Extraction of a Drugs of Abuse Panel from Whole Blood Using ISOLUTE® SLE+ Prior to UPLC-MS/MS Analysis

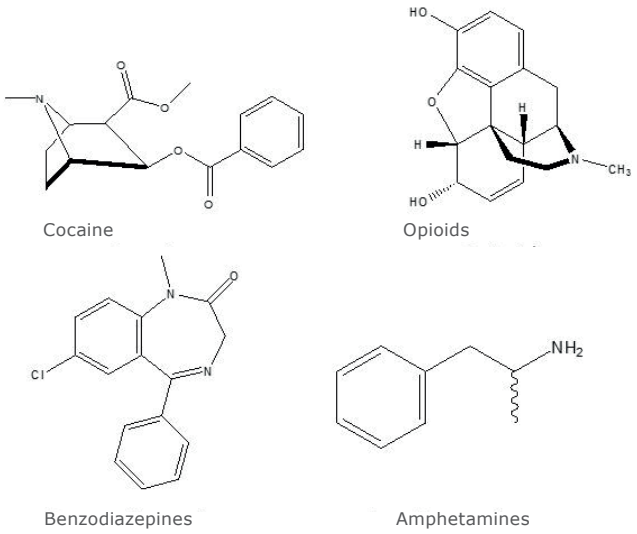

Figure 1. Example structures by class.

Introduction

This application note describes the extraction of 49 drugs of abuse from whole blood, prior to UPLC-MS/MS analysis. Figure 1. shows examples of these structures by class.

ISOLUTE® SLE+ Supported Liquid Extraction columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

This application note describes an effective and efficient ISOLUTE SLE+ protocol optimized the for 1 mL sample capacity column formats.

The simple sample preparation procedure delivers clean extracts, good recoveries and RSD values and LLOQ from 10 ng/mL.

Sample Preparation Procedure

Format

ISOLUTE® SLE+ 1 mL sample volume column, part number 820-0140-C

Sample Pretreatment

To 500 µL of whole blood, add 10 µL of ISTD. Allow to equilibrate and add 500 µL of 0.1% ammonium hydroxide (aq). Mix.

Sample Loading

Load 750 µL of the pre-treated whole blood onto the column and apply a pulse of vacuum or positive pressure (3–5 seconds) to initiate flow. Allow the sample to absorb for 5 minutes.

Analyte Extraction

Apply DCM/IPA (95/5, v/v, 2.5 mL) and allow to flow under gravity for 5 minutes. Collect in an appropriate glass tube containing 100 µL HCl in methanol (50 mM). This acts to stabilize free-base analytes in the solvent prior to evaporation. Apply a low vacuum or positive pressure (5–10 seconds) to elute any remaining extraction solvent, before applying the next aliquot.

Apply MTBE (2.5 mL) and allow to flow under gravity for 5 minutes. Apply a low vacuum or positive pressure (5–10 seconds) to elute any remaining extraction solvent, before applying the next aliquot.

Apply a final aliquot of MTBE (2.5 mL) and allow to flow under gravity for 5 minutes. Apply vacuum or positive pressure (5-10 seconds) to elute any remaining extraction solvent.

Post Elution and Reconstitution

Evaporate the extract to dryness in a stream of air or nitrogen using a TurboVap® LV (beginning at 8–10 psi or 1 L/min).

Reconstitute the extracts with 100 µL methanolic mobile phase (B) and vortex for 10 seconds before adding 400 µL aqueous mobile phase (A). Vortex for 10 seconds and transfer to suitable vials or a collection plate.

Analytes

2-OH-ethyl-flurazepam	Cocaine	Hydromorphone	Midazolam	PCP
6-MAM	Codeine	Ketamine	Morphine	Temazepam
7-amino-clonazepam	Diazepam	Lorazepam	Nitrazepam	THC-COOH
7-amino-flunitrazepam	Dihydrocodeine	LSD	Norbuprenorphine	Triazolam
Alprazolam	EDDP	MDA	Nordiazepam	Zaleplone
Amphetamine	Estazolam	MDEA	Norfentanyl	Zolpidem
Benzoylecgonine	Fentanyl	MDMA	Norketamine	Zopiclone
Bromazepam	Flunitrazepam	Mephedrone	Oxazepam	α-OH-alprazolam
Buprenorphine	Flurazepam	Methadone	Oxycodone	α-OH-triazolam
Clonazepam	Hydrocodone	Methamphetamine	Oxymorphone	

UPLC Conditions

Instrument

Waters ACQUITY UPLC with 20 µL loop

Column

Restek Raptor™ Biphenyl 2.7 µm (100 x 2.1 mm) with Raptor Biphenyl EXP guard cartridge

