Extraction of a Drugs of Abuse Panel from Human Urine Using Biotage® Mikro CX SPE Microelution Plates Prior to UPLC-MS/MS Analysis

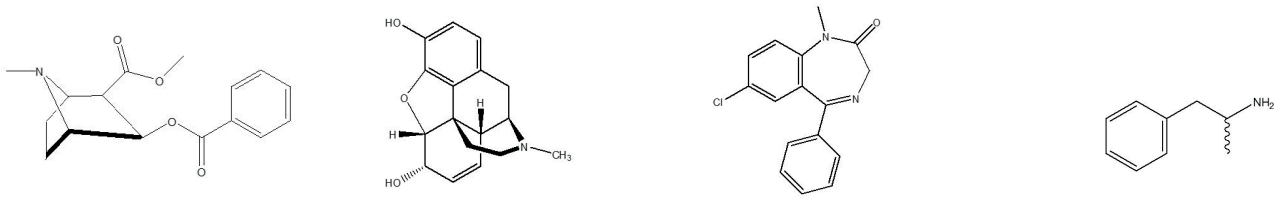

Figure 1. Example structures by class.

Introduction

This application note describes the extraction of mulitple drugs of abuse from human urine using Biotage® Mikro CX microelution plates, prior to LC/MS analysis.

The simple sample preparation procedure, based on a mixed-mode/strong cation exchange extraction mechanism, delivers clean extracts and analyte recoveries mostly greater than 70% with RSDs lower than 5% for most analytes. Linearity of greater than 0.999 is achieved for all analytes from 1–1000 pg/mL.

Mikro plate extraction allows for very low elution volumes and enhanced workflow efficiency.

Analytes

Amphetamine, Methamphetamine, 3,4-Methylenedioxyamphetamine (MDA), 3,4-Methylenedioxymethamphetamine (MDMA), 3,4-Methylenedioxy-N-ethylamphetamine (MDEA), Hydromorphone, Morphine, Benzoylecgonine (BZE), Oxymorphone, Dihydrocodiene, Oxycodone, Mephedrone, Norfentanyl, 7-amino-flunitrazipam, 7-amino-clonazepam, Hydrocodone, Codeine, 6-Monoacetylmorphine (6-MAM), Cocaine, Norketamine, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), Zaleplon, Zopiclone, Norbuprenorphine, Ketamine, Nitrazepam, Flunitrazepam, Clonazepam, α-OH-triazolam, Oxazepam, Estazolam, Temazepam, Zolpidem, Alprazolam, Methadone, Lorazepam, Bromazepam, α-OH-alprazolam, 2-OH-ethyl-flurazepam, Triazolam, Nordiazepam, Diazepam, Midazolam, Fentanyl, Flurazepam, Buprenorphine, Phencyclidine (PCP), Lysergic acid diethylamide (LSD).

Internal Standards

Amphetamine-D_5, Morphine-D_3, BZE-D_3, 6-MAM-D_3, Diazepam-D_5

Sample Preparation Procedure

Format

Biotage® Mikro CX Plate, 2 mg, part number 601-0002-LVP

Sample Pre-Treatment

Spike urine (1 mL) with internal standard solution and allow to equilibrate for 1 hour. Dilute sample with 100 mM NH_4OAC pH 5 (950 µL) and add β-glucuronidase (50 µL). Incubate at 60 °C for 2 hours.

Internal standard solution consisted of a 10 pg/ µL methanolic solution. 100 µL of this was added to 1 mL of urine to give a 1 ng/mL spike concentration.

Condition (Optional)

Condition wells with methanol (100 µL)

Equilibration (Optional)

Equilibrate wells with 4% phosphoric acid (aq) (100 µL)

Sample Loading

Load 400 µL of the pre-treated urine sample

Wash 1

Elute interferences with 4% phosphoric acid (aq) (100 µL)

Wash 2

Elute interferences with H_2O:MeOH (50:50, v/v, 100 µL)

Elution

Elute analytes with DCM:MeOH:NH_4OH (78:20:2, v/v, 30 µL)

Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry at 40 °C, 20 to 40 L/min, or a TurboVap® at 40 °C, 1.5 L/min, for approximately 5 minutes.

