Sample Preparation Techniques for Synthetic Benzodiazepines

Introduction

Synthetic benzodiazepines are becoming more abused and are considered “legal highs”. Many forensic laboratories are finding more and more of these compounds in postmortem and impairment cases. While synthetic benzodiazepine prevalence has not been widely broadcast as it is overshadowed by the opioid epidemic, this drug class is now the fourth most commonly identified illegal synthetic drug class, after synthetic cannabinoids, synthetic opioids, and synthetic cathinones (https://ndews.umd.edu/resources/dea-emerging-threat-reports).

Analytes

Clobazam, bromazepam, phenazepam, estazolam, clonazolam, prazepam, flubromazepam, etizolam, delorazepam, pyrazolam, diclazepam, nimetazepam

Methods

There are several different extraction methods that can be used to extract synthetic benzodiazepines from biological matrices such as urine and blood. This application note evaluates three common extraction techniques, detailing the methodology used, and comparing results in terms of analyte recovery and matrix effects.

One technique is **supported liquid extraction** using ISOLUTE® SLE+. This product, available in plate or cartridge format, employs the mechanism of a liquid-liquid extraction with a diatomaceous earth sorbent, allowing for complete separation of the aqueous and organic layers (see Figure 1).

The first step is loading samples onto the diatomaceous earth material. A five-minute wait time allows the aqueous sample to fully adsorb onto the sorbent. Next, an elution step follows using a water-immiscible organic solvent like dichloromethane (DCM), ethyl acetate (EA), or MTBE (tert-butyl methyl ether). This step targets the compounds of interest allowing them to elute off of the diatomaceous earth sorbent, while leaving behind any aqueous impurities and other unwanted components.

