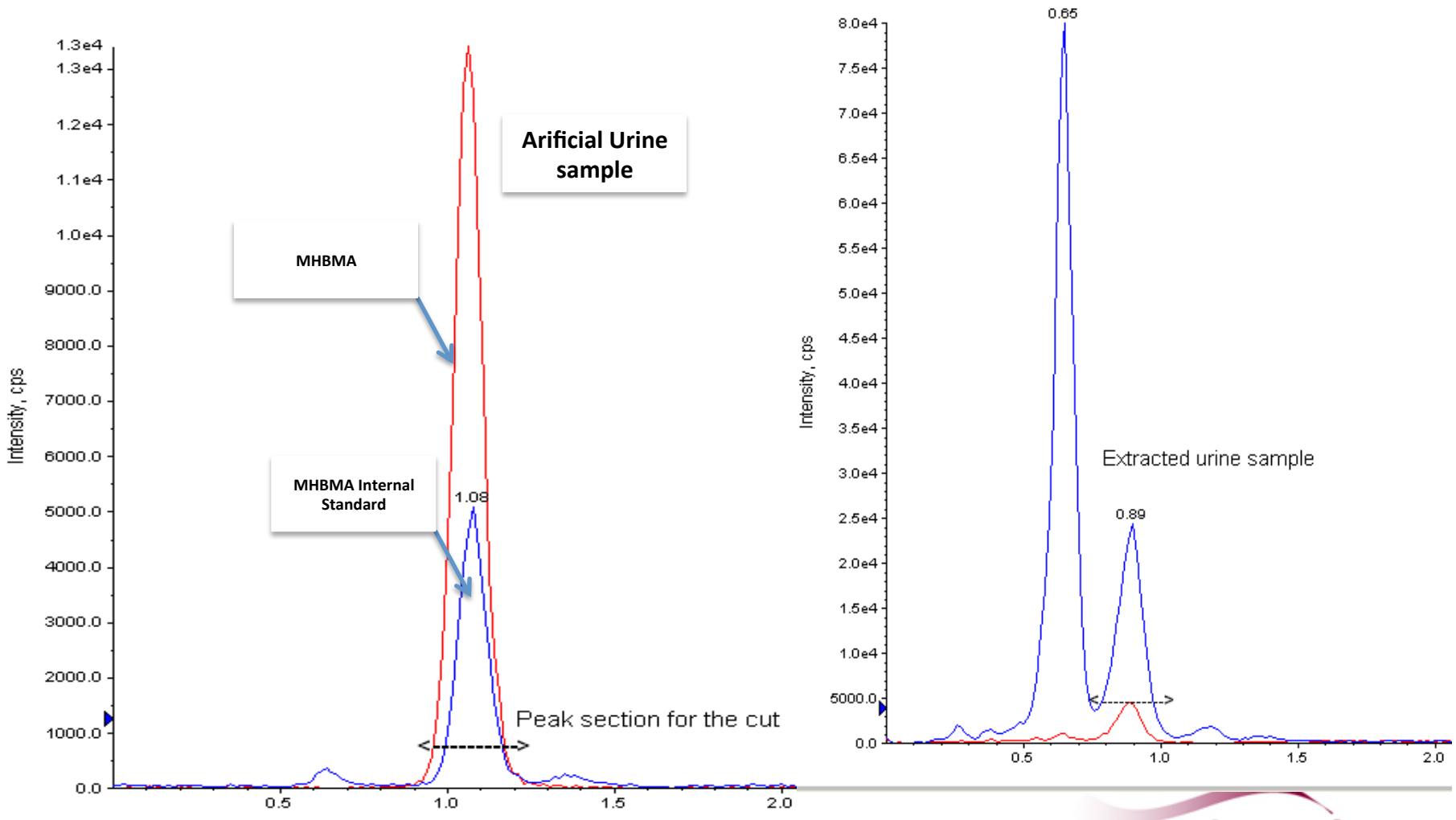
MHBMA Extracted Samples



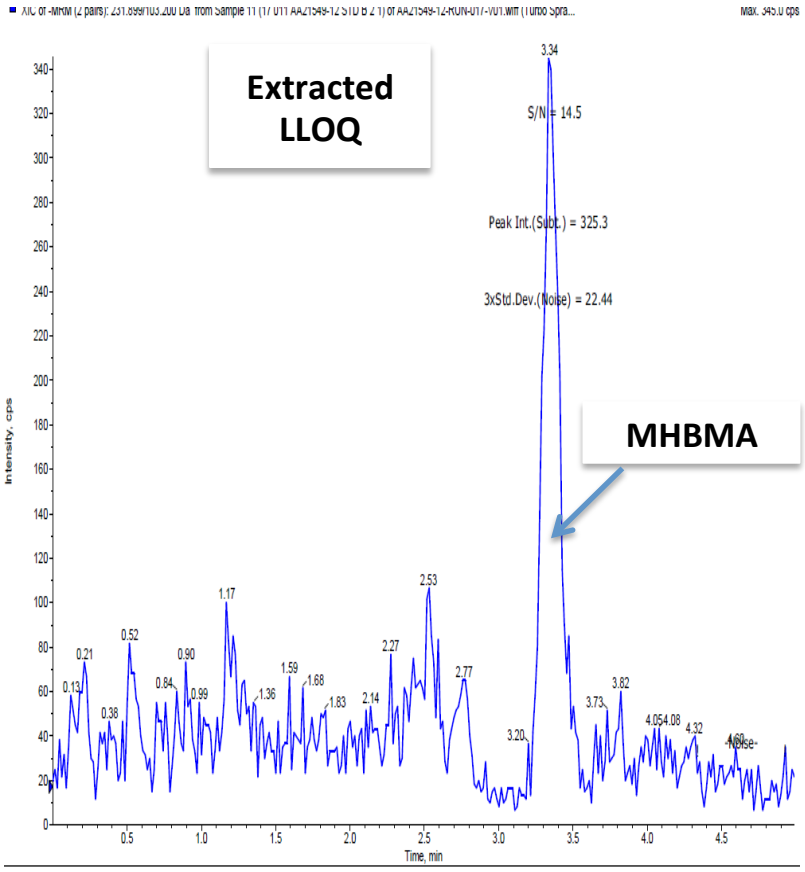


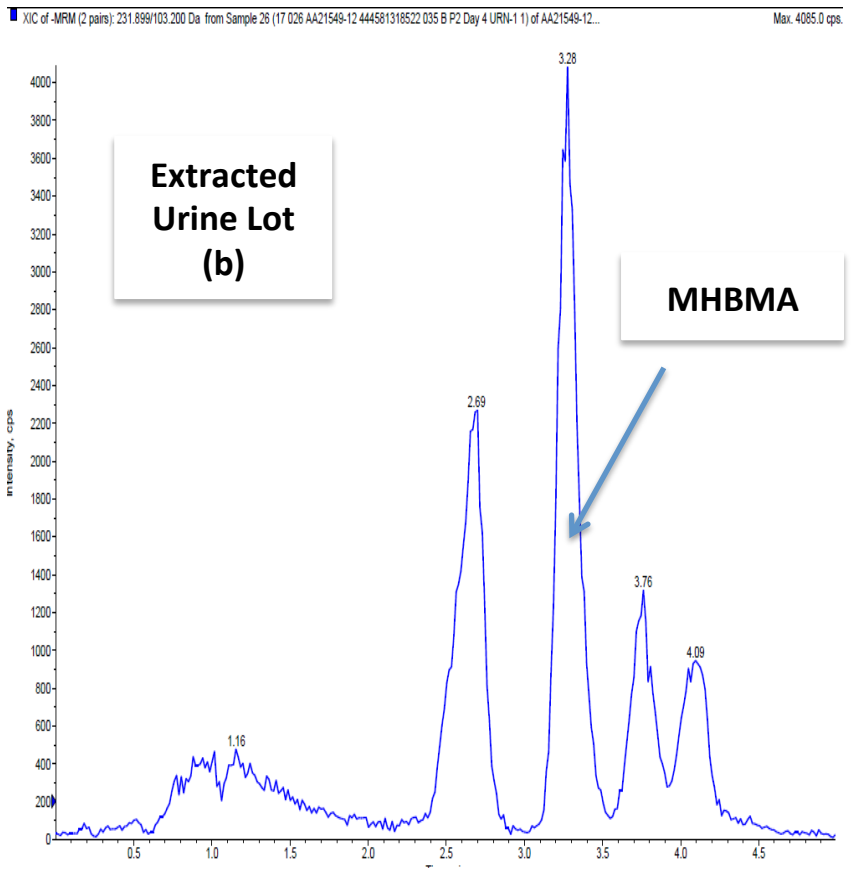


MHBMA heart cut on an anion exchange guard column

