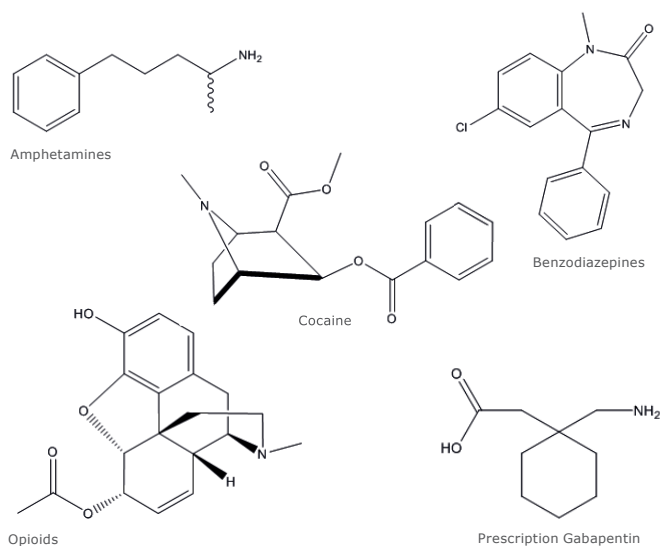


# Extraction of Illicit and Prescribed Drugs from Enzyme-Hydrolyzed Urine Using ISOLUTE® HYDRO DME+ Prior to UPLC-MS/MS Analysis



**Figure 1.** Example analyte structures by class.

## Introduction

This application note describes the extraction of 25 illicit and prescribed drugs from hydrolyzed urine using ISOLUTE® HYDRO DME+ Dual Mode Extraction plates prior to UPLC-MS/MS analysis.

ISOLUTE HYDRO DME+ products provide extremely efficient removal of matrix components and hydrolysis enzyme from urine samples, using a simple pass through workflow.

Because of the enhanced sample clean up delivered by ISOLUTE HYDRO DME+ products, analyte sensitivity is significantly increased compared to traditional dilute and shoot (D&S) techniques, resulting in reduced limits of quantitation (LOQ). ISOLUTE® HYDRO DME+ plates and columns are ideal for urinary drugs of abuse and pain management applications because the inclusion of Biotage® HYDRO frit technology means that urine samples can be hydrolyzed in-situ in the column, eliminating the need for post hydrolysis sample transfer steps.

The simple sample preparation procedure described delivers clean extracts and recoveries above 65% for the majority of analytes. The limits of quantitation all meet and exceed the sensitivity requirements set by SAMHSA and EWDTs for workplace testing applications.

## Analytes

Ecgonine methyl ester, pregabalin, morphine, oxycodone, amphetamine, gabapentin, codeine, 6-monoacetylmorphine, MDMA, hydrocodone, mephedrone, benzoylecgonine, ketamine, 7-aminoclonazepam, cocaine, norbuprenorphine, 7-aminoflunitrazepam, buprenorphine, PCP, EDDP, oxazepam, methadone, Zaleplon, flunitrazepam and ritalinic acid.

## Sample Preparation Procedure

### Format

ISOLUTE® HYDRO DME+ 400 mg Fixed Well Plate, part number: 970-0400-PZ01.

### Sample Pre-treatment

#### (hydrolysis using $\beta$ -Glucuronidase enzyme (*Helix pomatia*))

To 500  $\mu$ L of urine, add 25  $\mu$ L of internal standard mix at concentration 1 ng/ $\mu$ L and allow equilibration to take place at room temperature for 1 hour.

Apply 25  $\mu$ L of enzyme solution to 450  $\mu$ L of ammonium acetate (50 mM pH 5.0) and vortex briefly. Add this mix to the urine spiked with internal standard (as above) and vortex briefly.

Apply a 100  $\mu$ L aliquot of this sample (matrix/IS/enzyme/buffer mix) to the ISOLUTE HYDRO DME+ product and incubate for 2 hours at 60 °C.

## Extraction Procedure and Post-Extraction

Allow the sample to cool to room temperature and position a 96-well collection plate under the extraction plate. Add acetonitrile (600  $\mu$ L) onto the hydrolyzed urine sample. Perform 5x aspirate/dispense steps with an electronic 8-channel pipette to ensure sufficient mixing.