Mobile Phase

A: 2 mM ammonium formate (aq), 0.1 % formic acid

B: 2 mM ammonium formate (in methanol), 0.1 % formic acid

Flow Rate:

0.4 mL min

Injection Volume

10 µL (partial loop with overfill)

Sample Temperature

20 °C

Column Temperature

40 °C

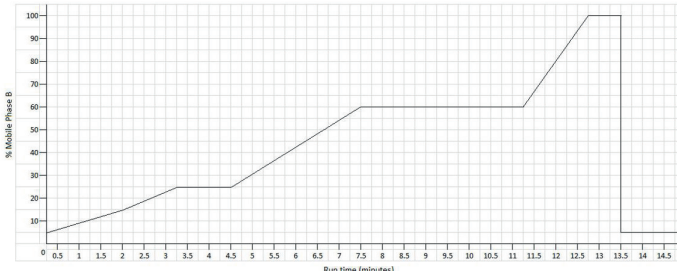

Figure 2. Graphical representation of LC gradient conditions.

Table 1. Gradient Conditions.

Time	% A	% B	Curve
0.00	95	5	6
2.00	85	15	6
3.25	75	25	6
4.50	75	25	6
7.50	40	60	6
11.25	40	60	6
12.75	0	100	6
13.50	0	100	6
13.51	95	5	6
15.00	95	5	6

Mass Spectrometry Conditions

Instrument

Premier XE triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Desolvation Temperature

450 °C

Ion Source Temperature

150 °C

Positive ions acquired in the multiple reaction monitoring (MRM) mode:

Table 2. MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Amphetamine	136.0 > 118.9	16	9
Amphetamine-D_5	141.0 > 123.9	16	9
Methamphetamine	150.0 > 90.9	22	17
MDA	180.1 > 105.0	16	23
MDMA	194.1 > 163.0	20	13
MDEA	208.2 > 163.0	22	13
Hydromorphone	286.2 > 185.1	44	29
Morphine	286.2 > 201.0	42	25
Morphine-D_3	289.2 > 201.0	42	25
BZE	290.1 > 168.0	30	18
BZE-D_3	293.1 > 171.0	30	18
Oxymorphone	302.2 > 198.1	34	37
Dihydrocodeine	302.2 > 199.1	42	33
Oxycodone	316.2 > 241.2	34	27
Mephedrone	178.1 > 160.0	35	12
Norfentanyl	233.1 > 84.0	25	19
7-amino-flunitrazepam	284.2 > 135.0	40	27
7-amino-clonazepam	286.2 > 121.0	40	30
Hydrocodone	300.2 > 199.1	46	33
Codeine	300.3 > 215.1	42	25
6-MAM	328.2 > 165.1	44	33
6-MAM-D_3	331.2 > 165.1	44	33
Cocaine	304.2 > 182.0	30	20
Norketamine	224.1 > 124.9	20	23
EDDP	278.2 > 234.2	26	30
Zaleplone	306.2 > 264.2	40	22
Zopiclone	389.2 > 245.1	20	17
Norbuprenorphine	414.3 > 101.0	55	42

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Ketamine	238.1 > 124.9	25	27
Nitrazepam	282.2 > 236.1	40	25
Flunitrazepam	314.2 > 268.2	40	25
Clonazepam	316.1 > 270.1	40	25
α-OH-triazolam	359.1 > 331.1	45	26
Oxazepam	287.2 > 241.0	30	21
Estazolam	295.2 > 267.2	40	24
Temazepam	301.1 > 255.1	30	22
Zolpidem	308.2 > 235.1	45	35
Alprazolam	309.2 > 281.2	40	26
Methadone	310.2 > 265.2	26	15
Lorazepam	321.1 > 275.1	30	22
Bromazepam	316.1 > 182.1	40	30
α-OH-alprazolam	325.2 > 297.1	40	25
2-OH-ethyl-flurazepam	333.2 > 109.0	40	27
Triazolam	343.0 > 308.1	45	27
Nordiazepam	271.1 > 139.9	40	28
Diazepam	285.2 > 154.0	40	27
Diazepam-D_5	290.2 > 154.0	40	27
Midazolam	326.2 > 291.2	45	29
Fentanyl	337.3 > 105.0	35	40
Flurazepam	388.2 > 315.1	35	23
Buprenorphine	468.3 > 468.3	55	5
PCP	244.2 > 158.9	20	15
LSD	323.8 > 222.8	30	25
THC-COOH-D_3	348.2 > 302.2	25	20
THC-COOH	345.2 > 299.2	25	19

Results

Method performance was assessed by spiking whole blood (375 µL) with 5 ng of the respective analytes, equating to a concentration of 13 ng/mL of each analyte when extracting 375 µL of pre-treated whole blood. The percentage analyte recoveries for the various drug classes can be seen in Figure 3. RSDs were all below 10% as shown in Figure 4.

