

**DEVELOPMENT AND VALIDATION OF LC-MS/MS METHODS  
WITH 96-WELL MIXED-MODE SOLID-PHASE EXTRACTION  
FOR THE ANALYSIS OF S-PHENYL MERCAPTURIC ACID AND  
3-HYDROXYPROPYL MERCAPTURIC ACID IN HUMAN URINE**

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# *Background (1)*

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- Benzene and acrolein represent two classes of compounds found in mainstream cigarette smoke (MSS)
  - Deliveries can be reduced by selective filtration
  - Extent of reductions in MSS can be measured analytically
  - Estimation of reductions in uptake by smokers of products with selective filtration versus uptake with conventionally filtered products requires human biomonitoring

## *Background (2)*

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- S-Phenylmercapturic acid (SPMA) has been used as biomarker for human exposure to benzene
  - Transformation of benzene to SPMA reported to be from 0.005 to 0.3% (Melikian *et al.*, *J. Chrom. B*, 2002)
  - About 10X higher intake in smokers versus nonsmokers (Wallace, *Environ. Health Perspect.*, 1996 Suppl 6)
  - Several LC-MS/MS methods reported recently

## *Background (3)*

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- 3-hydroxypropylmercapturic acid (3-HPMA) has been used as a biomarker for human exposure to acrolein
  - Acrolein formed endogenously through lipid peroxidation
  - Also found in automobile exhaust
  - About 3X higher urinary 3-HPMA concentrations in smokers versus nonsmokers using LC-MS/MS method (Mascher *et al.*, *J. Chrom. B*, 2001)

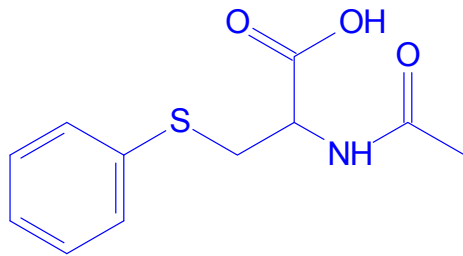
# *Analytical strategy*

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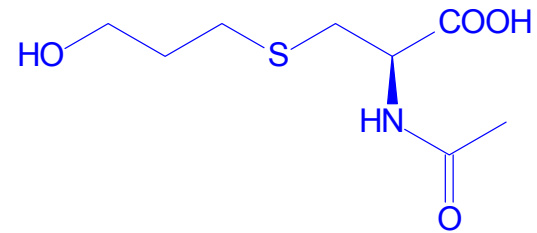
- Use deuterated analogs of SPMA (SPMA-d<sub>5</sub>) and 3-HPMA (3-HPMA-d<sub>3</sub>) as internal standards (ISTDs) to improve method reliability
- Use mixed-mode SPE for sample clean-up
- Use well-established automation strategy to improve sample analysis throughput and method robustness

# Structures of analytes and ISTDs

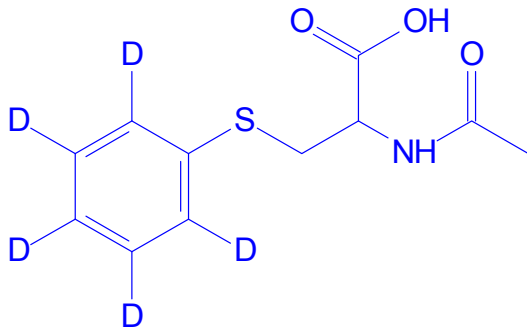
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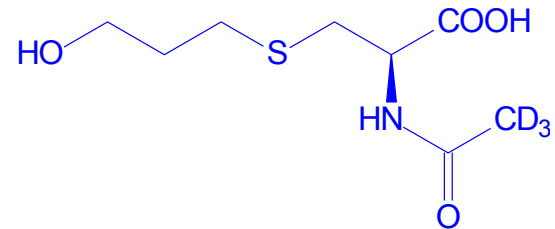
SPMA



3-HPMA



SPMA-*d*<sub>5</sub>



3-HPMA-*d*<sub>3</sub>

# *Sample preparation (1)*

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- Use Multiprobe liquid-handling station for taking urine aliquots (0.2 mL for SPMA and 0.1 mL for 3-HPMA) and transfer to 96-well plates
- Use Tomtec 96-well SPE work-station for the SPE (Oasis MAX cartridge) extraction and other sample preparation procedures

## *Sample preparation (2)*

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- The following steps are performed with the Tomtec
  - Precondition SPE cartridges with methanol and water.
  - Add pH 6.3 sodium acetate buffer to samples prior to SPE clean-up
  - After sample application, wash cartridges with water and then methanol.
  - Elute analytes and ISTDs with methanol containing 2% formic acid.

