

# Streamlined Sample Preparation of a Drugs of Abuse Panel in Human Hair Using ISOLUTE® SLE+ Prior to UPLC-MS/MS Analysis

## Introduction

This application note describes a streamlined procedure for sample pre-treatment and extraction of a panel of 49 drugs of abuse from human hair, using Biotage® Lysera for matrix micropulverization, prior to direct transfer to clean up using ISOLUTE® SLE supported liquid extraction.

Elimination of an evaporation step between the micropulverization and supported liquid extraction clean up stages provides a streamlined procedure for hair extraction. Analytes are quantified using LC-MS/MS.

The application note contains procedures optimized for the 400 µL capacity column format, and 200 µL and 400 µL supported liquid extraction plate formats, for higher throughput applications. The methodology delivers clean extracts and analyte recoveries mostly greater than 80% with RSDs lower than 10% for all analytes and LLOQ from 1 ng/mL.

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

## Analyses

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

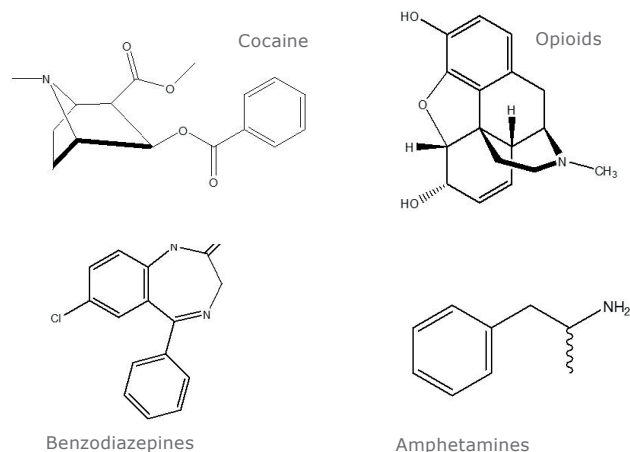


Figure 1. Example structures by class.

## Internal Standards

Amphetamine-D<sub>5</sub>, Morphine-D<sub>3</sub>, BZE-D<sub>3</sub>, 6-MAM-D<sub>3</sub>, Diazepam-D<sub>5</sub>

## Sample Preparation Procedure

### Format

ISOLUTE® SLE+ 400 µL capacity columns (p/n 820-0055-B)  
 ISOLUTE® SLE+ 200 µL capacity plates (p/n 820-0200-P01)  
 ISOLUTE® SLE+ 400 µL capacity plates (p/n 820-0400-P01)

### Matrix Preparation

Weigh 20 mg of hair into 2 mL Biotage® Lysera tubes containing 4 x 2.4 mm stainless steel beads. Add 1 mL methanolic 0.1% (v/v) NH<sub>4</sub>OH to each hair sample spiked with ISTD (1 ng/mL).

### Micropulverization Procedure

Biotage® Lysera: 3 x 5.3 m/sec for 3 minutes with a 20s dwell.

Centrifuge tubes for 10 minutes at 13,300 rpm.

### Post Micropulverization

Transfer supernatant directly to the appropriate ISOLUTE SLE+ product for clean up as described below.

	<b>ISOLUTE SLE+ 200 µL Plate, Part Number 820-0200-P01</b>	<b>ISOLUTE SLE+ 400 µL Plate, Part Number 820-0400-P01</b>	<b>ISOLUTE SLE+ 400 µL Columns, Part Number 820-0055-B</b>
<b>Sample loading</b>	Load 200 µL of supernatant directly to ISOLUTE®SLE+ sorbent. Allow the sample to absorb for 5 minutes.	Load 400 µL of supernatant directly to ISOLUTE®SLE+ sorbent. Allow the sample to absorb for 5 minutes.	Load 400 µL of supernatant directly to ISOLUTE®SLE+ sorbent. Allow the sample to absorb for 5 minutes.
<b>Analyte Extraction</b>	Apply DCM/IPA (95/5, v/v, 300 µL) allow to flow under gravity for 5 minutes. Apply a further aliquot of MTBE (300 µL) and allow to flow under gravity for 5 minutes. For complete removal apply a pulse of positive pressure at 10 psi (10–20 seconds).	Apply DCM/IPA (95/5, v/v, 600 µL) allow to flow under gravity for 5 minutes. Apply a further aliquot of MTBE (600 µL) and allow to flow under gravity for 5 minutes. For complete removal apply a pulse of positive pressure at 10 psi (10–20 seconds).	Apply DCM/IPA (95/5, v/v, 600 µL) allow to flow under gravity for 5 minutes. Apply a further aliquot of MTBE (600 µL) and allow to flow under gravity for 5 minutes. For complete removal apply a pulse of positive pressure at 10 psi (10–20 seconds).