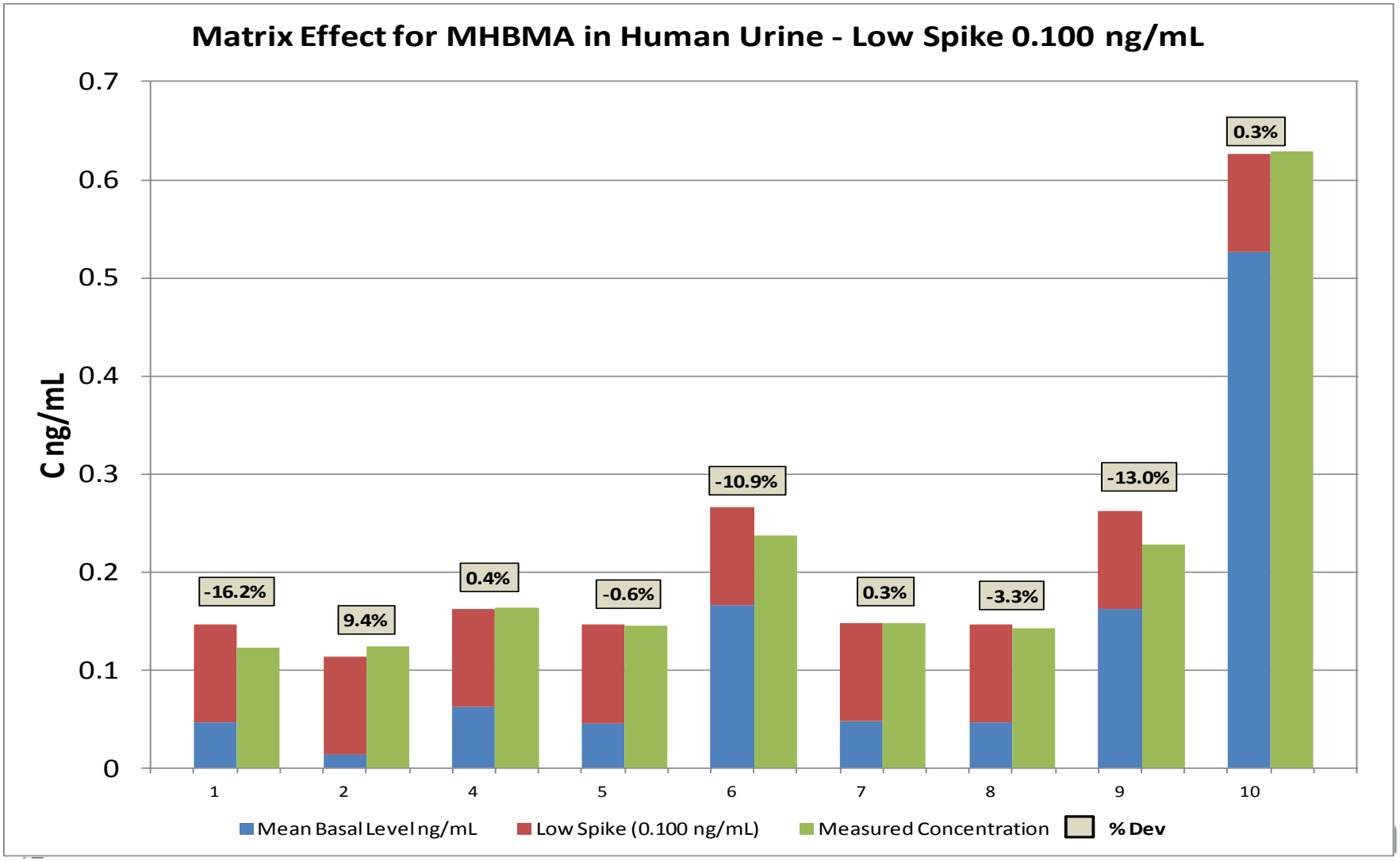


Examples of MHBMA Final Chromatography

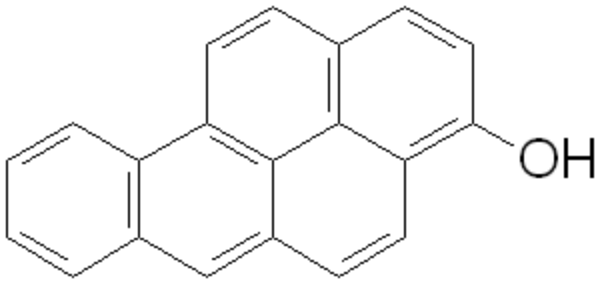




Accuracy of Matrix Effect for MHBMA in Human Urine