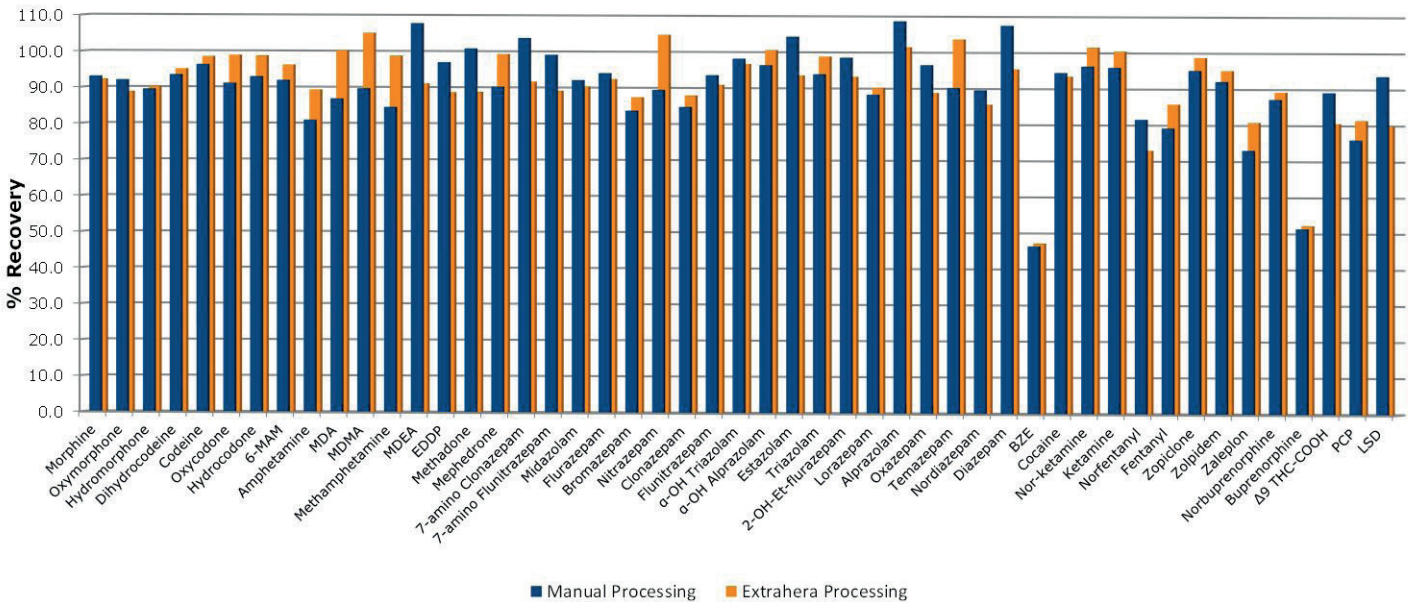

Figure 3. Percentage recovery of the application analytes.

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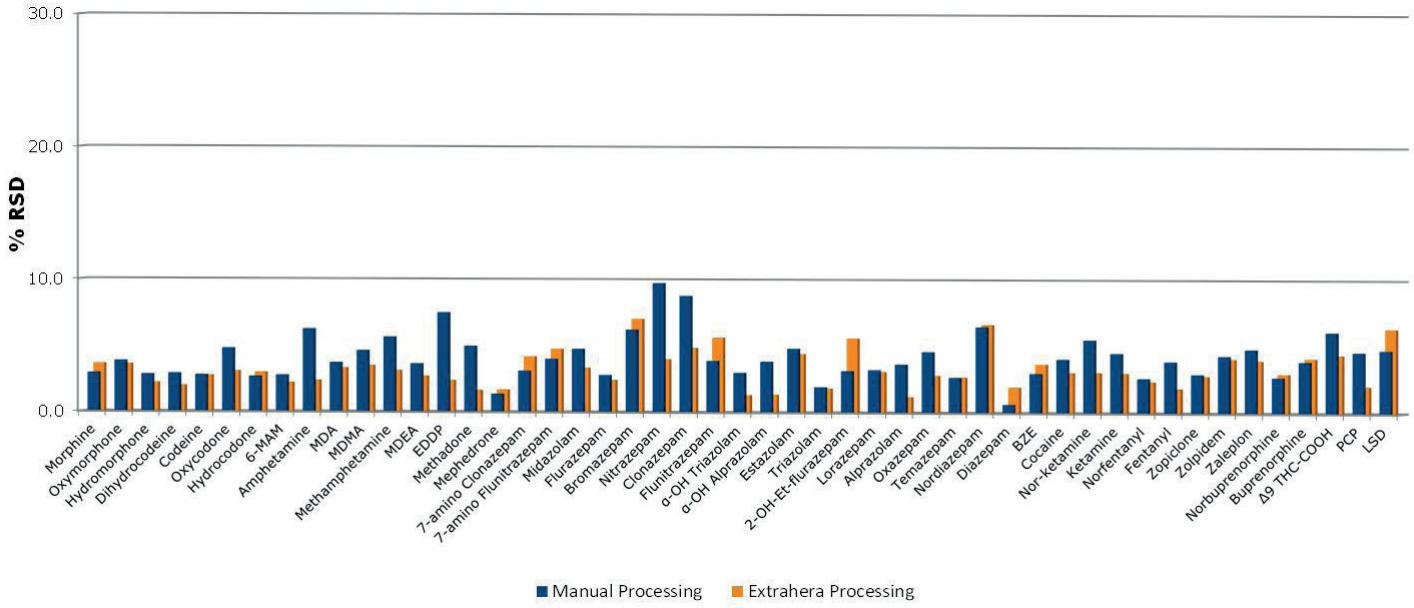