### Post Elution and Reconstitution

Evaporate extracts at 40 °C in the presence of 100 µL of 50 mM HCl in MeOH in order to avoid evaporative losses of amphetamines. For plate format, we used the Biotage® SPE Dry-96, and for column format we used the TurboVap® LV. Reconstitute using a mix of mobile phase A and mobile phase B (80/20, v/v, 200 µL).

### 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) 0.1% formic acid

B: 2 mM Ammonium Formate (MeOH) 0.1% formic acid

#### Flow Rate

0.4 mL/min

#### Injection Volume

5 µL

#### Column Temperature

30 °C

Table 1. UHPLC 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

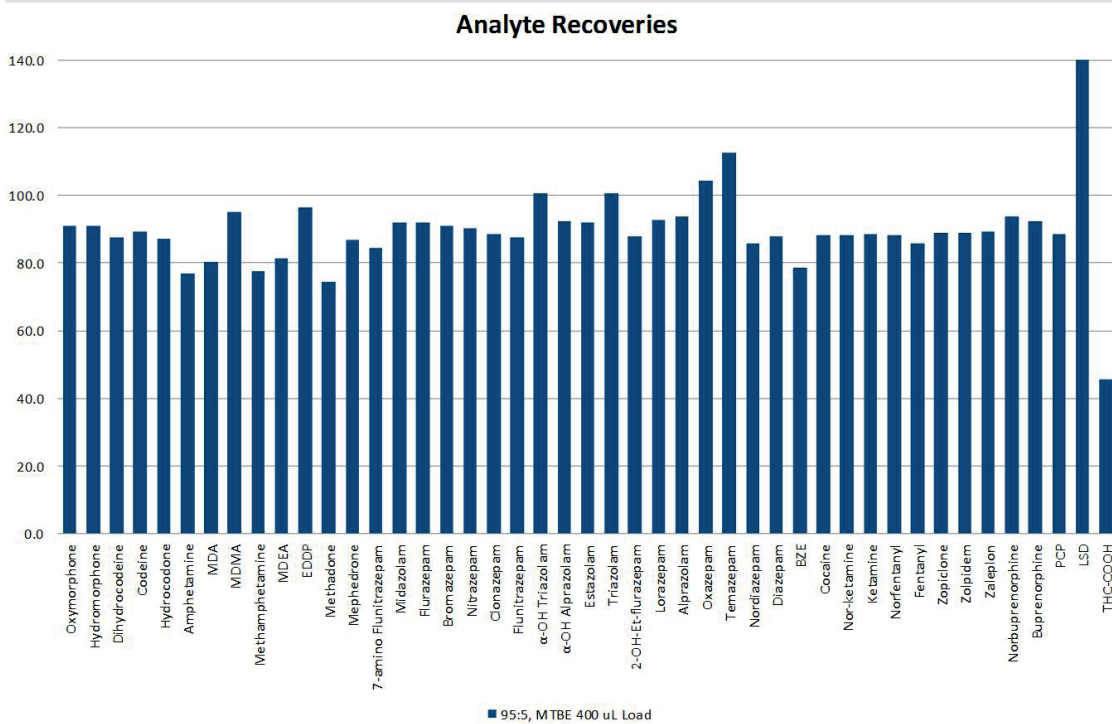


**Table 2.** MS conditions for target analytes in positive mode.

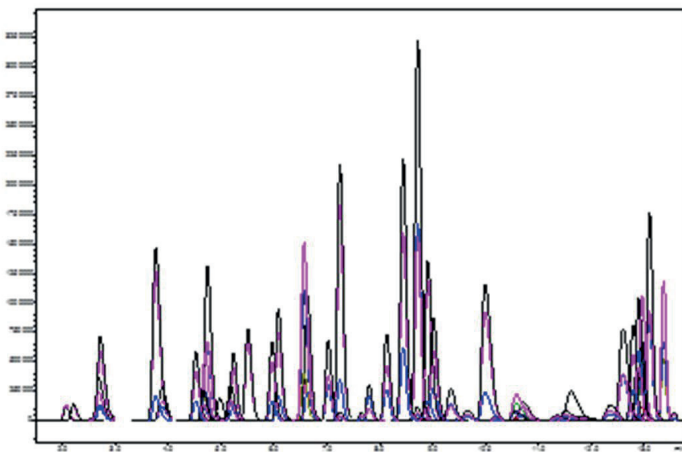
Analytes	MRM Transition	Collision Energy	Analytes	MRM Transition	Collision Energy
<b>Morphine-D<sub>3</sub></b>	289.0>201.1	-26.0	<b>LSD</b>	323.50>208.10	-29.0
	289.0>152.1	-50.0		323.50>223.25	-23.0
<b>Morphine</b>	286.0>152.1	-50.0	<b>7-Aminoflunitrazepam</b>	283.90>135.05	-30.0
	286.0>201.1	-25.0		283.90>227.05	-26.0
<b>Oxymorphone</b>	302.00>227.1	-30.0	<b>Zolpidem</b>	308.00>235.10	-35.0
	302.00>198.1	-45.0		308.00>263.10	-25.0
<b>Hydromorphone</b>	286.0>185.0	-30.0	<b>Buprenorphine</b>	468.10>396.25	-40.0
	286.0>157.0	-40.0		468.10>414.30	-35.0
<b>Amphetamine-D<sub>5</sub></b>	141.0>93.0	-15.0	<b>Fentanyl</b>	337.00>188.10	-20.0
	141.0>124.15	-20.0		337.00>105.00	-40.0
<b>Amphetamine</b>	136>91.05	-15.0	<b>Flurazepam</b>	388.00>315.00	-20.0
	136>119.1	-14.0		388.00>288.00	-26.0
<b>Methamphetamine</b>	150.0>90.95	-20.0	<b>PCP</b>	244.00>91.05	-35.0
	150>119.1	-14.0		244.00>159.15	-14.0
<b>MDA</b>	180>105	-20.0	<b>Midazolam</b>	325.90>249.10	-35.0
	180>77	-40.0		325.90>223.00	-40.0
<b>Dihydrocodiene</b>	302>119.05	-35.0	<b>Bromazepam</b>	315.80>182.10	-31.0
	302>171	-45.0		315.80>209.10	-27.0
<b>Codeine</b>	300.0>215.1	-25.0	<b>EDDP</b>	278.00>234.00	-30.0