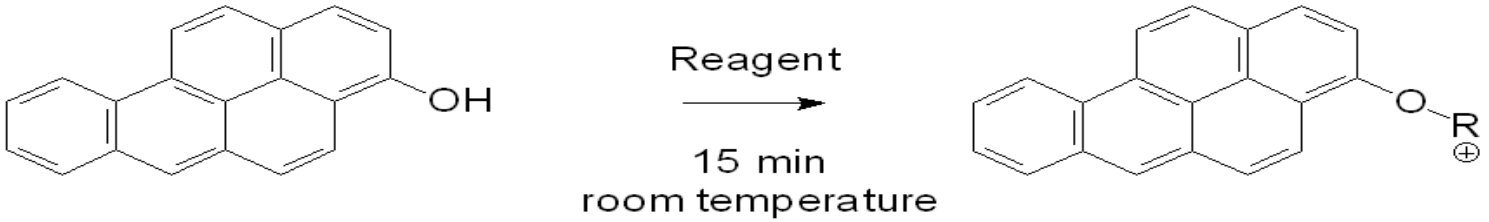


Sub-pg/mL Method Example



3-Hydroxybenzo[a]pyrene
$C_{20}H_{12}O$
268.31 Da

Derivatization of 3-OH-BaP



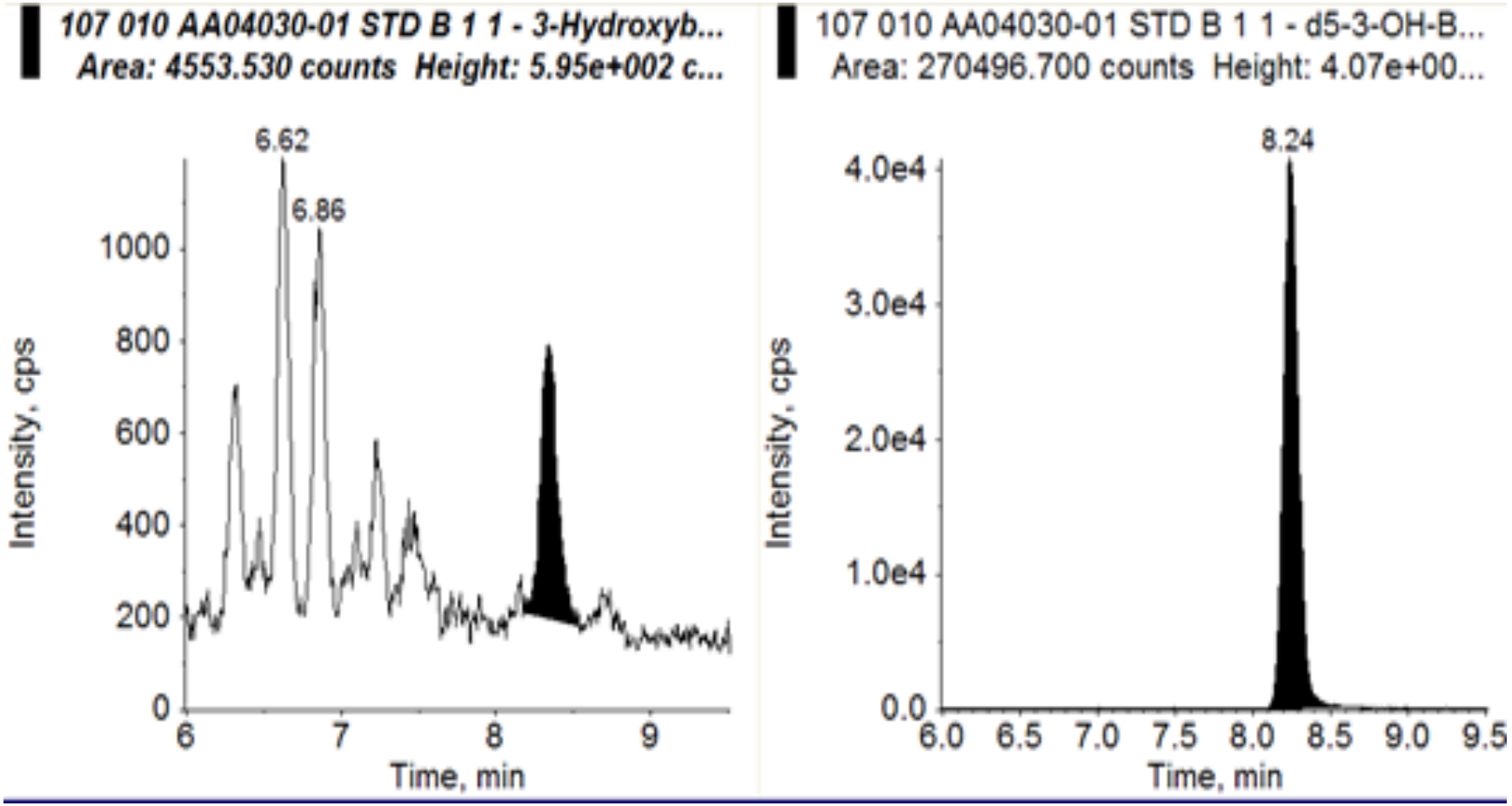
3-OH-BaP

Derivatized 3-OH-BaP

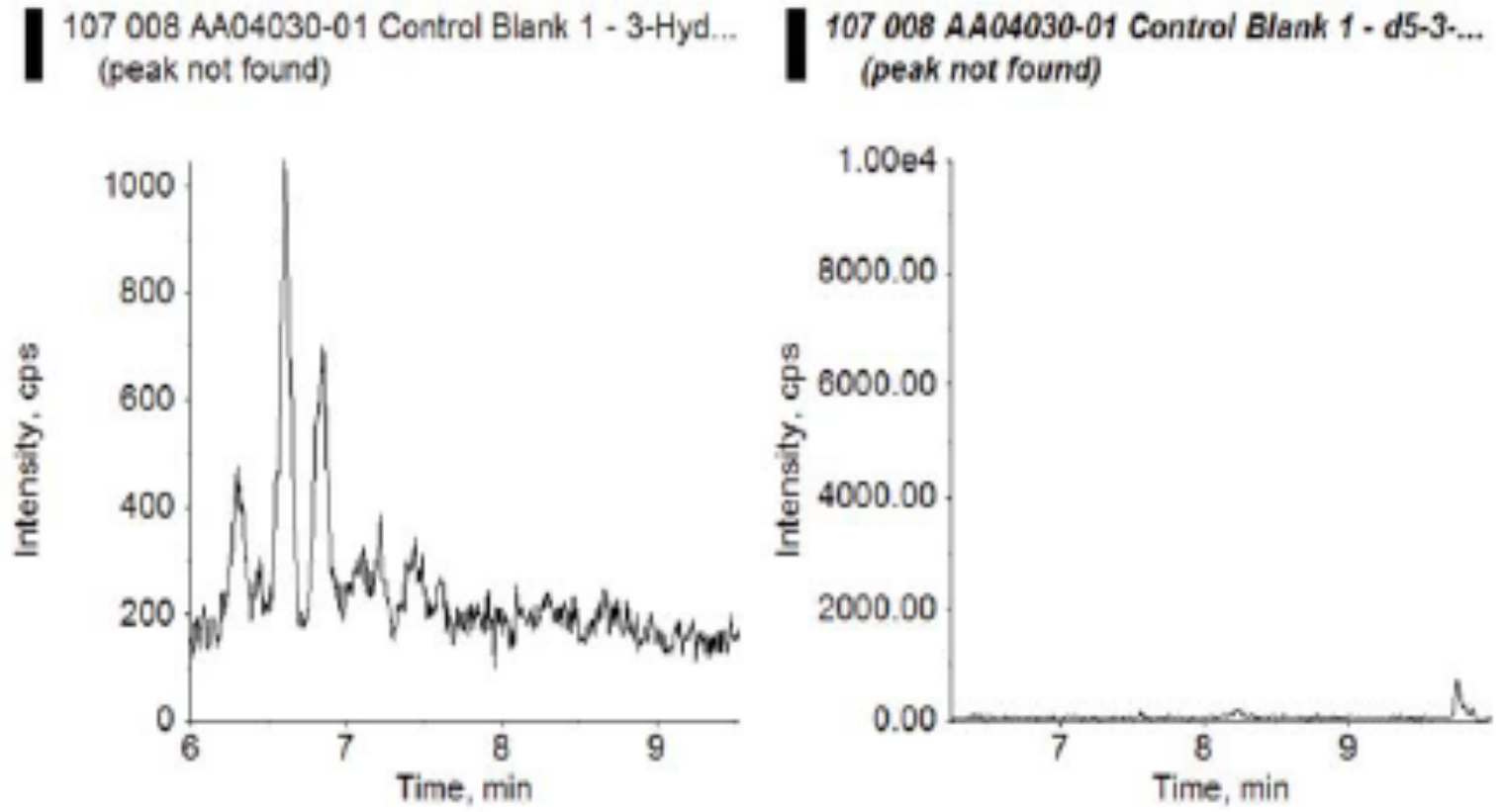
Derivatization added a new functional group which is easily ionized.