Figure 4. Percentage relative standard deviation of the application analytes.

Calibration Curves

Calibration curves were constructed by spiking whole blood from 1–500 ng/mL of each analyte prior to extraction. The respective internal standards were spiked at 50 ng/mL. Quadratic effect was observed at high concentrations for a number of analytes. However dilution and internal standards helped overcome this to achieve coefficient of determination (r^2) values greater than 0.99 for all analytes using the optimized extraction protocol. Representative curves are shown in Figures 5–8.

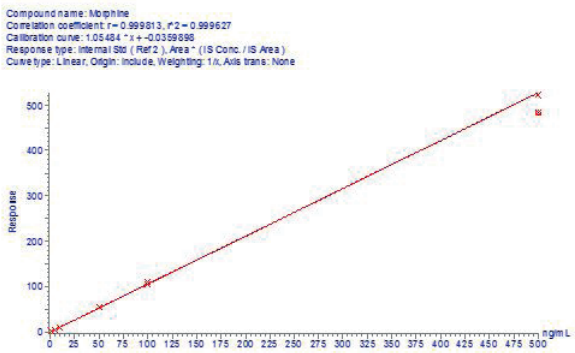

Figure 5. Calibration Curve for morphine.

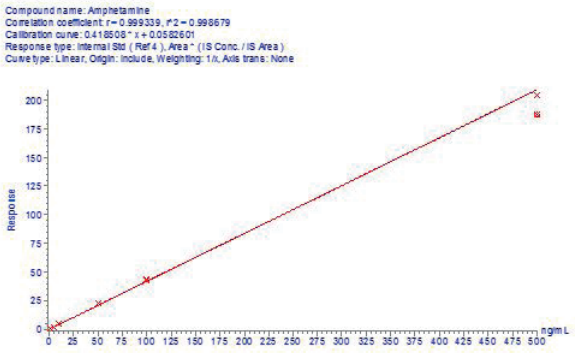

Figure 6. Calibration Curve for amphetamine.

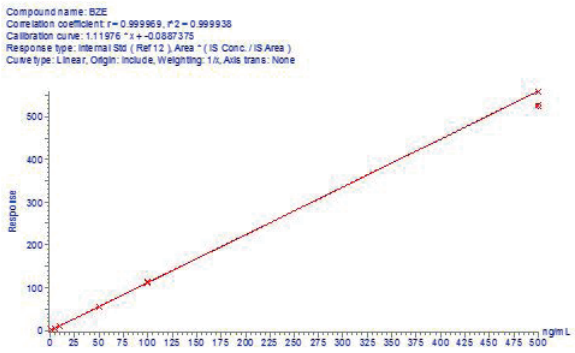

Figure 7. Calibration Curve for benzoylecgonine (BZE).

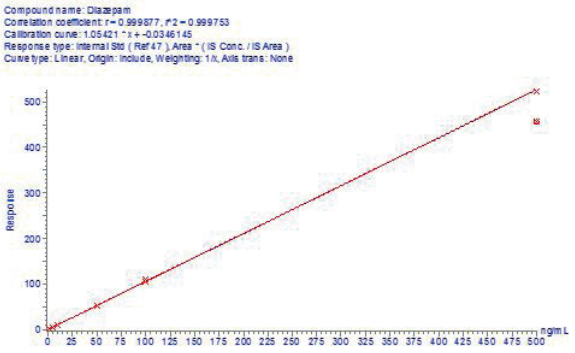

Figure 8. Calibration Curve for diazepam.

Table 3. Lower Limits of Quantitation (LLOQ) using optimized ISOLUTE® SLE+ extraction protocol.

