Automated Extraction of a Drugs of Abuse Panel from Human Urine Using Biotage® Extrahera™ LV-200 and Microelution SPE Prior to UPLC-MS/MS Analysis

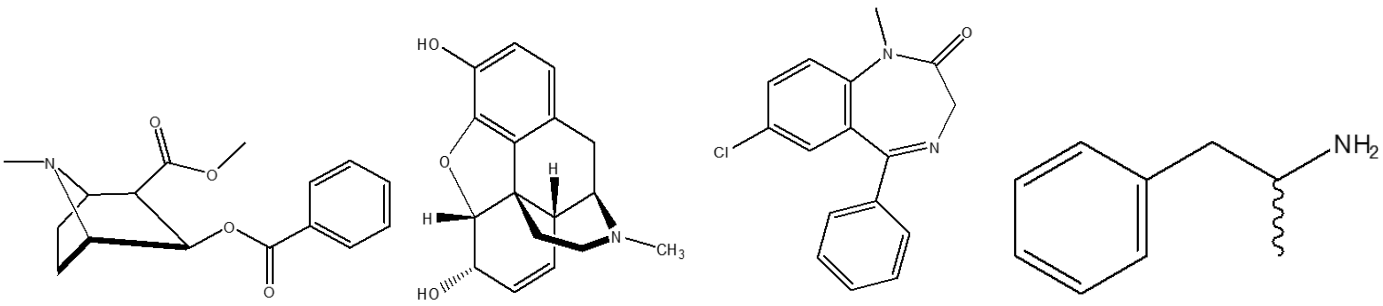


Figure 1. Example analyte structures by drug class.

Introduction

This application note describes the extraction of a multi-class drugs of abuse panel from human urine using Biotage® Mikro CX solid phase extraction microelution plates, prior to LC-MS/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 60% with RSDs lower than 5% for most analytes. Linearity of greater than 0.999 is achieved for all analytes from 1-1000 pg/mL.

The use of Biotage® Mikro SPE plates for extraction allows for low elution volumes and enhanced workflow efficiency.

This application note includes optimized conditions for automated processing of the Mikro plates (using Biotage® Extrahera™ LV-200, see appendix for settings) and manual processing (using the Biotage® PRESSURE+ 96 positive pressure manifold). Data generated using both processing systems is shown. Prior to analysis, extracts are evaporated using the TurboVap® 96 Dual.

Analytes:

Amphetamine, Methamphetamine, 3,4-Methylenedioxyamphetamine (MDA), 3,4-Methyl enedioxymethamphetamine (MDMA), 3,4-Methylenedioxy-N-ethylamphetamine (MDEA), Hydromorphone, Morphine, Benzoylecgonine (BZE), Oxymorphone, Dihydrocodeine, 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, 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, p/n 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.

Automated and Manual Processing Conditions:
Detailed automated processing conditions using the Biotage® Extrahera™ LV-200 system are included in the appendix.

To compare method performance, samples were also processed manually using a Biotage® PRESSURE+ 96 positive pressure manifold. Each step described below was processed at 6 to 9 psi using the adjustable flow setting. Drying steps were processed at 40 psi using the maximum flow setting.

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). On completion dry plate for 2 mins.

Wash 2:
Elute interferences with H_2O:MeOH (50:50, v/v, 100 µL). On completion dry plate for 2 mins.

Elution:
Elute analytes with DCM:MeOH:NH_4OH (78:20:2, v/v, 30 µL) into a 2 mL collection plate (p/n 121-5203)

Post Elution & Reconstitution:
Dry the extract in a stream of air or nitrogen using a TurboVap® 96 Dual at 25 °C, 60 L/min, plate height 46 mm.

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

Cover with a sealing mat, vortex mix and transfer to 1.5 mL LC/MS vial with 250 µL glass inserts topped with snap caps (LC/MS vials: Supelco p/n 854974; Snap Caps: VWR p/n 548-3206; Inserts: Agilent p/n 5183-2085).