Using a Biotage® Pressure+ 96 Positive Pressure Manifold, apply approximately 5 PSI of positive pressure to elute the acetonitrile. The samples may be analysed by UPLC-MS/MS without an evaporation step\*. Simply cover the collection plate with a sealing mat prior to transfer to the autosampler.

\*If increased analyte sensitivity is required, the samples may be evaporated using a Biotage® SPE Dry 96 at 40L/min at 40 deg C and reconstituted in a low solvent volume prior to UPLC-MS/MS analysis. If so, a 100  $\mu$ L volume of methanolic hydrochloric acid (50mM) should be added to each well prior to evaporation to prevent the loss of more volatile analytes such as amphetamine.

## UHPLC Conditions

### Instrument

Waters ACQUITY UPLC with 20  $\mu$ L loop

### Column

Restek Raptor™ Biphenyl 2.7  $\mu$ m (100 x 2.1 mm id) with Raptor™ Biphenyl EXP guard cartridge

### Mobile Phase

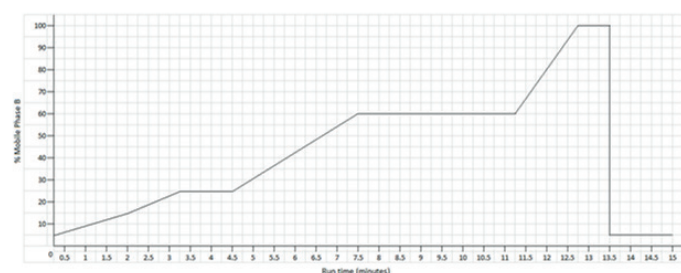
**A:** 2 mM ammonium formate (aq), 0.1 % formic acid

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

### Flow Rate

0.4 mL min

### Gradient Details



**Figure 2.** Graphical representation of LC conditions

**Table 1.** Gradient Conditions. Curve 6: Linear Gradient.

Time (min)	%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

### Column Temperature

40 °C

### Injection Volume

1  $\mu$ L (partial loop without overfill)

### Sample Temperature

20 °C

## MS/MS Conditions

### Instrument

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

### Source Temperature

150 °C

### Desolvation Temperature

450 °C

Positive ions acquired in the multiple reaction monitoring (MRM) mode are described in Table 2:

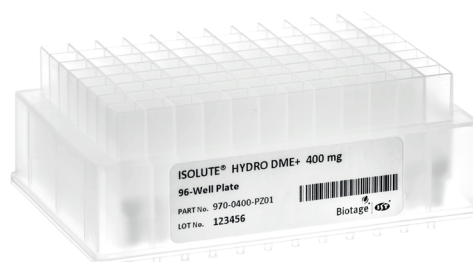
**Table 2.** MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Ecgonine Methyl Ester (EME)	182.2 > 82.0	50	15
Pregabalin	160.2 > 55.2	18	25
Morphine	286.2 > 201.0	42	25
Oxymorphone	302.2 > 198.1	34	37
Amphetamine	136.0 > 118.9	16	9
Gabapentin	172.3 > 137.1	23	15
Codeine	300.3 > 215.1	42	25
6-MAM	328.2 > 165.1	44	33
MDMA	194.1 > 163.0	20	13
Hydrocodone	300.2 > 199.1	46	33
Mephedrone	178.1 > 160.0	35	12
Ritalinic Acid	220.2 > 84.1	24	21
Benzoylcegonine (BZE)	290.1 > 168.0	30	18
Ketamine	238.1 > 124.9	25	27
7-amino-clonazepam	286.2 > 121.0	40	30
Cocaine	304.2 > 182.0	30	20
Norbuprenorphine	414.3 > 101.0	55	42
7-amino-flunitrazepam	284.2 > 135.0	40	27
Buprenorphine	468.3 > 468.3	55	5
PCP	244.2 > 158.9	20	15
EDDP	278.2 > 234.2	26	30
Oxazepam	287.2 > 241.0	30	21
Methadone	310.2 > 265.2	26	15
Zaleplon	306.2 > 264.2	40	22
Flunitrazepam	314.2 > 268.2	40	25