## *Sample preparation (3)*

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- Evaporate the samples to dryness with a SPE Dry-96 sample concentrator
- After dry down under nitrogen, the residue is reconstituted with 150  $\mu$ L of acetonitrile-water-formic acid (30:70:0.1 for SPMA and 10:90:0.1 for 3-HPMA) and analyzed using liquid chromatography (LC) with tandem mass spectrometric detection (MS/MS)

# *LC conditions*

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- Column: Genesis C18, 4.6 x 50 mm, 3  $\mu\text{m}$
- Column oven: 35°C
- Mobile phase:
  - For SPMA: Acetonitrile - 5 mM ammonium acetate - formic acid (40:60:0.2)
  - For 3-HPMA: Acetonitrile - water - formic acid (17:93:0.2)
- Flow rate: 1.0 ml/min
- Injection volume: 10  $\mu\text{l}$
- Run times: 2.5 min for SPMA and 2.0 min for 3-HPMA

# *MS/MS conditions for SPMA*

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- Mass Spectrometer: Sciex API 3000
- Ionization: ESI-
- Mode: MRM
- Ion spray voltage: -3500 V
- Ion spray temperature: 450 °C
- Curtain Gas Type: Nitrogen Setting: 9
- CAD Gas Type: Nitrogen Setting: 4
- Nebulizing Gas Type: Nitrogen Setting: 11
- Auxiliary Gas Flow: 8 L/minute

# *MS/MS conditions for 3-HPMA*

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- Mass Spectrometer: Sciex API 3000
- Ionization: ESI+
- Mode: MRM
- Ion spray voltage: 4500 V
- Ion spray temperature: 500°C
- Curtain Gas Type: Nitrogen Setting: 9
- CAD Gas Type: Nitrogen Setting: 4
- Nebulizing Gas Type: Nitrogen Setting: 11
- Auxiliary Gas Flow: 8 L/minute

# *MS/MS conditions*

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<b>Compound Name</b>	<b>Transition Monitored</b>	<b>Dwell Time (msec)</b>	<b>Collision Energy (eV)</b>	<b>Approximate Retention Time (min)</b>
SPMA	238.0→109.1	150	-17	0.95
ISTD	243.1→114.1	150	-17	0.95

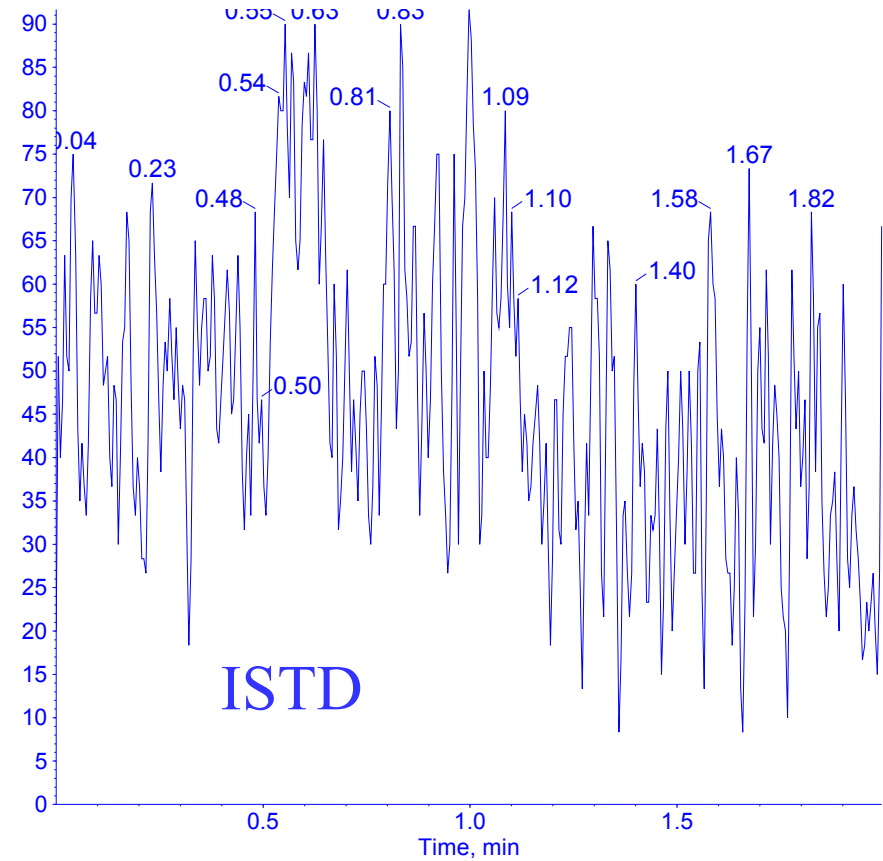
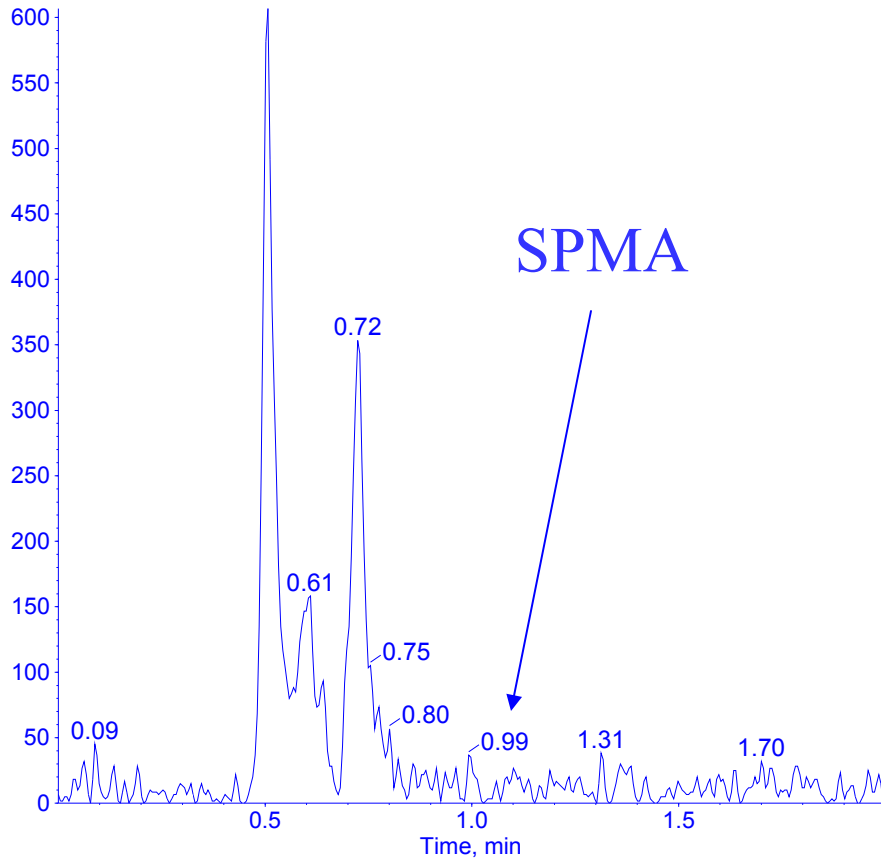
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<b>Compound Name</b>	<b>Transition Monitored</b>	<b>Dwell Time (msec)</b>	<b>Collision Energy (eV)</b>	<b>Approximate Retention Time (min)</b>
3-HPMA	221.1→117.2	150	21	0.8
ISTD	225.1→117.2	150	21	0.8

