Extraction of 45 Multi Class Drugs of Abuse from Hydrolyzed Urine Using ISOLUTE® SLE+ prior to LC-MS/MS Analysis

This application note describes a Supported Liquid Extraction (SLE) protocol for the extraction of various drugs of abuse from hydrolyzed urine prior to LC-MS/MS analysis.

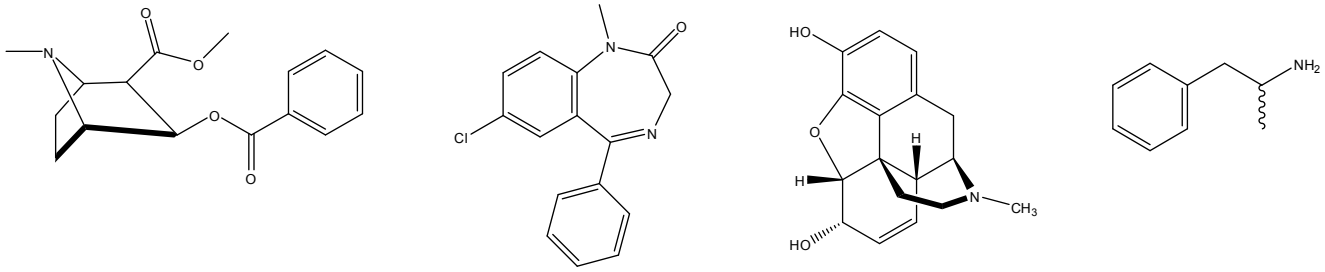

Figure 1. Example structures by class

Introduction

The method described in this application note achieves high reproducible recoveries for a wide range of drugs of abuse and metabolites from hydrolyzed urine. Hydrolyzed urine was extracted using the ISOLUTE SLE+ 200 supported liquid extraction plate and 1 mL column formats using 100 and 500 µL sample volumes, respectively. Sub ng/mL levels were achieved for all analytes using 100 µL of urine on the fixed well plate format.

ISOLUTE® SLE+ products provide clean, rapid, robust, efficient, high throughput and automatable extraction solutions for a wide range of analytes.

Analytes

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

Sample Preparation Procedure

SLE+ Format	ISOLUTE® SLE+ 200 supported liquid extraction plate, part number 820-0200-P01 or ISOLUTE SLE+ 1 mL sample volume supported liquid extraction columns, part number 820-0140-C.
Urine Hydrolysis	Take 1 mL urine and spike with internal standard (10 µL of Amphetamine-d3, Morphine-d3, Diazepam-d5, parent concentration 250 ng/mL; BZE-d3 and 6-MAM-d3, metabolite concentration 25 ng/mL). Add 1 mL of 100 mM ammonium acetate buffer (pH5) and 50 µL β-Glucuronidase enzyme (equivalent to approximately 4500 U/mL of urine). Hydrolyze at 60 °C for 2 hours.
Post Hydrolysis	Cool the hydrolyzed urine and add 10 µL of concentrated ammonium hydroxide (28–30% stock, aq) per mL of urine. Vortex mix thoroughly.