UHPLC Conditions

Instrument
Shimadzu Nexera UHPLC

Column
Restek Raptor™ Biphenyl 2.7 µm (100 x 2.1 mm) (p/n 9309A12)

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 Temp:
400 °C

DL Temp:
250 °C

Heat Block Temp:
300 °C

CID Gas Flow:
270 kPa

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

Analytes	MRM Transition	Collision Energy
Morphine-D3	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-D5	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
Dihydrocodeine	302>119.05 (302>171)	-35.0 -45.0
Codeine	300.0>215.1 (300.0>165)	-25.0 -40.0
6-MAM-D3	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-D3	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
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

Analytes	MRM Transition	Collision Energy
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-D5	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®

Results

Analyte recovery and extraction reproducibility

High (mostly › 60%) and very reproducible (RSD ‹ 5%) recoveries were achieved using the method described in this application note. Figure 2 below shows average recoveries (n=7) obtained by manual and automated processing procedures.

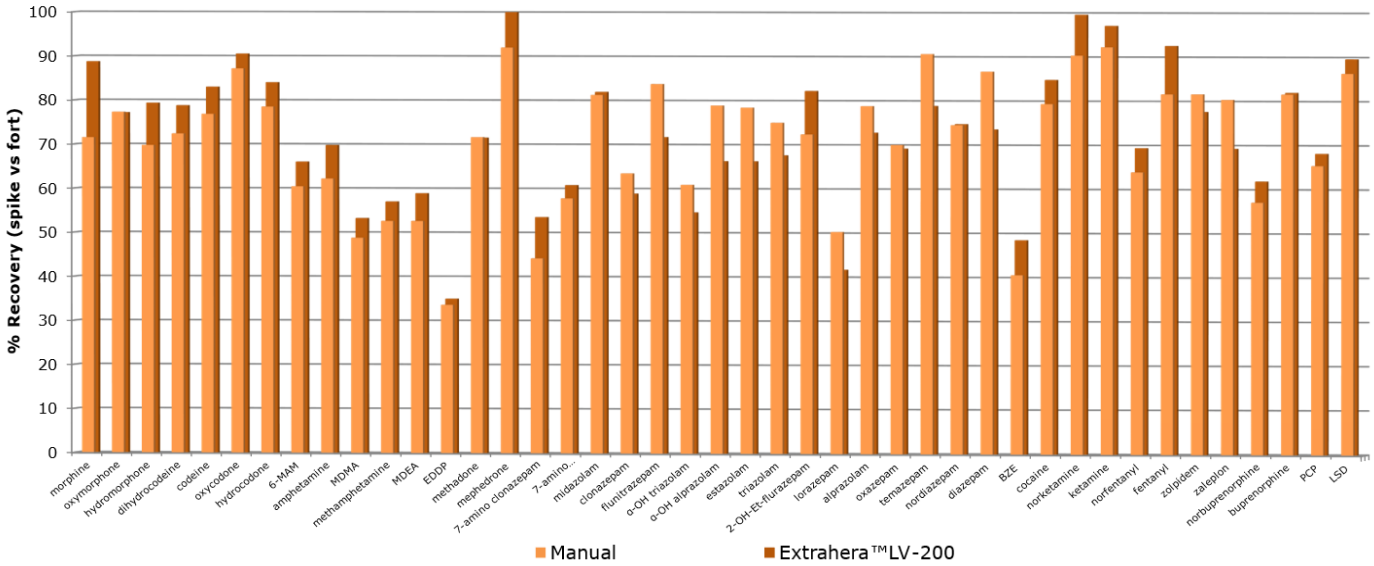


Figure 2. Analyte recoveries (1 ng/mL) using the optimized Biotage® Mikro CX protocol described in this application note. Recovery data comparing manual and automated processing is shown.

Linearity and Limit of Quantitation (LOQ)

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. Data obtained from manual and automated procedures was comparable.

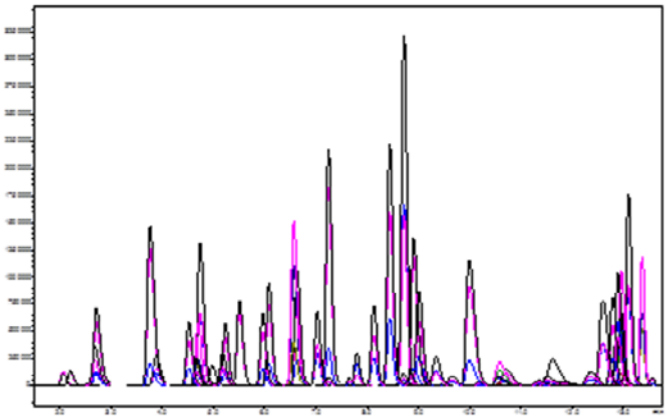


Figure 3. Representative chromatography for application analytes spiked at 1 ng/mL in urine.