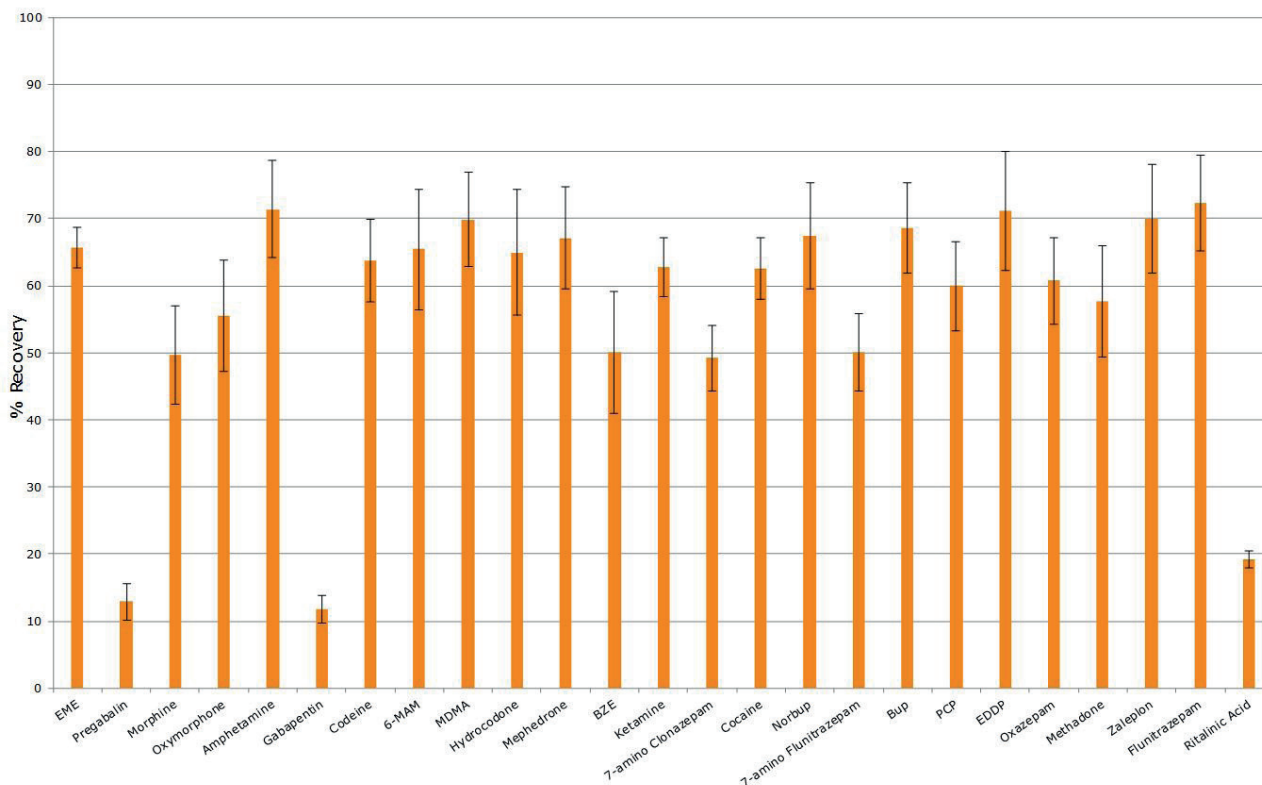
## Results

Analyte recovery, reproducibility, linearity and cleanliness studies were performed using intact urine from healthy volunteers.

Recovery data shown in Figure 3 demonstrates that this protocol provides extraction recovery of 65% or greater for the majority of analytes while simultaneously removing common urinary components. Where the recovery value was less than 65%, the sensitivity was more than sufficient to meet established cut off limits for drugs of abuse testing and prescription drug monitoring. RSD values were below 10%.