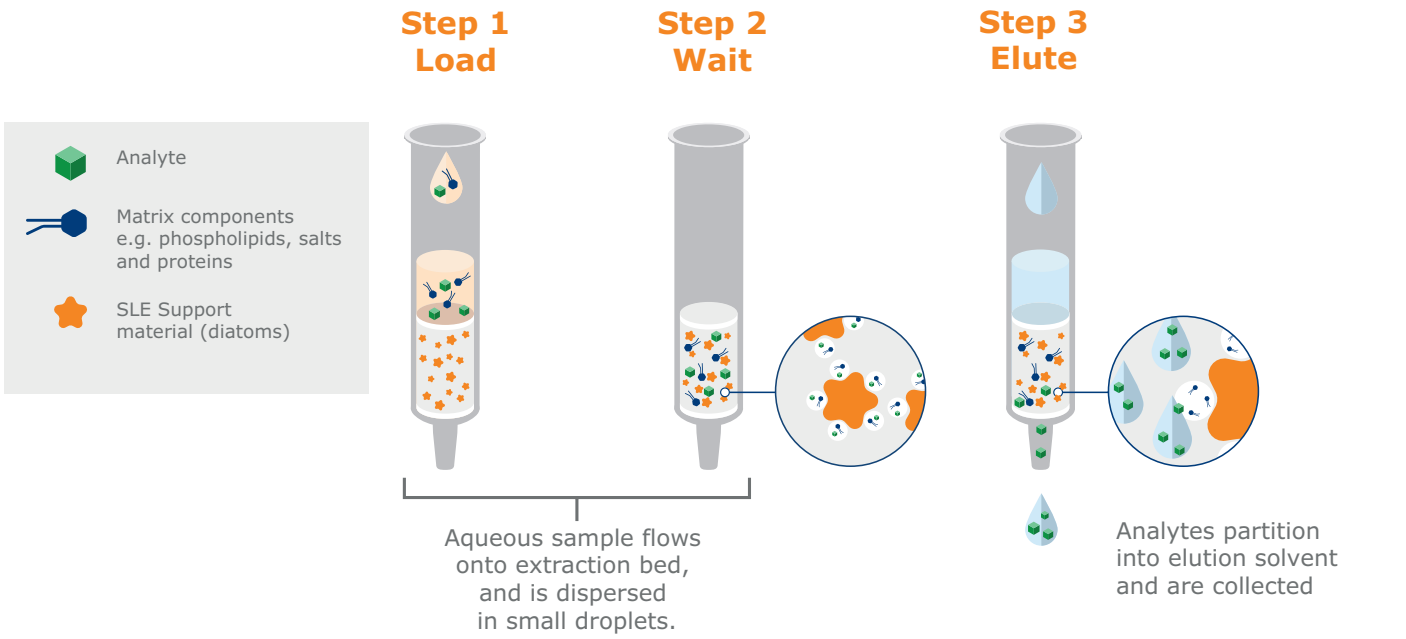

Figure 1. Typical workflow for ISOLUTE® SLE+.

Solid Phase Extraction (SPE) can also be used to isolate the synthetic benzodiazepine compounds. EVOLUTE® EXPRESS CX, a polymeric sorbent, was used for these compounds.
This technique utilizes a mixed-mode mechanism (non-polar interactions and cation exchange) to allow for additional sample cleaning without loss of analyte recovery. The polymeric sorbent is water-wettable, which permits the exclusion of the condition and equilibration steps and promotes less pre-elution plate drying time (see Figure 2).

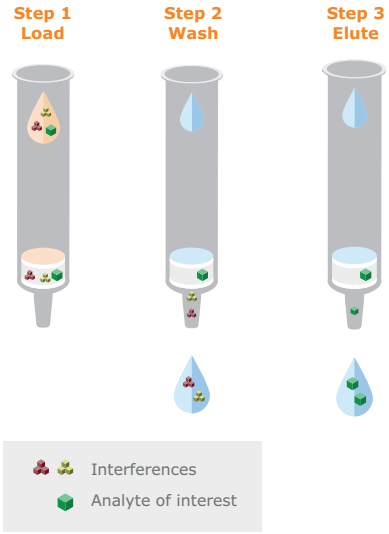

Figure 2. Typical workflow for solid phase extraction using EVOLUTE® EXPRESS.

Dual Mode Extraction (DME) using ISOLUTE® HYDRO DME+ is the simplest technique for extraction. This product, available in plate or cartridge format, uses two scavenging layers of sorbent to remove components of biological matrices, including pigments, salts, urea, and creatinine (see Figure 3).

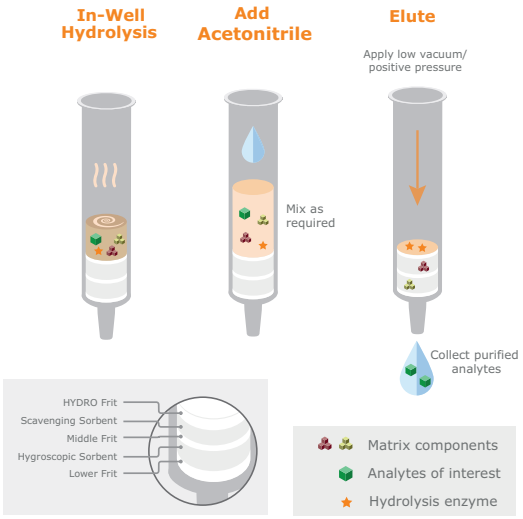

Figure 3. Typical workflow for ISOLUTE® HYDRO DME+.

The methods used for extraction of the synthetic benzodiazepine compounds from whole blood and urine samples are shown below. The samples can be extracted manually using a Biotage® VacMaster™ 96 or Biotage® Pressure+ 96. The extractions can also be automated using the Biotage® Extrahera Automated Sample Preparation System.

Table 1. ISOLUTE® SLE+ 400 µL Plate Methodology.

Sample volume	100 µL
Pretreatment	100 µL 1% NH4OH (aqueous)
Load sample	
Elution	2 x 750 µL dichloromethane **OR** 2 x 750 µL ethyl acetate **OR** 2 x 750 µL MTBE **OR** 2 x 750 µL 95:5 dichloromethane/isopropanol

Following elution, evaporate samples to complete dryness using a Biotage® SPE Dry 96 plate evaporator. Reconstitute in 100 µL 95:5 mobile phase A/mobile phase B.