Supported Liquid Extraction

Sample Loading	**ISOLUTE SLE+ 200 µL plate:** Load pre-treated sample (200 µL) to the 96 well-plate followed by a pulse of vacuum or positive pressure to initiate flow. Leave to absorb for 5 minutes. **ISOLUTE SLE+ 1 mL columns**: Load pre-treated sample (1 mL) to the columns followed by a pulse of vacuum or positive pressure to initiate flow. Leave to absorb for 5 minutes.
Elution	**ISOLUTE SLE+ 200 µL plate:** Apply 1 mL of dichloromethane/isopropanol (95/5, v/v) and allow to flow under gravity for 5 minutes. Pull through the remaining solvent with vacuum or positive pressure for 10–20 seconds. **ISOLUTE SLE+ 1 mL columns:** Apply 2.5 mL of dichloromethane/isopropanol (95/5, v/v) and allow to flow under gravity for 5 minutes. Apply a second 2.5 mL aliquot of dichloromethane/isopropanol (95/5, v/v)) and allow to flow under gravity for 5 minutes. Pull through the remaining solvent with vacuum or positive pressure for 10–20 seconds. Note: The addition of 100 µL of 50 mM HCl in methanol into the collection plate or culture tube is required prior to or post elution to stabilize multiple analytes (amphetamines, bath salts and ketamine) due to volatility issues during evaporation.
Post Elution	Evaporate to dryness at 40 °C in a stream of air or nitrogen using a SPE Dry.
Reconstitution	**ISOLUTE SLE+ 200 µL plate**: Reconstitute in 200 µL of reconstitution buffer. Cap with a sealing mat and vortex gently. **ISOLUTE SLE+ 1 mL columns:** Reconstitute in 200 µL of reconstitution buffer and vortex gently.

Biotage®

UPLC Conditions

Instrument	Waters ACQUITY UPLC 20 µL Loop
Column	ACQUITY UPLC BEH C18 column (1.7 µm, 100 x 2.1 mm id)
Mobile Phase	A: 2 mM ammonium acetate (aq) B: 2 mM ammonium acetate /methanol
Flow Rate	0.3 mL/min

Table 1. Gradient Conditions

Time	% A	% B	Curve
0	90	10	1
10	10	90	6
11.4	10	90	6
13.4	90	10	1

Curve 1. Conditions in line initiated immediately once previous time passed. i.e. 90:10 resumed at 11.4 minutes.

Curve 6. Linear Gradient

Injection Volume	10 µL (partial loop with overfill)
Sample Temperature	20 °C
Column Temperature	40 °C

Mass Spectrometry Conditions

Instrument	Waters Premier XE triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.
Desolvation Temperature	450 °C
Ion Source Temperature	120 °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-d5	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-d3	289.2 > 201.0	42	25
BZE	290.1 > 168.0	30	18
BZE-d3	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-d3	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
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
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-d5	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 > 101.0	55	42

Results

Recovery

Urine spiked with 2 ng of analytes (n=7), equating to 20 ng/mL or 4 ng/mL when extracting 100 or 500 µL of urine, respectively. RSD's (n=7) ranged from 0.1–9.6%.

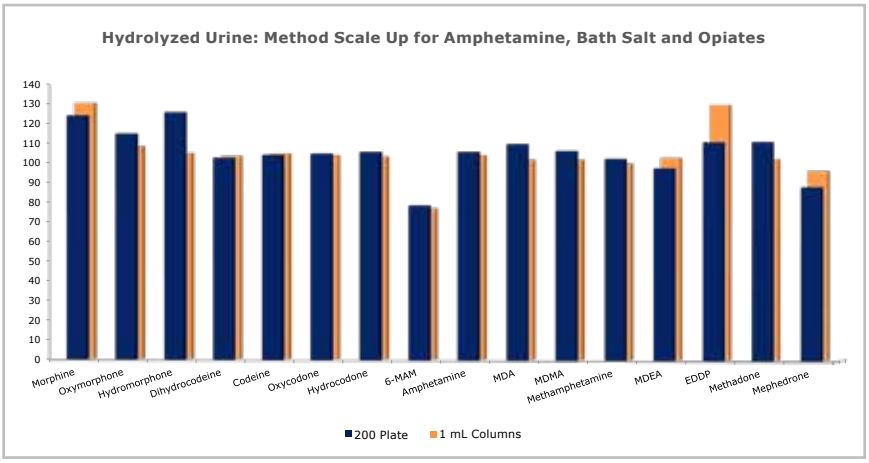

Figure 2. Recovery profile for amphetamines, bath salt and opiates from hydrolyzed urine using ISOLUTE® SLE+ 200 fixed well plates and 1 mL columns.