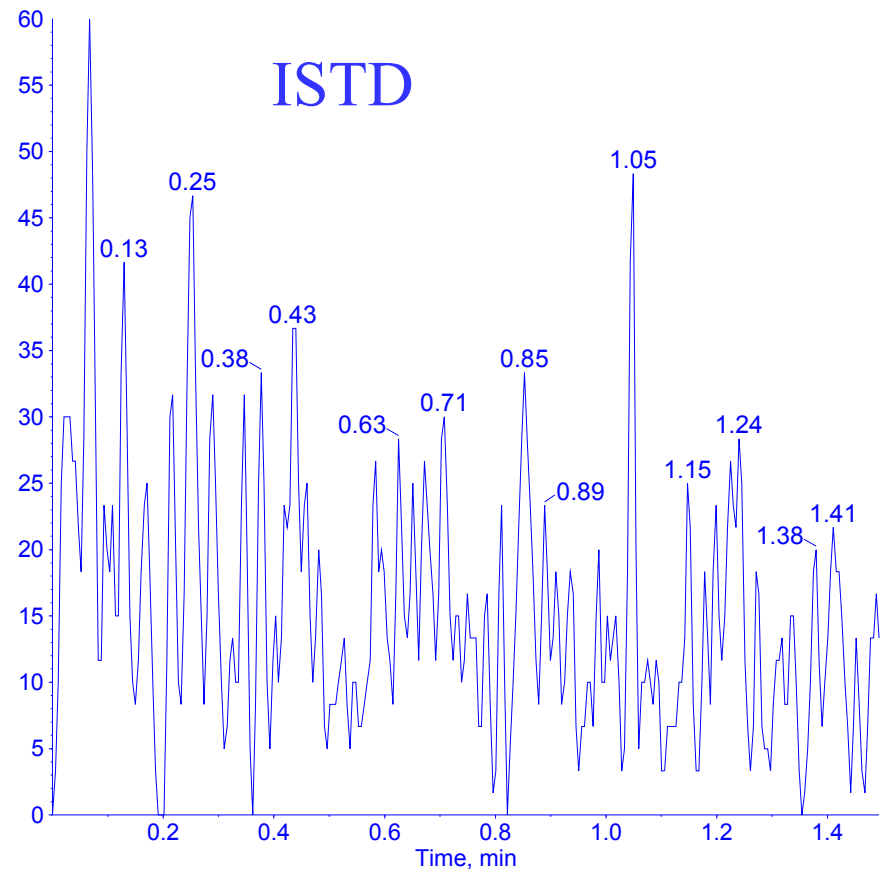
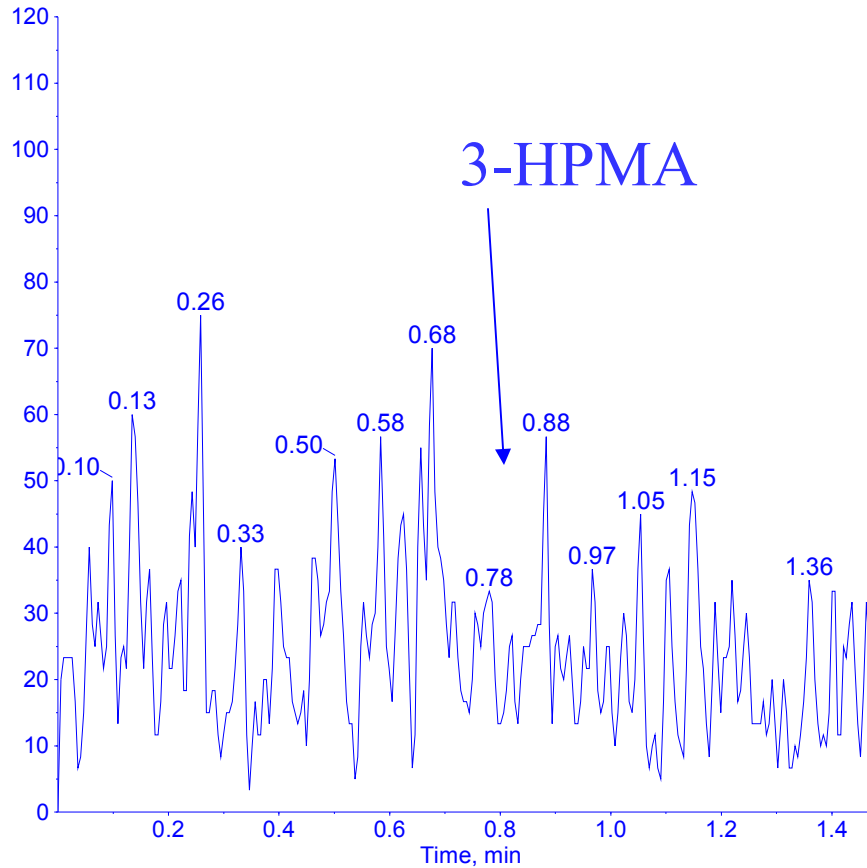
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- Quantitation
  - Regression type: Linear
  - Weighting:  $1/(\text{concentration})^2$