Table 3. Analyte calibration curve r^2 and LLOQ performance for the automated method.

Analyte	r^2	LLOQ (pg/mL)
Morphine	0.9997	50
Oxymorphone	0.9991	25
Hydromorphone	0.9994	25
Amphetamine	0.9994	50
Methamphetamine	0.9990	1
Dihydrocodeine	0.9995	10
Codeine	0.9996	5
6-MAM	0.9993	< 25
MDMA	0.9994	10
Oxycodone	0.9991	25
Mephedrone	0.9998	50
Hydrocodone	0.9993	50
MDEA	0.9994	10
Nor-Ketamine	0.9992	10
Nor-Fentanyl	0.9990	5
BZE	0.9997	5
Ketamine	0.9991	5
7-Aminoclonazepam	0.9990	100
Cocaine	0.9992	25
Norbuprenorphine	0.9998	250
LSD	0.9992	50
7-Aminoflunitrazepam	0.9991	100
Zolpidem	0.9995	5

Analyte	r^2	LLOQ (pg/mL)
Buprenorphine	0.9991	25
Fentanyl	0.9991	< 100
Flurazepam	0.9990	5
PCP	0.9992	10
Midazolam	0.9997	50
Bromazepam	0.9991	50
EDDP	0.9990	1
Lorazepam	0.9990	250
Oxazepam	0.9990	< 500
Nitrazepam	0.9991	50
Clonazepam	0.9990	< 250
a-OH-Triazolam	0.9992	25
2-OH-et-flurazepam	0.9998	50
Methadone	0.9994	10
a-OH-Alprazolam	0.9993	100
Nordiazepam	0.9996	50
Zaleplon	0.9991	25
Flunitrazepam	0.9992	25
Estazolam	0.9994	< 25
Temazepam	0.9997	< 250
Triazolam	0.9994	< 5
Alprazolam	0.9990	25
Diazepam	0.9993	25