Analyte	Estimated LLOQ (ng/mL) performed by Extrahera
Amphetamine	0.035
Methamphetamine	0.035
MDA	0.050
MDMA	0.015
MDEA	0.025
Hydromorphone	0.007
Morphine	0.035
BZE	0.055
Oxymorphone	0.035
Dihydrocodeine	0.010
Oxycodone	0.055
Mephedrone	0.125
Norfentanyl	0.055
7-amino-flunitrazepam	0.125
7-amino-clonazepam	0.125
Hydrocodone	0.055
Codeine	0.010
6-MAM	0.015
Cocaine	0.055
Norketamine	0.035
EDDP	0.025
Zaleplone	0.015
Zopiclone	0.035
Norbuprenorphine	0.015

Analyte	Estimated LLOQ (ng/mL) performed by Extrahera
Ketamine	0.055
Nitrazepam	0.010
Flunitrazepam	0.025
Clonazepam	0.025
α-OH-triazolam	0.035
Oxazepam	0.085
Estazolam	0.035
Temazepam	0.025
Zolpidem	0.055
Alprazolam	0.085
Methadone	0.035
Lorazepam	0.200
Bromazepam	0.055
α-OH-alprazolam	0.200
2-OH-ethyl-flurazepam	0.035
Triazolam	0.055
Nordiazepam	0.025
Diazepam	0.035
Midazolam	0.007
Fentanyl	0.025
Flurazepam	0.025
Buprenorphine	0.125
PCP	0.125
LSD	0.010
THC-COOH	5.000

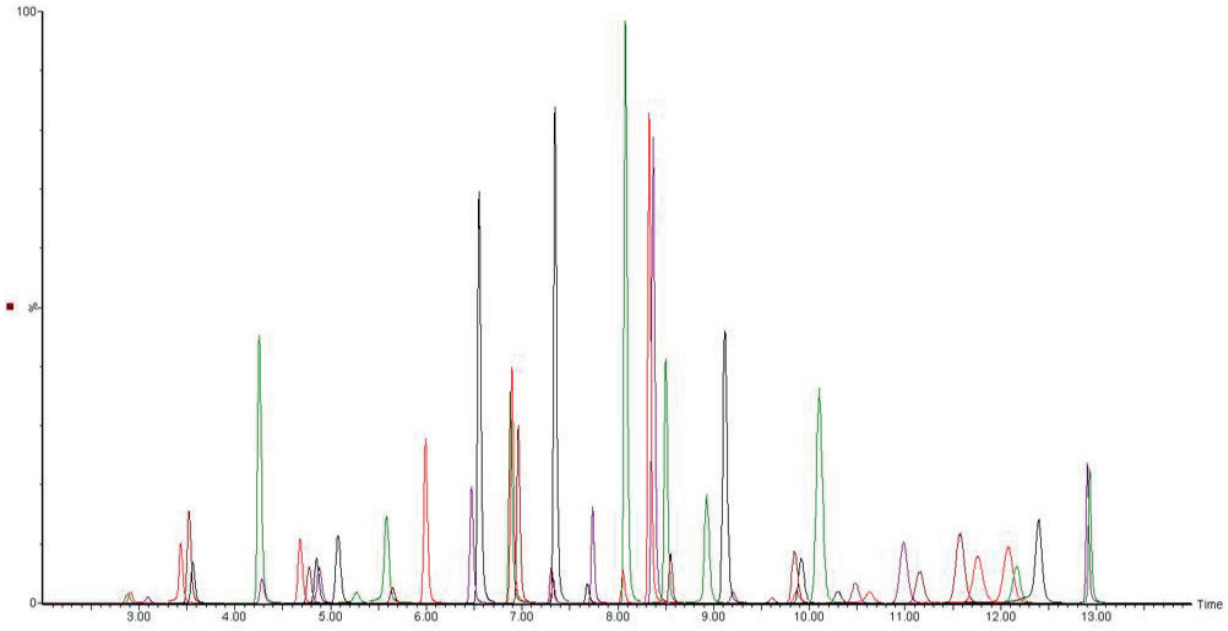

Figure 9. Overlaid MRM Chromatograms of application analytes at 50 ng/mL using the optimized extraction protocol.

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Extract Cleanliness

An experiment was performed to evaluate the level of residual phospholipids in the final extract. Phospholipids are interfering matrix components which can mask or otherwise interfere with the quantitation of the compounds of interest in LC-MS/MS.

When the optimized method is used, ISOLUTE® SLE+ 1 mL columns show very clean total ion chromatograms (see Figure 10), compared to whole blood matrix which had been precipitated with acetonitrile only prior to LC-MS/MS analysis.