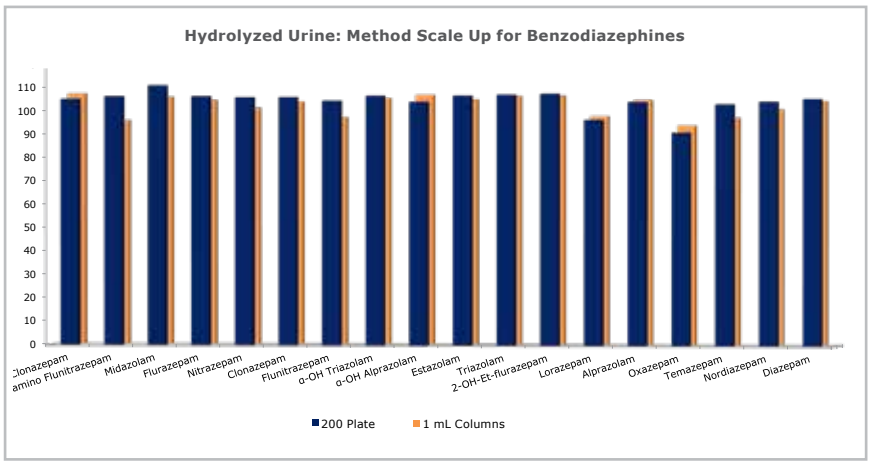

Figure 3. Recovery profile for benzodiazepines from hydrolyzed urine using ISOLUTE® SLE+ 200 fixed well plates and 1 mL columns.

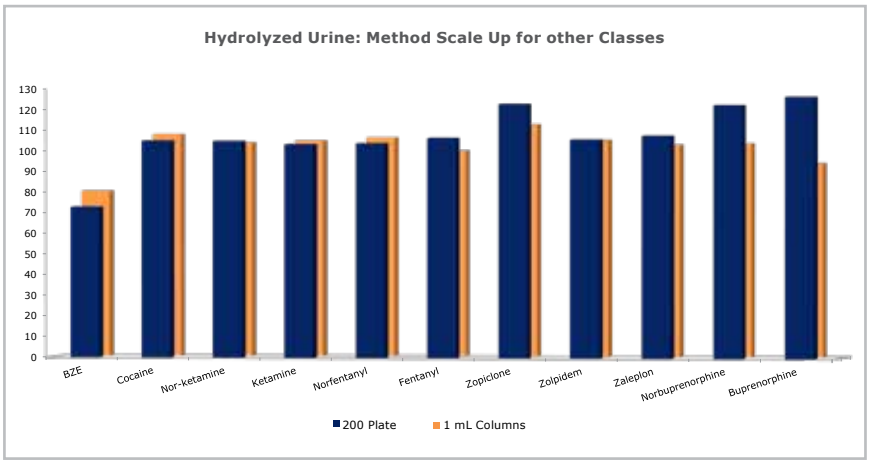

Figure 4. Recovery profile for multi class analytes from hydrolyzed urine using ISOLUTE® SLE+ 200 fixed well plates and 1 mL columns.

Calibration Curves

200 µL fixed well plate and 1 mL column processing: Calibration curves were generated using urine spiked at concentrations from 1–500 ng/mL. Good coefficients of determination were obtained for all analytes (r^2 › 0.99). Quadratic function was observed at the top end of the calibration curve for many analytes. Dilution of these samples was performed to improve linearity.