Table 2: EVOLUTE® EXPRESS CX 30 mg Plate Methodology.

Urine sample volume	100 µL
Pretreatment	100 µL 0.1% formic acid (aqueous)
Extraction	
Condition	NONE
Equilibrate	NONE
Load	
Wash 1	1 mL water
Wash 2	1 mL 0.1% formic acid (aq)
Wash 3	1 mL methanol **OR** 50:50 methanol/water
Plate Dry	1 min at 20 psi
Elution	2 x 750 µL 78:20:2 DCM/IPA/NH_4OH OR 2 x 750 µL 78:20:2 EA/ACN/NH_4OH

After elution, evaporate samples to complete dryness using a Biotage® SPE Dry 96 plate evaporator. Reconstitute in 100 µL 95:5 mobile phase A/mobile phase B.

Table 3. ISOLUTE® HYDRO DME+ Methodology.

Sample volume	100 µL
Load sample	
OPTIONAL	Add 10 µL formic acid to sample
Add Acetonitrile	600 µL
Mix	Pipette mix 5 times
Elution	Push sample through sorbent

Following elution, evaporate samples to complete dryness using a Biotage® SPE Dry 96 plate evaporator. Reconstitute in 100 µL 95:5 mobile phase A/mobile phase B.

Analytical Conditions

For all samples, following evaporation and reconstitution as described, LC-MS/MS analysis was performed using the conditions outlined below.

LC Parameters

Instrument
Shimadzu Nexera X2

LC Column
Restek Raptor Biphenyl 50 x 3 mm, 2.7 µm (Cat # 9309A52)

Column Temperature
40 °C

Mobile Phase A:
0.1% formic acid in water

Mobile Phase B:
0.1% formic acid in methanol

Table 4. Mobile phase gradient.

Time	% Mobile Phase B
0.01	5
0.5	10
5.25	70
7.5	95
7.7	95
7.75	5
9.25	STOP

Flow Rate
0.4 mL/min

Run Time
9.25 minutes

Injection Volume
2.5 µL

MS/MS Parameters

Instrument
SCIEX 5500 Triple Quadrupole

Source Gas
600 °C

Curtain Gas
30

Collision Gas (CAD)
8

Ionspray Voltage
1500 V

Ion Source Gas 1
50

Ion Source Gas 2
50

Entrance Potential
10

Positive Polarity
Table 3 shows the MS parameters for each compound in the panel

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Table 5. MS Parameters for all Synthetic Benzodiazepine Compounds.

Compound	Retention Time	Q1	Q3	Declustering Potential	Collision Energy	CXP
Clobazam	6.74	300.951	259.1	136	31	6
		300.951	224.1	136	45	10
Bromazepam	6.74	317.929	301.0	16	13	8
		317.929	259.0	16	35	16
Phenazepam	6.71	348.917	206.1	21	47	12
		348.917	184.1	21	41	8
Estazolam	6.86	295.000	267.0	96	33	4
		295.000	205.2	96	55	14
Clonazolam	6.57	353.962	308.1	181	37	14
		353.962	280.1	181	49	8
Prazepam	7.73	324.992	271.1	216	33	10
		324.992	140.1	216	47	10
Flubromazepam	6.57	334.904	226.1	66	39	12
		334.904	185.9	66	41	12
Etizolam	7.34	342.955	314.1	216	35	6
		342.955	259.1	216	47	8
Delorazepam	6.58	304.946	166.0	121	71	8
		304.946	140.2	121	71	26
Pyrazolam	6.51	355.938	206.2	156	47	12
		355.938	167.2	156	65	16
Diclazepam	7.15	318.927	154.1	141	39	10
		318.927	227.2	141	47	6
Nimetazepam	6.94	296.000	250.1	126	35	8
		296.000	221.2	126	47	8