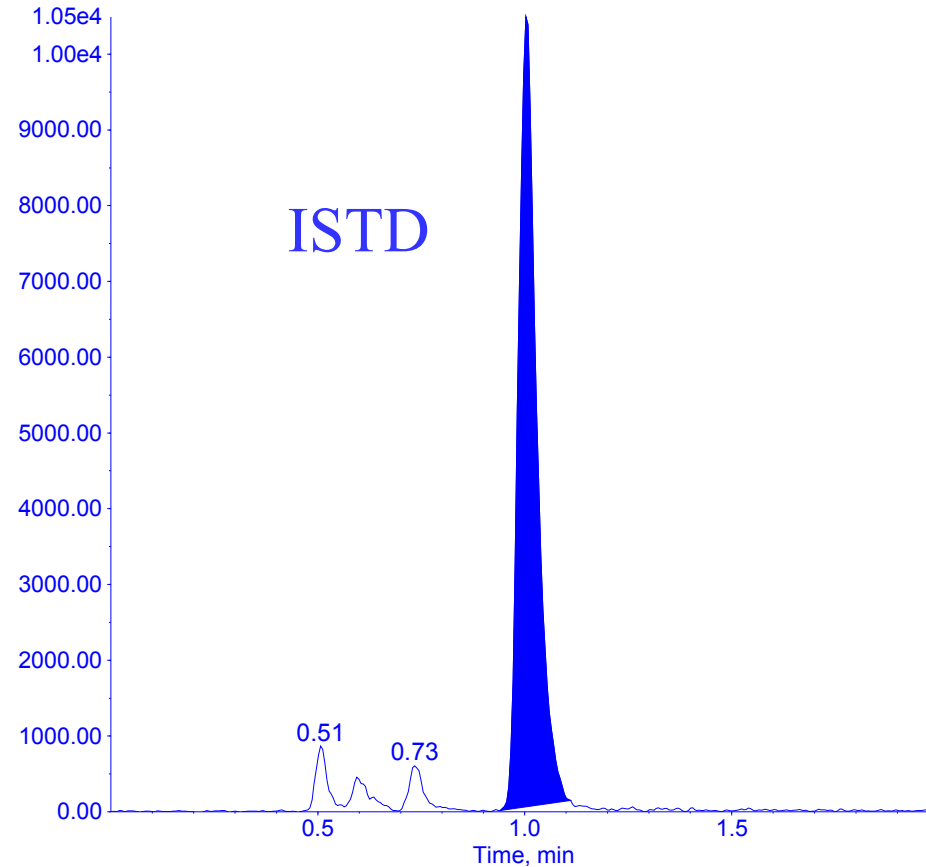
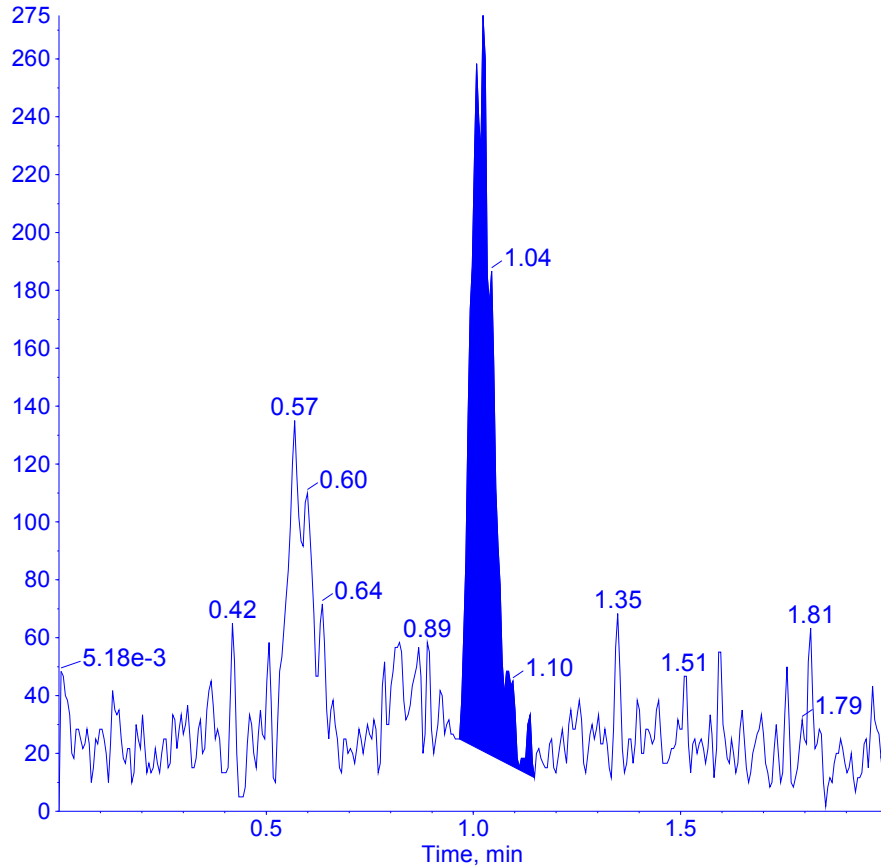
# Chromatogram of Blank Urine