Calibration Curves

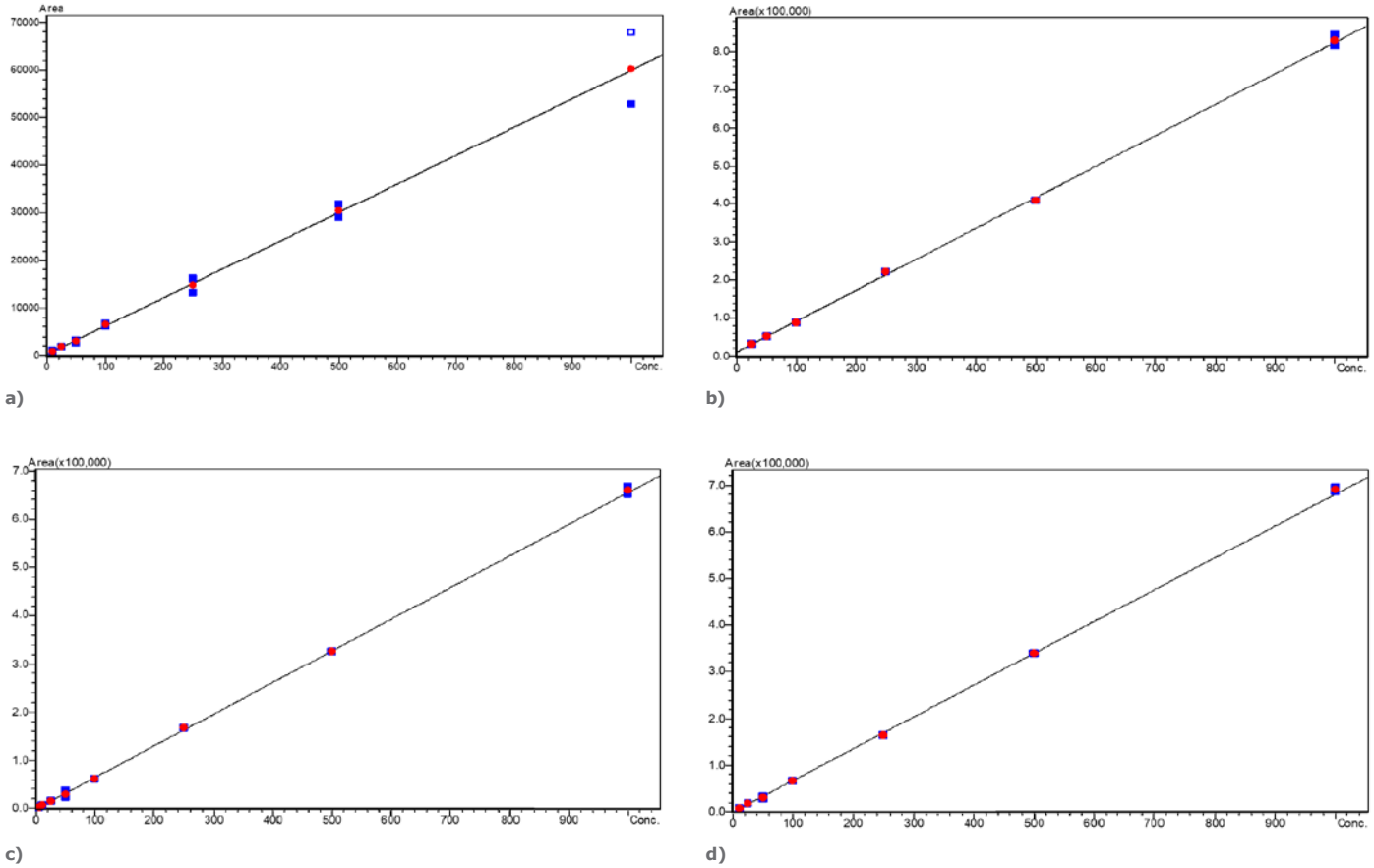


Figure 4. Calibration curves for Burprenorphine (a), Diazepam (b), 6-MAM (c) and Oxycodone (d) using the Biotage® Mikro CX plate to extract hydrolyzed human urine on the Extrahera™ LV-200.

Discussion and Conclusion

Biotage® Mikro CX solid phase extraction microelution plates provided robust automated extraction of a large multi-class drugs of abuse panel from hydrolyzed urine samples. Good, reproducible recoveries were achieved, with an overall automated processing time of ~25 minutes for 96 samples (excluding evaporation and transfer steps). Note: 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 direct injection onto the reversed phase analytical UPLC system. Note: due to the low reconsitution volume used, and issues with compatibility of the available autosampler, reconstituted samples were transfered to low volume inserts prior to injection.

Chemicals and Reagents

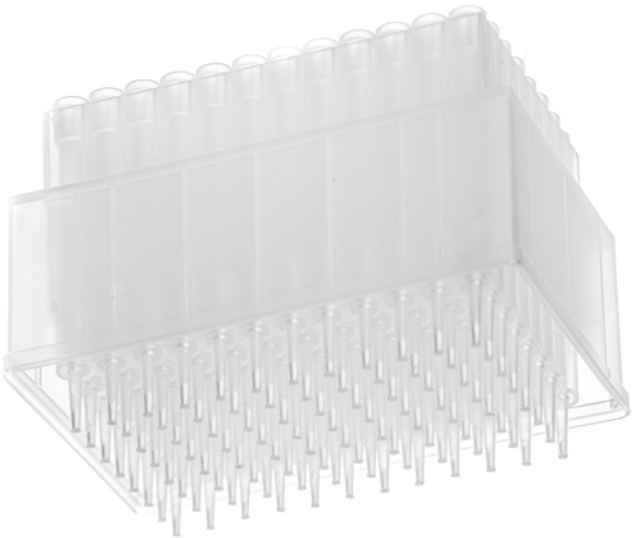
- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS), and dichloromethane (99.8%) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards, deuterated internal standards, ammonium acetate, ammonium formate, formic acid, phosphoric acid (49-51%) and ammonium 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 (aq), 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).
- 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 #	Description	Quantity
601-0002-LVP	Biotage® MIKRO CX Plate, 2mg	1
121-5203	Collection plate, 2 mL, Square	50
121-5204	Pierceable Sealing Mat	50
Automated Processing		
417000	Biotage® Extrahera™ LV-200	1
416920SP	Pipette Rack, LV/MV	1
417423SP	Pipette Rack, Short	1
417008	50 µL Clear Tips	960
417009	200 µL Clear Tips	960
Manual Processing		
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
Evaporation		
418000	TurboVap® 96 Dual	1