Results

For every method, a 10 ng/mL and a 100 ng/mL sample were run to ensure consistency over a concentration range. The recoveries and matrix effects shown are using a 10 ng/mL sample in whole blood or urine. When analyzing urine samples (Figure 4), the highest recoveries were obtained when using ISOLUTE® SLE+ with an ethyl acetate elution solvent. When using EVOLUTE® EXPRESS CX with a 100% methanol wash, bromazepam and clobazam were mostly washed away. ISOLUTE® HYDRO DME+ when used without formic acid had recoveries of 60–75% for all compounds.

When analyzing whole blood samples (Figure 5), the highest recoveries were obtained when using ISOLUTE® SLE+ with an MTBE or an ethyl acetate elution solvent. ISOLUTE® HYDRO DME+ when used with formic acid only resulted in recoveries of 10–20% for all compounds. Without using formic acid, recoveries were between 35–50% for all compounds.

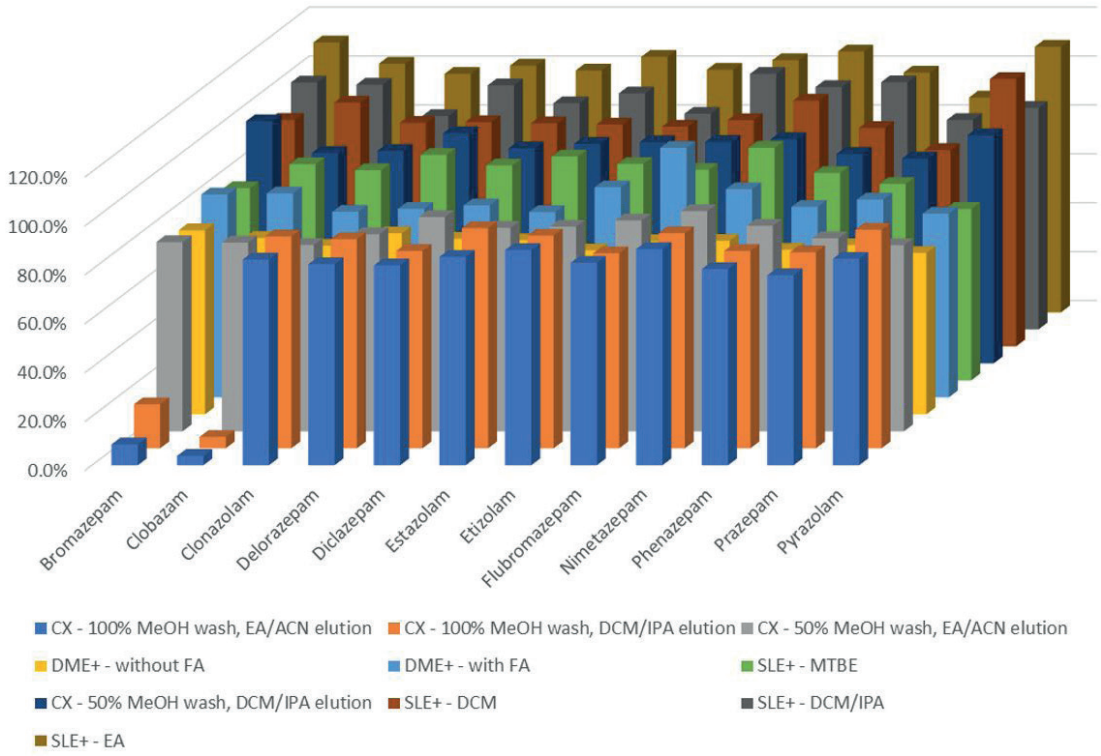

Figure 4. Recoveries of synthetic benzodiazepines from urine samples.