	300.0>165	-40.0		278.00>234.00	-45.0
<b>6-MAM-D<sub>3</sub></b>	331.0>165.1	-40.0	<b>Lorazepam</b>	320.80>275.00	-22.0
	331.0>211.1	-25.0		320.80>229.05	-30.0
<b>6-MAM</b>	328.0>165.1	-40.0	<b>Oxazepam</b>	320.80>229.05	-23.0
	328.0>211.1	-25.0		286.90>104.20	-35.0
<b>MDMA</b>	194.0>163.1	-15.0	<b>Nitrazepam</b>	286.90>104.20	-25.0
	194.0>105.0	-25.0		281.90>180.10	-35.0
<b>Oxycodone</b>	316.2>241.2	-20.0	<b>Clonazepam</b>	315.90>270.05	-25.0
<b>Mephedrone</b>	178.00>145.05	-20.0		315.90>214.05	-38.0
	178.00>144.00	-30.0	<b>a-OH-Triazolam</b>	358.90>331.10	-28.0
<b>Hydrocodone</b>	300.0>199.05	-30.0		358.90>239.05	-44.0
	300.0>171.1	-40.0	<b>2-OH-et-flurazepam</b>	332.90>211.10	-37.0
<b>MDEA</b>	208>163.05	-15.0		332.90>109.00	-27.0
	208>105.05	-25.0	<b>Methadone</b>	310.50>265.10	-16.0
<b>Nor-Ketamine</b>	223.9>125	-20.0		<b>a-OH-Alprazolam</b>	324.90>216.10
	223.9>179.05	-15.0	324.90>205.10		-46.0
<b>Nor-Fentanyl</b>	233.0>84.05	-20.0	<b>Nordiazepam</b>	270.90>140.05	-26.0
	233.0>56.05	-26.0		270.90>208.10	-28.0
<b>BZE-D<sub>3</sub></b>	293.00>171.05	-20.0	<b>Zaleplan</b>	305.90>236.15	-28.0
	293.00>77.00	-50.0		305.90>264.20	-22.0
<b>BZE</b>	289.90>168.05	-20.0	<b>Flunitrazepam</b>	313.90>268.10	-25.0
	289.90>105.00	-30.0		313.90>239.10	-35.0
<b>Ketamine</b>	237.90>125.00	-30.0	<b>Estazolam</b>	294.90>267.05	-20.0
	237.90>207.05	-14.0		294.90>205.05	-40.0
<b>7-Aminoclonazepam</b>	285.90>222.10	-25.0	<b>Temazepam</b>	300.90>255.05	-20.0
	285.90>121.10	-29.0		300.90>177.05	-39.0
<b>Cocaine</b>	304.00>182.05	-20.0	<b>Triazolam</b>	342.90>308.10	-27.0
	304.00>82.05	-30.0		342.90>239.05	-41.0
<b>Zopiclone</b>	388.90>245.05	-15.0	<b>Alprazolam</b>	308.90>281.00	-25.0
	388.90>217.00	-35.0		308.90>205.05	-40.0
<b>Norbuprenorphine</b>	414.00>101.25	-39.0	<b>Diazepam-D<sub>5</sub></b>	289.90>193.05	-32
	414.00>187.20	-38.0		289.90>258.00	-7.0
			<b>Diazepam</b>	285.10>193.05	-32.0
				285.10	-27.0