Improved fragmentation- MS/MS efficiency in positive ion mode.

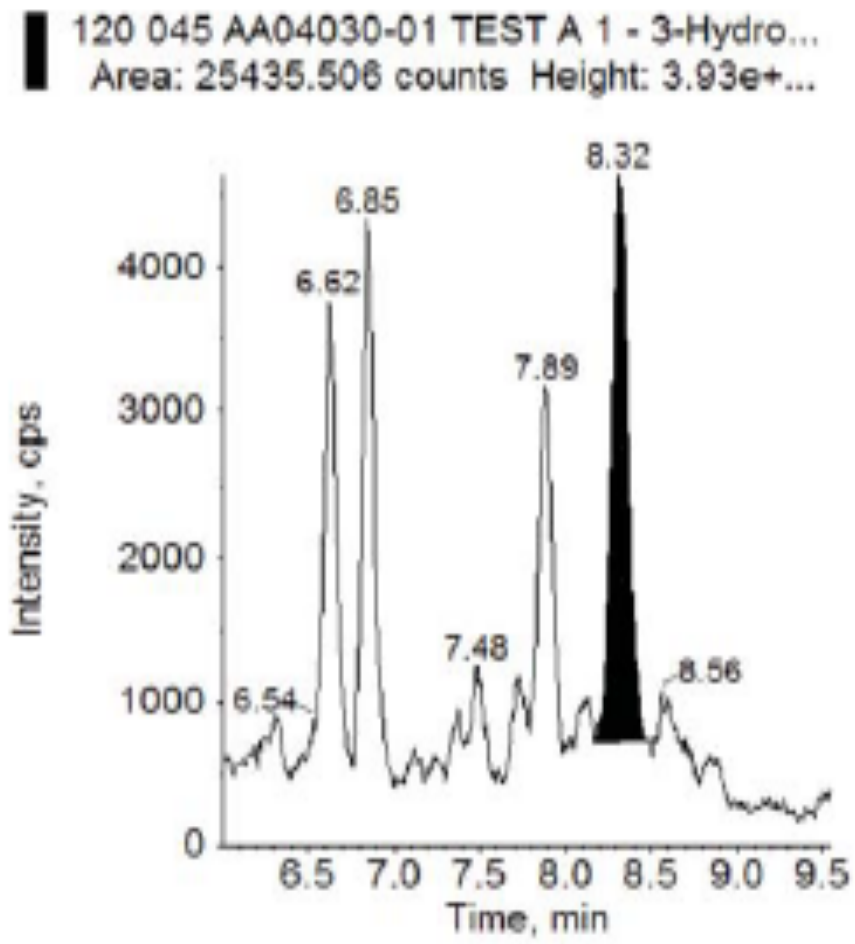
3-OH BaP LLOQ S/N= 23 (50 fg/mL in dog urine)

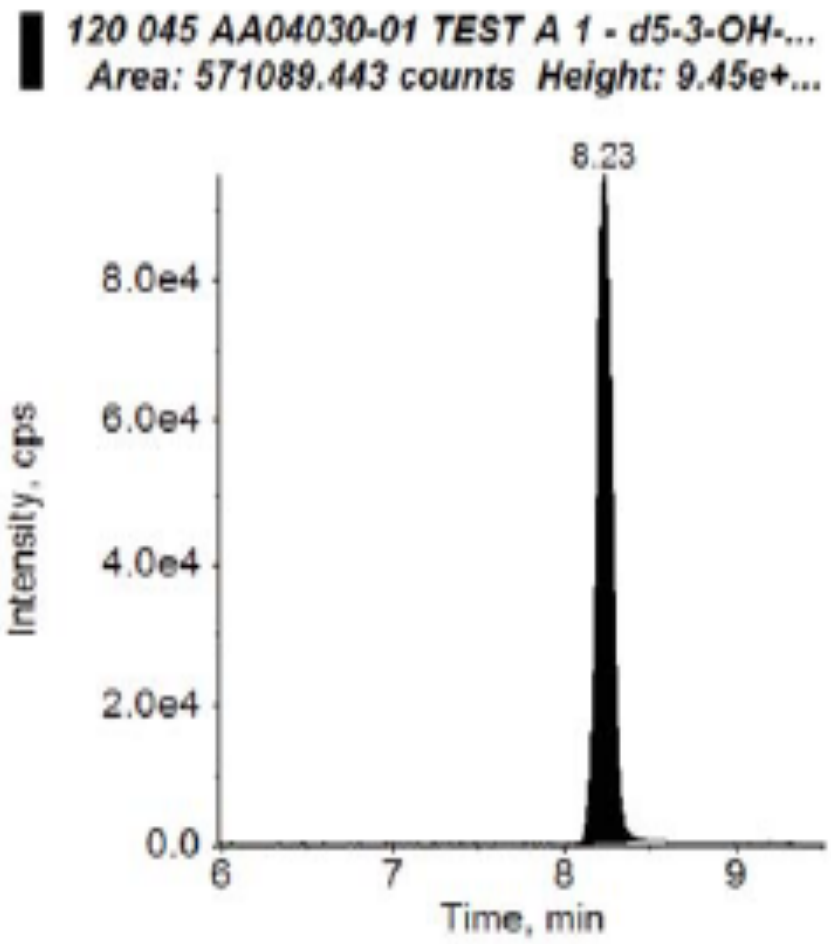


3-OH BaP Extracted Blank (in dog urine)