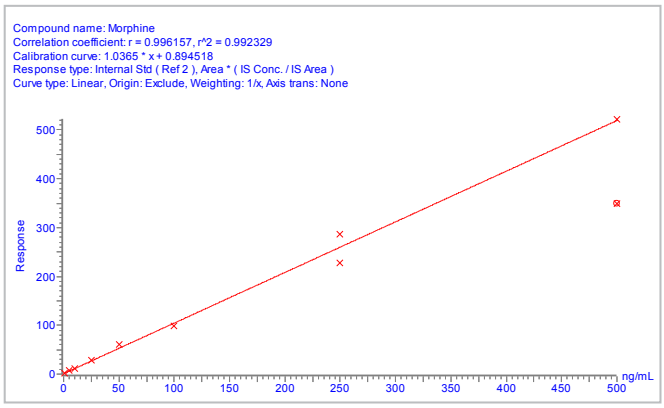

Figure 5. Calibration Curve for morphine using ISOLUTE® SLE+ 1 mL columns.

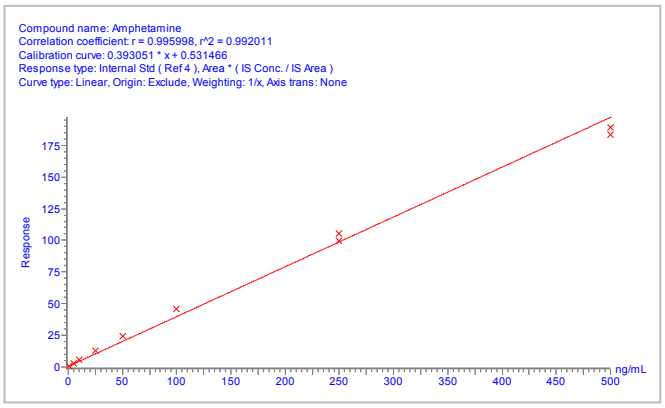

Figure 6. Calibration Curve for amphetamine using ISOLUTE SLE+ 1 mL columns.

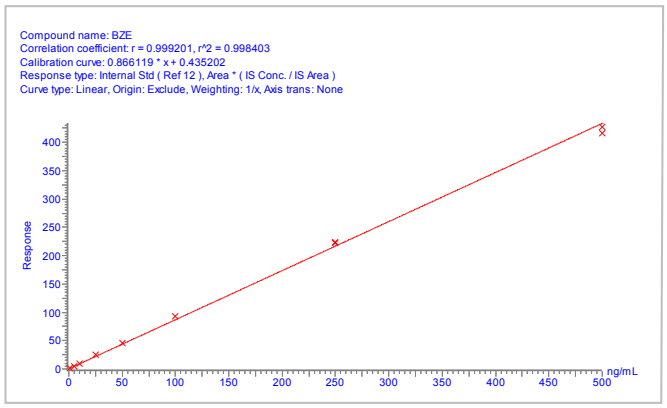

Figure 7. Calibration Curve for benzoylecgonine (BZE) using ISOLUTE® SLE+ 1 mL columns.

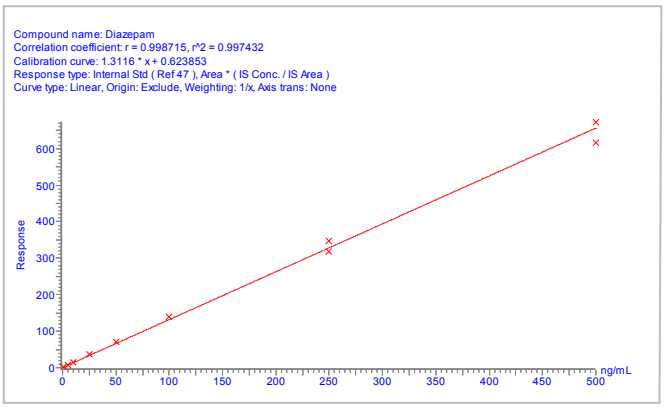

Figure 8. Calibration Curve for diazepam using ISOLUTE SLE+ 1 mL columns.