## Results

This simple sample preparation method delivers clean extracts and analyte recoveries mostly greater than 80% with RSDs lower than 10% for all analytes (see Figure 2), and LLOQs from 1 ng/mL (see table 3) for all ISOLUTE® SLE+ formats used.



**Figure 2.** Representative analyte recoveries using the optimized SLE protocol for the 400  $\mu$ L capacity column format (p/n 820-0055-B. Similar results were achieved using the 200  $\mu$ L and 400  $\mu$ L capacity plate formats.



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

Calibration curve performance was investigated from hair (analytes (1–1000 pg/mL) were added to methanolic NH<sub>4</sub>OH extraction solvent prior to micropulverization). Good linearity was observed for all analytes typically delivering  $r^2$  values greater than 0.99. Table 3. details linearity performance and associated LOQ for each analyte, using the 400  $\mu$ L capacity column format, p/n 820-0055-B. Similar results were achieved using the 200  $\mu$ L and 400  $\mu$ L capacity plate formats.

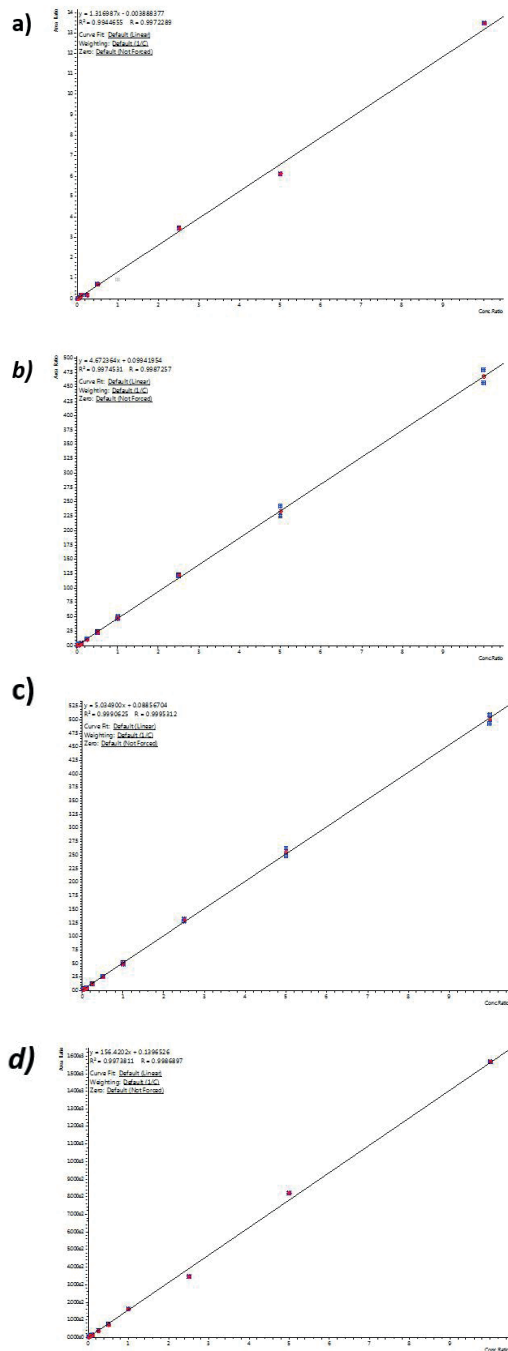


Figure 4. Calibration curves for Buprenorphine (a), 6-MAM (b), BZE (c) and Methamphetamine (d) extracted from human hair using the 400  $\mu$ L capacity column format loading 400  $\mu$ L of supernatant from the Biotage<sup>®</sup> Lysera tube. Similar results were achieved for the 200  $\mu$ L and 400  $\mu$ L capacity plate formats.

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

Analyte	400 $\mu$ L Load $r^2$	400 $\mu$ L Load LLOQ (pg/mL)