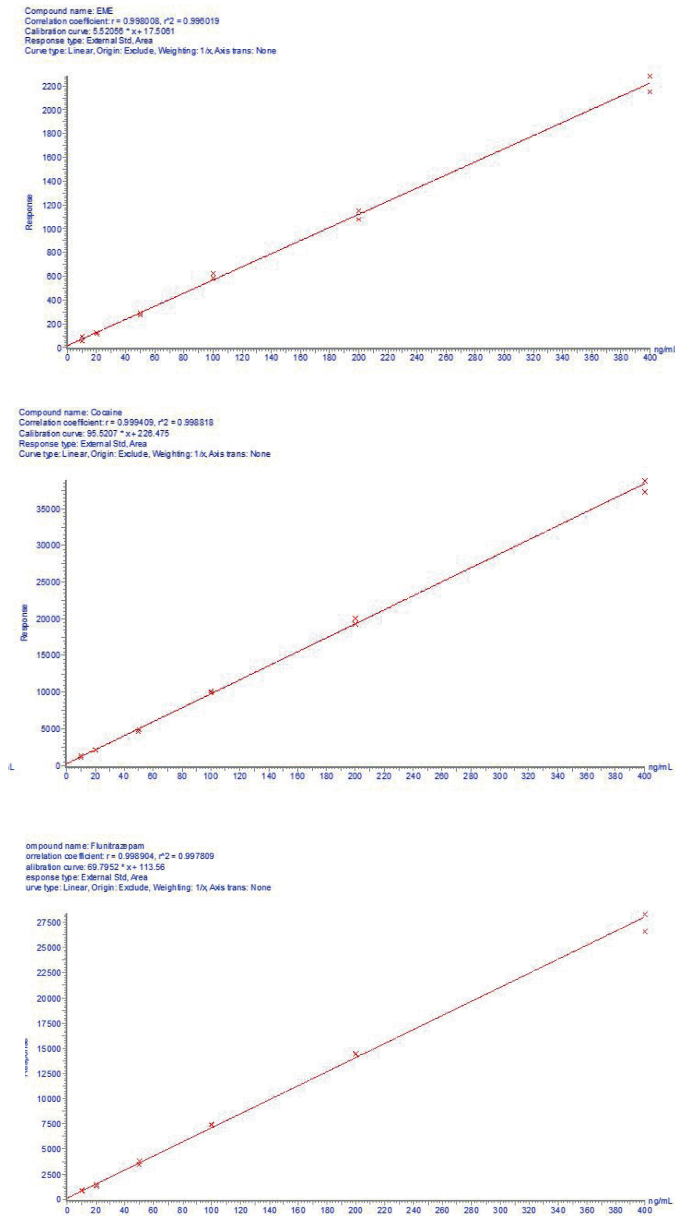


**Note:** The direct inject approach allows maximum sample throughput, however if greater signal is required, the samples may be evaporated and reconstituted in a low solvent volume prior to UPLC-MS/MS analysis (see page 1 for details).



**Figure 3.** Typical analyte recoveries and RSD (n=7, shown as error bars) for hydrolyzed urine following ISOLUTE® HYDRO DME+ processing and direct UPLC-MS/MS injection.

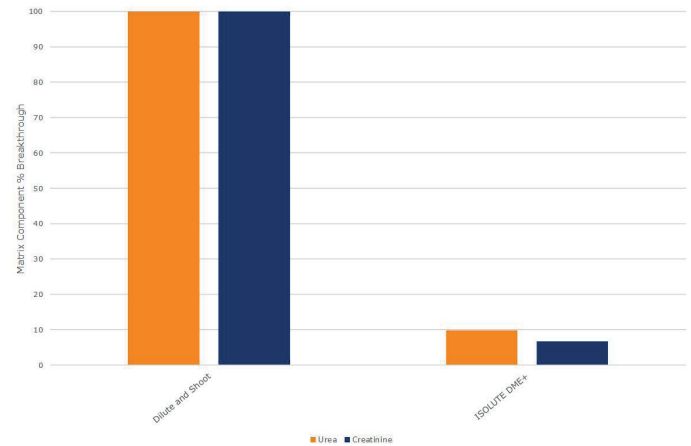
Following recovery determination, analytes were extracted from urine spiked before hydrolysis at levels 10, 20, 50, 100, 200 and 400 ng/mL to construct calibration curves. Representative curves are shown in Figure 4.