Appendix

Biotage® Extrahera™ Settings

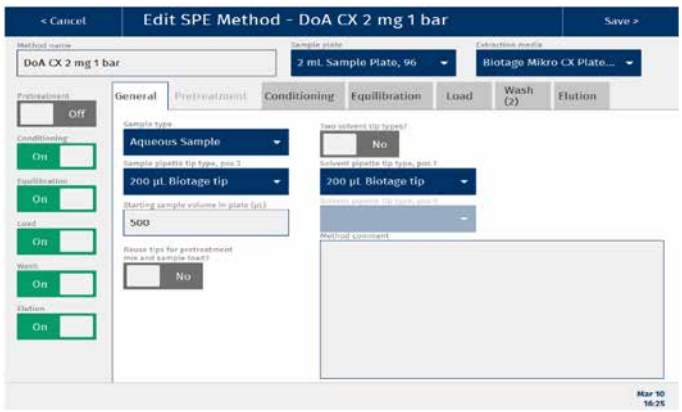
The method described in this application note was automated on the Biotage® Extrahera™ LV-200 using Biotage® Mikro CX plates.

This appendix contains the software settings required to configure Extrahera to run this method. As described in the main body of the application note, analyte recoveries, linearities and LOQs were comparable for both manually processed and automated methods. Reproducibility was slightly improved for samples extracted using the automated Extrahera™ LV-200 system.

Total time for extraction of 96 samples using this method was 25 minutes (excluding pre-extraction sample hydrolysis, and post extraction evaporation and reconstitution time).

Sample name:	DoA CX 2 mg 1 Bar
Sample plate/rack:	2 mL Sample Plate, 96
Extraction Media:	Mikro CX 96 Well Plate

"Sample" tab



Settings

Sample Type	Aqueous Sample
Starting Sample vol.	500
Method comment	

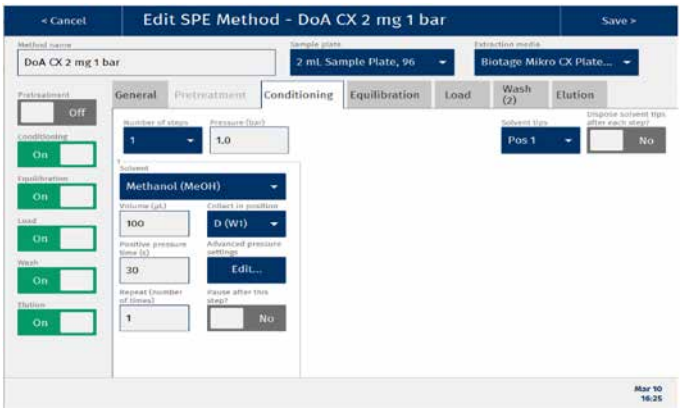
Pretreatment

No. of steps	0
Pause after last step	No
Dispose tips after last step	No

Solvent

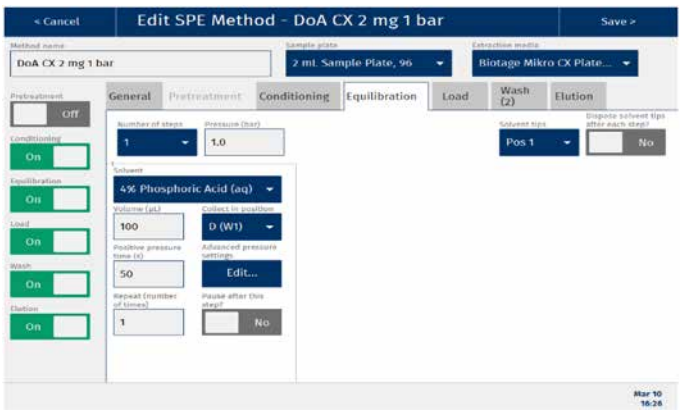
1				
2				
3				
4				
	1	**2**	**3**	**4**
Volume				
Time				