# Chromatogram of Blank Urine

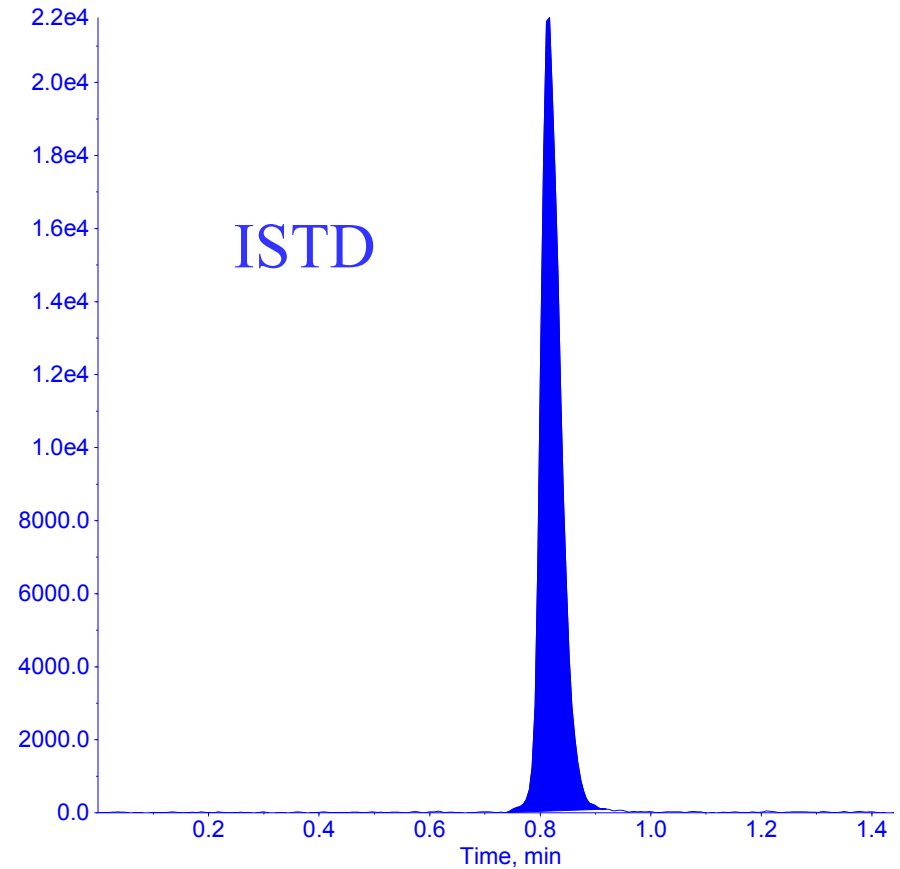
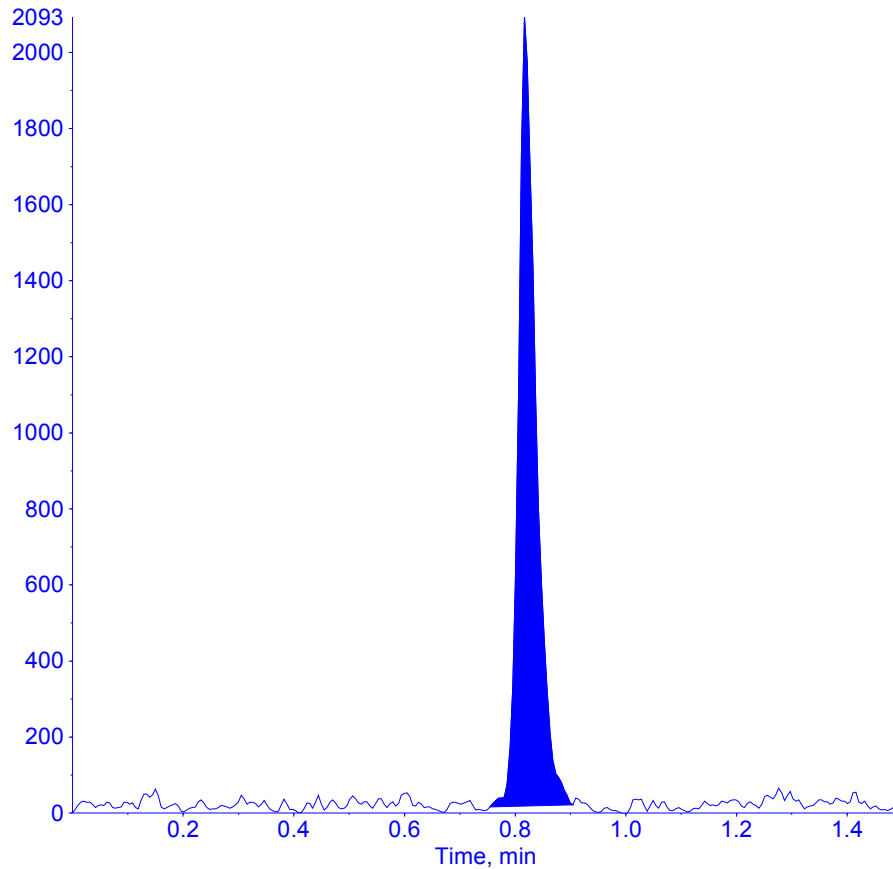