Reconstitute evaporated samples with H_2O:MeOH (90/10, v/v) containing 0.1% formic acid (30 µL).

Vortex mix and cover plate with a sealing mat prior to injection.

Processing Conditions

Biotage® Mikro plates were processed using a Biotage® PRESSURE+ Positive Pressure Manifold.

Settings: Condition, equilibrate, load, wash and elute steps: 7–9 psi (fine control setting).

UHPLC Conditions

Instrument
Shimadzu Nexera UHPLC

Column
Restek Raptor™ Biphenyl 2.7 µm (100 x 2.1 mm)

Mobile Phase
A: 2 mM ammonium formate (aq) containing 0.1% formic acid

B: 2 mM ammonium formate (MeOH) containing 0.1% formic acid

Flow Rate
0.4 mL/min

Injection Volume
5 µL

Column Temperature
30 °C

Table 1. HPLC Gradient.

Time (min.)	%A	%B
0	80	20
2.00	80	20
7.50	40	60
11.25	40	60
12.75	0	100
13.50	0	100
13.51	80	20
15.00	80	20

MS Conditions

Instrument
Shimadzu 8060 Triple Quadrupole MS using ES interface

Nebulizing Gas Flow
3 L/min

Drying Gas Flow
3 L/min

Heating Gas Flow
17 L/min

Interface Temperature
400 °C

DL Temperature
250 °C

Heat Block Temperature
300 °C

CID Gas Flow
270 kPa

Table 2. MS conditions for target analytes in positive mode.

Analytes	MRM Transition	Collision Energy
Morphine-D_3	289.0 > 201.1 (289.0 > 152.1)	-26.0 -50.0
Morphine	286.0 > 152.1 (286.0 > 201.1)	-50.0 -25.0
Oxymorphone	302.00 > 227.1 (302.00 > 198.1)	-30.0 -45.0
Hydromorphone	286.0 > 185.0 (286.0 > 157.0)	-30.0 -40.0
Amphetamine-D_5	141.0 > 93.0 (141.0 > 124.15)	-15.0 -20.0
Amphetamine	136 > 91.05 (136 > 119.1)	-15.0 -14.0
Methamphetamine	150.0>90.95 (150>119.1)	-20.0 -14.0
MDA	180 > 105 (180 > 77)	-20.0 -40.0
Dihydrocodiene	302 > 119.05 (302 >171)	-35.0 -45.0
Codeine	300.0>215.1 (300.0>165)	-25.0 -40.0
6-MAM-D_3	331.0 > 165.1 (331.0 > 211.1)	-40.0 -25.0
6-MAM	328.0 > 165.1 (328.0 > 211.1)	-40.0 -25.0
MDMA	194.0 > 163.1 (194.0 > 105.0)	-15.0 -25.0
Oxycodone	316.2 > 241.2	-20.0
Mephedrone	178.00 > 145.05 (178.00 > 144.00)	-20.0 -30.0
Hydrocodone	300.0 > 199.05 (300.0 > 171.1)	-30.0 -40.0
MDEA	208 > 163.05 (208 > 105.05)	-15.0 -25.0
Nor-Ketamine	223.9 > 125 (223.9 > 179.05)	-20.0 -15.0
Nor-Fentanyl	233.0 > 84.05 (233.0 > 56.05	-20.0 -26.0
BZE-D_3	293.00 > 171.05 (293.00 > 77.00)	-20.0 -50.0
BZE	289.90 > 168.05 (289.90 > 105.00)	-20.0 -30.0
Ketamine	237.90 > 125.00 (237.90 > 207.05)	-30.0 -14.0
7-Aminoclonazepam	285.90 > 222.10 (285.90 > 121.10)	-25.0 -29.0
Cocaine	304.00 > 182.05 (304.00 > 82.05)	-20.0 -30.0
Zopiclone	388.90 > 245.05 (388.90 > 217.00)	-15.0 -35.0

Table 2. Continued.