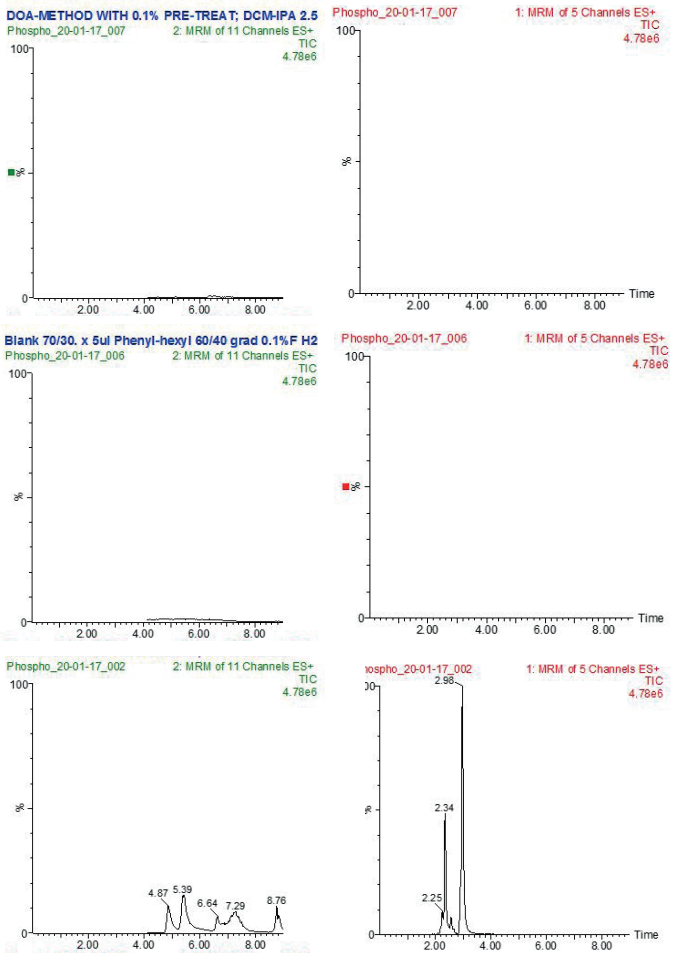

Figure 10. Total Ion Chromatograms of common phospholipid MRM transitions using the optimized extraction protocol (top), compared to blank 70/30 water/methanol (v/v) (middle) and precipitated whole blood (bottom).

Conclusions

The simple method described in this application note is suitable for extraction of a broad range of analyte classes, including THC-COOH from whole blood.

The method is easily automated using the Biotage® Extrahera™ Automation System, showing enhanced analyte recovery and reproducibility for many analytes compared to the manually processed method.

Additional Notes

1. Buprenorphine and BZE extraction recoveries are low compared to samples fortified with the analyte after extraction. However the LLOQ values in table 3 illustrate that this is not an obstacle to effective quantitation.
2. If increased sensitivity is desired on the THC-COOH quantitation, the final reconstitution volume can be modified to less than 0.5 mL.
3. Amphetamines, bath salts and ketamines can suffer loss on evaporation when drying in the more volatile free base form. To overcome this effect, 100 µL of 50 mM HCl in MeOH is added to the collection plate/culture tubes to convert to the corresponding HCl salt forms.

Reagent Preparation

All solvents were HPLC-grade.

- **0.1% ammonia hydroxide (aq)**: Add 0.1 mL of concentrated ammonium hydroxide (28–30%) to 99.9 mL HPLC grade water.
- **Aqueous Mobile Phase:** Weigh 126 mg and dissolve in 1 L UHPLC grade water. Add 1 mL concentrated formic acid.
- **Methanolic Mobile Phase:** Weigh 126 mg and dissolve in 1 L UHPLC grade methanol. Add 1 mL concentrated formic acid.
- **50 mM HCl in methanol:** Add 50 µL concentrated hydrochloric acid to 11.95 mL HPLC grade methanol. The hydrochloric acid stock is commercially available ~12M.

Ordering Information

Part Number	Description	Quantity
820-0140-C	ISOLUTE® SLE+ 1 mL Sample Volume Columns	30
414001	Biotage® Extrahera™ Automation System	1
415040	Configuration Kit 96 Positions Dual Flow	1
414141	Extrahera clear tips	960
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold (96 well)	1
C103199	TurboVap® LV Evaporator	1

Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using ISOLUTE® SLE+ 1 mL columns. Performance was comparable to manually processed samples, as demonstrated in Figures 3 and 4. For the majority of analytes, recovery and RSD is improved upon compared to manual processing. This appendix contains the software settings required to configure Extrahera to run this method. An importable electronic copy of this method for Extrahera can be downloaded from www.biotage.com

Using this automated procedure, 24 samples can be processed in a total of 40 min 43 secs.

