

# A Comparison of Current Methodologies for Extraction of Drugs of Abuse from Urine

Jillian Neifeld<sup>1</sup>, Jeremy Smith<sup>1</sup>, Stephanie Marin<sup>1</sup>, Mohamed Youssef<sup>1</sup>, Mario Merida<sup>1</sup>, and Elena Gairloch<sup>1</sup>  
<sup>1</sup>Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA

## Introduction

Urine is a very useful matrix in forensic laboratories, because it is easy to collect and can help determine if a drug has been used recently. There are many viable sample preparation options for urine samples, which include phospholipid depletion, supported liquid extraction, solid phase extraction, and more novel technologies, like dual-mode extraction. These sample preparation techniques each offer different levels of cleanliness and analyte recovery. Results from a drugs of abuse panel containing 100 compounds in urine were compared by LC-MS/MS using the different extraction techniques and popular hydrolysis procedures to identify practical considerations during method development.

## Methods

### Reagents and Materials

**Standards**  
 All standards were purchased from Cerilliant (Round Rock, TX). HPLC grade water and methanol (MeOH) were purchased from Sigma Aldrich (St. Louis, MO) in addition to reagent grade dichloromethane (DCM), formic acid, phosphoric acid (H<sub>3</sub>PO<sub>4</sub>), isopropanol, acetonitrile, and ammonium hydroxide (NH<sub>4</sub>OH). EVOLUTE<sup>®</sup> EXPRESS CX (30 mg bed) extraction plate (601-0030-PX01), ISOLUTE<sup>®</sup> SLE+ 400 µL extraction plate (820-0400-P01), ISOLUTE<sup>®</sup> HYDRO DME+ extraction plate (970-0400-PZ01), ISOLUTE<sup>®</sup> PLD+ extraction plate (918-0050-P01), Biotage<sup>®</sup> PRESSURE+ 96 position positive pressure manifold (PPM-96), and Biotage<sup>®</sup> SPE Dry 96 (SD-9600-DHS-NA) were supplied by Biotage. The LC column was provided by Restek (Bellefonte, PA).

### Sample Preparation

#### Urine Sample Preparation

A drug-free urine sample was donated. This urine was spiked with 100 ng/mL of the 98 drugs of abuse compounds. These DOA compounds included drugs from multiple drug classes: anticonvulsants, barbiturates, benzodiazepines, opioids, antidepressants, stimulants, hallucinogens, cannabinoids and antipsychotics.

#### Hydrolysis Parameters

Four different hydrolysis enzymes were tested: Campbell (Campbell Science, Rockford, IL), BG100 (Kura Biotech, Rancho Dominguez, CA), BGTurbo (Kura Biotech, Rancho Dominguez, CA), and IMCSzyme (IMCS, Irmo, SC). Sample size for all extraction techniques was 100 µL. For the Campbell and BG100 enzymes, 100 µL of 100 mM ammonium acetate buffer, pH 4.0, and 20 µL of enzyme was added to the urine sample. For the IMCSzyme, 25 µL of IMCS buffer, 55 µL of water, 10 µL of methanol, and 20 µL of enzyme was added to the urine sample. For the BGTurbo enzyme, 100 µL of 150 mM sodium phosphate buffer, pH 6.8, 55 µL of water, 10 µL of methanol, and 25 µL of enzyme was added to the urine sample.

### Extraction Procedures

After hydrolysis, extraction was performed. The phospholipid depletion (ISOLUTE<sup>®</sup> PLD+ extraction plate) protocol used 800 µL of acetonitrile, acetonitrile with 0.1% formic acid, or acetonitrile with 1% formic acid. The acetonitrile was added to the extraction plate first, followed by the sample. For the dual-mode extraction (ISOLUTE<sup>®</sup> HYDRO DME+ extraction plate) method, 600 µL of acetonitrile or acetonitrile with 0.1% formic acid was added to the urine sample following in-well hydrolysis. For the supported liquid extraction (ISOLUTE<sup>®</sup> SLE+ 400 µL extraction plate) method, a pretreatment of 100 µL 0.1% NH<sub>4</sub>OH was used. The samples were loaded, and after a 5-minute wait period, 2 aliquots of 750 µL of 95:5 dichloromethane/isopropanol was used for elution. The solid phase

extraction method (EVOLUTE<sup>®</sup> EXPRESS CX 30 mg extraction plate, Biotage) for the urine samples is shown in table 1.