Analytes	MRM Transition	Collision Energy
Norbuprenorphine	414.00 > 101.25 (414.00 > 187.20)	-39.0 -38.0
LSD	323.50 > 208.10 (323.50 > 223.25)	-29.0 -23.0
7-Aminoflunitrazepam	283.90 > 135.05 (283.90 > 227.05)	-30.0 -26.0
Zolpidem	308.00 > 235.10 (308.00 > 263.10)	-35.0 -25.0
Buprenorphine	468.10 > 396.25 (468.10 > 414.30)	-40.0 -35.0
Fentanyl	337.00 > 188.10 (337.00 > 105.00)	-20.0 -40.0
Flurazepam	388.00 > 315.00 (388.00 > 288.00)	-20.0 -26.0
PCP	244.00 > 91.05 (244.00 > 159.15)	-35.0 -14.0
Midazolam	325.90 > 249.10 (325.90 > 223.00)	-35.0 -40.0
Bromazepam	315.80 > 182.10 (315.80 > 209.10)	-31.0 -27.0
EDDP	278.00 > 234.00 (278.00 > 234.00)	-30.0 -45.0
Lorazepam	320.80 > 275.00 (320.80 > 229.05)	-22.0 -30.0
Oxazepam	320.80 > 229.05 (286.90 > 104.20)	-23.0 -35.0
Nitrazepam	286.90 > 104.20 (281.90 > 180.10)	-25.0 -35.0
Clonazepam	315.90 > 270.05 (315.90 > 214.05)	-25.0 -38.0
a-OH-Triazolam	358.90 > 331.10 (358.90 > 239.05)	-28.0 -44.0
2-OH-et-flurazepam	332.90 > 211.10 (332.90 > 109.00)	-37.0 -27.0
Methadrone	310.50 > 265.10	-16.0
a-OH-Alprazolam	324.90 > 216.10 (324.90 > 205.10)	-39.0 -46.0
Nordiazepam	270.90 > 140.05 (270.90 > 208.10)	-26.0 -28.0
Zaleplon	305.90 > 236.15 (305.90 > 264.20)	-28.0 -22.0
Flunitrazepam	313.90 > 268.10 (313.90 > 239.10)	-25.0 -35.0
Estazolam	294.90 > 267.05 (294.90 > 205.05)	-20.0 -40.0
Temazepam	300.90 > 255.05 (300.90 > 177.05)	-20.0 -39.0
Triazolam	342.90 > 308.10 (342.90 > 239.05)	-27.0 -41.0
Alprazolam	308.90 > 281.00 (308.90 > 205.05)	-25.0 -40.0
Diazepam-D_5	289.90 > 193.05 (289.90 > 154.00)	-32.0 -27.0
Diazepam	285.10 > 193.05 (285.10 > 154.00)	-32.0 -27.0

Biotage® SPE Dry Sample Concentrator System.

Results

High (mostly › 70%) and very reproducible (RSD ‹ 5%) recoveries were achieved using the method described in this application note using the Biotage® Mikro plate format.