Table 3. Estimated LOQs based on S/N ratios from 1 ng/mL extracted samples are:

Analyte	Estimated LOQ 200 µL Plate ng/mL	Estimated LOQ 1 mL Columns ng/mL
Amphetamine	< 0.3	< 0.2
Methamphetamine	< 0.15	< 0.1
MDA	< 0.2	< 0.1
MDMA	< 0.1	< 0.05
MDEA	< 0.2	< 0.1
Hydromorphone	< 0.2	< 0.2
Morphine	< 0.5	< 0.3
BZE	< 0.1	< 0.05
Oxymorphone	< 0.5	< 0.5
Dihydrocodeine	< 0.1	< 0.1
Oxycodone	< 0.5	< 0.5
Mephedrone	< 1	< 0.75
Norfentanyl	< 0.1	< 0.05
7-amino-flunitrazepam	< 0.5	< 0.25
7-amino-clonazepam	< 0.5	< 0.25
Hydrocodone	< 0.25	< 0.2
Codeine	< 0.25	< 0.2
6-MAM	< 0.25	< 0.2
Cocaine	< 0.1	< 0.05
Norketamine	< 0.2	< 0.1
EDDP	< 0.15	< 0.1
Zaleplone	< 0.2	< 0.15
Zopiclone	< 0.1	< 0.1
Norbuprenorphine	< 0.5	< 0.4
Ketamine	< 0.05	< 0.05
Nitrazepam	< 0.25	< 0.25
Flunitrazepam	< 0.5	< 0.2
Clonazepam	< 0.5	< 0.5
α-OH-triazolam	< 0.5	< 0.25
Oxazepam	< 0.2	< 0.2
Estazolam	< 0.2	< 0.1
Temazepam	< 0.1	< 0.1
Zolpidem	< 0.1	< 0.1
Alprazolam	< 0.15	< 0.1
Methadone	< 0.15	< 0.1
Lorazepam	< 0.75	< 0.5
α-OH-alprazolam	< 0.5	< 0.25
2-OH-ethyl-flurazepam	< 1	< 1
Triazolam	< 0.2	< 0.1
Nordiazepam	< 0.2	< 0.1
Diazepam	< 0.2	< 0.1
Midazolam	< 0.1	< 0.1
Fentanyl	< 0.15	< 0.1
Flurazepam	< 0.2	< 0.1
Buprenorphine	< 0.5	< 0.35

Additional Notes

Buffer Preparation

1. 100 mM Ammonium acetate buffer at pH5: Dissolve 7.708 g ammonium acetatec in H_2O and make up to 1L. Formic acid (concentration 98–100% Sigma Aldrich) was used to adjust the pH to 5.
2. 2 mM ammonium acetate aq: Weigh 0.15416 g and dissolve in H_2O. Dilute and make up to 1 L in H_2O .
3. 2 mM ammonium acetate in MeOH: Weigh 0.15416 g and dissolve in H_2O. Dilute and make up to 1 L in MeOH.
4. Dichloromethane/isopropanol (95/5, v/v): Take 95 mL of dichloromethane and add 5 mL of isopropanol
5. Reconstitution buffer: Take 80 mL of 2 mM ammonium acetate (aq) and add 20 mL of 2 mM ammonium acetate in MeOH

Blowdown Stability

Amphetamines, bath salts and ketamines suffer blow down issues when drying in the free base form. To combat this effect we added 100 µL of 50 mM HCl in MeOH to the collection plate/culture tubes to convert to the corresponding HCl salt forms.

Reconstitution volumes can be reduced in order to reach lower limits of quantitation, if required!

Ordering Information

Part Number	Description	Quantity
820-0200-P01	ISOLUTE SLE+ 200 Supported Liquid Extraction Plate	1
820-0140-C	ISOLUTE SLE+ 1 mL Supported Liquid Extraction Columns	30
121-9600	VacMaster-96 Sample Processing Manifold	1
PPM-96	Biotage PRESSURE+ 96 Positive Pressure Manifold.	1
SD-9600-DHS	SPE Dry sample evaporator	1
C103264	TurboVap 96	1

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