# Chromatogram of LLOQ



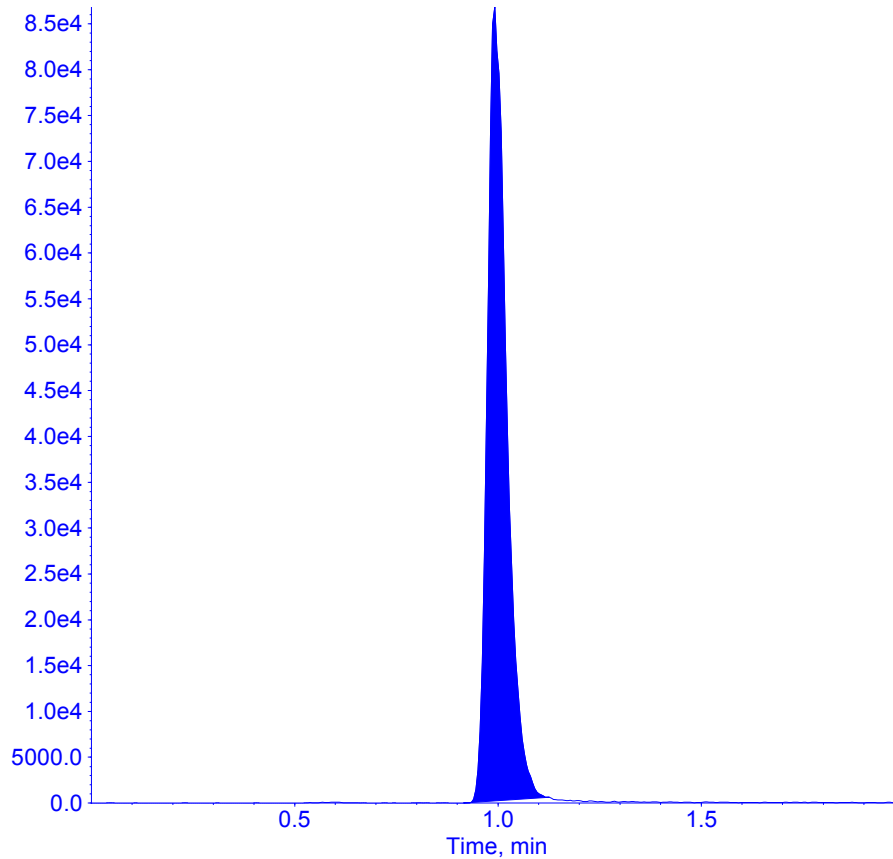
0.4 ng/mL SPMA

# Chromatogram of LLOQ

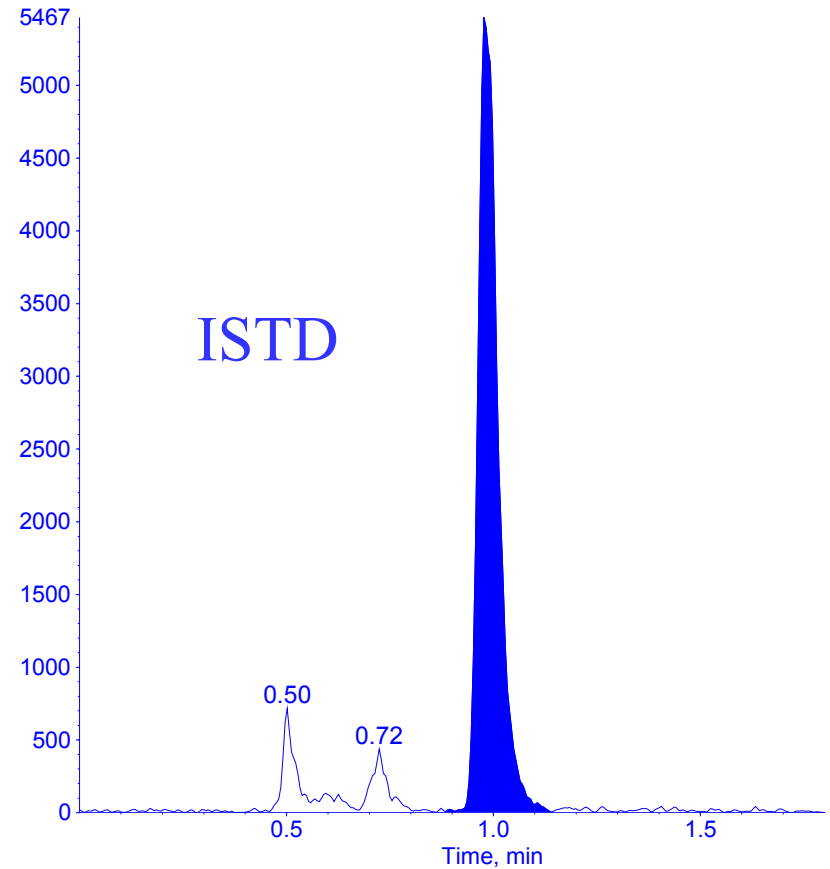