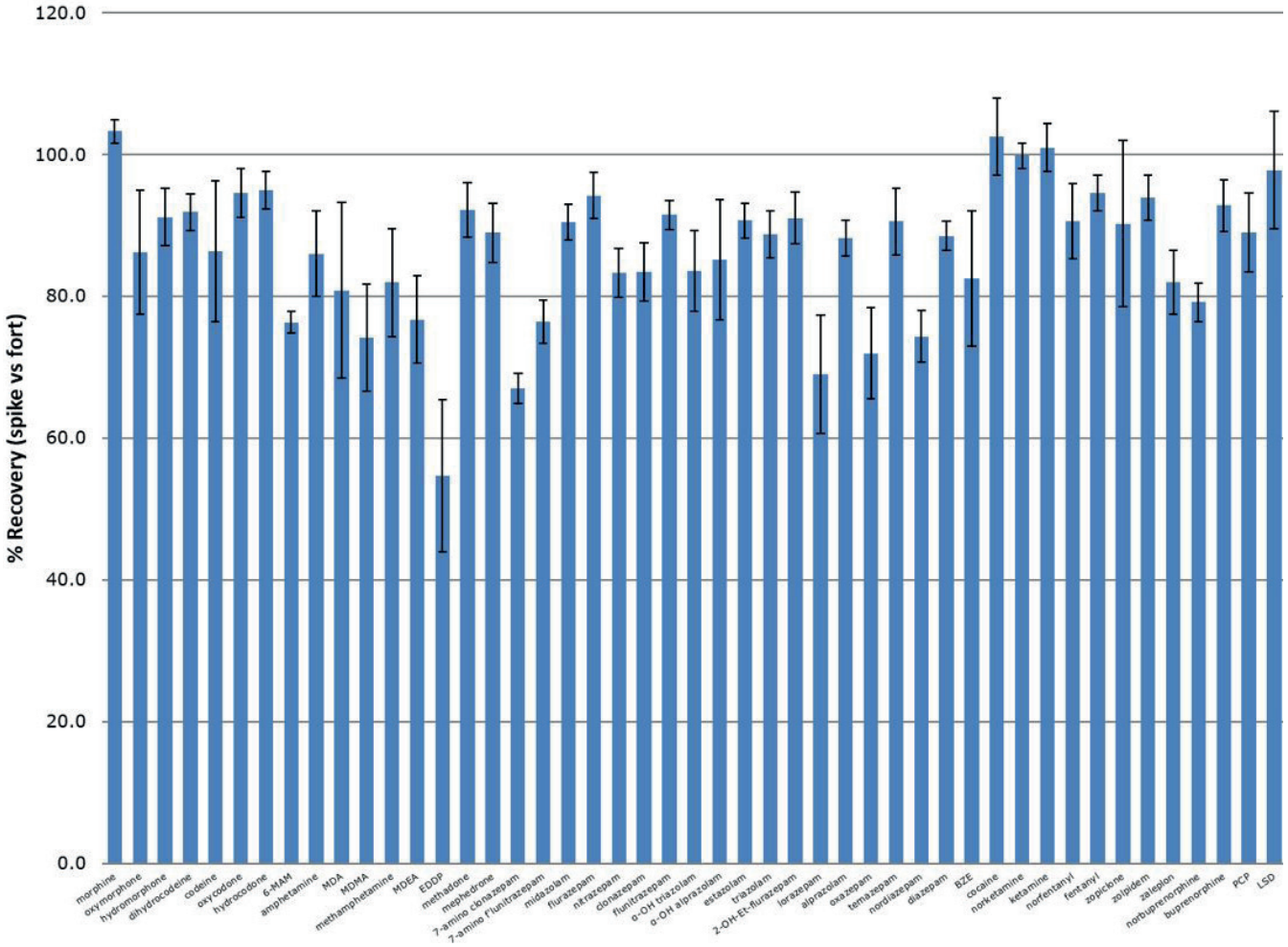

Figure 2. shows analyte recoveries (extracted from hydrolyzed urine spiked at 1 ng/mL) using the optimized Mikro CX protocol described in this application note.

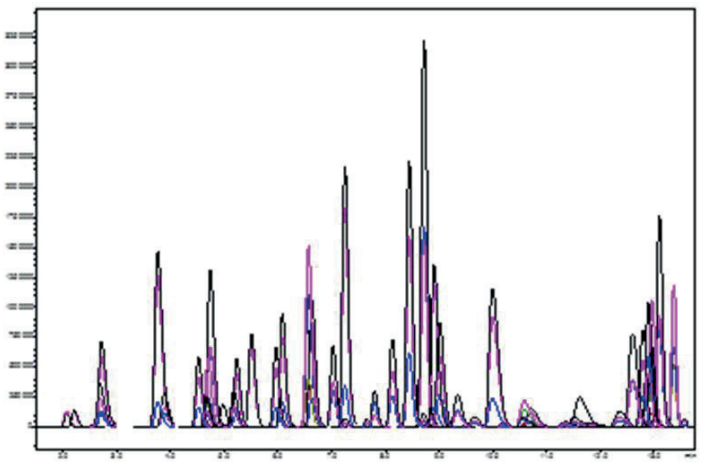

Figure 3. Representative chromatography for application analytes spiked at 1 ng/mL.

Calibration curve performance was investigated from plasma spiked between 1–1000 pg/mL. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.999. Table 3. details linearity performance and associated LOQ for each analyte.

Table 3. Analyte calibration curve r^2 and LOQ performance.