Step	Volume (µL)	Solvent	Time (min)	Pressure (psi)
<b>Sample Load</b>	ALL	Sample	1-2	2-4
<b>Wash #1</b>	1000	4% H <sub>3</sub> PO <sub>4</sub>	1-2	2-4
<b>Wash #2</b>	1000	50% Methanol (aq)	1-2	2-4
<b>Plate Dry</b>	N/A	N/A	1	20
<b>Elution</b>	2 x 750	DCM/MeOH/NH <sub>4</sub> OH [78:20:2]	2-3	2-4
<b>Plate Dry</b>	N/A	Quick Pulse	x2	20

**Table 1.** Biotage 96 Positive Pressure Processing Parameters.

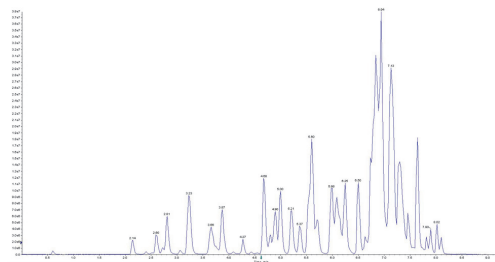
**Dry Down and Sample Reconstitution:** Elution solvent was collected into a collection plate. All samples were evaporated to dryness at 40°C with 20 L/min of nitrogen using a Biotage<sup>®</sup> SPE Dry. Extracts were then reconstituted with 100 µL of 90:10 mobile phase A/mobile phase B and analyzed via LC-MS/MS.

### Chromatography Parameters

UPLC	Parameter
<b>Column</b>	Restek Raptor Biphenyl 2.7 µm, 50 x 3.0 mm
<b>MPA</b>	0.1% Formic Acid (aq)
<b>MPB</b>	0.1% Formic Acid in MeOH
<b>Flow Rate</b>	0.45 mL/min
<b>Column Temp.</b>	40 °C
<b>Sample Temp.</b>	20 °C
<b>Injection Volume</b>	2.5 µL

**Table 2.** Shimadzu Nexera X2 UPLC.

The LC gradient started with 95% aqueous and gradually decreased to 5% aqueous over a 9.0-minute total run time. This allowed for full separation of all compounds in the panel. Figure 1 illustrates the TIC for the 98-compound panel.



**Figure 1.** TIC for 98-compound panel.

### Mass Spectrometry Parameters

**Instrument:** SCIEX 5500 triple quadrupole Mass Spectrometer with Turbo Ionspray<sup>®</sup> Ion interface (Foster City, CA). Optimized source parameters shown in table 3 (sMRM transition parameters not shown, but available upon request). Retention window for sMRM set at 45 seconds with target scan time at 2.85 seconds.

Ionization Spray Voltage	+1500(V)	CAD	Medium
<b>Source Temp</b>	600 °C	GS1	70
<b> Curtain</b>	30 (V)	GS2	70

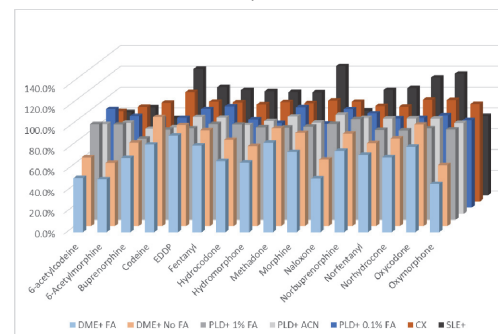
**Table 3.** SCIEX 5500 Triple Quadrupole ESI (+/-) Turbo Ionspray<sup>®</sup> Source Parameters.

## Results

### Extraction Recoveries

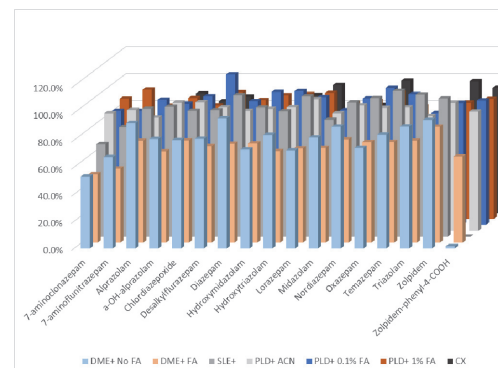
Recoveries varied greatly for some compounds, depending on the extraction technique as used. Figure 2 shows recoveries for the opiate/opioid compounds in the drugs of abuse panel for all extraction types. The four hydrolysis techniques tested did not

result in significant variation in recoveries (most compounds had recoveries within 20% for all enzymes tested).