Conditioning tab



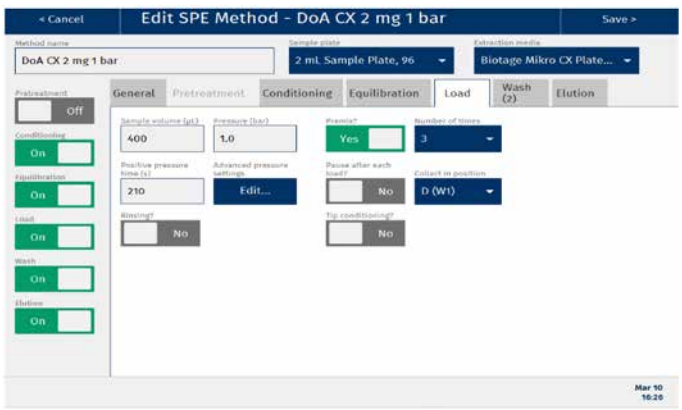
Pressure	1.0	
Pause after each load	No	
Volume	100Collect in position	D
Positive pressure time	30	
Advanced Pressure	No	
Number of times	1	
Solvent		
1	Methanol	
2		
3		
4		
Advanced settings		

Equilibration tab



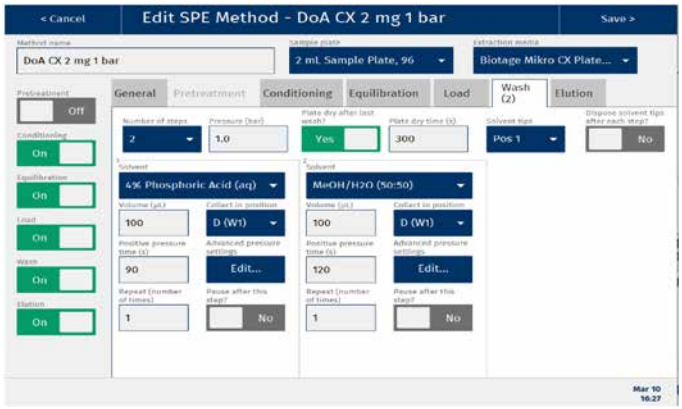
Pressure	1.0
Volume	100
Collect in position	D
Positive pressure time	50
Advanced Pressure	No
Number of times	1
Solvent	
1	4% Phosphoric Acid

Load tab



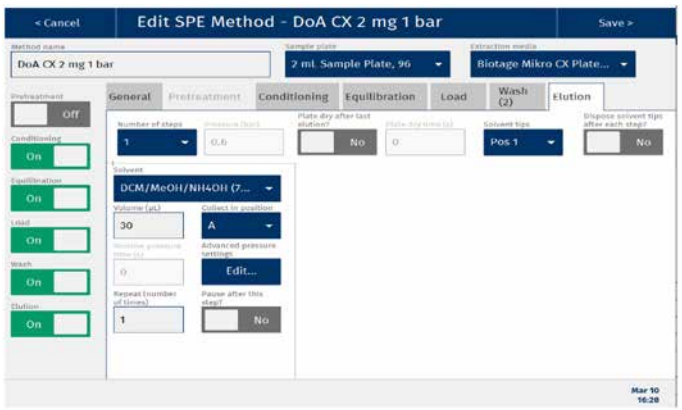
Pressure	1
Pause after each load	No
Volume	400
Collect in position	D
Positive pressure time	210
Premix	Yes
Number of times	3

Wash tab



Pressure	1
Volume	100
Collect in position	D
Positive pressure time	90
Advanced Pressure	No
Number of times	1
Wash 2	
Pressure	1
Volume	100
Collect in position	D
Positive pressure time	120
Advanced Pressure	No
Number of times	1
Plate Dry	YES
Solvent	
1	4% Phosphoric Acid
2	50:50 Water:MeOH

Elution tab



No. of steps	1
Pressure	
Plate Dry	No
Dry time	
Wait time (min)	
Solvent	
DCM:MeOH:NH4OH (78:20:2)	
1	
Volume	30
Position	A
Pressure time	0
Repeat	1
Pause	No
Advanced settings	