3-OH BaP Extracted Human Urine Sample Approximately 150 fg/mL





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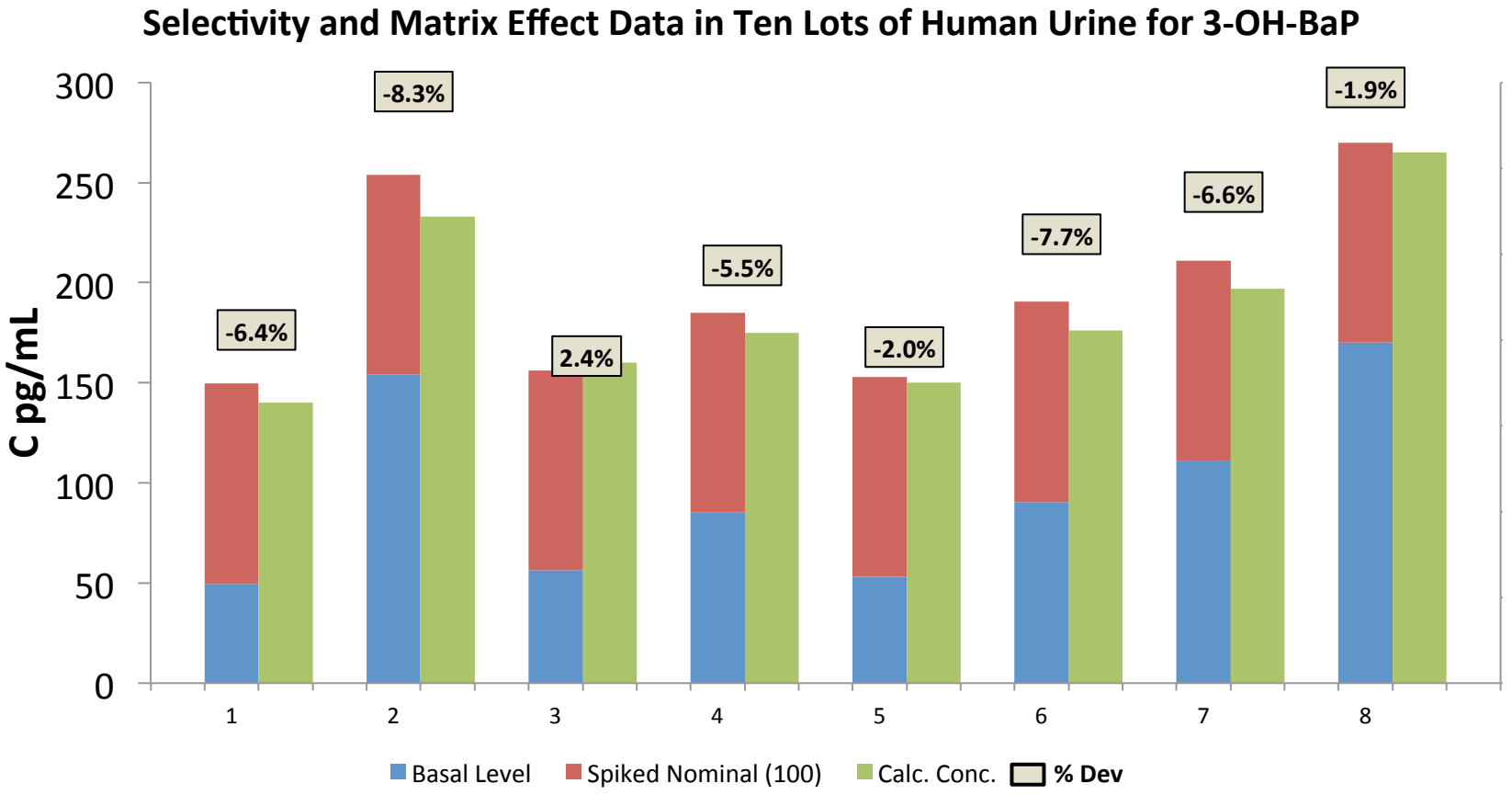
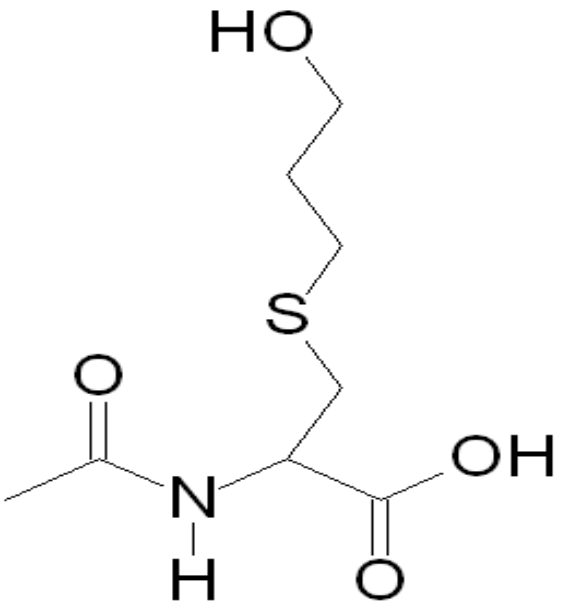
Accuracy of Matrix Effect Data in Human Urine



Summary for 3-OH-BaP Method

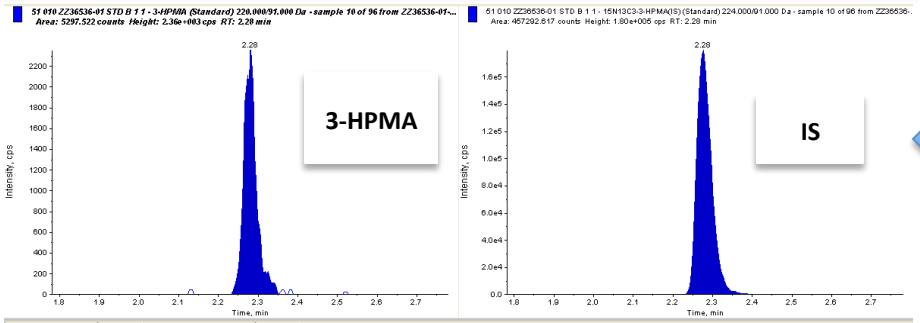
- An ultra-sensitive method for 3-OH-B[a]P with LLOQ of 50 fg/mL was developed and validated.
- Robustness was demonstrated by a batch passing rate over 98%.