Method Name:	DOA in whole blood
Sample Plate/Rack:	13 x 100 mm Test Tubes, 24
Extraction Media:	ISOLUTE® SLE+ 1 mL using whole blood

Settings

"Sample" Tab

Sample Type:	Whole Blood
Starting Sample Volume (µL)	500
Reuse sample tips?	No
Method comment:	

Whole Blood:0.1% NH_4OH(aq) using Columns

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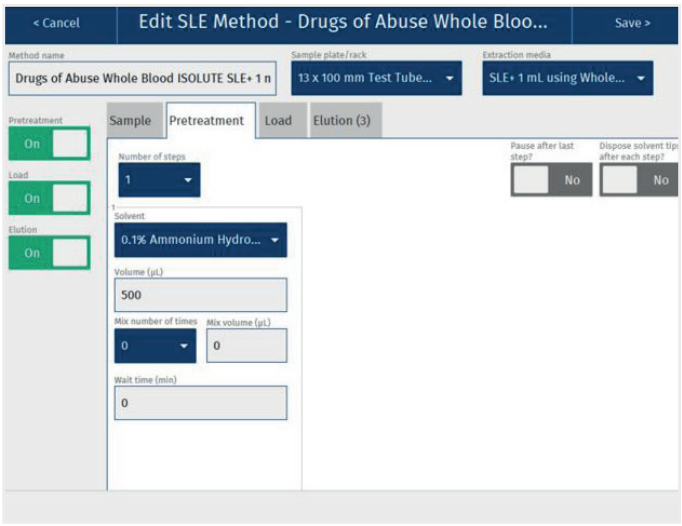
Screenshot



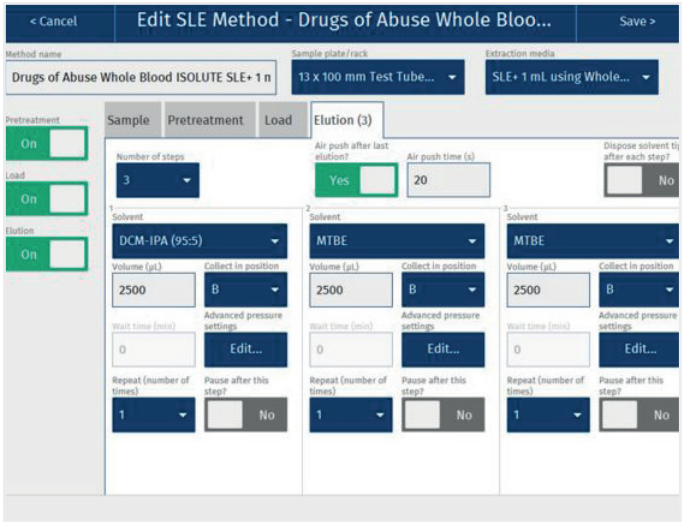
Settings

	Pre-treatment	Activated
	No. of steps	1
	Pause after last step	No
	Dispose tips after last step	No

	Solvent
1	0.1% Ammonium Hydroxide (aq)
2	
3	
4	

	1	2	3	4
Volume (µL)	500			
Mix number of times	0			
Mix volume (µL)	0			
Wait time (min)	0			

	Load	Activated
	Air Push Time (s)	20
	Pause after each load	No
	Volume (µL)	750
	Collect in position	D
	Air Push Time (s)	20
	Wait time (min)	5
	Premix	Yes
	Number of times	3



	Elution	Activated
	No. of steps	3
	Air push after last elution	Yes
	Air push time (s)	20
	Dispose tips after each step	No

	Solvent
1	DCM-IPA (95:5)
2	MTBE
3	MTBE
4	

	1	2	3	4
Volume (µL)	2500	2500	2500	
Collect in position	B	B	B	
Wait time (min)	N/A	N/A	N/A	
Repeat	1	1	1	
Pause	No	No	No	

'Advanced Settings'
0.5 bar for 300 s. Plate Dry OFF. This applies to all three elution steps

Solvent Properties

	Solvent Description
1	0.1% Ammonium Hydroxide (aq)
2	DCM-IPA (95:5)
3	MTBE
4	
5	
6	
7	
8	
9	
10	

Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type		**Refillable**					**Non Refillable**			
Capacity	N/A	N/A	N/A							
Aspiration flow rate (mL/min)	10	10	10							
Dispense flow rate (mL/min)	20	10	10							
Lower air gap flow rate (mL/min)	20	10	10							
Lower air gap volume (µL)	5	5	5							
Upper air gap flow rate (mL/min)	20	120	120							
Upper air gap volume (µL)	100	100	100							
Upper air gap dispense pause	300	300	300							
Conditioning?	Yes	Yes	Yes							
Conditioning number of times	2	2	2							
Conditioning flow rate (mL/min)	20	10	10							
Conditioning volume (%)	100	100	100							
Aspirate post dispense	Yes	Yes	Yes							
Chlorinated	No	Yes	No							
Serial dispense	No	No	No							