Morphine	0.998	1
Oxymorphone	0.998	1
Hydromorphone	0.997	1
Amphetamine	0.998	1
Methamphetamine	0.998	1
MDA	0.998	10
Dihydrocodeine	0.998	<1
Codiene	0.998	1
6-MAM	0.999	<1
MDMA	0.998	<1
Oxycodone	0.998	1
Mephedrone	0.996	1
Hydrocodone	0.998	1
MDEA	0.998	<1
Nor-Ketamine	0.996	1
Nor-Fentanyl	0.998	<1
BZE	0.999	<1
Ketamine	0.998	<1
7-Aminoclonazepam	0.998	1
Cocaine	0.997	<1
Zopiclone	0.998	5
Norbuprenorphine	0.998	50
LSD	0.998	1
7-Aminoflunitrazepam	0.999	1
Zolpidem	0.998	<1
Buprenorphine	0.996	1
Fentanyl	0.998	<1
Flurazepam	0.998	<1
PCP	0.998	1
Midazolam	0.997	1
Bromazepam	0.998	5
EDDP	0.999	<1
Lorazepam	0.998	10
Oxazepam	0.999	1
Nitrazepam	0.999	5
Clonazepam	0.998	5
a-OH-Triazolam	0.997	5
2-OH-et-flurazepam	0.998	10
Methadone	0.998	5
a-OH-Alprazolam	0.995	10
Nordiazepam	0.996	5
Zaleplon	0.997	1
Flunitrazepam	0.998	5
Estazolam	0.998	<1
Temazepam	0.998	5
Triazolam	0.998	1
Alprazolam	0.997	<1
Diazepam	0.997	1

## Ordering Information

Part Number	Description	Quantity
<b>19-060</b>	Biotage® Lysera	1
<b>19-649</b>	Bulk 2 mL Reinforced Tubes with Screw Caps	1000
<b>19-640</b>	Bulk 2.4 mm Metal Beads – 500 g	1
<b>820-0055-B</b>	ISOLUTE® SLE+ 400 µL Sample Volume Columns	50
<b>820-0200-P01</b>	ISOLUTE® SLE+ 200 µL Capacity Plate	1
<b>820-0400-P01</b>	ISOLUTE® SLE+ 400 µL Capacity Plate	1
<b>PPM-96</b>	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
<b>415000</b>	TurboVap® LV	1
<b>SD-9600-DHS</b>	Biotage® SPE Dry Sample Concentrator System	1

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