50 ng/mL 3-HPMA

# Chromatogram of ULOQ

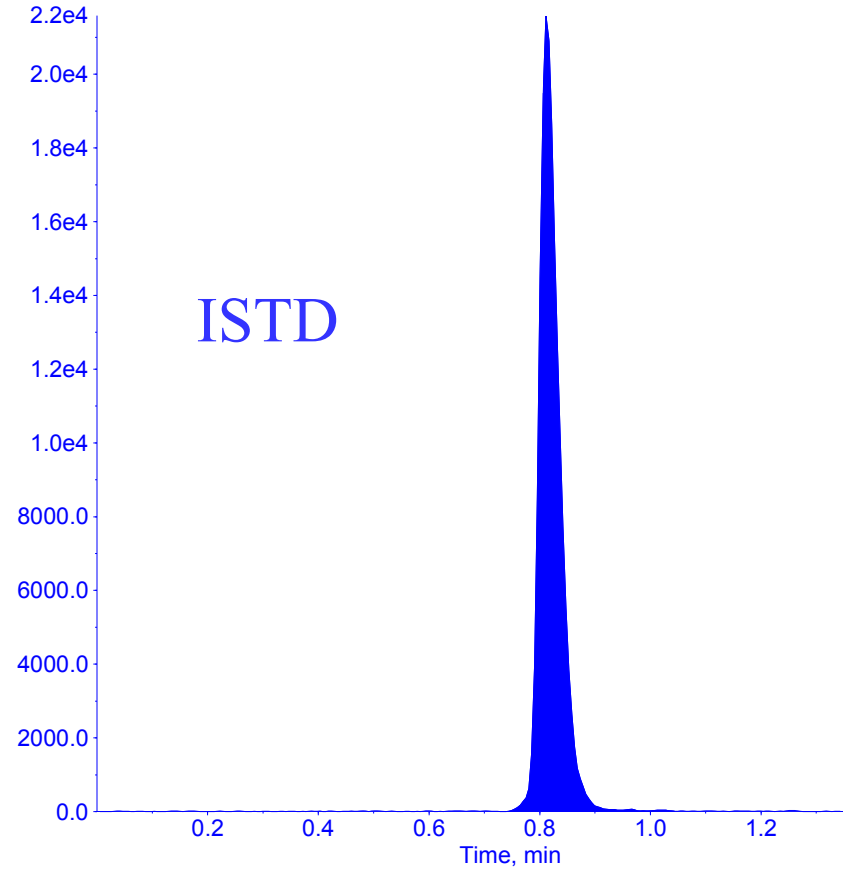
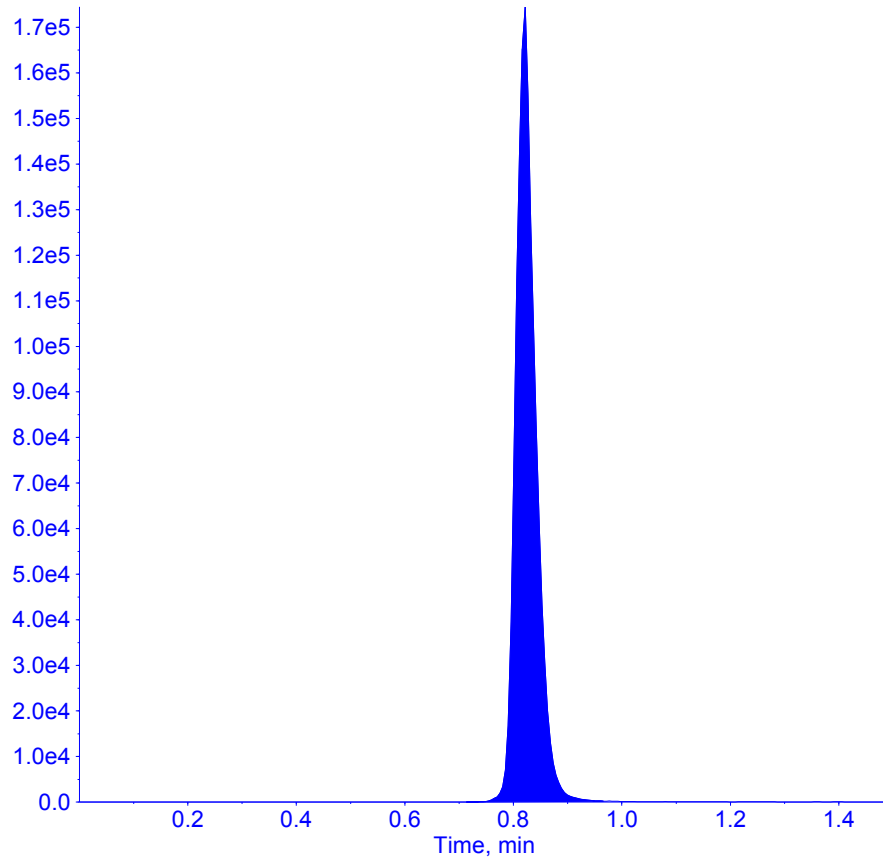