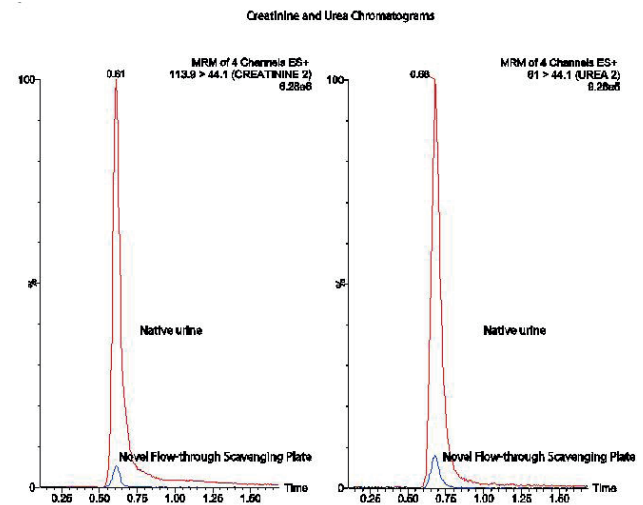
**Figure 4.** Calibration curves of application analytes EME, cocaine and flunitrazepam constructed following extraction of hydrolyzed urine using ISOLUTE® HYDRO DME+. Analyte concentrations are 10, 20, 50, 100, 200 and 400 ng/mL showing  $r^2$  values of greater than 0.99. Internal standard concentrations are at 50 ng/mL.

## Matrix Component Removal

Urea and creatinine, along with other urinary matrix components and hydrolysis enzyme, can have a detrimental effect on quantitation of desired analytes. Figures 5 and 6 illustrate the extent of removal of urea and creatinine from urine using ISOLUTE® HYDRO DME+ products, compared to the levels present in non-purified urine (as used in dilute and shoot (D&S) experiments).



**Figure 5.** Chart demonstrating urea and creatinine % breakthrough into sample extract with and without ISOLUTE® HYDRO DME+ clean up.



**Figure 6.** MRM chromatograms illustrating relative Creatinine (left) and Urea (right) content in hydrolyzed urine: (red) following ACN crash, (blue) post processing through ISOLUTE® HYDRO DME+.

## Chemicals and Reagents

- » Reference standards (including deuterated internal standards), ammonium acetate (reagent grade  $\geq 98\%$ ), ammonium formate (LC-MS grade), formic acid (LC-MS grade) and  $\beta$ -Glucuronidase enzyme (Type HP-2, aqueous solution  $\geq 100,000$  units/mL) were purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- » HPLC-grade solvents (acetonitrile, methanol) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- » Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- » Ammonium acetate (50 mM aq pH5) was prepared by adding 3.854 g of ammonium acetate to 1 L of deionized water. The pH was adjusted using formic acid (as above).
- » Mobile phase A (2 mM ammonium formate (aq), 0.1 % formic acid) was prepared by adding 126 mg of ammonium formate to 500 mL of purified water, adding 1mL of concentrated formic acid and making up to 1 L with purified water
- » Mobile phase B (2 mM ammonium formate (methanol), 0.1 % formic acid) was prepared by adding 126 mg of ammonium formate to 500 mL of HPLC grade methanol, adding 1mL of concentrated formic acid and making up to 1 L with HPLC grade methanol
- » 50 mM hydrochloric acid in methanol was prepared daily by adding 100  $\mu$ L of 12M concentrated hydrochloric acid to 23.9 mL of HPLC-grade methanol.

## Ordering Information

Part Number	Description	Quantity
<b>970-0400-PZ01</b>	ISOLUTE® HYDRO DME+ 400 mg Plate	1
<b>PPM-96</b>	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
<b>SD-9600-DHS-EU</b>	Biotage® SPE Dry 96 Sample Evaporator 220/240 V	1
<b>SD-9600-DHS-NA</b>	Biotage® SPE Dry 96 Sample Evaporator 100/120 V	1
<b>121-5203</b>	Collection Plate, 2 mL Square	50
<b>121-5204</b>	Pierceable Sealing Cap	50

## Additional information

All data shown in this application note was generated using real, intact matrix, obtained from human volunteers.

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