Analyte	r^2	LLOQ (pg/mL)
Morphine	0.9990	100
Oxymorphone	0.9991	25
Hydromorphone	0.9970	50
Amphetamine	0.9993	50
Methamphetamine	0.9990	50
Dihydrocodiene	0.9991	1
Codeine	0.9996	5
6-MAM	0.9993	5
MDMA	0.9993	5
Oxycodone	0.9991	25
Mephedrone	0.9998	50
Hydrocodone	0.9993	50
MDEA	0.9991	10
Nor-Ketamine	0.9993	5
Nor-Fentanyl	0.9990	5
BZE	0.9990	50
Ketamine	0.9991	5
7-Aminoclonazepam	0.9990	< 250
Cocaine	0.9990	50
Norbuprenorphine	0.9998	250
LSD	0.9993	10
7-Aminoflunitrazepam	0.9991	25
Zolpidem	0.9990	5
Buprenorphine	0.9991	25
Fentanyl	0.9991	50
Flurazepam	0.9990	1
PCP	0.9992	10
Midazolam	0.9995	50
Bromazepam	0.9991	50
EDDP	0.9990	1
Lorazepam	0.9990	250
Oxazepam	0.9990	< 500
Nitrazepam	0.9990	< 500
Clonazepam	0.9990	< 250
a-OH-Triazolam	0.9991	< 25
2-OH-et-flurazepam	0.9995	< 100
Methadrone	0.9990	< 500
a-OH-Alprazolam	0.9998	100
Nordiazepam	0.9996	50
Zaleplon	0.9998	5
Flunitrazepam	0.9990	< 25
Estazolam	0.9996	< 25
Temazepam	0.9997	< 250
Triazolam	0.9992	1
Alprazolam	0.9990	25
Diazepam	0.9993	25