200 ng/mL SPMA



# Chromatogram of ULOQ

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5000 ng/ml 3-HPMA

# Precision and accuracy of QC samples (Intraday)

SPMA	QC 0.400 ng/mL (LLOQ)	QC 1.20 ng/mL (Low)	QC 20.0 ng/mL (Mid)	QC 150 ng/mL (High)	*QC 400 ng/mL (Dilution)
N	6	6	6	6	6
Mean	0.430	1.24	20.6	154	402
% RSD	4.6	4.7	5.6	3.1	4.4
% DMT	7.5	3.3	3.0	2.7	0.5

3-HPMA	QC 50.0 ng/mL (LLOQ)	QC 116 ng/mL (Low)	QC 716 ng/mL (Mid)	QC 3816 ng/mL (High)	*QC 10116 ng/mL (Dilution)
N	6	6	6	6	6
Mean	49.2	114	772	4200	10400
% RSD	4.7	3.8	2.1	1.5	1.8
% DMT	-1.6	-1.7	7.8	10.1	2.8

\* Samples were diluted 10x prior to analysis.

# *Precision and accuracy of QC samples*

SPMA	QC 1.20 ng/mL (Low)	QC 20.0 ng/mL (Mid)	QC 150 ng/mL (High)
n	17	18	18
Mean	1.18	20.4	149
% RSD	6.5	3.7	4.7
% DMT	-1.7	2.0	-0.7

3-HPMA	QC 116 ng/mL (Low)	QC 716 ng/mL (Mid)	QC 3816 ng/mL (High)
n	18	18	18
Mean	113	710	3860
% RSD	4.5	6.9	6.7
% DMT	-2.6	-0.8	1.2

# *Precision and accuracy of calibration standards*

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SPMA	Calibration Level (ng/mL)							
	0.400	1.00	5.00	10.0	50.0	80.0	160	200
n	3	3	3	3	3	3	3	3
Mean	0.401	0.996	5.18	9.60	48.2	77.6	169	205
% RSD	2.8	7.7	3.3	2.4	8.0	4.7	6.8	9.5
% DMT	0.3	-0.4	3.6	-4.0	-3.6	-3.0	5.6	2.5

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3-HPMA	Calibration Level (ng/mL)							
	50.0	100	200	500	1000	2000	4000	5000
n	3	2	3	3	3	3	3	3
Mean	49.7	100	203	501	1010	2020	3970	4900
% RSD	1.2	NA	3.9	1.5	3.0	1.7	1.3	1.0
% DMT	-0.6	0.0	1.5	0.2	1.0	1.0	-0.8	-2.0

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# *Method stability - SPMA*

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## Condition

Stock standard solution in human urine  
Stock standard solution in human urine  
Stock internal standard solution in human urine  
Freeze-thaw in matrix  
Room temperature (benchtop) in matrix  
Processed-samples stored at 10°C  
Frozen storage stability at -10 to -30°C

## Minimum Stability

6 hours at room temperature  
12 days at 2 to 8°C  
6 hours at room temperature  
three cycles  
24 hours  
approximately 54 hours  
44 days

# *Method stability – 3-HPMA*

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## Condition

Stock standard solution stability 3-HPMA

Stock standard solution stability 3-HPMA-d<sub>3</sub>

Freeze-thaw in matrix

Room temperature (benchttop) in matrix

Processed-samples stored at 10°C

## Minimum Stability

6 hours at room temperature

18 hours at room temperature

three cycles

24 hours

approximately 49 hours

# *Extraction Recovery*

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- SPMA
  - 87.3% (RSD% = 5.0) at 1.2 ng/ml
  - 87.3% (RSD% = 3.2) at 20 ng/ml
  - 88.9% (RSD% = 2.9) at 150 ng/ml
- Overall recovery: 87.8%
- Overall recovery for SPMA- $d_5$ : 81.1%
- 3-HPMA- $d_3$ 
  - 49.3% (RSD% = 14) at 150 ng/ml
  - 52.3% (RSD% = 4.3) at 800 ng/ml
  - 47.6% (RSD% = 8.0) at 3500 ng/ml
- Overall recovery: 49.7%
- Background level of 3-HPMA prevented use for extraction recovery

# *Conclusions*

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- Sensitive, robust and fast LC-MS/MS methods were developed and validated to determine SPMA and 3-HPMA in human urine.
- Automation strategy and deuterated internal standards were used to significantly enhance the method efficiency and robustness

# *Acknowledgement*

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- Dr. Heiko Junga from Covance Laboratory is acknowledged for synthesizing and characterizing SPMA-d<sub>5</sub>.