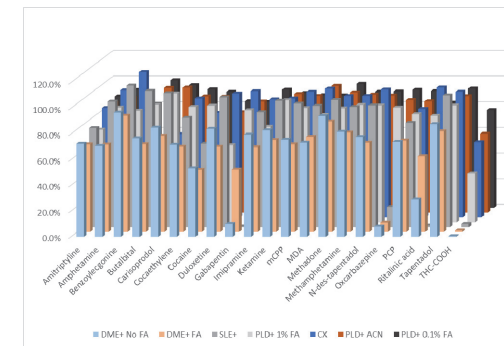
**Figure 2.** Variations in recoveries seen for opiate/opioid compounds using different extraction techniques and the IMCSzyme.

Figure 3 shows recoveries for the benzodiazepine compounds in the panel. As can be seen, recoveries varied greatly for this drug class. Zolpidem-phenyl-4-COOH had greater than 85% recovery when extracting via solid-phase extraction (EVOLUTE<sup>®</sup> EXPRESS CX) or phospholipid depletion (ISOLUTE<sup>®</sup> PLD+). However, when extracting with supported liquid extraction (ISOLUTE<sup>®</sup> SLE+) or dual mode extraction (ISOLUTE<sup>®</sup> HYDRO DME+) without formic acid, recoveries were less than 5%.



**Figure 3.** Variations in recoveries seen for benzodiazepine compounds using different extraction techniques and the IMCSzyme.

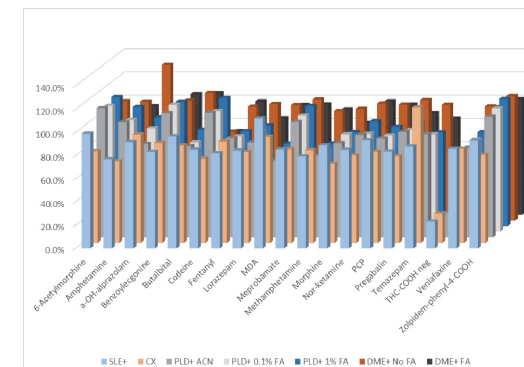
Figure 4 shows recoveries for other compounds (barbiturates, stimulants, antidepressants, antipsychotics) in the panel. As can be seen, recoveries varied greatly for these drug classes as well. Like, zolpidem-phenyl-4-COOH, gabapentin had much higher recoveries when extracting via solid-phase extraction (EVOLUTE<sup>®</sup> EXPRESS CX) or phospholipid depletion (ISOLUTE<sup>®</sup> PLD+). However, when extracting with supported liquid extraction (ISOLUTE<sup>®</sup> SLE+) or dual mode extraction (ISOLUTE<sup>®</sup> HYDRO DME+) without formic acid, recoveries were minimal. The barbiturates had recoveries of less than 30% when extracting with EVOLUTE<sup>®</sup> EXPRESS CX due to the cation exchange binding properties. However, with all other extraction techniques, the barbiturates had recoveries greater than 65%.



**Figure 4.** Variations in recoveries seen using different extraction techniques and the IMCSzyme.

### Extraction Matrix Effects

Matrix effects also varied greatly between extraction types tested. Figure 5 shows matrix effects for 19 of the 98 compounds tested. Some of the compounds when using the pass through SPE methods (ISOLUTE<sup>®</sup> HYDRO DME+ and ISOLUTE<sup>®</sup> PLD+) resulted in either ion suppression or ion enhancement, meaning these methods were not as clean as either the EVOLUTE<sup>®</sup> EXPRESS CX or ISOLUTE<sup>®</sup> SLE+ methods.



**Figure 5.** Variations in matrix effects seen using different extraction techniques and the IMCSzyme.

## Conclusions

- » ISOLUTE<sup>®</sup> HYDRO DME+ and ISOLUTE<sup>®</sup> PLD+ extraction techniques were the fastest and cheapest of all those tested.
- » The EVOLUTE<sup>®</sup> EXPRESS CX method was the most time-consuming extraction technique but resulted in recovery of all compounds with minimal matrix effects.
- » The ISOLUTE<sup>®</sup> SLE+ method resulted in clean extracts, but some compounds had little recovery when using this extraction technique.
- » The four extraction techniques all cleaned up the hydrolysis enzyme from the urine samples, resulting in little variation in recoveries and matrix effects seen using the different enzymes.