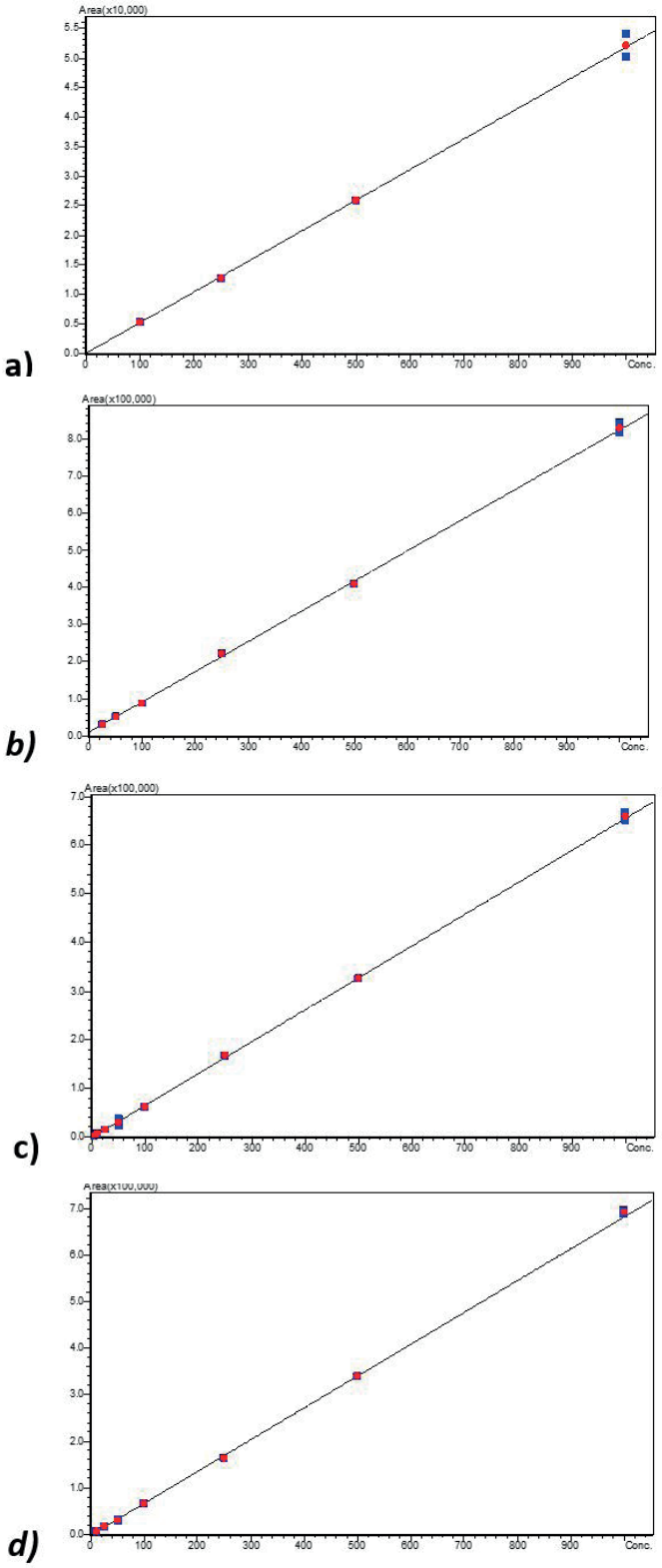

Figure 4. Calibration curves for Norburprenorphine (a), Diazepam (b), 6-MAM (c) and Oxycodone (d) using the Biotage® Mikro CX plate to extract hydrolyzed human urine.

Discussion and Conclusion

The Biotage® Mikro CX solid phase extraction microelution plate provided robust extraction of a large drugs of abuse panel from hydrolyzed urine samples. High, reproducible recoveries were achieved, with an overall processing time of approximately 22 minutes (including the evaporation step). An evaporation step was required in this application, as the elution solvent (DCM/MeOH/NH_4OH) which gave the highest analyte recoveries was not compatible with the reversed phase analytical UPLC system. However, due to the low elution volume, evaporation time for 96 samples was ~ 5 minutes.

Compared to the equivalent procedure using a 10 mg EVOLUTE® EXPRESS CX plate, reductions in total processing time (22 mins vs 33 mins) and organic solvent usage per sample (0.2 mL vs 1 mL total) were possible.

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS), and dichloromethane (99.8%) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards, deuterated internal standards, ammonium acetate, ammonium formate, formic acid, phosphoric acid (49-51%) and ammonium hydroxide (27–30%) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK).
- Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- Mobile phase A (2 mM ammonium formate (aq), 0.1% formic acid) was prepared by adding 0.126 mg of ammonium formate to 1 L purified water with 1 mL formic acid.
- Mobile phase B (2 mM ammonium formate (methanolic), 0.1% formic acid) was prepared by adding 0.126 mg of ammonium formate to 1 L ultra-pure MeOH with 1 mL formic acid.
- Internal standards (100 pg/µL) were prepared from a 10 ng/µL stock solution by adding 10 µL of each of to 950 µL of MeOH. 10 µL of this solution was then added to each calibration solution.
- Hydrolysis buffer 100 mM ammonium acetate was made by adding 0.3854 mg of ammonuim acetate to 50 mL of water (18.2 MOhm-cm).
- Hydrolysis enzyme β-Glucuronidase from Helix pomatia, Type HP-2; ≥100,000 units/mL purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- Equilibration and wash 1 solvent (4% phosphoric acid) was made by adding 4 mL of phosphoric acid to 96 mL of water (18.2 MOhm-cm).
- Wash 2 solvent (H_2O:MeOH (50:50, v/v)) was made up by measuring out 50 mL of water (18.2 MOhm-cm) and 50 mL of methanol and adding both to a bottle.
- Elution solvent (DCM:MeOH:ammonium hydroxide (78:20:2, v/v)) was made up by measuring out 78 mL of DCM (18.2 MOhm-cm) and 20 mL of methanol and adding both to a bottle with 2 mL ammonium hydroxide.
- Reconstitution solvent was made by measuring out 90 mL of purified water (18.2 MOhm-cm) and 10 mL of MeOH and adding them to the same bottle with 100 µL formic acid.

Additional Information

- All data shown in this application note was generated using human urine donated by healthy human volunteers.

Ordering Information

Part Number	Description	Quantity
601-0002-LVP	Biotage® Mikro CX Plate, 2 mg	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS	Biotage® SPE Dry Sample Concentrator System	1
121-5202	Collection Plate, 1 mL Square	50
121-5204	Pierceable Sealing Mat	50

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