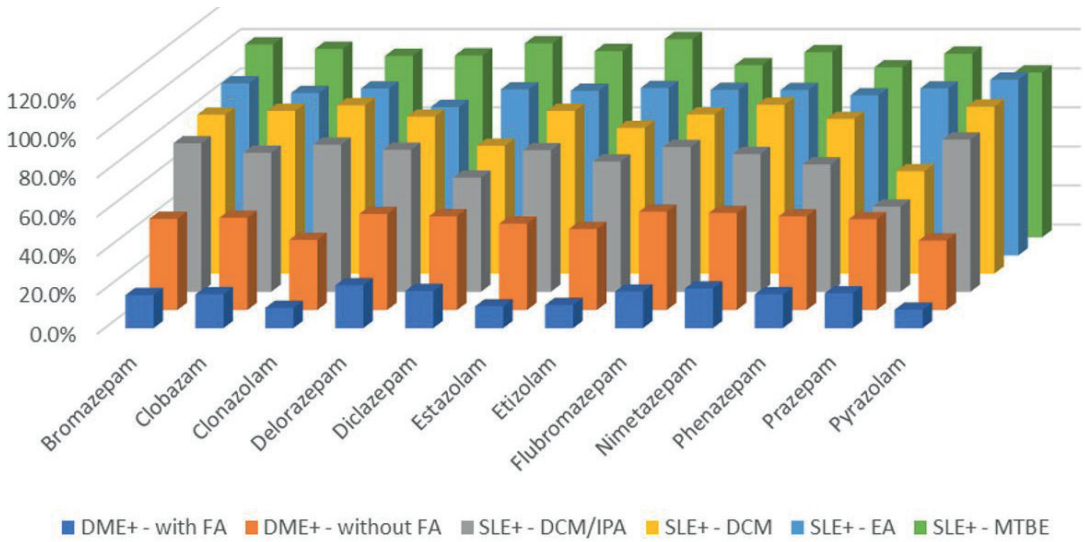

Figure 5. Recoveries of synthetic benzodiazepines from whole blood samples.

When looking at matrix effects of urine samples (Figure 6), ISOLUTE® HYDRO DME+ with formic acid resulted in the most suppression (dirtiest extracts). The cleanest extracts (least suppression) was seen when extracting with ISOLUTE® SLE+ using a dichloromethane or dichloromethane/isopropanol elution solvent or EVOLUTE® EXPRESS CX with a 50% methanol wash and an EA/ACN/NH_4OH elution solvent.

When looking at matrix effects of whole blood samples (Figure 7), the dirtiest extracts were seen when extracting using ISOLUTE® SLE+ with an MTBE elution solvent. Surprisingly, extracting using ISOLUTE® HYDRO DME+ resulted in the cleanest extracts (least suppression).