Structures of 3-HPMA

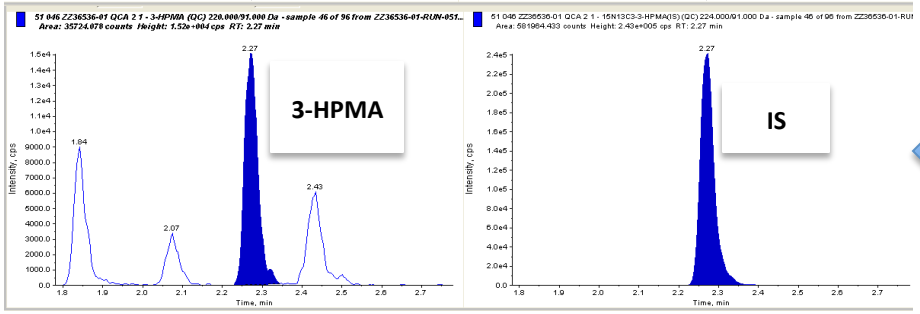


3-HPMA
N-Acetyl-S-(3-hydroxypropyl)cysteine
221.28 Da

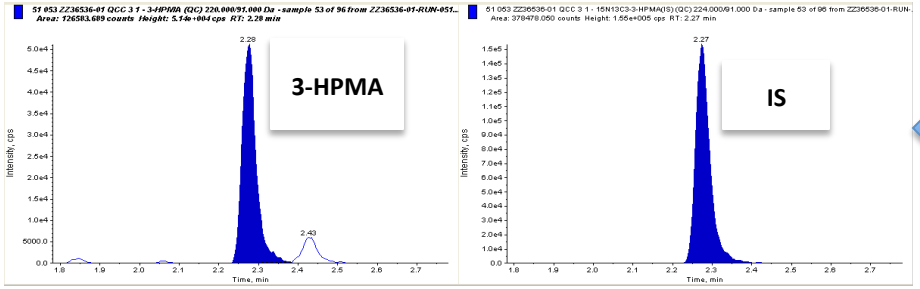
3-HPMA RP-UHPLC Chromatography Using the Stable labeled Internal standard