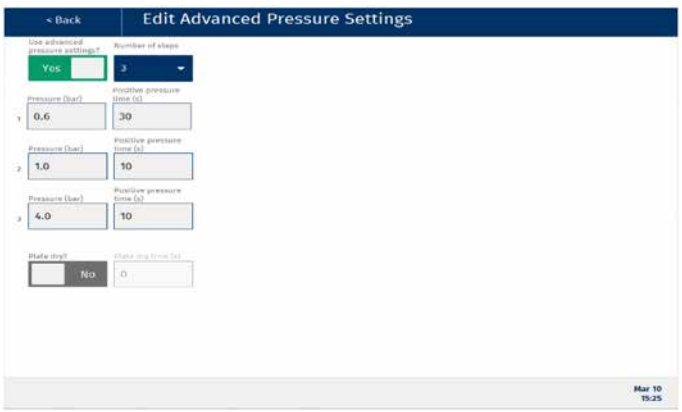
Advanced Pressure:
3 Steps; 0.6 bar for 30 seconds; 1 Bar for 10 seconds; 4.0 bar for 10 seconds

Solvent Properties

Solvent description	
1	Methanol
2	4% Phosphoric Acid
3	DCM/MeOH/NH4OH (78:20:2)
4	50:50 Water:Methanol

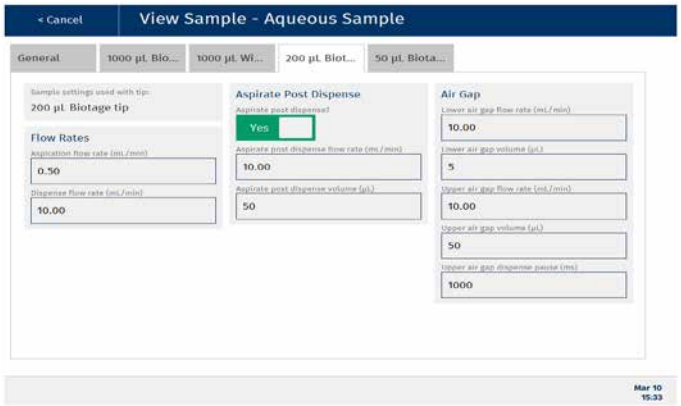
Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir type	Refillable					Non refillable				
Capacity										
Aspiration flow rate	5	1	0.6	5						
Dispense flow rate	10	10	10	10						
Lower air gap flow rate	10	10	10	10						
Lower air gap volume	5	5	5	5						
Upper air gap flow rate	10	10	10	10						
Upper air gap volume	140	140	50	140						
Upper air gap dispense pause	0	0	300	0						
Conditioning?	Yes	Yes	Yes	Yes						
Cond. Times	3	1	4	3						
Cond. Flow rate	5	3	4	5						
Chlorinated	No	No	Yes	No						
Serial dispense	No	No	No	No						

“Sample” screen



Sample name	Aqueous sample
Sample description	Default settings for Aqueous
Aspiration flow rate	0,5
Dispense flow rate	10
Lower air gap flow rate	10
Lower air gap volume	5
Upper air gap flow rate	10
Upper air gap volume	50
Upper air gap dispense pause	1000

“Extraction Media” screen

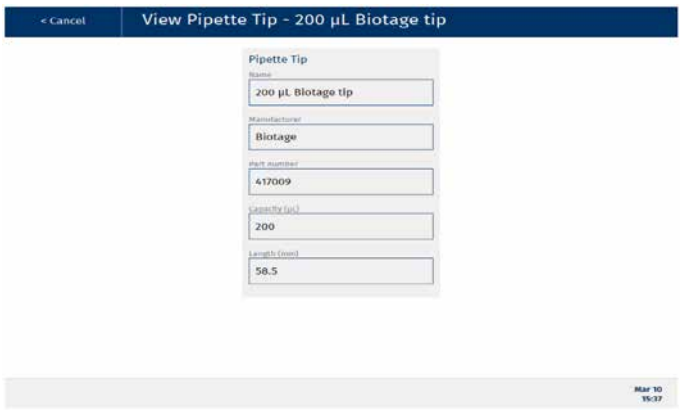


Name	Mikro CX Plate, 96
Manufacturer	Biotage
Part number	601-0002-LVP
Capacity volume	0
Format	96
Comment	
Solvent dispensation height	34,5
Sample dispensation height	34,5
Aspiration height	1

“Sample Plate/Rack” screen

Name	2 mL Sample plate, 96
Capacity volume	1800
Format	96
Aspiration height	-2
Pretreatment dispensation height	32

“Pipette tip” screen



Name	200µL Biotage tip
Manufacturer	Biotage
Part number	417009
Capacity	200
Length	58,5

Literature Number: AN964.v1