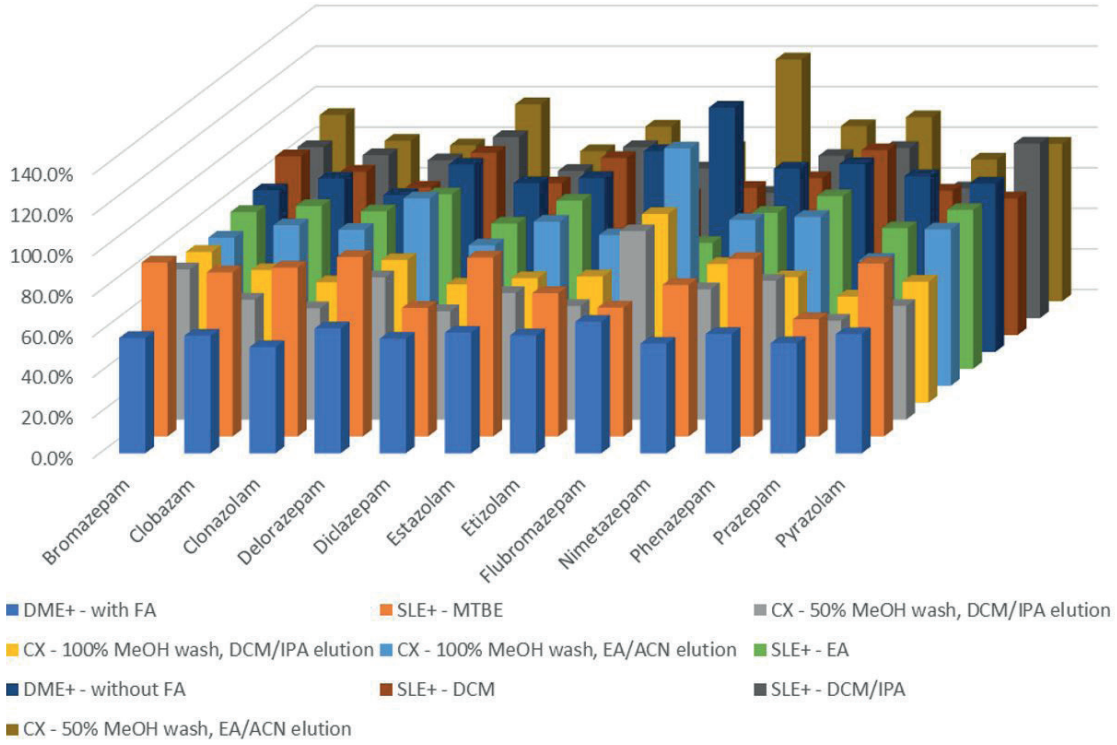

Figure 6. Matrix effects of synthetic benzodiazepines from urine samples.

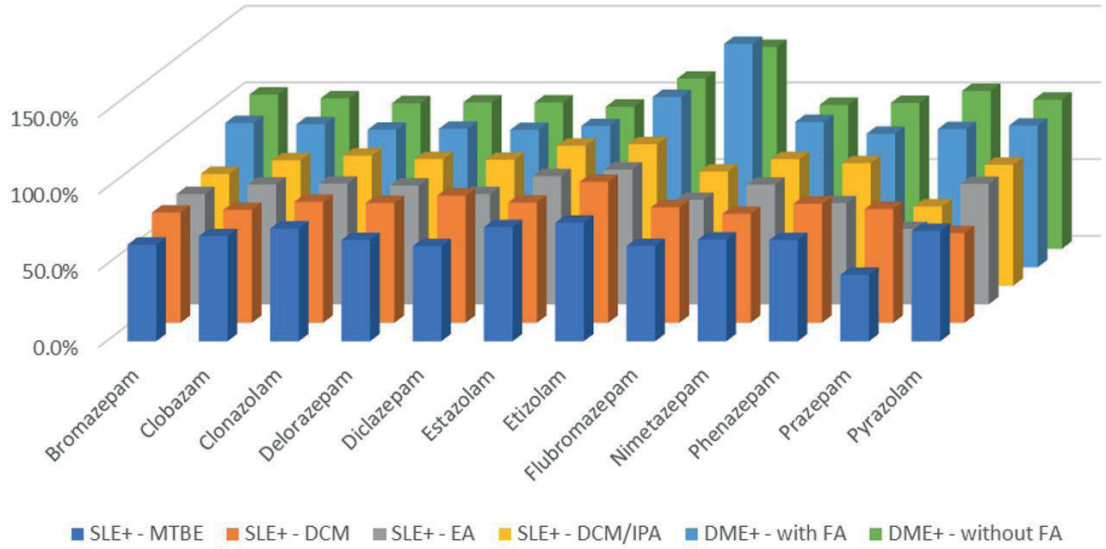

Figure 7. Matrix effects of synthetic benzodiazepines from whole blood.

Conclusion

There are several different extraction techniques that can be used to extract synthetic benzodiazepines from whole blood and urine samples. It is important to consider the compounds in the panel, the desired extract cleanliness, compound recoveries, and extraction time to determine which method best fits the application. If use of the same extraction technique for both whole blood and urine samples is desired, ISOLUTE® SLE+ using an ethyl acetate elution solvent results in recoveries over 75% and slight suppression for some compounds.

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