LLOQ in Artificial Urine

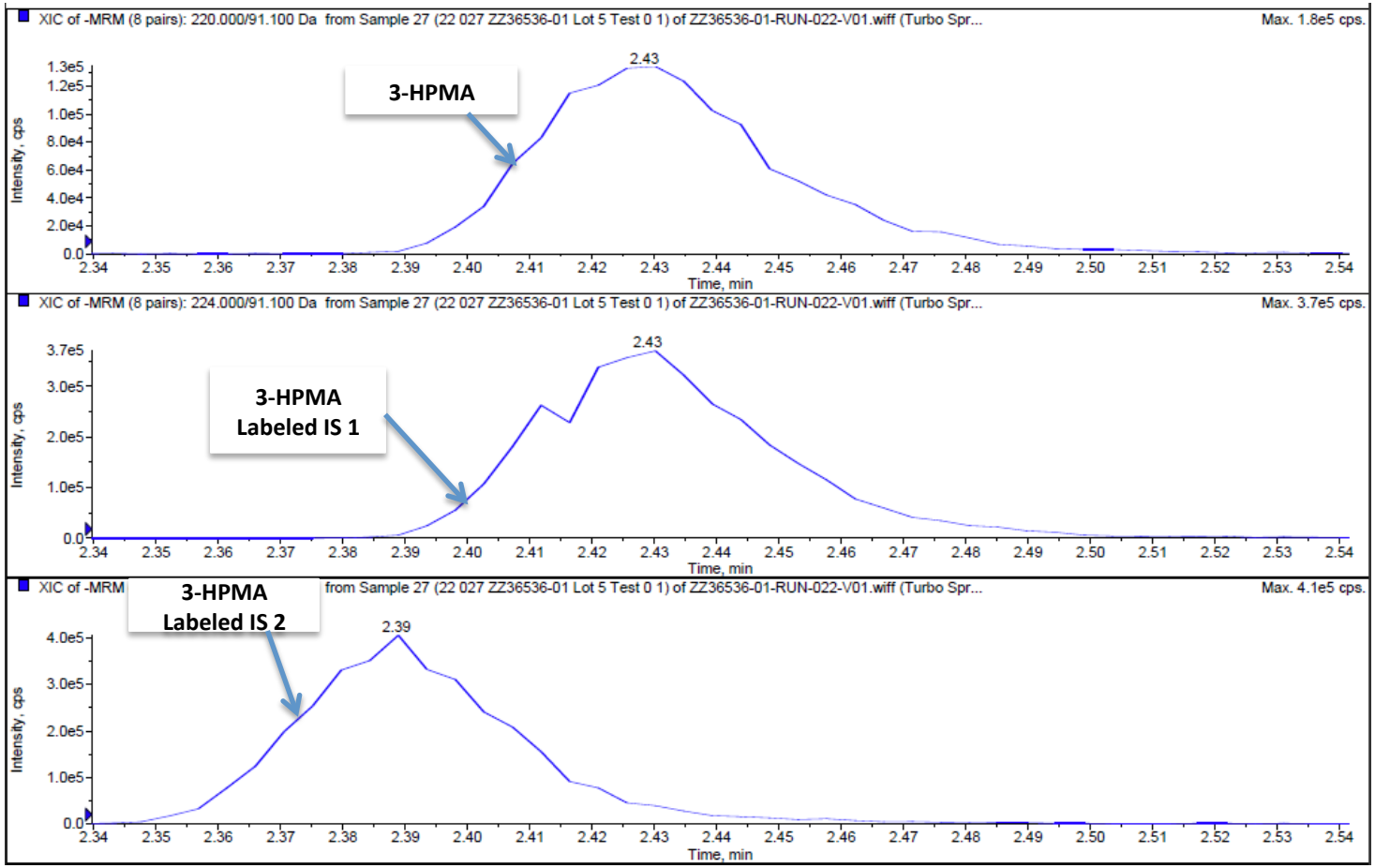


Low QC in Human Urine



High QC in Human Urine

3-HPMA UPLC RP chromatography using 2 stable labeled internal standards



Matrix Effect Test in Human Urine Comparing the Performance of Two Different Labeled Internal Standards

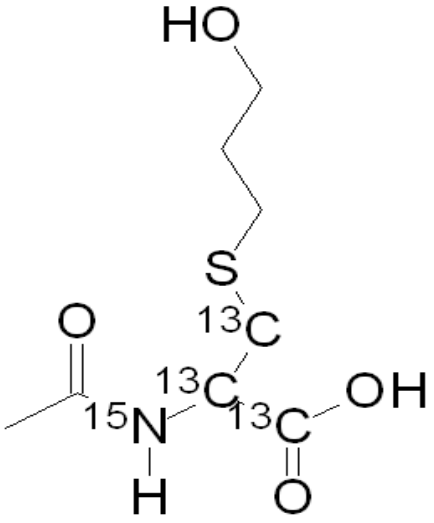
A

Lot#	Basal Level (ng/mL)	Low Spiked Nominal Conc. (ng/mL)	Expected Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Dev.	High Spiked Nominal Conc. (ng/mL)	Expected Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Dev.
1	813	20.0	833	768	-7.8	3750	4563	4660	+2.1
2	1160	20.0	1180	1150	-2.5	3750	4910	4840	-1.4
3	31.1	20.0	51.1	53.1	+3.9	3750	3781	3900	+3.1
4	225	20.0	245	256	+4.5	3750	3975	4010	+0.9
5	22.1	20.0	42.1	39.3	-6.7	3750	3772	3950	+4.7
6	1320	20.0	1340	1280	-4.5	3750	5070	5160	+1.8
7	1960	20.0	1980	1850	-6.6	3750	5710	5660	-0.9
8	440	20.0	460	446	-3.0	3751	4191	4130	-1.5

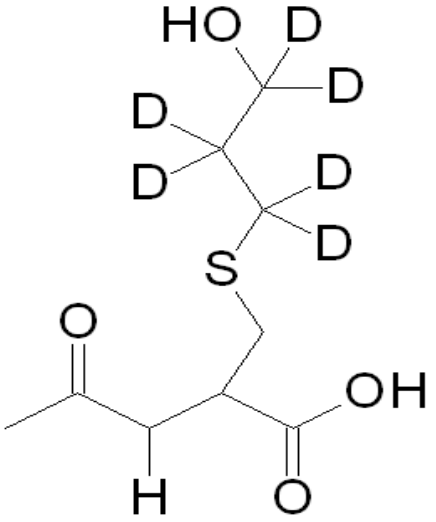
B

Lot#	Basal Level (ng/mL)	Low Spiked Nominal Conc. (ng/mL)	Expected Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Dev.	High Spiked Nominal Conc. (ng/mL)	Expected Conc. (ng/mL)	Calc. Conc. (ng/mL)	% Dev.
1	795	20.0	815	828	+1.6	3750	4545	4080	-10.2
2	*902*	20.0	922	860	-6.7	3750	4652	3500	**-24.8**
3	30.7	20.0	50.7	49.1	-3.2	3750	3781	3390	-10.3
4	227	20.0	247	268	+8.5	3750	3977	4140	+4.1
5	21.3	20.0	41.3	38.4	-7.0	3750	3771	3790	+0.5
6	1230	20.0	1250	1110	-11.2	3750	4980	4260	**-14.5**
7	*2330*	20.0	2350	2300	-2.1	3750	6080	6970	**+14.6**
8	*536*	20.0	556	572	+2.9	3750	4286	5270	**+23.0**

Structures of 3-HPMA Internal Standards



[^{15}N,$^{13}C_3$]-3-HPMA
225.25 Da



[$^{2}H_6$]-3-HPMA
226.32 Da

d_6-3HPMA failed to accurately quantify 3-HPMA matrix effect test in human urine when [$^{15}N^{13}C_3$]3-HPMA reliably tracked the analyte.

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3-HPMA Method Results

- A LC-MS/MS method for the simultaneous analysis of CEMA, 3-HPMA and HBMA with improved selectivity has been developed and validated.

Conclusion

- Two dimensional chromatography provided better selectivity for the assay of NNN and MHBMA.
- Derivatization provided an excellent solution to enhancing sensitivity to reach sub pg/mL levels 3-OH-BaP.
- ^{13}C and ^{15}N labeled internal standards which coelute with the analytes provide the most robust bioanalytical methods for 3-HPMA.

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- Veni Lapko
- Paul Brown

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