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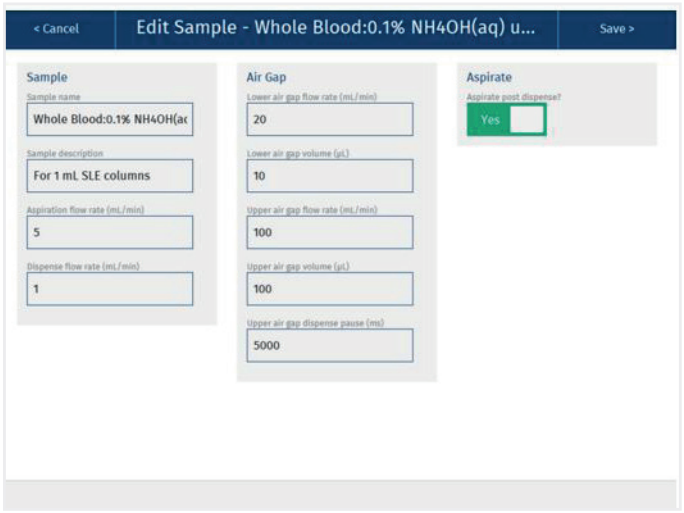
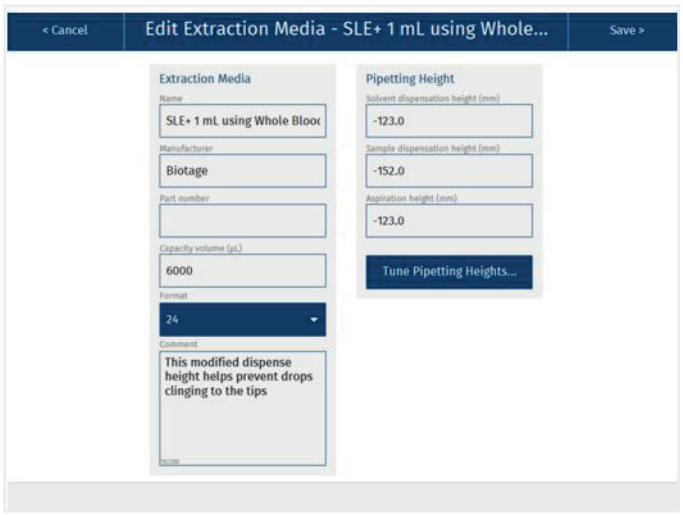
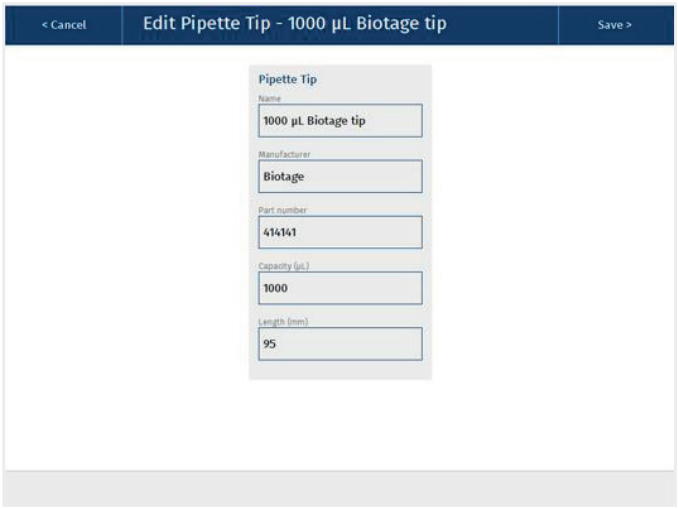


"Sample" Screen	
Sample name	Whole Blood
Sample description	For 1 mL SLE columns
Aspiration flow rate (mL/min)	5
Dispense flow rate (mL/min)	1
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	10
Upper air gap flow rate (mL/min)	100
Upper air gap volume (µL)	100
Upper air gap dispense pause	5000
Aspirate post dispense	Yes



"Extraction Media" Screen	
Name	ISOLUTE® SLE+ 1 mL using Whole Blood
Manufacturer	Biotage
Part number	820-0140-C
Capacity volume (µL)	6000
Format	24
Solvent dispensation height (mm)	-123.0
Sample dispensation height (mm)	-152.0
Aspiration height (mm)	-123.0
Comment	
This modified dispense height helps prevent drops clinging to the tips	

"Sample Plate/Rack" Screen	
Name	13 x 100 mm Test tubes, 24
Capacity volume (µL)	8000
Format	24
Aspiration height (mm)	-197.0
Pre-treatment dispensation height (mm)	-103.0



"Pipette tip" Screen	
Name	1000 µL Biotage Tip
Manufacturer	Biotage
Part number	414141
Capacity (µL)	1000
Length (mm)	95

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